IDENTIFICATION OF SOME LACTIC ACID BACTERIA FROM CO_2 PACKED MEAT BY 23S rRNA-TARGETED OLIGO NUCLEOTIDE PROBES

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INTRODUCTION

Storage of raw, cured or fermented meat and meat products in atmospheres of CO_2, CO_2/O_2 or in vacuum packs leads to a flora dominated to a greater or lesser extent by lactic acid bacteria (LAB). To evaluate the relative importance of different LAB to storage and/or fermentation processes and to determine the microbial consequences of changes in product formulation, processing parameters etc., it is important to have rapid, reliable, easy to perform identification tests. Present tests based on morphological and fermentation properties are time consuming and require experience in interpretation with small changes in medium composition and or growth conditions giving equivocal results. For example the ability to produce CO_2 in MRS (de Man, Rogosa and Sharpe) medium, is, in our hands, dependent on the brand of peptone used; while in Gibsons medium 3 weeks of incubation is recommended. The diagnostic test, production of NH_3 from arginine, may differ with the glucose concentration used (INGRAM, 1975). Furthermore, in newly isolated strains a high percent of deviation from the fermentation tests in the identification key in Bergey's manual has been reported (BORCH and MOLIN, 1988, CHAMPOMIER et al, 1987).

One possible alternative is the use of nucleic acid probes. HERTEL et al (1991) identified sequences of 23S rRNA specific for *Lactobacillus curvatus*, *L. pentosus/plantarum* and *L. sake* and synthesized the complementary oligonucleotides. The specificity of the probes was checked by dot blot and colony hybridization with strains of *L. curvatus*, *L. sake*, *L. pentosus* and *L. plantarum*. None of the other Gram-positive bacteria commonly found in meat reacted with the specific oligonucleotide probes.

We have tested the practicability of using such an approach in the identification of unknown LAB strains isolated in the course of a study of pork loins stored in packages with CO_2 and various small amounts of O_2, and compare the results with morphological and biochemical classification tests.

MATERIALS AND METHODS

Reference cultures: *L. curvatus* (NCDO 2739), *L. pentosus* (ATCC 8041), *L. sake* (MF 24) and *Leuconostoc carnosum* (NFC 2776).

Meat isolates: The LAB strains were isolated by swabbing the surface of pork loins after storage for two, four and six weeks at 4 °C in the dark. Appropriate dilutions of swab suspensions were plated on MRS agar (pH 5.7) and incubated at 20 °C. All strains were Gram-positive and catalase negative. Cell morphology was studied by phase contrast microscopy.

Biochemical tests

The following tests were performed:

- Gas production from glucose in Gibsons medium (GIBSON and ABEL-MALEK, 1945).

- Ammonia production from arginine as described by SCHILLINGER and LÜCKE (1987).
- Growth on acetate agar, pH 5.5 (ROGOSA et al, 1951).
- D and L lactic acid production enzymatically (Boehringer).

Hybridization

Oligonucleotide probes (15 - 20 bp) specific for *L. curvatus*, *L. pentosus/plantarum*, *L. sake* and a universal probe were synthesized from published sequences (HERTEL et al, 1991). The probes were labelled with a 3-end labelling kit (Amersham) using (α^{32}P)ddATP and their specificity tested by hybridization with the reference cultures.

The bacteria were streaked as single lines (approx 0,5 cm) directly on Colony Lift membranes (BioRad) with 50 strains per plate. The membranes were placed on MRS agar (pH 6.5) and incubated at 25 °C until growth was visible for all strains (usually 2-3 d). The membranes were then placed on filters soaked in 2xSSC (standard saline citrate) containing 5 % SDS, lysed in a microwave oven for 1-2 min and dried at 80°C for 10-15 min (BETZL et al, 1990). Hybridization was performed in an oven in rolling tubes. The composition of solutions, temperatures and length of prehybridization, hybridization and washes were as described by HERTEL et al (1991). The autoradiography detection (X-omatic Kodak film) took 2-3 days. For further hybridization with the same membranes the hybrids were denatured by washing high temperature (HERTEL et al, 1991).

Probes were also 3-end labelled with non-radioactive digoxigenin-ddUTP and the hybridized probes were detected with a DIG luminescent detection kit (Cat. No. 1362 372 and 1363 514, Boehringer Mannheim Biochemica). The manufacturers procedure was followed, except for the temperatures of hybridization and washes which were the same as for the radioactive hybridization.

RESULTS AND DISCUSSION

Figure 1 shows typical radioautograms of two membranes where each meat strain is represented by short straight lines. In the hybridization with the probe for *L. curvatus* (Figure 1 A), the strains that have a positive reaction are readily distinguishable.

