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Skin-Associated Bacillus, Staphylococcal and Micrococcal Species from the House Dust Mite, *Dermatophagoides pteronyssinus* and bacteriolytic enzymes

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Abstract

Dust mites produce bacteriolytic enzymes, one of which belongs to the NlpC/P60 superfamily comprising bacterial and fungal proteins. Whether this enzyme is derived from the mite or from mite-associated microbes is unclear. To this end, the bacteriology of mites per se, and carpet and mattress dust from a group of asthmatic children and their parents was investigated. Dust from parents' and children's mattresses yielded significantly more colony forming units (cfu) compared with dust from their corresponding carpets. Zymography demonstrated some dusts contained bacteriolytic enzymes, and in nine of the twelve dust samples from three of five houses examined, a prominent bacteriolytic band was obtained that corresponded to the mite band, although in one home, other lytic bands were detected. Fifty bacterial isolates were obtained from surface-sterilised, commercially obtained *Dermatophagoides pteronyssinus*. 16S rRNA, *tuf* and *rpoB* gene sequencing of nine Gram-positive isolates identified them as *Bacillus cereus*, *B. licheniformis*, *Staphylococcus aureus*, *S. epidermidis*, *S. capitis* and *Micrococcus luteus*, known human skin commensals. 16S rRNA sequence homologies of four of the nine isolates identified as *B. licheniformis* formed a distinct phylogenetic cluster. All species secreted lytic enzymes during culture although the lytic profiles obtained differed between the rods and the cocci, and none of the bands detected corresponded to those observed in dust or mites. In conclusion, mites harbour a variety of bacterial species often associated with human skin and house dusts contain bacteriolytic enzymes that may be mite-derived. The identification of a novel cluster of *B. licheniformis* isolates suggests an ecological adaptation to laboratory-reared *D. pteronyssinus*. It remains to be determined whether the previously described mite-associated 14K lytic enzyme is derived from a microbial source..

Key words: *Dermatophagoides pteronyssinus*, *Bacillus*, *Staphylococcus*, *Micrococcus*, bacteriolytic enzymes, house dust

Introduction

Mites are economically and medically important arachnids, which may cause food spoilage in agricultural industries, and disease in both animals and humans (Arlan, 1989; Fisher & Wilson 1977; Platt-Mills *et al.*, 1992). They have been shown to be particularly important in the development of allergic diseases (Platt-Mills *et al.*, 1992). As a result, their ecology and biology have been studied extensively, and a variety of allergenic proteins of importance to humans have been described. With regard to the clinically important house dust mite species such as *Dermatophagoides pteronyssinus* and *D. farinae*, a significant number of allergens have been shown to be digestive enzymes that are found in mite fecal pellets, and inhaled upon aerosolization (Chua *et al.*, 1988; King *et al.*, 1996; Lake *et al.*, 1991; Stewart *et al.*, 1992; Stewart *et al.*, 2006). Other mite enzymes are also present in these pellets but whether they are allergenic remains to be determined.

In this regard, we (Mathaba *et al.*, 2002) and others have reported that extracts of both whole mites and faecally-enriched growth medium contain several bacteriolytic enzymes, and it is possible that these play a role in both defence and nutrient utilisation (Barabanova & Zheltikova, 1985; Erban and Hubert, 2008). The cDNA of one of these enzymes was cloned, and the cDNA obtained was shown to encode a 14 kDa lytic enzyme belonging to the NlpC/P60 superfamily of endopeptidases (Mathaba *et al.*, 2002). The most significant homologies are observed with bacterial proteins from *Mycobacterium*, *Corynebacterium*, *Clostridium*, *Bacillus* and *Pseudomonas* species, and from entomopathogenic fungal species. Whether the mite protein originates from either bacteria or fungi associated with mites is unclear. Alternatively, if present in the genome of mites, the gene may have been acquired from such sources by horizontal gene transfer (Mathaba *et al.*, 2002).

With regard to the possibility that the enzyme is produced by mite-associated microbes or by those present in its environment, several studies have demonstrated the presence of bacterial species in house dusts that harbour mites, and include genera such as *Bacillus*, *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Klebsiella*, *Proteus*, *Corynebacterium*, *Nocardia* and *Mycobacterium* (Dawson, 1971; Oh *et al.*, 1986; Horak, 1987; Horak *et al.*, 1996). In addition, *Dermatophagoides* and *Psoroptes* mites have also been shown to contain *Bacillus*, *Staphylococcus* and coryneform species, and Gram-negative rods (Hogg & Lehane 1999; Mathieson & Lehane 1996; Oh *et al.*, 1986; Smrz and Trelova, 1995; Stefaniak and Seniczak, 1976). With regard to fungi in house dust and

mites, several species have been identified, and nine fungal species including *Eurotium amstelodami*, *Aspergillus penicilloides* and *Wallemia sebi* have been isolated from both the guts of surface-sterilised mites as well as from house dust (Oh *et al*, 1986; Hay *et al.*, 1992).

