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Isolation of a DNA Probe for *Lactobacillus curvatus*

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A genomic library of *Lactobacillus curvatus* DSM 20019 was constructed in bacteriophage λ gt11. A 1.2-kilobase DNA probe specific for *L. curvatus* was isolated from this library. When this probe was hybridized to DNA from *Lactobacillus* isolates from different sources classified by conventional techniques, differing degrees of hybridization were obtained. This could imply that these isolates may have been incorrectly classified.

Lactobacillus curvatus and *Lactobacillus sake* have been found to be the two dominant species of bacteria associated with the spoilage of vacuum-packed meats (21; E. S Gerber, M.S. thesis, University of Pretoria, 1984). They are able to grow at temperatures of 4°C or lower and cause spoilage of refrigerated meat products. This results in considerable losses to the meat industry. Meat spoilage is due to the production of lactic acid and off flavors. On the other hand, *L. curvatus* and *L. sake* appear to be of predominant importance in fermented meat products (10).

The taxonomic position of *L. curvatus* and *L. sake* has to date remained doubtful (5, 6, 19, 20). Differentiation between these two species is based on the fermentation patterns of five sugars (A. Kagermeier, Ph.D. thesis, Technical University, Munich, Federal Republic of Germany, 1981), of which melibiose and maltose are the most important (5, 6). Due to the variation in these patterns, differentiation between the two species is difficult and often arbitrary (5, 6, 21, 24). Because sugar fermentation of other lactic acid bacteria can be plasmid mediated (2, 7, 16, 17), this criterion cannot be regarded as a reliable means to distinguish between these organisms. *L. curvatus* and *L. sake* share 40 to 50% DNA homology, and they are not closely related to other *Lactobacillus* species (10).

To date, no DNA probes have been isolated for any *Lactobacillus* species. Such probes would be especially useful for the rapid identification and classification of the economically important representatives of this genus. Recombinant DNA technology has resulted in the development of sensitive techniques to identify bacteria.

DNA probes have been used for the identification of *Yersinia enterolitica* (9), *Salmonella typhi* (23), and *Bacillus subtilis* (11) as well as other bacterial species. In this paper we describe the isolation of a DNA probe for *L. curvatus*.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study are listed in Table 1.

DNA extraction. Cultures were grown in MRS broth (E. Merck AG, Darmstadt, Federal Republic of Germany) at 25 to 30°C. Ampicillin was added to cultures of *L. curvatus* during the late log phase, 1 h before harvesting, to a final concentration of 100 μg ml^{-1} to enhance protoplast formation in the presence of lysozyme. Bacteria were harvested by centrifugation, and the pellets were washed in saline-EDTA (0.15 M NaCl–0.01 M EDTA, pH 8.0) and suspended in a final concentration of 0.2× saline-EDTA after centrifugation. DNA was extracted as described by Marmur (14). Lysozyme (10 mg ml^{-1}) was added, and cultures were incubated at 37°C for 1 h. The saline-EDTA concentration was adjusted to 1×, sodium dodecyl sulfate was added to a final concentration of 1%, and the mixtures were incubated at 60°C for 1 h. DNA was phenol and chloroform extracted and ethanol precipitated, and the pellets were suspended in TE (10 mM Tris–1 mM EDTA, pH 8.0).

Genomic library construction. DNA for library construction (*L. curvatus* DSM 20019) was purified by CsCl-ethidium bromide density gradient centrifugation, dialyzed against TE, and ethanol precipitated (13). After *Eco*RI digestion at 37°C to yield fragments of 2 kilobases and smaller, this DNA was cloned into λ gt11 (Promega Biotec, Madison, Wis.). Ligations were performed at 16°C for 16 h, and the ligated DNA was in vitro packaged with commercially available packaging kits (Amersham Corp., Arlington Heights, Ill.).

Enzymes were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany, and were used in buffers according to the manufacturer's instructions. The library was amplified by plating the phages on *Escherichia coli* Y1090 and growing the organisms on LB plates containing 100 μg of ampicillin per ml as described by Huynh et al. (8). Recombinant phages were identified by growth on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside (8). The library was stored over chloroform at 4°C.

