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Carnobacterium divergens and *Carnobacterium maltaromaticum* as spoilers or protective cultures in meat and seafood: phenotypic and genotypic characterization

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Abstract

Carnobacterium, a genus of lactic acid bacteria, frequently dominate the microflora of chilled vacuum- or modified atmosphere-packed meat and seafood. In this study *Carnobacterium* isolates were characterized by phenotypic and molecular methods in order to investigate the association of species and intra-species groups with distinct kinds of meat and seafood.

Of 120 test strains, 50 originated from meat (beef and pork products, including 44 strains isolated during this study and 6 strains obtained from culture collections) and 52 from seafoods (cod, halibut, salmon, shrimps and roe products). In addition, 9 reference strains of *Carnobacterium* spp from other sources than meat and fish and 9 reference strains of lactic acid bacteria belonging to other genera than *Carnobacterium* were included. Numerical taxonomy relying on classical biochemical reactions, carbohydrate fermentation and inhibition tests (temperature, salt, pH, chemical preservatives, antibiotics, bacteriocins), SDS-PAGE electrophoresis of whole cell proteins, plasmid profiling, intergenic spacer region (ISR) analysis and examination of amplified-fragment length polymorphism (AFLP) were employed to characterize the strains.

The numerical taxonomic approach divided the carnobacteria strains into 24 groups that shared less than 89% similarity. These groups were identified as *Carnobacterium divergens* with one major cluster (40 strains) and 7 branches of one to four strains, *Carnobacterium maltaromaticum* (previous *C. piscicola*) with one major cluster (37 strains) and 9 branches of one to four strains and *Carnobacterium mobile* (three branches consisting in total of 4 strains). Branches consisting of references strains of the remaining *Carnobacterium* spp. were separated from clusters and branches of *C. divergens*, *C. maltaromaticum* and *C. mobile*. Isolates from the main clusters of *C. divergens* and *C. maltaromaticum* were found both in fresh and lightly preserved meat and seafood products. High phenotypic intra-species variability was observed for *C. divergens* and *C. maltaromaticum* but despite this heterogeneity in phenotypic traits a reliable identification to species levels was obtained by SDS-PAGE electrophoresis of whole cell proteins and by ISR based on 16S-23S rDNA intergenic spacer region polymorphism. With AFLP, two distinct clusters were observed for

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C. divergens but only one for *C. maltaromaticum*. The two *C. divergens* clusters were not identical to any of the clusters observed by numerical taxonomy.

A limited number of *C. divergens* and *C. maltaromaticum* isolates possessed a biopreservative potential due to their production of bacteriocins with a wide inhibition spectrum. This study serves as a base-line for further investigations on the potential role of species of *Carnobacterium* in foods where they predominate the spoilage microflora.

Keywords: Lactic acid bacteria; *Carnobacterium*; Spoilage; Antagonism; ISR; AFLP; Numerical taxonomy

Introduction

The genus of *Carnobacterium*, belong to the lactic acid bacteria (LAB) and consists of eight species, including *Carnobacterium alterfunditum*, *Carnobacterium divergens*, *Carnobacterium funditum*, *Carnobacterium gallinarum*, *Carnobacterium inhibens*, *Carnobacterium maltaromaticum*, *Carnobacterium mobile* and *Carnobacterium viridans*. Only *C. divergens* and *C. maltaromaticum* have been frequently isolated from a wide variety of sources such as humans [11], intestine, skin and gills of healthy fish (e.g. [24,59,60]), diseased fish [5] and various food products including seafood (e.g. [3,16,20,21,25,28,37,39,46,53], meat [30,47,64,66], poultry [2] and dairy products [50]).

C. divergens and *C. maltaromaticum* is frequently a predominating element of the microflora of chilled vacuum- or modified atmosphere-packed meat and seafood. An appreciable amount of research has been devoted to the application of carnobacteria for biopreservation of foods, i.e. the use of protective cultures that does not modify the sensory characteristics of products. Thus, bacteriocin-producing strains of *C. maltaromaticum* and *C. divergens* have been isolated from vacuum-packaged meat (e.g. [1,14]) and various seafoods (e.g. [18,54,56]). Bacteriocin production, however, is not a pre-requisite for the biopreservative efficacy of *Carnobacterium* and substantial antimicrobial activity in cold-smoked salmon has been documented for both bacteriocin positive and negative strains although activity may be enhanced by presence of bacteriocin [51,52].

Thus, strains of these species may be used as protective cultures in biopreserved meat and seafood. However, growth and/or presence of high numbers of *C. maltaromaticum* and/or *C. divergens* have been associated with sensory spoiled products, including cooked sliced ham inoculated with *C. maltaromaticum* [8], sterile beef inoculated with *C. divergens* or *C. maltaromaticum* and stored under vacuum and subsequently transferred to aerobic conditions [40], frozen/thawed and modified atmosphere packed fish [20,25,48], various lightly preserved seafoods [3,15,16,31,42] and high pressure processed squid mantle [53]. These bacteria may contribute to spoilage and knowledge about their potential in this regard is incomplete. It is presently not clear if distinct taxonomic clusters within the two species cause spoilage of meat and seafood in general or if they contribute to spoilage of specific types of products.

The objective of the present study was to characterize isolates of carnobacteria from meat and seafood and thereby identify strains or clusters of strains at species and sub-species levels that are potentially responsible for spoilage or appropriate for use as protective cultures. Isolates were characterized by a battery of phenotypic and DNA-based tests, including classical biochemical reactions, carbohydrate fermentation and inhibition tests (temperature, salt, pH, chemical preservatives, antibiotics, bacteriocins), SDS-PAGE electrophoresis of whole cell proteins, plasmid profiling, analysis of 16S-23S rDNA intergenic spacer region (ISR) polymorphism and examination of amplified-fragment length polymorphism (AFLP).

