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Lactobacillus Succession in the Piglet Digestive Tract Demonstrated by Plasmid Profiling

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Plasmid profiling was used to distinguish strains of lactobacilli inhabiting the digestive tract of piglets and the feces of sows. Fifteen plasmid profile types were detected among 328 isolates of lactobacilli. Plasmid profiling of lactobacilli permitted the following conclusions to be made: the maternal feces were a major source of lactobacilli colonizing the piglet digestive tract; the lactobacillus population of the gastric region of the piglet digestive tract was composed of lactobacillus strains different from those present in the rectal population; and a lactobacillus succession was observed in the digestive tract of piglets drawn from a single litter, and one plasmid profile type became dominant in the gastric region of these animals.

Lactobacilli are numerous in the digestive tract of pigs, constituting one of the principal bacterial groups inhabiting proximal regions of the tract (1). Some of these lactobacilli have the ability to colonize the epithelial surface lining the porcine esophagus and part of the stomach (the pars oesophagea), forming relatively thick bacterial layers (5, 13).

Observations made in a recent study (10) involving colonization of the digestive tract of piglets by specific strains of lactobacilli have raised the possibility that a succession of lactobacillus strains colonizes the piglet gastrointestinal tract during the first weeks of life. Culture and microscope investigations of digestive tract specimens demonstrated apparent qualitative and quantitative changes in lactobacillus colony and cell morphotypes in piglets of different ages. Confirmation of the occurrence of a biological succession involving lactobacillus types in the piglet digestive tract requires the use of a technique by which individual strains of lactobacilli, even within a species, can be distinguished. Plasmid profiling has been shown to be a useful technique to distinguish among strains of bacteria in medical, industrial, and environmental studies (2–4, 7). We have used this technique to demonstrate a lactobacillus succession occurring in the digestive tract of piglets.

MATERIALS AND METHODS

Porcine samples. The study was divided into two parts. In the first part, lactobacilli were cultured from esophagus washings, pars oesophagea washings, and stomach contents collected from four 1-day-old piglets and from the feces of their respective dams collected 1 day prior to farrowing. The sows had been held in separate stalls at the Institute for Grassland and Animal Production's Church Farm, Reading, United Kingdom, throughout gestation and were moved to farrowing stalls 2 to 3 days prior to the birth of the piglets. The pigs (crossbred Large White-Landrace) were fed a barley-soybean meal-based diet. Piglets had their teeth clipped and received an iron injection during the first day of life. The males were not castrated, and sows and piglets did not receive medication before or during the experimental period. Prophylactic use of antibiotics is not carried out at Church Farm. In the second part of this study, lactobacilli were cultured from pars oesophagea washings, stomach contents, and rectal contents of eight piglets drawn from a single litter. Two piglets (killed by intracardiac injection of pentobarbital) were examined at 1 day of age, and two each were examined at 4, 7, and 14 days. Lactobacilli were also cultured from maternal feces collected 1 day prior to farrowing.

Culture examination of specimens. Esophageal and pars oesophagea samples were rinsed with sterile distilled water and shaken in an additional sample of water containing glass beads (11). Stomach and rectal contents were suspended in sterile water to give a 1:10 (wt/vol) dilution. Epithelial, stomach, and rectal suspensions were diluted further in 10-fold steps in sterile distilled water, and samples of each dilution were spread-plated onto Rogosa agar (Oxoid Ltd., Basingstoke, United Kingdom) plates. After incubation at 37°C anaerobically for 48 h, lactobacilli were enumerated and 20 colonies were subcultured from the highest dilution of sample giving lactobacillus growth. The lactobacillus subcultures were stored in sterile milk at −20°C.

Plasmid profiling. Extracts of DNA were prepared from lactobacillus isolates as follows. (i) A 30-ml volume of MRS broth (Oxoid Ltd.), prewarmed and prereduced at 37°C, was inoculated with 1 ml of stationary-phase cells cultured in the same medium. (ii) The culture was incubated anaerobically at 37°C for 5 to 6 h until the lactobacilli were in the logarithmic phase of growth. (iii) The cells were harvested by centrifugation and washed in 10 ml of 0.01 M Tris buffer (pH 8.2), and the cells were suspended in 2.6 ml of the same buffer. (iv) The cells were lysed by the addition of 1 ml of lysozyme solution (6 mg/ml) and incubation for 25 min at 37°C, followed by the addition of 0.4 ml of sodium lauryl sulfate solution (10% solution in TE buffer [8]). (v) Chromosomal DNA was denatured by the addition of 160 μl of 3 N sodium hydroxide solution and gentle rocking of the preparations for 3 min. The preparations were neutralized by the addition of 1.5 ml of 2 M Tris buffer (pH 7.2). (vi) Removal of membrane-chromosome complexes was achieved by the addition of 0.95 ml of 20% sodium lauryl sulfate in TE buffer, followed immediately by 1.65 ml of 5 M sodium chloride solution. The lysates were left on ice at 4°C overnight. The

