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High Diversity among Feather-Degrading Bacteria from a Dry Meadow Soil

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ABSTRACT

The aim of this study was to determine the diversity of cultivable bacteria able to degrade feathers and present in soil under temperate climate. We obtained 33 isolates from soil samples, which clustered in 13 ARDRA groups. These isolates were able to grow on solid medium with pigeon feathers as sole carbon and nitrogen source. One representative isolate of each ARDRA group was selected for identification and feather degradation tests. The phylogenetic analysis of 16S rDNA gene fragments revealed that only 4 isolates were gram positives. Two other isolates belonged to the *Cytophaga–Flavobacterium* group, and the remaining to Proteobacteria. High keratinolysis activity was found for strains related to *Bacillus*, Cytophagales, Actinomycetales, and Proteobacteria. The 13 selected strains showed variable efficiency in degrading whole feathers and 5 strains were able to degrade maximum 40% to 98% of the whole feathers. After 4 weeks incubation, five strains grown on milled feathers produced more than 0.5 U keratinase per mL. Keratinase activities across the 13 strains were positively correlated with the percentage of feather fragmentation and protein concentration.

Introduction

Wild birds molt once or twice a year and abandoned feathers do not accumulate in nature, suggesting the existence of natural decomposers or users of feathers [19]. Feathers are constituted of almost pure keratin protein (90%), which is insoluble and undegradable by most proteolytic enzymes [31]. However, some microorganisms possess keratinolytic enzymes, which convert keratin into peptides [21]. Studies of feather-degrading microorganisms are mostly restricted to animal diseases and to biotechnology for processing large amounts of waste by-product at poultry-processing plants [19]. So far, only some species of saprophytic and parasitic fungi, thermophilic *Actinomycetes*, and *Bacillus* strains have been reported to be able to degrade feather keratin [19]. Most of these strains have been isolated from poultry waste using nutrient-rich medium and have been shown to degrade feathers at 50–60°C.

The ecological relevance of these thermophilic isolates in natural systems is unknown, but mesophilic bacteria might have a greater impact. Recently using a feather-meal

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medium, Sangali and Brandeli [23] isolated three strains, including two Gram negatives, able to degrade keratin at 30°C. It is not known how widely the ability to degrade feathers is distributed through the Bacteria because our basic knowledge of bacterial diversity and physiology is biased by limitations of culture methods [12]. Little research has been conducted to examine the impact of keratinolytic microorganisms in nature. Burtt and Ichida [4] found several keratinolytic *Bacillus* strains on the feathers of wild avian species. The analysis of keratinolytic assemblages could reveal an essential and undescribed community for the recycling of keratin and potentially affecting feather traits in wild birds [4, 7]. The identification of new feather-degrading isolates is necessary to develop culture-independent methods to analyze such communities. These efforts would also have potential application in biotechnology and fundamental biology.

The goal of our study was to investigate the diversity of cultivable keratinolytic bacteria active in the soil environment under temperate climate. We constituted a collection of feather-degrading soil bacteria using an adequate isolation culture medium. The strains isolated from soil were identified with 16S rDNA phylogeny. Their growth and degradation of feather were compared under controlled conditions. Fragmentation and production of dissolved proteins, amino acids, and keratinases were also analyzed.

Materials and Methods

Isolation, Purification, and Maintenance of Strains

In May 2001, feather-degrading strains were isolated from triplicate soil samples collected near the University of Lausanne (Switzerland) in a dry meadow. A volume of 200 μL of soil slurry (soil:sterile phosphate buffer 120 mM, 1:2 v/v) was streaked on feather-meal agar, containing 15 gL^{-1} of ground pigeon feather, 0.5 gL^{-1} NaCl, 0.3 gL^{-1} K_2HPO_4, 0.4 gL^{-1} KH_2PO_4, and 15 gL^{-1} agar and autoclaved 15 min at 120°C [23]. In another approach, UV sterilized pigeon feathers were allowed to degrade 3 weeks in litter bags placed in the first centimeter of the dry meadow soil. They were then collected and placed on the feather-meal agar medium for selection of feather-degrading bacteria. For subculturing, Caso-agar (Merck) medium was used for convenience since feather-meal medium needs handwork to reduce feather into powder. After 5 days of incubation at room temperature, single colonies were picked and transferred on Caso-agar medium. Isolates were purified by repeated subculturing on Caso medium and then stored at −20°C in Caso broth with 15% glycerol (v/v) until processing.