The study described here was, therefore, undertaken to determine whether bacterial species could be isolated from laboratory-reared *D. pteronyssinus* as a prelude to determining whether any produce the NlpC/P60 mite associated enzyme. Initially, standard microbiological techniques were used to identify such species, followed by molecular techniques. In addition, the number of colony forming units in house dust samples was investigated, as well as the presence of lytic enzymes in such samples. Gram-positive cocci and rods were identified in surface-sterilised mites and that the bacterial content of dusts was greater in mattresses from parents and their asthmatic children than in their carpets.. Bacteriolytic enzymes were present in some but not all house dust samples, one of which corresponded in size to a major band present in whole mite and spent growth medium, although it remains to be determined if this is the mite P60 superfamily member.

Materials and methods

All chemicals were obtained from BDH (Poole, England) or Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise indicated.

Bacterial colony forming units (cfu) in house dusts from Melbourne homes

House dusts (100mg) were extracted using 1 ml aliquots of three different buffers with shaking and then processed as described below using blood agar plates. The three buffers were phosphate buffered saline (PBS) containing 0.04 M EDTA (EDTA), PBS containing 10 mM cysteine (Cysteine), and PBS containing both 0.04 M EDTA and 10mM cysteine (EDTA/Cysteine). Buffers containing cysteine were used because of the redox potential likely to exist in mite guts and studies showing that some species such as micrococci associated with skin require sulphur-containing amino acids for growth (Farrior and Kloos, 1976). The numbers of bacterial cfu growing on blood agar plates incubated at 37°C under various atmospheric conditions were determined with 100 µl aliquots, after appropriate dilution using the spread plate method and a colony counter. Microaerophilic conditions for culturing plates were achieved using a candle jar, whereas anaerobic conditions were achieved in a chamber containing an 'Anaerogen' sachet (Oxoid, Basingstoke, Hampshire, UK).

Bacterial content of house dust mites

Live *Dermatophagoides pteronyssinus* mites, unused proprietary growth medium and spent growth medium were kindly donated by the Commonwealth Serum Laboratories (Melbourne, Australia). Mites were either used immediately or cultured on the unused growth medium in our laboratory at room temperature in sterile tissue culture flasks over saturated sodium chloride to control relative humidity. One hundred mg aliquots of the mites in sterile microcentrifuge tubes were sterilised with either 96% (v/v) ethanol or 5% (v/v) HCl followed by 70% (v/v) ethanol as described previously (Cazemier *et al.*, 1997; Smrz *et al.*, 1997) in order to remove any bacteria from the mite surfaces. Following centrifugation at 28,000 x *g* for 10 min at 4°C, the supernatants (sterilisation/wash buffers) were removed from the mites. The surface-sterilised mites were rinsed twice with sterile, distilled water and then homogenised using a hand-held microhomogeniser and 500 µl of one of the three different buffers described above. Three aliquots of each supernatant were inoculated onto each of three blood agar (BA) and nutrient agar (NA) plates and incubated at 37°C as described above. Plates were examined for distinct colony morphotypes and colonies restreaked onto fresh BA and NA plates. Aliquots of each extract were also inoculated into

tryptone soy broth for aerobes and thioglycolate for anaerobes, and incubated at 37°C. Cysteine-containing extractions were inoculated into cysteine-enriched cooked meat medium. Turbid broth cultures were then streaked onto BA and NA plates for discrete colony isolation. Gram-positive rods and cocci, with the exception of the putative staphylococcal species (maintained on P agar), were maintained on BA plates. Extracts were also prepared from unused growth medium and spent growth medium (SGM) by shaking suspensions continuously at 220 rpm for 1 h at 4°C. Aliquots of the suspensions were centrifuged at 28,000 x *g* for 15 min at 4°C and samples of each extract were plated out onto BA plates.

Phenotypic characterisation of bacteria from mites and mite growth medium materials

Phenotypic characterisation of the isolates was briefly performed using the Bergey's Manual of Systematic Bacteriology as a guide. Haemolysis, pigment production and colony morphology were noted. Gram-staining was performed and Gram-positive rods were tested for the production of catalase. Catalase-positive isolates were streaked onto sporulation agar plates, stained for spore formation using Schaeffer and Fulton, and Moeller spore stains, and the shapes and positions of the spores within the bacteria noted. The isolates were subsequently stained with malachite green to examine the presence of a swollen sporangium and parasporal bodies. The isolates were also subjected to a range of biochemical tests including casein decomposition and liquefaction of gelatin. For Gram-positive cocci, the arrangements of the cells were noted and the isolates subjected to biochemical tests including nitrate reduction, oxidase production, and aerobic or anaerobic fermentation of glucose.

