

Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia

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Summary

Simple living conditions and farming environment have been associated with reduced risk for allergic diseases such as atopy and asthma but the factors responsible for this effect remain unresolved. We examined the bacterial composition of house dusts obtained from Finnish and Russian Karelia, two adjacent areas with high and low occurrence of atopic diseases respectively. Two dust mixes, both composed of 10 randomly selected dust samples from 349 Finnish and 417 Russian Karelian households were studied for bacterial biomarkers (DNA, Limulus-active endotoxin, 3-OH fatty acids, muramic acid) and for 16S rRNA gene sequences. Overall, the DNA cloning revealed more taxons (94 different genera) of dust-borne bacteria than seen in any previous study on residential environments. Majority (67%) of the bacterial DNA clones in house dust from the low-allergy Russian Kareliarepresented Gram-positive bacteria (*Firmicutes* and *Actinobacteria*), predominantly *Staphylococcaceae* and *Corynebacteriaceae*. Russian Karelian dust showed up to 20-fold higher contents of muramic acid (marker of Gram-positive bacteria) and a sevenfold higher number of clones of animal-associated species, whereas in Finnish Karelian dust Gram-negatives (mainly *Proteobacteria*) predominated. Clones of plant-associated bacterial

species and of chloroplast, indicating plant biomass, were more numerous in Finnish than in Russian Karelian dust. In conclusion, this study revealed major disparities between Finnish and Russian house dusts. The higher bacterial content and the predominance of Gram-positive bacteria in Russian dust may have implications for occurrence of atopy.

Introduction

Many studies have indicated association of raised risk for atopic diseases with Western lifestyle and urbanization (Viinanen *et al.*, 2005; von Hertzen and Haahtela, 2006; Weinmayr *et al.*, 2007). Farming environment and simple living conditions (von Hertzen and Haahtela, 2006; von Hertzen *et al.*, 2006), in turn, appear to be associated with reduced risk for atopic diseases possibly due to heavy exposure to saprophytes in soil and vegetation.

Endotoxin (lipopolysaccharide), the major cell wall component of Gram-negative bacteria and a potent immunomodulator, has been suggested to confer protection against atopy and atopic diseases, although dose, route and timing of exposure may be important determinants here (reviewed in Gehring *et al.*, 2001; Liu, 2002; Liu and Murphy, 2003; Roy *et al.*, 2003). Gram-negative bacteria represent a major part of microbial communities in boreal soils (Neufeld and Mohn, 2005) but in freshwaters, Gram-positive *Actinobacteria* have been found to predominate (Gisi *et al.*, 1997; Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Sekar *et al.*, 2003; Haukka *et al.*, 2005). Gram-positive bacteria of the phylum *Firmicutes* comprise not only members of the normal flora of humans (Aas *et al.*, 2005; Gao *et al.*, 2007), but are also widely distributed in the environment and animals (Balows *et al.*, 1991; Nagase *et al.*, 2002).

Persistent exposure to microbial components in childhood via skin, respiratory tract and gut leads to tolerance through interaction with the innate immunity (van Oosterhout and Bloksma, 2005). Human exposure to non-pathogenic bacteria and bacterial components through their specific Toll-like receptors (TLRs) is crucial for the mucosal homeostasis and integrity (Rakoff-Nahoum *et al.*, 2004). The microorganisms need not to be viable, as non-viable bacteria have immunobiological properties similar to live bacteria (Hessle *et al.*, 2000).

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We reported earlier on the low prevalence of atopic diseases in Russian Karelia, where the risk of childhood atopy was fourfold lower as compared with the Finnish Karelia. This difference was not explained by geoclimatic and vegetative conditions or different genetic susceptibilities to atopy of the populations on the two sides of the border (Vartiainen *et al.*, 2002; von Hertzen and Haahtela, 2006). Living conditions and, e.g. exposure to saprophytes via drinking water (von Hertzen *et al.*, 2007) are fundamentally different on the two sides of the border.

Culturable bacterial diversity in indoor air is known to some extent (Andersson *et al.*, 1999; Macher, 2001; Górny and Dutkiewicz, 2002; Lee *et al.*, 2007), but the human exposure to microbial cell constituents, including the non-viables, via indoor air is largely unexplored. We describe in this paper the bacterial communities in house dusts of Finnish and Russian Karelia, areas with high and low occurrence of atopy (von Hertzen and Haahtela, 2006).

Results

This study is part of the Karelian Allergy Study (von Hertzen and Haahtela, 2006) carried out to examine the occurrence and risk/protective factors of atopy and atopic diseases among 367 randomly selected school children and their 357 mothers from Finnish Karelia and 446 such children with their 324 mothers from Russian Karelia. The geographically adjacent areas differ fundamentally with respect to living conditions, today resembling in Russian Karelia those in Finland c. 50 years earlier.

Dust samples were collected from 20 households in the Finnish ($n = 10$) and the Russian ($n = 10$) Karelia, combined in to lots of five dusts, and analysed for bacterial diversity by sequencing of the 16S rRNA gene clones obtained from the dust contained DNA. Figure 1 shows the phylum distribution of the clones ($n = 465$) isolated from the four composite dusts collected from the two sides of the border. When the independently selected 5 + 5 Russian and 5 + 5 Finnish Karelian dusts were compared, it is seen that the distributions of bacterial phyla among the DNA clones from the Russian composite dusts are similar and different from those of the Finnish dusts (Fig. 1). Similarly, the two composite dusts from the Finnish Karelian dusts had similar phylum distributions. Gram-positive taxa (*Actinobacteria* and *Firmicutes*) dominated in the bacterial populations of the dusts collected from the Russian Karelia (66.7% of all clones) but not in those from the Finnish side (34.2%, proportions test $P < 0.001$). Gram-negative bacteria in turn were more abundant in the Finnish Karelian house dusts (39.8%) than on the Russian side (23.9%, $P < 0.001$). Chloroplast 16S rRNA gene clones were obtained in significant quan-