PCR and Restriction Analysis

To extract genomic DNA, one colony of each isolate was resuspended in 50 μL of TE buffer [22] and boiled for 10 min. Samples were centrifuged 10 min at 8000 *g* and amplification of 16S rDNA by PCR were conducted using 5 μL of supernatant. Primers 63f (5′ CAGGCCTAACACATGCAAGTC 3′) and 1389r (5′ ACGGGCGGTGTGTACAAG 3′), corresponding to *Escherichia coli* 16S rRNA gene position, were used in a 50 μL reaction volume according to Osborn et al. [20]. Briefly, PCR products were amplified with 2.5 U of *Taq* DNA polymerase (Qiagen) and 100 ng of crude DNA. Reactions were initially denatured for 2 min at 94°C followed by 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min. PCR products were quantified using Hoechst dye and a Hoefler fluorimeter (Pharmacia).

Redundant isolates were checked using ARDRA (Amplified Ribosomal DNA Restriction Analysis) patterns [28]. An aliquot of 180 ng of PCR product (63f-1389r fragment) was digested with *CfoI* (Roche), according to manufacturer instructions. After electrophoresis on 2% agarose gel, band DNA positions were determined with the software Crosschecker 2.91 (J.B. Buntjer, Wageningen University). Band patterns were then compared by cluster analysis (Euclidean distance, UPGMA method) using the software S-PLUS 2000 (MathSoft, Inc.).

Sequencing and Phylogenetic Analysis

One representative isolate of each ARDRA group was picked for 16S rRNA gene partial sequencing. PCR products obtained with the primer set 63f-1389r (see above) were purified using the columns Wizard PCR preps DNA purification systems (Promega). Sequencing reaction was carried out in a 5-μL reaction volume with 15 ng of purified DNA, 1.5 μL of 1 μM primer 63f, 3 μL of BigDye Terminator 3.0 (ABI Prism, PE Applied Biosystems), and 0.5 μL of distilled water. PCR was run for 25 cycles under the following conditions: 96°C for 20 s, 50°C for 10 s, and 60°C for 4 min. An ABI PRISM 373 XL DNA sequencer (PE Applied Biosystems) was used for sequencing. Sequences were submitted to GenBank and received the accession numbers AY04487 to AY04492 and AF427156 to AF427162. Close relative accession numbers were *Bacillus macroides* dhr2 (AF157696), *B. megaterium* DMS 32 (X60629), *B. thuringiensis* bactisubtil (AF172711), *Arthrobacter ilicis* DMS 20138 (X83407), soil bacterium P16S841 (AF214140), *Cytophaga johnsoniae* DSM425 (M59053), Uncultured Cytophagale clone LD3 (AJ007872), *Sphingobacterium spiritivorum* ATCC33861 (M58778), Unidentified bacterium isolate SS5 (AJ223456), *Ochrobactrum grignosense* OgA9a (AJ242581), *Janthinobacterium lividum* BD17-1 (AF174648), Agricultural soil bacterium SI-8, *Pseudomonas fragi* ATCC4973 (AF094733), *P. agmydali* LMG 2123T (Z76654), Arsenite-oxidizing bacterium "*Alcaligenes fecalis* HLE" (AY027506), *Stenotrophomonas maltophilia* LMG 10857 (AJ131117), and *Serratia fonticola* DSM4576 (AJ233429). Sequences ranged from 470 to 499 base pairs in length (excluding

