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Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals

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Summary

Members of most *Chryseobacterium* species occur in aquatic environments or food products, while strains of some other species are pathogenic to humans and animals. A collection of 52 *Chryseobacterium* sp. strains isolated from diseased fish, one frog isolate and 22 reference strains were included in a polyphasic taxonomy study. Fourteen clusters of strains were delineated following the comparison of whole-cell protein profiles. Most of these clusters were confirmed when the phenotypic and RAPD profiles and the 16S rRNA gene sequences were compared. Fatty acid composition helped differentiate the *Chryseobacterium* strains from members of related genera. None of the fish isolates could be allocated to the two species previously reported from fish but two isolates belonged to *C. joostei*, while the frog isolate was identified as *Elizabethkingia meningoseptica*, a human pathogen previously included in the genus *Chryseobacterium*. Three clusters grouping from 3 to 13 isolates will probably constitute the core of new *Chryseobacterium* species but all other isolates occupied separate or uncertain positions in the genus. This study further demonstrated the overall high similarity displayed by most *Chryseobacterium* strains whatever the technique used and the resulting difficulty in delineating new species in the genus. Members of this bacterial group should be considered potential emergent pathogens in various fish and frog species, farming conditions and geographical areas.

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Abbreviation: RAPD, randomly amplified polymorphic DNA

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Introduction

The genus *Chryseobacterium* was proposed to accommodate six bacterial species previously included in the genus *Flavobacterium* [41]. Following an extensive

polyphasic study, the whole group of organisms related to the genera *Chryseobacterium* and *Flavobacterium* was included in the family *Flavobacteriaceae* [4,5]. The genus *Chryseobacterium* currently comprises nine valid species and a non-valid one representing a variety of ecological niches [3,20–22,41,44,46]. Five species, namely *C. indoltheticum* [10], *C. defluvii* [21], “*C. proteolyticum*” [44], *C. formosense* [46] and *C. daecheongense* [22] were isolated from various soil and aquatic environments. *Chryseobacterium* spp. strains are also part of the psychrotrophic and proteolytic bacterial population that causes a variety of defects in food products such as processed meats, chilled fish and shellfish, and dairy products [14,18]. The polyphasic study of a group of dairy isolates recently resulted in the description of *C. joostei* [19,20]. Other *Chryseobacterium* species are pathogenic to humans and animals. *Chryseobacterium gleum* and *C. indologenes* may cause nosocomial infections in humans, usually in neonates or immunocompromised patients; they can also be retrieved from hospital environments and equipments and are usually resistant to many disinfectants and antibiotics [3]. The 16S rRNA gene sequences of the putative new clinical species “*C. massiliae*” and “*Candidatus Chryseobacterium timonae*” have recently been deposited in GenBank [12]. Finally, *C. balustinum* [6,16] and *C. scopthalmum* [28,29] were isolated from diseased fish. *Chryseobacterium* spp. strains were also detected among the bacterial flora in the gut of insects by direct amplification and sequencing of 16S rRNA genes [13], but they have not been fully characterized. Until recently, two more bacterial species were allocated to the genus *Chryseobacterium*, i.e. *C. meningosepticum* and *C. miricola*. However, owing to their separate position compared to other *Chryseobacterium* species in 16S rRNA gene sequence comparison studies, their transfer to the new genus *Elizabethkingia* was proposed [23]. Hence, the new combinations *Elizabethkingia meningoseptica* and *E. miricola* will be used thereafter. The latter species was isolated from condensation water in the space station Mir [25] whereas the former is considered the most serious human pathogen in the group [3]. Since *E. meningoseptica* was also isolated from diseased birds [39], frogs, turtles and cats [11,15 and references therein,26], it may be the agent of zoonotic infections.

Over the last few years, fish pathology laboratories in Europe (Belgium, Finland, France) and Asia (Taiwan, Singapore) have isolated an increasing number of bacterial strains that shared the basic phenotypic characteristics of the genus *Chryseobacterium* and that were tentatively identified as *Chryseobacterium* sp., *C. indologenes* or *E. meningoseptica* using commercial identification galleries. In this paper, we present the results of the polyphasic study [42] of 52 fish isolates and one frog isolate.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. Except one frog isolate, all isolates were retrieved from diseased fish between 1994 and 2001. The host fish represented a variety of fish species, farming conditions, and geographical origins. About one-third of the strains were isolated from superficial lesions in otherwise apparently healthy fish while the other strains were isolated from internal organs of fish or frog suffering from hemorrhagic septicemia. Bacterial strains were isolated from samples inoculated on trypticase soy agar (bioMérieux sa, Marcy-l'Étoile, France) incubated at 22 or 26 °C for 3 days. After the basic common characteristics (i.e., Gram-negative, non-motile rods, producing bright yellow-orange flexirubin type pigments, a strong smell, catalase and oxidase, and yielding a *Chryseobacterium* spp. profile in API 20E and 20NE galleries) of these strains were identified, a retrospective screening of the culture collection of the Unité de Virologie et Immunologie Moléculaires yielded three poorly characterized fish isolates (i.e., JIP 105/82, JIP 108/83, and SAVU 12/90), tentatively labelled “*Flavobacterium*-like” or “*Flavobacterium* sp.” at the time of preservation, that shared the same basic characteristics and were consequently included in the study. The type strain of six valid *Chryseobacterium* species and of *E. meningoseptica* were also studied, as well as a second strain of each species. In the particular case of *C. scopthalmum*, a very high DNA homology had been demonstrated previously between the seven strains available [29]. Hence, all seven strains were included in this study in order to test the repeatability of the techniques used there after as well as their ability to reveal intra-specific variability. Since *Bergeyella zoohelcum*, *Riemerella anatipestifer* and *R. columbina* [40,41] form a distinct separate branch together with the *Chryseobacterium* and *Elizabethkingia* species in published 16S rRNA gene sequence similarity dendrograms [e.g., 3,4,20,22,25], the type strains of these three species were also included in the study. The other members of the *Chryseobacterium*–*Elizabethkingia*–*Riemerella*–*Bergeyella* branch were not studied, either because they were published after this study was completed (*C. defluvii* [21], *C. formosense* [46], *C. daecheongense* [22], *E. miricola* [25], *Kaistella koreensis* [24], *Sejongia antarctica* and *S. jeonii* [45]) or because they were not available (“*Haloanella gallinarum*” [Pham, unpublished] and “*Chryseobacterium proteolyticum*” [44]). However, their 16S rRNA gene sequences were included in the phylogenetic study (see below).

Table 1. Bacterial strains included in this study

Name as received	Strain ^a	Cluster number ^b	Sequence number ^c	Source ^d
<i>Elizabethkingia meningoseptica</i>	LMG 4027 ^T CIP 60.57 ^T	11	AY468445	Cerebrospinal fluid, premature infant, Massachusetts, USA, 1949
<i>Elizabethkingia meningoseptica</i>	LMG 12280	11	AY468480	Blood, newborn infant, Florida, USA, 1950
<i>Chryseobacterium scophthalmum</i>	LMG 13028 ^T CIP 104199 ^T	12		Gills, juvenile turbot (<i>Scophthalmus maximus</i>) with hemorrhagic septicemia, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13029	12		Gills, diseased turbot, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13030	12		Diseased turbot, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13031	12		Diseased turbot, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13032	12		Seawater, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13033	12		Seawater, Scotland, UK, 1987
<i>Chryseobacterium scophthalmum</i>	LMG 13034	12		Gills, diseased turbot, Scotland, UK, 1987
<i>Chryseobacterium balustinum</i>	LMG 8329 ^T CIP 103103 ^T		AY468447	Heart blood, dace (<i>Leuciscus leuciscus</i>), Dordogne river, Aquitaine, France, 1959
<i>Chryseobacterium balustinum</i>	LMG 12856	5	AY468481	Water, hospital, London, UK, 1978
<i>Chryseobacterium indoltheticum</i>	LMG 4025 ^T CIP 103168 ^T	10	AY468448	Marine mud
<i>Chryseobacterium indoltheticum</i>	LMG 13342	10	AY468444	London, UK
<i>Chryseobacterium gleum</i>	LMG 8334 ^T CIP 103039 ^T	14	AY468449	High vaginal swab, London, UK, 1979
<i>Chryseobacterium gleum</i>	LMG 12450	14		Wound swab, London, UK
<i>Chryseobacterium indologenes</i>	LMG 8337 ^T CIP 101026 ^T	5	AY468450	Human trachea at autopsy, 1958
<i>Chryseobacterium indologenes</i>	LMG 12452	5		No data available
<i>Chryseobacterium joostei</i>	LMG 18212 ^T CIP 105533 ^T	3		Raw cow milk, Ixopo district, Kwazulu-Natal, South Africa, 1981
<i>Chryseobacterium joostei</i>	LMG 18208	3	AY468479	Raw cow milk, Heidelberg district, Transvaal, South Africa, 1981
<i>Bergeyella zoohelcum</i>	LMG 8351 ^T CIP 103041 ^T			Human sputum, Nebraska, USA
<i>Riemerella anatipestifer</i>	LMG 11054 ^T CIP 82.28 ^T			Duck blood, USA
<i>Riemerella columbina</i>	LMG 11607 ^T CIP 106288 ^T			Pigeon palatine cleft, Germany, 1989
	JIP 105/82		AY468457	Skin lesion, Koi carp, (<i>Cyprinus carpio</i>), Ile-de-France, France, 1982 (Japan)
	JIP 108/83		AY468458	Extensive skin ulcer, snakehead (<i>Channa</i> sp.), France, 1983 (Thailand)

Table 1. (continued)

