

**STREPTOMYCETES IN INDOOR ENVIRONMENTS -
PCR BASED DETECTION AND DIVERSITY**

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ACADEMIC DISSERTATION

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ABSTRACT

Streptomycetes are a group of environmental bacteria present in almost all kinds of environments. They are common in soil, but also found in sediments, composts and fodder, aquatic habitats, and buildings. In buildings they have been associated with moisture conditions that enable microbial growth, and thus, considered as indicators of moisture and microbial damage. Streptomycetes are also potent inducers of inflammatory responses *in vitro* and *in vivo* and therefore, possible causes of adverse health effects in moisture damaged buildings.

A PCR based detection method was applied and tested with soil, building material and dust samples. According to the current sequence information, the PCR primers were specific for streptomycetes. The method proved to be specific also in experiments using potentially interfering microbes and environmental samples as template DNA. The results were confirmed by sequencing.

Streptomycetes were detected in 81 % of the dust samples (N=47) by PCR and mesophilic actinomycetes in 36 % of the samples by culture. The results of culture and PCR did not correlate well with each other, which may have several reasons. The amount of negative samples was higher by culture, indicating that the sensitivity of the method is poorer. The slow-growing streptomycetes were probably overgrown by other microbes and remained undetected. PCR amplification may have been affected by inhibiting agents present in environmental samples and co-purified with DNA. The amount of streptomycete -specific PCR amplification product was higher in dust samples collected from moisture-damaged houses than in dust from non-damaged residences ($p < 0.05$, Mann-Whitney test).

The diversity of streptomycetes in indoor environments was investigated by characterising *Streptomyces* strains isolated from indoor air (N=9), building materials (N=2), and house dust (N=15) by 16S rDNA sequencing. In a phylogenetic tree, the majority of these strains (16) affiliated with the *Streptomyces griseus* -cluster, which is the largest cluster of the genus, inhabiting many secondary metabolite-producing species. Another four strains grouped with members of the *Streptomyces albidoflavus* -cluster and a third group affiliated with *Streptomyces cyaneus* and with the model actinomycete *Streptomyces coelicolor* A3(2). Two isolates showed no clear affiliation to known sequences.

The results of a culture-independent approach, which included direct PCR amplification of the 16S rDNA from template DNA isolated from building materials, sequencing and sequence analysis, revealed that these sequences generally clustered with the same species than cultured strains. However, a somewhat broader diversity was detected indicating that this approach could be useful for detecting strains that are not easily cultured.

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Kuopio, December 2002

Helena Rintala

ABBREVIATIONS

| | |
|---------|---|
| ARDRA | Amplified ribosomal DNA restriction analysis |
| ATCC | American Type Culture Collection |
| CTAB | Cetyl-trimethylammonium bromide |
| DMSO | Dimethylsulphoxide |
| DTT | Dithiothreitol |
| EBI | European Bioinformatics Institute |
| EDTA | Ethylene diamine tetraacetate |
| EMBL | European Molecular Biology Laboratory |
| DGGE | Denaturing gradient gel electrophoresis |
| DG18 | Dichloran-glycerol agar |
| DNA | Deoxyribonucleic acid |
| DSMZ | German Collection of Microorganisms and Cell cultures |
| MEA | Malt extract agar |
| MVOC | Microbial volatile organic compounds |
| NCIB | National Collection of Industrial Bacteria |
| PCR | Polymerase chain reaction |
| PLFA | Phospholipid fatty acid |
| PKS | Polyketide synthase |
| RAPD | Randomly amplified polymorphic DNA |
| rDNA | Ribosomal DNA |
| RDP | Ribosomal Database Project |
| Rep-PCR | Repetitive intergenic DNA sequence PCR |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| SDS | Sodium dodecyl sulphate |
| TGGE | Temperature gradient gel electrophoresis |
| T-RFLP | Terminal restriction fragment length polymorphism |
| Tris | Tris (hydroxymethyl) aminomethane |
| TYG | Tryptone yeast extract glucose agar |
| VTT | Technical Research Center |

LIST OF ORIGINAL PUBLICATIONS

- I** Rintala H, Nevalainen A, Rönkä E, Suutari M. PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. *Molecular and Cellular Probes* 2001, 15:337-347.
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- III** Suutari M., Rönkä E, Lignell U, Rintala H, Nevalainen A. Characterisation of *Streptomyces* spp. isolated from water-damaged buildings. *FEMS Microbiology Ecology* 2002, 39:77-84.
- IV** Rintala H, Nevalainen A, Suutari M. Diversity of streptomycetes in water-damaged building materials based on 16S rDNA sequences. *Letters in Applied Microbiology* 2002, 34:439-443.

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ORIGINAL PUBLICATIONS

1 INTRODUCTION

Streptomycetes are Gram-positive, filamentous soil bacteria that undergo morphological differentiation during their life cycle. They normally occur as spores, but in the presence of sufficient moisture and nutrients, the spores can germinate and form vegetative mycelium. In response to environmental signals, such as a shortage of nutrients or water, the process of differentiation is set in motion, and spores resistant to desiccation and starvation are formed again. At the same time, the production of pigments, antibiotics and other secondary metabolites is initiated (Kutzner 1986, Williams *et al.* 1989).

Streptomycetes are common in soil, but also found in composts, fodder and aquatic habitats. Due to their characteristic life cycle, they are good survivors under the fluctuating growth conditions predominating in nature (Kutzner 1986). Streptomycetes have also been isolated from indoor environments, from air, building material and dust samples (Nevalainen *et al.* 1991, Andersson *et al.* 1999, Hyvärinen *et al.* 2002). They are considered as indicators of moisture conditions in buildings that are favourable for microbial growth (Samson *et al.* 1994).

Streptomycetes are known to be producers of many secondary metabolites, which have different biological activities, such as antibacterial, antifungal, antiparasitic, antitumor, and immunosuppressive actions (Demain 1999). They are not particularly pathogenic, although some species can also cause infections (Mishra *et al.* 1980), but they have been shown to be potent inducers of inflammatory responses *in vitro* and *in vivo* (Hirvonen *et al.* 1997b, Jussila *et al.* 2002).

Culture methods are currently used for the exposure assessment of microbes in indoor environments. However, it has been estimated that only 0.001-15 % of the environmental microbial population can be cultured (Amann *et al.* 1995), for indoor environments a value of 1% cultivability has been reported (Toivola *et al.* 2002). PCR-based detection methods are culture-independent and potentially more sensitive than culturing, and thus, can provide better tools for exposure assessment (Alvarez *et al.* 1994, Zhou *et al.* 2000, Roe *et al.* 2001). PCR based methods for detection of some indoor microbes, though not for streptomycetes, have been reported (Bartlett *et al.* 1997, Haugland *et al.* 1999a, Zhou *et al.* 2000, Cruz-Perez *et al.* 2001b). Since streptomycetes are frequently isolated from moisture damaged buildings, their enhanced detection will facilitate the assessment of their prevalence and possible connection to adverse health effects experienced by occupants of these buildings.

2 REVIEW OF LITERATURE

2.1 The genus *Streptomyces*

The streptomycetes are aerobic, Gram-positive bacteria, which produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthrospores. The substrate mycelium and spores can be pigmented, but also diffusible pigments are produced (Williams *et al.* 1989). On agar plates, they form lichenoid, leathery or butyrous colonies (Williams *et al.* 1989). The GC-content of the DNA is 69-78 % (Williams *et al.* 1989). L-diaminopimelic acid is the characteristic compound present in the cell wall peptidoglycan of streptomycetes (Lechevalier and Lechevalier 1970). The streptomycetes are able to utilise a wide range of organic compounds as a carbon source, including complex biological materials, such as cellulose and lignin, and can also utilise an inorganic nitrogen source (Kutzner 1986). They are also known as producers of many secondary metabolites, such as antibiotics and other bioactive compounds (Williams *et al.* 1989, Kutzner 1986). These bacteria are widely distributed in various habitats, including man-made environments.

2.1.1 Classification

The number of species in the genus *Streptomyces* is increasing continually. In 1997, 464 validly described species and 45 subspecies were reported (Hain *et al.* 1997), in September 2002 there were over 650 species listed in the German Collection of Microorganisms and Cell Cultures (DSMZ). Thus, the genus is the largest of the order *Actinomycetales* within the class *Actinobacteria* (Stackebrandt *et al.* 1997). The genera *Streptoverticillium* and *Kitasatospora* have been included in the genus *Streptomyces* (Witt and Stackebrandt 1990, Wellington *et al.* 1992), although the taxonomic position of *Kitasatospora* is unclear (Zhang *et al.* 1997).

The classification of streptomycetes was originally based on morphological and biochemical characterisation, later on physiological tests (Kutzner 1986, Williams *et al.* 1983, Goodfellow *et al.* 1987, Kämpfer *et al.* 1991, Goodfellow *et al.* 1992). Serological methods (Ridell *et al.* 1986), phage typing (Korn-Wendish and Schneider 1992) and protein profiling (Ochi 1995, Taguchi *et al.* 1997) have also been used in their classification. The application of genetic methods, such as DNA-DNA reassociation (Labeda 1992, Kim *et al.* 1999) and 16S rRNA gene sequence analysis (Gladek *et al.* 1985, Stackebrandt *et al.* 1992, Kim *et al.* 1996,

Takeuchi *et al.* 1996, Hain *et al.* 1997, Kataoka *et al.* 1997, Kim *et al.* 1999) has partly confirmed the phenotypic classification, but this approach has also provided new information. The review by Anderson and Wellington (2001) summarises all of the approaches to clarify the taxonomy of streptomycetes; however, this field remains somewhat confused.

According to all classifications, the streptomycetes can be divided into major, minor and single member clusters. The major clusters consist of six or more type strains and are considered as species groups and the minor clusters of two to five type strains could be considered as one species. The number and composition of clusters vary to a greater or lesser extent depending on the approach used, but the major clusters seem to be quite consistent (Anderson and Wellington 2001). The largest is the *Streptomyces albidoflavus* –cluster, which consists of 71 strains and has been divided into subclusters. The most important of these are *S. albidoflavus*, *Streptomyces anulatus* and *Streptomyces halstedii* subclusters (Williams *et al.* 1983).

2.1.2 Identification

A probabilistic identification matrix for streptomycetes, based on 41 characters like spore chain and spore morphology, pigmentation, physiological abilities, antibiosis and resistance to antibiotics, was developed by Williams *et al.* (1983). This scheme was applied to unknown isolates, 80 % of which could be assigned to a cluster. Kämpfer *et al.* (1991) revised the data using more characters and testing more strains and comparing the data with published genetic and chemotaxonomic data. Goodfellow *et al.* (1992) supplemented the method with rapid enzyme tests using fluorogenic substrates. Phage sensitivity and serological as well as cellular fatty acid characterisation can be useful for assigning an unknown isolate to the genus *Streptomyces*, however, these methods have difficulties to discriminate the species at the intragenus level (Anderson and Wellington 2001).

DNA based molecular methods have been used for species differentiation and the identification of streptomycetes. DNA-DNA reassociation is a method measuring the DNA relatedness of two organisms and has proved to be suitable for the investigation of relationships between closely related taxa, such as species (Stackebrandt and Goebel 1994). Strains belonging to the same species will generally have greater than 70 % DNA-DNA relatedness (Stackebrandt and Goebel 1994). The method has been used in numerous studies

dealing with streptomycetes; in some cases it has shown good correlation with the identification based on morphology and physiology, but in other cases there was no apparent correlation (Healy and Lambert 1991, Doering-Saad *et al.* 1992, Labeda 1992, Labeda and Lyons 1992, Kim *et al.* 1999, Bouchek-Mechiche *et al.* 2000). While DNA-DNA reassociation has shown to be useful in the identification of *Streptomyces* species, because of the instability of the genome, it should not be used alone, but in connection with other tests (Anderson and Wellington 2001).