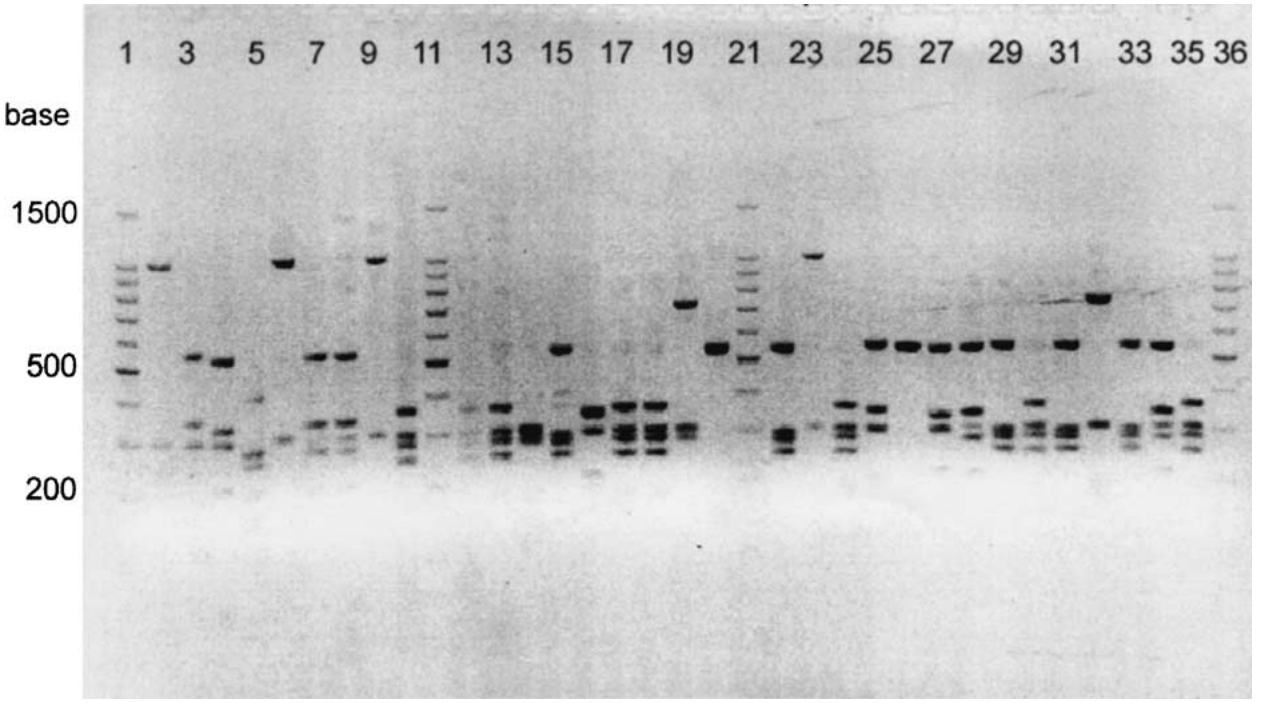

Fig. 1. Representative ARDRA gel of feather-degrading isolates. Lanes 1, 11, 21, and 36: 100 bp standard (Promega): Lanes 2 to 10: isolate (is.) 26-2, is. B, is. 28-22, is. 20, is. 7, is. 11-2, is.28-21, is. 28-1, is. 19. Lanes 12 to 20: is. 25-22, is. 23-1, is. 3-2, is. 24-1, is. C2, is. 25-21, is. 3-1, is. 24-25, is. 24-3. Lanes 22 to 35: is. 2-2, is. 25-1, is. 2-1, is. E, is. N, is. 24-2, is. 8-1, is. 5, is. NF, is. 18-2, is. 1, is. 4, is. 28-22, is. 25-22.

primer sequence), except for isolate J for which we obtained a 259 base pair sequence. To ensure reliable phylogenetic positioning at least 400 bp is desirable and sufficient, however it is possible to use partial sequences to identify organisms or to assign them to well-established phylogenetic groups, as long as the database contains sequences of close relatives [16].

The BLAST algorithm was used to search for homologous sequences in GenBank. Sequences were aligned and compared to similar database sequences using the Genetics Computer Group Inc. package (Madison, Wis.). Phylogenetic trees were inferred from Jukes–Cantor distances using the neighbor-joining method (software Phylip 3.572, [10]); the branching pattern was checked by 1000 bootstrap replicates.

Whole Feather Fragmentation

To test feather fragmentation by the representative isolates of each ARDRA group, gray pigeon feathers were collected on the two wings from six individuals, on the same row of overwing coverts. Feathers were thoroughly washed with tap water, rinsed 3 times with distilled water, dried overnight at 65°C, and weighed to the nearest microgram. Mean weight of feathers was 22.072 ± 0.107 mg. Each feather was placed in 8 mL of saline phosphate buffer and autoclaved 15 min at 120°C [23]. Each isolate was inoculated in 6 replicate tubes. Inoculations were adjusted to a final concentration of 1.6×10^6 cells per mL, after measurement with absorbance at 420 nm. Control tubes were not inoculated. Feather weight was not significantly different between the 14 groups of tubes (analysis of variance, $F_{13,60} = 0.18$, $p = 0.99$). After 3 weeks of incubation at 25°C and agitation at 200 rpm, the feather medium was filtered on GF/C membranes (Whatman) to collect feather fragments. This temperature was chosen since we aimed to look at the activity of bacteria occurring in temperate climate and isolated at 25°C. Boetius and Lochte [2] showed that optimal temperature, pH, and salinity of lytic enzymes are adapted to the environmental conditions where the bacteria naturally occur. Membranes were dried overnight at 65°C and weighed (dry weight) to the nearest microgram. Remaining feathers and fragments collected on GF/C membrane were reported as the percentage of weight compared to the initial dry weight of the feather. Statistical analysis was performed with the software JMP 4.0.4 (SAS Institute Inc.).

