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Enterococcus flavescens sp. nov., a New Species of Enterococci of Clinical Origin

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Four yellow-pigmented group D enterococci of uncertain taxonomic position were isolated from several humans with severe infections. The results of DNA composition, DNA-DNA hybridization, fatty acid content, and biochemical property studies demonstrated that these organisms were slightly related to other previously described yellow-pigmented enterococcal species and constitute a new species, for which we propose the name *Enterococcus flavescens*. The type strain of *E. flavescens* is strain CCM 439.

Until recently, the taxonomy of the group D streptococci was uncertain and not well defined. In 1984 Schleifer and Kilpper-Bälz proposed that the genus *Enterococcus* should include the species *Enterococcus faecalis* and *Enterococcus faecium* (17). Since then, other species have been transferred to this genus, and new species have also been described. The results of biochemical tests, DNA base composition determinations, DNA-DNA hybridization experiments, penicillin-binding protein determinations, and long-chain fatty acid content determinations have been used to describe as many as 17 different enterococcal species (1–3, 9, 11, 16, 17, 19).

Three yellow pigment-producing species belonging to the enterococcus group have been described previously; these are *Enterococcus casseliflavus*, *Enterococcus mundtii*, and *Enterococcus sulfureus* (2, 3, 12). These species have been isolated mainly from environmental materials (e.g., grass silage, plants, and soil, etc.); rarely, they have had a clinical origin (4, 16).

During a study on the taxonomy of enterococci, four strains of yellow-pigmented group D enterococci (CA-YPE strains) were isolated from clinical specimens. These organisms differed consistently from the previously described species. Biochemical tests, antibiotic susceptibility tests, and plasmid profiles of these human CA-YPE strains revealed several peculiarities (14). In this study, the results of DNA-DNA hybridization tests and fatty acid content determinations indicated that the four atypical CA-YPE strains, which were isolated from several humans with severe infections, occupy a unique taxonomic position and represent a new species, for which we propose the name *Enterococcus flavescens*.

MATERIALS AND METHODS

Strains. Reference strains were obtained from the American Type Culture Collection, Rockville, Md., and the Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia (Table 1). The CA-YPE strains were obtained from clinical specimens and have been described in detail elsewhere (14). Some strains of enterococci were obtained from J. Coyette, Liège, Belgium, and some strains (CA strains) were derived from the collection of the Cagliari Institute of Microbiology. Test strains of four recently described species were kindly provided by R. R. Facklam, Centers for Disease Control, Atlanta, Ga.

Biochemical tests. Biochemical tests were performed by using previously described methods (8, 12, 13, 16) and by using API 20 Strep and API 50CH systems (Ayerst Italiana) following the manufacturer's instructions. Additional tests were performed as described by Facklam (5). Bacteriolytic activity was determined in double-layer *Micrococcus luteus* agar plates as described previously by Pompei et al. (15).

Numerical taxonomy. The similarity coefficient which we used was the simple matching coefficient, and cluster analysis was performed by using the average linkage method (unweighted pair group using mathematical average) (18).

DNA base composition and DNA-DNA hybridization. DNA base compositions were estimated by subjecting preparations to thermal denaturation in standard saline citrate as described by Marmur and Doty (10), using a Beckman model DU70 spectrophotometer equipped with a jacketed cuvette chamber. DNA-DNA hybridization experiments were carried out by using the filter method under optimal (65°C) and suboptimal (75°C) conditions for reassociation, as described previously (7). Thermal binding indices (19) were also determined, as were the thermal elution midpoints of a homoduplex of *E. flavescens* CA 2^T (T = type strain) and heteroduplexes of *E. flavescens* strains CA 2^T and CA 3, strains CA 2^T and CA 4, and strains CA 2^T and CA 8.

Long-chain fatty acid analysis. Fatty acid methyl esters were prepared from packed freeze-dried cells as described by Farrow et al. (6) and were analyzed by using a Perkin-Elmer gas chromatograph.

RESULTS AND DISCUSSION

The strains which we used, their sources and characteristics, and their DNA base compositions are shown in Table 1. The guanine-plus-cytosine (G+C) contents of the CA-YPE strains ranged from 42 to 43 mol%. Numerical taxonomy, performed by using the simple matching coefficient and the average linkage methods, showed that the 39 strains which we studied could be clustered into several phena and that the CA-YPE strains constituted a homogeneous cluster (Fig. 1).