Name as received	Strain ^a	Cluster number ^b	Sequence number ^c	Source ^d
	SAVU 12/90		AY468459	Skin lesion, sea bass (<i>Dicentrarchus labrax</i>), Mediterranean coast, France, 1990
	UOF CO294		AY427792	Gills, brown trout (<i>Salmo trutta</i>), Finland, 1994
	UOF CR694 LMG 22906 CIP 108621	3		Tail lesion, Atlantic salmon (<i>Salmo salar</i>), Finland, 1994
	UOF CR1094 LMG 22907 CIP 108622	3	AY468451	Deep ulcerative dorsal lesion, Atlantic salmon, Finland, 1994
	UOF CM295		AY468452	Kidney, brown trout, Finland, 1995
	UOF CM895		AY468453	Kidney, Atlantic salmon, Finland, 1995
	UOF CR2995	4	AY468454	Deep ulcerative dorsal lesion, Atlantic salmon, Finland, 1995
	UOF CR4395	4	AY468455	Peduncle lesion, Atlantic salmon, Finland, 1995
	UOF CR4095		AY468483	Deep ulcerative dorsal lesion, Atlantic salmon, Finland, 1995
	UOF CO496 LMG 22914 CIP 108623	6		Jaw erosion, brown trout, Finland, 1996
	UOF CM1396 LMG 22905 CIP 108620	6	AY468456	Kidney, Atlantic salmon, Finland, 1996
	UOF CM1996 LMG 22915 CIP 108624	6		Kidney, Atlantic salmon, Finland, 1996
	UOF CM1796			Kidney, brown trout, Finland, 1996
	ZIL K4U	1		Skin ulcer, Koi carp, Belgium, 1996
	ZIL K5U	1		Skin ulcer, Koi carp, Belgium, 1996
	ZIL K5M1	1		Muscle lesion, Koi carp, Belgium, 1996
	ZIL K5M2	1		Muscle lesion, Koi carp, Belgium, 1996
	ZIL K5BV	1		Skin ulcer, Koi carp, Belgium, 1996
	ZIL K3.2 LMG 19173 CIP 108619	1	AJ874979	Tail lesion, Koi carp, Belgium, 1996 (Japan)
	ZIL K4.3	1		Tail lesion, Koi carp, Belgium, 1996 (Japan)
	ZIL K6.1	1		Skin lesion, Koi carp, Belgium, 1996 (Japan)
	ZIL K12.1	1		Mouth lesion, Koi carp, Belgium, 1996 (Japan)

Table 1. (continued)

Name as received	Strain ^a	Cluster number ^b	Sequence number ^c	Source ^d
	ZIL K13.1	1		Mouth lesion, Koi carp, Belgium, 1996 (Japan)
	JIP 16/96		AY468460	Internal organs, dwarf gourami (<i>Colisa lalia</i>), France, 1996 (Singapore)
	JIP 17/96 LMG 22908 CIP 108646	2	AY468461	Kidney, leopard corydoras (<i>Corydoras julii</i>), France, 1996 (USA)
	LPAA 3120	9	AY468462	Skin lesion, rainbow trout (<i>Oncorhynchus mykiss</i>), Brittany, France, 1996
	LPAA 3230	9		Skin lesion, rainbow trout, Brittany, France, 1996
	FRGDSA 1502/97		AY468463	Kidney, sturgeon (<i>Acipenser sturio</i>), Landes, France, 1997
	FRGDSA 4034/97		AY468464	Skin ulcer, rainbow trout, Landes, France, 1997
	FRGDSA 4580/97		AY468465	Siberian sturgeon (<i>Acipenser baeri</i>) fry, Landes, France, 1997
	LDA39 G1966 4a	13	AY468466	Skin lesion, Asian catfish (<i>Pangasius bocourti</i>), Kompong Chhnang, Cambodia, 1997
	LDA39 G1966 12a	13	AY468467	Fin lesion, Asian catfish (<i>Pangasianodon hypophthalmus</i>), Kompong Chhnang, Cambodia, 1997
	LDA39 G1966 12c	13	AY468468	Spleen, Asian catfish (<i>Pangasianodon hypophthalmus</i>), Kompong Chhnang, Cambodia, 1997
	LDA39 G1966 13a	13	AY468469	Skin lesion, walking catfish (<i>Clarias</i> sp.), Kompong Chhnang, Cambodia, 1997
	FRGDSA 11/99 LMG 22909 CIP 108647	1		Skin ulcer, Koi carp, Landes, France, 1999
	LDVH 33/99	8	AY468470	Goldfish (<i>Carassus auratus</i>), Hérault, France, 1999 (P. R. China)
	LDVH 1		AY468472	Internal organs, goldfish, Hérault, France, 2000 (P. R. China)
	LDVH 2 LMG 22910 CIP 108648	1		Internal organs, Koi carp, Hérault, France, 2000 (Japan)
	LDVH 3	8	AY468484	Skin ulcer, goldfish, Hérault, France, 2000 (Italy)
	LDVH 4	8	AY468473	Internal organs, goldfish, Hérault, France, 2000
	LDVH 5 LMG 22911 CIP 108649	1		Internal organs, goldfish, Hérault, France, 2000 (Italy)

Table 1. (continued)

Name as received	Strain ^a	Cluster number ^b	Sequence number ^c	Source ^d
	LDVH 42/00	7	AY468475	Internal organs, Malawi lake Cichlid, Hérault, France, 2000
	LDVH 43/00 LMG 22912 CIP 108650	2	AY468476	Internal organs, kuhli loach (<i>Acanthopthalmus kuhlii</i>), Hérault, France, 2000
	LDVH 44/00 LMG 22913 CIP 108651	2		Internal organs, Malawi lake Cichlid, Hérault, France, 2000
	LDVH 45/00	7		Internal organs, Malawi lake Cichlid, Vosges, France, 2000
	JG-Torno		AY468471	Liver, rainbow trout, Spain, 2000
	JG-2			Skin lesion, rainbow trout, Asturias, Spain, 2000
	JIP 13/00 (2)		AY468474	Muscle lesion, neon tetra (<i>Paracheirodon innesi</i>), France, 2000 (Hong Kong)
	JIP 06/01 (1)		AY468446	Internal organs, rainbow trout, Yvelines, France, 2001
	NTU 870424-IL CIP 108652	11	AY468477	Internal organs, cultured bullfrog (<i>Rana catesbeiana</i>) with hemorrhagic septicemia, Taiwan, 1986
	LMG 19914		AY468478	Apparently healthy cultured European eel (<i>Anguilla anguilla</i>), Belgium

^aCIP, Collection de l'Institut Pasteur, Paris, France; FRGDSA, strains provided by P. Daniel, then at Fédération Régionale des Groupements de Défense Sanitaire d'Aquitaine, Section Aquacole, Mont-de-Marsan, France; JG, strains provided by J.A. Guijarro, University of Oviedo, Oviedo, Spain; JIP, culture collection of the Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Jouy-en-Josas, France; LDA39, strains isolated by P. Girard and provided by M. Morand, Laboratoire Départemental d'Analyses du Jura, Lons-le-Saunier, France; LDVH, strains provided by N. Keck, Laboratoire Départemental Vétérinaire de l'Hérault, Montpellier, France; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; LPAA, strains provided by A. Abiven, Agence Française pour la Sécurité Sanitaire des Aliments Site de Brest, Laboratoire d'Études et de Recherches en Pathologie des Poissons, Plouzané, France; NTU, strain provided by H.-Y. Chung, National Taiwan University, Taipei, Taiwan; SAVU, strain provided by J.-C. Raymond, Service Aquacole Vétérinaire d'Urgence, Montpellier, France; UOF, strains provided by P. Rintamäki-Kinnunen, Department of Biology, University of Oulu, Oulu, Finland; ZIL, culture collection of the Laboratory for Ecology and Aquaculture, Zoological Institute, Leuven, Belgium.

^bNumbers of the clusters that emerged from SDS-PAGE analysis (Fig. 2).

^cOnly those 16S rRNA gene sequences that were determined in the course of this study are listed.

^dFish isolates are listed according to their year of isolation. The country where each strain was isolated is cited; for recently imported ornamental fish, the name of the country of origin is specified in parentheses when available.

Culture conditions and phenotypic study

All strains were cultivated on nutrient (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) or trypticase soy agar or in the corresponding broth at 26 °C, unless otherwise stated. The minimal standards proposed for describing new taxa of the family *Flavobacteriaceae* [4] were followed.

The morphology (i.e., shape, size, aspect of the edge, color), odor and consistency of the colonies were

recorded. Twenty-four-hour cultures in Anacker and Ordal's broth [1] were used to test bacterial motility using the hanging drop method and phase-contrast microscopy, to perform the Gram staining, and to record the bacterial morphology. The presence of flexirubin type pigments was tested by observing the color shift when a small mass of bacterial cells collected on agar and deposited on a glass slide was flooded with a 20% (w/v) KOH aqueous solution [4,31]. The type of respiratory metabolism was determined by inoculating

deep tubes of meat-liver medium containing 6% agar (BIO-RAD, Marnes-la-Coquette, France). The following characteristics were tested according to West and Colwell [43]: hydrolysis of sodium alginate; hydrolysis of starch, deoxyribonucleic acid, gelatin and Tween 80 (tested both on plain nutrient agar [containing 0.5% NaCl] and on the same medium enriched to 1.5% NaCl); production of indole; reduction of nitrate; and growth in 0.5%, 1.5%, 3%, 6%, 8%, and 10% NaCl nutrient broth. The oxidase reaction was performed on oxidase test discs (bioMérieux sa) and the presence of catalase was assessed using 3% (v/v) H₂O₂. Hemolysis was recorded on trypticase soy agar supplemented with 5% (v/v) sheep blood (bioMérieux sa). Trypticase soy agar was also used to test the temperature tolerance at 5, 26, 37, and 42 °C. Bacterial growth was tested on the following solid media: MacConkey no. 3 (Oxoid Limited, Basingstoke, England), thiosulfate-citrate-bile-sucrose (TCBS; Difco), brain–heart infusion (Difco), Mueller Hinton (Difco), marine 2216 (Difco), and cetrinide [9]. The following tests were performed according to Barrow and Feltham [2]: fermentative versus oxidative metabolism of glucose (O/F test); presence of arginine dihydrolase and of lysine and ornithine decarboxylases; hydrolysis of L-tyrosine and pigment production on 0.5% L-tyrosine nutrient agar; formation of a precipitate on 10% egg yolk nutrient agar; and acid production from carbohydrates in ammonium salt medium (the following carbohydrates were tested at a 1% final concentration: arabinose, cellobiose, glucose, ethanol, fructose, galactose, glycerol, lactose, maltose, mannitol, mannose, melibiose, saccharose, sorbitol, trehalose, and xylose). The following characteristics were tested according to Smibert and Krieg [35]: hydrolysis of aesculin and casein; methyl red test and Voges–Proskauer reaction; production of acid and alkaline phosphatases and of urease; and the reactions on Kligler iron agar (Difco), namely the fermentation of glucose and lactose and the production of gas and H₂S. Hydrolysis of elastin was tested according to Hsu et al. [17]. Growth on sodium citrate as the sole source of carbon was tested on Simmons' citrate agar (Difco). The production of L-phenylalanine deaminase was tested using the technique of Richard and Kiredjian [32]. All tests were read after 3 days of incubation, except for the acidification of sugars, the utilisation of citrate on Simmons' citrate agar, the degradation of aesculin, the production of urease, and the growth at 4 °C and on the various agar media, which were scored after 1 week of incubation. The reactions in API 20E, 20NE, and ZYM galleries (bioMérieux sa) were assessed following the producer's prescriptions except for the incubation temperature (26 °C) and time (48 h). The sensitivity of all strains to a selection of antibiotics was also investigated; it is the subject of another paper [27].