Milled Feather Degradation

Keratin solubilization was tested using milled feathers. Ventral body feathers from six pigeons were washed, dried as described above, cut in small pieces, and reduced in a coarse powder using a mortar and liquid nitrogen. A quantity of 10 mg of feather powder was resuspended in 5 mL of saline phosphate buffer and autoclaved as above. For each strain, three tubes were inoculated with 200 µL of a suspension of about 1.2×10^8 cell per mL (determined by measuring absorbance at 420 nm). Triplicate control tubes were not inoculated. Tubes were incubated 4 weeks

at 25°C with agitation. At the end of the experiment, cultures were prefiltered through GF/C membranes (Whatman) and then through 0.2 μm Durapore membranes (Millipore). Filters were discarded and filtrates were conserved to measured dissolved proteins, amino acids, and keratinase:

1. Total protein concentration was quantified with the Bio-Rad protein assay method, according to manufacturer protocol (Bio-Rad) and BSA (bovine serum albumin) as a standard (μg BSA per mL).
2. Amino acids were measured with a ninhydrin colorimetric method [13], using glycine as a standard (μg of glycine per mL).
3. Keratin azure (Sigma) hydrolyzing activity was estimated according to the method of Santos et al. [24], with 22 h incubation. Results were expressed as units of keratinase per mL, 1 unit of keratinase being defined as the activity required for 1.0 A_{595} unit increase in 3 h incubation [24].

Spearman rank correlation coefficients between results from whole and milled feather experiments were calculated using the software JMP 4.0.4 (SAS Institute Inc.).

Results

ARDRA and Phylogenetic Analysis of Isolates

A representative ARDRA gel is shown in Fig. 1. Thirty-three isolates were recovered from soil and degraded feathers. Analysis of ARDRA patterns grouped the isolates in 13 clusters (Fig. 2). For sequencing and feather degradation experiments, one random isolate was picked from each cluster: C2, E, J, N, 1, 2-2, 3-1, 3-2, 7, 8-1, 19, 20, and 24-25.

Phylogenetic analysis and identification of the strains are summarized in Figs. 3A and 3B. Based on the percentages of identity in 16S rRNA sequences, four isolates were gram positives. The isolates C2, E, and J shared less than 90% identity and were related to *Bacillus* species (99.3 to 99.8%). Isolate 20 was close to the Actinomycetale *Arthrobacter ilicis* (99.6%). Two isolates were related to the *Cytophaga–Flavobacterium* group: strain 24-25 was related to *Cytophaga johnsoniae* (98.3%), and isolate 7 was close to an uncultured Cytophagale clone LD3 (99.2%). Isolate 3-2 was 100% identical to an unidentified bacterium isolate SS5, which belongs to the *Ochrobactrum* genus of the α subdivision of the Proteobacteria. The sequences of strains N and 2-2 were respectively 100% identical to the sequences of *Janthinobacterium lividum* and the arsenite-oxidizing bacterium *Alcaligenes fecalis* HLE (β-Proteobacteria). Isolates 8-1, 19, 3-1, and 1 were related to the γ-subunit of Proteobacteria. The sequence of isolate 1 was 100% identical to the sequence of *Stenotrophomonas maltophilia*. Isolate 19 shared 99% identity with isolate 3-1. Both were related to *Pseudomonas* species: *P. fragi* and *P. agmydali*, respectively. Isolate 8-1 was related to *Serratia fonticola* (99.8%).

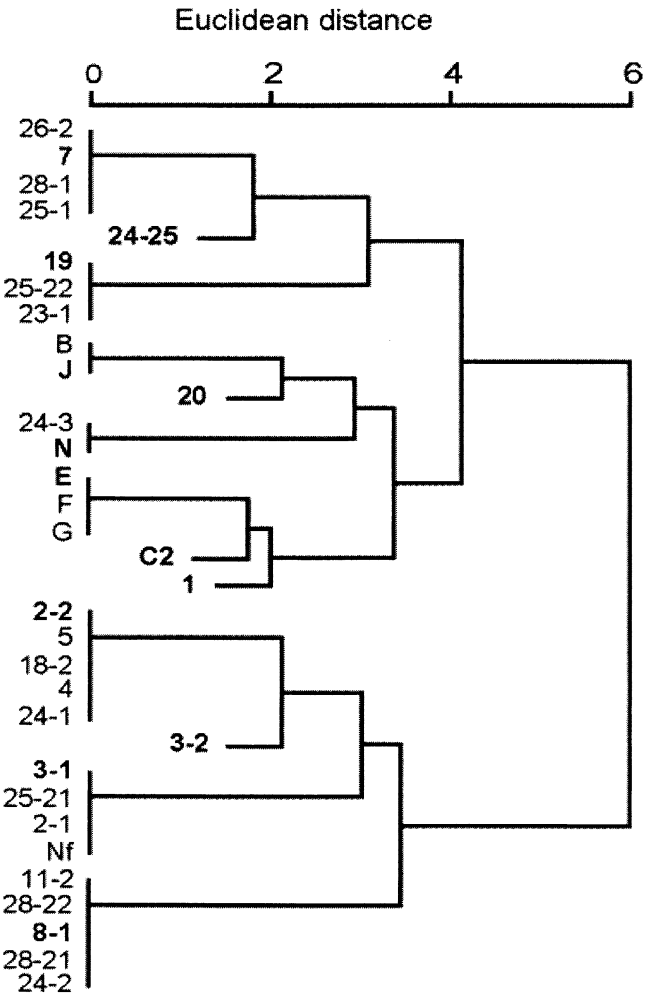

Fig. 2. Ward dendogram generated from ARDRA profiles of 33 isolates. Selected organisms for identification and testing are in bold.

