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Presumptive Fecal Streptococci in Environmental Samples Characterized by One-Dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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The use of fecal streptococci as fecal indicators requires better knowledge of the ecology of these bacteria. We isolated 371 presumptive fecal streptococci from environmental samples—domestic wastewater, forest industry wastewater, contaminated surface and seawater, well water, cow dung, bird droppings, and pristine waters—and clustered them according to their protein profiles in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Some clusters could be tentatively identified with the help of reference strains. Samples from each environment had a typical composition of streptococcus types. *Enterococcus faecalis* was present, but not as a dominating enterococcal species, in samples in which fecal contamination was probable. *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus mundtii* had protein profiles that were difficult to distinguish from each other. These bacteria were found in a variety of samples. *Enterococcus casseliflavus* and *Enterococcus gallinarum* had identical protein profiles. On the basis of the maximum temperatures for growth and pigment production, isolates of this protein profile group common in forest industry wastewaters were identified as *E. casseliflavus*. *Lactococcus lactis* subsp. *lactis* was also found in this environment. Nearly all strains from pristine waters belonged to protein profile groups which could not be identified with the aid of known *Aerococcus*, *Enterococcus*, *Lactococcus*, or *Streptococcus* strains. The maximum temperatures for growth and the results of fatty acid analysis were in general agreement within each protein profile group.

Fecal streptococci, in addition to coliform bacteria, are widely used as fecal indicators. Difficulties in differentiating between fecal streptococcal species complicate the gathering of detailed ecological information on them. Some epidemiological studies (2, 5, 6) encourage the use of these bacteria as fecal indicators in waters. Occasionally, however, routine enumeration methods yield high densities of typical colonies from samples in which fecal contamination is unlikely. Seasonal patterns in pristine and non-point-loading environments show maxima of presumptive fecal streptococci without concomitant coliform increases (14, 17). These strains should be identified and their ecology should be clarified. The recent rapid development in the taxonomy of the family *Streptococcaceae*, and especially of the genus *Enterococcus* (22), encouraged us to attempt the isolation and subsequent grouping of fecal streptococci from different environmental samples.

The protein profiles produced by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of whole cells of bacteria have been observed to correlate closely with DNA-DNA hybridization results and to be suitable for rapid bacterial identification (7, 11, 12, 20). SDS-PAGE offers the possibility of being a convenient and reliable basis for clustering similar strains and for their tentative identification when relevant reference strains are available.

In the present study, bacteria were isolated from environmental samples by using KF Streptococcus agar at the nonselective temperature of 35°C. After preliminary phenotypic characterization, isolates were characterized by SDS-PAGE. The protein profiles of environmental isolates were compared with each other and with the known protein profiles of *Aerococcus*, *Enterococcus*, *Lactococcus*, and *Streptococcus* species. The maximum temperature for growth (T_{max}) (16) was measured as a supplementary means of characterization. Fatty acid analysis was carried out on a subset of strains.

MATERIALS AND METHODS

Samples. The samples used for the enumeration of presumptive fecal streptococci are listed in Table 1. Water samples were taken manually into sterile bottles of borosilicate glass. Disposable spoons were used to collect fecal samples (composite samples of 10 bird droppings from snow in the area where a flock of wintering birds, mainly *Anas anas*, gathered; cow dung from a pile of manure). Water sources in pristine areas have been described previously (17). P. Martikainen (National Public Health Institute, Kuopio, Finland) kindly provided isolates from well waters.

Enumeration and isolation of bacteria. Presumptive fecal streptococci were enumerated on KF Streptococcus agar (Difco) at 35 ± 1°C for 44 ± 4 h (8). Thermotolerant coliform organisms were enumerated on mFC agar (Difco) at 44.5 ± 0.2°C for 22 ± 2 h, with the subsequent presumptive confirmation of isolates as *Escherichia coli* by incubation in lauryl tryptose tryptophan mannitol broth at 44.5°C for 22 ± 2 h (10). Production of gas and indole was recorded as a positive result.