Biotype 100 galleries

Utilization of carbon sources was investigated using Biotype 100 galleries (bioMérieux sa) that contained 99 pure carbon sources. A flask of Biotype medium 2 was inoculated with 2 ml of a bacterial suspension in distilled water calibrated at McFarland density index 4. The cupules were filled with 400 µl of inoculated medium and the galleries were incubated for 4 days at 30 °C. The galleries were checked daily for visible growth. Growth was scored visually by comparison with the aspect of the control cupule containing no carbon source. For the 10 strains that showed no visible growth at 30 °C, new galleries were inoculated and incubated at 25 or 37 °C.

Analysis of phenotypic data

Those characteristics that were positive or negative for all strains studied (see below) were not included in the analysis. Only those characteristics that gave variable results among the strains, and were thus considered discriminant, were included in the data analysis, with the following exceptions. Those characteristics that were tested using different methods or conditions (i.e., using a conventional procedure and one of the tests in API galleries, or on both plain and 1.5% NaCl nutrient agar) yielding identical results for all strains studied were included only once. This was the case for the hydrolysis of aesculin and Tween 80, and for the production of urease and indole. Conversely, when some strains yielded different results for a given characteristic using different tests, the different sets of data were included in the analysis. For instance, the results of the ONPG, PNPG, and no. 14 tests (in API 20E, 20NE and ZYM galleries, respectively) for the detection of β -galactosidase were all included. Also deleted from the analysis were those tests that proved less sensitive (i.e., that yielded a lower number of positive results) than the other tests used to detect the same characteristic. Hence, the conventional tests for nitrate reduction, hydrolysis of gelatin, and production of acid and alkaline phosphatases, that proved less sensitive than their counterparts in API galleries, were deleted from the analysis. Conversely, the API 20E test for bacterial growth on Simmons' citrate, less sensitive than its conventional counterpart, was deleted. The API 20E test for gelatin hydrolysis detected a lower number of positive strains than the corresponding test in API 20NE galleries and was consequently deleted from the analysis. Also deleted were the conventional tests performed on 1.5% NaCl nutrient agar for the hydrolysis of DNA and starch, since they were less sensitive than the same tests performed on plain nutrient agar.

The 111 differential phenotypic characteristics included in the analysis were grouped as follows: Group 1: four different colony types (yellow, brown, small and mucous); Group 2: three different growth temperatures (5, 37 and 42 °C); Group 3: 17 conventionally tested biochemical characteristics (growth on Simmon's citrate and acidification of the 16 above-mentioned carbohydrates); Group 4: 10 other conventionally tested biochemical characteristics (production of flexirubin type pigment, brown pigment on tyrosine agar, precipitate on egg yolk agar and phenylalanine deaminase; and hydrolysis of casein, DNA, starch, Tween 80, elastin, and tyrosine); Group 5: degradation of 18 substrates in APY ZYM galleries (i.e., all substrates except naphthol-AS-BI- β -D-glucuronide); Group 6: 17 characteristics in API 20E (ONPG) and API 20NE (i.e., all tests except fermentation of glucose, production of arginine dihydrolase and assimilation of caprate and malate) galleries; Group 7: assimilation of 38 substrates in Biotypel00 galleries; and Group 8: growth on/in four media (Mueller-Hinton, marine 2216 and cetrimide agars and 3% NaCl nutrient broth). For analysis, the results were expressed as positive/negative (colony types, most conventional biochemical tests, and tests in API 20E, 20NE and Biotype 100 galleries), or as intensities from 0 to 3 (growth on different media and at different temperatures, hydrolysis of tyrosine and production of pigment on tyrosine agar, production of phenylalanine deaminase, and formation of a precipitate on egg yolk agar) or from 0 to 5 (API ZYM galleries). A distance matrix was calculated for each group of characteristics using the BioNumerics software (Applied Maths, Sint Martens-Latem, Belgium). The Gower similarity coefficient was used for non-binary data while the simple matching coefficient and the Jaccard coefficient were used for binary data. A resulting distance matrix was calculated using the eight individual matrices.

Fatty acid methyl ester (FAME) analysis

Cultures were grown for 24 h at 28 °C on plates containing 30 g trypticase soy broth (BBL 11768, Becton Dickinson Microbiology Systems, Sparks, MD) supplemented with 15 g Bacto agar (Difco) per liter distilled water. Cells were saponified, methylated to fatty acid methyl esters (FAMES) and extracted following the recommendations of the commercial identification system MIDI (Microbial Identification System Inc., Newark, DEL). Fatty acid methyl esters were separated on a Agilent 6890A Series Gas Chromatograph with 7683 autoinjector and autosampler tray module (Agilent Technologies). Separation of FAMES was achieved with a fused-silica capillary column (25 m \times 0.2 mm) with cross-linked 5% phenylmethyl silicone (film thickness 0.33 μ m; HP Ultra 2). H₂ was serving as carrier gas.

Peak integration and identification was performed using the Hewlett–Packard Chemstation software (Hewlett–Packard GmbH) and the MIDI software.

Whole-cell protein analysis

Whole-cell protein extracts were prepared and SDS-PAGE was performed as described by Pot et al. [30]. Densitometric analysis, normalization and interpolation of protein profiles, and a numerical analysis (with the Pearson similarity coefficient and the unweighted pair group method using arithmetic averages [UPGMA]) were performed using the Gelcompar software package (Applied Maths, Sint Martens-Latem, Belgium), versions 3.1 and 4.0, respectively.

Randomly amplified polymorphic DNA analysis

Total cellular DNA of a 2-ml bacterial culture in late exponential growth phase was obtained using the Genomic DNA WIZARD Kit (Promega, Charbonnières-les-Bains, France). RAPD profiles were determined as described previously [37] using primers P1 (5'-CTG CTG GGA C-3') and P2 (5'-CGC CCT GCC C-3') in separate reactions. For each strain, the two RAPD profiles were merged and the resulting patterns were compared using the GelCompar software package (see above).

16S rRNA gene sequencing

The almost complete 16S rRNA gene of a selection of fish isolates and reference strains (see below) was amplified by PCR using two primers: 5'-AGAGTTT-GATYCTGGCTC-3' (position 8 forward of the *E. coli* 16S rRNA gene sequence) [8] and 5'-CNCGTCC TTCATCGCCT-3' (position 44 reverse of the *E. coli* 23S rRNA gene sequence) [7]. Amplification conditions were: 96 °C for 4 min followed by 30 cycles of 10 s at 96 °C, 30 s at 50 °C, and 2 min at 72 °C. PCR products were purified by a WIZARD PCR Purification Kit (Promega). Three 500–600-bp overlapping sequences were obtained by using the purified 16S rRNA gene PCR fragment as the template and the following sequencing primers: 5'-TATGCATTTACCKCTA-CA-3' (position 684 reverse), 5'-CTCGTTGCAGGACT TAAC-3' (position 1089 reverse) and 5'-GNTACCTT GTTACGACTT-3' (position 1492 reverse). The 16S partial and overlapping sequences were assembled and the resulting sequences were compared using the neighbor-joining method [33] and the model of Jukes and Cantor included in the PAUP programme [36]. The bootstrap analysis was performed with 1000 data sets.

Results

Phenotypic study

After 48-h incubation at 26 °C on trypticase soy agar, all strains produced shiny, smooth, circular, raised colonies with an entire edge. The colonies of most strains were 2–3 mm in diameter and colored bright yellow-orange by a non-diffusible, nonfluorescent, flexirubin type pigment. Conversely, the *E. meningoseptica*, *B. zoohelcum*, *R. anatipestifer*, and *R. columbina* reference strains, as well as the frog isolate NTU 87024-IL, produced very pale yellow or creamy-white colonies. The slightly darker, brownish orange pigment produced by the isolates UOF CR2995, UOF CR4395, and UOF CM1796 also belonged to the flexirubin type; numerous tiny crystals also grew in the agar under the colonies of the two former strains. These three strains, as well as the strain UOF CM895, produced smaller (i.e., about 1 mm in diameter) colonies than all other strains. The colonies of the strain LDVH 45/00 had a very mucous and ropy consistency, while those of all other strains had a creamy to butyrous consistency. Most strains emitted a rather strong, cheesy smell. Broth cultures were uniformly turbid. The cells of all strains were Gram-negative, uniformly shaped, non-sporeforming rods about 1–2 µm long and 0.3–0.5 µm wide with parallel sides and rounded ends. Neither gliding nor flagellar motility were observed.