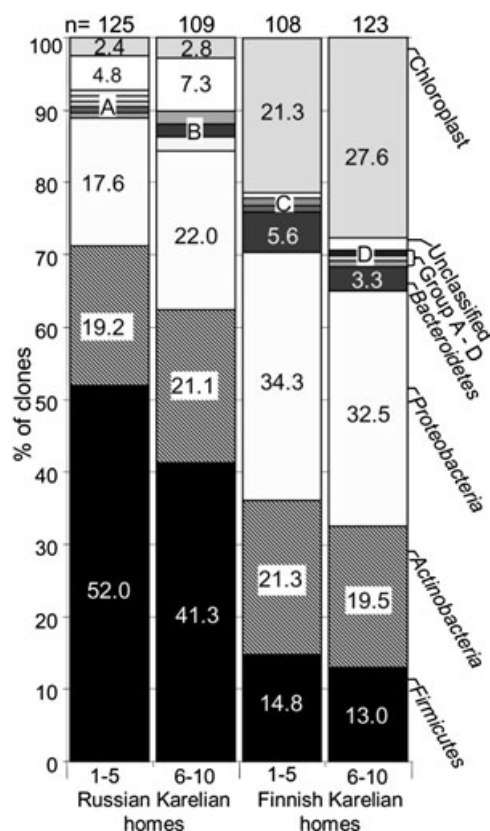


Fig. 1. Phylum distribution of 16S rRNA gene clones ($n = 465$) from the Russian and Finnish house dusts obtained using universal bacterial primers. Bacteria in four composite dusts, each consisting of five randomly selected homes from the Russian ($n = 5 + 5$) and the Finnish Karelia ($n = 5 + 5$), were analysed by cloning and sequencing of 16S rRNA genes from dust contained DNA. The names and proportions of the major phyla are shown in the figure. The minor phyla, representing $< 2\%$ of the clones, comprised the following A: *Planctomycetes* (0.8), *Acidobacteria* (0.8), *Gemmatimonadetes* (0.8), TM7 (0.8), *Cyanobacteria* (0.8); B: *Deinococcus* (1.8), *Bacteroidetes* (1.8), *Planctomycetes* (1.8); C: Unclassified (0.9), *Fusobacteria* (0.9), *Acidobacteria* (0.9); D: Unclassified (1.6), *Nitrospira* (0.8), *Gemmatimonadetes* (0.8), *Planctomycetes* (0.8).

tity (24.7%) from the Finnish dusts but not from the Russian dusts (3.0%, $P < 0.001$), indicating a higher amount of plant biomass origin in the Finnish dusts. A total of 94 different bacterial genera (Table S1) were found among the clones isolated from the Finnish and the Russian Karelian home dusts.

Firmicutes was the most prevalent bacterial phylum in the dusts collected from the Russian Karelian households (47% of all clones). Majority of the Firmicute clones ($n = 82$, Fig. 2) represented species not found from the Finnish Karelian dusts. The predominant family, representing 21.4% of all clones ($n = 234$) from the Russian side, was *Staphylococcaceae* (Table 1). These included species of *Staphylococcus* (12.4%), *Macrococcus* (3.4%), *Salinicoccus* (3.4%) and *Jeotgalicoccus* (1.7%) (Table 1).

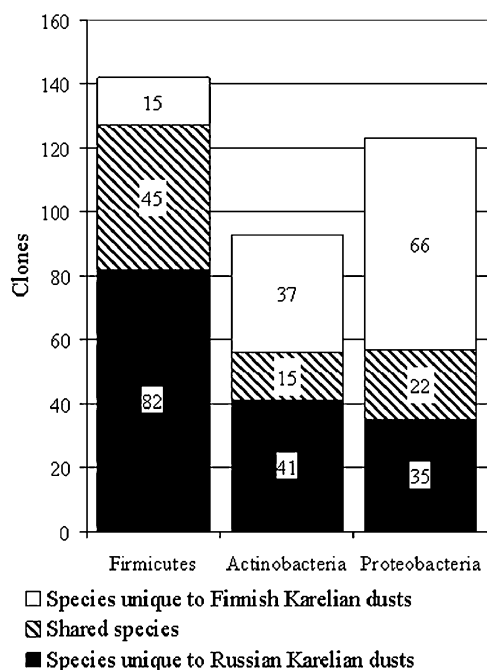


Fig. 2. Numbers of bacterial clones belonging to species unique to the Russian or the Finnish Karelian house dusts. The figure shows the clone numbers of the most abundant phyla found in the Russian and Finnish dusts (Fig. 1). The shaded areas represent clones of species shared by the Finnish and the Russian Karelian house dusts ($\geq 97\%$ sequence identity).

The house dust clones ($n = 251$) from the Finnish Karelia included fewer staphylococci (6.9%) than the Russian ($P < 0.001$). Only two out of the 17 staphylococcal species were found on both sides of the Finnish Russian border (Table 1).

A Total of 93 actinobacterial sequences were retrieved from the Karelian dusts. Figure 2 shows that only a minority of the dustborne *Actinobacteria* (16% of clones) on the two sides of Karelia represented shared species. *Corynebacteriaceae* clones were more frequent in the dusts from Russian (4.7%) than from the Finnish Karelia (1.7%, $P = 0.08$).

Gram-negative bacteria (*Proteobacteria*) were more common among the clones obtained from the Finnish (33%) than from the Russian (20%, $P < 0.001$) Karelian households. Major part of the proteobacterial sequences in the Finnish Karelian dusts represented plant-associated bacterial species as shown in Table 1. The presence of plant-associated taxa coheres with the prevalence of chloroplast 16S rRNA genes in the Finnish Karelian dusts, indicating plant biomass. *Rhizobiaceae* (4.3%) and *Acetobacteraceae* (3.0%) of the α -group and *Oxalobacteraceae* (5.6%) of the β -group were frequent on the Finnish side, whereas *Moraxellaceae* (3%) of the α -group, including the genus *Acinetobacter*, were most frequent on the Russian side (Table 1).

Out of the 465 bacterial clones obtained from the Russian and Finnish Karelian home dusts 242 were assigned to a cultured validly described bacterial species. A total of 238 of these clones belonged to the phyla *Firmicutes*, *Actinobacteria* or *Proteobacteria*. The species and their biotopes as reported in the literature (see Table S2) are summarized in Table 1. Human-associated species were found from both sides of the border, but only seven out of the 40 species found occurred on both sides. Animal-associated species were found in high numbers (27 clones, 14 species) from the Russian side whereas only one species was cloned from the Finnish side. In contrast, the Finnish Karelian dusts yielded higher numbers of plant-associated species (31 clones) than the Russian dusts (10 clones). Environmental species (soil, water, sewage, etc.) were similarly present in both Russian and Finnish Karelian home dusts (~ 40 clones on each side) but the species composition did not overlap.

The global features of the dusts collected from Karelian households were similar on both sides of the border (Table 2). The main difference was that the Russian Karelian dusts contained 5- to 20-fold more muramic acid, indicating higher content of biomass originating from Gram-positive bacteria. The numbers of 16S rRNA gene copies were also higher in these composite dust (up to threefold) compared with the Finnish Karelian counterpart.