Materials and methods

Origin of isolates

In addition to type and reference cultures, *Carnobacterium* strains were isolated from chilled meats and seafoods with different product characteristics (Tables 1 and 2). The seafood isolates have been characterized previously whereas the meat isolates, except for a few strains from vacuum packed meat, were obtained during the present study from various products. Type strains of all known species of *Carnobacterium* were included except *C. alterfunditum* LMG 14462^T and *C. funditum* LMG 14461^T that were unable to grow within the experimental design used and therefore omitted from this study. Reference strains of lactic acid bacteria belonging to other genera than the *Carnobacterium* were also included. The identity of these reference strains is listed in Table 1, footnote a.

Isolation of *Carnobacterium* from meat was done by preparing decimal dilutions with sterile peptone saline and plating on nitrite polymyxin agar (NP agar, as described by Davidson and Cronin [17] except that actidione was omitted as this compound was not commercially available). Enumeration and isolation of selected colonies was done after 3 d of incubation at

Table 1. Source and identity of the *Carnobacterium* strains included in this study[a]

Phenotypic cluster[b]	Species	Strain (**source**)
A	*C. inhibens*	*DSMZ 13024*^{T} (**Atlantic salmon intestine**)
B	*C. viridans*	*DSMZ 14451*$^{T\,c}$ (**refrigerated bologna sausage VP**[d])
C	*C. maltaromaticum*	5.27 (**beef aerob**)
D	*C. gallinarum*	*LMG 9841*^{T} (**ice slush around chicken carcasses**)
E	*C. divergens*	8.11 (**pork MAP**), 8.18 (**pork processed**)
F	*C. divergens*	8.16 (**pork processed**)
G	*C. divergens*	1.4, 2.5, 4.26, 5.30, 5.33, 5.39 (**beef aerob**), 8.3, 9.3, 9.5 (**beef MAP**), LMG 9199^T, LMG 11390, LMG 11391, LMG 11392 (**beef VP**), 4.12[e], 5.23, 6.1 (**pork aerob**), 5.2, 5.5, 7.4 (**pork MAP**), NCFB 2855 (**pork VP**) 8.17, 8.19, 8.25, 8.27 (**pork processed**), N14 (**halibut sugar-salted**), ML1-94, ML1-98, ML1-100, ML1-102, ML1-112, ML1-114, ML1-115 (**salmon fresh MAP**), 3b-5bA, 3b-6bA (**salmon cold-smoked**), N18, N20 (**salmon sugar-salted**), 0vb-3, 5va-1[e], 5vb-1, 8v7[e] (**shrimps**)
H	*C. maltaromaticum*	3.1, 6.2 (**beef aerob**), 4.7 (**beef MAP**), 3.18[e] (**pork aerob**), 5.3, 5.4, 8.6, 8.8 (**pork MAP**), 7.6, 8.22 (**pork processed**), *DMRI 4039* (**ham boiled**), F29-1, F41-1 (**lumpfish roe**), FM-C4, FM-C5, FM-C9, FM-C13[e], FM-C20, FM-C22 (**cod thawed MAP**), N1, N3, N5, N7 (**halibut sugar-salted**), ML1-95[e], ML1-96, ML1-101 (**salmon fresh MAP**), OB6-1b, OB6-2b (**salmon thawed MAP**), R1.7, R2.1, R2.10, 3T1, 3T4, A9a, A10a, A10b, A10f (**salmon cold-smoked**)
I	*C. divergens*	1.1[e] (**beef aerob**), 3.12, 5.18 (**pork aerob**), N21 (**salmon sugar-salted**)
J	*C. maltaromaticum*	N15 (**halibut sugar-salted**)
K	*C. maltaromaticum*	LMG 9840 (**meat VP**), *DMRI 4013* (**salami containing cheese**)
L	*C. maltaromaticum*	9.4[e] (**beef MAP**), *LMG 11393* (**beef VP**), F46-1 (**lumpfish roe**), ML1-97 (**salmon fresh MAP**)
M	*C. maltaromaticum*	*DMSZ 20342*^{T} (**milk**)
N	*C. maltaromaticum*	8.1 (**beef MAP**)
O	*C. maltaromaticum*	9.6 (**beef MAP**)
P	*C. maltaromaticum*	*DMRI 4024* (**ham boiled**), OB6-3 (**salmon thawed MAP**)
Q	*C. divergens*	9.7 (**beef MAP**)
R	*C. divergens*	5.11 (**pork aerob**)
S	*C. mobile*	5kb-7 (**shrimps**)
T	*C. divergens*	5.19 (**pork aerob**)
U	*C. divergens*	7.8, 7.9 (**pork processed**), ML1-99 (**salmon fresh MAP**)
V	*C. maltaromaticum*	N26 (**halibut sugar-salted**)
X	*C. mobile*	5kb-5[e], 5kb-6[e] (**shrimps**)
Y	*C. mobile*	*LMG 9842*^{T} (**irradiated chicken meat**)

[a]Reference strains not listed in this table include *Desemzia incerta* DSMZ 20581^T; *Enterococcus faecalis* DSMZ 20478^T; *Lactobacillus curvatus* subsp. *curvatus* LMG 17303, LMG 17299; *Lactobacillus plantarum* DSMZ 20174^T; *Lactobacillus sakei* subsp. *carnosus* LMG 17302^T, LMG 21529; *Lactobacillus sakei* subsp. *sakei* LMG 9468^T and *Weissella viridescens* LMG 3507^T.

[b]See Fig. 1.

[c]Strains listed in italics are not included in Table 2.

[d]Abbreviations: MAP = modified atmosphere packaged; VP = vacuum packaged; LMG = BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Gent, Belgium; NCFB = National Collection of Food Bacteria, UK; DSMZ = Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; DMRI = Danish Meat Research Institute, Roskilde, Denmark.

[e]4.12 = LMG 22903, 5va-1 = LMG 21335, 8v7 = LMG 19743, 3.18 = LMG 22901, FM-C13 = LMG 22899, ML1-95 = LMG 22898, 1.1 = LMG 22900, 9.4 = LMG 22902, 5kb-5 = LMG 21342, 5kb-6 = LMG 21341.