Purification of genomic DNA from the nine mite-derived isolates

The genomic DNA of the isolates was purified using a commercial kit (Masterpure™ Gram-positive DNA, Epicentre, USA). An aliquot of stationary-phase culture (1 ml) was centrifuged at 28,000 x *g* at 4°C for 10 min. The cells obtained were resuspended in 150 µl of TE buffer and 1 µl of Ready-Lyse Lysozyme was added, and the sample incubated at 37°C for 30 min. Proteinase K (50 µg) in 150 µl of Gram-Positive Lysis Solution was then added to the sample. Following incubation at 65°-70°C for 15 min, samples were cooled to room temperature before 175 µl of MPC Protein Precipitation Reagent were added. Protein removal by centrifugation at 28,000 x *g* at 4°C for 10 min was followed by the addition of 5 µg of RNase A and the sample incubated at 37°C for 30 min. The genomic DNA was precipitated with 500 µl isopropanol, washed with 500 µl of 70% (v/v) ethanol, air-dried and reconstituted in 35 µl TE buffer.

Identification of mite-derived isolates by 16S rRNA, *tuf* and *rpoB* gene sequencing

The 16S rRNA gene sequences in the DNA samples were amplified by PCR using the primer pair FD1 (5' CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG 3') and RD1 (5' CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC 3') as previously described (Weisburg *et al.*, 1991). The identity of isolates 2 to 5 was verified by *rpoB* sequencing using primer pair *rpoBF* (5' AGGTCAACTAGTTCAGTATGGAC 3') and *rpoBR* (5' AAGAACCATAACCGGCAACTT 3') as previously described (Xiao *et al.*, 2009). Amplicons were sequenced by the Lotterywest State Biomedical Facility Genomics (Royal Perth Hospital, Perth, Australia), using the protocol recommended by Applied Biosystems (Mulgrave, Melbourne) (<http://www.lsbfg.uwa.edu.au/procedures/protocols>). As these *rpoB* primers failed to generate PCR products for the Gram-positive rod Isolate 1 and coccus Isolate 6, PCR was performed using primer pair *rpoBBc1* (5' TGATGCTGATATTTTCGATTGAATC 3') and *rpoBBc2* (5' TTTTCACATTGAACTTCTTCTACTAC 3') (based on *Bacillus cereus* 03BB102 *rpoB* GI:225862154 gene sequence) and primer pair *rpoBM1* (5' TTGAAGTACCAGATGTGGGTGAC 3') and *rpoBM2* (5' ATCCACAAGACGTACCTGAG 3') (based on *Micrococcus luteus* NCTC 2665 *rpoB* GI:239916571 gene sequence), respectively, and PCR conditions of 95°C for 15 min, 94°C for 1 min, 44°C for 45 sec, 68°C for 1 min and 72°C for 10 min for thirty cycles. The identities of the Gram-positive cocci Isolates 7, 8 and 9 were determined by real-time PCR analysis of the *tuf* sequence and performed using the primer pair *tufF* (5' GGCCGTGTTGAACGTGGTCAAATCA 3') and *tufR* (5' TTACCATTTCAGTACCTTCTGGTAA 3') as previously described (Martineau *et al.*, 2000). The 320 bp amplicons obtained were sequenced by PathWest Laboratory Medicine (Perth, Western Australia) as previously described (Martineau *et al.*, 2001). In all cases, sequencing was performed using the forward and the reverse primer used to amplify the product. Amplicon sequences were compared with nucleotide sequences in GenBank using the Basic Local Alignment Search Tool (BLASTn) search algorithm (Altschul *et al.*, 1990). Phylogenetic analyses were performed using the neighbour-joining algorithm and the Kimura two-parameter model in MEGA 5.0 (Tamura *et al.*, 2011), and the phylogenetic distances were calculated with 1,000 bootstrap replications as described previously (Porwal *et al.*, 2009). The sequences were deposited with Genbank.