All strains were positive for the following conventional tests: strictly aerobic metabolism; production of catalase and oxidase; growth in 0.5% and 1.5% NaCl nutrient broth; growth at 26 °C; oxidative metabolism of glucose in O/F test; and growth on brain–heart infusion, trypticase soy, sheep blood trypticase soy, and nutrient agars. In API ZYM galleries, all strains hydrolyzed the following substrates to varying degrees: 2-naphthyl-phosphate, 2-naphthyl-caprylate, L-leucyl-2-naphthylamide, L-valyl-2-naphthylamide, 2-naphthyl-phosphate, and naphthol-AS-BI-phosphate. All strains were negative for the following conventional tests: methyl red test and Voges–Proskauer reaction; growth in 6%, 8%, and 10% NaCl nutrient broth; production of indole; fermentative metabolism of glucose in O/F test; the four tests on Kliger iron agar; growth on MacConkey and TCBS agars; hydrolysis of sodium alginate; and hemolysis of sheep blood. All strains were also negative (i) for glucose fermentation, production of arginine dihydrolase, and assimilation of caprate and malate in API 20NE galleries; (ii) for the production of arginine dihydrolase, lysine and ornithine decarboxylases, H₂S, tryptophane deaminase and acetoin (Voges–Proskauer test), and for the nine O/F reactions in API 20E galleries; and (iii) for the hydrolysis of naphthol-AS-BI-β-D-glucuronide in API ZYM galleries. The results differed among the strains for all other tests, either

conventional or included in API galleries. In Biotype 100 galleries, the type strains of *B. zoohelcum*, *R. anatipestifer* and *R. columbina*, as well as the fish isolates UOF CM895, UOF CM1796 and FRGDSA 4034/97 were unable to assimilate any of the 99 substrates whatever the temperature used. All other strains were able to assimilate from 4 up to 22 substrates in Biotype 100 galleries; in all, 38 substrates out of 99 were assimilated by at least one strain.

The results of the analysis of phenotypic data are presented as a dendrogram calculated by unweighted pair group average linkage (UPGMA) (Fig. 1).

Comparison of whole-cell protein patterns, randomly amplified polymorphic DNA patterns and phenotypic characteristics

The results of the SDS-PAGE analysis of protein profiles of all strains studied are presented in Fig. 2 and those of the RAPD analysis in Fig. 3. The comparison of Figs. 1–3 showed that some fish isolates and reference strains were clustered whereas the others exhibited independent profiles and occupied isolated positions in the dendrograms. Fourteen clusters comprising 2–13 strains were most distinctly delineated in SDS-PAGE, while RAPD either confirmed some clusters (i.e., 1, 2, 5, 7, 9, 12 and 14) or differentiated the isolates within others (clusters 3, 4, 6, 8, 10, 11 and 13). Identical RAPD profiles were displayed by the isolates sharing the same origin (e.g., the 10 ZIL isolates, the 7 *C. scophthalmum* strains, and the members of clusters 7 and 9) and even by some strains whose origins were different (e.g., the members of clusters 1, 2, 5, 6 and 14). Conversely, some isolates displayed different RAPD profiles though sharing a common origin (e.g., the four LDA39 G1966 strains and the five UOF CR strains). The phenotypic study confirmed the clustering resulting from the comparison of protein profiles, except for clusters 1, 2, 3, 5, 12 and 14 whose members displayed varying degrees of phenotypic heterogeneity (Fig. 1). Some clusters were delineated in the same way by the SDS-PAGE and phenotypic analyses (i.e., clusters 4, 10, 11 and 13) or by the SDS-PAGE and RAPD analyses (clusters 1, 2, 5, 12 and 14), while clusters 7, 8 and 9 were delineated in the same way by all three methods. Cluster delineation was made difficult by the overall high similarity of most isolates and reference strains in all techniques used.

FAME analysis

All fish isolates also shared an overall very similar fatty acid composition, consistent with that of the reference strains of most *Chryseobacterium* species and of *B. zoohelcum* (Table 2) and with published data for

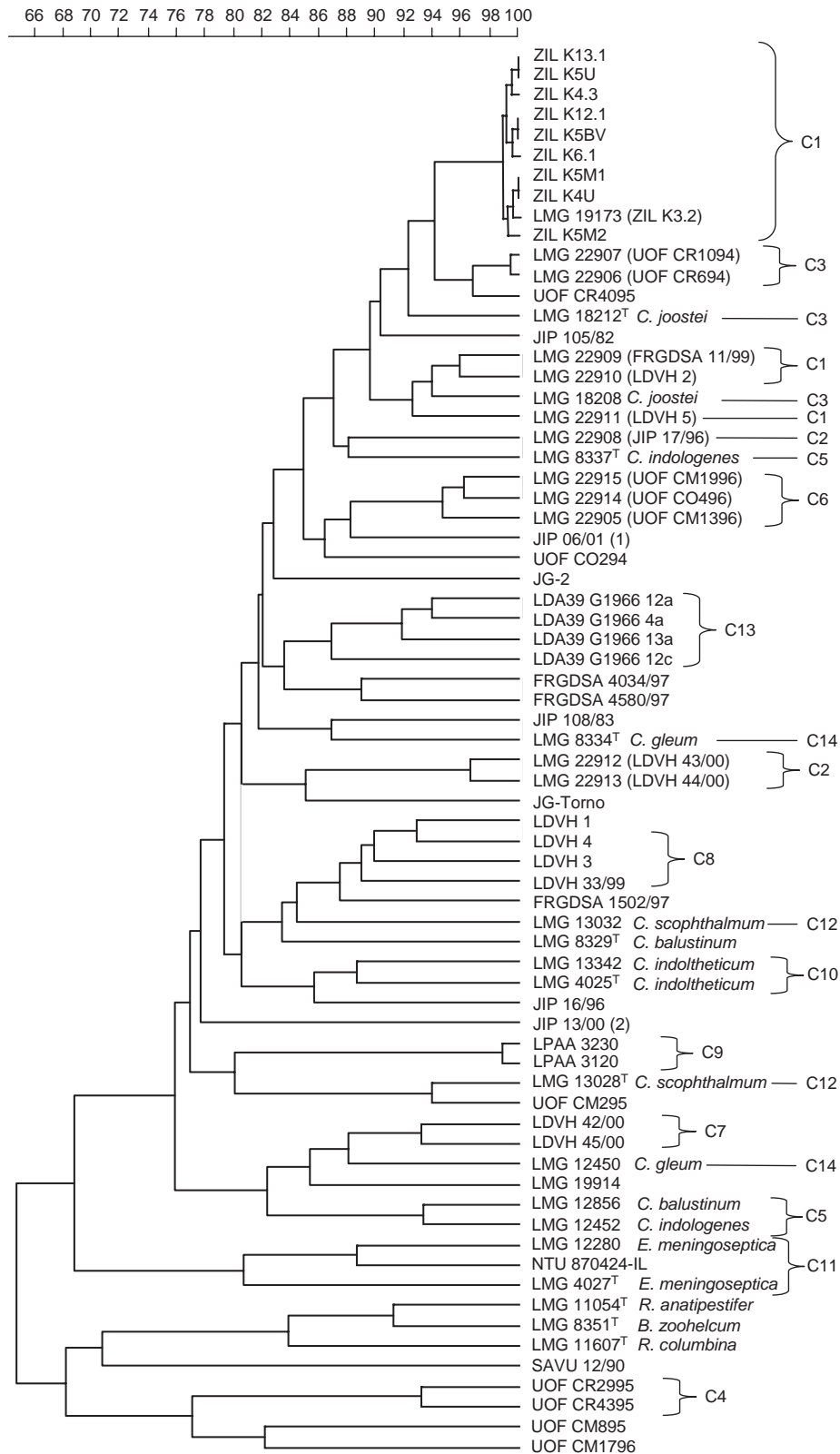


Fig. 1. Comparison of the 111 differential phenotypic characteristics of the 70 bacterial strains studied (only two among the seven *C. scophthalmum* strains were included in the phenotypic analysis) and deduced dendrogram obtained by the unweighted pair group method using arithmetic averages (UPGMA). A distance matrix was calculated for each group of characteristics. The eight distance matrices were then combined using the BioNumerics software. The scale bar indicates the correlation values ($\times 100$). The position of members of the clusters (C1–C14) that emerged from SDS-PAGE analysis (Fig. 2) is indicated.