DNA-DNA hybridization. Plaque hybridization was essentially as described by Maniatis et al. (13). Phage dilutions were plated on *E. coli* Y1090, and the plaques were transferred to nitrocellulose. After prehybridization, filters were incubated with nick-translated DNA (22) and hybridized at 65°C for 16 h. Hybridization was in a solution containing 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.5% dextran sulfate, 0.5% sodium dodecyl sulfate, and 5× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] bovine serum albumin, 0.02% [wt/vol] ficoll 400, 0.02% [wt/vol] polyvinylpyrrolidone 40). Blots were washed twice in 2× SSC for 20 min at room temperature and then once in 0.1× SSC containing 0.1% sodium dodecyl sulfate at 65°C for 20 min. Autoradiography was at −70°C for at least 12 h with Kodak X-Omat R film and Du Pont Lightning Plus intensifying screens.

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TABLE 1. Bacterial strains

Organism	Strain	Source
E. coli	DSM 1328	DSM[a]
L. alimentarius	DSM 20181	DSM
L. bavaricus	83, 429	FMP[b]
L. brevis	DSM 20054	DSM
L. carnis	DSM 20624	DSM
L. curvatus	DSM 20019	DSM
L. curvatus	DSM 20010	DSM
L. curvatus	DSM 20496	DSM
L. curvatus	DSM 20499	DSM
L. curvatus	1704, 2321, 1674	SMP[c]
L. curvatus	2108, 1568, 1581, 2114	SMP
L. curvatus	453, 289	FMP
L. divergens	DSM 20623	DSM
L. divergens	60	FMP
L. farciminis	DSM 20184	DSM
L. plantarum	DSM 20205	DSM
L. sake	DSM 20017	DSM
L. sake	DSM 20497	DSM
L. sake	DSM 20498	DSM
L. sake	85, 86, 93, 441, 489	FMP
L. sake	2308, 2160, 1699	SMP
L. sake	1687, 2291	SMP
L. viridescens	DSM 20410	DSM

[a] DSM, German Collection of Microorganisms.
[b] FMP, Fermented meat products. The final identification was made by DNA-DNA hybridization (Kagermeier, Ph.D. thesis).
[c] SMP, Spoiled processed meat products. Classification into biogroups was according to variations in sugar fermentation patterns (10).

DNA from positive bacteriophage plaques was extracted by the rapid lysis method of Davis et al. (3). DNA was digested to completion with *Eco*RI, and fragments were separated by electrophoresis through 0.8% agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in 0.089 M Tris–0.089 M boric acid–0.002 M EDTA (pH 8.0). After Southern transfer (25) to nylon membranes, hybridization was performed as described above.

RESULTS

Library construction. A genomic library of *L. curvatus* DNA was constructed in the expression vector λ gt11. A titer of 2.5×10^{11} PFU ml^{-1} was obtained, of which 64% was recombinant phages.

Probe isolation. It was attempted to isolate an *L. curvatus*-specific DNA sequence by hybridizing total *L. sake* DNA to the *L. curvatus* genomic library. It was assumed that plaques which did not produce an autoradiographic signal after hybridization contained *L. curvatus*-specific sequences. Cross-hybridizing DNA between the two species would result in strong autoradiographic signals. Extensive hybridization was obtained between *L. sake* and *L. curvatus* DNA, illustrating the high degree of DNA homology between these two species (data not shown). Because of this it was not possible to identify areas on the membranes where no hybridization occurred, thus rendering this approach unsuitable for probe isolation. With this method no *L. curvatus*-specific sequence was isolated.

Subsequently we attempted to isolate a probe by using a technique which detects repetitive DNA sequences (15). Depending on the number of repeats present, various intensities of hybridization signal can be obtained. This technique has been successfully used in the isolation of a *Plasmodium falciparum* probe (1). Dilutions of the *L. curvatus* library were plated, blotted onto nitrocellulose, and probed with total labeled *L. curvatus* DNA. Seventy-four clones giving a strong hybridization signal were isolated.