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TABLE 1. Sample sources used for the isolation of the presumptive fecal streptococci

Source	Presumptive fecal streptococci (CFU/100 ml or CFU g^{-1})	Thermotolerant coliforms (CFU/100 ml or CFU g^{-1})[a]
Frozen bird droppings	3.8×10^9	7.5×10^6 (87)
Frozen cow manure	8.7×10^6	1.1×10^6 (100)
Domestic sewage		
Untreated	2.6×10^4	4.9×10^4 (97)
Treated	4.0×10^4	6.5×10^4 (100)
Sea water outside of Helsinki	2.0×10^3	2.4×10^4 (100)
Kotojärvi brook, non-point loading	1.7×10^4	4.5×10^2 (92)
Forest industry wastewater		
Savon Sellu Co.	3.1×10^4	3.7×10^3 (33)
Kajaani Co.	3.8×10^2	8.0×10^4 (100)
Pristine brooks		
Pyhälammi	4	0
Käyrälammi	6	1 (100)
Pieni-Toivakka	20	1 (100)
Vaunulammi	>100	0
Kilpilammi	18	0
Takalammi	8.6	7 (14)
Pajusto	12	0
Teeressuonoja 1	79	24 (92)
Teeressuonoja 2	66	45 (100)

[a] The percentage of presumptive *E. coli* of the thermotolerant coliform bacteria is given in parentheses.

Whenever possible, 30 colonies of presumptive fecal streptococci were isolated from each sample. At least three successive single-colony isolations were carried out with brain heart infusion agar (BHI agar; Difco) and incubation at 35°C overnight. For short time periods, strains were stored as stab cultures in 50% BHI agar at room temperature, and for longer time periods, they were stored on glass beads at −70°C as described by Kirsop and Snell (13).

Physiological tests. Physiological tests were carried out by using anaerobically grown inocula for API 50CHS (Bio Merieux SA, Marcy-l'Etoile, France) according to the manufacturer's instructions. In addition, growth at 10°C for 6, 7, and 10 days, growth at 45°C for 2 days, hydrolysis of L-arginine, hydrolysis of esculin on bile esculin agar at 35°C, hydrolysis of hippurate and tolerance to 6.5% sodium chloride, resistance to 60°C for 30 min, liquefaction of gelatin, and tolerance and reduction of potassium tellurite were tested (1, 4, 18, 19).

Numerical identification on the basis of physiological tests. Numerical identification on the basis of the principles of Dybowski and Franklin (3) and Willcox et al. (21) was carried out by using the known characteristics of species as described in the literature and by using test results for reference strains obtained simultaneously while testing the environmental isolates. The results of phenotypic identifications were regarded as preliminary because duplicate cultures did not yield satisfactorily reproducible results when inoculated from different precultures. The unsatisfactory reproducibility could partly be explained by variations in the inoculum sizes of these often fastidious organisms, which have a strong tendency to aggregate. Another problem was the low level of discrimination between some species, which complicated numerical identification. Because of these problems, the results of numerical identification on the basis of physiological tests were used only as additional information for evaluation of protein profile clusters. The reference strains used in the physiological tests and SDS-PAGE are listed in Table 2.

SDS-PAGE. Since the growth of many environmental isolates was poor on solid medium, cells for SDS-PAGE were collected by centrifugation from 1 ml of BHI broth incubated overnight at 35°C (5 to 7 min in a Heamofuge; Heraeus Sepatech, Am Kalkberg, Osterode, Germany). Cells were carefully resuspended with 100 μl of phosphate buffer (9). Lysozyme solution (3 μl; L-6876; 0.1 mg/ml in phosphate buffer; Sigma) and 2.5 μl of mutanolysin solution (M-9901; 5,000 IU/ml in phosphate buffer; Sigma) were added. After 45 min at room temperature, 50 μl of sample buffer (10 ml of a solution containing 30 g of Tris base and 30.34 g of $NaH_2PO_4 \cdot H_2O$ in 500 ml of distilled water, 10 ml of 10% [wt/vol] SDS, 0.4 ml of EDTA [0.5 mol $liter^{-1}$; pH 7.5], 1.0 ml of 2-mercaptoethanol, 10 ml of 87% glycerol, and 10 mg of bromophenol blue [Riedel-de Haen 32712]) was added and the samples were assayed as described by Olkkonen and Bamford (18). The Protean II (Bio-Rad, Richmond, Calif.) electrophoresis apparatus was used according to the manufacturer's instructions, and gels were stained with Coomassie brilliant blue (Serva blue R-250). Gels were scanned with a computing laser densitometer (Molecular Dynamics A300 with ImageQuant version 3.0 software [ImageQuant, Sunnyvale, Calif.]). Densitograms were normalized by using bacteriophage φ6 structural proteins with known molecular weights (15) and the position of lysozyme included in each sample.