The sequence analysis of the genes coding for the ribosomal subunits (16S, 23S, and 5S rRNA), in particular the 16S rRNA gene, has become an important tool in bacterial identification, since it provides information about the phylogenetic placement of species (Woese 1987, Brenner *et al.* 2001). The DNA sequences of the ribosomal genes are highly conserved, but the genes also contain variable regions, which sometimes can be useful for species discrimination (Stackebrandt and Goebel 1994, Rosselló-Mora and Amann, 2001). Kataoka *et al.* (1997) utilised the hypervariable region of the 16S rRNA gene to create an index for *Streptomyces* species identification. However, the 16S rDNA sequence information alone is not sufficient for species identification (Rosselló-Mora and Amann, 2001). Also repetitive intergenic DNA sequences (rep-PCR) (Sadowsky *et al.* 1996), and PCR-RFLP of the 65-kDa heat shock protein gene (Steingrube *et al.* 1997) have been used for the classification and identification of pathogenic and other clinically important *Streptomyces* species.

2.1.3 Isolation methods

There is no single correct way to selectively isolate streptomycetes from environmental samples. Some pre-treatment methods have been used for the enrichment, such as heating or treating with ammonia and sodium hypochlorite, chloramine or CaCO₃, these being based on the fact that streptomycete spores are more resistant to these treatments than Gram-negative bacteria (Kutzner 1986, Goodfellow and Simpson 1987).

Further selectivity can be achieved by using selective nutrient sources in the cultivation media. In contrast to many other bacteria, streptomycetes are able to utilise many biopolymers and are satisfied with an inorganic nitrogen source like nitrate (Kutzner 1986, Goodfellow and Simpson 1987, Williams *et al.* 1989). Isolation media containing starch or glycerol as the

carbon source and nitrate, casein or arginine as the nitrogen source have proven to be the most effective growth media for selective isolation of streptomycetes (Kutzner 1986).

The addition of antifungal agents to the isolation media suppresses the growth of fungal species on the plates, however antibacterial antibiotics cannot be used to reduce the amount of other bacterial isolates because they will also inhibit the growth of many streptomycetes. Cycloheximide (50-100 µg/ml), as well as pimaricin and nystatin (10-50 µg/ml) have been used (Kutzner 1986).

Mesophilic streptomycetes are usually cultivated at temperatures from 22 to 37 °C for 14 days and thermophilic species at 40 to 55 °C for 5 days (Kutzner 1986, Goodfellow and Simpson 1987, Williams *et al.* 1989). Most streptomycetes are neutrophilic, and the isolation media commonly have a neutral pH, thus, if acidophilic strains are to be isolated, the pH of the medium can be adjusted to 4.5, and for alkalophilic strains to pH 10-11. However, some species may also show remarkable adaptation to a wide pH range (Suutari *et al.* 2000).

2.1.4 Molecular detection methods

Stackebrandt *et al.* (1991) and Mehling *et al.* (1995) used 16S rDNA sequencing to determine those regions suitable for detection of streptomycetes, and proposed a genus-specific probe and primers targeting the 16S rRNA gene. However, all of them do not perfectly match all of the streptomycete sequences currently available. Huddleston *et al.* (1997) described a method for the molecular detection of streptomycin-producing streptomycetes in soil with DNA probes targeting the streptomycin biosynthetic genes. Roberts and Crawford (2000) applied randomly amplified polymorphic DNA (RAPD) PCR for the development of a strain-specific DNA probe for *Streptomyces lydicus*.

2.1.5 Occurrence of streptomycetes in natural and man-made environments

Streptomycetes are able to produce extracellular enzymes (Morosoli *et al.* 1997), which can decompose various materials. As reviewed by Williams (1985), streptomycetes have been implicated in the biodeterioration of silicone and natural rubber, wool, PVC plastic, cotton, jute fibre, wood, hay and grain. Jendrossek *et al.* (1997) isolated 50 rubber-degrading

actinomycetes, from which 33 were identified as streptomycetes. Mesophilic actinomycetes, many of which belong to the genus *Streptomyces*, have been frequently isolated from all kinds of building materials, especially from ceramic materials, paints and glues (Hyvärinen *et al.* 2002). They are able to grow and proliferate on plasterboards (Murtoniemi *et al.* 2002).

Soil

Soil is the most common habitat of streptomycetes, those bacteria can be found in all kinds of soils (Kutzner 1986, Williams *et al.* 1989). It has been observed that the streptomycetes in soil grow attached to surfaces, such as plant residues or fungal hyphae (Mayfield *et al.* 1972), and they may have an important ecological role in the degradation of litter in soil (Khan and Williams 1975, Kutzner 1986, Goodfellow and Simpson 1987). The largest concentrations of streptomycetes can be found in the organic horizon (Hagedorn 1976).

In most soils, streptomycetes comprise 1-20 % of the total viable count (Kutzner *et al.* 1986), and 64-97 % of the cultivable actinomycetes (Xu *et al.* 1996, Wang *et al.* 1999). The more arid the soil and cooler the climate, the higher the percentage of the streptomycetes in the total count of actinomycetes (Xu *et al.* 1996). In coniferous forest soil in Finland, streptomycetes represented 5 % of the viable count (Elo *et al.* 2000). Plate counts of streptomycetes in acidic soils, peat and mine waste ranged from 10^3 - 10^6 cfu / g dry wt soil (Khan and Williams 1975).

Streptomycetes may play a role in promoting plant growth, through control of root pathogens or in some indirect way, since some species are able to produce antifungal compounds (Yuan and Crawford 1995, Hamby and Crawford 2000). Streptomycetes are commercially used as biocontrol agents (Raatikainen 1995). On the other hand, *Streptomyces* spp. are the causative agents of potato scab, a plant disease, which causes significant financial losses to the agricultural community (Takeuchi *et al.* 1996).

Composts and fodder

Streptomycetes are found in composts and fodder, especially in self-heated hay or grain (Lacey and Crook 1988). During the early stages of composting or self-heating, mesophilic species are present, but these are replaced by thermotolerant species like *Streptomyces albus* or *Streptomyces griseus*, and with increasing temperature, the real thermophilic species take their place (Goodfellow and Simpson 1987). Thermophilic strains were isolated from

composts in numbers ranging from 10^3 to 10^5 cfu / g dry weight, and in mouldy hay from 10^5 to 10^7 cfu / g dry weight (Goodfellow *et al.* 1987). The strains were assigned to clusters *Streptomyces thermovulgaris*, *Streptomyces thermoviolaceus*, *Streptomyces macrosporus*, *Streptomyces megasporus* and *Streptomyces thermolineatus* (Goodfellow *et al.* 1987).

Aquatic habitats

Streptomycetes have been isolated from fresh water as well as marine environments, although, it has been a subject of debate, whether they are indigenous, or have been washed off from the surrounding soils (Cross 1981, Goodfellow and Simpson 1987). Moran *et al.* (1995) showed that in coastal marsh sediment, streptomycetes accounted for 2-5 % of the microbial community, and were an indigenous population.

Occasionally, streptomycetes also grow in drinking water reservoirs affecting the water quality by causing earthy odours, which are due to their production of volatile secondary metabolites, such as geosmin and methyl *iso*-borneol (Bentley and Meganathan 1981, Cross 1981, Wood *et al.* 1985).

2.1.6 Secondary metabolites

According to one definition, microbial secondary metabolites are substances that are not needed for the growth or other essential processes in the cell (Vining 1990). Secondary metabolites are mainly produced by microbial genera inhabiting soil and undergoing morphological differentiation, such as actinobacteria, bacilli and fungi (Vining, 1990). There are over 23 000 known microbial secondary metabolites, 42 % of which are produced by actinobacteria, 42 % by fungi, and 16 % by other bacteria (Lazzarini *et al.* 2000).

The streptomycetes are very potent producers of secondary metabolites. Out of the approximately 10 000 known antibiotics, 45-55 % are produced by streptomycetes (Demain 1999, Lazzarini *et al.* 2000). The secondary metabolites produced by them have a broad spectrum of biological activities; *e.g.* antibacterial (streptomycin, tetracycline, chloramphenicol), antifungal (nystatin), antiviral (tunicamycin), antiparasitic (ivermectin), immunosuppressive (rapamycin), antitumor (actinomycin, mitomycin C, anthracyclines), enzyme inhibitory (clavulanic acid), diabetogenic (bafilomycin, streptozotocin). Spore

pigments also have structures similar to those of other secondary metabolites and are synthesised by the same kinds of mechanisms (Metsä-Ketelä *et al.* 1999, Nakano *et al.* 2000). Some of the important secondary metabolites are listed in Table 1.

The genes coding for the proteins responsible for the synthesis of secondary metabolites are often clustered. These clusters include the genes for the actual biosynthesis, but also determinants for regulation and self-resistance (Pissowotzki *et al.* 1991). Sometimes the genes are located in plasmids and it is apparent that horizontal transfer of genes coding for secondary metabolites can take place, even in soil (Egan *et al.* 1998, Omura *et al.* 2001). At present, there are at least two completely sequenced *Streptomyces* genomes, the genome of *Streptomyces coelicolor* (Bentley *et al.* 2002) and that of *Streptomyces avermitilis* (Omura *et al.* 2001). Both species possess several secondary metabolite gene clusters that are spread over the whole genome, although a remarkable part of them is located near the ends of the chromosome (Omura *et al.* 2001). The ends of the streptomycete chromosome are less stable than the core region around the replication origin (Volff and Altenbuchner 1998), which means that the characteristics coded by genes at the end regions are more often subject to change.

The production of secondary metabolites is affected by the availability of nutrients. In fermentation experiments, the production of antibiotics is increased by the presence of a non-preferred carbon source, or by phosphate starvation (Aharonowitz and Demain 1978, McDowall *et al.* 1999). The source and availability of nitrogen can also influence the production of secondary metabolites (Aharonowitz 1980). The regulation mechanisms of the production of secondary metabolites are not yet fully understood, but they seem to be linked to the regulation of sporulation. Both processes are induced by hormone-like regulatory factors, such as A-factor by *S. griseus* (Horinouchi and Beppu 1992). The streptomycetes have a complex regulatory apparatus, 12 % of the proteins coded by the genes of *S. coelicolor* are predicted to have regulatory functions (Bentley *et al.* 2002).

Table 1. Examples of secondary metabolites produced by streptomycetes, their biological activities and the producing species known so far.

| Compound | Biological activity | Species | Reference |
|-----------------|---|---|---|
| Avermectin | Antiparasitic | <i>S. avermitilis</i> | Burg <i>et al.</i> 1979 |
| Anthracyclines | Antitumor | <i>S. galileus</i> | Fujii and Ebizuka 1997 |
| Bafilomycin | ATPase-inhibitor of micro-organisms, plant and animal cells | <i>S. griseus</i> <i>S. halstedii</i> | Werner <i>et al.</i> 1984 Frändberg <i>et al.</i> 2000 |
| Chloramphenicol | Antibacterial, inhibitor of protein biosynthesis | <i>S. venezuelae</i> | Bewick <i>et al.</i> 1976 |
| Hygromycin | Antimicrobial, immunosuppressive | <i>S. hygrosopicus</i> | Omura <i>et al.</i> 1987 Uyeda <i>et al.</i> 2001 |
| Lincomycin | Antibacterial, inhibitor of protein biosynthesis | <i>S. lincolnensis</i> | Peschke <i>et al.</i> 1995 |
| Mitomycin C | Antitumor, binds to double-stranded DNA | <i>S. lavendulae</i> | Mao <i>et al.</i> 1999 |
| Rapamycin | immunosuppressive, antifungal | <i>S. hygrosopicus</i> | Vezina <i>et al.</i> 1975 |
| Streptomycin | Antimicrobial | <i>S. griseus</i> | Egan <i>et al.</i> 1998 |
| Streptozotocin | Diabetogenic | <i>S. achromogenes</i> | Herr <i>et al.</i> 1967 |
| Tetracyclines | antimicrobial | <i>S. aureofaciens</i> <i>S. rimosus</i> | Saleh <i>et al.</i> 1985 Hansen <i>et al.</i> 2001 |
| Valinomycin | Ionophor, toxic for pro- and eukaryotes | <i>S. griseus</i> | Andersson <i>et al.</i> 1998 |

2.1.7 Adverse effects on human health

Infections

Streptomyces somaliensis has been identified as one of the causal agents of actinomycetoma in different parts of the world (McNeil and Brown 1994). Also the species *S. griseus*, *S. albus*, *S. rimosus*, *S. lavendulae*, *S. violaceoruber*, and *S. coelicolor*, have been isolated from clinical samples (Mishra *et al.* 1980, McNeil and Brown, 1994). Streptomycetes have been isolated from sputum, wounds, skin, blood, brain, tonsils, and dental caries (McNeil and Brown, 1994). *Streptomyces* species have been isolated from patients with acquired immunodeficiency syndrome; this may represent an increasing problem in today's world (Holtz *et al.* 1985, Ahmed *et al.* 1996).