DNA was extracted from all of these clones and blotted onto nylon membranes for hybridization with *L. sake* and *L. curvatus* DNA. When total *L. sake* DNA was hybridized to membranes containing DNA from these clones, three of the clones did not hybridize. These three clones were isolated and termed Lc24, Lc45, and Lc47. However, when Lc24, Lc45, and Lc47 DNA was hybridized to blots of digested *L. sake* DNA, different degrees of hybridization were obtained with Lc24 and Lc47. No hybridization was obtained with Lc45 on DNA from *L. sake* DSM 20017 (Fig. 1A, lane 3). This clone hybridized strongly to *L. curvatus* DSM 20019 DNA (Fig. 1A, lane 4). The hybridization pattern obtained suggests that the insert in Lc45 contains a repetitive element. Hybridization to λ (Fig. 1A, lane 1) and *E. coli* (Fig. 1A, lane 5) was expected, because Lc45 DNA contains sequences homologous to those of these organisms (LacZ and λ DNA). The size of Lc45 was found to be 1.2 kilobases (data not shown).

To determine the extent of possible cross-hybridization between λ gt11, *L. sake*, and *L. curvatus*, λ gt11 DNA was labeled and hybridized to blots of digested *Lactobacillus* DNA. No hybridization to *L. sake* DNA was obtained (Fig. 1B, lane 3), but a low level of background hybridization was obtained with *L. curvatus* DNA (Fig. 1B, lane 4).

Lc45 was tested for its ability to differentiate between *L. sake* and *L. curvatus* strains isolated from processed meat. Lc45 DNA was also hybridized to a number of *Lactobacillus* isolates classified into different species according to sugar fermentation reactions. This probe detected *L. curvatus* DNA (Fig. 2, lanes 9 through 15; Fig. 3, lanes 8 through 12) as well as DNA from organisms classified as *L. sake* (Fig. 2, lanes 16 to 20) and distinctly related lactobacilli (Fig. 2, lanes 1 through 8).

The intensity of hybridization of Lc45 to *L. curvatus* 2114 (Fig. 2, lane 15) was considerably lower than that obtained with the other *L. curvatus* strains. No hybridization was obtained with a strain originally classified as *L. curvatus* 2108 (Fig. 2, lane 12). However, various degrees of hybridization were obtained with strains classified as *Lactobacillus plantarum* DSM 20205 (Fig. 2, lane 2), *L. viridescens* DSM 20410 (Fig. 2, lane 4), and *L. farciminis* DSM 20184 (Fig. 2,

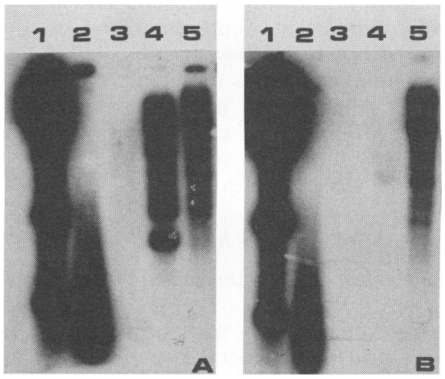

FIG. 1. Hybridization to Southern blots of restriction endonuclease-digested λ, *E. coli*, and *Lactobacillus* DNA. (A) Hybridization of Lc45 DNA. Lanes: 1, *Hin*dIII-digested λ; 2, Lc45; 3, *L. sake*; 4, *L. curvatus*; 5, *E. coli*. DNA in lanes 2 to 5 was digested with *Eco*RI. (B) Hybridization of λ gt11 DNA. Lanes 1 to 5 were loaded as in panel A. Autoradiography was for 12 h at −70°C with an intensifying screen.