Visual comparison of protein patterns was carried out for the reference strains (Table 2) and the environmental isolates. Cluster analysis of the densitograms was used to group and to compare a subsample of environmental isolates and the 37 reference strains. The cluster analysis, which with replicates included 450 operational taxonomic units, was run by using the correlation coefficient as the measure of similarity and average linkage for grouping (UPGMA) (23).

T_{max}. T_{max} was measured on KF Streptococcus agar (Difco) by using a Gradiplate W10 (Biodata, Helsinki, Finland) temperature gradient incubator, according to the manufacturer's instructions. The temperatures at points of interest were obtained by converting distance readings to temperatures by an empirical formula obtained by calibrating the instrument with a series of Grant's thermocouples (type HT) and a series 1200 Squirrel meter/logger (Grant Instruments, Cambridge, United Kingdom).

Fatty acid analysis. Microbial ID, Inc. (Newark, N.J.), kindly carried out the fatty acid profile analysis and the two-dimensional principal component analysis for 47 environmental isolates and 20 type strains.

RESULTS

The grouping of 371 environmental isolates together with the reference strains was based on visual comparison of gel patterns. This grouping, which was liable to subjectivity, was compared with cluster analysis of the densitograms of a random subset of 108 environmental isolates and the reference strains. The numerical analysis of densitograms agreed with the visual grouping. The only exceptions were fecal isolates visually grouped with *Streptococcus bovis* (supported by the numerical identification on the basis of physiological tests), which the numerical analysis of densitograms placed into different taxa.

Seven protein pattern groups were formed (Fig. 1). Each

TABLE 2. Reference strains used in the present study

Code	Species	Source[a]
64390	*Aerococcus viridans* 20340^T	DSM
61290	*Enterococcus avium* SE 11[b]	L. A. Devriese
153892	*Enterococcus avium* 20679^T	DSM
63990	*Enterococcus casseliflavus* 20680[b]	DSM
80290	*Enterococcus casseliflavus*	A. H. Havelaar
63290	*Enterococcus cecorum*	L. A. Coykendall
61190	*Enterococcus columbae* Li 52	L. A. Devriese
60690	*Enterococcus durans* SP90[b]	L. A. Devriese
79690	*Enterococcus durans*	A. H. Havelaar
153992	*Enterococcus durans* 20633^T	DSM
79290	*Enterococcus faecalis*	A. H. Havelaar
61490	*Enterococcus faecalis* CCM 5613[b]	L. A. Devriese
154092	*Enterococcus faecalis* 20478^T	DSM
61390	*Enterococcus faecium* SP 183[b]	L. A. Devriese
79490	*Enterococcus faecium*	A. H. Havelaar
154192	*Enterococcus faecium* 20477^T	DSM
60390	*Enterococcus gallinarum* SC 25[b]	L. A. Devriese
154292	*Enterococcus gallinarum* 20628^T	DSM
60890	*Enterococcus hirae* S 185[b]	L. A. Devriese
154392	*Enterococcus hirae* 20160^T	DSM
64090	*Enterococcus malodoratus* 20681^T	DSM
60290	*Enterococcus mundtii* SL55[b]	L. A. Devriese
63390	*Enterococcus mundtii* 4838^T	DSM
63490	*Enterococcus pseudoavium* 5632^T	DSM
63590	*Enterococcus raffinosus* 5633^T	DSM
64190	*Enterococcus saccharolyticus* 20726^T	DSM
63690	*Enterococcus solitarius* 5634^T	DSM
111990	*Lactococcus garviae* 20684^{Tb}	DSM
79890	*Lactococcus lactis*	A. H. Havelaar
112090	*Lactococcus lactis* subsp. *cremoris* 20069^T	DSM
111690	*Lactococcus lactis* subsp. *hordniae* 20450^T	DSM
111590	*Lactococcus lactis* subsp. *lactis* 20481^{Tb}	DSM
111790	*Lactococcus plantarum* 20686^T	DSM
111890	*Lactococcus raffinolactis* 20443^T	DSM
60990	*Streptococcus alactolyticus* NCDO 1091[b]	L. A. Devriese
154492	*Streptococcus alactolyticus* 20728^T	DSM
62290	*Streptococcus anginosus* ATCC 33397^T	L. A. Coykendall
61790	*Streptococcus bovis* I MGETHE[b]	L. A. Coykendall
61890	*Streptococcus bovis* II/1 CDC 018[b]	L. A. Coykendall
61990	*Streptococcus bovis* II/2 MG 565[b]	L. A. Coykendall
62090	*Streptococcus bovis* ATCC 33317^T	L. A. Coykendall
60590	*Streptococcus bovis* 357	L. A. Devriese
	Streptococcus bovis	A. H. Havelaar
60790	*Streptococcus canis* 257	L. A. Devriese
63090	*Streptococcus cricetus* HS6^T	L. A. Coykendall
63790	*Streptococcus equinus* 20558^T	DSM
80190	*Streptococcus equinus*	A. H. Havelaar
62690	*Streptococcus ferus* 8S1^T	L. A. Coykendall
62790	*Streptococcus ferus* HD3	L. A. Coykendall
60490	*Streptococcus hyointestinalis* S57	L. A. Devriese
63890	*Streptococcus intermedius* 20573^T	DSM
62890	*Streptococcus mutans* NCTC 10449^T	L. A. Coykendall
62990	*Streptococcus rattus* FA1^T	L. A. Coykendall
61090	*Streptococcus salivarius* ATCC 13419	L. A. Devriese
61590	*Streptococcus salivarius* AC 002	L. A. Coykendall
61690	*Streptococcus salivarius* CDC 013	L. A. Coykendall
154592	*Streptococcus salivarius* subsp. *salivarius* 20560^T	DSM
63190	*Streptococcus sobrinus* SL1^T	L. A. Coykendall