Inflammatory disorders

A serious pulmonary disease, hypersensitivity pneumonitis, or extrinsic allergic alveolitis, with farmer's lung disease being the most well-known example, can be caused by thermophilic actinomycetes, including some members of the genus *Streptomyces* (Lacey and Crook 1988, Kotimaa 1990). Skin reactions have also been reported in farmers who have become sensitised to these microorganisms (Spiewak *et al.* 2001).

An epidemic of a respiratory disease, resembling allergic alveolitis, took place in a small community in Finland in 1978. Repeated exposures to tap water aerosols that were heavily contaminated with actinomycetes, including streptomycetes was discovered to have been responsible for the disease (Ojanen *et al.* 1983).

Inflammation potency of streptomycetes in vitro and in vivo

In vitro experiments with mouse and human macrophages and human epithelial cells have shown that spores of different fungi and bacteria isolated from moisture damaged buildings are potent inducers of inflammatory responses (Hirvonen *et al.* 1997b, Jussila *et al.* 1999, Huttunen *et al.* 2002). Streptomycetes induced the highest responses in a comparison with fungi or other bacteria (Huttunen *et al.* 2002). The biological activity was not dependent on the viability of the spores (Hirvonen *et al.* 1997c). Damp building material, which supports

streptomycete growth, also appears to have an effect on the induction of inflammatory responses (Murtoniemi *et al.* 2001, Roponen *et al.* 2001).

Mice exposed to *Streptomyces californicus* spores via intratracheal instillation show both acute inflammation of the lungs and systemic immunotoxicity, especially in the spleen (Jussila *et al.* 2002).

2.2 Molecular methods for the investigation of environmental microbes

With currently used cultivation methods, only a small part of the microbial diversity is detected. The cultivability values reported range from 0.001 to 15%, depending on the environment (Amann *et al.* 1995). On the other hand, PCR amplification of 16S rRNA genes from environmental samples has revealed that 7-64 % of the amplified sequences originated from uncultured microorganisms (Kuske *et al.* 1997, Zhou *et al.* 1997). Comparisons of amounts of total and cultivable microbes in the indoor environment have shown that the cultivable part of the microbial community ranges from 1 to 10% (Palmgren *et al.* 1986, Toivola *et al.* 2002).

2.2.1 Bacterial 16S rRNA genes as a target

The 16S rRNA gene has been widely used for phylogenetic and diversity studies for several reasons. It consists of conserved and variable regions, which allows the development of primers and probes with variable levels of specificity (Woese 1987). The conserved regions carry information about phylogenies at the higher taxonomic levels, since they have evolved slowly and are highly similar among the different taxa, whereas the variable regions have undergone more mutations during evolution, and are more useful for classification at the intraspecies level (Woese 1987). The rRNA genes are essential, and therefore present in all organisms. Finally, after many years of intensive sequencing, the current sequence databases comprise a huge amount of ribosomal DNA sequences, encompassing cultured but also many uncultured species. There were about 65 000 bacterial 16S rDNA sequences in the European Molecular Biology Laboratory (EMBL) database and 16 000 in the Ribosomal Database Project (RDP) in September 2002 (Stoesser *et al.* 2002, Maidak *et al.* 2001).

2.2.2 DNA isolation from environmental samples

Target DNA isolation is the first step in DNA-based applications and the isolation method used should meet the following criteria: lysis of target cells, and sufficient yield of high molecular weight DNA free from contaminants that could affect the subsequent applications (Wilson 1997). There are two possible strategies to isolate the microbial DNA: direct DNA extraction, or isolation of the microbial fraction, and subsequent DNA extraction. Direct DNA isolation may yield higher quantities of DNA (Kozdrój and van Elsas, 2000). Cell lysis can be achieved by different enzymatic (lysozyme), physico-chemical (heating, chemicals like SDS, freeze-thaw) or mechanical (bead-beating, grinding in liquid nitrogen) treatments (Frostegård *et al.* 1999, Miller *et al.* 1999). Different types of microbes possess different sensitivities to the lysis methods, with spores of bacteria and fungi being more resistant than vegetative cells or mycelium (von Wintzingerode *et al.* 1997). Bead beating has been shown to be the most effective method for the disruption of bacterial and fungal spores (Kuske *et al.* 1998, Haugland *et al.* 1999b, Haugland *et al.* 2002). Environmental samples but also microbes themselves contain many different substances, which are co-isolated and can interfere with the subsequent analyses (Frostegård *et al.* 1999, Cruz-Perez *et al.* 2001a). The inhibitory substances are efficiently removed with different gel filtration resins, spin columns and agarose gel electrophoresis (von Wintzingerode *et al.* 1997, Miller 2001).

2.2.3 PCR based applications

PCR based applications intended for the analysis of microbial communities include PCR amplification of environmental DNA with universal, group or species -specific primers and subsequent separation of the products using one technique or another. Restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), and terminal restriction fragment length polymorphism (T-RFLP) are based on the length polymorphism of PCR amplified and restriction enzyme digested DNA fragments. In the T-RFLP, only the fluorescently labelled 5'-terminal fragment is detected. The separation of PCR amplified DNA sequences with denaturing gradient gel electrophoresis (DGGE) is based on the decreased mobility of the partially molten DNA fragments in a linear gradient of denaturants, such as urea and formamide, in polyacrylamide gel. Temperature gradient gel electrophoresis (TGGE) utilises the same principle in a temperature gradient. DNA fragments

that differ in their sequences have different motilities. These methods have been widely used for the analysis of microbial community structure and diversity, with the studies being mainly focused on natural environments, such as soils, sediments and water. These techniques have been reviewed by Head *et al.* (1998), Muyzer (1999), Tiedje *et al.* (1999), Hill *et al.* (2000).

Few applications for the PCR based detection of a single species in indoor environments have been reported. They have targeted bacteria in air (Alvarez *et al.* 1994, Alvarez *et al.* 1995, Stärk *et al.* 1998) and surface (Buttner *et al.* 2001) samples, or fungi in air or dust samples (Bartlett *et al.* 1997, Roe *et al.* 2001). There are a few publications dealing with the PCR detection of streptomycetes in environmental samples, targeting streptomycin biosynthesis genes (Huddleston *et al.* 1997) or other genomic sequences (Roberts and Crawford 2000).

However, PCR methods have their own limitations and problems to be solved, the most important of which is probably the inhibition of the PCR reaction due to co-purified substances (von Wintzingerode *et al.* 1997, Cruz-Perez *et al.* 2001a,b). DNA isolated from an environmental sample is usually highly multi-template, demanding high primer specificity. Even with careful design of the primers, the PCR amplification may be biased by favourable amplification of certain templates due to the properties of the genes themselves, or their flanking sequences (Hansen *et al.* 1998, Polz and Cavanaugh 1998). This can be minimised by addition of chemicals, such as acetamide (Reysenbach *et al.* 1992). Another problem in multi-template PCR is the formation of chimeric DNA molecules. These are DNA molecules that are combined from different initial sequences. It has been reported that up to 32 % of PCR products may be chimeric (Wang and Wang 1997, von Wintzingerode *et al.* 1997). This is a problem especially with the highly conserved ribosomal genes (von Wintzingerode *et al.* 1997).

2.2.4 *In situ* -techniques

Whole cell *in situ* hybridisation is based on the detection of whole cells with fluorogenic probes targeting the rRNA genes. The cells are fixed with alcohol or formaldehyde to allow the probe to access the cell and hybridise to its target, and the fluorescence is measured. Both the amount of target cells and their spatial distribution can be recorded (Amann *et al.* 1995, Head *et al.* 1998). *In situ* -PCR applies the PCR technique to fixed and permeabilised cells. Fluorescent nucleotides are used for the amplification to facilitate the detection of the PCR

products. The advantage of this technique is that low copy number genes can be detected (Hodson *et al.* 1995, Vaid and Bishop 1999). *In situ* RT-PCR can be similarly used for investigation of gene expression *in situ*.

2.2.5 Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acids are components of the microbial cell membrane and are present in most living cells. After cell death, phospholipids are rapidly metabolised, so they can serve as indicators for active biomass. In addition, certain fatty acids are indicative of specific groups of microbes. PLFA profiling provides a culture-independent approach for determining the composition and changes in microbial populations (Zelles 1999, Hill *et al.* 2000)

2.3 Moisture-damaged buildings

2.3.1 Microbes in moisture-damaged buildings

Moisture damage in buildings can result from leakage of roofs or water pipelines, and condensation, among other reasons. Once a building material becomes wet, it provides a basis for microbial growth, which starts if the water activity (a_w) of the material reaches approximately 0.7 (Flannigan *et al.* 1996). There is hardly any material that will not support microbial growth if it is moist enough; the essential nutrients are provided by the material itself, organic dust, or other microbes. A wide variety of microbes, including filamentous fungi, yeasts and bacteria, have been isolated from building material, air, and dust samples from moisture damaged buildings (Hyvärinen *et al.* 2002).

Building materials

The fungal genera frequently found in moisture-damaged building materials are *Acremonium*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Cladosporium*, *Penicillium*, *Phialophora*, and *Stachybotrys* (Hyvärinen *et al.* 2002). Yeasts are also present. The bacteria include different Gram-negative bacteria, such as *Agrobacterium*, Gram-positive bacteria, such as *Bacillus* and *Arthrobacter*, actinobacteria, such as *Mycobacterium*, *Nocardiosis*, *Micrococcus* and *Cellulomonas* (Andersson *et al.* 1997, Vuorio *et al.* 1999, Peltola *et al.* 2001a). Spore-forming

actinomycetes, mostly *Streptomyces* spp. have also been isolated from moisture-damaged building materials (Andersson *et al.* 1997, Hyvärinen *et al.* 2002). With respect to the different material types, actinobacteria seem to occur more frequently on ceramic materials, paints and glues (Hyvärinen *et al.* 2002). They often coexist with *Acremonium* and *Aspergillus versicolor* on these materials.

Indoor air

Fungal genera dominating in indoor air are *Penicillium*, *Cladosporium* and *Aspergillus* (Waegemaekers *et al.* 1989, Verhoeff *et al.* 1992, Flannigan 2001). Both Gram-negative and Gram-positive bacteria have been isolated from indoor air (Ross *et al.* 2000, Flannigan 2001). The genera frequently found are *Micrococcus*, *Staphylococcus*, *Bacillus*, *Pseudomonas* and various actinobacteria, including *Streptomyces* (Hameed *et al.* 1999, Pastuszka *et al.* 2000, Flannigan 2001). Toxin producing strains of *Bacillus*, *Streptomyces* and *Nocardiosis* have been isolated (Andersson *et al.* 1998, Peltola *et al.* 2001b). Streptomycetes are found in the indoor air of moisture-damaged buildings but not in urban reference buildings, and thus they can be considered as indicators for moisture damage (Nevalainen *et al.* 1991, Samson *et al.* 1994).