Preparation of mite materials, house dusts and bacterial isolates for bacteriolytic enzyme determination

A previously prepared whole mite extract (10% w/v) was solubilised with 0.01 M phosphate buffer, pH 6.2 containing 50 mM EDTA, 2 mM DDT and 50 units ml⁻¹ aprotinin (Bayer Australia, Sydney,

Australia) for 1 h at 4°C with gentle rocking. Carpet and mattress dust samples from six homes (forming part of a collection obtained from a previously described allergen avoidance study performed in Melbourne (Hill *et al.*, 1997)) were extracted at 25% (w/v) as described previously (Mathaba *et al.*, 2002) as were the spent growth medium and unused growth medium. Extracted proteins were recovered by centrifugation at 28,000 x *g* for 5 min at 4°C and filtered (0.2 µm; Pall Gelman Laboratory, Ann Arbor, USA). Bacteria isolated from whole mites and house dusts were grown in tryptone soy broth at 37°C to an optical density (OD)_{600nm} of 1.0. Bacterial cells were removed by centrifugation at 28,000 x *g* for 15 min at 4°C the culture media containing secreted proteins (SP) were filtered and stored at –20°C until required for zymographic analysis.

Zymographic analyses

Bacteriolytic activity was demonstrated using substrate SDS-PAGE (zymography) as described previously (Mathaba *et al.*, 2002). Extracts were heated at 100°C for 5 min in SDS sample buffer devoid of DTT and electrophoresed on 13% (w/v) homogeneous polyacrylamide gels containing 2% (w/v) autoclaved *M. lysodeikticus* at 20 mA for 3.5 h at room temperature (RT). Following electrophoresis, gels were rinsed in deionised water for 30 min followed by 0.1 M phosphate buffer, pH 6.2 containing 3.2 mM DTT and 1% (v/v) Triton X-100 for 18 h at 37°C, with gentle agitation. Gels were then rinsed with deionised water, stained with 0.01% (w/v) methylene blue in 0.01% (w/v) KOH for 5 min, destained in deionised water and then photographed as described previously (Rashid *et al.*, 1995). The apparent molecular weights of the lytic bands were determined by comparison to prestained standards containing phosphorylase b (97K), serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), lysozyme (14.4K) and aprotinin (6.5K) (Bio-Rad, Sydney Australia).

Statistical Analyses

CFU obtained from the various house dust samples were converted to logs and statistical differences in the geometric means were determined initially by one way analysis of variance using Excel for Mac with the Statplus software addin and then Student's T test, where differences were detected and Bonferroni's correction as appropriate.

Results

Bacterial content of house dust samples

Bacteria were readily detected in all house dust samples, with all buffers used in the extraction process within 18 hr incubation. Several trial runs were performed to obtain countable cfu, and a dilution of 1 in 10,000 was found to be optimal. The number of cfu and types of colony morphologies obtained from dust samples from different homes appeared similar. When the cysteine extracted cfu values were examined, a significant difference in geometric mean cfu's was obtained for all dust samples cultured under microaerophilic conditions (mean, 229; $p=0.00002$) compared with aerobic (mean, 58) but not anaerobic conditions (Table 1). When the three extraction conditions were compared for each of the different atmospheric conditions, the mean geometric EDTA/cysteine extract cfu value (mean, 85) was significantly lower than that obtained using cysteine (mean, 232, $p=0.00457$) but not EDTA after Bonferroni's correction (mean 256, $p=0.018$) under microaerophilic conditions. Dusts from the parents' rooms and their children's rooms yielded similar numbers of cfu but for both parents and their children, significantly lower total numbers of cfu were obtained from carpet dusts compared with mattress dusts (Table 1, $p=0.000261$, <0.00001 , respectively). In general, the numbers of cfu obtained from the lounge carpet were intermediate between those obtained from children's and parents' bedroom carpets (Table 1).

Bacterial content of dust mites

Bacterial and fungal colonies grew on BA plates inoculated with surface-sterilised whole mite extracts, but not on plates inoculated with UGM or SGM, or the reagents used for surface sterilisation of the mites (ethanol or HCl, data not shown). Extractions were performed on five occasions over a period of 6 months using the buffers described above and bacterial colonies isolated and purified by subculturing on appropriate media as described. *Staphylococcus*, *Micrococcus* and *Bacillus* species were routinely identified. A total of 50 isolates were eventually obtained from a number of extractions, but 9 morphologically distinct Gram-positive rods and cocci were selected for detailed characterisation given their ease and frequency of isolation. Isolates 1 to 5 were Gram-positive spore-forming rods that tested negative for parasporal bodies but Isolate 1 demonstrated a swollen sporangium. All five of the rod isolates hydrolysed casein and gelatine, and grew under aerobic conditions. Isolates 6 to 9 were found to be Gram-positive cocci. Isolate 6 and Isolate 7 produced pale orange and buttercup pigmented colonies, respectively, whereas Isolates 8 and 9 did not. Isolates 6 and 8 tested positive for oxidase

production and fermented glucose under aerobic conditions. Isolate 9 was positive for nitrate reduction, and fermented glucose under anaerobic conditions. Colonies with morphological characteristics similar to the above were also found in carpet and mattress dusts from parents and children (data not shown).