[a] L. A. Devriese, Faculty of Veterinary Medicine, Gent, Belgium; A. L. Coykendall, University of Connecticut Health Center, Farmington; and A. H. Havelaar, Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands, kindly provided the majority of reference strains. DSM, Deutsche Sammlung von Microorganismen und Zellkulturen.

[b] T_{max}s are indicated in Fig. 1.

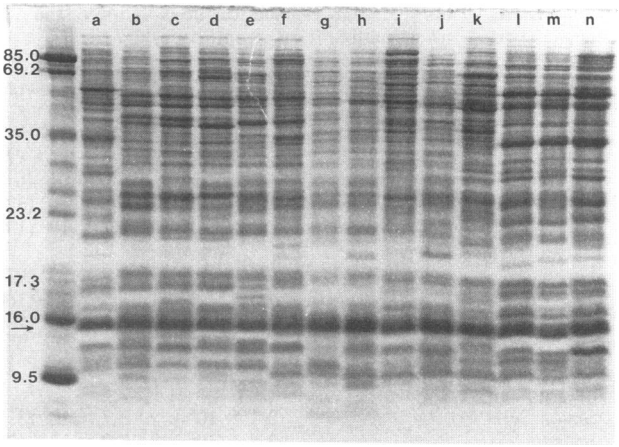

FIG. 1. SDS-PAGE analysis of isolates of different protein profile groups. Lanes: a, *Streptococcus alactolyticus*; b, *E. faecium*; c, *E. hirae*; d, *E. durans*; e, *E. mundtii*; f, *E. faecalis*; g and h, group VI isolates from the two subclusters; i, group VII isolate; j, *E. casseliflavus*; k, *L. lactis* subsp. *lactis*; l, *S. bovis* group I; m, *S. bovis* group II/1; n, *S. bovis* group II/2. Molecular masses are expressed in kilodaltons, and the position of lysozyme is indicated by an arrow.

environment had a characteristic composition of protein patterns (Table 3).

The group with the largest protein pattern (group I) included the reference strains *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus mundtii*. In many gels the resolution was good enough to allow separation of *E. faecium* and *E. hirae*, but some gels were less clear.