Indoor dust

The same fungal genera as found in indoor air, such as *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium* and *Penicillium* are frequently found in indoor dust (Miller *et al.* 1988, Wickman *et al.* 1992, Beguin 1995, Beguin and Nolard 1996, Koch *et al.* 2000). In addition, species not so often found in air samples, such as *Aureobasidium*, *Fusarium*, *Mucor*, *Phoma* and *Wallemia* have been detected (Miller *et al.* 1988, Beguin 1995, Beguin and Nolard 1996, Ren *et al.* 1999). Bacteria have also been cultivated from indoor dust. The genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Staphylococcus*, *Streptomyces*, *Nocardiosis* and other actinomycetes have been identified (Andersson *et al.* 1999, Smedje and Norbäck 2001, Macher 2001a, Peltola *et al.* 2001a).

2.3.2 Health effects

Moisture and microbial damage in buildings has been associated with different adverse health effects. They include irritation of eyes and mucous membranes, infections of the upper respiratory tract, increased risk of asthma, and unspecific symptoms, such as headache and tiredness (Husman 1996, Verhoeff and Burge 1997, Peat *et al.* 1998, Bornehag *et al.* 2001). Some individuals may also develop allergies (Waegemaekers *et al.* 1989, Husman 1996, Garrett *et al.* 1998, Reijula 1998). Chronic diseases like allergic alveolitis are reported from occupational environments where there is heavy exposure to the spores of fungi and actinomycetes (Lacey and Crook 1988, Husman 1996). The exposure to moisture damage in the work place has been shown to increase the production of proinflammatory cytokines and nitric oxide in nasal lavage fluid, and the levels of those compounds decrease during absence from the work place (Hirvonen *et al.* 1999). Moisture damage has also been associated with clusters of autoimmune diseases (Myllykangas-Luosujärvi *et al.* 2002). In one case study, occupants of a dwelling heavily contaminated with actinomycetes suffered from rheumatoid symptoms, which became less severe when they left the building (Lorenz *et al.* 2002).

Although the association between moisture damage in buildings and the adverse effects on the health of the occupants is evident, the causal agents of the health effects remain obscure. People living or working in buildings with moisture damage are exposed to microbial spores and cells, and to parts of the cell envelopes, such as endotoxin or β - 1,3 -glucan (Rylander *et al.* 1998). Microbes also can produce secondary metabolites, some of which are volatile (MVOC, microbial volatile organic compounds) (Sunesson *et al.* 1997). Some metabolites are toxic or otherwise biologically active and can be associated with the microbial particles (Hirvonen *et al.* 1997a, Hintikka and Nikulin 1998). However, the relative importance of these different exposing agents in relation to the adverse health effects is not known.

2.3.3 Exposure assessment

A proper exposure assessment is necessary for the risk evaluation of the health effects of environmental agents. This includes environmental sampling and quantitative determination of the agents of interest. Samples of airborne particles can be taken with impactors, where the microbes are impacted on the surface of agar, and cultivated for enumeration and identification. Also impingers that collect the air sample in a liquid, or different kinds of

filtering devices can be used. The performance and application of the various samplers has been reviewed *e.g.* by Willeke and Macher (1999).

According to Flannigan (1997), dust reflects the microbial flora of the building, although the viable counts are higher in dust than in the air. Dust samples can be collected with vacuum cleaners from carpets, mattresses, floors or other surfaces, or from the dust bag of the cleaner. Settled dust can also be collected with other techniques (Macher 2001b).

Microbes associated with building materials can be collected with swab sampling or by pressing adhesive tape on the material if it is not possible to take a sample from the material. Microbial growth has traditionally been assessed by direct microscopy or cultivation (Macher 1999).

Cultivable microbes can be identified to the genus or species level using standard microbiological methods. These include microscopical examination of colony morphology and colour, diffusible pigments and the characteristics of reproductive forms, such as conidia and spores. Under standardised growth conditions, many isolates can be identified to the genus level with this approach. However, species identification often requires more laborious methods, including physiological testing, analysis of biochemical markers and DNA sequencing (Rosselló-Mora and Amann 2001).

However, a substantial part of the microbes in environmental samples are not cultivable, and therefore, cannot be identified by methods based on culturing of the strains. Methods based on DNA techniques are not dependent of the viability of the microbes, and they can be designed to work at different levels of specificity, detecting a whole group of microbes, a genus, or for example, a biosynthetic gene. PCR based methods for the detection and identification of microbes are widely used in clinical microbiology and food hygiene (Tang and Persing 1999). Applications in environmental microbiology, especially in the soil environment are also increasing (Tiedje *et al.* 1999, Hill *et al.* 2000, Ranjard *et al.* 2000). A few methods have been published for applications in indoor environments, aiming at the detection of major fungal species, such as *Stachybotrys chartarum* and *Aspergillus fumigatus* (Haugland *et al.* 1999a, Cruz-Perez *et al.* 2001b, Roe *et al.*, 2001, Zhou *et al.* 2000).

Need for non-culture detection methods for streptomycetes

Streptomycetes are bacteria, which have an enormous capacity for the production of secondary metabolites that have a broad spectrum on biological activities (Demain 1999). They are saprophytic and not highly pathogenic, but are associated with inflammatory disorders of the airways, and possibly other symptoms of an autoimmune character (Lacey and Crook 1988, Ojanen *et al.* 1983, Lorenz *et al.* 2002, Huttunen *et al.* 2002). They are non-fastidious in their growth requirements and well adapted to life under fluctuating environmental conditions (Kutzner *et al.* 1986), such as those present on building materials. They are considered as indicators of moisture damage, since they have been isolated from moisture damaged buildings, but not from reference buildings (Nevalainen *et al.* 1991). Their reported concentrations in the indoor air have not been especially high, < 10-150 cfu / m³ (Nevalainen *et al.* 1991), but this may be a methodological artefact.

About 0.001-15 % of the environmental microbes can be detected by cultivation (Amann *et al.* 1995), but their potential bioactivity is not dependent on their viability (Hirvonen *et al.* 1997c). Therefore, culture-independent methods are needed for the proper assessment of the microorganisms to which people living or working in moisture damaged buildings are exposed. PCR based methods have been shown to be sensitive and specific tools for the detection of microbes in different matrices (Zhou *et al.* 2000, Buttner *et al.* 2001, Roe *et al.* 2001). However, applications for the detection of streptomycetes in samples from indoor environments have not been previously reported.

3 AIMS OF THE STUDY

This study was conducted to develop a PCR-based method for the detection of streptomycetes in moisture-damaged buildings and to investigate the diversity of *Streptomyces* species found in these indoor environments. It consists of four component studies that are also addressed in the four original publications listed on page 7. The objectives of these component studies were as follows:

1. To develop a PCR-based detection method specific for streptomycetes and applicable to environmental samples taken from moisture-damaged buildings.
2. To compare culture and PCR methods in the detection of streptomycetes in indoor environments.
3. To characterise *Streptomyces* species isolated from moisture-damaged buildings.
4. To investigate the diversity of streptomycetes in moisture-damaged buildings using DNA-based techniques.

4 MATERIALS AND METHODS

4.1 Samples

4.1.1 Building materials

The building materials analysed in studies I and IV were collected during study visits to moisture damaged buildings by the personnel of the Laboratory of Environmental Microbiology of the National Public Health Institute (KTL), Kuopio, Finland. Two materials, which were culture-positive for actinobacteria, were selected for further testing. The materials were painted plaster from an interior wall of a dwelling and linoleum floor tile from the ground floor of an office building.

4.1.2 House dust samples

The house dust samples for study II were collected from regular dust bags of vacuum cleaners in a study investigating the connection between microbial condition and moisture damage in homes. Subsamples were taken for the evaluation of the streptomycete-PCR method and analyses of streptomycete diversity in house dust. The homes were visited by a civil engineer trained to look for moisture damage, and visible signs of moisture and microbial damage were recorded according to a checklist (Nevalainen *et al.* 1998). Based on the severity of the moisture damage, the homes were divided into moisture-damaged or non-damaged residences (Haverinen *et al.* 2001).

4.1.3 Soil samples

Soil samples used for the determination of primer specificity in study I originated from a Norway spruce forest stand in south-east Finland (soil A) and from a Scots pine forest stand in central Finland (soil B). The samples were taken from the humus layer, stored at 4 °C for 2-4 weeks, and then at -20 °C until DNA isolation.

4.2 Microbial strains and cultivation

4.2.1 Microbial strains

Microbial strains used in studies I - III and their sources are listed in Table 2. *Streptomyces* strains VTT E-99-1326 - 1336 were obtained from the culture collection of the Laboratory of Environmental Microbiology, KTL. These strains were originally isolated from the indoor air of moisture damaged buildings using an Andersen six-stage impactor, or from building materials with moisture and microbial damage. These and other strains were used for specificity testing of the streptomycete-PCR in study I, for the preparation of standard spore suspension in study II, and for the characterisation of *Streptomyces* strains isolated from moisture damaged buildings in study III as indicated in Table 2.

4.2.2 Cultivation of pure strains

Streptomyces isolates from the VTT collection used in studies I - III were maintained and grown on tryptone-yeast extract-glucose (TYG) agar (Bacto Plate Count Agar, Difco). *Streptomyces* strains obtained from the DSMZ and other microbial strains used for PCR specificity testing in study I were maintained and grown on the cultivation media recommended by their sources.

For the isolation of chromosomal DNA in studies I - III, the microbes were grown in 10 ml of the appropriate medium at the given temperature (Table 1., Study I) under agitation (200 rpm). The fungi were cultivated for 72 h, the streptomycetes and other bacteria for 48 h, except *M. lylae*, *B. megaterium*, *B. subtilis*, *B. fermentas*, *E. coli*, *P. aeruginosa* and *P. oleovorans* that were cultivated for 16 h. The *Streptomyces*-strains isolated from dust in study II were grown on TYG agar at 20 - 23 °C for 14 days for the chromosomal DNA extraction.

Table 2. Microorganisms used in this study.

| Species | Strain* | Study |
|--|---------------|------------|
| Streptomycetes | | |
| <i>Streptomyces albus</i> | DSM 40313 | I |
| <i>Streptomyces anulatus</i> | VTT E-99-1331 | I, III |
| <i>Streptomyces californicus</i> | VTT E-99-1326 | I, II, III |
| <i>Streptomyces thermovulgaris</i> | DSM 40444 | I |
| <i>Streptomyces thermoalkalitolerans</i> | DSM 41741 | I |
| <i>Streptomyces</i> sp. | VTT E-99-1327 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1328 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1329 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1330 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1332 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1333 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1334 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1335 | I, III |
| <i>Streptomyces</i> sp. | VTT E-99-1336 | I, III |
| Other actinobacteria | | |
| <i>Amycolatopsis methanolica</i> | DSM 44096 | I |
| <i>Cellulomonas uda</i> | DSM 20108 | I |
| <i>Kineococcus aurantiacus</i> | DSM 7487 | I |
| <i>Kitasatospora setae</i> | DSM 43861 | I |
| <i>Micrococcus lylae</i> | DSM 20315 | I |
| <i>Mycobacterium murale</i> | DSM 44340 | I |
| <i>Mycobacterium novocastrense</i> | DSM 44203 | I |
| <i>Promicromonospora citrea</i> | DSM 43110 | I |
| <i>Saccharopolyspora spinosa</i> | DSM 44228 | I |
| <i>Thermocrispum municipale</i> | DSM 44069 | I |
| Microbes used for the DNA mixture | | |
| <i>Bacillus megaterium</i> | VTT E-70007 | I |
| <i>Bacillus subtilis</i> | ATCC 4944 | I |
| <i>Brevibacterium fermentas</i> | NCIB 9943 | I |
| <i>Escherichia coli</i> | 89M | I |
| <i>Pseudomonas aeruginosa</i> | ATCC 10145 | I |
| <i>Pseudomonas oleovorans</i> | W16 | I |
| <i>Aspergillus niger</i> | VTT D-77020 | I |
| <i>Lipomyces starkeyi</i> | DSM 70295 | I |
| <i>Penicillium chrysogenum</i> | VTT D-74021 | I |

* DSMZ, German Collection of Micro-organisms and Cell Cultures, ATCC, American Type Culture Collection, NCIB, National Collection of Industrial Bacteria, VTT, Culture Collection of the Technical Research Centre, Finland, Biotechnology and Food Research.