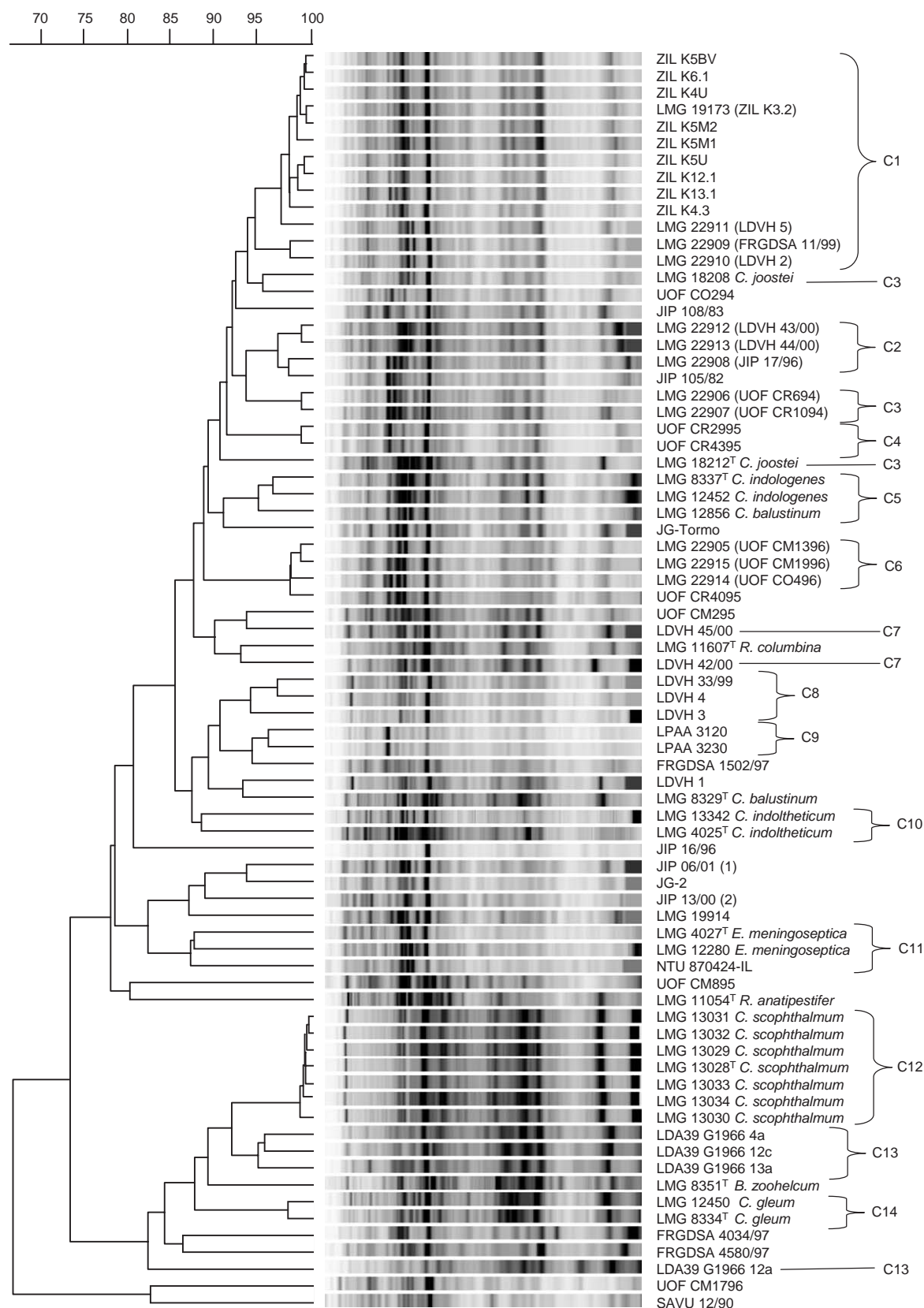


Fig. 2. Comparison of whole-cell protein profiles of the 75 strains studied and deduced dendrogram obtained by the UPGMA. The scale bar indicates the correlation values (Pearson's coefficient, $\times 100$). The clusters (C1–C14) that emerged from the analysis are indicated.

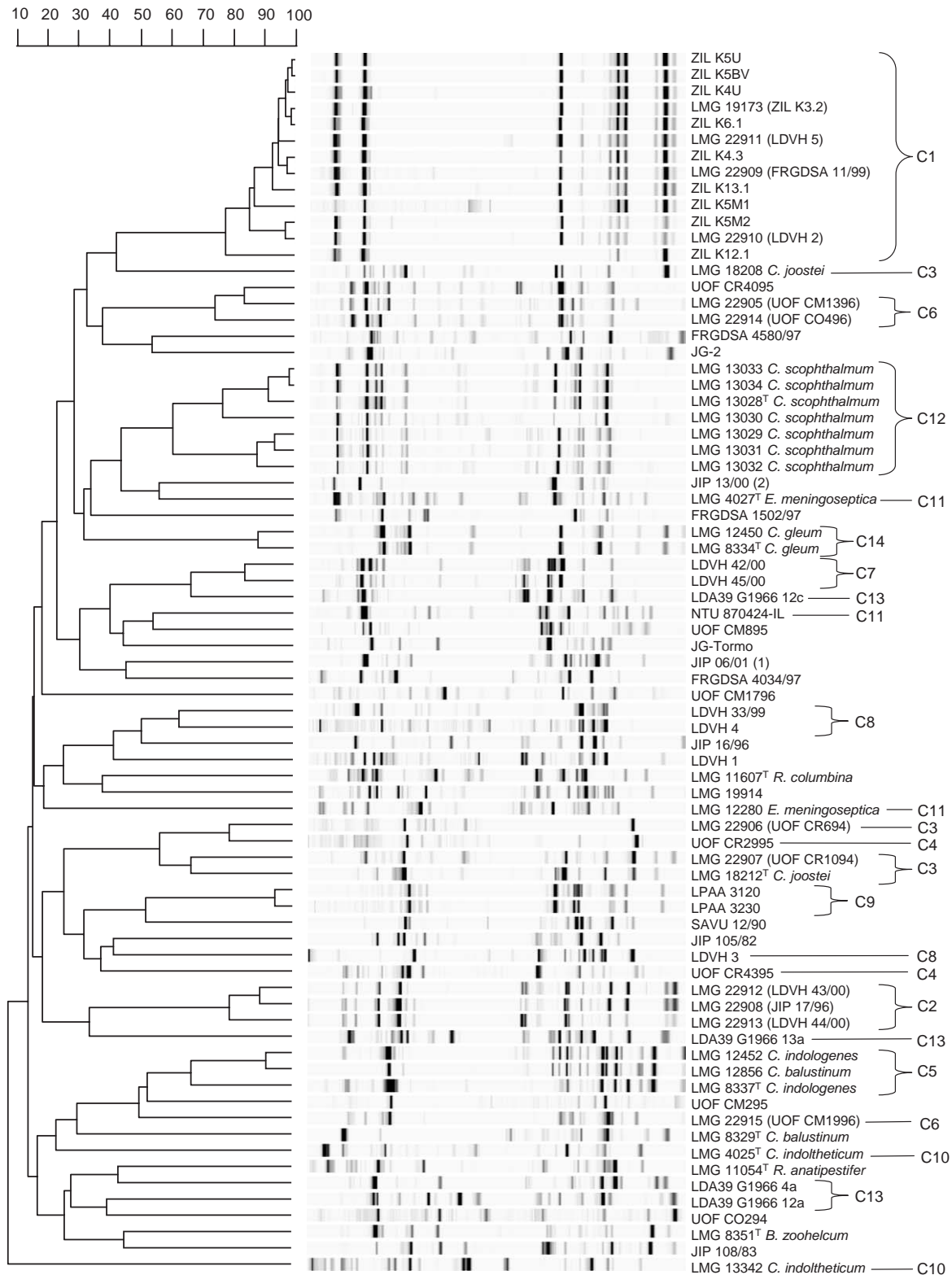


Fig. 3. Comparison of the RAPD profiles of the 75 strains studied and deduced dendrogram obtained by the UPGMA. The scale bar indicates the correlation values (Pearson's coefficient, $\times 100$). The position of members of the clusters (C1–C14) that emerged from SDS-PAGE analysis (Fig. 2) is indicated.

other members of the family *Flavobacteriaceae* [5], although slight quantitative differences occurred and some strains contained additional minor components. In contrast, the reference strains of the *Elizabethkingia*, *Riemerella*, *Kaistella* and *Sejongia* species and of the recently described species *C. defluvii*, *C. formosense* and *C. daecheongense* displayed rather distinct fatty acid profiles. The fatty acid composition of the *Riemerella* strains had previously been determined using the same growth conditions than those used in this study [34,40] whereas somewhat different growth media and temperatures were used for the other organisms [21–25,45,46]. The major components included 15:0 iso, 17:0 iso 3-OH, 17:1 ω 9c iso and summed feature 4 (that comprises 15:0 iso 2-OH, 16:1 ω 7c, 16:1 ω 7t or any combination of these fatty acids). A dendrogram based on the percentages of the major peaks of the FAMES patterns was generated using Euclidian distance as similarity coefficient and UPGMA as clustering algorithm (data not shown). Four major groups of strains were delineated. Two small groups only comprised the members of SDS-PAGE clusters 4 and 11 (i.e., the two *E. meningoseptica* reference strains and the frog isolate NTU 870424-IL), respectively. A larger group included the members of clusters 6 and 9, the *B. zoohelcum* type strain and several unclustered isolates. The fourth and largest group comprised the members of SDS-PAGE clusters 1, 2, 3, 5, 7, 8, 10, 12, 13 and 14, the *C. balustinum* type strain and several unclustered isolates. The type strains of the two *Riemerella* species and the isolates UOF CM895, UOF CM1796 and FRGDSA 4034/97 were not grouped and occupied separate branches at the base of the dendrogram.

16S rRNA gene sequence analysis

The 16S rRNA gene sequences determined in this study are listed in Table 1. Since the sequences already deposited in the GenBank nucleotide database (www.ncbi.nlm.nih.gov) for the type strains of *C. balustinum*, *C. indoltheticum*, *C. gleum*, *C. indologenes* and *E. meningoseptica* were short and/or of poor quality, they were determined again in this study, together with the 16S rRNA gene sequences of a second strain of *C. balustinum*, *C. indoltheticum*, *C. joostei* and *E. meningoseptica*. Fig. 4 shows the phylogenetic tree resulting from the comparison of the 16S rRNA gene sequences of a selection of fish isolates, of the reference strains and of several other relevant strains (i.e., the type strains of “*C. proteolyticum*” [44], *C. defluvii* [21], *E. miricola* [25], “*C. massiliae*” [12], *C. formosense* [46], *C. daecheongense* [22], “*Haloanella gallinarum*” [Pham, unpublished], *Kaistella koreensis* [24], *Sejongia antarctica* and *S. jeonii* [45], as well as a *Chryseobacterium* sp. from the gut of cockroach [13]). Owing to their short

size (less than 800 bp), the 16S rRNA gene sequences of the strains LDVH 33/99 (AY468470) and LDVH 4 (AY468473) were not included in the analysis. The overall level of sequence similarity between members of the genus *Chryseobacterium* was high, reaching up to 97% between the different species. Even higher levels of 16S rRNA gene sequence similarity (i.e., 97.2–97.9%) were recently published for closely related *Chryseobacterium* species [20–22,44,46]. The sequence of the strain SAVU 12/90 was the most divergent compared to those of all other strains, and the primers used in this study were not able to amplify the 16S rRNA gene of the strain UOF CM1796. The sequence of the strain FRGDSA 4034/97 was clustered with those of the two newly described *Sejongia* species [45] (bootstrap value higher than 80%) while the sequence of the frog isolate NTU 870424-IL was clustered with those of the *E. meningoseptica* and *E. miricola* type strains. All other isolates which 16S rRNA gene was sequenced clearly fell within the main branch grouping all other *Chryseobacterium* species, although only few of them (e.g., UOF CR1094) were highly related to reference strains (Fig. 4).