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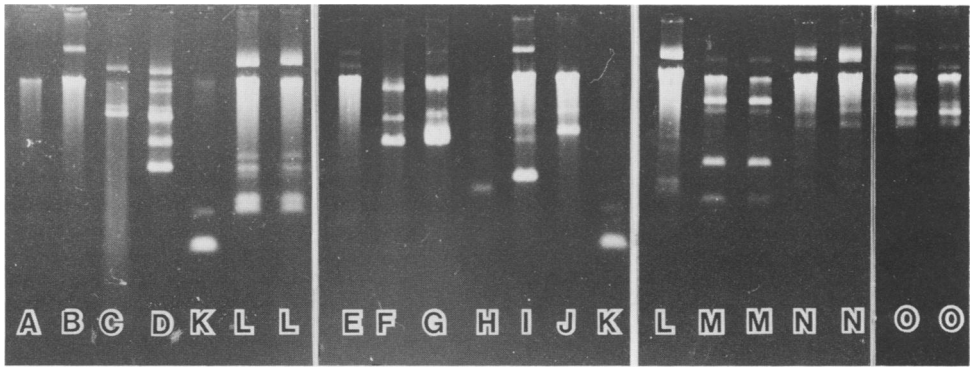

FIG. 1. Plasmid profiles of lactobacilli isolated from porcine specimens. Profile types are labeled alphabetically.

preparations were centrifuged at 27,000 × *g* for 30 minutes at 5°C, and the clear supernatant was retained. (vii) Supernatants were incubated at 37°C after the addition of 0.4 ml of RNase solution (2 mg/ml), and proteins were then removed by the addition of 8 ml of phenol saturated with TES buffer (8) and centrifugation at 9,750 × *g* for 30 min. Two extractions were then made with chloroform-isoamyl alcohol (24:1) with centrifugation at 1,935 × *g* for 5 min. (viii) DNA was precipitated by the addition of 0.4 ml of 3 M sodium acetate solution and 16 ml of cold (−20°C) ethanol. The preparations were held at −20°C overnight. (ix) The precipitated DNA was collected by centrifugation at 27,000 × *g* for 30 min at −10°C and dissolved in 100 μl of TE buffer. (x) The DNA was concentrated by using the Geneclean kit method and the manufacturer's instructions (BIO 101 Inc., La Jolla, Calif.). Electrophoresis was carried out with 0.7% agarose gels and Tris-borate buffer (8) in a GNA-100 gel electrophoresis apparatus (Pharmacia, Uppsala, Sweden) at 30 mA for 2 h.

In the first part of the study, plasmid profiles were obtained for 128 strains of lactobacilli isolated from sow feces and piglet digestive tract. In the second part of the study, plasmid profiles for 200 strains of lactobacilli isolated from piglet digestive tract samples and sow feces were determined.

Plasmid profiles of lactobacilli isolated from porcine samples were stable when the bacteria were stored at −20°C: repeat DNA extractions of lactobacillus isolates made at intervals during a 12-month storage period gave plasmid profiles identical to those obtained when they were first isolated and tested.

Identification to species of lactobacillus isolates. Representative lactobacillus strains belonging to each plasmid profile group and 62 plasmid-free isolates (profile type A) were identified to species, using API 50CH strips and following the manufacturer's instructions (API System, La Balme les Grottes, France).

In vitro adherence assay. Representative strains of each of the plasmid profile groups colonizing the pars oesophagea of the animals drawn from a single litter (second part of the study) were tested for their ability to adhere to pars oesophagea epithelial cells collected from neonatal pigs. The piglets used as a source of epithelial cells had not suckled the dam, and the cells were therefore free of adhering lactobacilli (10). The adherence assay was carried out as described previously (10), and semiquantitative observations comparing the strains were recorded: 20 to 50 lactobacillus cells adhering per epithelial cell (+++), 10 to 19 lactobacilli per epithelial cell (++), 5 to 9 lactobacilli per epithelial cell (+), and absence of adherent lactobacillus cells (−).