To test whether the quality of the dusts was affected by the method of sampling (vacuuming in Finland, sweeping in Russia) the bacterial composition of dusts collected in parallel from the same homes (in Finland) by the two methods (vacuuming and sweeping) was analysed. The results show that the vacuumed samples contained more muramic acid, DNA and 16S rRNA gene copies than the swept samples ($P > 0.50$), but the difference was twofold or less. This indicates no significant bias introduced by the method of sampling. The mean values of 3-OH fatty acids were higher in the vacuumed as compared with swept samples, but only twofold (Table 3). Therefore, the 5- to 20-fold higher muramic acid contents of the Russian Karelian dusts (swept) is not explained by the method of collection (sweeping versus vacuuming).

The dust content of 3-OH ($C_{10:0}$ - $C_{16:0}$) fatty acids was twofold higher in the Russian side compared with the Finnish side of Karelia. As these fatty acids are mainly found in Gram-negative endotoxins, which were similar on both sides of the border, the higher 3-OH fatty acid content may originate also from Gram-negative bacteria other than those containing Limulus-active endotoxin.

When mycobacterial 16S rRNA gene copies were measured by *Mycobacterium*-specific qPCR similar numbers ($\sim 2 \times 10^7$ 16S rRNA gene copies per gram) were obtained for both composite dusts, corresponding to 0.2% to 1.1% of the total bacterial 16S rRNA genes (Table 2). This result

Table 1. The biotopes of the bacterial species identified among the 16S rRNA gene clones isolated from Finnish and Russian house dust or both.

Biotope ^a	n	Species identification of the clones based on sequence identity of $\geq 97\%$
Russian dust:	Human	28 <i>Acinetobacter baumannii</i> (3), <i>Ac. schindleri</i> (2), <i>Aerococcus viridans</i> (1), <i>Corynebacterium afermentans</i> (2), <i>Coryne. amycolatum</i> (1), <i>Coryne. appendicis</i> (1), <i>Coryne. aurimucosum</i> (1), <i>Coryne. riegliei</i> (1), <i>Coryne. tuscaniae</i> (1), <i>Eubacterium rectale</i> (1), <i>Kocuria palustris</i> (3), <i>Lactobacillus crispatus</i> (2), <i>Luteococcus sanguinis</i> (2), <i>Micrococcus lylae</i> (2), <i>Mycobacterium aubagnense</i> (1), <i>Roseburia intestinalis</i> (1), <i>Staphylococcus haemolyticus</i> (1), <i>Staph. lugdunensis</i> (1), <i>Streptococcus parasanguinis</i> (1)
	Animal	27 <i>Caryophanon latum</i> (1), <i>Clostridium disporicum</i> (1), <i>Cl. estertheticum</i> (1), <i>Exiguobacterium mexicanum</i> (1), <i>Kocuria carniphila</i> (2), <i>Lactobacillus sobrius</i> (1), <i>Macroccoccus caseolyticus</i> (2), <i>Macroccoccus bovis</i> (4), <i>Planococcus rifietoensis</i> (1), <i>Psychrobacter faecalis</i> (1), <i>Sphingomonas panni</i> (1), <i>Staphylococcus cohnii</i> (3), <i>Staph. sciuri</i> (2), <i>Staph. xylosus</i> (6)
	Vegetation	5 <i>Enterococcus casseliflavus</i> (1), <i>Microbacterium phyllosphaerae</i> (1), <i>Microbacterium testaceum</i> (1), <i>Okibacterium fritillariae</i> (1), <i>Sphingomonas melonis</i> (1)
	Environment	36 <i>Acinetobacter lwoffii</i> (1), <i>Bacillus benzoovorans</i> (1), <i>Bacillus psychrodurans</i> (1), <i>Brevibacterium antiquum</i> (1), <i>Carnobacterium maltaromaticum</i> (1), <i>Clostridium frigidis</i> (1), <i>Cl. roseum</i> (1), <i>Halomonas boliviensis</i> (1), <i>Jeotgaliococcus halotolerans</i> (1), <i>Jeotgaliococcus psychrophilus</i> (3), <i>Legionella wroelsiensis</i> (1), <i>Leucobacter aridicollis</i> (1), <i>Microbacterium hydrocarbonoxydas</i> (1), <i>Nakamurella multipartita</i> (1), <i>Nocardioides ganghwensis</i> (1), <i>Nocardioides oleivorans</i> (1), <i>Paenibacillus amylolyticus</i> (1), <i>P. chondroitinus</i> (1), <i>Paracoccus aminophilus</i> (1), <i>Paracoccus carotinifaciens</i> (1), <i>Planococcus antarcticus</i> (2), <i>Pseudomonas veronii</i> (1), <i>Rhodoferax fermentans</i> (1), <i>Salinicoccus roseus</i> (6), <i>Sphingopyxis taejonensis</i> (1), <i>Trichococcus collinsii</i> (2), <i>Variovorax paradoxus</i> (1)
	Other	1 <i>Corynebacterium casei</i> (1)
Finnish dust:	Human	20 <i>Corynebacterium glucuronolyticum</i> (1), <i>Coryne. sundsvallense</i> (1), <i>Enterobacter hormaechei</i> (1), <i>Kytococcus sedentarius</i> (1), <i>Mycobacterium houstonense</i> (1), <i>Propionibacterium acnes</i> (2), <i>Pseudomonas luteola</i> (1), <i>Ps. mosselii</i> (1), <i>Rothia nasimurium</i> (2), <i>Staphylococcus capitis</i> (2), <i>Staph. caprae</i> (2), <i>Staph. schleiferi</i> (3), <i>Staph. warneri</i> (1), <i>Streptococcus salivarius</i> (1)
	Animal	1 <i>Dietzia maris</i> (1)
	Vegetation	23 <i>Agrobacterium tumefaciens</i> (3), <i>Agrobacterium rubi</i> (2), <i>Ancylobacter rudongensis</i> (1), <i>Bradyrhizobium betae</i> (1), <i>Methylobacterium radiotolerans</i> (1), <i>Pantoea agglomerans</i> (1), <i>Phyllobacterium ifriqiense</i> (1), <i>Phyllobacterium myrsinacearum</i> (1), <i>Pseudomonas cichorii</i> (2), <i>Rhizobium huautlense</i> (1), <i>Rhizobium leguminosarum</i> (2), <i>Rhodococcus fascians</i> (3), <i>Sphingomonas azotifigens</i> (1), <i>Xanthomonas campestris</i> (1), <i>Xanthomonas theicola</i> (2)
	Environment	30 <i>Arthrobacter nicotianae</i> (1), <i>Bacillus altitudinis</i> (1), <i>Brevundimonas diminuta</i> (1), <i>Brevundimonas vesicularis</i> (2), <i>Devosia riboflavina</i> (1), <i>Duganella zoogloeoides</i> (5), <i>Enhydrobacter aerosaccus</i> (1), <i>Friedmanniella spumicola</i> (1), <i>Janthinobacterium lividum</i> (1), <i>Massilia plicata</i> (1), <i>Methylobacterium adhaesivum</i> (2), <i>Methylobacterium organophilum</i> (2), <i>Microbacterium deminutum</i> (1), <i>Nocardioides aquiterrae</i> (1), <i>Pseudomonas putida</i> (1), <i>Pseudoxanthomonas japonensis</i> (1), <i>Rhodoferax ferrireducens</i> (1), <i>Shinella granuli</i> (1), <i>Sporosarcina globispora</i> (1), <i>Sphingomonas aurantiaca</i> (1), <i>Sphingomonas dokdonensis</i> (1), <i>Telluria mixta</i> (1), <i>Williamsia maris</i> (1)
	Other	2 <i>Arthrobacter arilaitensis</i> (1), <i>Microbacterium liquefaciens</i> (1)
Russian and Finnish dust:	Human	23 <i>Corynebacterium tuberculostearicum</i> (1/2), <i>Massilia timonae</i> (1/1), <i>Micrococcus luteus</i> (1/2), <i>Staphylococcus epidermidis</i> (1/2), <i>Staph. hominis</i> (7/1), <i>Streptococcus mitis</i> (1/1), <i>Streptococcus pseudopneumoniae</i> (1/1)
	Animal	16 <i>Lactococcus lactis</i> (2/2), <i>Staphylococcus aureus</i> (1/3), <i>Staph. equorum</i> (7/1)
	Vegetation	13 <i>Curtobacterium flaccumfaciens</i> (1/2), <i>Erwinia billingiae</i> (1/1), <i>Kocuria rhizophila</i> (1/2), <i>Stenotrophomonas rhizophila</i> (1/2), <i>Streptococcus thermophilus</i> (1/1)
	Environment	13 <i>Actinoplanes brasiliensis</i> (1/1), <i>Frigoribacterium faeni</i> (2/2), <i>Mycoplasma bullata</i> (2/1), <i>Nitrobacter vulgaris</i> (1/1), <i>Stenotrophomonas maltophilia</i> (1/1)