Discussion

Clustering of reference strains

High DNA homologies had been reported between the two reference strains of *C. indologenes* [38], *C. scopthalmum* [29], *C. gleum* [38] and *C. joostei* [19] included in this study. Although the two strains of the three former species differed by several phenotypic traits, they shared identical fatty acid, protein and RAPD profiles and were consequently grouped in clusters 5, 12 and 14, respectively (Figs. 1–3). Conversely, the protein profiles of the two *C. joostei* strains occupied separate positions in the corresponding dendrogram (Fig. 2) (as previously reported [19]), although visual observation of the profiles actually revealed only limited differences. The two *C. joostei* strains also differed in phenotypic [20 and this study] and RAPD profiles but they displayed similar fatty acid profiles [19 and this study] and a very high DNA homology [19]. The isolates UOF CR694 and CR1094 from Atlantic salmon in Finland were closely related to the *C. joostei* type strain in fatty acid, SDS-PAGE, RAPD and phenotypic analyses. The two *C. joostei* reference strains and UOF CR1094 also shared more than 99.5% of their 16S rRNA gene sequence (Fig. 4). Moreover, the G+C content of the two fish isolates was similar to that of the *C. joostei* reference strains (i.e. 37–38 mol% G+C; L. Gardan, personal communication) and preliminary DNA-DNA hybridization experiments using the S1 nuclease technique showed that the two fish isolates and

Table 2. Fatty acid composition (%) of the bacterial strains included in this study

Cluster	Name and strain number	13:0 iso	Unknown13.566 ^a	15:0 iso	15:0 iso 3-OH	15:0 anteiso	16:0	16:0 3-OH	Unknown16.580 ^a	17:0 iso	17:0 iso 3-OH	17:1 iso ω 9c	SF4 ^b
1	ZIL K4U	Tr	Tr	34.5	3.3	Tr	1.3	1.1	1.7	1.2	21.5	21.7	11.5
	ZIL K5U	Tr	Tr	34.0	3.4	Tr	1.1	1.1	1.7	1.1	21.3	21.8	12.3
	ZIL K5M1	Tr	Tr	34.2	3.4		1.2	1.0	1.7	1.4	20.9	22.2	12.0
	ZIL K5M2	Tr	Tr	35.0	3.4	Tr	1.2	1.0	1.7	1.4	20.5	22.1	11.5
	ZIL K5BV	Tr	1.0	34.8	3.3		1.2	1.0	1.8	1.3	20.7	22.3	11.4
	LMG 19173 (ZIL K3.2)	Tr	1.0	34.7	3.3		1.1	1.0	1.6	1.4	20.4	22.5	11.9
	ZIL K4.3	Tr	1.0	36.2	3.5		1.2	1.1	1.9	1.4	21.2	20.7	10.7
	ZIL K6.1	Tr	1.0	34.9	3.2	Tr	1.3	1.0	1.8	1.4	19.7	22.9	11.0
	ZIL K12.1	Tr	1.0	35.1	3.4		1.3	1.1	1.8	1.4	21.2	21.5	11.1
	ZIL K13.1	Tr	1.0	34.8	3.3		1.4	1.0	1.7	1.6	21.0	22.3	10.9
	LMG 22909 (FRGDSA 11/99)	Tr	Tr	37.5	3.0	Tr	1.3	1.1	1.5	1.8	20.1	21.5	9.7
	LMG 22910 (LDVH 2)	Tr		35.5	3.0	Tr	1.2	1.2	1.6	1.3	20.7	22.6	10.9
	LMG 22911 (LDVH 5)	Tr		36.5	3.0	Tr	1.4	1.2	1.7	1.5	20.6	21.9	10.1
2	LMG 22908 (JIP 17/96)	Tr	1.3	31.1	3.3		1.3	1.1	1.8	1.5	22.5	20.5	14.6
	LMG 22912 (LDVH 43/00)	Tr	Tr	30.5	3.4		1.4	1.4	1.5	1.5	25.3	18.1	15.4
	LMG 22913 (LDVH 44/00)	Tr	Tr	30.5	3.6		1.8	1.4	1.4	1.5	25.0	18.0	14.8
3	<i>C. joostei</i> LMG 18212 ^T	Tr	1.1	34.5	2.4	Tr	1.0	1.0	1.6	1.1	18.4	25.8	11.4
	<i>C. joostei</i> LMG 18208	1.0	1.2	37.9	2.5		Tr	1.2	1.3	Tr	19.4	21.9	11.2
	LMG 22906 (UOF CR694)	Tr	1.1	34.3	2.8	Tr	1.4	1.0	1.7	1.1	20.1	22.9	11.5
	LMG 22907 (UOF CR1094)	Tr	1.0	35.2	2.8	Tr	1.0	1.0	1.8	1.1	19.3	23.9	11.1
4	UOF CR2995	6.0	Tr	45.8	3.1	7.4	1.3	2.1	1.5	Tr	14.8	3.2	11.0
	UOF CR4395	5.5	1.2	45.3	3.1	6.8	1.5	1.8	1.7	Tr	15.0	3.6	11.4
5	<i>C. indologenes</i> LMG 8337 ^T	Tr	1.2	29.5	2.8		1.0		1.7	Tr	20.4	28.4	13.1
	<i>C. indologenes</i> LMG 12452	Tr	1.7	30.2	2.7		1.0	1.1	1.6	Tr	18.9	27.0	12.5
	<i>C. balustinum</i> LMG 12856	Tr	1.7	32.3	2.5	Tr	1.0	1.1	1.6	Tr	18.5	27.0	10.8
6	LMG 22914 (UOF CO496)	1.7	1.3	43.0	2.9	Tr	1.0	1.1	1.7	Tr	20.0	14.9	9.2
	LMG 22905 (UOF CM1396)	1.0	1.3	39.0	3.0	Tr	1.0	1.1	1.7	Tr	19.6	18.2	11.6
	LMG 22915 (UOF CM1996)	1.1	1.2	39.4	3.0	Tr	1.0	1.0	1.8	Tr	19.4	18.1	11.9

Table 2. (continued)

Cluster	Name and strain number	13:0 iso	Unknown13.566 ^a	15:0 iso	15:0 iso 3-OH	15:0 anteiso	16:0	16:0 3-OH	Unknown16.580 ^a	17:0 iso	17:0 iso 3-OH	17:1 iso ω 9c	SF4 ^b
7	LDVH 42/00	Tr		33.3	2.6	Tr	1.6	1.0	1.6	1.6	21.2	23.4	12.0
	LDVH 45/00	Tr	1.0	30.5	2.4	Tr	1.4	1.1	1.5	1.2	20.2	25.9	13.0
8	LDVH 33/99	1.0		36.5	3.2	Tr	1.3	1.0	1.6	Tr	15.8	22.8	12.3
	LDVH 3	Tr		29.6	2.6	1.0	1.2	1.8	1.4	1.5	16.3	24.1	15.5
	LDVH 4	1.4		40.2	3.0	1.0	1.5	1.4	1.6	1.3	16.8	20.8	9.9
9	LPAA 3120	1.8	1.0	44.0	3.0	3.0	Tr	1.1	1.6		13.4	15.9	10.8
	LPAA 3230	2.1	1.0	44.7	2.9	3.1	Tr	1.3	1.6		13.3	15.9	10.7
10	<i>C. indoltheticum</i> LMG 4025 ^T	Tr	1.6	32.4	2.2	5.4	1.0	1.2	1.5	Tr	15.3	24.1	10.8
	<i>C. indoltheticum</i> LMG 13342	Tr	1.7	35.2	2.5	5.1	1.0	1.3	1.1	1.0	15.1	19.8	10.9
11	<i>E. meningoseptica</i> LMG 12279 ^T	1.4	1.5	41.4	3.5	2.3	Tr	2.2	1.7	Tr	16.3	7.0	18.1
	<i>E. meningoseptica</i> LMG 12280	Tr	2.0	36.8	3.0	2.2	Tr	2.2	1.7	Tr	16.6	8.1	22.0
	NTU 870424-IL	1.0	Tr	44.0	3.0	1.5	Tr	1.8	1.6	Tr	17.6	8.7	17.0
12	<i>C. scophthalmum</i> LMG 13028 ^T	Tr	3.1	36.0	2.8	Tr	1.0	1.2	1.3	1.0	17.1	21.8	11.1
	<i>C. scophthalmum</i> LMG 13032	Tr	3.5	34.8	3.0	Tr	1.1	1.4	1.8	1.1	17.2	21.1	12.0
13	LDA39 G1966 4a	Tr	2.9	30.3	2.5		1.6	1.2	1.8	1.4	19.6	25.4	11.8
	LDA39 G1966 12a	Tr	2.1	33.9	2.7		1.9	1.3	1.9	1.2	20.7	21.1	12.2
	LDA39 G1966 12c		2.5	30.5	2.8		1.2	1.1	1.8	1.1	20.9	24.7	12.7
	LDA39 G1966 13a	Tr	2.4	35.6	2.6		1.4	1.4	1.8	1.0	18.8	21.8	11.3
14	<i>C. gleum</i> LMG 8334 ^T	Tr	1.6	31.6	2.6	Tr	1.0	1.1	1.9	1.0	19.1	25.0	12.9
	<i>C. gleum</i> LMG 12450	Tr	1.9	29.8	2.3	Tr	1.3	1.2	1.6	1.3	19.2	26.5	12.7
	<i>C. balustinum</i> LMG 8329 ^T	Tr	1.6	32.4	2.7	Tr	1.6	1.4	1.3	1.0	16.9	27.2	9.2
	<i>C. daecheongense</i>	1.6	1.5	51.2	2.0	1.0	1.8	Tr	1.0	3.0	15.7	7.6	10.3
	<i>C. defluvii</i>	2.8	Tr	58.5	2.6	3.2	1.3	Tr	Tr	2.0	14.1	4.8	8.4
	<i>C. formosense</i>	3.6	Tr	52.2	1.8	2.1	1.5	Tr	1.0	2.3	10.9	4.3	6.5
	<i>E. miricola</i>	2.0	1.5	46.4	3.0	1.0	1.2	3.0	1.3	Tr	15.3	6.6	17.0
	<i>R. columbina</i> LMG 11607 ^T	12.1	0.5	48.1	3.3	20.6	Tr	Tr	Tr		6.5		1.8
	<i>R. anatipestifer</i> LMG 11054 ^T	9.3	1.0	60.6	5.2	5.9	Tr		1.5		11.7		
	<i>B. zoohelcum</i> LMG 8351 ^T	1.5	1.8	47.8	4.0		Tr		1.4		13.5	17.5	9.0
	<i>K. koreensis</i>	10.0		52.0	3.0	11.0					9.0	8.0	2.0