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TABLE 1. Strains and DNA base compositions of enterococcal species

Species	Strain	Source and/or comments	G+C content (mol%)
Enterococcus flavescens	CA 2^T (= CCM 439^T)	Human (sepsis)	42
	CA 3 (= CCM 440)	Human (abscess)	42
	CA 4	Human (abscess)	43
	CA 8 (= CCM 441)	Human (osteomyelitis)	42
Enterococcus casseliflavus	ATCC 25788^T	Plant (MUT 20)	43[a]
	ATCC 25789	CCM 2479	45[a]
	ATCC 14436		43[b]
	ATCC 12755	R. Hugh 491, motile	42
Enterococcus mundtii	CCM 2801		39
	CA 61	Human (urine)	39
Enterococcus gallinarum	CCM 2518		40
Enterococcus faecium	ATCC 19434^T		38[a]
	CA 108	Human (hemoculture)	38
	CA 15	Human (hemoculture)	37
	CA 120	Human (genital swab)	38
Enterococcus durans	ATCC 19432^T		38[b]
	ATCC 6056		38
	ATCC 11576		39
	S271	J. Coyette	39
Enterococcus hirae	ATCC 9790		37
	ATCC 8043^T		38[b]
	ENT 86		38
	SEG	J. Coyette	38
	A51b	J. Coyette	37
Enterococcus faecalis	ATCC 27959		39
	ATCC 19433		39[b]
	ATCC 11700	CCM 1875	39
	CCM 2541	*E. faecalis* subsp. *zymogenes*	38
	CCM 2497		38
	CA 176	Human (urine)	39
	CA 18b	Human (urine)	39
Enterococcus avium	CA 8S	Human (urine)	40
	CA 15S	Human (urine)	39
	CA 20	Human (urine)	40
	CA 103b	Human (urine)	40
Enterococcus pseudoavium	SS 1277	R. R. Facklam	40[c]
Enterococcus raffinosus	SS 1278	R. R. Facklam	40[c]
Enterococcus solitarius	SS 1279	R. R. Facklam	38[c]
Enterococcus malodoratus	SS 1266	R. R. Facklam	40[c]
Enterococcus sulfureus	NCDO 2379		38

[a] Data from reference 4.
[b] Data from reference 2.
[c] Data from reference 1.

TABLE 2. Results of DNA-DNA hybridization studies performed with *E. flavescens* and various enterococcal species

Species	Strain	% Homology with [^{32}P]DNA from *E. flavescens* CA 2^T	
		Optimum temp	Stringent conditions
Enterococcus flavescens	CA 2^T	100	100
	CA 3	96	72.7
	CA 4	71.6	49.8
	CA 8	104	99.7
Enterococcus casseliflavus	ATCC 25788^T	19.8	12.9
	ATCC 25789	36.9	19.5
	ATCC 14436	10.5	25.7
	ATCC 12755	8.7	<2
Enterococcus mundtii	CCM 2801	2.4	<2
	CA 61	4.8	<2
Enterococcus gallinarum	CCM 2518	6.2	<2
Enterococcus faecium	ATCC 19434^T	5.6	<2
	CA 108	12.6	<2
	CA 15	3.3	<2
	CA 120	7.2	<2
Enterococcus durans	ATCC 19432^T	7.7	<2
	ATCC 6056	9.1	<2
	ATCC 11576	7.2	<2
	S271	5.6	<2
Enterococcus hirae	ATCC 9790	7.2	<2
	ATCC 8043^T	6.4	<2
	ENT 86	8.1	<2
	SEG	2.9	<2
	A51b	<2	<2
Enterococcus faecalis	ATCC 27959	2.9	<2
	ATCC 19433^T	4.2	<2
	ATCC 11700	3.8	<2
	CCM 2541	13.4	<2
	CCM 2497	5.8	<2
	CA 176	1.1	<2
	CA 18b	2.7	<2
Enterococcus avium	CA 8S	7.5	<2
	CA 15S	8.5	<2
	CA 20	8.3	<2
	CA 103b	13.6	<2
Enterococcus pseudoavium	SS 1277	4.4	<2
Enterococcus raffinosus	SS 1278	3.1	<2
Enterococcus malodoratus	SS 1279	6.7	<2
Enterococcus solitarius	SS 1266	5.4	<2

This group was linked to the nearest species (*E. casseliflavus*) at a similarity level of 87%, while the similarity level between the CA-YPE strains and most other species of the genus *Enterococcus* was 73% (the exceptions were *Enterococcus avium*, *Enterococcus pseudoavium*, *Enterococcus malodoratus*, and *Enterococcus raffinosus*). It is worth noting that *Enterococcus hirae* and *Enterococcus durans* are linked at a similarity level of 88% and that these two species are linked to *E. faecium* at a similarity level of 86%.

The results of the DNA-DNA hybridization studies demonstrated that the four CA-YPE strains were closely related to each other. The ΔT_m values supported the hypothesis that strains CA 2^T, CA 3, CA 4, and CA 8 should be included in the same species (ΔT_m is the difference between the melting point of a homoduplex and the melting point of a heteroduplex) (data not shown). In contrast, the CA-YPE strains were not closely related to any of the other *Enterococcus* strains studied which belonged to different species (Table 2).