25 °C under microaerophilic conditions (CampyGen Oxoid). After enumeration the colonies were transferred by replica plating using paper filter (Whatman # 5) to Rogosa agar (Oxoid) and incubated under microaerophilic conditions at 25 °C for 5 d and to tetrazolium chloride agar and incubated aerobically at 30 °C for 2 d. Colonies on the original NP enumeration plates that did not grow on Rogosa agar and formed red colonies on the tetrazolium containing agar medium was transferred from the NP plates and sub-cultured in APT broth (Difco), examined under phase-contrast microscopy in order to select cultures containing rod-shaped cells and subsequently sub-cultured twice on APT agar before storage in glycerol freeze medium at −80 °C.

Phenotypic characterization

The isolates were analysed by 180 unit characters, including the tests listed in Table 3.

Table 2. Distribution of *Carnobacterium* isolates in meat and fish products as well as characteristics of the products from which they were isolated

Product	Storage	Product characteristics			Microbial counts		Number of isolates distributed on species			References
	Temp., time	pH	% NaCl[d]	Preservative agents	TVC[a]	NP[a]	*C. divergens*	*C. maltaromaticum*	*C. mobile*	
Beef (Fresh)										
Aerob	2 °C, 24 h	5.8	ND[e]	—	7.2±0.4	7.3±0.3	7	3	—	[f]
MAP[a]	5 °C, 8 d	5.8	ND	29–31% CO_2	ND	5.3±1.3	4	4	—	[f]
VP[a]	—	—	—	—	—	—	4	1	—	BCCM
Pork (Fresh)										
Aerob	2 °C, 24 h	5.9	ND	—	7.0±0.8	6.8±0.8	7	1	—	[f]
MAP	5 °C, 8 d	5.9	ND	19–21% CO_2	ND	7.8±1.3	4	4	—	[f]
VP	—	—	—	—	—	—	1	—	—	[66]
Pork (Processed)										
MAP	5 °C, 10 d	6.3	3.5±0.8	564–13393 ppm lactic acid	ND	6.2±0.0	6	—	—	[f]
VP	5 °C, 30–35 d	6.4	4.5±0.2	3501–4951 ppm lactic acid 0–390 ppm acetic acid	ND	8.0±1.4	2	2	—	[f]
Lumpfish roe, VP	5 °C, 90 d	5.4	3.5–4.5	1000 ppm lactic acid 129–252 ppm acetic acid	7.2±0.1	7.0±0.2	—	3	—	[3,4]
Cod										
Thawed, MAP[b]	1.6 °C, 20 d	6.7	ND	33% CO_2	5.7	ND	—	6	—	[25]
Halibut										
Sugar-salted, VP	5–10 °C, 7–26 d	6.5	4–5	—	8.1–8.5	7.3±0.7	1	6	—	[22,28,38,39]
Salmon										
Fresh, MAP	0 °C, 20–22 d	6.3	0.1	43% CO_2	7.1±0.3	ND	8	4	—	[g]
Thawed, MAP[c]	2.0 °C, 21–34 d	6.3	0.1	51% CO_2	5.9±0.4	6.8±0.4	—	3	—	[20]
Cold-smoked, VP	5 °C, 21–42 d	6.2	3.6–5.7	—	6–7.1	6.1–7.1	2	9	—	[31,54,70]
Sugar-salted, VP	5–10 °C, 7–19 d	6.1–6.2	4–5	—	7.6–7.9	6.6–7.0	3	—	—	[22,28,38,39]
Shrimp										
Cooked, brined, MAP	0–8 °C, 35–246 d	5.7–5.9	2.3–3.3	735–767 ppm benzoic acid 2849–3251 ppm citric acid 632–768 ppm sorbic acid 14–15% CO_2	8	~8	4	—	3	[15,16]
Total							53	46	3	

[a]Abbreviations: MAP = modified atmosphere packaged; VP = vacuum packaged; WPS = water phase salt; TVC = total viable count, NP = nitrite polymyxin.
[b]Previously stored at −21±0.5 °C.
[c]Previously stored at −30.8±0.3 °C.
[d]Water phase salt.
[e]Not determined.
[f]This study.
[g]Dalgaard, unpublished.