4.2.3 Cultivation of microbes from environmental samples

Microbes from building materials (study IV) were cultivated as described earlier (Hyvärinen *et al.* 2002). The samples were weighed, and dilution buffer solution (42.5 mg ml⁻¹ KH₂PO₄, 250 mg ml⁻¹ MgSO₄ x 7 H₂O, 8 mg ml⁻¹ NaOH, 0.02 % (v/v) Tween 80) was added. The samples were sonicated for 30 minutes (FinnSonic M03/m, FinnSonic Ltd, Lahti, Finland), and shaken in a reciprocal shaker (KS125 basic, IKA Laboteknik), 400 rpm, for 60 min. Serial ten-fold dilutions were made and plated out on TYG agar supplemented with 0.05 % cycloheximide for bacteria. The plates were cultivated at room temperature (20 - 25 °C) for 5 days for the total bacterial count and for 14 days for mesophilic actinomycetes.

The dust samples in study II were treated as follows. To 5 g of dust, 45 ml of dilution buffer was added, and the sample was shaken as described above without sonication. After removal of hair and other fibrous material by sieving, serial dilutions were plated out on TYG agar supplemented with 200 mg l⁻¹ natamycin for the cultivation of bacteria. The remaining suspension was stored at -20 °C until DNA isolation. The agar plates were incubated as described for the material samples. Presumptive actinomycete colonies were picked on the basis of colony morphology with a sterile needle and streaked out on TYG agar to obtain pure cultures.

4.3 Molecular methods

4.3.1 DNA isolation

Pure cultures

Cells used in studies I and III were harvested from a 10 ml culture by centrifugation (3000 × g, 20 min) and suspended in 2 volumes of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 1 mg wet weight of microbial cells corresponding to 1 µl of TE. The suspension was incubated at 65 °C (bacteria) or 100 °C (fungi) for 20 min for cell lysis. The cell debris was removed by centrifugation (10 000 × g), and the supernatant was extracted once with buffered phenol and once with chloroform: isoamylalcohol (24:1 v/v). After centrifugation, the aqueous phase was collected in a clean tube, and the DNA was precipitated by adding 1/10

volume of 3 M potassium acetate (pH 4.8) and two volumes of ethanol. The DNA precipitate was collected by centrifugation at $10\,000 \times g$, dissolved in sterile, deionised water and stored at $-20\text{ }^{\circ}\text{C}$. An aliquot was analysed by agarose gel electrophoresis (1.5 %) with known amounts of phage λ DNA to assess the DNA concentration.

In study II, the spores from 2 TYG agar plates were suspended in two ml of sterile deionised water with a sterile loop. The spore suspension was stored at $-20\text{ }^{\circ}\text{C}$ until DNA isolation. The DNA was isolated as follows. The spores were suspended in 300 μl of TENS buffer (50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1 % SDS, pH 8.0), (Kuske *et al.* 1998) and 300 mg of quartz beads were added. The mixture was incubated 10 min at $65\text{ }^{\circ}\text{C}$, and shaken in a laboratory shaker (Vortex genie-2, Scientific Industries Inc., New York, USA) at maximal speed for 20 minutes. The cell debris was removed by centrifugation (5 min, $10\,000 \times g$) and the supernatant was extracted with chloroform-isoamylalcohol (24:1). Then the DNA was purified with the Wizard DNA Clean up System spin column (Promega, Madison, WI, USA).

Environmental samples

In studies I and IV, 2 g of 0.2-0.8 mm glass beads (Merck, Darmstadt, Germany), and 2.1 ml lysis solution (0.33 M Tris-HCl pH 8.0, 1mM EDTA, 70 mM NaCl, 1 % (w/v) CTAB, 1 % (w/v) SDS) were added to 2 g of building material. After 20-min incubation at $65\text{ }^{\circ}\text{C}$, the tube was shaken in a laboratory shaker (Vortex genie-2, Scientific Industries Inc., New York, USA) at maximal speed for 30 minutes. The cell debris was removed by centrifugation ($10\,000 \times g$) and the supernatant was extracted with chloroform-isoamylalcohol (24:1). Thereafter, the DNA was purified with the Wizard DNA Clean up System spin column (Promega, Madison, WI, USA) and agarose gel electrophoresis.

From the soil samples in study I, the DNA was isolated as from the building materials, with the exception that the samples were first washed with 2 % sodium hexametaphosphate, pH 8.5, and Crombach buffer (0.33 M Tris-HCl, 1 mM EDTA, pH 8.0) to reduce the levels of humic compounds.

The dust samples in study II were processed as follows. The sieved 1:10 dilution (5 g dust in 45 ml of dilution buffer) was filtered through a Ultipor N₆₆ nylon filter (Pall), pore size 0.45 μm . One quarter of the filter was taken to the DNA isolation, and the rest was stored in a

sterile petri-dish at -20°C . The filter was cut to small pieces with a sterile knife. The pieces were placed in a 2 ml tube; 800 mg quartz beads and 600 μl TENS buffer were added. After that, the sample was treated as described above for pure cultures in study II.

4.3.2 PCR

Primer design

PCR primers targeting the *Streptomyces* 16S rRNA gene were designed in study I. *Streptomyces* 16S rDNA sequences were obtained from public sequence databases, aligned with GCG-Pileup (Wisconsin Package, version 10.0, Genetics Computer Group, Madison, WI, USA), and suitable primer candidates were searched for manually. Candidate primer sequences were evaluated by the Fasta sequence comparison algorithm (Pearson and Lipman 1988) against EMBL (Stoesser *et al.* 2002) and GenBank (Benson *et al.* 2002) databases, and by the probe_match option of the Ribosomal Database Project (RDP) (Maidak *et al.* 2001). Primers having a perfect match only to streptomycete sequences were further tested for their PCR performance with the Oligo® v. 5.0 primer analysis package (National Biosciences Inc., Plymouth, MN, USA). The primers used in this study are listed in Table 3.

Table 3. Primers used in this study.

| Primer | Sequence (5' → 3') | Position* | Purpose | Reference |
|-------------|----------------------------|-----------|------------|----------------------------|
| StrepB | ACA AGC CCT GGA AAC GGG GT | 139-158 | PCR | Study I |
| StrepE | CAC CAG GAA TTC CGA TCT | 640-657 | PCR | Study I |
| StrepF | ACG TGT GCA GCC CAA GAC A | 1194-1212 | PCR | Study I |
| PA | AGA GTT TGA TCC TGG CTC AG | 7-26 | PCR | Edwards <i>et al.</i> 1989 |
| PH | AAG GAG GTG ATC CAG CCG CA | 1506-1525 | PCR | Edwards <i>et al.</i> 1989 |
| M13/pUC fw | GTA AAA CGA CGG CCA GT | | sequencing | MBI Fermentas |
| fw488 | TGC CAG CAG CCG CGG TAA TA | 488-507 | sequencing | Study III |
| fw1055 | TGT TGG GTT AAG TCC CGC AA | 1055-1074 | sequencing | Study III |
| M13/pUC rev | CAG GAA ACA GCT ATG AC | | sequencing | MBI Fermentas |
| rev560 | CCG CCT ACG AGC TCT TTA | 543-560 | sequencing | Study III |
| rev1074 | TTG CGG GAC TTA ACC CAA CA | 1055-1074 | sequencing | Study III |

* numbers refer to *Streptomyces ambofaciens* 16S rRNA gene sequence (Pernodet *et al.* 1989)

PCR amplifications

PCR reaction components used in the different studies are summarised in Table 4. The PCR reactions in studies I, III, and IV were carried out in 0.5 ml reaction tubes in a final volume of 50 µl overlaid with 50 µl of sterile mineral oil (Sigma Chemical Co., St. Louis, MO, USA) or in 0.2 ml reaction tubes in a volume of 25 µl in study II. The PCR amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) in studies I, III, and IV or TGradient thermocycler (Biometra GmbH, Goettingen, Germany) in study II. Negative controls containing no template DNA and positive controls were included in each set of PCR amplifications. All reactions in studies I and II were run in duplicate and repeated twice.

The cycling parameters for the primer specificity testing and PCR amplification from environmental samples using the *Pfu* DNA polymerase and PTC-100 thermal cycler in studies I, III and IV were: an initial denaturation of 5 min at 98 °C, 30 cycles of 45 s denaturation at 95 °C, 40 s primer annealing, and 2 min primer extension at 72 °C, followed by a final extension at 72 °C for 10 min. The annealing temperature used was 54 °C for the primers StrepB/StrepE and 58 °C for the primer pairs StrepB/StrepF and PA/PH. Primer extension times of 2 min for the Strep-primers and 3 min for the PA/PH primers were used. For the DyNzyme™ DNA polymerase in study I, the cycling was performed as follows; after an initial denaturation of 5 min at 98 °C, 30 cycles of 1 min denaturation at 95 °C, 40 s primer annealing at 52 °C, and 2 min primer extension at 72 °C were performed, followed by a final extension at 72 °C for 10 min.

The PCR amplifications from dust samples using the TGradient thermocycler and *Pfu* DNA polymerase in study II were performed according to following scheme; the initial denaturation step of 2 min at 95 °C was followed by 35 cycles of 45 s denaturation at 95 °C, 30 s annealing, and 2 min 10 s (StrepB/StrepF) or 1 min 10 s (StrepB/StrepE) primer extension at 72 °C, and a final extension of 5 min at 72 °C. The annealing temperature was set at 64 °C for the primers StrepB/StrepF and 54 °C for the primers StrepB/StrepE.

Table 4. PCR reaction components used.

| Template DNA | Study | Primers | Conc. of nucleotides | Reaction conditions | Additives | Enzyme |
|--------------------------------|-----------|--|----------------------|-----------------------------|-----------|-----------------------------|
| DNA from pure cultures | I, II | 0.2 μ M StrepB 0.2 μ M StrepE 0.2 μ M StrepF | 100 μ M | <i>Pfu</i> reaction buffer* | | <i>Pfu</i> DNA polymerase |
| Microbial DNA mixture | I | 0.6 μ M StrepB 0.6 μ M StrepE 0.6 μ M StrepF | 100 μ M | DyNazyme™ reaction buffer** | 10% DMSO | DyNazyme™ II DNA polymerase |
| DNA from environmental samples | I, II, IV | 0.2 μ M StrepB 0.2 μ M StrepE 0.2 μ M StrepF | 100 μ M | <i>Pfu</i> reaction buffer* | | <i>Pfu</i> DNA polymerase |
| DNA from pure cultures | III | 0.6 μ M PA 0.6 μ M PH | 100 μ M | <i>Pfu</i> reaction buffer* | 10% DMSO | <i>Pfu</i> DNA polymerase |

* 1 x *Pfu* reaction buffer contained 20 mM Tris-HCl, pH 8.2, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 1.0% Triton X-100, 100 ng ml⁻¹ BSA.

** 1 x DyNazyme™ II DNA polymerase reaction buffer contained 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100.

4.3.3 *Bst*YI restriction enzyme digestion

PCR amplification products obtained with StrepB/StrepF in studies I and II were subjected to restriction endonuclease digestion. 5 U of *Bst*YI (New England Biolabs, Beverly, MA, USA) was directly added to 10 μ l of the PCR reaction and incubated at 60 °C for 3 h.

Plasmid DNA carrying the StrepB/StrepF PCR fragment (study I) was digested with *Bst*YI in a reaction mixture containing 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM DTT and 2 U *Bst*YI in a total volume of 10 μ l. The reaction was performed at 60 °C for 2 hours. The results were analysed in 1.5 % agarose gels that were run for 60 min at 80 V.