The T_{max}s of environmental isolates compared with the T_{max}s of reference strains (Fig. 2) offered an independent method of confirming the groupings on the basis of protein profiles. The isolates of protein pattern group I were divided into two major T_{max} groups. Because the reference strain of *E. faecium* had a T_{max} of 49.7°C and because the gel data suggested *E. faecium* as the most probable identification, all isolates of the cluster with a T_{max} of about 50°C could belong to this species. The numerical identification obtained on the basis of physiological tests divided these strains into different species. However, all isolates identified as *E. faecium* belonged to this protein pattern-T_{max} group. All the strains of the group with a lower T_{max} which, on the basis of gel data, could be distinguished from other species of this multispecies protein pattern group were identical to *E. hirae*. This identification was supported by the T_{max} (46.4°C) of the type strain of *E. hirae*. Because the T_{max} of *E. durans* was lower, 43.2°C, it was not a probable species of this group. On the other hand, *E. mundtii* (T_{max}, 46.9°C) is a possible species of this group. The numerical identification obtained on the basis of physiological tests identified the vast majority of these strains as *Lactococcus garviae*. This result can be regarded as erroneous and to be due to a few discriminating characteristics between typical *L. garviae*, *E. faecium*, and *E. hirae* (both enterococci differed from *L. garviae* in 5 of the 64 tests analyzed).

Group II included *Enterococcus faecalis* as the only reference strain, and the majority of strains included in this group were identified to be this species by numerical identification on the basis of physiological tests (Fig. 2).

Group III included the reference strains of *Enterococcus*

TABLE 3. Protein profile groups of bacteria from different sources

Protein pattern group[a]	No. (%) of isolates from:								
	Domestic wastewater	Seawater	Brook, non-point loading	Well water	Cow dung	Bird droppings	Pristine waters	Forest industry wastewater	Total
I	21	20	35	4	6	4		17	107 (29)
II	7	8		2	1	4	4		26 (7)
III	1	1				4		31	37 (10)
IV					21	10			31 (8)
V	2							23	25 (7)
VI				5			84		89 (24)
VII				1			44		45 (12)
Outlier	2				1	2	4	2	11 (3)
Total	33	29	35	12	29	24	136	73	371 (100)

[a] Group I includes the reference strains of *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii*. *E. faecalis* was the only reference strain of group II. Group III included the reference strains of *E. casseliflavus* and *E. gallinarum*, but the yellow pigment and T_{max}s of the environmental isolates support their identification as *E. casseliflavus*. In group IV, different *S. bovis*, *S. equinus*, and *S. saccharolyticus* strains yielded variable protein patterns, and numerical analysis of densitograms clustered only 2 of the 10 strains tested as *S. bovis*. Others were clustered as *S. ferus*, *S. rattus*, *S. hyointestinalis*, *E. solitarius*, or *Aerococcus* sp. In group V *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* had identical protein patterns, but the latter did not grow on the KF Streptococcus agar used for the preliminary isolation. No reference strain analyzed produced a protein profile identical to that of group VI and VII isolates. Group VI isolates resembled group II isolates, but the two groups were not identical.

casseliflavus and *Enterococcus gallinarum*. The T_{max}s of most strains clustered around the T_{max} of *E. casseliflavus* (45.7°C; Fig. 2), whereas the T_{max} of *E. gallinarum* was higher (48.4°C). The numerical identification obtained on the basis of physiological tests was in agreement with this identification even if the discrimination between *E. casseliflavus* and *E. gallinarum* was low.

