Abstract

**Background:** Child-care facilities appear to provide daily opportunities for exposure and transmission of bacteria and viruses. However, almost nothing is known about the diversity of microbial contamination in daycare facilities or its public health implications. Recent culture-independent molecular studies of bacterial diversity in indoor environments have revealed an astonishing diversity of microorganisms, including opportunistic pathogens and many uncultured bacteria. In this study, we used culture and culture-independent methods to determine the viability and diversity of bacteria in a child-care center over a six-month period.

**Results:** We sampled surface contamination on toys and furniture using sterile cotton swabs in four daycare classrooms. Bacteria were isolated on nutrient and blood agar plates, and 16S rRNA gene sequences were obtained from unique (one of a kind) colony morphologies for species identification. We also extracted DNA directly from nine representative swab samples taken over the course of the study from both toy and furniture surfaces, and used "universal" 16S rRNA gene bacterial primers to create PCR-based clone libraries. The rRNA gene clones were sequenced, and the sequences were compared with related sequences in GenBank and subjected to phylogenetic analyses to determine their evolutionary relationships. Culturing methods identified viable bacteria on all toys and furniture surfaces sampled in the study. *Bacillus* spp. were the most commonly cultured bacteria, followed by *Staphylococcus* spp., and *Microbacterium* spp. Culture-independent methods based on 16S rRNA gene sequencing, on the other hand, revealed an entirely new dimension of microbial diversity, including an estimated 190 bacterial species from 15 bacterial divisions. Sequence comparisons and phylogenetic analyses determined that the clone libraries were dominated by a diverse set of sequences related to *Pseudomonas* spp., as well as uncultured bacteria originally identified on human vaginal epithelium. Other sequences were related to uncultured bacteria from wastewater sludge, and many human-associated bacteria including a number of pathogens and opportunistic pathogens. Our results suggest that the child-care facility provided an excellent habitat for slime-producing Pseudomonads, and that diaper changing contributed significantly to the bacterial contamination.

**Conclusion:** The combination of culture and culture-independent methods provided powerful means for determining both viability and diversity of bacteria in child-care facilities. Our results provided insight into the source of contamination and suggested ways in which sanitation might be improved. Although our study identified a remarkable array of microbial diversity present in a single daycare, it also revealed just how little we comprehend the true extent of microbial diversity in daycare centers or other indoor environments.
Background

Child-care facilities appear to provide a setting with many opportunities for exposure and transmission of bacteria and viruses [1-4]. Preschool aged children are often sick with illnesses of unknown origins, and young children have not yet mastered the sanitary cleaning habits present among most adults in our society. Moreover, children have had less exposure to microorganisms, making them more likely to catch and transmit pathogens or opportunistic pathogens, and perhaps more likely to suffer the effects from contact in densely populated facilities. Recent studies of microbial diversity in indoor environments using molecular methods have revealed considerable bacterial contamination and underscored how little we know about such contamination [5-7]. Understanding the potential public health risks in daycare centers requires a better understanding of microbial diversity in these settings. This is particularly important given the increasing reliance of working parents on daycare facilities for childcare [4].

Culture-based studies of human indoor environments have shown that significant levels of bacteria are present in seemingly innocuous areas such as office buildings, residential homes, and children’s schools and daycare centers [8-10]. According to these surveys, low DNA G+C content, Gram-positive bacteria, such as Bacillus cereus, Bacillus licheniformis, Brevibacillus brevis and Staphylococcus spp. along with a few Gram negative species including Chryseomonas spp. and Pantoea spp. tend to predominate [8,11,12]. Indoor culturing studies have also identified the presence of bacteria from the order Actinomycetes, including Rhodococcus fascians, Arthrobacter pascens, and Corynebacterium spp. [8,11].

