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ORIGINAL PAPER

M.-L. Suihko · R.M. Kroppenstedt · E. Stackebrandt

# Occurrence and characterization of actinobacteria and thermoactinomycetes isolated from pulp and board samples containing recycled fibres

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**Abstract** The aim of this study was to characterize the actinobacterial population present in pulps and boards containing recycled fibres. A total of 107 isolates was identified on the basis of their pigmentation, morphological properties, fatty acid profiles and growth temperature. Of the wet pulp and water sample isolates ($n = 87$), 74.7% belonged to the genus *Streptomyces*, 17.2% to *Nocardiopsis* and 8.0% to thermoactinomycetes, whereas all the board sample isolates ($n = 20$) were thermoactinomycetes. The identification of 53 isolates was continued by molecular methods. Partial 16S rDNA sequencing and automated ribotyping divided the *Streptomyces* isolates ($n = 31$) into 14 different taxa. The most common streptomycetes were the mesophilic *S. albidoflavus* and moderately thermophilic *S. thermocarboxydus*. The *Nocardiopsis* isolates ($n = 11$) belonged to six different taxa, whereas the thermoactinomycetes were mainly members of the species *Laceyella sacchari* (formerly *Thermoactinomyces sacchari*). The results indicated the probable presence of one or more new species within each of these genera. Obviously, the drying stage used in the board making processes had eliminated all members of the species *Streptomyces* and *Nocardiopsis* present in the wet recycled fibre pulp samples. Only the thermotolerant endospores of *L. sacchari* were still present in the final products. The potential of automated ribotyping for identifying actinobacteria was indicated, as soon as comprehensive identification libraries became available.

M.-L. Suihko (✉)
VTT Biotechnology, P.O.Box 1500,
02044 VTT, Finland
E-mail: maija-liisa.suihko@vtt.fi
Tel.: +358-20-7225133
Fax: +358-20-7117071

R.M. Kroppenstedt · E. Stackebrandt
DSMZ—Deutsche Sammlung von Mikroorganismen
und Zellkulturen GmbH, Mascheroder Weg 1b,
38124 Braunschweig, Germany

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## Introduction

Actinobacteria are a group of morphologically and physiologically diverse, Gram-positive bacteria having DNA with a high GC (> 55 mol%) content [8]. The morphological diversity ranges from micrococci to branched filament-forming species. The physiological diversity of actinobacteria is demonstrated by the wide range of metabolic products which they synthesize and secrete. Many of these products are antibiotics with the ability to inhibit the growth of other microorganisms, but some may also be plant, animal or human toxic substances [4, 36]. Members of the thermoactinomycetes resemble mycelium-forming actinobacteria and grow in similar conditions to them, but they are phylogenetically very different and currently belong to the family *Bacillaceae* [5]. The natural habitat of most mesophilic actinobacteria is the aerobic zone of soil, where they live by decomposing a wide variety of organic substrates, but members of many genera have also been isolated from fresh- and seawater sediments. Thermophilic species are found widely distributed in nature but are most numerous in moulding hay and cereal grains, decaying vegetable material and composts [15]. Actinobacteria may also be present in samples from water-damaged houses [3, 23, 33] as well as in recycled fibres (newspapers, magazines, milk and juice packages, corrugated cases, plastic coated wrapping papers, craft papers), which are used in increasing amounts as raw material in paper mills [28, 30].

Recycled fibres are mainly used in the production of paper and board for non-food use, e.g. in newspapers, coreboards, napkins and toilet and kitchen rolls, which usually contain recycled fibres from 50 to 100% [34], but they have also been used for over 25 years in food packages [13]. The microbiological quality of recycled

fibre products is controlled by the use of biocides in the process and by heat treatment (80–120°C) and drying at the end of the process. However, these operations are not sufficient to eliminate all microorganisms, especially spore-forming, thermophilic or thermotolerant bacteria occurring in pulps [17, 28]. The current legislation includes clear requirements for the chemical quality of raw materials and food packages, whereas requirements for controlling microorganisms are usually inadequate. This is mainly due to the lack of relevant data, especially about the organisms present in recycled fibres. The existing data are mainly restricted to bacilli [24, 31] and filamentous fungi [29, 35]. Very little is known especially about actinobacteria and their harmful metabolites [2]. The filament-forming actinobacteria are able to cause problems in paper making processes by clogging pipes and by the development of odour in the final products similar to those caused by fungi [14, 17, 35].

The aim of this study was to isolate and characterize the actinobacterial population occurring in paper mill pulps and end products with special reference to the content of recycled fibres, using the automated ribotyping approach for rapid assessment of the identity of these microorganisms.

## Materials and methods

### Samples and isolation

A total of 36 samples was taken from pulps/water and products of seven different mills (coded from A to G) using recycled fibres and of two mills (coded H and I) using virgin fibres (Table 1). The samples were analysed as described by Suihko and Skyttä [30]. Actinobacteria were isolated from actinomyces isolation agar (Difco 212168) after incubation at 30°C (mesophilic isolates) or 45°C (thermophilic or thermotolerant isolates) for 7–14 days. The amounts were expressed as the dilution numbers of the agar plates from which the colonies were picked. The detection limit was 10 cfu $g^{-1}$ dry weight (d.w.) or $ml^{-1}$. From the board samples, 2–4 pieces (about 1 $cm^2$) were also placed directly on agar plates.