Table 3. Summarization of phenotypic tests included in this study

Test	Medium/method	Incubation, temperature	References or suppliers
Metabolism of carbohydrates			
Oxidation–fermentation test	Hugh & Leifson medium	3 d, 25 °C	Standard method
Gas from glucose or gluconate	Modified MRS	8 d, 25 °C	[66]
Acetoin formation	BM-broth	7 d, 25 °C	[73]
La-broth final pH	La-broth	7 d, 25 °C	[66]
Acid from 49 carbohydrates and derivates	API 50 CHL	7 d, 25 °C	BioMerieoux
Additional biochemical reactions			
NO_3 reduction	Nitrate-medium	3 d, 25 °C	NIT1, NIT2; BioMerieoux
Arginine hydrolysis (with 0.1% or 2.0% glucose)		7 d, 25 °C 28 d, 5 °C	[38]
Reduction of tetrazolium chloride		2 d, 30 °C	[73]
Various physiological and chemical tests			
Gram test	KOH		Standard method
Catalase	H_2O_2		Standard method
Oxidase	Dryslide		Becton and Dickinson
Microscopy: cell morphology and motility	APT broth	1 d, 25 °C	Difco
Growth on Rogosa acetate agar	Rogosa agar	5 d, 25 °C	[66]
Growth on STA agar	STA agar	3 d, 25 °C	Oxoid
Growth at 0, 5, 15, 35 and 45 °C	APT broth	28 d	Difco
Growth with 2, 6.5, 8, 10 and 15% NaCl	APT broth, pH 5.8	28 d, 25 °C	Difco
Growth with 4000, 8000 ppb acetate	APT broth, pH 5.8	28 d, 25 °C	Difco, J.T. Baker
Growth with 8000, 16,000 ppb citrate	APT broth, pH 5.8	28 d, 25 °C	Difco, Sigma
Growth with 10,000, 20,000 ppb lactate	APT broth, pH 5.8	28 d, 25 °C	Difco, Sigma
Growth with 4000, 8000 ppb ascorbate	APT broth, pH 5.8	28 d, 25 °C	Difco, Merck
Growth with 100, 200 ppb $NaNO_2$	APT broth, pH 5.8	28 d, 25 °C	Difco, Merck
Growth with 2000, 4000 ppb Na-benzoate	APT broth, pH 5.8	28 d, 25 °C	Difco, Merck
Growth with 2000, 4000 ppb K-sorbate	APT broth, pH 5.8	28 d, 25 °C	Difco, Merck
Growth with 4000 or 8000 ppb propionate	APT broth, pH 5.8	28 d, 25 °C	Difco, Merck
Growth with 100, 200 ppb phenol in liquid smoke	APT broth, pH 5.8	28 d, 25 °C	Difco, Scansmoke, PB1200, Denmark
Sensitivity towards antibiotics			
Vancomycin (5 and 30 μg)	MH-agar	3 d, 25 °C	Oxoid
Ampicillin (2 and 25 μg)	MH-agar	3 d, 25 °C	Oxoid
Penicillin (1 and 10 U)	MH-agar	3 d, 25 °C	Oxoid
Erythromycin (5 and 30 μg)	MH-agar	3 d, 25 °C	Oxoid
Gentamicin (10 and 200 μg)	MH-agar	3 d, 25 °C	Oxoid
Tetracycline (5 and 50 μg)	MH-agar	3 d, 25 °C	Oxoid
Furazolidone (50 μg)	MH-agar	3 d, 25 °C	Oxoid
Antagonistic effect against			[9]
Listeria monocytogenes (Scott A)	APT agar	44 h[a], 30 °C	Difco
L. innocua (LMG 11387)	APT agar	44 h, 30 °C	Difco
Brochothrix thermosphacta (LMG 17208)	APT agar	44 h, 30 °C	Difco
Staphylococcus aureus (NCTC 8325-4, ATCC 25923)	APT agar	44 h, 30 °C	Difco
Weisella viridescens (LMG 3507)	APT agar	44 h, 30 °C	Difco
Lactobacillus sakei subsp. *carnosus* (LMG 21529	APT agar	44 h, 30 °C	Difco
Lactobacillus curvatus subsp. *curvatus* (LMG 17303)	APT agar	44 h, 30 °C	Difco
C. divergens-pool (9 strains)	APT agar	44 h, 30 °C	Difco
C. maltaromaticum-pool (10 strains)	APT agar	44 h, 30 °C	Difco
Sensitivity towards bacteriocinproducers			[9]
C. divergens NCFB 2855 (Divergicin A)	APT agar	22 h[b], 30 °C	Difco
P. acidilactici PAC-1.0 (Pediocin PA1)	APT agar	22 h, 30 °C	Difco
L. lactis subsp. *lactis* ATCC 11454 (Nisin)	APT agar	22 h, 30 °C	Difco
B. campestris DSM 4772 (Brochocin C)	APT agar	22 h, 30 °C	Difco
L. sakei DSM 6333 (sakacin A)	APT agar	22 h, 30 °C	Difco
Three *Carnobacterium* strains (unknown)	APT agar	22 h, 30 °C	Difco

[a]Incubation of producer and indicator strains.
[b]Incubation of indicator strains.

Data from phenotypic tests was pair-wise compared. Physiological and biochemical characteristics were coded as 0 for negative and 1 for positive and analysed by hierarchic clustering using the software package BioNumerics™ version 3.5 (Applied Maths, Kortrijk, Belgium). Similarity was computed with the simple matching coefficient (S_{SM}) and agglomerative clustering was done using UPGMA. Reproducibility was calculated by use of the average error probability (p), as described by Sneath and Johnson [67] and using duplicates of 11 randomly selected isolates.

Protein-based identification

After incubation of cells for 24 h on Man Rogosa Sharpe (MRS) agar (Oxoid), whole-cell protein extracts were prepared and SDS-PAGE was performed as described previously [57]. A densitometric analysis, normalization and interpolation of the protein profiles, and a numerical analysis were performed using the Gelcompar software package version 3.1 and 4.0, respectively (Applied Maths, Kortrijk, Belgium). Identification was performed by numerical and visual comparison of the protein patterns with a database of normalized fingerprints derived from reference strains for almost all known species of lactic acid bacteria [58]. BioNumerics™ ver. 3.5 software was used to calculate Pearson correlation coefficient (r) between strains and conducting cluster analysis by the UPGMA algorithm.

Plasmid profiling

The procedure described by Leisner et al. [41] was followed. Band sizes of plasmid profile patterns were calculated by plotting migration positions of the bands of *Escherichia coli* strains V517 and 39R861 which contain plasmids of known sizes as well as migration positions of each band pattern on logarithmic paper.

Intergenic spacer region (ISR) analysis

This analysis was based on 16S-23S rDNA intergenic spacer region polymorphism according to Kabadjova et al. [32]. Ready-To-Go™ PCR Beads and restriction enzymes (*HinfI* and *HindIII*) were obtained from Amersham Biosciences, whereas primers (23S/p10, $tRNA^{Ala}$) were obtained from TAG Copenhagen, Denmark. DNA was isolated from bacterial cells grown in APT (25 °C, 24 h) by use of DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. Band sizes of ISR patterns were calculated by the same procedure as for plasmid profile patterns by comparing with migration positions of a 20 fragment DNA ladder ranging from 100 bp to 12.000 bp (Ready-load™ plus DNA ladder, Invitrogen).

Amplified-fragment length polymorphism (AFLP)

The fingerprinting of whole genome DNA by PCR was performed according to Vos et al. [72] and DNA was prepared using the method of Gevers et al. [23]. Briefly, purified total DNA was digested by the *Eco*RI and *Tag*I restriction enzymes and fragments were amplified by PCR using the following primer combinations: E01: 5′-GACTGCGTACCAATTCA-3′ and T03: 5′-CGATGAGTCCTGACCGAG-3′, E01 and T01: 5′-CGATGAGTCCTGACCGAA-3′ as well as T03 and E03: 5′-GACTGCGTACCAATTCG-3′. PCR products were separated on a high-resolution polyacryalamide gel using a DNA sequencer. Fragments that contained an adaptor specific for the halfsite created by the *Eco*RI enzyme was visualized due to the 5′-end labeling of the corresponding primer with the fluroscent dye FAM. The resulting electrophoretic patterns were normalized using the GeneScan 3.1 software (Applera, USA) and tables of peaks, containing fragments of 50–536 base pairs, were transferred into the BioNumerics™ ver. 3.5 software (Applied Maths, Belgium). For numerical analysis, data intervals were delineated between the 75- and 500-bp bands of the internal size standard. The three primer combinations gave a reproducibility of 72–75% and similarity between strains was calculated using a Dice coefficient and clustering was completed using UPGMA algorithm. AFLP patterns were compared with profiles of LAB reference taxa.