Recently, culture-independent molecular studies have greatly expanded our understanding of the bacterial diversity that can be present in indoor environments. The molecular methods we performed in this study included PCR amplification of 16S rRNA genes conducted on DNA extracted directly from our environmental samples. Culture-independent methods have been able to offer a much more complete view of the bacteria present in ordinary everyday surroundings such as indoor pools, shower curtains, and airplane bathrooms; these same methods should prove equally effective for use in daycare settings [5-7]. In some cases, culture-independent methods have identified the source of illness when the microbes were unknown or not currently culturable [5,13].

In an environment so potentially rich in microbial diversity, culturing methods readily identify bacteria with known growth requirements and these methods are necessary to prove the viability of microorganisms in the environment. However, previous work has shown that <1% of bacterial species in a given environment are culturable suggesting that a vast majority of the true diversity may be missed if studies rely solely on culturing [14,15]. Indeed, the development of culture-independent methods based on the 16S rRNA gene used in conjunction with phylogenetic analysis has revealed an abundant array of previously unknown and uncultured microbes, including many new bacterial divisions [13-18]. The 16S rRNA gene is particularly useful for molecular analysis and identification of organisms due to its high level of information content, conserved nature, and its presence in all cellular microorganisms [15]. Researchers have also begun to use culture-independent methods to study human biology [19-22], complex diseases [13,23], and human environments [5-7]. Collectively these studies have exposed a remarkable array of microorganisms, many of them with no cultured representatives. In the case of human environments, many potentially opportunistic pathogens have been identified [5-7].

In this study, we surveyed the bacterial diversity present in a daycare facility using both culture and culture-independent methods to analyze samples taken from various toys and surfaces (e.g., counter-tops). This allowed us to gauge the overall complexity of bacterial diversity, determine viability of bacteria, and see how the diversity and abundance changed over time. A total of four rooms were sampled over a six-month period. Sampling was alternated between two toddler rooms and two infant rooms every one to two weeks. Of these samples, DNA was successfully extracted directly from nine swabs, and the samples were subjected to both culture and culture-independent analysis. The facility tested in this case had specific disinfection protocols in place for daily cleaning of the rooms and washing of the toys that the children have played with or come into contact with during the course of the day. Cleaning protocols (e.g., cleaning surfaces with 10% bleach) were followed diligently by the staff in this daycare facility, which placed a high premium on cleanliness.

Results

Table 1 details the total number of cultured isolates obtained over the course of the study based on the 16S rRNA gene sequence analysis and visible colony morphology. The lysozyme extraction protocol effectively isolated bacterial DNA from all the colonies picked from plates. Typical DNA yields for bacterial colonies were in the range of 0.6 to 82.4 ng μl⁻¹. Table 2 shows the results of culturing on the environmental swab samples taken between September 2005 – April 2006. For all days sampled except October 6, 2005, colonies grew on either blood or nutrient agar plates. This means that there were large numbers of viable bacteria consistently present on the surfaces and toys sampled at the daycare center. We isolated as many as
29 putative bacterial species. We considered it a potentially different species of bacteria if it had a unique 16S rRNA gene sequence or a clearly distinct morphology or both (Table 2). Bacillus species were the most commonly culture-isolated bacteria, followed by Staphylococcus spp. Culture methods identified at least 29 viable bacterial species on toy and furniture surfaces over the 6 months of the study (Table 1). Species of Bacillus were isolated every day of sampling (Table 2), and we identified as many as 15 different distinct morphologies over the course of the study (Table 1).

The lysozyme DNA extraction protocol proved effective for direct swab extractions and yielded DNA in the range of 5.1 to 14.7 ng µl⁻¹. Bead-beating methods are typically preferred for isolating environmental DNA because the mechanical shearing of cells by the beads helps extract DNA from particularly “tough” bacterial cells such as bacterial vegetative cells (e.g., Pseudomonas putida), bacterial endospores (e.g., Bacillus spp.), and fungal conidia (e.g., Fusarium moniliforme) [24]. However, our attempts with bead-beating methods failed to isolate sufficient DNA from the swabs (data not shown), whereas the lysozyme method yielded sufficient amounts of DNA for PCR.