### Preliminary identification

The isolates were grown on various International Streptomyces Project (ISP) media (DSM 65, DSM 83, DSM 252, DSM 535) or other diagnostic media [10, 27]. Pigmentation and morphology were observed after incubation for 14 days at 28°C (mesophilic isolates) or at 45°C (thermophilic isolates). The isolates were examined by light microscopy as described by Shirling and Gottlieb [27], Greiner-Mai et al. [12] and Korn-Wendisch and Kutzner [20]. Phenotypic identification followed keys described by Hütter [16] and in *Bergey's Manual of Systematic Bacteriology* [37]. Fatty acid methyl esters (FAMEs) were determined by the Microbial Identification System (MIDI, Newark, USA) according to the instructions of the manufacturer.

### Automated ribotyping

Except for sample preparation, ribotyping was performed using the standard method of the automated ribotyping device RiboPrinter® System (DuPont Qualicon™, Wilmington, DE, USA) as described by Bruce [6]. Cells were grown in 30 ml of Trypticase Soy Broth (BBL 11768) in an Erlenmeyer flask on a rotary shaker (120 rpm) for 3 days, or longer if required, at 28 or 45°C. Mycelium was collected by vacuum filtration

**Table 1** Samples studied and numbers of actionobacterial and thermoactinomycetes isolates detected using preliminary identification tests

| Samples | | | | | Number of isolates detected | | | |
|---|---|---|---|---|---|---|---|---|
| Mill | Code | No. | Type | Recycled fibre (%) | *Streptomyces* | *Nocardiopsis* | Thermoactinomycetes | Total |
| Pulps and water | | | | | | | | |
| A | A1–A5 | 5 | Wet pulp | 95–100 | 29 | 4 | 4 | 37 |
| B | B1–B4 | 4 | Wet pulp | 100 | 10 | 4 | 0 | 14 |
| C | C1–C4 | 4 | Wet pulp | 100 | 0 | 0 | 0 | 0 |
| D | D1–D3 | 3 | Wet pulp | 85–100 | 17 | 3 | 2 | 22 |
| D | Dw | 1 | Water | Unknown | 9 | 4 | 1 | 14 |
| H | h1–h3 | 3 | Dry pulp | 0 | 0 | 0 | 0 | 0 |
| I | i1–i3 | 3 | Dry pulp | 0 | 0 | 0 | 0 | 0 |
| Products | | | | | | | | |
| A | a1–a5 | 5 | Coreboard | 45–75 | 0 | 0 | 4 | 4 |
| B | b1–b2 | 2 | Coreboard | 75 | 0 | 0 | 4 | 4 |
| C | c1–c2 | 2 | Kitchen roll | Unknown | 0 | 0 | 0 | 0 |
| D | d | 1 | Food packaging board | 55 | 0 | 0 | 5 | 5 |
| E | e | 1 | Food packaging board | 50–60 | 0 | 0 | 2 | 2 |
| F | f | 1 | Food packaging board | 50–60 | 0 | 0 | 1 | 1 |
| G | g | 1 | Food packaging board | 50–60 | 0 | 0 | 4 | 4 |
| Total | | 36 | | | 65 | 15 | 27 | 107 |
| Pulp and water samples | | | | | 65 (74.7%) | 15 (17.2%) | 7 (8.0%) | 87 |

using cellulose nitrate filters of pore size Ø 0.45 µm (Sartorius AG, Göttingen, Germany). A loop of mycelium was suspended in 120 µl of sample buffer (DuPont Qualicon), the suspension was mixed thoroughly (Pellet Peste Mixer®, DuPont Qualicon) and 30 µl was transferred to a sample carrier after which the standard procedure was followed. Alternatively, the mycelium was collected from cultures grown on cellulose nitrate filters placed onto the surface of the agar plate. The restriction enzymes used were *Eco*RI and *Pvu*II (DuPont Qualicon). The automated system generated a pattern for each strain using proprietary algorithms. Each batch included six marker lanes including a total of 30 molecular markers, which the system used for selection of a ribogroup already existing in the database or for creation of a new one and for calculation of the similarities between different patterns. A ribogroup is defined as a set of closely related patterns (threshold similarity 0.96) that are mathematically indistinguishable from one another by the system. The isolates are called ribotypes and they have the same code as the relevant ribogroup (here coded Eco-number or Pvu-number). To ensure reproducibility, the patterns of all new ribogroups were analysed three times.

In order to compare the riboprint patterns they were transferred to the BioNumerics programme (Applied Maths, Sint-Martens-Latem, Belgium) and analysed by clustering methods using Pearson correlation and UPGMA. In addition, the riboprint patterns of 37 relevant type strains (received from DSMZ) from the VTT RiboPrint database were included (Figs. 1, 2, 3). The same reference strains were also used for partial sequencing of the 16S rRNA gene.