Molecular identification of the mite-derived Gram-positive isolates

16S rRNA gene sequencing was performed to identify the mite-derived isolates, and Figures 1a-c show phylogenetic trees for the Gram-positive rods and cocci obtained. The sequence homologies for each of the isolates were below the accepted cut-off value for species identification, namely, 98% (Table 2) (Woo *et al.*, 2009). However, the percent homologies of five of the isolates were consistent with the *Bacillus* genus, homologies of three of the four coccal species (Isolates 7 to 9) were consistent with the *Staphylococcus* genus, and one of the cocci (Isolate 6) was found to be closely related to *Micrococcus luteus*. Of the *Bacillus* species, Isolate 1 was provisionally identified as *B. cereus*, given its homology with the closest-matched species whereas the remaining four were provisionally identified as *B. licheniformis*, although with low homology (<85%) (Fig. 1a and Table 2). Further studies were performed using *rpoB* and *tuf* gene sequence homology analyses (Table 2). Isolate 1 was confirmed as *B. cereus*, and Isolates 2 to 5 were identified as *B. licheniformis* (Fig. 1b and Table 2). The finding of a relatively low homology of Isolates 2 to 5 with an ATCC strain of *B. licheniformis* was confirmed by more detailed phylogenetic analyses, where it was shown that the four *B. licheniformis* 16S rRNA sequences formed a cluster (Fig. 1c) distinct from the 13 clusters of *B. licheniformis* strains published previously (Porwal *et al.*, 2009). Subsequent analyses of isolates using *tuf* gene sequence homology showed that Isolates 7, 8 and 9 were *Staphylococcus aureus*, *S. epidermidis* and *S. capitis*, respectively (Table 2 and Supplementary Material 1). Although alternative probes for *M. luteus* identification have not been previously reported, analysis of Isolate 6 using the *rpoB* gene showed a 99% match with *M. luteus* NCTC 2665 (Table 2 and Supplementary Material 2).

Zymographic analyses

Extracts of house dust mites, mite material, house dusts and bacteria isolated from whole mite and house dusts revealed the presence of bacteriolytic enzymes (Fig. 2a-d). Whole mite and spent growth medium extracts produced a strong lytic band with an apparent mol wt of about 14K (Fig. 2a-d), slightly larger than the band seen with hen's egg white lysozyme, and a weak band below. Culture supernatants containing secreted proteins from *B. cereus* (Isolate 1) produced a faint lytic

band at about 66kDa, while *B. licheniformis* 1-4 (Isolates 2 - 5) produced a pair of prominent lytic bands at approximately 30-40kDa (Fig. 2a). The supernatants from *B. licheniformis* 2 also showed weak lytic bands at 14-22K and a very weak lytic band was observed at about 14K in supernatants from *B. licheniformis* 1 and *B. licheniformis* 3 (Fig. 2a). The Gram-positive cocci showed a different spectrum of lytic bands compared with that seen with the Gram-positive rods. As shown in Fig. 2b, supernatants from *S. aureus* (Isolate 7) contained very prominent lytic bands ranging from >21.5 to >97.4K, while these lytic bands were much weaker in supernatants from *M. luteus* (Isolate 6) and *S. epidermidis* (Isolate 8). A prominent lytic band was observed at about 45kDa in the supernatant from *S. capitis* (Isolate 9) (Fig 2b), as shown in the same figure. Zymographic analyses of 12 extracts prepared from carpet and mattress dusts are shown in Fig. 2c and 2d. Lytic bands were observed in 9 of the 20 carpet and mattress dust samples from 3 of the 5 homes examined, with varying intensities. A band corresponding in position to a prominent mite 14kDa band were detected in 9 samples, and a 22K band, together higher molecular weight bands, were detected in samples other than the child's mattress dust from one home (Fig. 2d). However, lytic band profiles comparable to those observed with the Gram-positive rods and cocci were not detected.

Discussion

Consistent with data from other studies, carpet and mattress dusts were shown to contain significant numbers of bacteria. The cfu's obtained from Melbourne mattress dusts from parents', but particularly children's beds, were significantly higher than those obtained from the corresponding bedroom carpets. It is likely these differences reflect intervention by the childrens' parents, and that carpets tend to be cleaned more frequently than mattresses. The extraction buffer additives and atmospheric conditions used in the cfu study did not appear to markedly influence the mean counts obtained, with the exception of a reduced geometric mean cfu when extraction was performed with EDTA/Cysteine, and dusts cultured under microaerophilic conditions compared with the EDTA and Cysteine buffer, and the elevated cfu's obtained with the Cysteine buffer under microaerophilic conditions compared with this buffer under aerobic conditions. However, it is not clear at present whether the differential conditions used influenced the growth of particular bacterial species, but this is likely. Although dust-derived colonies were not specifically identified to species, based on previous culture-based studies, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Mycobacterium* spp. and *Acinetobacter* spp. would be well represented (Dawson 1971; Horak 1987; Horak *et al.*, 1996; Tsukamura *et al.*, 1985).