a. As reported in the literature (see Table S2).

In parenthesis the number of clones assigned to each species (for the species found from both dusts as: Russian dust/Finnish dust).

matches with the proportion of mycobacterial sequences recovered as clones from the same dusts (0.4%), indicating the absence of bias introduced by PCR or cloning.

Discussion

Farming environment and simple living conditions have consistently been associated with reduced risk of atopy and atopic disease (von Hertzen and Haahtela, 2006; von Hertzen *et al.*, 2006), although the precise nature of factors responsible for this protection is unknown. This study revealed major qualitative and quantitative dispari-

ties in the bacterial contents of house dusts between the Finnish and the Russian Karelia, irrespective of the proximity of the areas and similarity of vegetative and climatic conditions. The dusts collected from the Russian side were two times richer in Gram-positives as compared with the households on the Finnish side. The prevalence of staphylococci and particularly of species associated with animals in the Russian Karelian dusts was striking and indicates more intense contacts between humans and animals. The percentages of households with cats are high in Russian Karelia, e.g. 73% of the Russian children at the age of 1–9 years were reported by their parents to

Table 2. Total bacterial and mycobacterial DNA content and muramic acid, 3-OH (C10-C16) fatty acids and Limulus-active endotoxin concentrations in Finnish and Russian Karelian house dusts.

Homes:	Finnish		Russian	
	1–5	6–10	1–5	6–10
DNA, $\mu\text{g g}^{-1}$	58	55	53	90
Bacterial 16S rRNA gene, copies per gram	6.4×10^9	8.8×10^9	1.6×10^{10}	1.8×10^{10}
Mycobacterial 16S rRNA gene, copies per gram	7.3×10^7 (1.1%) ^a	3.6×10^7 (0.4%) ^a	3.6×10^7 (0.2%) ^a	5.7×10^7 (0.3%) ^a
Muramic acid, ng mg^{-1}	3.0	11.0	59.2	69.7
3-OH C10-C16 fatty acids, nmol mg^{-1}	0.029	0.12	0.13	0.18
Endotoxin (Limulus), ng mg^{-1}	2.8	10	2.5	5.9

a. Mycobacterial 16S rRNA gene copy number/total bacterial 16S rRNA gene copy number.

have a cat as compared with 36% in Finland (von Hertzen and Haahtela, 2006). In addition to these pets, there are numerous stray cats in the area. Pet ownership in childhood has indeed been connected to lower risk of allergic disease also elsewhere (Svanes *et al.*, 2003; Pohlabein *et al.*, 2007). In this study, we identified several animal-associated bacterial species in house dusts collected in the Russian Karelian homes. This could connect to the lower allergy risk in this area, compared with the adjacent areas in Finland.

Not only staphylococci but also dustborne corynebacteria were more frequent in the Russian households than in their counterparts in the Finnish side. Interestingly, two out of the four corynebacterial clones in the Finnish dust were identified as *Corynebacterium tuberculostrictum* (predominant resident of human skin), whereas in the Russian dust, 10 out of the 11 corynebacterial clones represented other species. The family *Corynebacteriaceae* comprises the genus *Mycobacterium*, and several closely related genera which possess mycolic acids in their cell wall (Boone *et al.*, 2001). Of importance is the high content of cytosine-guanine oligonucleotide repeats (CpG) in *Corynebacteriaceae*, which displayed in murine models potent immunomodulatory, antiallergic and even

tolerogenic properties (Horner and Raz, 2003; Moseman *et al.*, 2004). In this study, the proportion of DNA clones representing the high G+C DNA of mycobacteria (~0.5% of clones) was confirmed by an independent technique, *Mycobacterium*-specific qPCR, indicating that the high G+C DNA of mycobacteria were not underrepresented in the clone libraries due to selective PCR amplification of low G+C DNA (Reysenbach *et al.*, 1992; Dutton *et al.*, 1993).

As to the Gram-negative bacteria (mainly *Proteobacteria*), major disparities between the households of Russian and Finnish side of Karelia were in the occurrence of the nitrogen-fixing nodule bacterium *Rhizobium* present on the Finnish but not the Russian side. Interestingly, despite the geographical and climatic proximity, only a minor fraction of the clones, 18% of *Proteobacteria*, 16% of *Actinobacteria* and 32% of *Firmicutes* represented species found from both sides of the border. For instance *Acinetobacter*, characteristic of soil, fresh water and animal sheds, was found in the households of the Russian but not on the Finnish side. A recent report showed that *Acinetobacter*, along with *Lactococcus*, reduced allergic reactions in mice and induced a Th1-polarized response in human dendritic cells (Debarry *et al.*, 2007).