Antagonistic activity of isolates and sensitivity towards bacteriocins

The deferred inhibition test [9] was used to detect antagonistic activity by the *Carnobacterium* strains included in this study against the bacterial target cultures listed in Table 3. These target cultures included a pool of 9 strains of *C. divergens* consisting of 7 cluster G strains, 1 cluster E strain and 1 cluster Q strain and a pool of 10 strains of *C. maltaromaticum* consisting of 4 cluster H strains, 1 cluster C strain, 1 cluster K strain, 1 cluster O strain, 1 cluster P strain, 1 cluster V strain and one strain that was not included in the numerical taxonomic study. Phenotypic clusters are listed in Fig. 1.

The sensitivity of the *Carnobacterium* strains towards various known bacteriocin producers was also examined by use of the deferred inhibition test. The producer strains and the corresponding bacteriocins are listed in Table 3. These producers included three *C. divergens* cultures (1.1, 2.5 and ML1-102) for which the observed antagonistic activity was presumed to be due to bacteriocins although this has not been investigated in details.

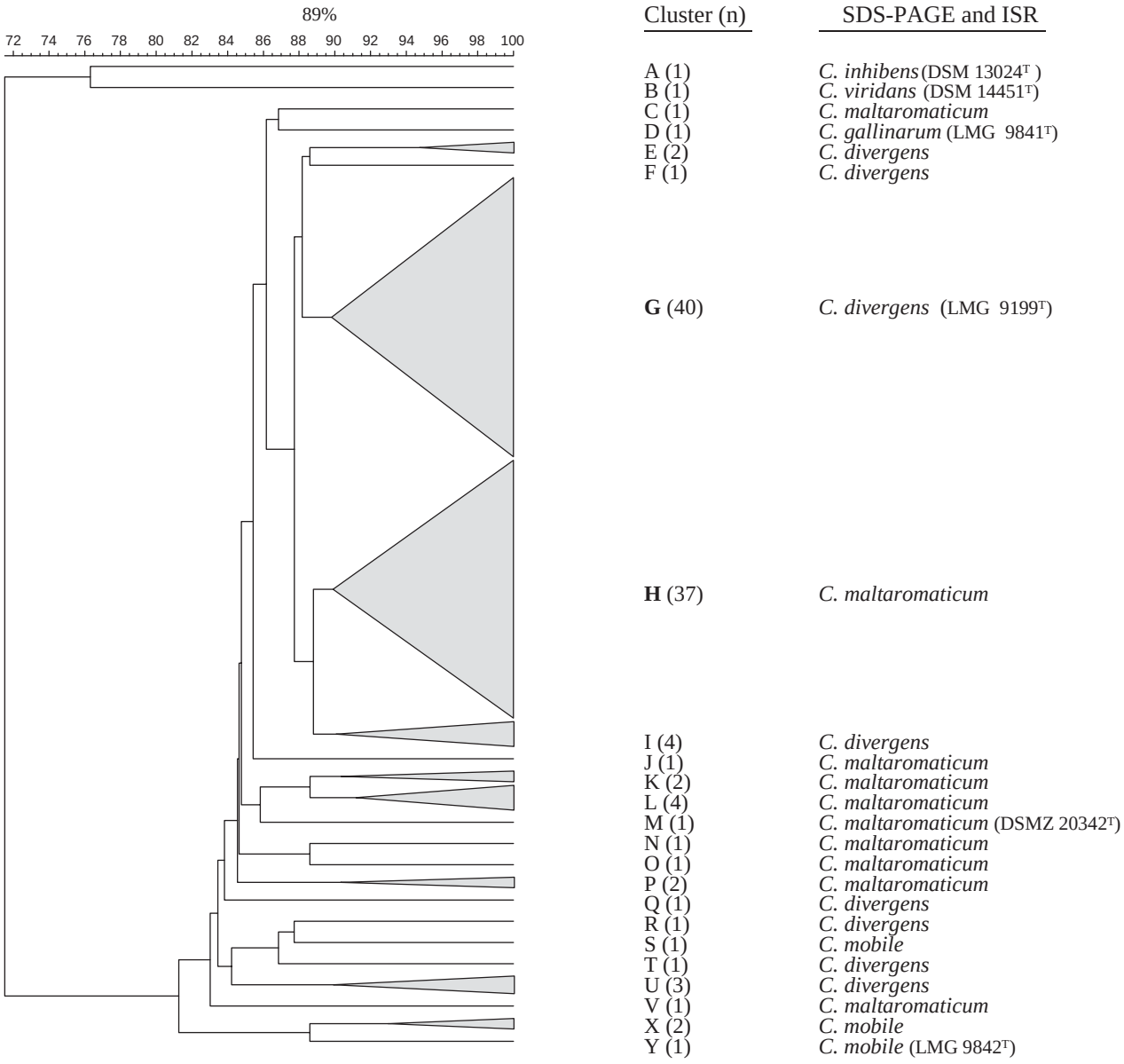


Fig. 1. Dendrogram, obtained from phenotypic characteristics of 108 carnobacteria isolates from meat (including strains from BCCM and NCFB) and seafood products and 6 reference strains. Clustering was performed with simple matching and UPGMA.

Results

Isolation of *Carnobacterium* from meat products

Thirty isolates of *C. divergens* and 14 isolates of *C. maltaromaticum* were obtained from beef, pork and processed pork. All isolates originated from the end of product shelf life as listed by the manufacturer. Isolates from vacuum packed beef and pork were not obtained in this study but originated from culture collections (Table 2).