Growth rates of lactobacillus strains. To compare the growth rates of lactobacillus plasmid profile types, representative strains colonizing the pars oesophagea of the animals drawn from a single litter were examined as follows. Volumes, 50 ml, of MRS broth, prewarmed and prereduced at 37°C in an anaerobic glovebox, were inoculated with stationary-growth-phase cells. Aliquots, 1 ml, of culture were removed at intervals for determination of the A_{600} of light (CE292 digital spectrophotometer; Cecil Instruments, Cambridge, England). Growth rates for the strains were expressed as the slope (from regression curve equation) of the linear part of the growth curve obtained by plotting absorbance values against time of incubation.

Cell mass of lactobacillus cultures. To compare the cell mass produced by lactobacillus plasmid profile types from a finite amount of nutrients, representative strains colonizing the pars oesophagea of the animals drawn from a single litter were used to inoculate 10-ml volumes of MRS broth that were incubated at 37°C for 24 h under anaerobic conditions. Lactobacillus cells, harvested by centrifugation, were lyophilized and then weighed to obtain the dry weight of cells produced by each strain.

Examination of strains for inhibitor production. Repre-

TABLE 1. Plasmid profile types of lactobacilli detected in sow feces and piglet (1 day of age) digestive tract samples

Litter	Specimen	Plasmid profile types detected[a]
1	Sow feces	1A, 1B, 2C, 2D, 1E, 1G
	Piglet esophagus	3A, 1B, 1F, 1H, 1I, 1J
	Piglet pars oesophagea	7A, 1B
	Piglet stomach contents	2A, 4B, 2C
2	Sow feces	5A, 1C, 1D, 1K
	Piglet esophagus	3A, 4B, 1E
	Piglet pars oesophagea	6B, 1D, 1F
	Piglet stomach contents	4A, 3B, 1E
3	Sow feces	2A, 1B, 4C, 1D
	Piglet esophagus	8A
	Piglet pars oesophagea	2A, 4B, 1D, 1H
	Piglet stomach contents	4A, 1B, 1C, 1D, 1E
4	Sow feces	2A, 1B, 4C, 1D
	Piglet esophagus	1A, 2B, 1C, 3D, 1H
	Piglet pars oesophagea	1A, 3C, 1D, 1G, 2I
	Piglet stomach contents	1A, 1B, 3C, 1D, 2E

[a] Number of isolates (of eight) belonging to each profile type cultured from the specimen.