Group IV was named as *S. bovis* on the basis of a visual

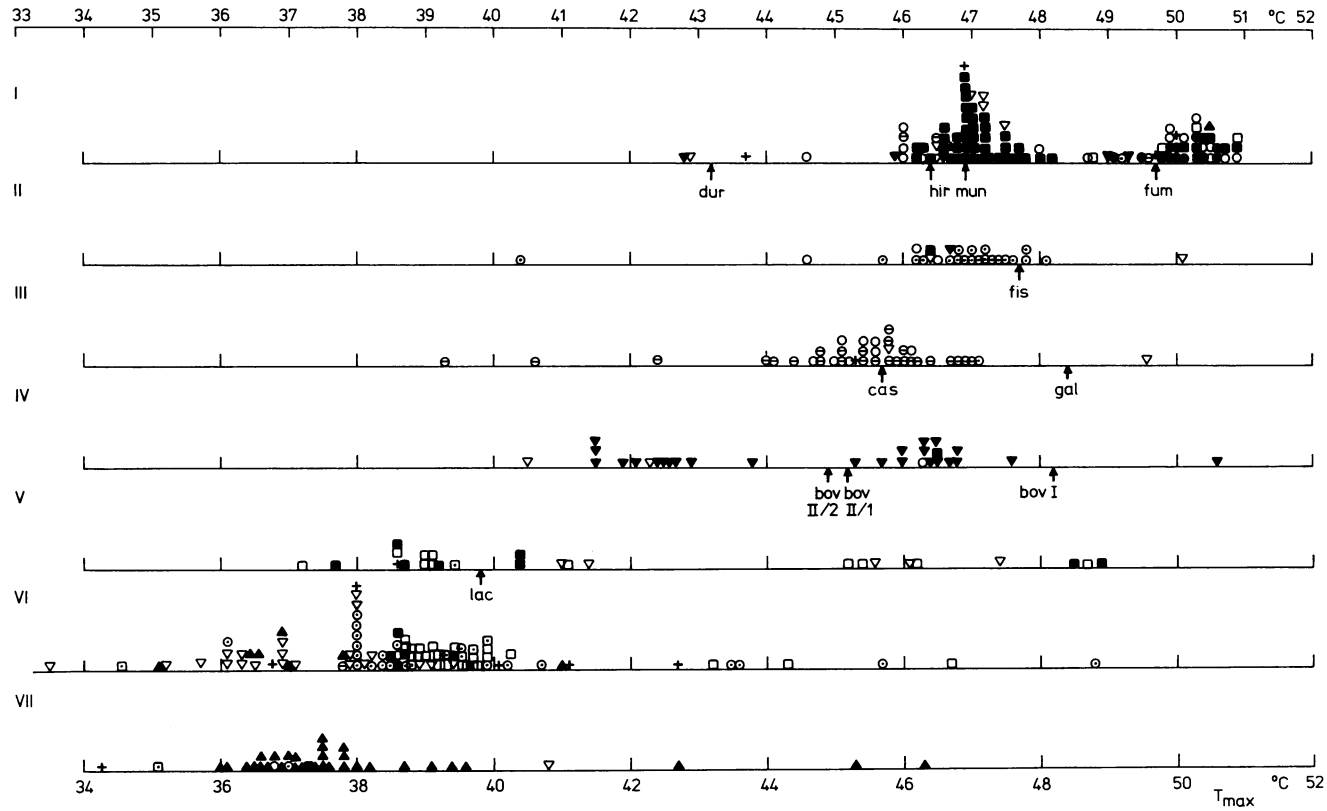

FIG. 2. T_{max}s of environmental strains with different protein profile patterns (Table 3). The arrows indicate the position on the T_{max} scale of reference strains grouped to each protein profile group (dur, *E. durans*; hir, *E. hirae*; mun, *E. mundtii*; fum, *E. faecium*; fis, *E. faecalis*; cas, *E. casseliflavus*; gal, *E. gallinarum*; bov, *S. bovis*; lac, *L. lactis* subsp. *lactis*; the exact strains are indicated in Table 2, footnote *b*). The preliminary identification results on the basis of phenotypic test results are as follows: *E. faecalis* (⊙), *E. faecium* (●), *E. casseliflavus* (⊖), *Enterococcus* spp. (○), *S. bovis-S. equinus* (▼), *Streptococcus* spp. (▽), *L. garviae* (■), *L. lactis* subsp. *lactis* (⊡), *Lactococcus plantarum* (▲), *Lactococcus* spp. (□), unidentified (+).