Phenotypic characterization

Sixty six out of the original 180 unit characters were excluded from the final data matrix either because they showed no separation value (41 characters) or were not reproducible (i.e. showed less than 90% reproducibility). The remaining 114 unit characters distinguished the strains at an 89% similarity level with a significance of 99%. This allowed classification of the six *Carnobacterium* species studied into 24 distinct clusters consisting of *C. divergens* (eight clusters), *C. gallinarum* (one cluster),

C. inhibens (one cluster), *C. maltaromaticum* (ten clusters), *C. mobile* (three clusters), and *C. viridans* (one cluster) (Fig. 1). It should be noted, that the isolates in cluster S and X identified as *C. mobile* were confirmed to be non-motile and thus differed from the type strain [16].

One major cluster consisting of several strains was assigned for each of the two species *C. divergens* (cluster G) and *C. maltaromaticum* (cluster H), whereas the remaining clusters belonging to these two species consisted at most of four strains and in general they contained only one or two strains (Fig. 1). Phenotypic characters that contributed to differentiation of the major clusters of *C. divergens* and *C. maltaromaticum* from each other and from some of the minor clusters are included in Table 4. The major clusters of *C. divergens* and *C. maltaromaticum* were distributed on a number of meat and seafood products and thus showed no particular association with specific chilled foods or specific product characteristics (Tables 1,2).

Protein-based identification

It was possible to assign all strains to known *Carnobacterium* species and this identification matched the classification obtained by cluster analysis of phenotypes and ISR analysis (Fig. 1). No match was, however, obtained by comparison of sub-species clusters established by analyzing phenotypic characters and SDS-PAGE profiles of *Carnobacterium* strains.

Plasmid profiling

Approximately half of the *C. divergens* isolates contained no plasmids (53%) and the same result was obtained for *C. maltaromaticum* (62%). For the major phenotypic clusters of strains within the two species the presence of plasmids of different sizes showed considerable variation (Table 4). Twenty-seven percent of the *C. divergens* strains contained at least one plasmid and the same did 34% of the *C. maltaromaticum* strains. Only a minority of strains showed two plasmids bands or more, including 20% of *C. divergens* and 4% of *C. maltaromaticum*. It was confirmed that the three *C. mobile* strains isolated from brined shrimps all contained several identical plasmids [16] that differed from the plasmid pattern observed for the *C. mobile*-type strain LMG 9842^T. The majority of the observed plasmids had a large size (>25–30 kb) as shown in Table 3 for the major clusters of *C. maltaromaticum* and *C. divergens*.

ISR analysis

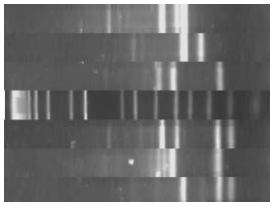
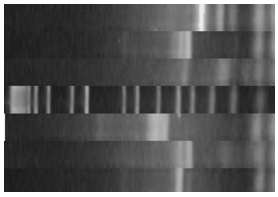
The RFLP patterns resolved the 83 isolates analysed into six species of *Carnobacterium* as shown in Table 5. The species identification was in agreement with

Table 4. Selected characteristics of *C. maltaromaticum* and *C. divergens*.

Tests	% positive			
	C. divergens		*C. maltaromaticum*	
Biochemical/fermentation tests	Cluster G	Cluster I	Cluster H	Cluster L
Production of acetoin	100	100	100	100
Acid from inulin	0	0	0	0
Acid from α-methyl-D-glucoside	0	0	8	25
Acid from α-methyl-D-mannoside	0	0	76	100
Acid from D-turanose	0	0	0	0
Acid from D-xylose	0	0	3	25
Sensitivity towards (>1 mm)				
Brochocin-C producer	100	100	100	100
Divergicin A producer	79	0	100	100
Nisin producer	100	100	100	100
Pediocin PA-1 producer	92	100	95	100
Sakacin-A producer	100	100	100	100
Inhibition of (zones >1 mm)				
B. thermosphacta[a] (LMG 17208)	0	0	0	75
L. monocytogenes (Scott A)	21	50	95	100
S. aureus (8325-4)	3	0	0	0
C. divergens-pool[a]	28	75	14	50
C. maltaromaticum-pool	8	50	49	100
Plasmids <25–30 kb	11	25	11	0
Plasmids >25–30 kb	42	25	31	0
No plasmids	56	50	58	100

[a]Inhibition zones >5 mm.

Table 5. ISR analysis based on 16S-23S ISR fragments (RFLP) of *C. maltaromaticum*, *C. divergens*, *C. mobile, C. gallinarum, C. inhibens* and *C. viridans* digested with *Hind III* (a) or with *HinfI* (b)

	Species	Strains (*n*)	*Hind* III fragment size (bp)
(a)			
	*C. maltaromaticum*	41	425, 470, 560
	C. divergens	31	350, 425
	C. mobile	4	270, 520, 620
	Ladder	—	100–12,000
	C. gallinarum	1	180, 250, 470, 560
	C. viridans	1	270, 520, 600, 660
	C. inhibens	1	200, 270, 425
	Species	Strains (*n*)	*Hinf* I fragment size (bp)
(b)			
	*C. maltaromaticum*	41	<100, 100, 200, 260, 340
	C. divergens	31	<100, 100, 420, 460
	C. mobile	4	<100, 100, 260, 460
	Ladder	—	100–12,000
	C. gallinarum	1	<100, 100, 590, 650
	C. viridans	1	<100, 100, 240, 420, 490
	C. inhibens	1	<100, 100, 460

Kabadjova et al. [32] although the band sizes differed in the two studies.

AFLP

The 30 analysed *C. divergens* strains were divided into two groups consisting of 12 and 18 strains (Fig. 2). Representatives of the major phenotypic cluster G within this species were distributed in each of the two AFLP groups. The primer combinations were not able to identify sub-groups of strains within the 25 *C. maltaromaticum* isolates and four *C. mobile* isolates analysed (Fig. 2).