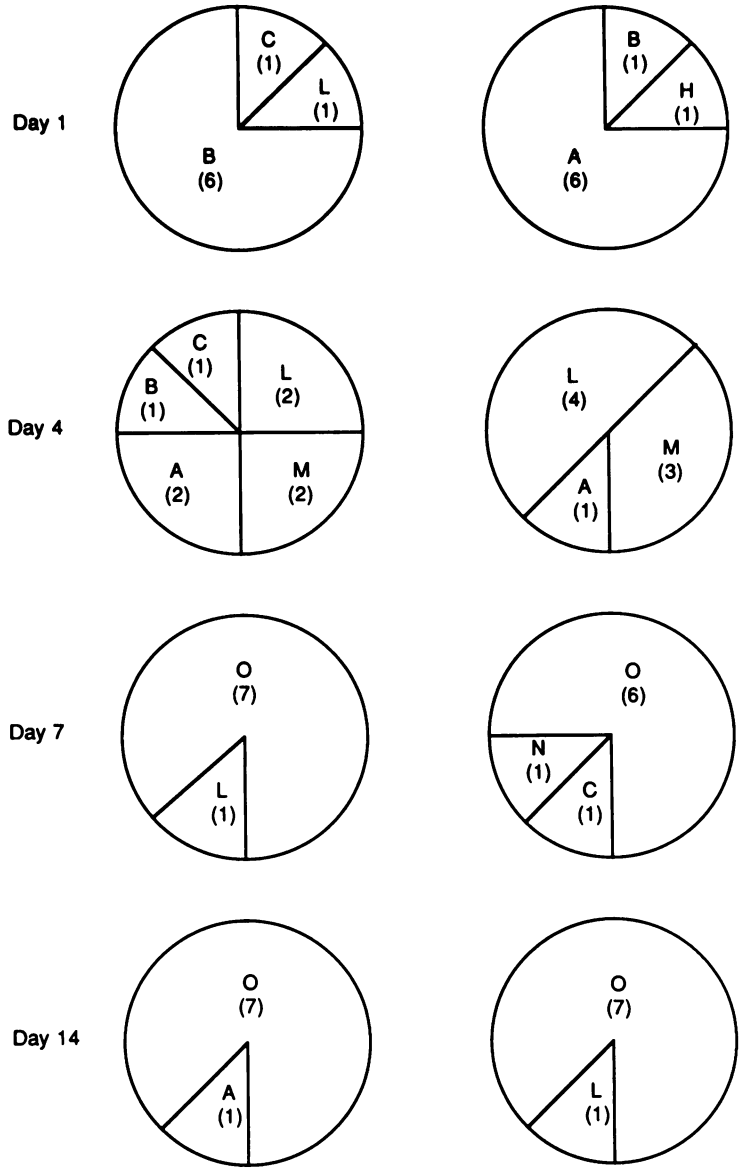

FIG. 2. Plasmid profile types as proportions of the total lactobacillus population inhabiting the pars oesophagea surface in the piglet stomach. Each pie chart represents eight lactobacillus isolates from a piglet. The letters indicate a plasmid profile type, and the numbers in parentheses give the number of isolates belonging to that plasmid profile type. Two piglets were examined at 1, 4, 7, and 14 days after birth.

sentative strains of each plasmid profile group colonizing the pars oesophagea of the animals drawn from a single litter were tested for the production of substances inhibitory to members of other profile groups. Lactobacilli to be tested as producers of inhibitors were stabbed into MRS agar plates and incubated anaerobically for 48 h, and the plates were overlaid with 5 ml of MRS medium containing 0.65% agar and to which had been added 100 μl of broth culture of the strain to be tested for sensitivity to inhibitor production. The plates were examined after a further 24-h incubation to observe zones of inhibition surrounding the strains stabbed into the agar. Zones with sharply demarcated edges indicate the production of inhibitors other than lactic acid. Inhibitor production tests were carried out with MRS medium adjusted to give a range of pH values (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) since inhibitor production by lactobacilli is known to be influenced by culture pH (9).

RESULTS

Lactobacillus populations in porcine samples. Sow feces contained 10^8 to 10^9 lactobacilli per g (wet weight). Esophageal washings contained about 10^6 lactobacilli per ml. The lactobacillus population associated with the pars oesophagea increased as the piglets grew older: 10^7 per ml of washings at day 1, and 10^8 per ml thereafter. Piglet stomach and rectal populations of lactobacilli showed a similar trend: 10^7 per g of contents at day 1, and 10^9 per g in later samples.

Comparison of plasmid profile types in sow feces and piglet digestive tract. Examination of 128 lactobacillus strains isolated from sow feces and the digestive tract of 1-day-old piglets born to these sows detected 11 profile types subsequently designated A to K (Fig. 1). Although 24% of the strains were plasmid-free (profile type A), plasmid profiling was found to distinguish between strains of lactobacilli