4.3.4 Cloning and sequencing

The PCR products obtained from environmental samples in studies I and IV were purified from 1 % agarose gels with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The

fragments were ligated into *Sma*I digested and dephosphorylated pUC19 vector (MBI Fermentas, Hanover, MD, USA) using T4 DNA ligase (MBI Fermentas). The ligation mixture was transformed into *Escherichia coli* XL10-Gold Ultracompetent Cells (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

For the sequencing of the 16S rRNA gene of the *Streptomyces* spp. VTT E-99-1326 - 1336 in study III, the PCR-amplified gene fragments were purified from agarose gel, ligated as described for the environmental PCR products, and transformed into *E. coli* DH5 α .

In studies I, III and IV, plasmid DNA from transformants was isolated with the Wizard Plus SV minipreps kit (Promega, Madison, WI, USA). Sequencing was performed in the A.I.Virtanen Institute, University of Kuopio, Finland, using the Thermosequenase Fluorescent Labeled Primer Cycle Sequencing kit with 7-deaza-GTP, RPN 2538 (Amersham Pharmacia Biotech, Uppsala, Sweden), and A.L.F. or A.L.F. express DNA sequencer (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The sequencing primers (A.I.Virtanen Institute) used are listed in Table 3.

In study II, the PCR fragments of the actinomycete strains isolated from dust were purified from agarose gels and directly sequenced using the primers StrepB and StrepE. The sequencing was performed in the DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, University of Helsinki, Finland.

4.3.5 DNA sequence analysis

The DNA sequences were compared to other prokaryotic sequences in the EMBL database with the fasta program (Pearson and Lipman 1988) available from the EBI homepage (www.ebi.ac.uk/services/index.html). The sequences obtained in this work were aligned with the 16S rDNA sequences of *Streptomyces* type strains obtained from databases by using GCG-Pileup. The Phylip package (Felsenstein 1989) was used for the construction of phylogenetic trees. Distance matrices were calculated with the Kimura-2-parameter (Kimura 1980) algorithm of the dnadist-programme, and phylogenetic trees were constructed with the neighbor-joining method (Saitou and Nei 1987). *Arthrobacter globiformis* was used as an outgroup. Bootstrap analysis (500 replicates) was done to assess the significance of the obtained tree by using the programs seqboot, dnadist, neighbor and consense of the Phylip package. Treeview (Page 1996) was used to display the trees.

5 RESULTS

5.1 Development and evaluation of the PCR detection method for streptomycetes

Three primers were selected for further testing, the forward primer StrepB, and the reverse primers StrepE and StrepF. The target areas of the primers along the 16S rRNA gene are bp 139 – 158 (StrepB), bp 640 – 657 (StrepE), and bp 1194 – 1212 (StrepF). The size of the amplification product is 519 bp for StrepB/StrepE, and 1074 bp for StrepB/StrepF.

5.1.1 Specificity

There were 386 *Streptomyces* sequences in the databases containing all three primer sites (last search November 2002). Of these sequences, 370 (96 %) matched perfectly to all primers. The results of the sequence comparisons and experimental PCR analyses of selected *Streptomyces* strains with the primer sets are summarised in Table 5. All streptomycetes identified to the species level and having mismatches to the primers are listed. In addition, the searches revealed four sequences of *Streptomyces* sp. having 2 - 7 mismatches to StrepB, and one sequence having one mismatch to StrepE and one to StrepF. Table 5 also shows similar sequence comparison and PCR analysis results for a number of closely related actinobacterial species identified in the database searches as well as other common indoor micro-organisms.

The primer specificity was further investigated using DNA isolated from two soil and two moisture-damaged building materials as the template. The amplified PCR fragments were cloned in a pUC19 vector and sequenced. Ten clones from each material amplified with StrepB/StrepE were sequenced, and all of them showed greater than 96 % sequence similarity with *Streptomyces* 16S rDNA. The sequencing results of the StrepB/StrepF fragment were more variable. All the sequences amplified from the building material RM1, and 58 % of the RM2-sequences had greater than 95 % sequence similarity with streptomycetes and tested positive for the *Bst*YI digestion. The other 42 % of the sequences showed similarity to *Cellulomonas* 16S rDNA sequences, and were *Bst*YI negative. Of the soil sequences, 73 % showed similarity to streptomycete rDNA, and produced positive results with *Bst*YI, and 27 % were *Bst*YI negative, showing 90 - 92 % sequence similarity to either uncultured organisms or other actinomycetes than streptomycetes.

Table 5. Sequence comparison and PCR analysis results for selected streptomycetes, related actinobacteria and other common indoor microbes.

| Strain | Mismatches / position (5'→3') | | | PCR amplification | | <i>Bst</i> YI digestion |
|--|-------------------------------|-------------|---------------|-------------------|----------|-------------------------|
| | StrepB | StrepE | StrepF | StrepB/E | StrepB/F | |
| <i>Streptomyces californicus</i> VTT E-99-1326* | 0 | 0 | 0 | + | + | + |
| <i>Streptomyces albus</i> DSM 40313 | 0 | 1 /14 | 0 | + | + | + |
| <i>Streptomyces thermovulgaris</i> DSM 40444 | 1 /2 | 0 | 0 | + | + | + |
| <i>Streptomyces thermoalkalitolerans</i> DSM 41741 | 1 /2 | 0 | 0 | + | + | + |
| <i>Streptomyces thermonitrificans</i> DSM 40579 | 1 /2 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces thermosacchari</i> K122 | 1 /5 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces thermoviolaceus</i> AB106 | 1 /12 | 0 | 1 /11 | n.t. | n.t. | n.t. |
| <i>Streptomyces cattleya</i> JCM 4925 | 6 /2,5,6,9,16,20 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces thermolineatus</i> DSM 41451 | 6 /5,8,9,16,17,20 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces megasporus</i> DSM 41476 | 7 /2,5,6,9,16,19,20 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces macrosporus</i> DSM 41449 | 7 /2,5,6,9,16,19,20 | 0 | 0 | n.t. | n.t. | n.t. |
| <i>Streptomyces ushikuensis</i> KM 4927 | 8 /2,5,6,7,9,16,19,20 | 3 /6,15,16 | 0 | n.t. | n.t. | n.t. |
| <i>Amycolatopsis methanolica</i> DSM 44096 | 2 /2,10 | 2 /14,15 | 4 /2,8,14,15 | - | - | - |
| <i>Cellulomonas uda</i> DSM 20108 | 1 /2 | 3 /14,15,18 | 3 /2,4,8 | - | + | - |
| <i>Kineococcus aurantiacus</i> DSM 7487 | 2 /5,7 | 2 /14,15 | 4 /8,16,17,19 | - | - | - |
| <i>Kitasatospora setae</i> DSM 43861 | 2 /8,17 | 0 | 0 | + | - | - |
| <i>Micrococcus lylae</i> DSM 20315 | 2 /2,8 | 2 /14,15 | 3 /2,4,8 | - | + | + ^a |
| <i>Mycobacterium murale</i> DSM 44340 | 4 /2,8,9,16 | 2 /14,15 | 4 /2,8,14,15 | - | - | - |

| | | | | | | |
|--|-----------------|-----------------|-----------------|----------------|----------------|------|
| <i>Mycobacterium novocastrense</i> DSM 44203 | 4 /2,8,9,16 | 2 /14,15 | 4 /2,8,14,15 | - | - | - |
| <i>Promicromonospora citrea</i> DSM 43110 | 2 /2,8 | 2 /14,15 | 3 /2,4,8 | - | + | - |
| <i>Saccharopolyspora spinosa</i> DSM 44228 | 2 /2,8 | 2 /14,15 | 5 /2,8,14,15,17 | - | - | - |
| <i>Thermocrispum municipale</i> DSM 44069 | 1 /2 | 2 /14,15 | 3 /2,8,15 | - | - | - |
| <i>Bacillus megaterium</i> VTT E-70007 | 8 ^b | 4 ^b | 3 ^b | - ^c | - ^c | n.t. |
| <i>Bacillus subtilis</i> ATCC 4944 | 6 ^b | 4 ^b | 3 ^b | - ^c | - ^c | n.t. |
| <i>Brevibacterium fermentas</i> NCIB 9943 | 5 ^b | 2 ^b | 3 ^b | - ^c | - ^c | n.t. |
| <i>Escherichia coli</i> 89M | 7 ^b | 6 ^b | 5 ^b | - ^c | - ^c | n.t. |
| <i>Pseudomonas aeruginosa</i> ATCC 10145 | 8 | 8 | 5 | - ^c | - ^c | n.t. |
| <i>Pseudomonas oleovorans</i> W16 | 12 ^b | 7 ^b | 7 ^b | - ^c | - ^c | n.t. |
| <i>Aspergillus niger</i> VTT D77020 | 12 ^b | 11 ^b | 12 ^b | - ^c | - ^c | n.t. |
| <i>Lipomyces starkeyi</i> DSM 70295 | n.a. | n.a. | n.a. | - ^c | - ^c | n.t. |
| <i>Penicillium chrysogenum</i> VTT D-74021 | n.a. | n.a. | n.a. | - ^c | - ^c | n.t. |

* representative of the 370 out of 386 sequences from *Streptomyces* spp. found in a search of the EMBL and GenBank databases in November 2002 that were 100% homologous to all three primers

^a digestion revealed different fragment lengths

^b no sequence available, deduced from the sequence of another strain of the same species

^c determined by PCR amplification from a mixture of DNA isolated from pure cultures of these strains

n.a. not available

n.t. not tested

5.1.2 Detection limit

The detection limit of the PCR reaction determined with serial ten-fold dilutions of DNA isolated from *Streptomyces* sp. VTT E-99-1328 in study I was 10 pg / reaction for StrepB/StrepE and 1 pg / reaction for StrepB/StrepF.

The detection limit of the primers StrepB/StrepF in the slightly modified PCR protocol in study II, was determined using DNA isolated from serial ten-fold dilutions of a spore suspension of *Streptomyces* sp. VTT E-99-1326. The DNA was isolated in the same way as from the environmental samples resulting in 50 µl of DNA solution, 1 µl of which was used for the PCR. The lowest amount of spores that produced a visible band in agarose gel electrophoresis was 30 spores in the sample.

5.2 Comparison of PCR and cultivation methods for the detection of streptomycetes in house dust

The presence of mesophilic actinomycetes including streptomycetes in dust samples (n = 47) was determined by culture and PCR. Of the samples, 38 (81 %) tested positive in the PCR, and 17 (36 %) were actinomycete-positive by culture. The cultivation and PCR methods did not correlate with each other in their abilities to detect streptomycetes. Three of the 9 PCR-negative samples were culture-positive, and 24 of the 30 culture-negative samples were PCR-positive.

The calculated detection limit of the culture method was 450 cfu g⁻¹, and for the PCR method 30 spores per gram dust. According to the 16S rDNA sequence comparison, the actinomycete isolates were all members of the genus *Streptomyces*. To investigate the possible presence of PCR-inhibitors in the PCR-negative samples, they were subjected to PCR amplification with the universal bacterial primers pA/pH. All samples produced an amplification band with pA/pH, indicating that they were streptomycete-negative, but did not contain PCR-inhibitors, although two samples required a further purification step with a Wizard spin column.

According to the two-level classification, 38 (81 %) of the houses (n = 47) had some moisture damage. The amount of streptomycete-specific PCR product, measured as intensity of the band in agarose gel electrophoresis, was significantly higher in dust samples originating from

moisture-damaged homes compared to non-damaged homes ($p < 0.05$, Mann-Whitney test). Streptomyces were detected in 36 % of the samples originating from moisture-damaged houses by cultivation and in 81 % by PCR. However, from the nine non-damaged sites, only one dust sample was culture-positive, but five were PCR-positive. When binary data (presence or absence of moisture damage or streptomyces) and Fisher's exact test were used for the statistical analysis, no statistically significant correlation between moisture damage and the occurrence of streptomyces was observed ($p = 0.127$ for culture, and $p = 0.054$ for PCR).