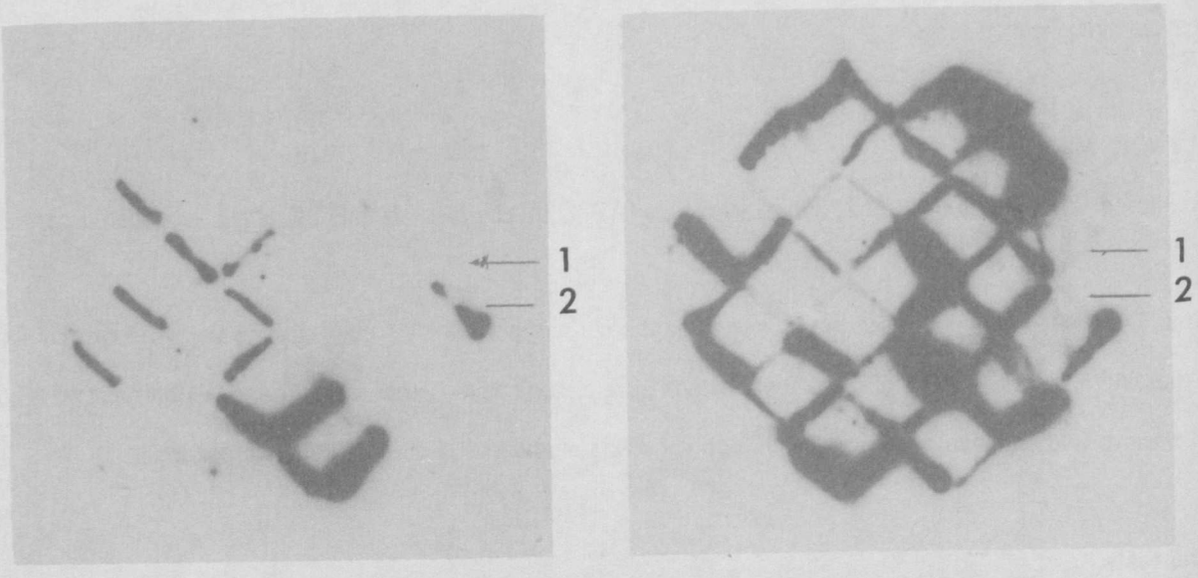

Figure 1. Radioautograms of duplicate membranes where each LAB strain is represented by short straight lines. Membrane A was hybridized with an oligo-probe specific for *Lactobacillus curvatus* while B was hybridized with a general probe (see Materials and Methods).

However, the intensity of the reaction varied somewhat, the reason being that not all the strains grew at the same rate on the membrane, some formed barely visible straks while others formed thick lines. After autoradiography the membranes were stripped of the specific probes and hybridized with the general probe (Fig 1B). For some strains the reaction with the general probe was weak or missing (arrow 1) because of little available rRNA. To ensure that these do not give false negative hybridizations with the specific probe, they were streaked out and hybridized a second time. In some cases hybridization with the specific probe gave a stronger reaction than with the general probe (arrow 2). This can be due to differences in the efficiency of labelling, in the stringency in washing or in the loss of RNA during stripping of the first probe.

Of the 96 LAB strains 25 hybridized with *L. sake*, 15 with *L. curvatus* and 2 with *L. pentosus/L. plantarum*. Of the strains that hybridized with *L. sakes* 5 did not produce NH_3 from arginine, but otherwise the identities coincided with the morphology and the biochemical tests (Table 1). MONTEL and CHAMPOMIER (1987) reported that *L. sake* produced NH_3 at low glucose concentrations wheras KANDLER and WEISS (1986) stated that *L. sake* did not produce NH_3 (without specifying the medium). In view of these different reports it is possible that the 5 strains that hybridize with the specific probe but do not produce NH_3 are in fact *L. sake*, but further fermentation tests are required.

Table 1 shows that for *L. curvatus* and *L. pentosus/L. plantarum* the morphology and the biochemical tests are identical and that only production of NH_3 from arginine differentiated between *L. sake* and *L. curvatus*. The two species are genetically closely related and not easy to separate by conventional tests (HERTEL et al, 1991). The great majority of the rest of the strains seem to be *Leuconostoc spp.*, being cocci, producing gas from glucose but not NH_3 from arginine and exclusively the D isomer of lactic acid. About 10% of the strains did not seem to fit into any of the groups but none coincided with *Carnobacterium* spp. which give no growth on acetate agar and produced the L isomer of lactic acid.

Table 1. Classification of lactobacillus strains

	Biochemical tests				Probes		
	Morphology	CO_2 from glucose	NH_3 from arginine	Isomer of lactic acid	*Lactobacillus sake*	*Lactobacillus curvatus*	*Lactobacillus pentosus*
REFERENCE STRAINS							
Lactobacillus curvatus	rods	-	-	D+L	-	+	-
Lactobacillus pentosus	rods	-	-	D+L	-	-	+
Lactobacillus sake	rods	-	+	D+L	+	-	-
Leuconostoc carnosum	rods	+	-	D	-	-	-
NUMBER OF MEAT STRAINS							
20	rods		+	D+L	+	-	-
5	rods	-	-	D+L	+	-	-
15	rods	-	-	D+L	-	+	-
2	rods	-	-	D+L	-	-	+
43	cocci	+	-	D	-	-	-

All strains grew on acetate agar

Using chemiluminescent detection probes labelled with digoxigenin, positive hybridization reactions have been obtained with RNA

isolated from pure cultures, but to date in colony hybridization the signals have been too weak. It is possible that this method can be improved, but it seems that ^{32}P labelling is more sensitive for colony hybridization with such short probes.

CONCLUSION

Results from the identification of the LAB probes were available within 4 days, and by streaking out 50 strains on the same plate it is possible to test many strains in a short time compared with the 3 weeks or so required for reliable biochemical tests. The coincidence of the strains with the biochemical tests indicates that the hybridizations are specific. The availability of probes of other common meat LAB will make classification of LAB fast, easy and reliable if somewhat more expensive.

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