Figure 1 and 2 show the results of phylogenetic analysis of selected sequences from the 453 clones obtained from the nine libraries relative to sequences from cultured and uncultured bacteria from other studies. Based on our survey of nine 16S rRNA gene PCR-clone libraries we identified as many as 190 bacterial species (1% divergence; 141 at the more conservative 3% divergence level), most of them with no cultured representatives. The clone library sequence coverage ranged from 48% to 65% (average 54%) for the nine clone libraries. Most sequences found in both groups appeared to be uncultured bacterial species. Members of the Pseudomonadaceae and the Oxalobacteraceae predominated in the clone libraries (Fig. 3). Pseudomonads were particularly abundant and were on every surface sampled on every sampling occasion (Fig. 3).

Since the main purpose of this study was to identify the types of organisms in the daycare center, one-directional sequencing of the first part of the small-subunit rRNA gene, which includes the most variable part of the gene, was enough for our purposes. Sequencing bi-directionally would have been ideal in terms of reducing error, but would have doubled the sequencing costs and added little to our understanding of the diversity. Also, we trimmed the sequences to around ~500 bp in length, and edited our most problematic part of the reads. Any errors that remained would have had little impact on the phylogenetic analysis. However, we have made glycerol stocks of all the clone libraries we created, which are available from the authors upon request.

The alignment of the edited and trimmed sequences proved straightforward, and the alignment was checked by confirming complementary base-pairing in known stem regions of the alignment. A total of 78 sequences were deposited in GenBank, including sequences from both culture isolates (Table 1) and representative clone library sequences used in the phylogenetic analysis (Fig. 1, 2). Approximately 500 nucleotide positions, corresponding to E. coli positions 25 to 534, were used for all the phylogenetic analyses. There was strong support for the majority of the phylogenetic relationships as judged by both Bayesian posterior probabilities (Fig. 1, 2) and MP bootstrap support (not shown). Bayesian, MP and ML methods produced highly similar tree topologies. The differences in tree topologies produced by the various methods were in regions of the trees not supported by either posterior probabilities (< 0.5) or MP bootstrap values (<50%). The phylogenetic analysis allowed us to easily identify the position of our cloned environmental sequences within known bacterial divisions (Fig. 1, 2). Using this information, and the Fastgroup II dereplication information, we were able to assess the relative abundance of various sequences in clone libraries and these are presented in Figure 3.

Discussion
Our combination of culturing and culture-independent techniques revealed a remarkable diversity of bacteria contaminating every surface sampled in the daycare facility. Bacillus spp. were particularly common (Table 1 and 2). Bacillus endospores disperse rapidly through the air and are ubiquitous in soils and other environments [25,26] so their abundant presence here was not alarming. Staphylococcus species were also repeatedly isolated, as were species of Pseudomonas and Microbacterium (Table 1 and 2). A number of the isolates were potential pathogens and opportunistic pathogens, including Enterococcus faecalis [27], Moraxella osloensis [28], and Staphylococcus haemolyticus [29]. E. faecalis has become a particular problem in hospitals [27]. Species belonging to these genera of bacteria are commonly found on skin, nostrils, or even as part of the normal gut microbiota [30]. Normal shedding of these surfaces, along with the attached bacteria, may explain their abundance in indoor human environments [9,30].

Our culturing results appeared highly similar to culture-based studies of other indoor environments [8,9]. Specifically we found ~90% of the same bacterial genera as another culture-based study of a daycare setting [8]. Most of these studies sampled airborne bacteria in environments, such as daycare centers, schools, and office build-
ings [8,9,11,12]. Our results suggest diversity found in air-sampling studies is very similar to that of surface sampling methods and may be a reasonable substitute for costly air-sampling methods at least in terms of determining microbial diversity.