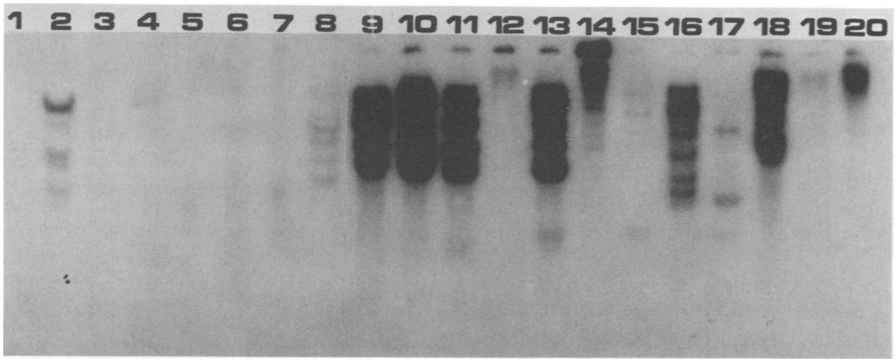

FIG. 2. Hybridization of Lc45 DNA to a Southern blot of *Eco*RI-digested DNA from different lactobacilli. Lanes: 1, *L. brevis*; 2, *L. plantarum*; 3, *L. alimentarius*; 4, *L. viridescens*; 5 and 6, *L. divergens* 60 and DSM 20623, respectively; 7, *L. carnis* DSM 20624; 8, *L. farciminis*; 9 to 15, *L. curvatus* 1704, 2321, 1674, 2108, 1568, 1581, and 2114, respectively; 16 to 20, *L. sake* 2308, 2160, 1699, 1687, and 2291, respectively. Electrophoresis and hybridization were as described in the text. The blot was autoradiographed for 12 h at −70°C with one intensifying screen.

lane 8). No hybridization was obtained with Lc45 on *L. divergens* DSM 20623 and 60 (Fig. 2, lanes 5 and 6), *L. carnis* DSM 20624 (Fig. 2, lane 7), and *L. alimentarius* DSM 20181 (Fig. 2, lane 3). When Lc45 was hybridized to DNA from other isolates of *L. sake* and *L. curvatus* from the German Collection of Microorganisms (DSM strains), hybridization was obtained to one authentic strain of *L. sake* (DSM 20489) (Fig. 3, lane 2). Low-intensity bands were obtained with two other *L. sake* strains (Fig. 3, lanes 5 and 6) as well as to a *L. bavaricus* isolate (Fig. 3, lane 14). Strong hybridization was obtained with *L. curvatus* DSM 20010, 20496, and 20499 (Fig. 3, lanes 8, 11, and 12), whereas Lc45 hybridized weakly to *L. curvatus* isolates 453 and 289 (Fig. 3, lanes 9 and 10).

DISCUSSION

The organisms used in this study were classified as *L. plantarum*, *L. divergens*, *L. viridescens*, *L. alimentarius*, *L. brevis*, *L. farciminis*, *L. carnis*, *L. sake*, and *L. curvatus* by using sugar fermentation patterns and other conventional physiological criteria (10).

Problems with the classification of this genus, especially of *L. sake* and *L. curvatus*, have been well documented (5, 6, 10, 19, 20, 21, 24). Another complicating factor in classifying these two species is the relatively high DNA homology of 40 to 50%. Definitive identification is therefore dependent on a number of time-consuming tests.

At present no methods are available to distinguish between *L. sake*, *L. curvatus*, *L. bavaricus*, and other lactobacilli on an accurate, rapid, and reliable basis. DNA probes have been used successfully for the rapid identification of a number of bacterial taxons (12, 18). Probes have proved to be highly specific and suitable for detecting small quantities of DNA in routine applications. The probe described in this paper is specific for *L. curvatus* DNA. Lc45 did not hybridize to DNA from *L. sake* type culture DSM 20017 or to a number of other *L. sake* isolates. Results obtained with this probe emphasize existing doubts as to the accuracy of current criteria used to classify these organisms. Lc45 did not hybridize to a strain classified physiologically as *L. curvatus* (strain 2108), suggesting that this strain may not be *L. curvatus*. On the other hand Lc45 detected DNA from strains 2308, 2160, 1699, and 2291 classified as *L. sake*. All of the above strains were classified according to conventional techniques (21). Hybridization was also obtained with iso-