<i>S. antarctica</i>	2.5	Tr	13.6	1.0	15.2			Tr		5.6	21.3	2.7
<i>S. jeonii</i>	2.9	Tr	12.2	1.3	24.2			Tr		4.4	8.6	2.6
JIP 105/82	Tr	1.6	32.6	2.8	Tr	1.3	1.0	2.2	1.3	21.6	21.2	12.4
UOF CO294	Tr	Tr	32.6	3.1	2.2	1.6	1.0	1.5	1.3	20.3	19.9	11.5
UOF CM295	Tr	1.6	39.0	2.8	1.9	1.2	1.2	1.5	Tr	17.7	18.2	11.7
UOF CM895	Tr		26.2	8.7	4.5	3.0	3.7	Tr	Tr	16.2	5.0	20.5
UOF CR4095	1.0	1.7	36.7	3.3	Tr	1.0	1.3	1.7	Tr	22.1	14.6	14.3
UOF CM1796	Tr	Tr	54.0	6.6	1.0	Tr	Tr	1.9	Tr	12.6	17.2	2.7
JIP 108/83	1.0	1.7	40.5	3.1	Tr	1.2	Tr	2.0	1.6	20.6	14.6	11.4
SAVU 12/90	Tr	0.8	38.1	5.5	2.3	Tr		1.5		21.6	12.3	12.2
JIP 16/96	1.0	1.0	43.9	2.3	5.1	Tr	Tr	1.2		12.1	20.9	7.6
FRGDSA 1502/97	Tr	1.7	33.7	3.2	Tr	2.2	1.2	1.8	2.3	17.6	23.8	10.1
FRGDSA 4034/97	15.2	Tr	32.6	2.4	19.4			Tr		6.9	6.5	2.1
FRGDSA 4580/97	4.5	1.8	50.7	2.5	5.6	2.4	3.2	1.2		11.9	4.4	8.8
JG-Torno	Tr		33.0	3.0		1.3	1.0	1.6	1.6	22.5	25.5	9.3
JG-2	1.3		41.4	2.7	1.3	1.0	1.0	1.6	Tr	19.1	16.7	11.0
LDVH 1	1.3	Tr	41.2	3.2	1.0	1.2	1.2	1.5	1.1	17.1	19.1	9.7
JIP 13/00(2)	1.5		44.7	2.4	Tr	1.5	1.2	1.5	1.2	23.7	10.2	10.9
JIP 06/01(1)	1.1		39.9	3.2	Tr	1.3	1.2	1.6	1.0	21.8	16.0	11.2
LMG 19914	Tr		33.4	2.7	Tr	1.3	1.4	1.5	1.1	20.6	22.7	13.9

The fatty acid compositions of the type strains of several related organisms (i.e., *C. defluvi* [21], *C. formosense* [46], *C. daecheongense* [22], *E. miricola* [23,25], *Kaistella koreensis* [24], *Sejongia antarctica* and *S. jeonii* [45]) were included for comparison; some of these studies used different growth media and temperature. The fatty acid composition of “*C. proteolyticum*” [44] has not been determined. The strains that were grouped in 14 clusters by SDS-PAGE analysis are listed first.

Fatty acid percentages amounting to less than 1% of the total fatty acids in all strains were not included; therefore, the percentages do not total 100%. Tr, trace (less than 1%); blank, not detected. Only major components were included in the table. Other, minor fatty acids also occurred in the following strains: all members of cluster 4 contained 1.0% of 16:0 iso 3-OH; *C. indologenes* LMG 12452 contained 1.0% each of 18:1 ω 5c and SF5; “*C. balustinum*” LMG 12856 contained 1.0% of SF5; the strain LDVH 3 contained 1.0% each of 16:0 iso 3-OH and 17:0 2-OH; the strain LPAA 3230 contained 1.0% of 18:1 ω 5c; *C. indoltheticum* LMG 4025^T contained 1.0% each of 16:0 iso 3-OH and 18:1 ω 5c; *C. indoltheticum* LMG 13342 contained 2.7% of 17:0 2-OH and 1.0% of 18:1 ω 5c; *E. meningoseptica* LMG 12280 and the strain NTU 870424-IL each contained 1.0% of 16:1 ω 5c; *C. formosense* contained 1.0% of 16:0 iso 3-OH; *R. columbina* LMG 11607^T contained 1.9% of 13:0 anteiso, 1.2% of 14:0 iso and 1.0% each of 16:0 iso 3-OH and 17:0 2-OH; *R. anatipestifer* LMG 11054^T contained 1.3% of 14:0 iso and 1.6% of 16:0 iso 3-OH; *B. zoohelcum* LMG 8351^T contained 1.5% each of 18:1 ω 5c and SF5; *K. koreensis* contained 2.0% each of 14:0 iso and 16:0 iso 3-OH; *S. antarctica* contained 3.2% of 13:0 anteiso, 1.5% of 14:0 iso, 2.6% of 15:0, 1.9% of 15:0 2-OH, 6.6% of 15:1 anteiso A, 2.8% of 16:0 iso, 5.1% of 16:0 iso 3-OH, 3.6% of 16:1 iso H, 3.3% of 17:0 2-OH, 2.5% of 17:1 anteiso ω 9c and 1.5% of 18:1 ω 5c; *S. jeonii* contained 1.0% of 12:0 iso, 3.6% of 13:0 anteiso, 5.0% of 14:0 iso, 1.5% of 15:0, 1.9% of 15:0 2-OH, 5.7% of 16:0 iso, 9.0% of 16:0 iso 3-OH, 9.1% of 16:1 iso H, 2.3% of 17:0 2-OH and 1.9% of 17:1 anteiso ω 9c; the strain UOF CO294 contained 1.5% of 17:0 2-OH; the strain UOF CM295 contained 1.1% of 17:0 2-OH; the strain UOF CM895 contained 3.2% of 15:0, 1.5% of 15:1 anteiso A and 1.7% of 17:0 2-OH; the strain UOF CM1796 contained 1.0% of 15:0; the strain SAVU 12/90 contained 1.8% of 15:1 anteiso A; the strain JIP 16/96 contained 1.0% of 18:1 ω 5c and 1.4% of SF5; the strain FRGDSA 4034/97 contained 5.9% of 13:0 anteiso, 1.5% of 15:0 2-OH, 1.1% of 16:1 ω 5c, 1.3% of 17:0 2-OH, 1.7% of 18:1 ω 5c and 1.1% of SF5; and the strain FRGDSA 4580/97 contained 1.1% of 14:0 and 1.0% of 17:0 2-OH.

^aUnknown fatty acid; numbers indicate equivalent chain length.

^bFatty acids that could not be separated by gas chromatography using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 4 contains 15:0 iso 2-OH and/or 16:1 ω 7c and/or 16:1 ω 7t. Summed feature 5 contains 17:1 iso I and/or 17:1 anteiso B.