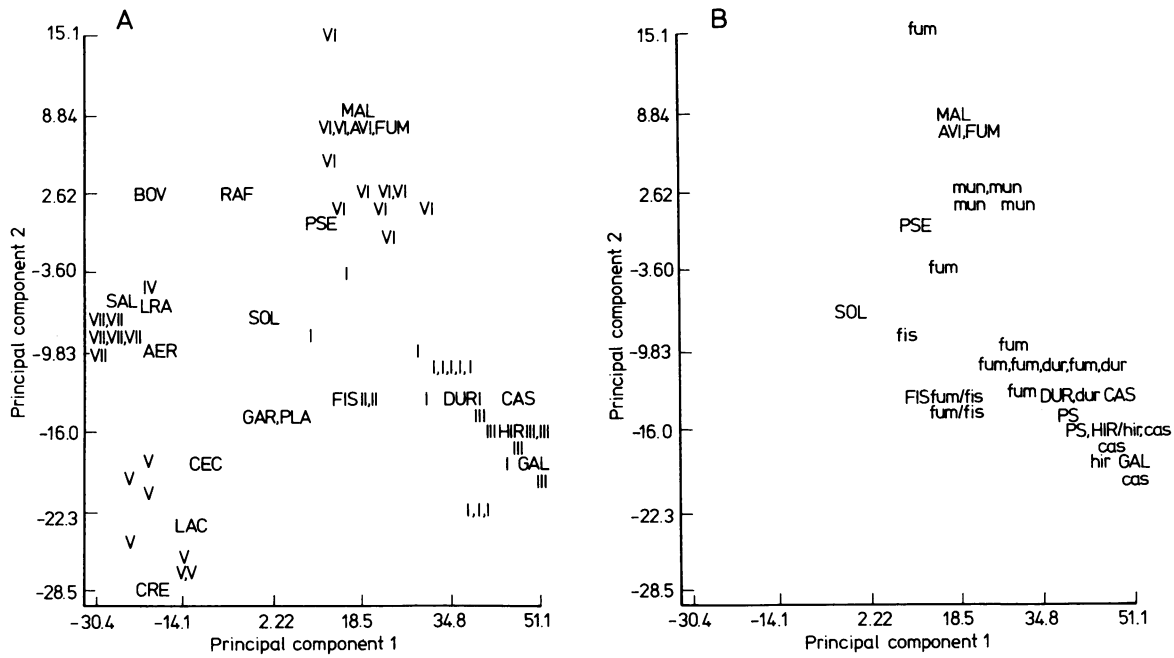

FIG. 3. Two-dimensional principal component analysis of fatty acids. (A) Comparison with protein profile groups. (B) Identification results on the basis of fatty acid analysis (unidentified isolates are not indicated). Roman numerals indicate protein profile groups (Table 3), capital letters indicate type strains, and lowercase letters identify results obtained on the basis of fatty acid analysis. AER, *A. viridans*; AVI, *Enterococcus avium*; BOV, *S. bovis*; CAS, *E. casseliflavus*; CEC, *E. cecorum*; CRE, *L. lactis* subsp. *cremoris*; DUR, *E. durans*; FIS, *E. faecalis*; FUM, *E. faecium*; GAL, *E. gallinarum*; GAR, *L. garviae*; HIR, *E. hirae*; LAC, *L. lactis* subsp. *lactis*; LRA, *L. raffinolactis*; MAL, *Enterococcus malodoratus*; PLA, *L. plantarum*; PS, *Pseudomonas vesicularis*; PSE, *Enterococcus pseudoavium*; RAF, *E. raffinosus*; SAL, *S. salivarius* subsp. *salivarius*; SOL, *Enterococcus solitarius*.

comparison of the gels. The strains with a T_{max} of greater than 44°C might be *S. bovis* because the reference strains *S. bovis* I, II/1, and II/2 had T_{max}s that were approximately in this range. In addition, the numerical identification obtained on the basis of physiological tests supported identification of this protein profile group as *S. bovis*.

The reference strains of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* belonged to protein pattern group V. The wide range of T_{max}s within this protein pattern group makes the homogeneity of this cluster questionable. The reference strain of *L. lactis* subsp. *lactis* had a T_{max} of about 40°C. The vast majority of isolates of this protein profile group were identified to the genus *Lactococcus*, and many were identified as *L. lactis* subsp. *lactis* by numerical identification on the basis of physiological tests.

The protein profile of group VI differed only slightly from the protein profile of group II. Many strains of this group were identified as *E. faecalis* by numerical identification on the basis of physiological tests. The low T_{max}s of these strains, however, indicate that they differed from *E. faecalis*. Group VI strains had two subclusters of protein profiles. Some isolates of this group were identified as different lactococci in the numerical identification on the basis of physiological tests.

The group VII strains were phenotypically uniform, but the numerical identification as *Lactococcus plantarum* on the basis of physiological tests is questionable since the reference strain had a different protein profile. No reference strain included in the present study had a protein profile that resembled the protein profiles of these strains. Strains of clusters VI and VII had low T_{max}s.