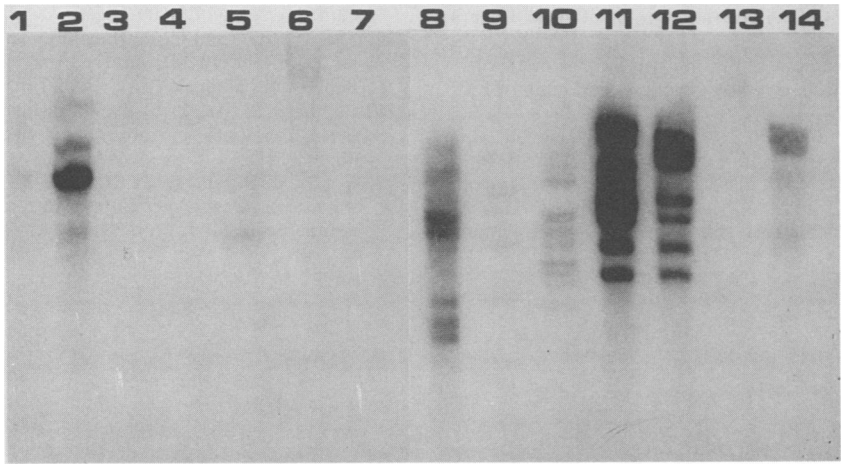

FIG. 3. Hybridization of Lc45 DNA to a Southern blot of restriction endonuclease-digested *L. sake*, *L. curvatus*, and *L. bavaricus* DNA. Lanes: 1, *L. sake* DSM 20497; 2, *L. sake* DSM 20498; 3 to 7, *L. sake* 85, 86, 93, 441, and 489, respectively; 8, *L. curvatus* DSM 20010; 9 and 10, *L. curvatus* 453 and 289, respectively; 11 and 12, *L. curvatus* DSM 20496 and 20499, respectively; 13 and 14, *L. bavaricus* 83 and 429, respectively. Hybridization was as described in Materials and Methods. Autoradiography was for 12 h at −70°C.

lates classified as *L. plantarum*, *L. viridescens*, and *L. farciminis*.

This probe did not detect DNA from *L. brevis*, *L. alimentarius*, two strains of *L. divergens*, and one strain of *L. carnis*, suggesting that these are indeed different species not related to *L. curvatus*.

The possibility that Lc45 is a repetitive probe needs to be investigated. A repetitive DNA probe would have certain advantages, including an increased sensitivity of detection and species specificity.

Repetitive DNA can be detected by standard hybridization techniques with a sensitivity only slightly less than that needed to detect genomic DNA. This has allowed the detection of as little as 25 pg of *P. falciparum* DNA, corresponding to 50 μl of a 0.001% parasitemic blood (1).

Libraries containing short sequences of DNA have been used routinely to detect organisms present in low concentrations below the detectability of conventional techniques. The length of Lc45 is 1.2 kilobases. Because of the specificity of Lc45 for *L. curvatus* DNA, we intend to identify short sequences suitable for synthetic repetitive probes as has been done for *P. falciparum* (4). A methodology based on this probe suitable for the rapid identification and differentiation between *Lactobacillus* species in the processed meat industry is currently being developed.