### 16S rDNA sequence analysis and PCR reactions

The partial 16S rRNA gene sequences (ca. 450 nucleotides between 10 and 550 bp according to *E. coli* numbering) were determined by direct sequencing of PCR-amplified 16S rDNA. The primers 10–30F (5′ GAGTTTGATCCTGGCTCAG 3′) and 1500R (5′ AGAAAGGAGGTGATCCAGCC 3′) were used for PCR and the primer 530R (5′ GKATTACCGCGGCKGCTG 3′) for partial sequencing. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of the PCR product were carried out as described previously [25]. Purified PCR products were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer. The sequences were compared with those of the type strains as well as with 16S rDNA reference sequences obtained from the Ribosomal Database Project [22].

## Results

### Occurrence of actinobacteria and thermoactinomycetes

Except for the wet pulps and end products (kitchen rolls) of mill C, all samples containing recycled fibres harboured actinobacteria or thermoactinomycetes, whereas all of the virgin fibre dry pulps used as a reference were free of these bacteria (Table 1). In almost all samples the number of streptomycetes ($10^4$–$10^6$ cfu $g^{-1}$ d.w.) exceeded that of *Nocardiopsis* isolates ($10^2$–$10^4$ cfu $g^{-1}$ d.w.). The numbers of thermoactinomycetes in wet pulp samples were even lower (up to $10^2$ cfu $g^{-1}$ d.w.). Because of the high number of rapidly developing streptomycetes colonies, those of *Nocardiopsis* and thermoactinomycetes may have been overgrown and thus their numbers were probably underestimated. In the absence of competition, such as in board samples, these taxa were frequently detected, although their numbers were still low ($<10$ cfu $g^{-1}$ d.w.). In many cases they were detected only when samples were placed directly on agar plates.

### Preliminary screening

On the basis of the morphological studies, pigmentation and fatty acid profiles of the isolates were affiliated with the genera *Streptomyces* (65 isolates), *Nocardiopsis* (15) and *Laceyella* (formerly *Thermoactinomyces*) (27) (Table 1). The identification of the majority of *Laceylla* isolates was very good (only three isolates had a similarity index of $<0.500$) on the basis of fatty acid profiles, whereas only a few isolates of *Streptomyces* and *Nocardiopsis* could be identified reliably to the species level (similarity index $>0.500$) (Table 2). Most of the mesophilic streptomycetes (28/44, 64%) indicated *S. coelicolor* (*S. albidoflavus*) and almost all thermophilic isolates were related to *S. thermoviolaceus*.

### Characterization by automated ribotyping

A total of 31 strains of *Streptomyces*, 11 of *Nocardiopsis* and 11 of *Laceyella* isolates was selected for further studies. Automated ribotyping with *Pvu*II (*Eco*RI) digestion divided them into 26 (22), 9 (9) and 9 (7) ribogroups (ribotypes), respectively (Table 2). Most of the isolates generated satisfactory patterns with both enzymes (data not shown), but the discrimination power of *Pvu*II was slightly higher than that of *Eco*RI. In addition, *Eco*RI digestion was often incomplete or generated fewer fragments (bands) than the *Pvu*II digestion. As the riboprint identification database provided by the manufacturer was too incomplete to affiliate patterns to taxon names, identification was continued by partial 16S rRNA gene sequencing.