5.3 Characterisation of *Streptomyces* isolates from moisture damaged buildings

The 16S rRNA gene of *Streptomyces* strains isolated from indoor air and building materials (N=11), and from house dust (N=15) was partially sequenced. Sequence comparisons with sequences in the EMBL database revealed that the 1520 bp sequences of the indoor air and building material isolates (VTT E-99-1326 - 1336) had > 99 % sequence similarity to sequences of known species, such as *S. griseus*, *S. albidoflavus* and *S. coelicolor*. The 520 bp sequences of the dust isolates had > 97 % sequence similarity with known species, except B352 that had 96 % similarity to *S. albidoflavus*.

Figure 1. shows the phylogenetic affiliations of the streptomycete strains investigated in this study based on a 120 bp (nt. 159 - 278) alignment of the 16S rRNA gene sequences. The sequences of the streptomyces isolated from indoor air, building materials and house dust formed four groups in the phylogenetic tree. The largest group of 16 strains affiliated with sequences of *S. anulatus* and *S. halstedii*, two further groups with four strains in each of them affiliated with *S. albidoflavus* and *S. tendae*. Two strains isolated from dust clustered with *S. exfoliatus* and *Streptomyces* sp. YIM 26 sequences (Figure 1.).

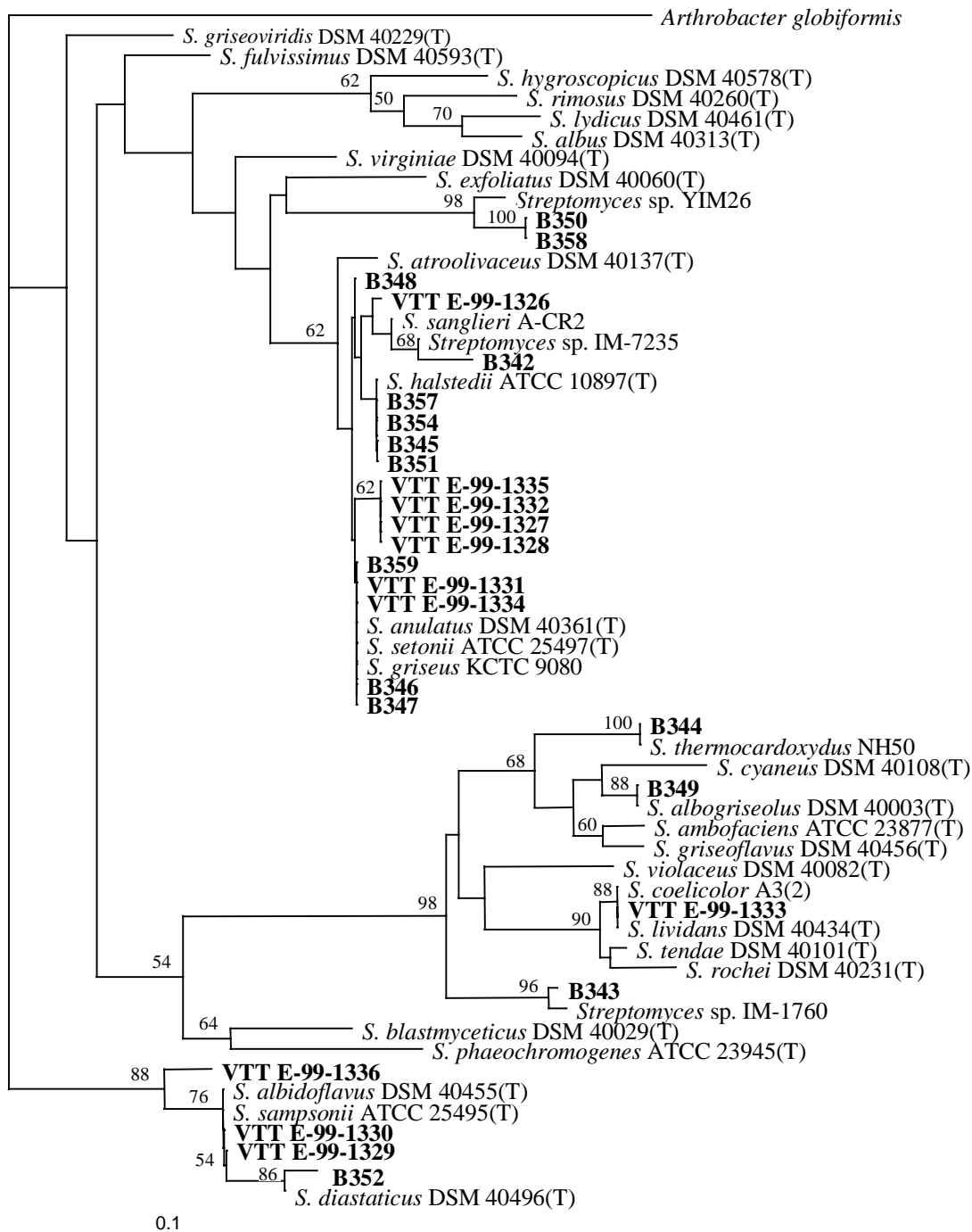


Figure 1. Neighbor-joining tree based on a 120 bp (bp 159 - 278) alignment of 16S rDNA sequences obtained from *Streptomyces* spp. isolated from indoor environments, and from public databases. B, *Streptomyces* isolates from dust; VTT-E-99-, *Streptomyces* strains isolated from indoor air and building materials. Scale bar = 10% dissimilarity. *Arthrobacter globiformis* was used as an outgroup to root the tree. Bootstrap values are given as percent out of 500 trees. Values that are greater than 50 are indicated.

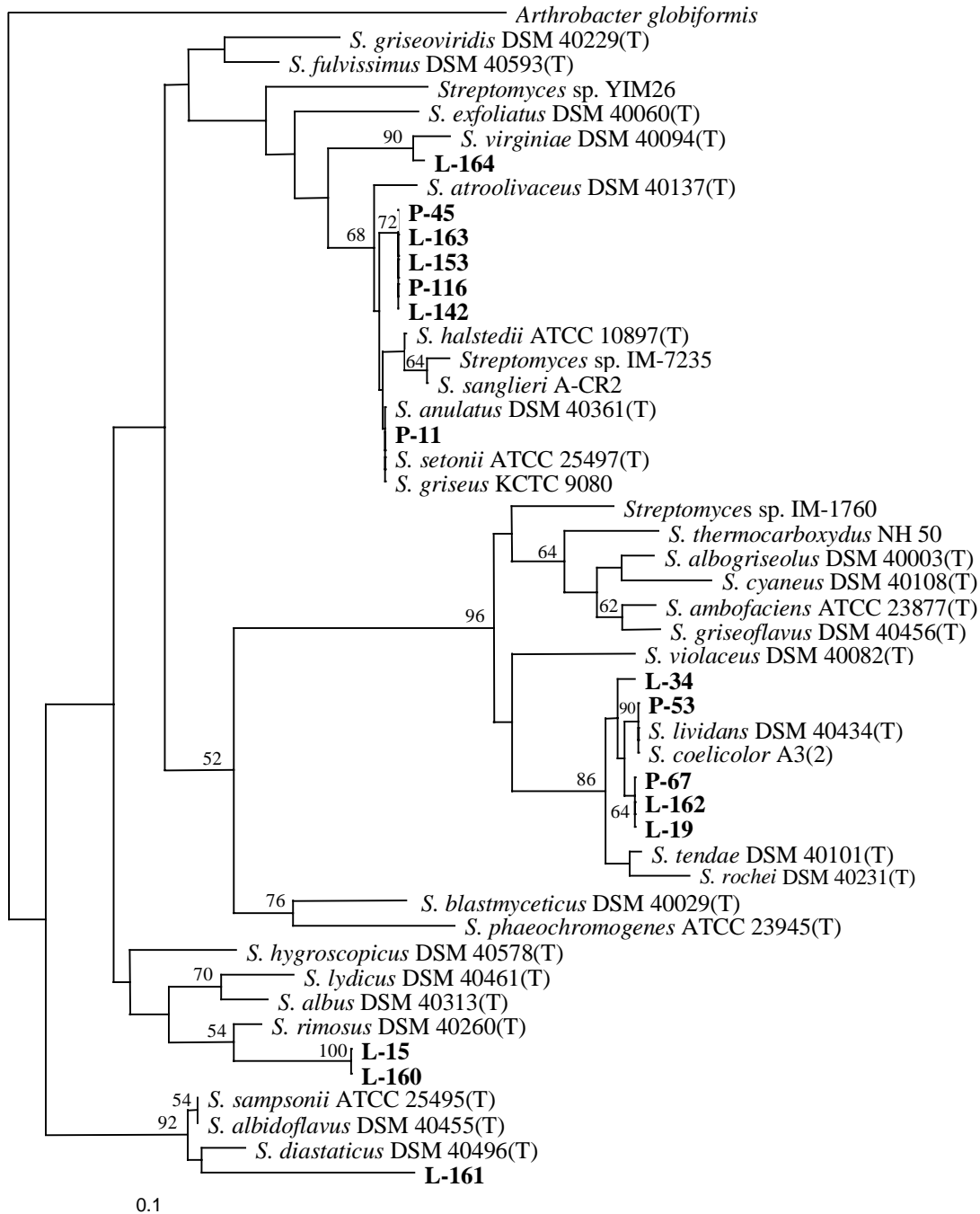


Figure 2. Neighbor-joining tree based on a 120 bp (bp 159 - 278) alignment of 16S rRNA gene sequences of *Streptomyces* strains obtained from public databases and sequences of PCR amplified environmental clones. L, sequences amplified from linoleum; P, sequences amplified from plaster. Scale bar = 10% dissimilarity. *Arthrobacter globiformis* was used as an outgroup to root the tree. Bootstrap values are given as percent out of 500 trees. Values that are greater than 50 are indicated.

5.4 Diversity of streptomycetes based on 16S rDNA sequences

A culture-independent approach was used to investigate the diversity of streptomycetes in building materials. DNA was isolated directly from the building material and subjected to PCR amplification with the primer sets StrepB/StrepE and StrepB/StrepF. The amplification products were cloned in pUC19 vector and sequenced. In sequence comparisons with sequences in the EMBL database, the clones had 95 - 100 % sequence similarities with known streptomycete sequences.

A phylogenetic tree was constructed based on a 120 bp (bp 159 - 278) alignment of the cloned PCR amplicons and known streptomycete sequences. The streptomycete 16S rDNA sequences amplified from DNA isolated from building materials affiliated with several clusters in the phylogenetic tree. Most of the clones affiliated with two branches, one including *S. anulatus*, *S. setonii* and *S. halstedii*, and another including *S. lividans* and *S. tendae*. Other sequevars were also present; some of them being distantly related to sequences obtained from databases (Figure 2.).

DNA sequence accession numbers

The *Streptomyces*-sequences were submitted to the EMBL sequence database. The accession numbers for the building material sequences are AJ344118 - AJ344132. The accession numbers of the *Streptomyces* isolates VTT E-99-1326 - 1336 are AF429390 - AF429400, respectively.

6 DISCUSSION

6.1 Development and evaluation of the PCR-based detection method

6.1.1 Primer design

The 16S rRNA gene was chosen as the target gene for the PCR primers in the PCR assay, aiming at the detection of as many as possible of the over 650 described *Streptomyces* -species (www.dsmz.de/species/). The advantages of rRNA genes as targets for PCR detection assays are that the genes are essential for the cells, and they are conserved among the prokaryotes (Kim *et al.* 1993). With streptomycetes, which have a highly unstable genome (Volf and Altenbuchner 1998), it is important that the rRNA genes are situated in the stable core part of the genome (Bentley *et al.* 2002).