Table 3. Comparison of the bacterial compositions of dusts collected by vacuuming and sweeping.

	Vacuuming	Sweeping	P-value
DNA, $\mu\text{g g}^{-1}$ (7 dusts)			
Mean (SEM)	212 (73)	150 (74)	0.580
Median (range)	208 (36–395)	112 (47–292)	
Bacterial 16S rRNA gene, 10^9 copies g^{-1} (7 dusts)			
Mean (SEM)	3.9 1.3	2.9 (1.6)	0.646
Median (range)	3.4 (1.5–7.4)	1.3 (1.3–6.1)	
Muramic acid, ng mg^{-1} (12 dusts)			
Mean (SEM)	21.6 (6.16)	10.6 (0.88)	0.575
Median (range)	18.7 (8.0–44.9)	10.1 (8.5–14.6)	
3-OH (C10-C16) fatty acids, nmol mg^{-1} (12 dusts)			
Mean (SEM)	0.08 (0.012)	0.04 (0.004)	0.008
Median (range)	0.08 (0.052–0.12)	0.04 (0.028–0.056)	

Paired dust samples were collected from the same homes (in Finland) by two methods: by vacuuming ($n = 6$) and by sweeping ($n = 6$). The table shows the contents of DNA, bacterial 16S rRNA gene copies, muramic acid, 3-OH fatty acids and Limulus-active endotoxin in the dusts.

Exposure of the innate immune system to non-pathogenic bacteria, specifically by TLRs, plays a pivotal role in the maintenance of mucosal epithelial homeostasis and the protection against mucosal epithelial injury (Rakoff-Nahoum *et al.*, 2004). The importance of TLR2, the receptor for components of Gram-positive bacteria and the fungal cell wall (Heine and Lien, 2003), on the development of tolerance (i.e. reduced risk for atopic disease) among children was recognized in several studies (Lauener *et al.*, 2002; Eder *et al.*, 2004).

Recent studies indicated association of muramic acid or fungal extracellular polysaccharides and wheezing/asthma among children (van Strien *et al.*, 2004; Douwes *et al.*, 2006). Interestingly, barn dust extract, administered via inhalation, was recently shown to be able to inhibit the development of markers of atopic asthma in mice, whereas lipopolysaccharide, administered at a concentration similar to that found in the barn dust (800 ng ml⁻¹), conferred no protection against atopic asthma in the murine model (Peters *et al.*, 2006). Endotoxin appeared thus not to be the primary component in barn dust responsible for protection (Peters *et al.*, 2006).

There is no standardized, generally accepted dust sampling procedure making comparisons between dusts difficult, particularly in the context of different human cultures. In this study we could only get dusts obtained by sweeping from the homes in Russian Karelia. However, we showed results indicating that this has unlikely biased interpretation of the present results. All dusts were sieved before analysis and only fine dust was used further.

Cloning of dust borne DNA allowed us the detection and enumeration of the unculturable bacteria. Gram-positive bacilli, cocci and actinobacteria (*Staphylococcus*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Arthrobacter*) have dominated in culture studies of indoor air (Andersson *et al.*, 1999; Górny and Dutkiewicz, 2002). The DNA cloning in this paper revealed a wider diversity (94 different genera) of dustborne bacteria than seen in any previous study on residential environments. Górny and Dutkiewicz (2002) reported 42 different bacterial genera from homes ($n > 100$) in Poland, and Andersson and colleagues (1999) found 17 bacterial genera from schools and day-care centres in Finland. Rintala and colleagues (2008) isolated DNA clones representing > 100 bacterial genera from office environments in Finland. Lee and colleagues (2007) isolated 16S rRNA gene clones representing 18 different bacterial genera from indoor surfaces of a child-care facility. Twelve of these genera were also found in our study. Another strength here was the study setting, which allowed us to assess the quality and quantity of environmental saprophytic exposure in two adjacent areas with fundamentally different living conditions (von Hertzen *et al.*, 2006).

In conclusion, high occurrence of Gram-positive bacteria, and particularly animal-associated staphylococci, was found in the house dust from Russian Karelia, an area with low occurrence of atopy and atopic disease. Our findings provide an impetus for further examination of the potential use of TLR2 ligands, particularly members of the phyla *Actinobacteria* and *Firmicutes*, in allergy prevention strategies.

Experimental procedures

House dust

House dust samples from living room and bedroom floors were collected by the participating mothers in Finland and Russia in September and October 2003 according to written instructions using a vacuum cleaner, or if not possible in Russian Karelia, by sweeping. All samples were sieved (diameter 1 mm). The obtained homogenous dusts were combined in to four lots each originating from five randomly selected homes in the Finnish (two composite dusts) and the Russian Karelia (two composite dusts). For sampling method validation, see the description below.

DNA isolation and determination of DNA concentration

DNA was isolated from 40 to 50 mg of dust and DNA concentration measured as described elsewhere (Pakarinen *et al.*, 2007).

Quantitative PCR

Real-time qPCR, using the primers pE and pF' (Edwards *et al.*, 1989) targeted for the 16S rRNA gene of *Eubacteria*, was carried out as described by Ekman and colleagues (2007). *Mycobacterium*-specific qPCR using specific primers and hybridization probes was carried out as described by Pakarinen and colleagues (2007).

DNA cloning and sequence analysis

About 15–25 ng of DNA isolated from dust was PCR amplified using the primers pA and pH' (Edwards *et al.*, 1989), targeted for the 16S rRNA gene of *Eubacteria* and DyNAzyme II Taq polymerase enzyme (Finnzymes, Espoo, Finland).

DNA cloning and sequencing was purchased service from the University of Helsinki Institute of Biotechnology DNA Laboratory. The PCR products were cloned into *E. coli* using PCR Cloning Kit (Qiagen, Valencia, CA, USA), plasmid DNA was extracted using MultiScreen 96PLASMID Plates (Millipore, Billerica, MA, USA), and inserts were re-amplified with vector primers and purified with MultiScreen 384PCR-plates. Sequencing was done using the primer pD' (Edwards *et al.*, 1989), BigDye Terminator cycle sequencing kit and ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences ($n = 465$, length 220–484 nt) were deposited in the EMBL sequence database (accession numbers

AM943126–AM943359 for the Russian and AM942777–AM943007 for the Finnish Karelian dust DNA clones). The sequences were aligned using ClustalW2 (Larkin *et al.*, 2007), and analysed using the Library Compare and Sequence Match tools at the Ribosomal Database Project II (Cole *et al.*, 2007) and BLAST search at the National Center for Biotechnology Information website (McGinnis and Madden, 2004).