Whole Feather Fragmentation

After an incubation period of 3 weeks, the fragmentation of feathers differed among replicates (Table 1). The percentage of remaining feather ($\chi^2 = 43.9$, DF = 13, $p < 0.001$), the percentage of GF/C fraction ($\chi^2 = 33.6$, DF = 13, $p < 0.0014$), and the concentration of bacteria in liquid medium ($\chi^2 = 59.4$, DF = 13, $p < 0.001$) were significantly different among strains. Strains E, J, 24-25, 19, 1, and 20 displayed at least one replicate with a degradation superior to 50% (Table 1). Growth was higher in strains

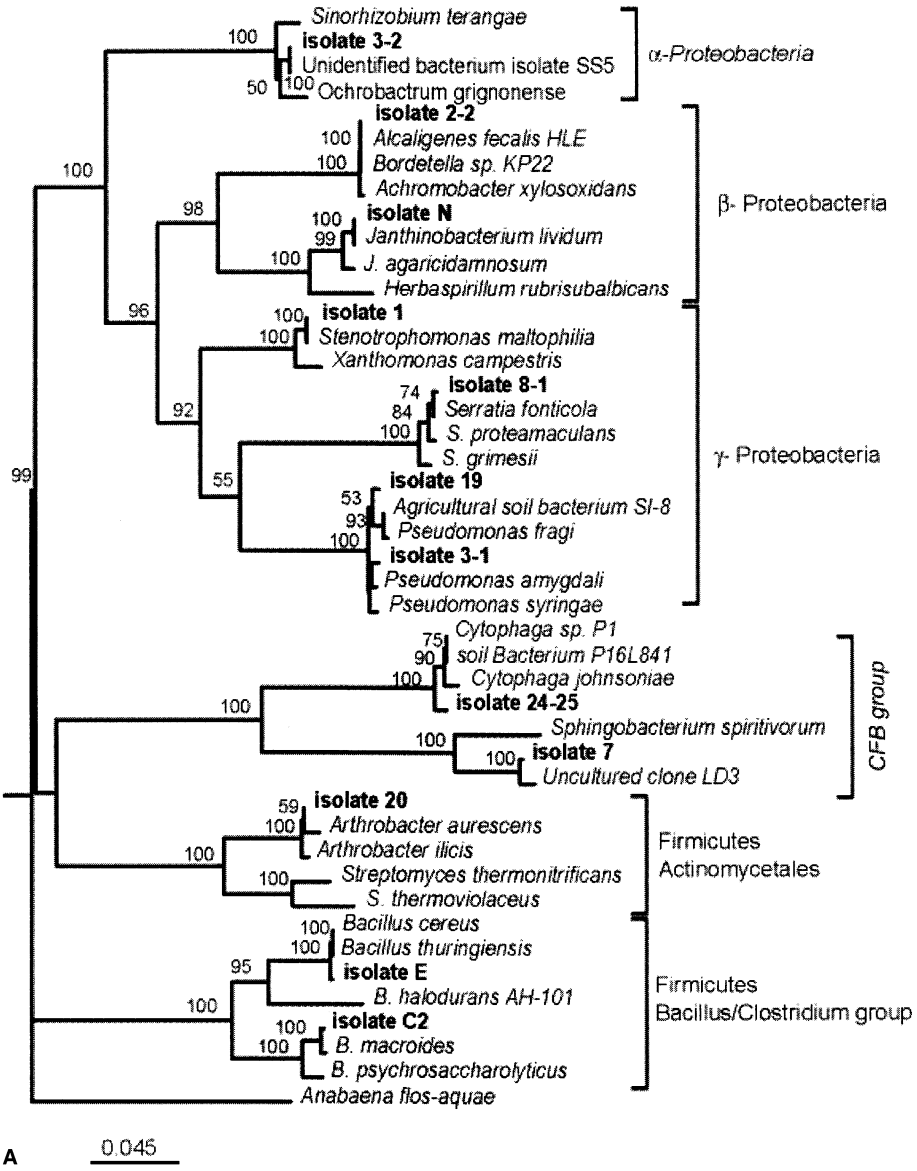

Fig. 3. Phylogenetic relationships of the 16S rDNA gene fragments sequenced from representative isolates. The bar indicates a Jukes–Cantor distance of 0.02 and bootstrap values greater than 50% are indicated. (A) Phylogenetic position of isolates 1, 2-2, 3-1, 3-2, 7, 8-1, 19, 20, 24-25, C2, E, N; the tree is unrooted with *Anabaena flos-aquae*. (B) Phylogenetic position of isolates C2, E, and J among the *Bacillus* genus; the tree is unrooted with *Arthrobacter ilicis*.