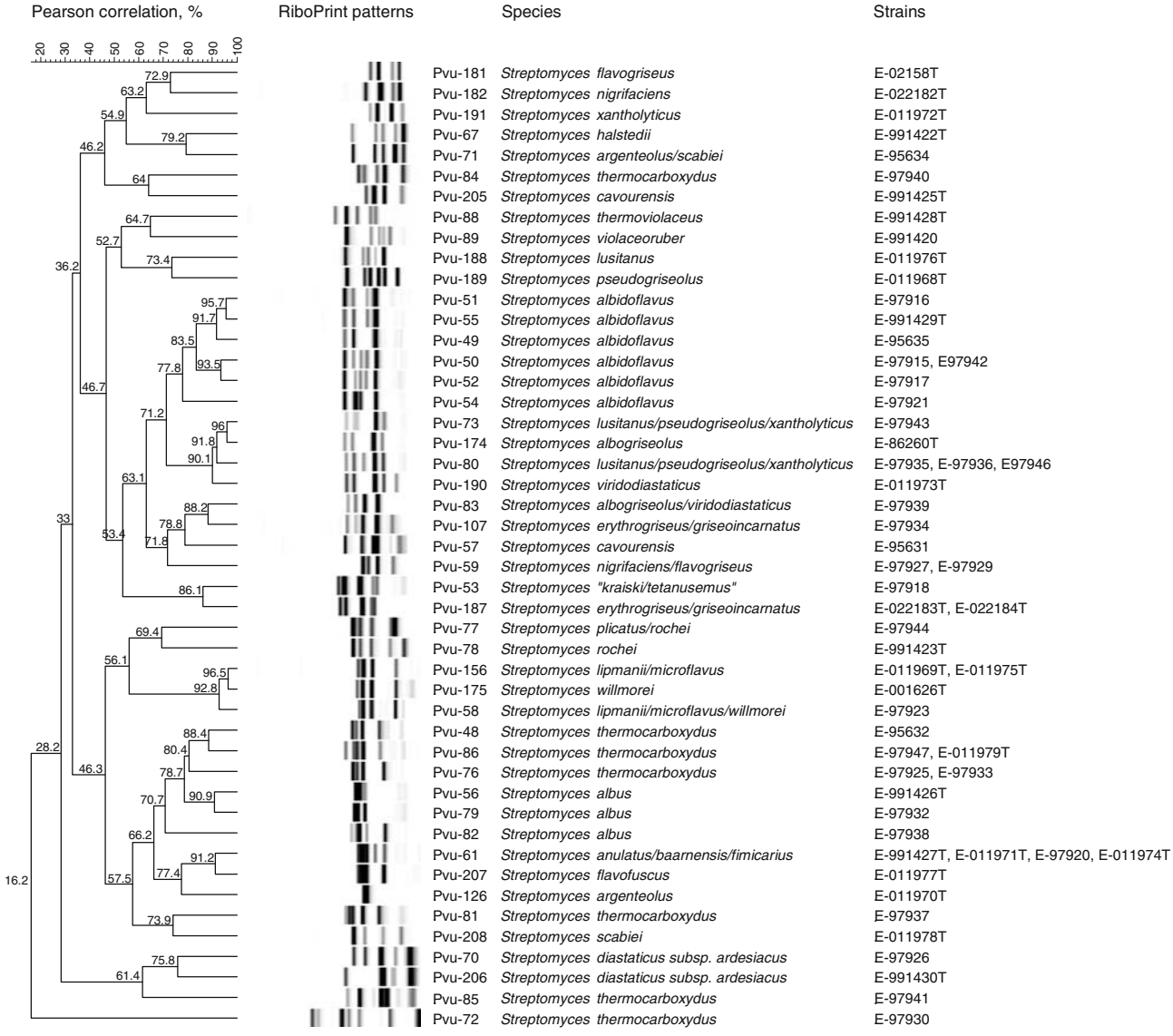


**Fig. 1** Clustering of the generated riboprint patterns (*Pvu*II digestion) of *Streptomyces* isolates and 29 type strains (T)

The clustering of riboprint patterns of mill isolates and relevant type strains (indicated by partial sequencing) is presented in Figs. 1, 2, and 3. Surprisingly the type strains of *Streptomyces anulatus, S. baarnensis* and *S. fimicarius* matched to the same ribogroup (Pvu-61, Fig. 1) and could not be separated by this method, though they are considered to be different species. The same was found for the type strains of *S. microflavus* and *S. lipmanii* (Pvu-156) as well as of *S. erythrogriseus* and *S. griseoincarnatus* (Pvu-187), respectively.

## Identification by partial 16S rDNA sequencing

The *Streptomyces* isolates formed at least 14 different taxa (Table 2). The mesophilic *S. albidoflavus* (*coelicolor*) and the thermophilic *S. thermocarboxydans* were the most common species thriving in all three mills (A, B and D), the pulp of which contained actinobacteria. Of these, *S. albidoflavus* occurred most frequently in different samples of mill A, whereas *S. thermocarboxydans* was most abundant in samples of mill D. The results of ribotyping and sequence analysis were in agreement for *S. albidoflavus* isolates (Fig. 1). As a moderately thermophilic organism, *S. thermocarboxydans* strains were isolated from both the mesophilic (30°C) and thermophilic (45°C) growth conditions used. Only four of eight riboprint patterns of *S. thermocarboxydans* mill isolates clustered with the type strain E-011979$^T$ of the species (Pvu-86, Fig. 1), indicating that not all of them are typical members of this species. The other bacterium, isolated frequently from samples of mill D, was closely related (99.5% similarity) to *S. lusitanus*, *S. pseudogriseolus* and *S. xantholyticus*. However, the riboprint pattern similarities of the type strains of these species were only distantly similar to the patterns of the paper