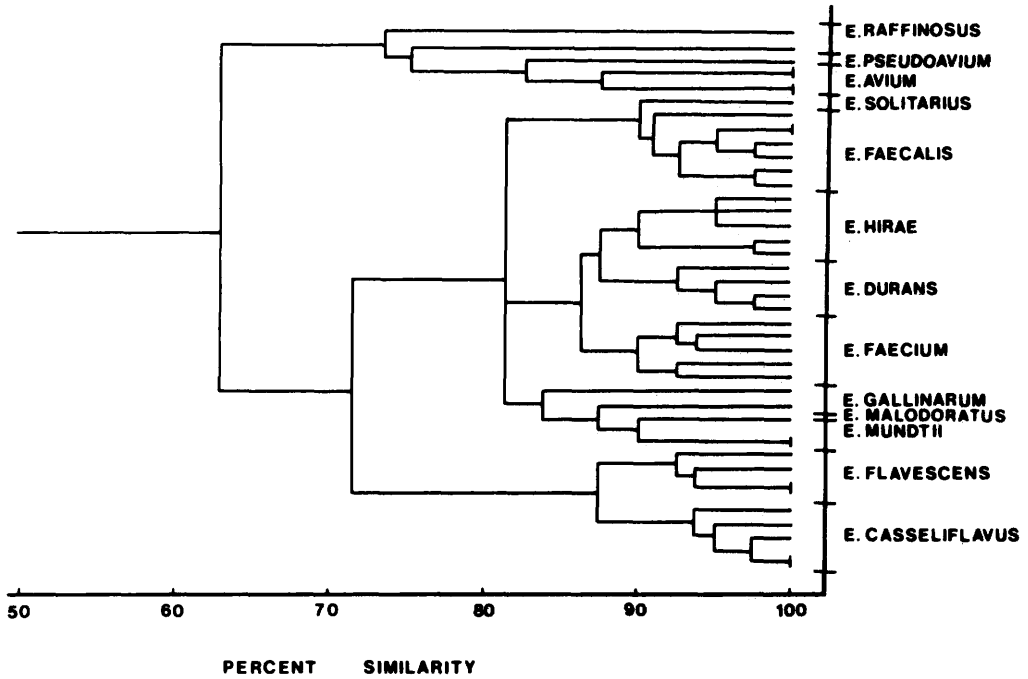

FIG. 1. Dendrogram of phenotypic relationships among the enterococcal strains studied, based on simple matching coefficients and unweighted average-linked clustering.

E. casseliflavus ATCC 25789 appeared to be the closest relative, but the hybridization value which we determined was far below the level of DNA homology that is consistent with a relationship at the species level. Moreover, when we determined the hybridization values under stringent conditions, the distance between the CA-YPE strains and the other enterococcal species notably increased. Therefore, the CA-YPE strains are not closely related to any of the previously described species of enterococci, although they belong to the same genus. We propose that these organisms belong to a new species, *Enterococcus flavescens* sp. nov.

Description of *Enterococcus flavescens* sp. nov. *Enterococcus flavescens* (fla. ve' scens. L. adj. *flavescens*, showing a yellow color). Cells are ovoid and occur mostly in pairs or short chains. Surface colonies on blood or nutrient agar are circular and smooth with entire edges. Nonhemolytic. Catalase negative. Facultatively anaerobic. Yellow pigmented. Motile. Antigenic extracts of all strains react with group D antiserum. All strains hydrolyze esculin, are Voges-Proskauer, arginine dehydrolase, β-galactosidase, leucine arylamidase, and pyrrolidonylarylamidase positive, and produce acid from L-arabinose, D-xylose, galactose, D-fructose, D-mannose, mannitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, D-raffinose, inulin, gluconate, rhamnose, and β-gentiobiose. All strains are amylase, protease, pyruvate, β-glucuronidase, and alkaline phosphatase negative and fail to produce acid from glycerol, erythritol, ribose, L-xylose, adonitol, α-methyl-xyloside, L-sorbose, dulcitol, *myo*-inositol, sorbitol, α-methyl-mannoside, melezitose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, and 2- and 5-ketogluconate. Variable results are obtained for growth at 10°C (one strain is able to grow), growth at 45°C (one strain is not able to grow), and the litmus milk test. With regard to bacteriolytic activity patterns, the CA-YPE strains fall into lyogroup EIII, just as *E. casseliflavus* does (Table 3).