20, 24-25, 2-2, 1, 3-1, 19, 3-2, and 7. Isolate N, which produced a violet pigment, formed a visible biofilm on the rachis of each replicate feather. We were not able to estimate this attached biomass. Strain 8-1 did not appear to degrade feathers.

Milled Feather Degradation

Activity of keratinase and concentrations of dissolved protein and amino acid differed among strains (Fig. 4). The highest quantity of proteins was present in the filtrates of strains E, 1, 20 24-25, and J with 19.8 to 9.9 μg of protein per mL (Fig. 4). The culture of the strain 8-1 did not release any detectable dissolved protein after 4 weeks of incubation. For the strains 20, E, and 1, we measured 27.3 to 15.5 μg of amino acids per mL of filtrate (Fig. 4). Strains E, 24-25, and J showed the highest keratin azure hydrolysis (1.29 to 0.88 U keratinase per mL).

Comparisons between whole and milled feather experiments showed that the percentage of remaining feather was positively correlated with GF/C fragments, proteins, amino acids, and keratinase activities as well as bacterial densities. Keratinase activity was positively correlated with the quantity of proteins; finally amino acid concentrations were positively correlated with protein concentrations and bacterial densities (Table 2).

Discussion

During the past decade several thermophilic feather-degrading bacteria have been isolated from soils and poultry

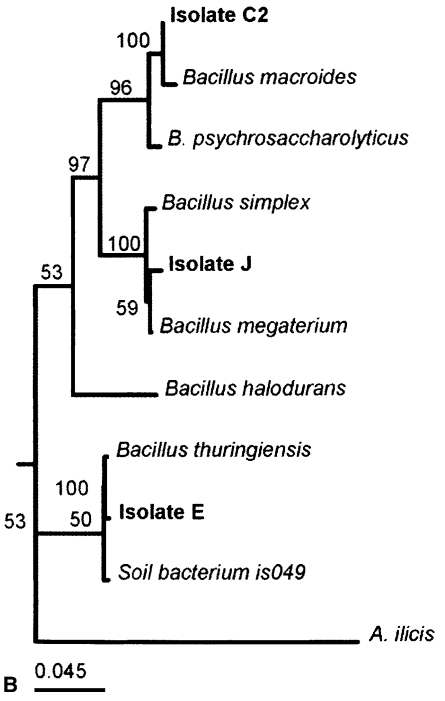

Fig. 3. Continued.

wastes [19]. These isolates are mostly confined to the gram-positives: genera *Bacillus* and *Streptomyces* (Table 3). Gram-positive bacteria have already been shown to represent an important part of the soil microbial communities [25]. In our study, we obtained from the meadow soil two *Bacillus*-related isolates (E and J) demonstrating a high feather-degrading activity at 25°C. Isolate J matched closely to *Bacillus megaterium*. Another feather-degrading *B. megaterium* has been isolated by EI-Shora et al. [8]. It seems that keratinolysis is commonly found among *Bacilli*. However, Burtt and Ichida [4] isolated from wild birds some *Bacillus* species that did not show any keratinolytic activity. In our study the *Bacillus*-related strain C2 showed the weakest keratinolytic activity. We did not isolate any *Streptomyces* sp., which may result from the fact that *Streptomycetes* are slow growing and were probably overgrown by other strains. However, strain 20 was related to *Arthrobacter* sp., which also belongs to the Actinomycetales. Up to now, the genus *Arthrobacter* had never been associated with keratinolytic activity.

Keratinolysis may not be limited to gram-positive bacteria. Recently, Sangali and Brandelli isolated the Gram-negative *Vibrio* sp. strain Kr2 [23] and the Cytophagale *Chryseobacterium* sp. Kr6 (in press) from a poultry environment; furthermore, *Fervidobacterium pennavorans* of the order Thermotogales is also a feather-degrader [11]. Our results demonstrate that many other gram-negative bacteria are able to degrade feathers and that there is a high and undescribed diversity among keratinolytic bacteria colonizing abandoned feathers in soil. The keratinolytic function is present in firmicutes as well as in Cytophagales and Proteobacteria.