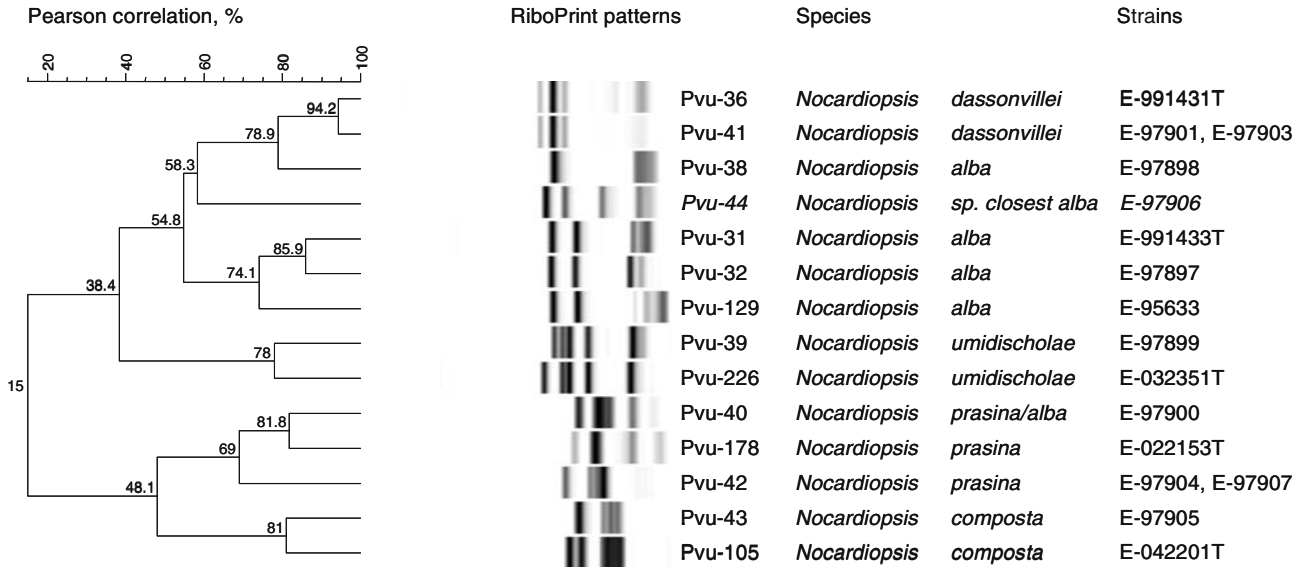


**Fig. 2** Clustering of the generated riboprint patterns (*Pvu*II digestion) of *Nocardiopsis* isolates and five type strains (T)

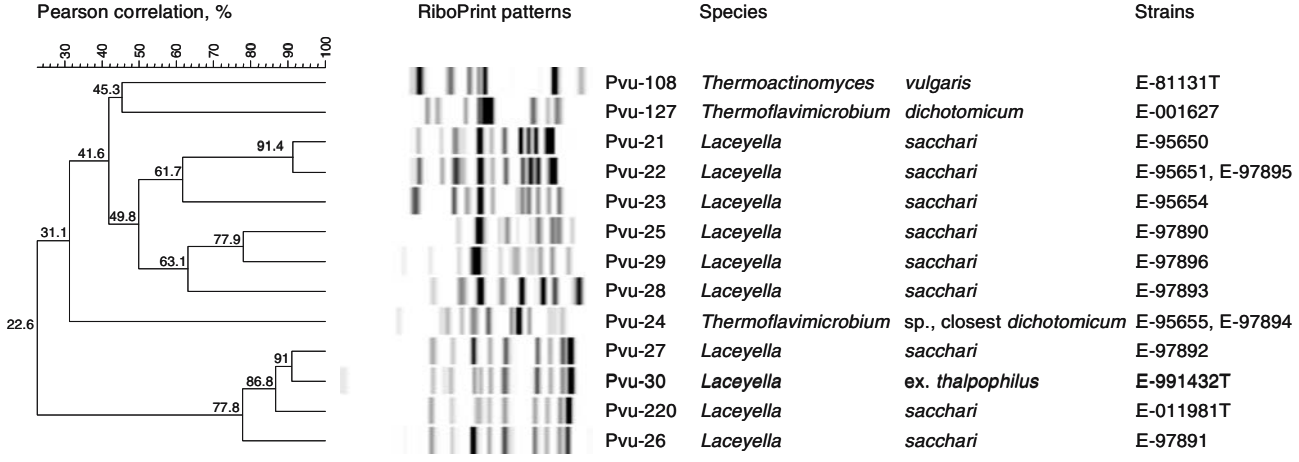


**Fig. 3** Clustering of the generated riboprint patterns (*Pvu*II digestion) of thermoactinomycetes isolates, three type strains (T) and *T. dichotomicum* E-001627 (DSM 43308)

mill isolates, which formed two closely related riboprints (Pvu-73 and Pvu-80). In many cases, the affiliations of strains to validly named species on the basis of sequence analysis and riboprint analyses were not in accordance, which may indicate the presence of novel species.

The *Nocardiopsis* isolates formed six clusters (Table 2), five of which grouped with the type species *N. alba*, *N. composta*, *N. dassonvillei*, *N. prasina* and *N. umidischolae* (Fig. 2). Two isolates (E-97906 and E-97900) were equidistantly related to *N. alba* and *N. prasina* by 16S rRNA gene sequencing and the riboprint patterns showed differences from those of the type strains of the respective species; thus they may represent new species.

Due to the unambiguous results of FAME and ribotyping, only five thermoactinomycetes isolates were selected for partial sequencing (Table 2, Fig. 3). Three sequences confirmed their preliminary identification as *Laceyella sacchari*, but the sequence similarity (95.5% to *Thermoflavimicrobium dichtomicum*, formerly *Thermoactinomyces dichtomicus*) of two strains (E-95655 and E-07894) indicated a new species within one of the genera described recently as a result of the taxonomic dissection of the genus *Thermoactinomyces* [39]. Most of the mill isolates did not cluster with the type strain of *L. sacchari* (Fig. 3), indicating significant genomic heterogeneity of this species.