TABLE 3. Bacteriolytic activities of *E. flavescens* and some other species of enterococci

Species	Bacteriolytic activity on the following media[a]:						Lyogroup
	THD	THO + 0.02% SA	TA + 0.04% BS	TA + 0.05% SUR	TA (pH 9.6)	SSA + Ml-Ca	
Enterococcus flavescens	2+	–	1+	2+	–	2+	EIII
Enterococcus casseliflavus	3+	–	1+	2+	–	2+	EIII
Enterococcus gallinarum	2+	2+	–	1+	1+	2+	EVI
Enterococcus mundtii	2+	1+	1+	–	2+	–	EVII
Enterococcus faecium	3+	1+	1+	2+	–	3+	EII
Enterococcus faecalis	3+	3+	2+	–	4+	2+	EI

[a] Abbreviations: THD, Todd-Hewitt medium (Difco); THO, Todd-Hewitt medium (Oxoid); TA, tryptose agar; SSA, streptococcus selective agar (Biolife); SA, sodium azide; BS, bile salts; SUR, suramin; Ml-Ca, *Micrococcus luteus* Ml-Ca used as a substrate for lysis.

TABLE 4. Long-chain fatty acid compositions of *E. flavescens* strains and *E. casseliflavus* ATCC 25789

Strain	Fatty acid content (%)[a]				
	$C_{14:1}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}$ $\Delta 11$	$C_{18:0}$
Enterococcus casseliflavus ATCC 25789		6.5	41.0	47.9	4.4
Enterococcus flavescens					
CA 2^T	0.6	11.3	30.1	39.6	14.5
CA 3		12.6	24.0	33.2	14.4
CA 4	0.4	9.7	25.6	40.5	13.7
CA 8		4.5	35.7	48.1	8.5

[a] Examples of abbreviations for fatty acids: $C_{16:0}$, saturated hexadecanoic acid; $C_{16:1}$, monounsaturated hexadecanoic acid. None of the strains contained $C_{15:0}$ acid, $C_{16:1}$ acid, or cyclo-C_{19} acid (*cis*-11,12-methylenoctadecanoic acid).

The major fatty acids are hexadecanoic acid and monounsaturated octadecenoic acid ($\Delta 11$); no unsaturated hexadecanoic fatty acids are detected (Table 4). A minor, but not significant, difference in fatty acid composition occurs in strain CA 8. *E. flavescens* is distinguished from *E. casseliflavus* by being unable to ferment ribose and by lacking hemolysis on sheep blood. Other tests for separating *E. flavescens* from *E. faecium*, *E. mundtii*, and *E. sulfureus* are shown in Table 5. The type strain is strain CA 2 (= CCM 439).

Description of the type strain. The description of the type strain is the same as the description of the species.

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TABLE 5. Differential characteristics of *Enterococcus flavescens*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, *Enterococcus gallinarum*, *Enterococcus sulfureus*, and *Enterococcus faecium*

Characteristic	*E. flavescens*	*E. casseliflavus*	*E. mundtii*	*E. gallinarum*	*E. faecium*	*E. sulfureus*
Group D antigen	+[a]	+	+	+	+	−
Acid produced from:						
Adonitol	−	−	−	−	−	−
Amidon	−	−(V)	−(+)	+(−)	−	ND
L-Arabinose	+	+	+	+	+	−
Glycerol	−	V	V	V	V	−
Glycogen	−	−	−	−(+)	−	−
Gluconate	+	+	−	+	V	+
Inulin	+	+	−(+)	+	−	−
Mannitol	+	+	+	+	+(−)	−
α-Methyl-D-mannoside	−	+(−)	+(−)	−(+)	−(+)	ND
α-Methyl-D-glucoside	+	+	−	+	−	+
D-Raffinose	+	+(−)	+(−)	+	−	+
Rhamnose	+	+(−)	+(−)	−(+)	−(+)	−
Ribose	−	+	+	+	+	+
Sorbitol	−	−	V	−	−	−
Sucrose	+	+	+	+	V	+
D-Tagatose	−	−(+)	−	+	−(+)	−
D-Turanose	−	+(−)	−	+	−	ND
D-Xylose	+	+	+	+	−	−
Hydrolysis of hippurate	−	−	−	+	−	−
Production of:						
Arginine dehydrolase	+	V	+	+	+	−
α-Galactosidase	+	+	V	+	−	+
β-Galactosidase	+	+	+	+	+	+
β-Glucuronidase	−	−	−	V	−	−
Yellow pigment	+	+	+	−	−	+
Motility	+	+	−	+	−	−
Hemolysis[b]	−	α	−	β[c]	V	ND

[a] +, positive; −, negative; V, variable; +(−), most strains are positive; −(+), most strains are negative; ND, not determined.
[b] On Columbia CNA blood agar (sheep blood).
[c] On horse blood.

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