Zymography studies showed that both mattress and carpet dusts from 3 of the 5 homes sampled contained bacteriolytic enzymes, and of the 9 positive samples from these homes, all contained a prominent lytic band in the 14kDa region with a much less intense band below. As both of these bands were present in mite extracts, it is possible that they originate from mites, but whether either corresponded to the mite-derived lytic enzyme belonging to the NlpC/P60 superfamily, mite-derived lysozyme (Erbert & Hubert, 2009) or other peptidoglycan degrading enzymes is unclear. In addition to this prominent band, both carpet and mattress dusts from one of the three homes demonstrated a prominent band at 21.5 kDa, together with lytic bands above this, but these were not seen in any of the mite extracts examined and, thus, their origin is unknown.

Nine of the Gram-positive isolates obtained from *D. pteronyssinus* mites were studied in detail because of their prominence and morphological similarities with those obtained from house dust. A combination of 16S rRNA, *rpoB* and *tuf* sequencing was required to identify them to the species level, a finding consistent with data from studies showing that 16S rRNA gene sequencing may not be reliable for some species (Fox *et al.*, 1992; Dahllof *et al.*, 2000; Blackwood *et al.*, 2004; Ghebremedhin *et al.*, 2008; Woo *et al.*, 2009). Four of the five mite-derived rod isolates were *B.*

licheniformis and one was *B. cereus*. Of the three coccal isolates, three were staphylococcal species and one was identified as *M. luteus* after confirmatory analysis targeting the *rpoB* gene. Most of the 9 species identified are likely derived from human skin scales as they are known human commensals (Evans *et al.*, 1978; Glass 1973; Stackebrandt *et al.*, 1995), and skin scales are used as a food source (Colloff, 2009). Although this material does not, to our knowledge, form part of the proprietary medium used to propagate *D. pteronyssinus*, the establishment of the commercial source may have necessitated some form of dander. Such species are routinely found in house dusts, and 45 to 88% of species isolated from mattress and floor dusts are of human origin (Lee *et al.*, 2007; Pakarinen *et al.*, 2008; Rintala *et al.*, 2008; Taubel *et al.*, 2009).

Each of these species secreted bacteriolytic enzymes into the growth medium, although the profiles varied for the rods and cocci. Coccal species, particularly *S. aureus* were dominated by bands between 30-100 kDa, whereas rods were dominated by 2 bands in the 30-40 kDa region, and a number of weak low molecular weight bands. However, none of the bacterial species isolated appeared to produce a prominent 14 kDa band equivalent to that seen in mite extracts, suggesting that these species were not its source. The specific identities of the lytic bands produced by these bacterial isolates is unknown but an extensive body of data demonstrate that bacteria are capable of secreting glucosidases, peptidases and amidases that may cleave peptidoglycan, resulting in lysis.

The identification of the mite-associated species described above is, in the main, consistent with data from both culture-dependent and -independent studies. For example, in both *D. farinae* and *D. pteronyssinus* from diverse locations, *Bacillus* and *Staphylococcus spp.* predominate, with culturable Gram-positive spp such as *Kokuria* and Gram-negative spp. such as *Rhizobium* and *Enterobacteriaceae* being less commonly reported (Oh *et al.*, 1986; Hubert *et al.*, 2012a). Where mite-derived 16S rRNA clones have been examined, the diversity of mite-associated bacterial species increases markedly, particularly with regard to Gram-negative species. Here, bacteria such as *Bartonella*, *E. coli*, *Pseudomonas*, and *Acinetobacter* (Valerio *et al.*, 2005; Hubert *et al.*, 2012a) have been reported, although Gram-positive coccal spp. clones have also been detected. In addition to the identification of these species, clones originating from the arthropod symbiont, *Cardinium*, have also been detected in mites including *D. pteronyssinus* (Kopecky *et al.*, 2013). Such results are also consistent with previous reports showing that mite extracts contain bacterial

components such as lipopolysaccharide (Trevidi *et al.* 2003) and it is possible they also contain peptidoglycan as well as other bacterial components.