## Discussion

Actinobacteria or thermoactinobacteria were common in samples containing recycled fibres, whereas they were completely lacking in the samples containing only virgin

**Table 2** Identification of actinobacterial isolates selected for further studies

| Strain | Source | Colour | °C | FAME | | Riboprint patterns | | Partial 16S rDNA sequencing | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Profile | Sim. | *Pvu*II | *Eco*RI | Sim. (%) | Closest species |
| *Streptomyces* isolates | | | | | | | | | |
| E-95635 | A3 | Yellow | 30 | *S. coelicolor* | 0.323 | Pvu-49 | Eco-906 | 99.8 | *S. albidoflavus* |
| E-97915 | A5 | Yellow | 30 | *S. coelicolor* | 0.671 | Pvu-50 | Eco-957 | 100 | *S. albidoflavus* |
| E-97942 | $D_W$ | Yellow | 30 | *S. coelicolor* | 0.470 | Pvu-50 | Eco-907 | NA | NA |
| E-97917 | A2 | Yellow | 30 | *S. albidoflavus* | 0.887 | Pvu-52 | Eco-907 | 100 | *S. albidoflavus* |
| E-97921 | A5 | Yellow | 30 | *S. coelicolor* | 0.102 | Pvu-54 | Eco-907 | 100 | *S. albidoflavus* |
| E-97916 | B3 | Yellow | 30 | *S. coelicolor* | 0.091 | Pvu-51 | Eco-908 | 100 | *S. albidoflavus* |
| E-97918 | D1 | Yellow | 30 | *S. coelicolor* | 0.843 | Pvu-53 | Eco-909 | 99.1//100 | *S. albidoflavus* //"*kraiski*"/ "*tetanusemus*" |
| E-97927 | A2 | Yellow | 30 | *S. halstedii* | 0.362 | Pvu-59 | Eco-915 | 100/99.7 | *S. anulatus* group/*S. halstedii* |
| E-97929 | B4 | Grey | 30 | *S. halstedii* | 0.561 | Pvu-59 | Eco-915 | 100 | *S. nigrifaciens*/*flavogriseus* |
| E-97920 | B2 | Yellow | 30 | *S. coelicolor* | 0.289 | Pvu-61 | Eco-913 | 100//99.5 | *S. baarnensis*/*fimicarius*/*flavofuscus*// *anulatus* |
| E-95631 | A1 | Yellow | 30 | *S. anulatus* | 0.534 | Pvu-57 | Eco-912 | 99.1 | *S. cavourensis* |
| E-95634 | A2 | Grey | 30 | *S. anulatus* | 0.625 | Pvu-71 | Eco-922 | 100 | *S. argenteolus*/*scabiei* |
| E-97923 | B4 | Yellow | 30 | *S. anulatus* | 0.196 | Pvu-58 | Eco-914 | 100 | *S. lipmanii*/*microflavus*/*willmorei* |
| E-97943 | $D_W$ | Grey | 30 | *S. coelicolor* | 0.446 | Pvu-73 | Eco-926 | 99.5 | *S. lusitanus*/*pseudogriseolus*/ *xantholyticus* |
| E-97930 | D1 | Grey | 30 | *S. anulatus* | 0.467 | Pvu-72 | Eco-916 | 100 | *S. thermocarboxydus* |
| E-97926 | A5 | Grey | 45 | *S. violaceoruber* | 0.325 | Pvu-70 | Eco-920 | 99.8 | *S. diastaticus* subsp. *ardesiacus* |
| E-97939 | B2 | Grey | 45 | *S. anulatus* | 0.207 | Pvu-83 | Eco-917 | 100 | *S. albogriseolus*/*viridodiastaticus* |
| E-97932 | A1 | Light yellow | 45 | *S. thermoviolaceus* | 0.430 | Pvu-79 | Eco-911 | 98.9 | *S. albus* |
| E-97938 | A2 | White | 45 | *S. thermoviolaceus* | 0.272 | Pvu-82 | Eco-911 | 98.9 | *S. albus* |
| E-97935 | D2 | Grey | 45 | *S. thermoviolaceus* | 0.507 | Pvu-80 | Eco-925 | 99.5 | *S. lusitanus*/*pseudogriseolus*/ *xantholyticus* |
| E-97946 | $D_W$ | Grey | 45 | *S. thermoviolaceus* | 0.372 | Pvu-80 | Eco-925 | 99.5 | *S. lusitanus*/*pseudogriseolus*/ *xantholyticus* |
| E-97936 | D3 | Grey | 45 | *S. thermoviolaceus* | 0.348 | Pvu-80 | Eco-926 | 99.5 | *S. lusitanus*/*pseudogriseolus*/ *xantholyticus* |
| E-97933 | B2 | Grey | 45 | *S. thermoviolaceus* | 0.546 | Pvu-76 | Eco-927 | 100 | *S. thermocarboxydus* |
