SHORT COMMUNICATION

Sub-divisions Within the Genus *Leuconostoc* as Shown by RNA/DNA Hybridization

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RNA/DNA hybridization studies were carried out using material from strains of the different species of *Leuconostoc* and of *Lactobacillus confusus* and *Lactobacillus viridescens*. The non-acidophilic species *Leuconostoc mesenteroides*, its sub-species *dextranicum* and *cremoris*, *Leuconostoc lactis* and *Leuconostoc paramesenteroides* form a single group. This is unrelated to *Leuconostoc oenos* and also to the two lactobacilli. There is only a low relationship between the two lactobacilli.

INTRODUCTION

At present the genus *Leuconostoc* comprises six species. Of these, *Leuconostoc oenos* is found in wine and related habitats, while the other five species – *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, *Leuconostoc cremoris*, *Leuconostoc lactis* and *Leuconostoc paramesenteroides* – are found on plants and in dairy products but cannot live in the acidic environment favoured by *Leuc. oenos*. *Leuconostoc oenos* has some fundamental differences in enzymes from the other species (Garvie, 1975; Gasser & Hontebeyrie, 1977) and is unrelated to these by DNA/DNA homology studies (Garvie, 1976; Hontebeyrie & Gasser, 1977). It has been suggested that two species of lactobacilli – *Lactobacillus confusus* and *Lactobacillus viridescens* – which occur in the same habitat as the leuconostocs should be transferred from the lactobacilli to the leuconostocs (Sharpe *et al.*, 1972). This communication reports a study of RNA/DNA hybridization within this group of eight species to learn more of the relationships between them.

RNA/DNA hybridization has been used to study the relationships between different genera in Gram-negative bacteria (de Smedt *et al.*, 1980) and in *Rhodococcus* and related genera (Mordarski *et al.*, 1980) but has not been used to study the lactic-acid bacteria. RNA is being used in bacterial systematics because there is evidence that it has been conserved in structure over long periods of time (Woese *et al.*, 1975). Palleroni *et al.* (1973) have suggested that the different RNA groups found within the present genus *Pseudomonas* should each have generic status. If RNA groups in the lactic-acid bacteria are established they might help to unravel some of the relationships between the species (and genera) of this important group of organisms. They might also show if the lactic-acid bacteria form a natural group or whether similarities have arisen because of environmental influence rather than by evolution.

METHODS

Strains. All the strains used were selected from the National Collection of Dairy Organisms. They were grown in MRS broth at 30 °C, except *Leuc. oenos* which was grown in acidic tomato broth at 22 °C (Garvie & Mabbitt, 1967).

Preparation of DNA. DNA was prepared as described by Garvie (1976). Labelled DNA was extracted from cells grown in media containing [methyl-³H]thymidine (0.1 µCi ml⁻¹, 3.7 kBq ml⁻¹).

Preparation of randomly labelled RNA. For RNA extraction, cells were grown in medium of the same composition as cells used for DNA extraction. [^3H]Uracil ($1\ \mu\text{Ci ml}^{-1}$, $37\ \text{kBq ml}^{-1}$) was added to the medium when labelled RNA was required. Cells were harvested after overnight growth at $30\ ^\circ\text{C}$. They were washed in water and deep frozen. Cells were thawed and suspended at a concentration of $1\ \text{g wet packed cells ml}^{-1}$ in $0.01\ \text{M-Tris/HCl}$ buffer pH 7.0 containing $0.01\ \text{M-KCl}$, $0.01\ \text{M-MgCl}_2$, lysozyme ($1.0\ \text{mg ml}^{-1}$) and deoxyribonuclease ($10\ \mu\text{g ml}^{-1}$). The mixture was incubated at $37\ ^\circ\text{C}$ for 30 min, cooled and 1% (w/v) sodium dodecyl sulphate was added. The method of Moore & McCarthy (1967) was followed for the rest of the extraction.

Pulse-labelled RNA. *Lactobacillus confusus* NCDO 1586 and *Leuconostoc mesenteroides* NCDO 523 were grown to the mid-exponential phase and then $1\ \mu\text{Ci } [^3\text{H}]\text{uracil}$ was added per ml culture. NCDO 1586 was left for 6 min and NCDO 523 for 30 min at $30\ ^\circ\text{C}$. The cultures were then cooled rapidly by pouring on to an equal volume of frozen broth and kept at $4\ ^\circ\text{C}$ while cells were harvested. Preparation of RNA followed the previous procedure.

Other methods. Sucrose density gradient centrifugation, estimation of purity of RNA, hybridization with DNA, and competition between 16S and 23S RNA and estimation of T_{me} followed the techniques described elsewhere (Garvie & Farrow, 1982).

RESULTS

Purity of RNA and DNA. The contamination of the nucleic acid preparations with the other species of nucleic acid and with protein was less than 5%. Chemical estimation of impurity was impractical at this level and the purity of the majority of preparations was judged by absorbance readings.