With regard to the *Bacillus* species isolated in this and other studies, *B. cereus* appears to be a consistent feature, as is *B. subtilis*. However, mite-associated *B. licheniformis* isolates have not been routinely reported, although it is the predominant *Bacillus* spp. in dusts from animal sheds and mattresses of farm children (Vogel *et al.*, 2008). *B. licheniformis* isolates are known to be highly variable within their 16S rRNA gene sequences, so much so, that it has been possible to identify 10 distinct clusters based on 131 isolates, with sequence homologies ranging from 80 to 98% (Porwal *et al.*, 2009). When the *B. licheniformis* isolates described here were compared with a representative sample from the Porwal *et al.* (2009) data set, the 4 mite-associated isolates formed a distinct phylogenetic cluster. This clustering of bacterial spp. to specific mite species or related mite groupings based on 16S rRNA sequences has also been reported for *Cardinium*, *Bartonella* and *Sphingobacteriales* spp. (Hubert *et al.*, 2012a; Kopecky *et al.*, 2013) indicating some selective advantage for the mite such as nutrient utilisation.

In this regard, laboratory studies, where bacterial communities associated with *Tyrophagus putrescentiae* were studied, demonstrate that the transfer of this mite species from a non-fungal to a fungal (*Fusarium* spp.) based diet results in a shift in the bacterial community profile towards a domination by *Bacillaceae* spp., in particular, *B. galactosidilyticus* (Hubert *et al.*, 2012b). These authors hypothesised that the introduction of *Bacillus* spp. into a fungal environment facilitates the degradation and utilisation of mycelium as a food source due to the release of chitinases, since these enzymes are a feature of a number of *Bacillus* spp. including *B. cereus* and *B. licheniformis*. These findings suggest that mites other than *T. putrescentiae* might also rely on such species to aid in the digestion of environmental fungal species. Whether the *B. licheniformis* cluster isolated from mites secrete chitinase is as yet unknown but, in support, commercial house dust mite extracts are known to contain chitinases (Post *et al.*, 2012).

In conclusion, we have demonstrated the presence of several species of Gram-positive bacteria within the house dust mite, all of which are common on human skin as well as in house dusts. Mite extracts were dominated by the presence of an unidentified 14 kDa bacteriolytic enzyme which was also present in mattress and carpet dusts, suggesting a mite origin. None of the secreted bacteriolytic enzymes from the mite-associated bacterial isolates corresponded to the

mite or dust lytic enzymes, and the origin of the previously described P60-related 14 kDa mite enzyme remains to be determined. Of the 9 bacterial isolates from mites, 4 were *B. licheniformis* which, on the basis of 16S rRNA gene sequencing, formed a distinct phylogenetic cluster suggesting some selective advantage for *D. pteronyssinus*, perhaps in dealing with fungal food sources.

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Figure legends

Fig. 1 Phylogenetic trees constructed based on **(a)** 16S rRNA gene sequences showing the relationship between house dust mite-derived Isolates 1 to 9 and different but phylogenetically related species of *Bacillus*, *Micrococcus* and *Staphylococcus* with *Lactobacillus delbrueckii* as the outgroup; **(b)** rpoB gene sequences showing the relationship between house dust mite-derived Isolates 1 to 5 and different but phylogenetically related species of *Bacillus*, with *Lactobacillus acidophilus* as the outgroup and **(c)** 16S rRNA gene sequences showing the relationship between house dust mite-derived Isolates 2 to 5 and 45 isolates representing the phylogenetic grouping of *B. licheniformis* delineated by Porwal *et al.* (2009). Phylogenetic analyses were performed using the neighbour-joining algorithm with the Kimura two-parameter model in MEGA 5.0. The numbers at the nodes are percent values obtained with 1,000 bootstrap replications.

Fig. 2 Zymographic analysis of bacteriolytic enzymes in whole mite extract (WME), spent growth medium extract (SGME), secreted proteins (SP) from *B. cereus* (Bc), *B. licheniformis* (Bl) 1-4, *M. luteus* (Ml), *S. aureus* (Sa), *S. epidermidis* (Se) and *S. capitis* (Sc), extracts prepared from house dusts including children's mattress dust extracts (CMDE) 1, 4 and 5, parents' mattress dust extracts (PMDE) 1, 4 and 5, children's carpet dust extracts (CCDE) 1, 4 and 5, parents' carpet dust extracts (PCDE) 1, 4 and 5. Buffer (0.01 M phosphate buffer, pH 6.2 containing 50 mM EDTA, 2 mM DDT and 50 units ml⁻¹ aprotinin) and unused growth medium extract (UGME) were included as negative controls. Hen egg white lysozyme (HEWL) was used as a relative size marker for the 14 kDa lytic enzyme

Table 1 **Bacterial content of extracts prepared from carpet and mattress dusts from Melbourne homes**