| E-97925 | A5 | Grey | 45 | *Streptovertivillium* | 0.321 | Pvu-76 | Eco-927 | 100 | *S. thermocarboxydus* |
| E-97937 | A2 | Grey | 45 | *S. thermoviolaceus* | 0.136 | Pvu-81 | Eco-927 | 100 | *S. thermocarboxydus* |
| E-95632 | B2 | NA | 45 | *S. coelicolor* | 0.334 | Pvu-48 | Eco-927 | 100 | *S. thermocarboxydus* |
| E-97940 | D2 | Grey | 45 | *Streptovertivillium* | 0.138 | Pvu-84 | Eco-929 | 100 | *S. thermocarboxydus* |
| E-97941 | D3 | Grey | 45 | *S. thermoviolaceus* | 0.212 | Pvu-85 | Eco-930 | 100 | *S. thermocarboxydus* |
| E-97947 | $D_W$ | Grey | 45 | *S. thermoviolaceus* | 0.133 | Pvu-86 | Eco-931 | 100 | *S. thermocarboxydus* |
| E-97934 | D1 | Grey | 45 | *S. violaceoruber* | 0.437 | Pvu-107 | Eco-932 | 100 | *S. erythrogriseus*/*griseoincarnatus* |
| E-97944 | $D_W$ | Grey | 45 | *S. thermoviolaceus* | 0.660 | Pvu-77 | Eco-924 | 100 | *S. plicatus*/*rochei* |
| *Nocardiopsis* isolates | | | | | | | | | |
| E-95633 | B2 | Light yellow | 30 | *N. alba* | 0.425 | Pvu-129 | Eco-759 | 99.8 | *N. alba* |
| E-97897 | A1 | Scant white | 30 | *N. dassonvillei* | 0.096 | Pvu-32 | Eco-759 | 99.8 | *N. alba* |
| E-97898 | B2 | Scant white | 30 | *N. alba* | 0.002 | Pvu-38 | Eco-763 | 99.8 | *N. alba* |
| E-97901 | D1 | Light yellow | 30 | *N. dassonvillei* | 0.186 | Pvu-41 | Eco-766 | 100 | *N. dassonvillei* |
| E-97903 | $D_W$ | Yellow/braun | 30 | *N. dassonvillei* | 0.336 | Pvu-41 | Eco-762 | 100 | *N. dassonvillei* |
| E-97900 | D1 | Light yellow | 30 | *N. dassonvillei* | 0.018 | Pvu-40 | Eco-765 | Bad/98.6 | *N. prasina*/*alba* |
| E-97899 | B2 | Light yellow | 30 | *N. dassonvillei* | 0.055 | Pvu-39 | Eco-764 | 99.8 | *N. umidischolae* |
| E-97905 | A1 | Scant white | 45 | *N. dassonvillei* | 0.006 | Pvu-43 | Eco-768 | 100 | *N. composta* |
| E-97904 | $D_W$ | Scant white | 45 | *N. dassonvillei* | 0.007 | Pvu-42 | Eco-767 | 100 | *N. prasina* |
| E-97907 | D1 | Light yellow | 45 | *N. dassonvillei* | 0.003 | Pvu-42 | Eco-767 | 100 | *N. prasina* |
| E-97906 | A5 | Scant white | 45 | no match | no match | Pvu-44 | Eco-769 | 100/98.6 | *N. prasina*/*alba* |
| Thermoactinomycetes isolates | | | | | | | | | |
| E-95650 | A2 | White | 45 | *L. sacchari* | 0.719 | Pvu-21 | Eco-751 | 99.6 | *L. sacchari* |
| E-95651 | a4 | White | 45 | *L. sacchari* | 0.657 | Pvu-22 | Eco-751 | NA | NA |
| E-97895 | $D_W$ | White | 45 | *L. sacchari* | 0.818 | Pvu-22 | Eco-751 | NA | NA |
| E-95654 | b2 | White | 45 | *L. sacchari* | 0.856 | Pvu-23 | Eco-752 | NA | NA |
| E-97890 | d | White | 45 | *L. sacchari* | 0.916 | Pvu-25 | Eco-754 | NA | NA |
| E-97891 | d | Light brown | 45 | *L. sacchari* | 0.365 | Pvu-26 | Eco-755 | 99.6 | *L. sacchari* |
| E-97892 | f | Light brown | 45 | *L. sacchari* | 0.770 | Pvu-27 | Eco-755 | NA | NA |
| E-97893 | g | White | 45 | *L. sacchari* | 0.804 | Pvu-28 | Eco-756 | 99.6 | *L. sacchari* |
| E-97896 | D2 | White | 45 | *L. sacchari* | 0.752 | Pvu-29 | Eco-758 | NA | NA |
| E-95655 | b2 | Light yellow | 45 | *T. vulgaris* | 0.182 | Pvu-24 | Eco-753 | 95.5 | *Thermoflavimicrobium dichotomicum* |
| E-97894 | g | Sandy | 45 | *T. vulgaris* | 0.005 | Pvu-24 | Eco-753 | 95.5 | *Thermoflavimicrobium dichotomicum* |