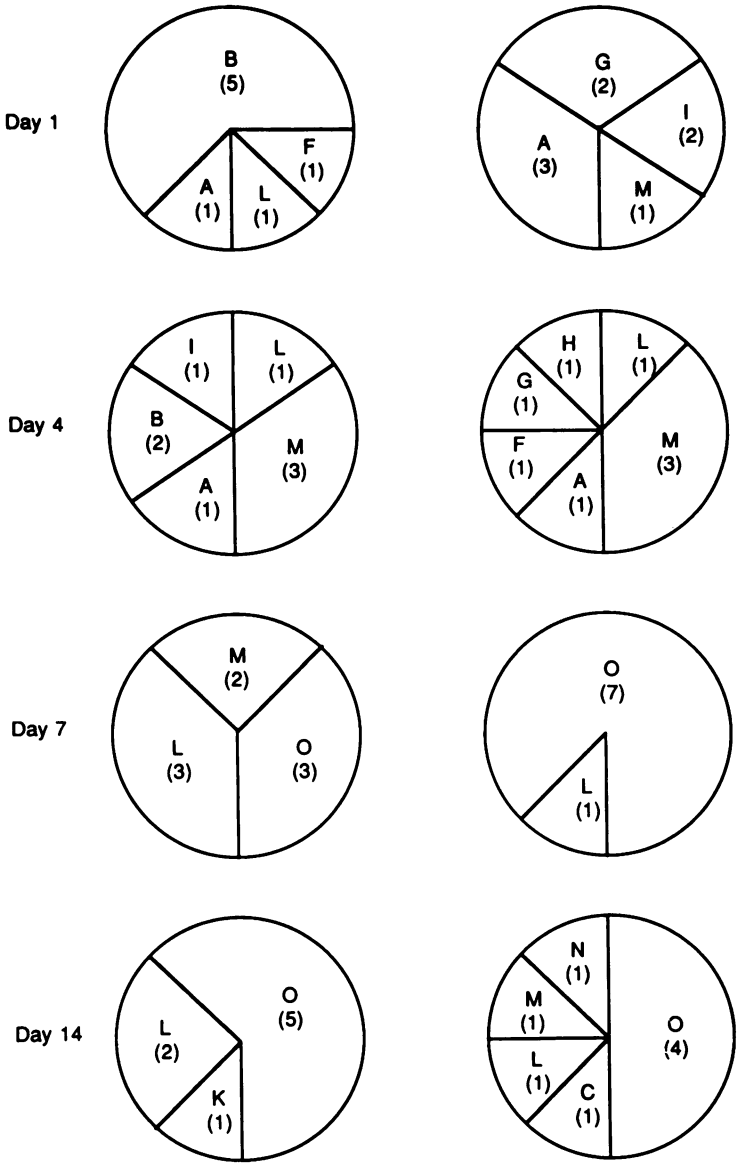

FIG. 3. Plasmid profile types as proportions of the total lactobacillus population inhabiting the stomach contents of the piglet stomach. Other details are as in the legend to Fig. 2.

within a species. Thus, plasmid profile types C, F, G, and K were *Lactobacillus acidophilus*; types B, E, H, I, and J, were *L. fermentum*; and type D was *L. delbrueckii*. Plasmid-free strains (plasmid profile type A) were *L. acidophilus*, *L. fermentum*, *L. brevis*, and *L. crispatus*. Plasmid profiling also revealed that at least some of the lactobacillus types were common to both the sows' feces and the piglet digestive tract, suggesting that one was the source of the other (Table 1).

Plasmid profile B was the only lactobacillus type common to all four piglets examined in this part of the study (Table 1). The profile types detected in esophageal and pars oesophagea samples were also detected in stomach contents in keeping with the hypothesis that epithelium-associated lactobacillus populations serve as inoculum for the gastric contents (5).

Demonstration of a lactobacillus succession in a litter of piglets. Plasmid profiling discriminated between strains of lactobacilli inhabiting the digestive tract (pars oesophagea, stomach contents, and rectal contents) of piglets, of the same litter, sampled at 1, 4, 7, and 14 days after birth. Twelve plasmid profile types were detected among 192 strains tested. Profile types D, E, and J were not detected in this litter, but additional profile types (L, M, N, and O) were present (Fig. 1). Types M, N, and O were *L. acidophilus* and type L was *L. fermentum*. A succession of lactobacillus strains was shown to colonize each of the three digestive tract sites that were examined as the piglets grew older (Fig. 2, 3, and 4). The succession was particularly striking when the lactobacillus population of the pars oesophagea was examined (Fig. 2). The lactobacillus population inhabiting this site was quite different between the two piglets examined at 1 day of age. At 4 days, however, the two piglets examined showed similarity in the plasmid profile types present (types A, L, and M). By 7 days after birth, a radical change in the lactobacillus population had occurred since