In our study, the Cytophagale isolate 24-25 showed a significant feather degradation activity. Cytophagales are known to be involved in the degradation of complex macromolecules [26]. Cottrell and Kirchman [6] found

Table 1. Whole feather fragmentation by isolates

	Bacteria. 10^6.ml^{-1}[a]		% remaining feather[b]		% of GF/C fraction[c]	
Isolate	Median	Range	Median	Range	Median	Range
C2	4	1–31	95	78–97	1	0–4
J	23	8–81	80	0–94	3	0–83
E	23	9–37	42	15–84	42	7–69
20	150	134–236	90	40–81	2	1–11
24-25	135	46–469	81	39–91	9	3–36
7	30	6–207	94	73–96	1	0–4
3-2	60	15–179	93	74–96	1	0–11
N	20	9–35	95	89–97	1	0–4
2-2	103	65–376	93	59–94	1	0–20
1	93	50–429	92	54–94	1	0–19
3-1	61	41–201	95	90–97	1	1–2
19	28	20–526	93	21–96	1	0–47
8-1	10	4–27	95	93–98	1	0–4
Control	0	0–0	96	95–97	0	0–1

[a] Final densities of bacteria.
[b] percentage of remaining feather.
[c] percentage of feather fragments collected on GF/C membrane.

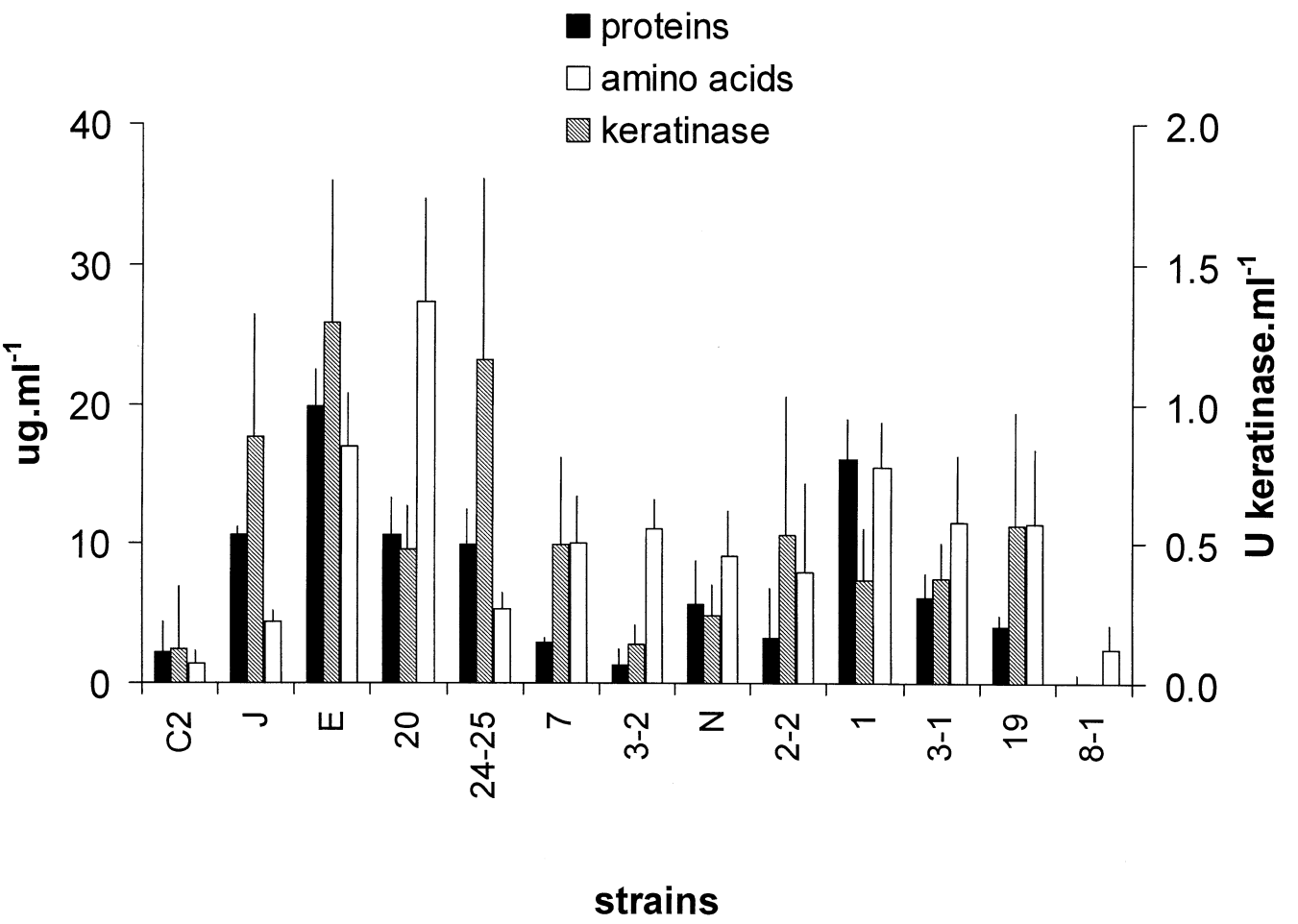

Fig. 4. Degradation of milled feathers by various strains and release of dissolved proteins (μg of BSA per mL), keratinase activity (units per mL), and amino acids (μg of glycine per mL) in the filtrate (average and standard deviation of concentrations of triplicates).

that the *Cytophaga–Flavobacteria* group accounted for most of the cells consuming proteins and chitin in estuarine and coastal environments.