*NA* Not analysed

fibres. The exceptions were pulps of mill C, which may have been treated with growth inhibitors. Their microbial diversity was significant, representing at least 14 different taxa within the genus *Streptomyces*, six within *Nocardiopsis* and two within thermoactinomycetes (endospore-forming bacteria related to the genus *Bacillus*). The drying stage used in the board making processes had eliminated all members of the species *Streptomyces* and *Nocardiopsis* present in the wet recycled fibre pulp samples. Only the thermotolerant endospores of thermoactinomycetes persisted to the final products, as they may survive heating for 30 min at 90°C [15].

Although all streptomycetes isolates were reliably identified to the genus level on the basis of morphology, identification of species was difficult. Currently there are about 550 validly described *Streptomyces* species [4, http://www.bacterio.cict.fr/] exhibiting a non-discriminatory pattern of fatty acids composed mainly of iso/anteiso-15:0, iso-16:0 and iso/anteiso-17:0. As a result, individual species are not definable by individual fatty acids but show overlapping patterns [37], as was also observed in this study. This overlap is not only restricted to the FAME profiles, but it is also evident in the results of molecular analyses. In this study, several type strains of *Streptomyces* fell into the same ribogroup, indicating highly similar riboprint fingerprints. Similarly, several partial 16S rDNA sequences (ca. 450 nt) of mill isolates were highly related to more than one type of strain. This situation makes routine identification of certain streptomycetes to the species level almost impossible and calls for a reclassification of a large number of *Streptomyces* species. In order to solve these taxonomic ambiguities, DNA/DNA hybridization studies and chemotaxonomic tests need to be performed, which is laborious, expensive and out of reach for a routine industrial identification service. On the basis of quite recent hybridization results [21], e.g. *S. lipmanii* and *S. willmorei* were reclassified as later synonyms of *S. microflavus*, which is in good agreement with our ribotyping results. In this study, the most common *Streptomyces* species were *S. albidophilus* and *S. thermocarboxydus*, occurring in all three mills of which the pulps contained actinobacteria. *S. thermocarboxydus* has been isolated from soil and it is moderately thermophilic, growing between 20 and 55°C [19]. The common human pathogen of this genus, *S. griseus*, was not detected in these samples.

*Nocardiopsis* isolates represented six different species. *N. composta* has recently been isolated from the air surrounding a composting facility [18], and *N. umidischolae* was found in indoor dust of a water-damaged school [23]. The human pathogenicity of the recently described species is not yet known, but at least *N. dassonvillei* belongs to the health hazard risk group 2 [4, 36] and may be able to cause diseases. In addition, members of one or two new species were probably present in the samples.

The taxonomic classification of the genus *Thermoactinomyces* has been revised and it has been transferred from the phylum and class Actinobacteria to the phylum Firmicutes class ''Clostridia'' [5, 9, 38]. Furthermore, quite recently three new genera (*Laceyella*, *Thermoflavimicrobium* and *Seinonella*) were reclassified from the genus *Thermoactinomyces* [39]. Thus, the most common thermoactinomycete in this study is now called *L. sacchari* (formerly *Thermoactinomyces sacchari* and *T. thalpophilus*), but two strains may represent new species within one of these genera. The detected numbers of thermoactinomycetes in wet pulp samples were low, but it was possible by ribotyping to trace the path of contamination from pulps containing recycled fibres to board samples. Spores of thermoactinomycetes have been implicated in various forms of hypersensitivity pneumonitis, especially farmer's lung disease [15].

In this study, the automated ribotyping method was applied to the identification of actinobacteria and morphologically related strains. This may support partial 16S rDNA sequencing as soon as a comprehensive, curated and public identification database becomes available for RiboPrint® patterns and possibly other more effective enzymes are used. The standardization of the system allows combination of the results of different instruments and locations [32], as also noticed in this study when the patterns of some type strains were generated by two instruments, one at VTT in Finland and the other at DSMZ in Germany (data not shown). The different cultivation conditions used in these laboratories did not affect the results either. This study concentrated on the analysis of filament-producing organisms not previously analysed. Thus, the suitability of different restriction enzymes as well as the optimal treatment of these organisms needs to be evaluated. Different restriction enzymes have differing discrimination power, as cleavage results in different sets of DNA fragments and different riboprints. Whereas initially *Eco*RI was the only enzyme used by the RiboPrinter® System, other enzymes have recently been introduced. The discrimination power of *Pvu*II among *Listeria monocytogenes* isolates has been reported to be slightly higher than that of *Eco*RI [1, 7, 11], which was also noticed among actinobacteria analysed in this study. The same conclusion was recently reached by Ritacco et al. [26], who applied automated ribotyping for the dereplication of *Streptomycetes* and detection of specific biosynthetic genes.

It can be concluded that discrimination power of automated ribotyping is sufficient for identification purposes, it is reproducible and the data can be stored for later use. It is rapid, simple to carry out, standardized, objective, labour saving and versatile. The disadvantages of the system are that it is very expensive to purchase, the running costs are high and effective use of the system requires good characterization and identification databases. The current DUP identification database (Release 12.2 © 2000 Qualicon, last update August 2004) also includes several *Pvu*II digested patterns for streptomycetes, but these could not identify the isolates of this study. The inclusion of fingerprints generated in this study into the RiboPrint identification database of VTT will be extremely useful in future for reliable and

rapid identification of actinobacteria and morphologically related taxa.

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