Endotoxin measurements

Biologically active endotoxin was measured using a validated kinetic chromogenic Limulus Amebocyte Lysate assay (Kinetic QCL; Lonza, Walkersville, MD, USA) according to the European Standard EN 14031 (2003), as described in detail earlier (Hyvärinen *et al.*, 2006).

Measurements of muramic acid and 3-OH fatty acids

A sample of about 5 mg of dust was accurately weighed for determination of 3-OH FAs and muramic acid. The sample preparation of 3-OH FAs was done slightly modifying the method presented by Sebastian and Larsson (2003) and the preparation of muramic acid modifying the method presented by Sebastian and Larsson (2003). In our methods, hexane was used instead of heptane. The muramic acid samples were diluted to 150 µl of chloroform prior to the analysis by gas chromatography mass spectrometry (GC-MS). In the preparation of 3-OH FAs, no water was used in the first extraction.

The analyses of muramic acid were performed with a Thermo PolarisQ ion trap MS equipped with a Thermo Trace GC-ultra GC with a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm) from J&W Scientific. The injection volume of the samples was 1 µl, the temperature of the injector was 280°C, helium was used as carrier gas at a constant flow of 1.0 ml min⁻¹ and the GC was operated in splitless mode. The temperature program of the GC was following: 90°C for 0.5 min, 40°C min⁻¹ to 290°C and hold 290°C for 2.5 min. Positive electron ionization was performed at an ionization energy of 70 eV and the temperature of the ion source was 220°C. The selected reaction monitoring method was used to monitor transition from the one fragment ion of muramic acid derivate, m/z 213.1 → 127.1 and one from the fragment ion of ¹³C-labelled muramic acid derivate, the internal standard, m/z 221.1 → 132.1.

The analyses of 3-OH FAs were performed with Agilent 5973N Inert quadrupole MS equipped with an Agilent 6890N GC with an HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 µm) from J&W Scientific. The injection volume of the samples was 2 µl, the temperature of the injector was 280°C, helium was used as carrier gas at a constant flow of 1.0 ml min⁻¹ and the GC was operated in splitless mode. The temperature program of the GC was following: 90°C for 1 min, 20°C min⁻¹ to 280°C and hold 280°C for 1 min. Positive electron ionization was performed at an ionization energy of 70 eV and the temperature of the ion source was 200°C. The single ion monitoring method was used to monitor one fragment ion of silylated 3-OH FAs, m/z 175 and m/z 178 for internal standard.

Sampling method validation

To verify the comparability of the results on both sides of the border, we collected additionally six paired dust samples from the same floors (2 paired samples from apartment houses, 3 from row houses, 1 from a single house) using both vacuuming and sweeping according to a written protocol, and analysed for DNA, 16S rRNA gene copy number, muramic acid and 3-OH-(C_{10:0}–C_{16:0}) fatty acid concentrations.

Statistical analysis

All analyses for statistically significant differences between bacterial proportions were performed with the RDP Library Compare tool at the Ribosomal Database Project II website (<http://rdp.cme.msu.edu/comparison/comp.jsp>). For other comparisons, Student's *t*-test and the Mann–Whitney *U*-test was used (SAS Program, version 6.12 (SAS Institute, Cary, USA)). *P*-values < 0.05 were considered significant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genus distribution of the 16S rRNA gene clones isolated from Finnish and Russian Karelian house dusts. The 16S rRNA gene sequences ($n = 465$) were assigned to bacterial genera using the RDP Library Compare program (Cole *et al.*, 2007).

Table S2. Bacterial species identified among the 16S rRNA gene clones isolated from Finnish and Russian Karelian house dusts and their reported habitats.

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Table S1. Genus distribution of the 16S rRNA gene clones isolated from Finnish and Russian Karelian house dusts. The 16S rRNA gene sequences (n=465) were assigned to bacterial genera using the RDP Library Compare program (Cole et al., 2007).

Genus	Russia	Finland
	Clones	
Acidobacterium	1	1
Acinetobacter	6	0
Aerococcus	1	0
Aeromicrobium	0	1
Agrobacterium	0	1
Alloiococcus	1	0
Anaerococcus	1	0
Arthrobacter	0	2
Bacillus	2	2
Brevibacterium	1	1
Brevundimonas	1	4
Carnobacterium	1	0
Caryophanon	1	0
Chitinophaga	1	2
Chondromyces	0	1
Chryseomonas	0	1
Clostridium	5	0
Conexibacter	0	1
Corynebacterium	11	4
Cryptanaerobacter	1	0
Curtobacterium	1	2
Deinococcus	2	0
Devosia	0	1
Dietzia	0	1
Duganella	0	4
Enhydrobacter	0	1
Enterococcus	1	0
Eubacterium	1	0
Exiguobacterium	1	0
Flavimonas	1	0
Flavobacterium	1	2
Friedmanniella	0	1
Frigoribacterium	1	2
Fusobacterium	0	1
Gemmatimonas	1	1
Halomonas	2	0
Helcococcus	1	0
Hyphomicrobium	1	0
Isosphaera	0	1
Janthinobacterium	0	1
Jeotgalicoccus	4	0
Kocuria	6	2
Kytococcus	0	1

Lactobacillus	3	1
Lactococcus	2	2
Legionella	1	0
Leifsonia	0	1
Leptolyngbya	1	0
Leucobacter	1	0
Luteococcus	2	0
Macrococcus	8	0
Marinobacter	1	0
Marmoricola	0	1
Massilia	1	2
Megamonas	0	1
Methylobacterium	0	5
Microbacterium	3	3
Micrococcus	3	2
Mycobacterium	1	1
Nakamurella	1	1
Nannocystis	0	1
Nocardioides	2	2
Okibacterium	1	0
Paenibacillus	2	0
Pantoea	0	1
Paracoccus	2	0
Pedobacter	0	3
Peptoniphilus	0	1
Planococcus	4	0
Polaromonas	1	0
Propionibacterium	0	2
Pseudomonas	1	4
Pseudoxanthomonas	1	1
Psychrobacter	1	0
Rhizobium	0	6
Rhodococcus	0	3
Rhodoferax	1	1
Rhodopila	0	1
Roseburia	1	0
Rothia	0	2
Ruminococcus	0	1
Salinicoccus	8	1
Sphingomonas	2	2
Sphingopyxis	1	0
Sporosarcina	0	1
Staphylococcus	29	15
Stenotrophomonas	4	3
Streptococcus	5	4
Thermomonas	1	0
Tissierella	1	0
TM7	1	0
Trichococcus	3	0
Vagococcus	1	0
Williamsia	0	1

Xanthomonas	0	2
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Table S2. Bacterial species identified among the 16S rRNA gene clones isolated from Finnish and Russian Karelian house dusts and their reported habitats.