Antagonistic activity of isolates

Fourteen strains of *C. divergens*, four strains of *C. maltaromaticum* and all four strains of *C. mobile* did not inhibit any of the indicator strains studied.

A majority of the remaining *C. maltaromaticum* strains showed a limited inhibition (zones less than 5 mm) of the indicator strains of *Listeria innocua* (results not shown) and *L. monocytogenes* (see Table 4 for results for major clusters) whereas relative few strains of *C. divergens* showed limited inhibition of these indicators (Table 4). The pool of *C. divergens* indicator strains were inhibited by 16 strains of *C. divergens* including 28% of the cluster G strains (Table 4) and 12 strains of *C. maltaromaticum* including 14% of the cluster H strains (Table 4). The pool of *C. maltaromaticum* strains were inhibited by 5 strains of *C. divergens* including 8% of the cluster G strains (Table 4) and 25 strains of *C. maltaromaticum* including 49% of the cluster H strains (Table 4). The remaining indicator strains were all inhibited by less than four strains of either *C. divergens* or *C. maltaromaticum*.

Very few strains showed a broad inhibitory spectrum. One *C. maltaromaticum* cluster L strain isolated from meat inhibited all indicator strains to some degree except both of the *Staphylococcus aureus* indicator strains and *Lactobacillus sakei* subsp. *carnosus* (LMG 21529). There was no correlation between antagonistic activity and the possession of plasmids (data not shown).

Sensitivity towards bacteriocins

All strains were sensitive to the nisin producer *Lactococcus lactis* subsp. *lactis* ATCC 11454 (see Table 4 for major clusters) whereas 17 strains of *C. divergens* (including the divergicin A producer; five strains which clustered together within cluster G and all four cluster I strains), one strains of *C. maltaromaticum* and one strain of *C. mobile* (5 kb-7) were resistant to the *C. divergens* NCFB 2855 divergicin A producer. Only four strains of *C. divergens* and five strains of *C. maltaromaticum* were resistant towards the *Pediococcus acidilactici* PAC-1.0 pediocin PA-1 producer and one strain of *C. maltaromaticum* was resistant towards the *Brochothrix campestris* DSMZ 4712 brochocin-C producer and the *Lactobacillus sakei* DSMZ 6333 sakacin-A producer.

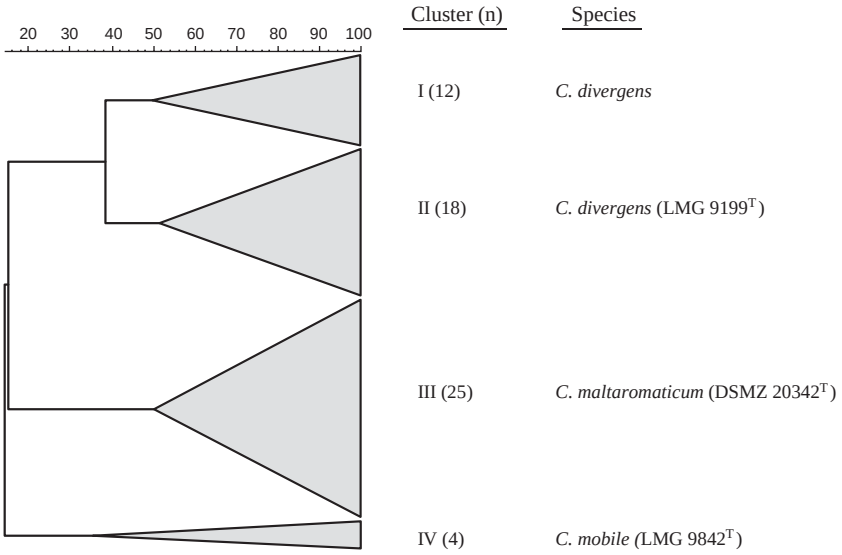


Fig. 2. Dendrogram, obtained from AFLP, of 59 *Carnobacterium* strains. Clustering of data from three primer combinations was performed with the software BioNumerics using the Dice similarity coefficient and UPGMA.

Discussion

The present study focused on *Carnobacterium* from various meat and seafood products and included a larger number of *C. divergens* and *C. maltaromaticum* isolates than previous studies [24,33]. The 52 seafood isolates have been studied previously and identified as *C. divergens* (7 isolates) and *C. maltaromaticum* (14 isolates) but 60% of the studied seafood isolates were initially not identified at the species level (see references in Table 2). Several of these isolates were characterized by a limited number of biochemical tests including their fermentation of inulin and mannitol but not α-methyl-D-glucoside and α-methyl-D-mannoside. The present study confirmed previous identification of these isolates and showed the isolates not previously identified at the species level to be *C. divergens* (11 isolates) or *C. maltaromaticum* (17 isolates). In addition, we found isolates from brined shrimps to be *C. mobile* although they were confirmed to be non-motile.

None of the phenotypic tests included in the present study clearly differentiated *C. divergens* and *C. maltaromaticum* at the species level (Table 4). This was surprising as simple identification keys relying, e.g. on fermentation of inulin, mannitol, α-methyl-D-glucoside and α-methyl-D-mannoside and use of the API 50 CH system has been suggested previously [12]. Lai and Manchester [33] when using the fermentation medium (SBM) of Wilkinson and Jones [73] however found, that fermentation of α-methyl-D-glucoside was the only test that clearly differentiated *C. divergens* and *C. maltaromaticum*. We compared the API 50 CH system and the SBM fermentation medium for ten *C. maltaromaticum* isolates (results not shown). For these isolates inulin and α-methyl-D-glucoside were fermented by 80% and 100% in the SBM medium but only by 0% and 20% of the ten isolates when the API 50 CH system was applied. Clearly, simple identification keys must be used with caution and the procedures used to evaluate acid production from carbohydrates must be taken into account.

