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Nucleic Acid Relatedness Studies on the Genus *Carnobacterium* and Related Taxa

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None of the species *Carnobacterium carnis, C. divergens, C. mobile* or *C. gallinarum* showed significant DNA–DNA homology between themselves and other lactic acid bacteria. Nevertheless, the *Carnobacterium* species were found to belong to the same ribosomal RNA homology cluster. The species in this cluster were distant from the other bacteria tested.

INTRODUCTION

Collins *et al.* (1987) described a new genus, *Carnobacterium,* incorporating four species – *C. piscicola, C. divergens, C. mobile* and *C. gallinarum.* All had been isolated from meat, fish or poultry. *C. piscicola* had been described previously as *Lactobacillus piscicola* (Hiu *et al.*, 1984). The high DNA homology between *Lactobacillus carnis* strains (Shaw & Harding, 1986) and *C. piscicola* led to the proposal that *L. carnis* should be named *C. piscicola. C. divergens* included strains formerly called *Lactobacillus divergens* (Holzapfel & Gerber, 1983). *C. mobile* and *C. gallinarum* are the names proposed for one of the groups of 'atypical lactobacilli' described by Thornley & Sharpe (1959).

Peptidoglycan containing *meso*-diaminopimelic acid (mDAP) type is a feature common to all these taxa. It is also found in a few species of *Lactobacillus* and *Brochothrix.* Given the importance of this chemotaxonomic marker in phylogenetic studies (Stackebrandt & Woese, 1981) it is pertinent to study genetic relationships between all these taxa.

The aim of the present work was to determine the degree of relatedness between strains of *Carnobacteria* by measuring DNA–DNA homologies and RNA cistron similarities.

METHODS

Strains and growth conditions. The sources of the strains tested are given in Table 1. Strains were cultivated in MRS broth or All Purpose Tryptone (APT; Merck).

Isolation of DNA. After 18 h growth, cells were harvested and lysed according to the method described by Rocourt *et al.* (1982), except that the lysozyme concentration was increased (to 300 mg per 100 ml of lysate) and 500 units of mutanolysin (Sigma) were added. DNA was extracted and purified as described by Brenner *et al.* (1982).

DNA–DNA hybridization. DNA was labelled by nick-translation with [^{3}H]ATP (925 GBq mmol^{-1}), [^{3}H]GTP (625 GBq mmol^{-1}), [^{3}H]CTP (977 GBq mmol^{-1}) and [^{3}H]TTP (1·53 TBq mmol^{-1}) (Amersham). DNA–DNA hybridization was done at 60 °C following treatment with S_1 nuclease and precipitation with trichloroacetic acid (Grimont *et al.*, 1980).

Isolation of ribosomal 16S and 23S RNA. Ribosomal RNA (rRNA) was labelled *in vivo* and extracted according to the method described by Kilpper-Bälz & Schleifer (1981), with the following modifications. Cells were cultivated in 400 ml APT broth (Merck) supplemented with 18·5 MBq [5,6-^{3}H]uracil (1·48 TBq mmol^{-1}; Amersham). They were harvested at the beginning of the stationary phase and suspended in 0·04 M-Tris/0·02 M-acetate buffer (7·2) containing 10 mM vanadyl ribonucleoside complex (VRC; Gibco BRL). After thawing overnight at 18 °C in the cylindrical chamber of an Xpress cells were disrupted by passage through the press at a pressure of 150 MPa. Nucleic acids were extracted with phenol/chloroform and precipitated by NaCl (0·5 M) and ethanol.

Abbreviation: mDAP, *meso*-diaminopimelic acid.

Table 1. *Designation and source of strains*

[T], type strain; ATCC, American Type Culture Collection, Rockville, Md., USA; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, FRG; NCDO, National Collection of Dairy Organisms, Reading, UK; Shaw, Dr B. G. Shaw, Institute of Food Research, Bristol, UK.

Name as received	Reference number	Isolated from:
Lactobacillus carnis = *Carnobacterium piscicola*	LV61 Shaw	Vacuum-packed beef
Lactobacillus divergens = *Carnobacterium piscicola*	LV14 Shaw	
Lactobacillus divergens = *Carnobacterium divergens*	LV6 Shaw	
Lactobacillus divergens = *Carnobacterium divergens*	LV60 Shaw	
Carnobacterium piscicola[T]	NCDO 2762	Salmonid fish
Carnobacterium gallinarum	NCDO 2766	Chicken meat
Carnobacterium mobile	NCDO 2765	Irradiated chicken meat
Lactobacillus plantarum[T]	ATCC 14917	Pickled cabbage
Lactobacillus yamanashiensis[T]	DSM 20444	Apple juice from cider press
Lactobacillus vaccinostercus[T]	DSM 20634	Cow dung
Lactobacillus pentosus	NCDO 363	Cow silage
Lactobacillus maltaromicus[T]	DSM 20342	Malt-flavoured milk
Lactobacillus agilis[T]	DSM 20509	Sewage
Lactobacillus sharpae	DSM 20205	Sewage
Lactobacillus ruminis[T]	DSM 20403	Bovine rumen
Pediococcus pentosaceus	DSM 20336	Beer yeast
Leuconostoc mesenteroides	DSM 20343	Fermenting olives
Brochothrix thermospacta[T]	ATCC 11509	Fresh pork sausage
Brochothrix campestris[T]	ATCC 43574	Soil
Sporolactobacillus inulinus	DSM 20348	Chicken feed

After separation on a 2·8 (w/v) polyacrylamide slab gel for 16 h (30 V, 15 mA) ribosomal RNAs were localized by the method of Hassur & Whitlock (1974), and purified by electro-elution for 6 h at 100 V in elution buffer consisting of 0·5 M-ammonium acetate, 1 mM-EDTA (pH 8).