Species identified	Habitat
<u>From Russian dust only</u>	
Firmicutes	
<i>Aerococcus viridans</i>	human clinical sp. (Bosley et al., 1990)
<i>Bacillus benzoovorans</i>	soil (Tzeneva et al., 2004)
<i>Bacillus psychrodurans</i>	soil (Abd El-Rahman et al., 2002)
<i>Carnobacterium maltaromaticum</i>	environment (Leisner et al., 2007)
<i>Caryophanon latum</i>	cattle manure (Trentini, 1978)
<i>Clostridium disporicum</i>	swine manure (Leung & Topp, 2001)
<i>Clostridium estertheticum</i>	vacuum packed meat (Collins et al., 1992)
<i>Clostridium frigidis</i>	antarctic lake (Spring et al., 2003)
<i>Clostridium roseum</i>	anaerobic compost (Chang et al., 2006)
<i>Enterococcus casseliflavus</i>	vegetable foods (Abriouel et al., 2007)
<i>Eubacterium rectale</i>	human intestine (Aminov et al., 2006)
<i>Exiguobacterium mexicanum</i>	<i>Artemia</i> cysts (López-Cortés et al., 2006)
<i>Jeotgalicoccus halotolerans</i>	halophilic sp. (Yoon et al., 2003)
<i>Jeotgalicoccus psychrophilus</i>	halophilic sp. (Yoon et al., 2003)
<i>Lactobacillus crispatus</i>	human skin. (Gao et al., 2006)
<i>Lactobacillus sobrius</i>	swine intestine (Konstantinov et al., 2006)
<i>Macrococcus bovicus</i>	animal skin (Kloos et al., 1998)
<i>Macrococcus caseolyticus</i>	animal skin (Kloos et al., 1998)
<i>Macrococcus lamae</i>	animal skin (Mannerova et al., 2003)
<i>Paenibacillus amylolyticus</i>	soil (Holt, 1986)
<i>Paenibacillus chondroitinus</i>	soil (Shida et al., 1997)
<i>Planococcus antarcticus</i>	antarctic lake (Reddy et al., 2002)

<i>Planococcus rifietoensis</i>	pigeon faeces (Suresh et al., 2007)
<i>Salinicoccus roseus</i>	halophilic sp. (Ventosa et al., 1990)
<i>Staphylococcus cohnii</i>	animal skin (Nagase et al., 2001)
<i>Staphylococcus equorum</i>	animals (Meugnier et al., 1996)
<i>Staphylococcus haemolyticus</i>	human skin (Gao et al., 2006)
<i>Staphylococcus lugdunensis</i>	human skin (Nagase et al., 2001)
<i>Staphylococcus sciuri</i>	animal skin (Nagase et al., 2001)
<i>Staphylococcus xylosus</i>	animal skin (Nagase et al., 2001)
<i>Streptococcus mitis</i>	human oral (Aas et al., 2005)
<i>Streptococcus parasanguinis</i>	human skin (Gao et al., 2006)
<i>Trichococcus collinsii</i>	hydrocarbon contaminated soil (Liu et al., 2002)
Actinobacteria	
<i>Brevibacterium antiquum</i>	soil (Gavrish et al., 2004)
<i>Corynebacterium afermentans</i>	human skin (Gao et al., 2006)
<i>Corynebacterium amycolatum</i>	human skin (Gao et al., 2006)
<i>Corynebacterium appendicis</i>	human skin (Gao et al., 2006)
<i>Corynebacterium aurimucosum</i>	human skin (Gao et al., 2006)
<i>Corynebacterium casei</i>	cheese (Brennan et al., 2001)
<i>Corynebacterium riegelii-like</i>	human clinical (Funke et al., 1998)
<i>Corynebacterium tuscaniae</i>	human clinical (Riegel et al., 2006)
<i>Kocuria carniphila</i>	meat (Tvrzová et al., 2005)
<i>Kocuria palustris</i>	human skin (Gao et al., 2006)
<i>Leucobacter aridicollis</i>	chromium polluted sludge (Morais et al., 2004)
<i>Luteococcus sanguinis</i>	human clinical (Collins et al., 2003)
<i>Microbacterium hydrocarbonoxydans</i>	oil-contaminated soil (Schippers et al., 2005)
<i>Microbacterium phyllosphaerae</i>	grass phyllosphere, soil litter (Behrendt et al., 2001)
<i>Microbacterium testaceum</i>	plants (Zinniel et al., 2002)

<i>Micrococcus lylae</i>	human skin (Kloos & Musselwhite, 1975)
<i>Mycobacterium aubagnense</i>	human clinical (Adékambi et al., 2006)
<i>Nocardioides oleivorans</i>	crude oil (Schippers et al., 2005)
<i>Okibacterium fritillariae</i>	plant seeds (Evtushenko et al., 2002)
Proteobacteria	
<i>Acinetobacter baumannii</i>	environment and human clinical (Garrity, 2005)
<i>Acinetobacter lwoffii</i>	environment (Garrity, 2005)
<i>Acinetobacter schindleri</i>	human clinical (Nemec et al., 2001)
<i>Halomonas boliviensis</i>	halophilic sp. (Quillaguamán et al., 2004)
<i>Legionella worsleiensis</i>	water (Dennis et al., 1993)
<i>Paracoccus aminophilus</i>	soil (Garrity, 2005)
<i>Paracoccus carotinifaciens</i>	soil (Tsubokura et al., 1999)
<i>Pseudomonas veronii</i>	natural mineral water (Elomari et al., 1996)
<i>Psychrobacter faecalis</i>	pigeon faeces (Kämpfer et al., 2002)
<i>Rhodoferrax fermentans</i>	freshwater and sewage (Garrity, 2005)
<i>Sphingomonas melonis</i>	melon fruit pathogen (Buonaurio et al., 2002)
<i>Sphingomonas panni</i>	from a wipe at an animal clinic (Busse et al., 2005)
<i>Sphingopyxis taejonensis</i>	natural mineral water (Lee et al., 2001)
<i>Variovorax paradoxus</i>	mountain snow (Segawa et al., 2005)