The biochemical and physiological heterogeneity of *C. divergens* and *C. maltaromaticum* observed in the present study concur with previous less extensive studies of *Carnobacterium* [24,33]. Clearly, phenotypic testing is time consuming but when a larger number of phenotypic tests are used and data evaluated by numerical taxonomy this allow isolates of *Carnobacterium* to be identified at the species level with the same degree of confidence as obtained, e.g. by SDS-PAGE electrophoresis of whole cell proteins (Fig. 1), ribotyping [43], PCR assays using 16S rDNA-directed primers [2,44] or by ISR analysis (this study, [10,32,65]). In the present study we used the ISR analysis developed by Kabadjova et al. [32] and confirmed the ability of this method to identify isolates of *Carnobacterium* at the species level (Table 5). Using different PCR equipments and different batches of reagents we, however, found variable RFLP patterns for identical isolates (results not shown). Thus, analysis of references strains was imperative for species identification of *Carnobacterium* isolates by this method.

It is clear that molecular typing techniques in addition to other modern typing techniques will be most useful for further elucidating the possible presence of subspecies within *C. divergens* and *C. maltaromaticum*. Typing by numerical taxonomy [33] and by pyrolysis mass spectrometry [45] or Fourier transform infrared

spectroscopy [35] identified some corresponding subgroups within *C. maltaromaticum* or *C. divergens*. This was, however, in contrast to a comparison by Montel et al. [49] between numerical phenetic and DNA:DNA relatedness data. The presence of sub-species within *C. divergens* has also been suggested by use of the RAPD-PCR technique [34]. Surprisingly AFLP analysis in the present study did only to a limited extent demonstrate multiple stable intra-species groups in *C. divergens* and not at all in *C. maltaromaticum* although the technique is generally used for this purpose [71]. The present study has therefore not demonstrated whether the observed major *C. maltaromaticum* phenotypic cluster H is in fact monophyletic although this was shown not to be so for the major *C. divergens* cluster G.

The two major clusters (G and H) did not show association to any particular source of food (Table 1) and the primary habitat of *C. divergens* and *C. maltaromaticum* is not known. *C. maltaromaticum* (and in a few cases also *C. divergens*) were members of the bacterial flora found on various fish species, including fresh water fish [24], bass and catfish [5], Arctic charr [59,61–63], salmon [60], trout [68], cod and wolf fish [62]. In addition, in one case *C. maltaromaticum* was isolated from human pus caused by a traumatic amputation of a hand and, as the amputation occurred in a water sawmill, water might have been the source of the bacteria [11]. Both *C. divergens* and *C. maltaromaticum* are isolated from sources that cannot be immediately linked to the aquatic environment such as milk, cheese [26,50] and meat products ([2,30,47,49,64], this study).

For the remaining species of carnobacteria only a few isolates are described and therefore their primary habitate cannot be assessed with any great degree of accuracy. *C. funditum* and *C. alterfunditum* are isolated from Antarctic lakes and *C. alterfunditum* is also isolated from rainbow trout [68], *C. inhibens* from intestine of Atlantic salmon, *C. mobile* from shrimps and meat products and *C. gallinarum* and *C. viridans* from meat products.

Both *C. divergens* and *C. maltaromaticum* are able to grow in a wide variety of refrigerated raw and processed meat and seafood products stored aerobically, vacuum-packaged or packaged under MAP conditions (Tables 1,2). They survive freezing and are able to grow and produce tyramine in products after thawing [20,25]. They grow readily in presence of preservation additives such as acetic acid, lactic acid (this study, Table 2), ascorbic acid (this study, results not shown), $NaNO_2$ (information from the product manufacturer) added to processed pork meat (this study, Table 2), presence of benzoic, citric and sorbic acids in brined shrimps [16] as well as in the presence of smoke components [13,31,54,71], this study) including phenols in concentrations at least up to $14.6 \pm 3.7\,mg\,kg^{-1}$ [36]. Both *C. divergens* and *C. maltaromaticum* therefore appears to possess an extensive potential spoilage domain and it seems important to evaluate the actual spoilage potential of the two species.

The reason(s) for the observed discrepancy between the ability of *Carnobacterium* spp. to predominate a large range of meat and seafood products and their documented ability to spoil a limited number of these products is not known. Strains belonging to one or both of these two species produce compounds of importance for the sensory quality of foods including compounds with a disagreeable odour such as ammonia (from arginine, e.g. [38]), acetate [6], diacetyl [29], 3-methylbutanal, 2-methylproponal, 3-methylbutanol and 2-methylpropanol [8]. *C. divergens* may also produce hydrogen peroxide [6] that causes green discoloration of meat.

Carnobacterium spp. may be potential spoilage organisms in meat products. Thus, isolates of *C. maltaromaticum* are able to spoil inoculated beef stored under vacuum at 2 °C and subsequently in air at 7 °C [40] as well as cooked sliced ham [8]. *C. divergens* produced odours (acid, buttery and slightly sulphurous) in normal as well as high pH beef stored in vacuum at 4 °C for 30 days [7]. Furthermore *C. viridans* may spoil vacuum-packaged bologna sausage, presumably as a post-pasteurization contaminant [27,55].

Carnobacterium spp. may add a buttery/rubbery odour to cold-smoked salmon although the products were not sensory rejected [29,42,69]. Others have found that sensory rejection of this product was due to other groups of bacteria or autolytic changes [31,54]. *Carnobacterium* spp. probably contributes to spoilage of chilled, cooked and brined MAP shrimp [16]. Their ability for metabolizing arginine [38] that is present in high amounts in shrimps will result in formation of ammonia with a negative effect on the sensory quality of this product.

In conclusion *C. divergens* and *C. maltaromaticum* are known to be able to grow under a wide range of storage conditions. Thus, *C. divergens* and *C. maltaromaticum* might be spoilage organisms associated with certain products of meat and seafood or this ability may instead be limited to some lineages within these species. We are currently testing whether selected isolates belonging to the two major phenotypic clusters of *C. divergens* and *C. maltaromaticum* are able to spoil shrimps products and pork meat. In addition we are examining whether the few strains obtained in this study that showed a potential for biopreservation, including some of the *C. maltaromaticum* strains belonging to cluster L (Table 4), might be spoilage organisms in selected meat or seafood products. One study by Duffes [19] showed that vacuum-packed, refrigerated cold-smoked salmon was not spoiled by addition of bacteriocin-producing *C. divergens* or *C. maltaromaticum*.

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