The eluate was purified by extraction with phenol and precipitation with ethanol. After centrifugation at 10000 *g*, the RNA pellet was dissolved in 2 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-trisodium citrate, pH 7·0) and stored at −20 °C. The concentration was determined spectrophotometrically at 260 nm. The specific activity of ^{3}H-labelled RNA from *C. piscicola* was 6000 c.p.m. (μg RNA)$^{-1}$.

DNA–RNA hybridization. Nitrocellulose filters (Sartorius) were loaded with 100 μg DNA. DNA was denatured by 30 min treatment with an equal volume of 1 M-NaOH, then, neutralized by addition of 1 vol. 1 M-HCl, 1 vol. 1 M-Tris/HCl (pH 8) and 2 vols 2 × SSC. This solution was allowed to pass through nitrocellulose under vacuum. Filters were baked in an oven for 3 h at 80 °C. DNA–rRNA hybridization and $T_{m(e)}$ values were determined according to the methods described by De Ley & De Smedt (1975).

RESULTS AND DISCUSSION

DNA–DNA homologies between strains of *C. piscicola*, *C. divergens* and other species belonging to the genera *Carnobacterium*, *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Brochothrix* are shown in Table 2. It is apparent that *C. piscicola* and *C. divergens* are distantly related to other species of lactic acid bacteria (i.e. $\leqslant 22\%$ homology). Moreover, the four species of the genus *Carnobacterium* share low homology towards DNA of *C. piscicola* and *C. divergens* (homology values $<15\%$). In view of these low values, it is difficult to conclude if these species are genuinely related at the genus level.

Results of DNA–rRNA hybridizations are shown in Table 3. Hybrids are characterized by the $\Delta T_{m(e)}$ value towards RNA of *C. piscicola*. This value is the most useful taxonomic parameter to measure RNA cistron similarity (De Vos *et al.*, 1985). It is apparent that all the *Carnobacterium* species belong to the same RNA homology group as revealed by the low range of $\Delta T_{m(e)}$, i.e. from 1 to 3 °C. Several authors (Gillis & De Ley, 1980; Stackebrandt *et al.*, 1981; Kilpper-Bälz & Schleifer, 1981) suggest that strains for which the $\Delta T_{m(e)}$ is less than 6 °C can be included in the same RNA homology group. The closest neighbour of *C. piscicola*, at a $\Delta T_{m(e)}$ of 5 °C, is *Lactobacillus plantarum*. No close genetic relationship was found between *C. piscicola*

Table 2. *DNA–DNA relatedness between strains of C. piscicola, C. divergens and other lactic acid bacteria*

Source of unlabelled DNA	Percentage relatedness with labelled DNA from: *C. piscicola* LV61	*C. divergens* LV6
C. piscicola = *L. carnis* LV61	100	8
C. piscicola = *L. carnis* LV14	68	NT
C. piscicola NCDO 2762	80	6
C. divergens LV6	8	100
C. divergens LV60	10	82
C. gallinarum NCDO 2766	12	17
C. mobile NCDO 2765	10	8
L. plantarum ATCC 14917	4	2
L. yamanashiensis DSM 20444	7	3
L. vaccinostercus DSM 20634	5	NT
L. pentosus NCDO 363	6	2
L. maltaromicus DSM 20342	9	22
L. agilis DSM 20509	10	2
L. sharpae DSM 20205	11	NT
L. ruminis DSM 20403	7	2
P. pentosaceus DSM 20336	6	5
Leuc. mesenteroides DSM 20343	13	9
B. thermosphacta ATCC 11509	8	10
S. inulinus DSM 20408	11	NT

NT, Not tested

Table 3. *Peptidoglycan type and nucleic acid relatedness of Carnobacterium spp. and other lactic acid bacteria*

Filter-bound DNA from:	Peptido-glycan type	DNA G+C content (mol%)*	$\Delta T_{m(e)}$ of heteroduplex with 23S RNA from *C. piscicola* LV6
C. piscicola = *L. carnis* LV61	mDAP	35	0
C. piscicola = *L. carnis* LV14	mDAP	35	2
C. piscicola NCDO 2762, type strain	mDAP	34	0
C. divergens LV6	mDAP	35	3
C. divergens LV60	mDAP		2
C. gallinarum NCDO 2766	mDAP	37	1
C. mobile NCDO 2765	mDAP	37	1
L. plantarum ATCC 14917	mDAP	45	5
L. yamanashiensis DSM 20444	mDAP	33	7·5
P. pentosaceus DSM 20336	L-lys-L-ala-D-asp	38	7·5
L. vaccinostercus DSM 20634	mDAP	36	8
L. pentosus NCDO 363	mDAP	46	11·5
L. maltaromicus DSM 20342	mDAP	36	12
B. thermosphacta ATCC 11509	mDAP	36	13
Leuc. mesenteroides	L-ser-L-lys-L-ala2	37	14
L. agilis DSM 20509	mDAP	44	20

* Data from Bergey's Manual of Systematic Bacteriology (Sneath, 1986) and from Collins *et al.* (1987).

and other species containing mDAP in their cell wall except *Lactobacillus yamanashiensis*, which shows a $\Delta T_{m(e)}$ of 7·5 °C.

These genetic findings support the proposal of Collins *et al.* (1987) for the recognition of a new genus named *Carnobacterium*. The four member species, though sharing low overall genomic

similarity, form a phylogenetically coherent group, with the following characters: low G+C percentage (35 to 37%); mDAP in the cell wall; production of mainly L-lactate; no growth on acetate agar and synthesis of C18:1 (Δ9:10) instead of C18:1 (Δ11:12) (Collins *et al.*, 1987). Only these two latter features differentiate this genus from *Lactobacillus*.

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