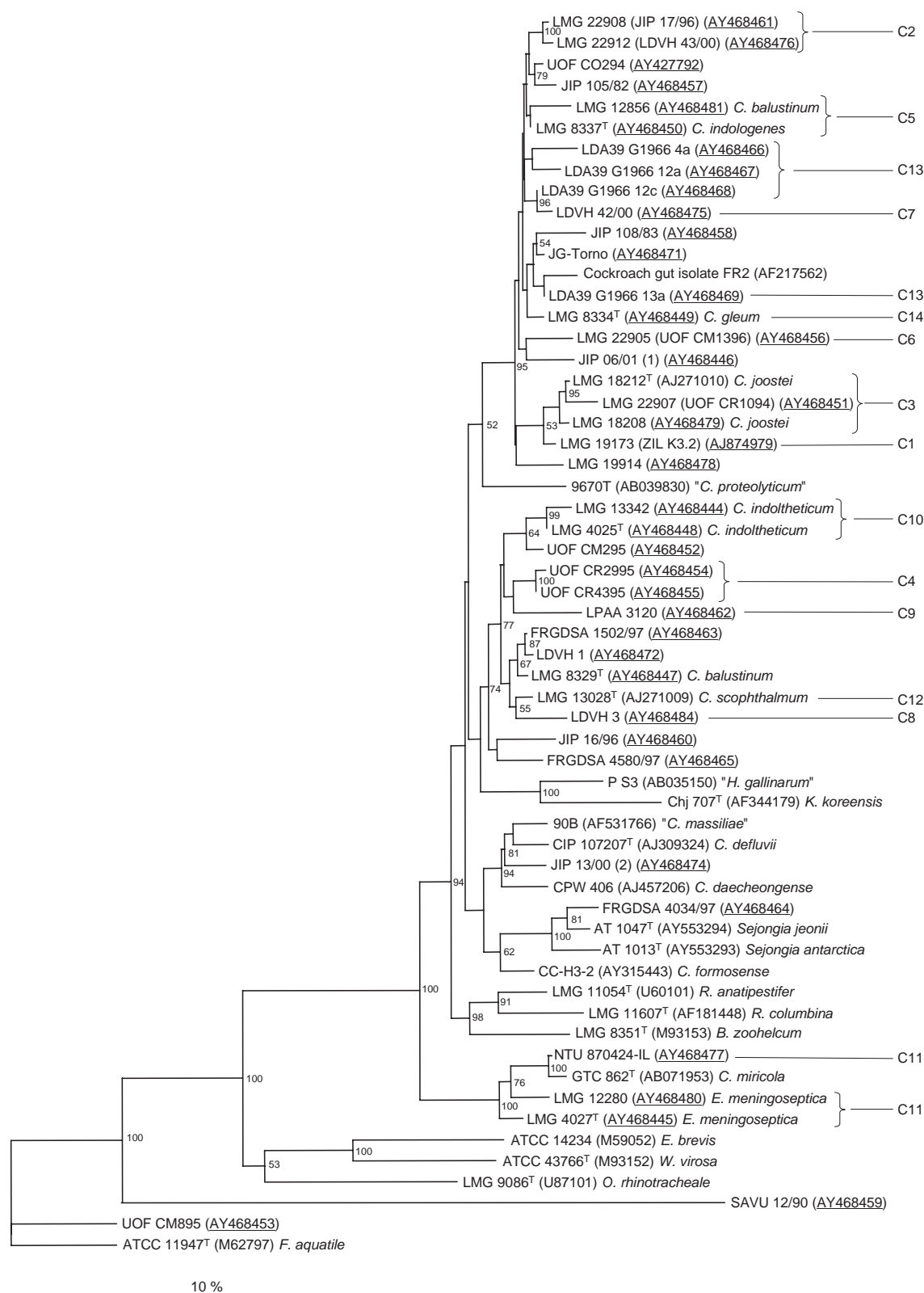


Fig. 4. Distance tree showing the phylogenetic relationships of a selection of fish isolates and other related members of the *Chryseobacterium*–*Elizabethkingia*–*Bergeyella*–*Riemerella* branch. The tree was constructed using the 16S rRNA gene sequences, the model of Jukes and Cantor and the neighbor-joining method included in the PAUP software [36]. *Flavobacterium aquatile* and the fish isolate UOF CM895 were used as outgroups. Bootstrap values (percentages of 1000 replicates) of > 50% are shown. GenBank accession numbers are shown in parentheses. The underlined numbers correspond to those 16S rRNA gene sequences that have been determined in this study; the other sequences were retrieved from GenBank. Bar, 10% sequence divergence. The position of members of the clusters (C1–C14) that emerged from SDS-PAGE analysis (Fig. 2) is indicated.

the *C. joostei* type strain shared 73–80% DNA relatedness with a ΔT_m (thermal stability of heteroduplexes) value of 3.3 °C (data not shown). Hence, the isolates UOF CR694 and CR1094 represent strains of *C. joostei* and can be considered members of cluster 3 together with the 2 *C. joostei* reference strains although all four strains were actually not grouped by any analysis (Figs. 1–3).

No DNA homology data were available for the couples of reference strains of *C. balustinum*, *C. indoltheticum* and *Elizabethkingia meningoseptica* included in this study. All techniques, including the sequencing of the 16S rRNA gene (Fig. 4), actually grouped the “*C. balustinum*” strain LMG 12856 with the reference strains of *C. indologenes* within cluster 5, while the type strain of *C. balustinum* remained isolated. These results demonstrated that the strain LMG 12856 had been wrongly attributed to *C. balustinum* and that it actually most likely belongs to *C. indologenes*. The two reference strains of *C. indoltheticum* and *E. meningoseptica* were grouped in clusters 10 and 11, respectively, when their protein and phenotypic profiles were compared, but they displayed different RAPD patterns (Figs. 1–3) and slightly different fatty acid patterns (Table 2). The frog isolate NTU 870424-IL was also included in cluster 11 following SDS-PAGE and phenotypic analyses; since its fatty acid profile and 16S rRNA gene sequence were very similar to those of the *E. meningoseptica* type strain, this isolate most probably belongs to this species.

Clustering of new isolates and delineation of potential new taxa

Besides the clusters that comprised *Chryseobacterium* reference strains, our interest focussed on those clusters that included at least three fish isolates originating from at least two different origins, i.e. clusters 1, 2 and 6. The ten ZIL isolates in cluster 1, retrieved from the same koi carp farm in Belgium (and, for some of them, from the same fish), exhibited a very high level of homogeneity by all techniques used in this study (Figs. 1–3 and Table 2) and presumably represented different isolates of the same clone. Hence, the protein, fatty acid, RAPD and phenotypic analyses performed in this study were highly repeatable. The three other isolates in cluster 1 (i.e., LDVH 2, LDVH 5 and FRGDSA 11/99) had been isolated in France from skin ulcers or internal organs of recently imported koi carp and goldfish (Table 1). They displayed identical RAPD and fatty acid patterns but slightly different protein and phenotypic profiles compared to the 10 ZIL isolates (Figs. 1–3, Table 2).

Cluster 2 comprised three strains isolated in France from internal organs of three different tropical ornamental fish (Table 1). The two strains originating from

the same facility (i.e., LDVH 43/00 and 44/00) shared identical protein and phenotypic profiles while the profiles of the strain JIP 17/96 were slightly different (Figs. 1 and 2). The three strains displayed identical fatty acid composition (Table 2) and RAPD profiles (Fig. 3), and the strains LDVH 43/00 and JIP 17/96 also shared a very high 16S rRNA gene sequence similarity (Fig. 4). Although the protein profile of the strain JIP 105/82 was very similar to those of members of cluster 2, its RAPD and phenotypic profiles were quite different.

Cluster 6 contained three isolates from external lesions or internal organs of Atlantic salmon and brown trout reared in Finland (Table 1). The three strains exhibited very similar protein, fatty acid and phenotypic patterns (Figs. 1, 2 and Table 2). Visual comparison showed that the apparently divergent RAPD profile of the strain UOF CM1996 was actually rather similar to the profile of the two other strains (Fig. 3). Although the strain UOF CR4095 displayed protein, fatty acid and RAPD profiles very similar to those of members of cluster 6, it was not included in the cluster owing to significant phenotypic discrepancies (Fig. 1).

Interestingly, none of the fish isolates included in this study could be attributed to the two *Chryseobacterium* species previously reported from fish, i.e. *C. balustinum* and *C. scophthalmum*. However, two isolates from external lesions of Atlantic salmon reared in Finland were unexpectedly attributed to *C. joostei*, a bacterial species only reported from raw cow milk in South Africa to date. As a result of this study, 18 among the 35 isolates studied could be clustered or allocated to existing *Chryseobacterium* species while the other strains occupied separate or uncertain positions in the genus *Chryseobacterium*, a situation strikingly similar to that resulting from the polyphasic study of a large collection of dairy isolates in South Africa [19]. Hence, our study confirmed that the delineation of clusters of strains that could constitute new species is particularly difficult in the genus *Chryseobacterium*. However, clusters 1, 2 and 6, grouping 3–13 isolates originating from 2 or 3 different geographical areas or facilities, were distinctly delineated and probably constitute the core of three new *Chryseobacterium* species. Extensive DNA–DNA hybridization experiments and the identification of differential phenotypic traits will be necessary to confirm their specific status.

The three fish isolates UOF CM895, UOF CM1796, and SAVU 12/90 differed clearly from all other isolates and reference strains studied in most characteristics although their API 20E and 20NE profiles were similar to those of members of the genera *Chryseobacterium* and *Elizabethkingia*. Comparison of the 16S rRNA gene sequences of UOF CM895 and SAVU 12/90 with the GenBank nucleotide database using BLAST-n searches revealed that they still belong to the family

Flavobacteriaceae and that their closest relatives are members of the genera *Flavobacterium* and *Vitellibacter*, respectively.

Ecology and pathological significance of the isolates

Although some members of the genus *Chryseobacterium* had already been reported from freshwater environments (Table 1), this study demonstrated that *Chryseobacterium* strains are widely distributed in fish farms and may be readily isolated from diseased fish. Except the frog isolate NTU 870424-IL, all strains included in this study were retrieved from fish and represented an interesting variety of geographical, zoological (i.e., various fish taxa), and environmental (i.e., wild, farmed, or ornamental freshwater fish species) sources. This study also demonstrated that *Chryseobacterium* strains belonging to different clusters may occur in the same fish farm. For instance, the five UOF strains originating from the same salmon farm in Finland (noted CR in the strain number [Table 1]) were divided between clusters 3 (i.e., *C. joostei*) and 4 or remained isolated. Conversely, strains belonging to the same cluster were retrieved from different fish farms or facilities; for instance, cluster 6 contained isolates from the Finish salmon farms CO and CM. Although all fish isolates originated from diseased individuals and about half of them were isolated from internal organs, their virulence for fish has not been demonstrated unequivocally. Preliminary experimental infection studies using one of the salmon isolates from Finland suggested that this strain was only moderately virulent for salmon (P. Rintamäki-Kinnunen, personal communication). The fact that a number of isolates had been retrieved from recently imported fish (Table 1) suggests that the bacteria they harboured actually were opportunistic pathogens which took advantage from the severe stress resulting from international transport. Since this study was completed, however, the authors isolated (or received from other laboratories) a significant number of additional *Chryseobacterium* sp. strains from diseased fish, demonstrating that *Chryseobacterium* strains potentially represent emergent pathogens in fish.

Most isolates included in this study exhibited high levels of resistance to many antimicrobial agents. This result, that is the subject of another paper [27], is consistent with published data dealing with human clinical *Chryseobacterium* and *Elizabethkingia* isolates [3]. Hence, the possible contamination of immunocompromised humans by resistant strains originating from fish or frogs farmed for ornamental, food or research purpose should not be underestimated. In this study, the bullfrog isolate NTU 870424-IL from Taiwan was clearly identified as *E. meningoseptica*. Since this study was completed, similar strains have repeatedly

been isolated from the same frog species in Taiwan (S.-C. Chen, personal communication) and a bacterial strain retrieved from a farmed clawed frog (*Xenopus laevis*) in France was also identified as *E. meningoseptica* (strain CIP 108653; N. Keck and J.-F. Bernardet, unpublished data). These reports confirm published occurrences of this human pathogen in various frog species [11,15,26]. Moreover, the first isolations of *E. meningoseptica* from fish (i.e., koi carp [strain CIP 108654] and snake fish [*Erpetoichthys calabaricus*]) were reported recently (N. Keck, personal communication). Again, the clawed frog and the two fish had recently been imported in France, from a natural pond in South Africa and from facilities in China and Africa, respectively.

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