LITERATURE CITED

1. **Barker, R. H., Jr., L. Suebsaeng, W. Rooney, G. C. Alecrim, H. V. Dourado, and D. F. Wirth.** 1986. Specific DNA probe for the diagnosis of *Plasmodium falciparum* malaria. Science **321:** 1434–1436.
2. **Chassy, B. M., E. M. Gibson, and A. Giuffrida.** 1978. Evidence for plasmid-associated lactose metabolism in *Lactobacillus casei* subsp. *casei*. Curr. Microbiol. **1:**141–144.
3. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Franzen, L., R. Shabo, H. Perlman, H. Wigzell, G. Westin, L. Aslund, T. Persson, and U. Petterson.** 1984. Analysis of clinical specimens by hybridization with probe containing repetitive DNA from *Plasmodium falciparum*. A novel approach to malaria diagnosis. Lancet **i:**525–528.
5. **Hastings, J. W., and W. H. Holzapfel.** 1987. Numerical taxonomy of lactobacilli surviving radurization of meat. Int. J. Food Microbiol. **4:**33–49.
6. **Hastings, J. W., and W. H. Holzapfel.** 1987. Conventional taxonomy of lactobacilli surviving radurization of meat. J. Appl. Bacteriol. **62:**209–216.
7. **Hofer, F.** 1977. Involvement of plasmids in lactose metabolism in *Lactobacillus casei* suggested by genetic experiments. FEMS Microbiol. Lett. **1:**167–170.
8. **Huynh, T. V., R. A. Young, and R. W. Davis.** 1985. Constructing and screening cDNA libraries in λ gt10 and λ gt11, p. 49–78. *In* D. M. Glover (ed.), DNA cloning, vol. 1. A practical approach. IRL Press, Arlington, Va.
9. **Jagow, J., and W. E. Hill.** 1986. Enumeration by DNA colony hybridization of virulent *Yersinia enterocolitica* colonies in artificially contaminated food. Appl. Environ. Microbiol. **51:** 441–443.
10. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing Gram-positive rods, p. 1208. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergy's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
11. **Krauss, J., W. Ludwig, and K. H. Schleifer.** 1986. A cloned 23S rRNA gene fragment of *Bacillus subtilis* and its use as a hybridization probe of conserved character. FEMS Microbiol. Lett. **33:**89–93.
12. **Kuritza, A. P., C. E. Getty, P. Shaughnessy, R. Hesse, and A. A. Salyers.** 1986. DNA probes for identification of clinically important *Bacteroides* species. J. Clin. Microbiol. **23:**343–349.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Marmur, J.** 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. **3:**208–218.
15. **McPheat, W. L., and T. McNally.** 1987. Isolation of a repeated DNA sequence from *Bordetella pertussis*. J. Gen. Microbiol. **133:**323–330.
16. **Morelli, L., M. Vescovo, and V. Bottazzi.** 1982. Characterization of plasmid DNA molecules in *L. acidophilus* strain D137. Ann. Microbiol. **32:**99–106.
17. **Nes, I. F.** 1984. Plasmid profiles of ten strains of *Lactobacillus plantarum*. FEMS Microbiol. Lett. **21:**359–361.
18. **Nur, I., J. M. Bove, C. Saillard, S. Rottem, R. M. Whitcomb, and S. Razin.** 1986. DNA probes in detection of spiroplasmas and mycoplasma-like organisms in plants and insects. FEMS Microbiol. Lett. **35:**157–162.
19. **Reuter, G.** 1970. Lactobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischerzeugnissen. Fleischwirtschaft **50:**954–962, 1397–1399.
20. **Reuter, G.** 1975. Classification problems, ecology and some biochemical activities of lactobacilli of meat products, p. 221–229. *In* J. C. Carr, C. V. Cutting, and G. C. Whiting (ed.), Lactic acid bacteria in beverages and foods. Academic Press, Inc., New York.
21. **Reuter, G.** 1981. Psychrotrophic lactobacilli in meat products, p. 253–258. *In* J. H. B. Christian, G. Hobbs, T. A. Roberts, and N. Skovgaard (ed.), Psychrotrophic microorganisms in spoilage and pathogenicity. Academic Press, Inc., New York.
22. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase 1. J. Mol. Biol. **113:** 237–251.
23. **Ruben, F. A., D. J. Kopecko, K. F. Noon, and L. S. Baron.** 1985. Development of a DNA probe to detect *Salmonella typhi*. J. Clin. Microbiol. **22:**600–605.
24. **Shaw, B. G., and C. D. Harding.** 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb, and bacon. J. Appl. Bacteriol. **56:**25–40.
25. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
26. **Wirth, D. F., and D. Pratt.** 1982. Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proc. Natl. Acad. Sci. USA **79:**6999–7003.