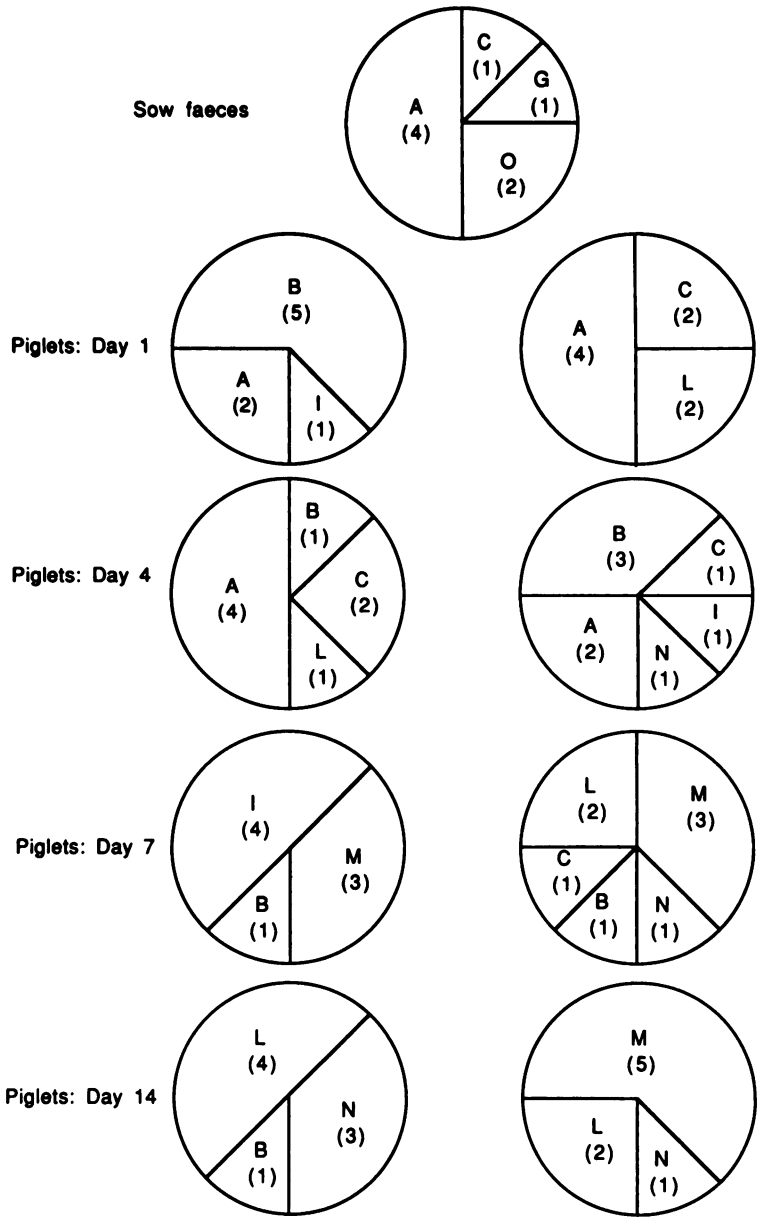

FIG. 4. Plasmid profile types as proportions of the total lactobacillus population inhabiting the rectal contents of piglets. Other details are as in the legend to Fig. 2. Plasmid profile types detected in maternal feces are also shown.

profile type O was now the numerically dominant type in both animals examined. The population remained stable at least until day 14, when the majority of the lactobacillus isolates still belonged to profile type O (Fig. 2). In general, the lactobacillus population of the stomach contents reflected that of the pars oesophagea, although it was somewhat more diverse in lactobacillus components (Fig. 3). The most numerous lactobacillus plasmid profile types in the rectal contents were not the same as those dominating the gastric region: type O strains were not detected in rectal contents at days 7 and 14, though these were the numerically dominant bacteria on the pars oesophagea at this time (Fig. 2 and 4).

Comparison of plasmid profile types participating in the lactobacillus succession. Comparisons of growth rate, bacterial cell mass after 24-h culture, and adhering ability were made between representative strains of each of the profile types colonizing the pars oesophagea of the piglets drawn from a single litter. These comparisons were made to attempt to identify characteristics of lactobacillus plasmid profile type O that enabled this strain to become dominant in the pars oesophageal habitat. The results of these experiments are given in Table 2. In comparison to other profile types, the type O strain adhered well to epithelial cells in vitro (ranking first equal with strain N), grew rapidly in broth medium (ranking third of seven strains tested), and produced dense turbidity in broth (cell mass from 24-h culture ranking second). The profile type N strain adhered well to epithelial cells (ranking first equal) but grew more slowly (ranking fifth) than the type O strain in broth medium and produced less cell mass (ranking fourth) than strain O. The profile type H strain grew the fastest of any of the seven strains tested but

TABLE 2. Comparison of cell mass, growth rate, and adhering ability of representative strains of plasmid profile groups

Profile type[a]	Cell mass (mg, dry wt)[b]	Growth rate[c]	Adherence[d]
B	22.6	0.362	−
C	28.8	0.342	−
H	26.1	0.410	−
L	26.5	0.408	++
M	15.4	0.160	+
N	26.1	0.348	+++
O	27.8	0.381	+++

[a] Profile type A, a heterogeneous group of plasmid-free isolates, was not included.