Although the culture-based methods discovered a number of bacterial species and confirmed the viability of bacteria on surfaces in the daycare facility, these methods identified only a small fraction of the true bacterial diversity (~3%). The culture-independent portion of our analysis revealed a whole new dimension of largely unexplored microbial diversity present in a daycare center environment. Similar to other culture-independent studies in human environments [5-7], we uncovered an extraordinary diversity of bacteria from 16 bacterial divisions or sub-divisions that included many bacterial species without cultured representatives (Fig. 1, 2, 3).

The largest proportion of sequences found in the clone libraries came from two groups: the Pseudomonadaceae and the Oxalobacteraceae (Fig. 3). Pseudomonads comprise an extremely diverse array of bacteria that grow on numerous carbon sources and are often associated with spoilage [31]. Many of them produce biofilm “slime layers” that serve as environmental protection and make them resistant to both antibiotics [32] and cleaning regimens [33]. Moreover, this same slime production ability appears to protect them from the mammalian immune system [32]. A number of Pseudomonads, such as P. stutzeri, are known opportunistic pathogens [34] and have also been implicated in hospital acquired infections [35,36]. P. aeruginosa is also known to be resistant to antibiotics [37].

The predominance of a diverse array of Pseudomonads in the daycare appears to be quite consistent with the nature of the environment. The constant spillage of food and liquids, spread over every surface reachable by children, would make a perfect growth medium for Pseudomonads [31]. This particular daycare center had very rigorous cleaning policies. However, the natural resistance of Pseu-

Table 1: Unique morphologies present in culture results, and their identification through sequencing

| Organism          | Sequence Length | % Identity | GenBank Accession | Morphology          |
|-------------------|-----------------|------------|-------------------|---------------------|
| Acinetobacter sp. | 741             | 98         | EF409307          | Acinet. 1           |
| Bacillus licheniformis | 720        | 99         | EF409308          | Bacillus M1         |
| Bacillus megaterium | 739        | 99         | EF409309          | Bacillus M2         |
| Bacillus sp.      | 498             | 97         | EF409310          | Bacillus M3         |
| Bacillus sp.      | 754             | 100        | EF409311          | Bacillus M4         |
| Bacillus sp.      | 724             | 99         | EF409312          | Bacillus M5 (Nutrient) |
| Bacillus sp.      | 748             | 99         | EF409313          | Bacillus M6         |
| Bacillus sp.      | 730             | 100        | EF409314          | Bacillus M7         |
| Bacillus sp.      | 664             | 99         | EF409315          | Bacillus M8         |
| Bacillus sp.      | 741             | 89         | EF409316          | Bacillus M9         |
| Bacillus sp.      | 677             | 98         | EF409317          | Bacillus M10        |
| Bacillus sp.      | 721             | 99         | EF409318          | Bacillus M11        |
| Bacillus subtilis | 759             | 99         | EF409319          | Bacillus M12        |
| Bacillus subtilis | 759             | 100        | EF409320          | Bacillus M5a        |
| Bacillus subtilis | 736             | 99         | EF409321          | Bacillus M13        |
| Bacillus subtilis | 722             | 99         | EF409322          | Bacillus M14        |
| Bacillus subtilis | 718             | 99         | EF409323          | Bacillus M15        |
| Brevibacillus sp. | 711             | 99         | EF409324          | Brevi. M1a          |
| Brevibacterium sp. | 695         | 99         | EF409325          | Brevi. M1b          |
| Enterococcus faecalis | 764        | 99         | EF409326          | Enterococ. M1       |
| Exiguobacterium sp. | 767         | 99         | EF409327          | Exiguobac. M1       |
| Microbacterium aurum | 289        | 99         | EF409328          | Microbac. M1a       |
| Microbacterium esteraromaticum | 327 | 98 | EF409329 | Microbac. M1b |
| Moraxella osloensis | 750       | 98         | EF409330          | Morax. M1           |
| Pseudomonas stutzeri | 682       | 99         | EF409331          | Pseudom. M1         |
| Staphylococcus aureus | 752       | 99         | EF409332          | Staph. M1           |
| Staphylococcus aureus | 727       | 100        | EF409333          | Staph. M2           |
| Staphylococcus epidermidis | 721 | 100        | EF409334          | Staph. M3           |
| Staphylococcus haemolyticus | 703 | 99 | EF409335 | Staph. M4 |