Our results showed that some Proteobacteria species also degrade feathers: Among the γ-Proteobacteria, the *Stenotrophomonas*-related isolate 1 significantly degraded

Table 2. Spearman correlation coefficients between experiments examining whole feather fragmentation versus milled feather degradation

	% GF/C fragments[a,d]	Bacterial densities[a,e]	Proteins[b,f]	Amino acids[b,g]	Keratinase[b,h]
% remaining feathers[a,c]	**−0.6799**	**−0.5721**	**−0.7714**	**−0.5677**	**−0.8779**
	$p = 0.008$	$p = 0.033$	$p = 0.001$	$p = 0.034$	$p < 0.001$
% GF/C fragments[a,d]		0.3602	**0.6976**	0.3326	0.4873
		$p = 0.206$	$p = 0.006$	$p = 0.245$	$p = 0.077$
Bacterial densities[a,e]			0.4488	**0.5837**	0.4956
			$p = 0.107$	$p = 0.028$	$p = 0.072$
Proteins[b,f]				**0.6403**	**0.6689**
				$p = 0.014$	$p = 0.009$
Amino acids[b,g]					0.4163
					$p = 0.139$

Significant correlations are indicated in bold.
[a] Whole feather experiment.
[b] Milled feather.
[c] Percentage of remaining feather.
[d] Percentage of fragments collected on GF/C membranes.
[e] Final bacterial densities.
[f] Protein concentration.
[g] Amino acid concentration.
[h] Keratinase activities.

Table 3. List of feather-degrading isolates and their origins

Organism	Origin	Reference
Bacillus licheniformis PWD-1	Poultry waste	[30]
Bacillus licheniformis Carlsberg NCIMB6816	—	[9]
Bacillus licheniformis	—	[8]
	Soil	[15]
	Wild bird	[4]
Bacillus subtilis	Wild bird	[4]
	Poultry waste	[14]
	Soil	[32]
Bacillus pumilus	Wild bird	[4]
	Poultry waste	[9]
Bacillus circulans	—	[8]
Bacillus megaterium	—	[8]
Streptomyces pactum DSM 40530	Collection culture	[1]
Streptomyces fradiae ATCC 14544	Soil	[18]
Streptomyces thermonitrificans MG104	Agricultural soil	[17]
Streptomyces graminofaciens	—	[27]
Streptomyces thermoviolaceus SD8	Lake	[5]
Streptomyces albidoflavus S.K1-02	Hen house soil	[3]
Fervidobacterium pennavorans DSM7003	Hot springs	[11]
Vibrio sp. *Kr2*	Poultry soil	[23]

pigeon feathers. *Pseudomonas*-related strains 19 and 3-1 were also able to degrade feathers, as well as the β-Proteobacteria isolate 2-2 (related to the genus *Achromobacter*). The isolate N, another of the β-Proteobacteria, showed some keratinolytic activity. No previous work related keratinolytic activities among these genera.

Measurements of whole feather fragmentation were variable. This was probably due to slight structural differences in feathers (stiffness, pigment concentration, degradation by lice, age, and sex of the individuals) which may have interfered with the degradation. Nevertheless, there was a strong negative correlation between dissolved keratinase activities in milled feather medium and percentage of remaining whole feather. This result suggests that the level of whole feather fragmentation could be associated with levels of keratinase activity, although further experiments are required to corroborate the patterns found between these two different sets of experiments.

Most of our isolates seemed to belong to opportunistic species, which do not need keratin to survive in soil. Even if not essential for the individual cell, hydrolysis of high-molecular-weight compounds is an essential first step in the degradation of organic matter in nature [6]. As the primary source of soil protease [29], *Bacillus* isolates may play a key role in feather recycling. However, one might expect that in the soil feathers are degraded by a consortium of bacteria and fungi, which act in synergy or compete for keratin [19].

This study revealed that the diversity of feather-degrading bacteria is significantly greater than previously described. We showed that keratinolysis was present in phylogenetic groups that had never been associated previously with this function. It also suggests that improved isolation or culture-independent techniques are needed to explore this functional group and its ecological role in the environment. The strains that we obtained in this study should provide a starting point to investigate the distribution and activity of feather-degrading bacteria in soil and on bird plumage.

Acknowledgments

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