Site sampled	Extraction conditions and geometric mean number of cfu (x10 ⁵)									⁶ P=
	<i>Aerobic</i>			<i>Microaerophilic</i>			<i>Anaerobic</i>			
	¹ EDTA	² Cysteine	³ EDTA/ Cysteine	EDTA	⁴ Cysteine	⁵ EDTA/ Cysteine	EDTA	Cysteine	EDTA/ Cysteine	
Child's carpet	22	13	9	151	168	12	16	63	15	<0.00001
Child's mattress	395	205	136	644	524	266	147	432	249	
Parents' carpet	208	45	88	282	122	126	204	177	54	0.000261
Parents' mattress	361	295	235	521	650	244	630	269	275	
Lounge carpet	52	19	74	77	99	46	161	51	39	

¹One way analysis of variance, all EDTA extraction cfu's obtained under aerobic, anaerobic and microaerophilic conditions (df=89); p=0.1075

²One way analysis of variance, all Cysteine extraction cfu's obtained under aerobic, anaerobic and microaerophilic conditions (df=89); p=0.04925

³One way analysis of variance, all EDTA/Cysteine extractions obtained under aerobic, anaerobic and microaerophilic conditions (df=89); p=0.7321

⁴Geometric mean cysteine extraction cfu under microaerophilic conditions significantly greater than mean cfu of EDTA extractions cultured under aerobic conditions, p=0.00002

⁵One way analysis of variance, EDTA, cysteine and EDTA/Cysteine extraction cfu's examined under microaerophilic conditions (df=89); p=0.0387; Geometric mean EDTA/cysteine extract cfu significantly lower than cysteine extract cfu's, p=0.00457

⁶Comparing significance of geometric mean of all extraction cfu's for childrens' carpet dust versus mattress dust, and parents' carpet versus mattress dust, df=106

Table 2 Identification of the nine mite-derived Gram-positive isolates by 16S rRNA, *tuf* and *rpoB* sequence analyses

Mite-derived bacterial isolate No	Identity based on 16S <i>rRNA</i> sequencing		Identity based on <i>tuf</i> gene sequencing		Identity based on <i>rpoB</i> gene sequencing		Final designation of isolate
	Species identity ^a	% Similarity	Species identity	% Similarity	Species identity	% Similarity	
1	<i>B. cereus</i> (KC814644) ^b	97	NA ^c		<i>B. cereus</i> (KC814653)	99	<i>B. cereus</i>
2	<i>B. licheniformis</i> (KC814648)	81	NA		<i>B. licheniformis</i> (KC814657)	98	<i>B. licheniformis</i> 4
3	<i>B. licheniformis</i> (KC814646)	81	NA		<i>B. licheniformis</i> (KC814655)	98	<i>B. licheniformis</i> 2
4	<i>B. licheniformis</i> (KC814647)	80	NA		<i>B. licheniformis</i> (KC814656)	98	<i>B. licheniformis</i> 3
5	<i>B. licheniformis</i> (KC814645)	81	NA		<i>B. licheniformis</i> (KC814654)	99	<i>B. licheniformis</i> 1
6	<i>M. luteus</i> (KC814649)	96	NA		<i>M. luteus</i> (KC814658)	99	<i>M. luteus</i>
7	<i>S. aureus</i> (KC814650)	96	<i>S. aureus</i> (KC814659)	99	NA		<i>S. aureus</i>
8	<i>S. epidermidis</i> (KC814651)	97	<i>S. epidermidis</i> (KC814660)	99	NA		<i>S. epidermidis</i>
9	<i>Staphylococcus</i> sp. (KC814652)	97	<i>S. capitis</i> (KC814661)	99	NA		<i>S. capitis</i>

^aSpecies with the highest % similarity are listed; ^bGenBank accession numbers for 16S *rRNA*, *tuf* and *rpoB* gene sequences are shown in parentheses;

^cNot applicable. *B.* = *Bacillus*; *M.* = *Micrococcus*; *S.* = *Staphylococcus*

a)

b)

(c)

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Supplementary material 1.

Neighbour-joining tree constructed based on *tuf* sequences showing the relation between house dust mite-derived Isolates 7, 8 and 9 and other different but phylogenetically related species of *Staphylococcus*. Phylogenetic analyses were performed using the neighbour-joining algorithm with the Kimura two-parameter model in MEGA 5.0. The numbers at the nodes are bootstrap values greater than 50% with 1,000 bootstrap replications. *Streptococcus pyogenes* was used as the outgroup

Supplementary material 2.

Neighbour-joining tree constructed based on *rpoB* sequences showing the relation between house dust mite-derived Isolate 6 and other different but phylogenetically related species that previously belonged to the *Micrococcus* genus. Phylogenetic analyses were performed using the neighbour-joining algorithm with the Kimura two-parameter model in MEGA 5.0. The numbers at the nodes are bootstrap values greater than 50% with 1,000 bootstrap replications. *Staphylococcus aureus* was used as the outgroup