The sequence differences in the 16S rRNA gene are located in the so-called variable regions V1-V8. The V2 and V3 the regions show the greatest differences between streptomycetes and *E. coli*, while the V2 region shows the greatest variability within the streptomycetes (Pernodet *et al.* 1989, Stackebrandt *et al.* 1991). According to Stackebrandt *et al.* (1991), the variable regions V2, V6 and V7 are suitable for the discrimination of *Streptomyces* species. Sequence analysis of the highly variable V2 region has been applied for *Streptomyces* species identification; however, it was not able to distinguish all of the species (Kataoka *et al.* 1997). Both of the primer pairs designed in this work, StrepB/StrepE and StrepB/StrepF amplified the V2 region. In addition, the variable regions V6 and V7 are included in the fragment amplified with the primers StrepB/StrepF. Thus, the gene fragments amplified with these primers contain sufficient information for diversity studies; however, the 16S rDNA sequence diversity provides no information about the functional diversity of species (Metsä-Ketelä *et al.* 2002).

Two of the primers developed in this work, StrepB and StrepE partially overlap with published *Streptomyces* -specific primers (Mehling *et al.* 1995). However, the 3' ends of the primers are different. The previously reported primers have some disadvantages compared to those developed in this work. Many of the primers have ambiguous nucleotides, perhaps due to the limited sequence information available at the time of their publication. Two of them also target the V7 variable region. A previously reported *Streptomyces* -specific probe

(Stackebrandt *et al.* 1991) targets a conserved region around a streptomycete -specific nucleotide at position 6 from the 5' end of the probe, which represents the only difference to hundreds of other species. Use of this probe requires very stringent hybridisation conditions.

6.1.2 PCR specificity

DNA isolated from environmental samples mostly contains DNA of many known and unknown species. The specificity of the PCR is affected by multiple factors, such as the primers, the properties of the gene regions flanking the target site, the annealing temperature in the PCR reaction, and the reaction conditions (von Wintzingerode *et al.* 1997). In this work, the primer specificity was determined by multiple approaches. These included: 1) DNA sequence comparisons with the existing databases of target and related non-target species; 2) Experimental analyses of pure cultures of micro-organisms representing possible false positives, and also multitemplate mixtures of non-target micro-organisms abundant in indoor environments; and 3) Sequencing of PCR amplified DNA from environmental samples. The primer pair StrepB/StrepF amplified DNA from pure cultures of *C. uda*, *P. citrea*, and *M. lylae*, all of which can be discriminated from streptomycetes by the *Bst*YI restriction enzyme digestion. The primer pair StrepB/StrepE tested positive with *Kitasatospora setae* (two mismatches to StrepB). However, the taxonomic position of the genus *Kitasatospora* is still somewhat ambiguous, and there have been attempts to include them in the genus *Streptomyces* (Wellington *et al.* 1992) or to revise the genus (Zhang *et al.* 1997). The DNA sequencing of the environmental amplification products confirmed the specificity of the PCR assay for streptomycetes. Although non-streptomycete sequences were detected among the amplicons, these were *Bst*YI-negative. The *Bst*YI-negative sequences from soil had only low similarities to sequences in databases, indicating that they could represent previously uncultured organisms or be PCR artefacts, such as chimeric sequences (Wang and Wang 1997, von Wintzingerode *et al.* 1997).

Some thermophilic streptomycetes, including *S. thermolineatus*, *S. macrosporus* and *S. megasporus*, have several mismatches to StrepB and are not likely to be detected with the designed primers. These species, however, are reported to belong to a phylogenetically distinct clade (Kim *et al.* 1996). They are commonly isolated from composts and heated hay or grain (Kim *et al.* 1996), and their prevalence or significance in indoor environments is not yet known.

6.1.3 PCR detection limit

The detection limit of the PCR reaction with the primer pair StrepB/StrepF reported in this work was < 1 pg DNA/reaction (corresponding to approximately 30 cells) (study I), or 30 spores/sample (study II). The reported detection limits of indoor fungi range from two fungal spores/sample using real time PCR (Haugland *et al.* 1999a) to 2 spores/reaction using conventional PCR (Zhou *et al.* 2000). Cruz-Perez *et al.* (2001a,b) reported a sensitivity of < 20-23 spores/reaction in real-time PCR. All these experiments were carried out with pure cultures. Roe *et al.* (2001) used the method developed by Haugland *et al.* (1999a) for quantification of *Stachybotrys chartarum* in dust samples, and found that small amounts of spores (< 10 spores/sample) in dust were not reliably quantified. In addition, all PCR primers for fungi mentioned above, target the genes coding for ribosomal RNAs, which in fungi are present in copy numbers of hundreds (Borsuk *et al.* 1982), while streptomycetes have less than ten copies (Fogel *et al.* 1999). Hence, the results obtained in this work are comparable with those obtained with spore-forming fungi in indoor environments.

6.1.4 PCR inhibition

Inhibition of PCR amplification by substances co-extracted with DNA, is a well-known problem with environmental and clinical samples as well as with pure cultures (von Wintzingerode *et al.* 1997, Tang and Persing 1999, Cruz-Perez *et al.* 2001a,b). This can be avoided by dilution of the template DNA, further purification by agarose gel electrophoresis or various columns and resins intended for DNA purification, or addition of substances like bovine serum albumin to the PCR reaction mixture. In this work, PCR inhibition was observed with two dust samples, although the DNA was purified twice with a Wizard spin column. Ten-fold dilution of the template did not affect the amplification, but a third purification with the Wizard spin column enabled the PCR amplification. PCR inhibition can be identified by addition of an internal positive control to each PCR reaction (Tang and Persing 1999). In this work, each sample PCR negative for *Streptomyces* was tested for inhibitors separately using the PA/PH universal primer set. However, in routine PCR the use of an internal positive control is more convenient.

6.2 Comparison of PCR and culture

It has been reported that only 0.001 - 15 % of the microbial population in environmental samples is detectable by culture (Amann *et al.* 1995). Generally, streptomycetes spores are readily cultivable from environmental samples, however, medium selectivity can affect their growth (Goodfellow and Simpson 1987). PCR based methods are not dependent on viability of the target organisms, however, they also suffer from problems. These include inhibition caused by co-extracted substances, and in diversity studies, differential amplification and formation of PCR-artefacts, which have to be taken into account during the design of the assays (von Wintzingerode *et al.* 1997).

The PCR method was more sensitive than the culture method in the detection of streptomycetes in house dust samples, since 81% of the 47 dust samples tested positive by PCR and 36% by culture. This is in accordance with previous works, which have used PCR detection of bacterial and fungal spores in indoor environments (Zhou *et al.* 2000, Buttner *et al.* 2001, Roe *et al.* 2001). In addition, the PCR method was more specific than the culture method used in this work. The culture method identified the colonies simply as actinomycetes, which are not routinely identified to the genus level, whereas the PCR method indicated the presence of the genus *Streptomyces*.

There was no correlation found between culture and PCR results. There may be several reasons for this including the different error sources inherent in both methods. The different detection limits of the methods make presence /absence comparisons difficult. The cultivation method used in this work was a routine method used in our laboratory for the detection of total counts of bacteria. It was not specifically designed for the detection of streptomycetes. The medium used has been shown to be suitable for streptomycetes (Suutari *et al.* 2002), but it can also support the growth of other bacteria. The presence of mesophilic actinomycetes was recorded after incubation at 20-25 °C for 14 days. After these long incubations the plates were often overgrown, and slow-growing actinomycetes may have remained undetected. On the other hand, the sensitivity of the PCR method may have been affected in some instances by inhibitors present in the samples and co-purified with the DNA (von Wintzingerode *et al.* 1997, Cruz-Perez *et al.* 2001a). The impurities in the DNA may lower the PCR amplification efficiency without inhibiting it totally, which can be determined by using an internal positive control in the PCR reaction, but not with the inhibition control used in this study.

6.3 Diversity of streptomycetes in indoor environments

The diversity of streptomycetes in buildings was investigated by PCR amplification of 16S rDNA sequences directly from building materials and by amplifying the 16S rDNA sequences of *Streptomyces* isolates cultured from indoor air, building materials and dust. The 16S rRNA gene is highly conserved, but a few variable regions are present, which have value in examining the diversity within the genus *Streptomyces* (Stackebrandt *et al.* 1991, Kataoka *et al.* 1997). The most highly variable region, which extends from nucleotide position 158 to 203 in the 16S rRNA gene (Pernodet *et al.* 1989), was included in the PCR amplicons created in this study.

In contrast to the 16S rRNA genes, which are located in the conserved core part of the *Streptomyces* chromosome, genes coding for morphological and physiological characters, such as pigments and production of extracellular enzymes, are often located in the chromosome arms, which can undergo dramatic rearrangements (Bentley *et al.* 2002). Therefore, the 16S rDNA sequence diversity does not necessarily reflect the diversity of other properties, and depending on the application, other genes could be used. Metsä-Ketelä *et al.* (2002) compared the phylogenetic trees obtained from 16S rRNA and polyketide synthase (PKS) gene (involved in biosynthesis of secondary metabolites) sequences of 99 actinomycetes isolated from soil, and found no correlation between the two trees.

Interestingly, a great part of the 16S rDNA sequences obtained in this work clustered with the sequences of *S. griseus*, *S. anulatus* and *S. setonii* in the phylogenetic tree. These species belong to the *Streptomyces albidoflavus* -cluster, which is the largest group of *Streptomyces* species. This group is known to include many producers of secondary metabolites (Anderson and Wellington 2001), and was the largest group of secondary metabolite producing isolates investigated by Metsä-Ketelä *et al.* (2002). These findings suggest that this cluster may be one of the most important groups of streptomycetes in moisture damaged buildings both in terms of their abundance and also in terms of their production of secondary metabolites that could pose as exposing agents to building occupants.

The culture-independent approach revealed 16S rDNA sequences that clustered with the same database sequences as the cultured isolates, but also sequences that were only distantly related to known sequences. Hence, somewhat greater diversity was detected with this approach.

7 CONCLUSIONS

1. The PCR method applied in this work proved to be specific for the detection of streptomycetes and was successfully used for the detection of streptomycetes in building materials and dust samples.
2. Occurrence of streptomycetes in house dust correlated with observed moisture damage in the houses. The results of PCR detection of streptomycetes and culturing of mesophilic actinomycetes in dust samples did not correlate with each other. However, all the strains isolated from dust and identified as mesophilic actinomycetes were streptomycetes according to their 16S rDNA sequences. Streptomycetes were detected in 81 % of the dust samples by PCR and actinomycetes in 36 % of the samples by culture. Thus, PCR based detection seems to be a more sensitive and accurate method for detection of streptomycetes in house dust than culture. This proposal requires further validation in future experiments.
3. According to their 16S rRNA sequences, the *Streptomyces* -isolates from indoor environments are mostly members of the *Streptomyces albidoflavus* -cluster, which includes the subclusters *S. albidoflavus*, *S. anulatus* and *S. halstedii*. However, other species were also present. The species diversity in buildings seems to reflect the species diversity in the soil and other natural habitats, and the assessment of the importance of each individual species in relation to the adverse health effects suffered by occupants of moisture-damaged buildings needs further research.
4. 16S rRNA gene sequences directly PCR amplified from DNA of building material samples generally affiliated with the same branches in the phylogenetic tree than the sequences of the cultured isolates. However, also sequences more distantly related to known 16S rDNA sequences of streptomycetes were detected, indicating that this approach could be useful for detecting species that are not readily cultivable.

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Rintala H, Nevalainen A, Rönkä E, Suutari M.

PCR primers targeting the 16S rRNA gene for the specific detection of streptomyces

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II

Rintala H, Hyvärinen A, Paulin L, Nevalainen A.

Detection of streptomyces in house dust - comparison of culture and PCR methods

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III

Suutari M., Rönkä E, Lignell U, Rintala H, Nevalainen A.

Characterisation of *Streptomyces* spp. isolated from water-damaged buildings

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IV

Rintala H, Nevalainen A, Suutari M.

**Diversity of streptomycetes in water-damaged building materials based on
16S rDNA sequences**

Letters in Applied Microbiology 2002, 34:439-443