Presence of 16S and 23S RNA. RNA from both *Leuc. mesenteroides* NCDO 523 and *Lb. confusus* NCDO 1586 contained 16S and 23S RNA and also a small amount of low molecular weight material. Most experiments were made using total randomly labelled RNA rather than a single molecular species for reasons discussed elsewhere (Garvie & Farrow, 1982). There was about 50% competition for hybridization with DNA between the 16S and 23S RNA from the same strain, indicating that the 23S material had fragmented and was present in the 16S peak.

Table 1. Percentage hybridization between nucleic acids of some strains of heterofermentative lactic-acid bacteria

Source of unlabelled DNA		^3H -labelled nucleic acid from <i>Leuc. mesenteroides</i> NCDO 523			^3H -labelled nucleic acid from <i>Lb. confusus</i> NCDO 1586		
Species	NCDO no.	DNA*	Pulse-labelled RNA	Randomly labelled RNA	DNA*	Pulse-labelled RNA	Randomly labelled RNA
<i>Leuconostoc mesenteroides</i>	523	100	100	100	13	40	25
<i>Leuc. mesenteroides</i>	768	48	70	126	15	42	28
<i>Leuc. dextranicum</i>	529	110	172	116	16	39	20
<i>Leuc. cremoris</i>	543	66	161	103	13	37	22
<i>Leuc. lactis</i>	533	58	32	79	18	—	23
<i>Leuc. lactis</i>	546	49	33	121	18	24	35
<i>Leuc. paramesenteroides</i>	803	6	36	92	28	32	58
<i>Leuc. paramesenteroides</i>	871	19	17	130	26	25	51
<i>Leuc. paramesenteroides</i>	2036	—	18	104	—	17	26
<i>Leuc. oenos</i>	1674	15	8	34	4	12	11
<i>Leuc. oenos</i>	1707	14	7	32	—5	13	11
<i>Lactobacillus confusus</i>	1586	15	35	37	100	100	100
<i>Lb. confusus</i>	930	20	25	39	82	74	69
<i>Lb. confusus</i>	1975	14	33	45	86	115	69
<i>Lb. viridescens</i>	403	23	14	50	50	—	29
<i>Lb. viridescens</i>	1655	12	—	29	32	—	25

—, Not tested. * Results from Garvie (1976).

Fig. 1. Graphs showing the elution of rRNA/DNA hybrids from membrane filters.

Table 2. *Melting temperatures calculated from the elution of rRNA/DNA hybrids from membrane filters*

Source of unlabelled DNA		Source of ³ H-labelled rRNA	
Species	NCDO no.	<i>Leuc. mesenteroides</i> NCDO 523	<i>Lb. confusus</i> NCDO 1586
<i>Leuc. mesenteroides</i>	523	77.5	—
<i>Leuc. mesenteroides</i>	768	77.0	—
<i>Leuc. dextranicum</i>	529	76.5	—
<i>Leuc. cremoris</i>	543	78.0	—
<i>Leuc. lactis</i>	546	76.0	—
<i>Leuc. paramesenteroides</i>	803	69.5	—
<i>Lb. confusus</i>	1586	—	78.0

Hybridization between DNA and RNA. DNA/DNA hybridization is normally expressed as a percentage, taking the amount of labelled DNA attached to homologous unlabelled DNA as 100%. The same method of calculation has been adopted with rRNA and mRNA so that a comparison of the information from the different procedures can be made (Table 1). The results show that *Leuc. oenos* has a low relationship to *Leuc. mesenteroides* and to *Lb. confusus*. Hybridization using pulse-labelled RNA gave the same pattern of relationship as DNA, linking *Leuc. mesenteroides* with *Leuc. dextranicum* and *Leuc. cremoris* but not with other species. In contrast, hybridization to rRNA showed that all the non-acidophilic leuconostocs belong to a single group. *Lactobacillus confusus* showed a relationship only between strains of the same species.

Estimation of $T_{m(e)}$. $T_{m(e)}$ values were not estimated for any hybrids where the c.p.m. values were very low. The elution curves of DNA/rRNA hybrids formed amongst the non-acidophilic leuconostocs are shown in Fig. 1. The estimated $T_{m(e)}$ values are given in Table 2.

DISCUSSION

Pulse-labelled RNA has seldom been used in hybridization experiments, and it was not found to have any advantage over DNA, rather the reverse. Pulse-labelled RNA has a low

label and is highly contaminated with ribosomal RNA (Gillespie, 1968). The pulsing time is short and the label can only reflect the messenger RNA being made at one particular time and under one set of growth conditions. Pulsing is unsatisfactory in bacteria which do not multiply rapidly, e.g. *Leuc. mesenteroides*, although this is a problem which it might be possible to overcome. Pulsing has to be prolonged with the slower growing organisms or the label will be too low; on the other hand, RNA other than mRNA, may carry label if the incubation time is increased. By contrast, randomly labelled RNA is believed to be slow to evolve and to show the ancient origins of the bacteria (Woese *et al.*, 1975). It has none of the disadvantages associated with pulse-labelled RNA.

The high hybridization between RNA of *Leuc. mesenteroides* and the DNA of *Leuc. dextranicum* and *Leuc. cremoris*, *Leuc. lactis* and *Leuc. paramesenteroides* shows that these species form a natural group of bacteria only distantly related at any level to the other species examined. More work is needed amongst the lactic-acid bacteria before these results can be fully assessed.

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