Since the data base used for fatty acid analyses did not include all the relevant taxa, identification was not always possible. Therefore, two-dimensional principal component analysis of fatty acid analysis results was used for comparison with the protein profile results (Fig. 3). Fatty acid analysis divided the strains into lactococci, enterococci, and streptococci and into several subgroups, sometimes without clear boundaries. The multispecies protein profile group I was not homogeneous when it was analyzed by using fatty acids (Fig. 3A), and even those strains identified as *E. faecium* did not form a tight cluster (Fig. 3B). They were associated with *E. durans*, *E. faecalis*, and group VI isolates. *E. hirae* and *E. durans* were closely associated with each other and with *E. casseliflavus* and *E. gallinarum*. Principal component analysis of the fatty acids confirmed that protein profile group V isolates are closely related to *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris*. Protein profile group VII formed a tight cluster in the proximity of *Streptococcus salivarius* subsp. *salivarius*, *Lactococcus raffinolactis*, and *Aerococcus viridans*. The *S. bovis* type strain was less firmly associated with group IV isolates than with group VII isolates.

DISCUSSION

On the basis of the clusters of protein profiles, it was possible to tentatively classify presumptive fecal streptococci isolated from different environments (Table 3).

Group I isolates (*E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*) were observed in all the environments studied and were often the dominating group. Because of the wide distribution of these strains, a more accurate identification would be necessary. On the basis of the T_{max}s, it was

possible to observe two subgroups. The T_{max} of reference strains indicated that the group with a T_{max} of about 50°C could be *E. faecium* and the group with a lower T_{max} could be *E. hirae*. All the isolates from the brook affected by non-point loading belonged to the cluster designated *E. hirae*, whereas about half of the isolates from forest industry wastewaters and from waters contaminated by human wastes clustered with *E. faecium*. *E. faecalis* was present in polluted water samples, but not as the dominating enterococcus. *E. casseliflavus* (*E. gallinarum* grows poorly on the media used) and *L. lactis* subsp. *lactis* (*L. lactis* subsp. *cremoris* grows poorly on the media used) were characteristic of forest industry wastewaters. To our knowledge, their association with forest industry wastewaters has not been reported earlier. The isolates from frozen fecal samples could not be reliably identified in the present study but were tentatively designated *S. bovis*. Isolates of protein pattern groups VI and VII were primarily observed in pristine waters. The isolates in groups VI and VII were not identical to any of the reference strains with which they were compared, but those in group VI closely resembled *E. faecalis*.

The two-dimensional principal component analysis of the fatty acid analysis results clustered enterococci, lactococci, and streptococci into different groups (Fig. 3). It confirmed that group V consists of lactococci, that group VII isolates are homogeneous and separated from enterococci, and that groups II, III, and VI form subclusters within enterococci. The two-dimensional principal component analysis of the fatty acid analysis results distributed widely strains belonging to protein profile group I. The data base of the fatty acid analysis results enabled the identification of some strains (Fig. 3B). Even those strains identified as *E. faecium* on the basis of fatty acid analysis seemed to be heterogeneous. It can be concluded that the fatty acid analysis was in general agreement with the groupings on the basis of protein profiles, but further work is needed to define the boundaries between species.

It is worth noting that the multispecies protein pattern groups included those species that are known to be closely related. *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii* form a distinct cluster, with intragroup homology being from 98.7 to 99.5% in the reverse transcriptase sequencing of small-subunit 16S rRNA (22). Williams et al. (22) have also shown that *E. faecalis* is only distantly related to other enterococci, which is in agreement with its distinct protein pattern compared with those of other enterococci. However, group VI isolates may be closely related to *E. faecalis*. The protein patterns of *E. casseliflavus* and *E. gallinarum* were identical, which is in agreement with the observation of Williams et al. (22), who showed that the 16S rRNA homology between the two strains is 99.8%.

It seems quite likely that unidentified isolates in groups VI and VII include species that have not so far been defined taxonomically. Their origins in pristine and well waters imply that they might originate from vegetation, invertebrates, or vertebrates and, therefore, have no value in the assessment of health risks or less value than species known to grow in human and homoiothermic animal intestines. An increase in the incubation temperature to about 41°C would exclude them as well as lactococci and some streptococci with low T_{max}s. However, some strains belonging to protein profile groups V and III (probably *L. lactis* subsp. *lactis* and *E. casseliflavus*) with no value as fecal indicators tolerate the elevated incubation temperature.

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