From Finnish dust only

Firmicutes

<i>Bacillus altitudinis</i>	high altitude air (Shivaji et al., 2006)
<i>Sporosarcina globispora</i>	soil and water (Holt, 1986)
<i>Staphylococcus capitis</i>	human skin (Gao et al., 2006)
<i>Staphylococcus caprae</i>	human skin (Gao et al., 2006)
<i>Staphylococcus schleiferi</i>	human skin (Gao et al., 2006)
<i>Staphylococcus warneri</i>	human skin (Gao et al., 2006)

<i>Streptococcus salivarius</i>	human oral (Aas et al., 2005)
Actinobacteria	
<i>Actinoplanes brasiliensis</i>	soil (Parenti & Coronelli, 1979)
<i>Arthrobacter arilaitensis</i>	cheese (Irlinger et al., 2005)
<i>Arthrobacter nicotianae</i>	soil, sewage and air (Holt, 1986)
<i>Corynebacterium glucuronolyticum</i>	human skin (Gao et al., 2006)
<i>Corynebacterium sundsvallense</i>	human skin (Gao et al., 2006)
<i>Dietzia maris</i>	intestine and skin of carp, soil (Rainey et al., 1995)
<i>Friedmanniella spumicola</i>	activated sludge (Maszenan et al., 2005)
<i>Kytococcus sedentarius</i>	human skin (Levenga et al., 2003)
<i>Microbacterium deminutum</i>	soil (Kageyama et al., 2006)
<i>Microbacterium liquefaciens</i>	dairy products (Holt, 1986)
<i>Mycobacterium houstonense</i>	human clinical (Schinsky et al., 2004)
<i>Nocardioides aquiterrae</i>	groundwater (Yoon et al. 2004)
<i>Propionibacterium acnes</i>	human skin (Gao et al., 2006)
<i>Rhodococcus fascians</i>	plants (Holt, 1986)
<i>Rothia nasimurium</i>	human skin (Gao et al., 2006)
<i>Williamsia maris</i>	sea water (Stach et al., 2004)
Proteobacteria	
<i>Acidisphaera rubrifaciens</i>	acidic water and mine drainage (Hiraishi et al., 2000)
<i>Agrobacterium rubi</i>	<i>Rubus</i> plant (Garrity, 2005)
<i>Ancylobacter rudongensis</i>	plants (Xin et al., 2004)
<i>Bradyrhizobium betae</i>	<i>Beta vulgaris</i> roots (Rivas et al., 2004)
<i>Brevundimonas diminuta</i>	water, human and animal clinical (Garrity, 2005)
<i>Brevundimonas vesicularis</i>	water (Garrity, 2005)
<i>Devosia riboflavina</i>	soil (Nakagawa et al., 1996)
<i>Duganella zoogloeoides</i>	wastewater (Hiraishi et al., 1997)

<i>Enhydrobacter aerosaccus</i>	lake water (Garrity, 2005)
<i>Enterobacter hormaechei</i>	human clinical (O'Hara et al., 1989)
<i>Janthinobacterium lividum</i>	soil & water (Garrity, 2005)
<i>Massilia plicata</i>	soil (Zhang et al., 2006)
<i>Methylobacterium adhaesivum</i>	water (Gallego et al., 2006)
<i>Methylobacterium organophilum</i>	lake sediment (Garrity, 2005)
<i>Methylobacterium radiotolerans</i>	plants (Garrity, 2005)
<i>Pantoea agglomerans</i>	plants (Garrity, 2005)
	root nodules of <i>Astragalus algerianus</i> and <i>Lathyrus</i>
<i>Phyllobacterium ifriqiyense</i>	numidicus (Mantelin et al., 2006)
<i>Phyllobacterium myrsinacearum</i>	plants (Garrity, 2005)
<i>Pseudomonas cichorii</i>	plants (Kiba et al., 2006)
<i>Pseudomonas luteola</i>	human clinical (Garrity, 2005)
<i>Pseudomonas mosselii</i>	human clinical (Dabboussi et al., 2002)
<i>Pseudomonas putida</i>	soil & water (Garrity, 2005)
<i>Pseudoxanthomonas japonensis</i>	soil (Thierry et al., 2004)
<i>Rhizobium huautlense</i>	Sesbania herbacea plant (Wang et al., 1998)
<i>Rhizobium leguminosarum</i>	plants (Garrity, 2005)
<i>Rhodoferrax ferrireducens</i>	sediment (Finneran et al., 2003)
<i>Shinella granuli</i>	sewage (An et al., 2006)
<i>Sphingomonas aurantiaca</i>	dust (Busse et al., 2003)
<i>Sphingomonas azotifigens</i>	<i>Oryza sativai</i> roots (Xie & Yokota, 2006)
<i>Sphingomonas dokdonensis</i>	soil (Yoon et al., 2006)
<i>Stenotrophomonas maltophilia</i>	soil, water, food, human clinical (Garrity, 2005)
<i>Telluria mixta</i>	soil (Garrity, 2005)
<i>Xanthomonas campestris</i>	plants (Garrity, 2005)
<i>Xanthomonas theicola</i>	tea plant (Garrity, 2005)

From Both dusts

Firmicutes

<i>Lactococcus lactis</i>	raw milk (Holt, 1986)
<i>Roseburia intestinalis</i>	human faeces (Duncan et al., 2002)
<i>Staphylococcus hominis</i>	human skin. (Gao et al., 2006)
<i>Streptococcus pseudopneumoniae</i>	human clinical (Arbique et al., 2004)
<i>Streptococcus thermophilus</i>	plants (Michaylova et al., 2007)

Actinobacteria

<i>Corynebacterium tuberculostearicum</i>	human skin (Gao et al., 2006)
<i>Curtobacterium flaccumfaciens</i>	plants (Araújo et al., 2002)
<i>Frigoribacterium faeni</i>	dust, environment (Kämpfer et al., 2002)
<i>Kocuria rhizophila</i>	<i>Typha angustifolia</i> rhizoplane (Kovács et al., 1999)
<i>Micrococcus luteus</i>	human skin (Kloos & Musselwhite, 1975)
<i>Nakamurella multipartita</i>	activated sludge (Yoshimi et al., 1996)
<i>Nocardioides ganghwensis</i>	sediment (Yi & Chun, 2004)

Proteobacteria

<i>Agrobacterium tumefaciens</i>	plants (Garrity, 2005)
<i>Erwinia billingiae</i>	plants (Garrity, 2005)
<i>Massilia timonae</i>	human clinical (La Scola et al., 1998)
<i>Mycoplana bullata</i>	soil (Garrity, 2005)
<i>Nitrobacter vulgaris</i>	soil & water (Garrity, 2005)
<i>Stenotrophomonas rhizophila</i>	plants (Wolf et al., 2002)

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