[b] Dry weight of lactobacillus cells harvested from 10 ml of MRS broth after 24 h of incubation. Mean of duplicate cultures.

[c] Growth rate expressed as the slope of the regression curve obtained when absorbance values from the logarithmic growth phase of cultures were plotted against time of incubation. Mean of duplicate cultures.

[d] Adherence of lactobacillus cells to epithelial cells harvested from piglet pars oesophagea. +++, 20 to 50 bacteria per epithelial cell; ++, 10 to 19 bacteria per cell; +, 5 to 9 bacteria per cell; −, absence of adherent bacteria.

did not adhere in vitro to epithelial cells and produced a cell mass similar to that of strain N. The profile type C strain produced the largest cell mass when cultured in broth medium but grew slowly (ranking sixth) and did not adhere to epithelial cells. All of the profile types that assumed prominence at some stage of the succession on the pars oesophageal surface (L, M, N, and O) had some ability to adhere to epithelial cells in vitro.

None of the representative strains produced substances that inhibited the growth of the other profile types when tested under in vitro conditions.

DISCUSSION

Plasmid profiling of lactobacillus isolates allowed discrimination between strains inhabiting the porcine digestive tract. Most of the plasmid profile types detected in piglet digestive tract samples were also detected in the feces of sows in the piggery. Thus, maternal feces are a major source of lactobacilli colonizing the neonatal piglet and probably contribute to a "piggery microflora" that contaminates the young animals soon after birth. Plasmid profile types F, H, I, and J were only detected in piglet specimens. These types may have been minor numbers of the total lactobacillus population of sow feces and therefore not detected in our study because only numerically dominant strains were examined.

The collection of profile types present in the gastric region of the piglets drawn from a single litter was different from that inhabiting the rectal contents. The difference was so marked that culture examination of the rectal contents or feces would not have provided information about the lactobacillus population of the proximal digestive tract. Savage (12) has theorized that examination of the feces of an animal provides little useful ecological information about the digestive tract. The detection of different lactobacillus collections in the gastric and rectal regions of the piglet digestive tract reinforces this point.

Profile type O became the dominant lactobacillus strain inhabiting the pars oesophagea at 7 days after the birth of the piglets in which a lactobacillus succession was observed. We do not known what bacterial characteristics determine whether a lactobacillus strain will be a successful colonizer of the digestive tract ecosystem. The in vitro tests (growth rate, cell mass, and adherence) that we carried out to compare the profile types were an attempt to obtain information on possible colonization factors. Although in vitro experiments can give misleading results as far as predicting the outcome of bacterial interactions in a natural ecosystem is concerned (6), we believe that plasmid profile type O dominated the pars oesophageal habitat for the following reasons. In comparison with other lactobacillus strains, it had the potential to grow rapidly under optimal conditions (as evidenced by its growth in MRS medium) and could thus outnumber competing strains, was efficient in converting nutrients to cellular components (cell mass), and could adhere well to epithelial cells, a prerequisite to colonization of the pars oesophagea surface. Pedersen and Tannock (10) observed in experiments in which specific strains of lactobacilli were used to inoculate newborn piglets that, as long as the strain used as inoculum continued to colonize the esophageal or pars oesophageal surface, the bacteria persisted in the digestive tract, albeit in low numbers.

Inhibitory substances were not produced by the lactobacillus strains under the in vitro conditions that we utilized. We assume, therefore, that they were not involved in the lactobacillus succession occurring on the pars oesphageal epithelial surface.

The lactobacillus succession we observed in the piglets explains why Pedersen and Tannock (10) were unable to establish specific lactobacillus strains in the digestive tracts of newborn piglets by dosing the animals with bacteria on a single occasion. Although all of the lactobacillus strains were able to colonize the digestive tract for the first few days of life, they were supplanted by other lactobacilli better adapted to life in the changing gastrointestinal ecosystem of the developing piglet. This phenomenon is part of the natural biological succession in the piglet digestive tract that we have demonstrated by plasmid profiling of lactobacillus isolates.

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