1 Length of sequence used in BLAST search
2 Based on BLAST alignment
3 Abbreviations for each morphology type; cross-referenced in Table 2
domonads to cleaning may actually have served to increase their abundance relative to other bacteria. Given the abundance of Pseudomonads in our clone libraries, it is somewhat surprising that we did not find more species growing on nutrient agar plates (Table 2). Another published culture-based study of a daycare facility showed a lack of diversity on nutrient agar plates [8]. However, growth on media other than blood or nutrient agar was not tried. It is possible that had a media specifically designed to culture Pseudomonads been selected, our results may have been different. In addition, our inability to grow these bacteria on agar plates may be a result of the fact that so many of the Pseudomonas spp.-related sequences came from uncultured bacteria (1–3% divergence from cultured species; Fig. 2).

The other most consistently abundant group, based on clone library sequence analysis, included a large collection of uncultured species in the Oxalobacteraceae family. According to the research literature, the Oxalobacteraceae include numerous bacterial species with diverse habitats. For example, many Collimonas and Herbaspirillum spp. are

| Date        | Plate type | Total Plates | % Growth | Organisms detected          |
|-------------|------------|--------------|----------|-----------------------------|
| 30-Sep-2005 | Nutrient   | 6            | 66.7     | N/A                         |
|             | Blood      | 4            | 75.0     | N/A                         |
| 6-Oct-2005  | Nutrient   | 12           | 0.0      | N/A                         |
|             | Blood      | 12           | 33.3     | N/A                         |
| 14-Oct-2005 | Nutrient   | 14           | 16.7     | N/A                         |
|             | Blood      | 0            | 0.0      | N/A                         |
| 21-Oct-2005 | Nutrient   | 24           | 79.2     | Bacillus sp. (Bacillus M7)   |
|             | Blood      | 0            | 0.0      | Bacillus sp. (Bacillus M2, M11) |
| 28-Oct-2005 | Nutrient   | 10           | 80.0     | N/A                         |
|             | Blood      | 6            | 83.3     | N/A                         |
| 3-Nov-2005  | Nutrient   | 13           | 84.6     | Bacillus sp. (Bacillus M7)   |
|             | Blood      | 12           | 83.3     | Bacillus sp. (Bacillus M11)  |
| 15-Nov-2005 | Nutrient   | 22           | 81.8     | Bacillus sp. (Bacillus M7)   |
|             | Blood      | 16           | 56.3     | Bacillus sp. (Bacillus M3, M11, 14) |
| 18-Jan-2006 | Nutrient   | 16           | 56.3     | N/A                         |
|             | Blood      | 0            | 0.0      | N/A                         |
| 25-Jan-2006 | Nutrient   | 16           | 68.8     | N/A                         |
|             | Blood      | 16           | 75.0     | N/A                         |
| 3-Feb-2006  | Nutrient   | 12           | 75.0     | Bacillus sp. (Bacillus M2, M7, M10), Staphylococcus sp. (Staph. M1) |
|             | Blood      | 16           | 81.3     | Bacillus sp. (Bacillus M1, M2, M3, M14), Staphylococcus sp. (Staph. M2), Moraxella sp. (Morax. M1), Brevibacterium sp. (Brevi. M1b), Brevibacillus sp. (Brevi. M1a) |
| 15-Feb-2006 | Nutrient   | 10           | 90.0     | Bacillus sp. (Bacillus M10), Staphylococcus sp. (Staph. M3) |
|             | Blood      | 10           | 80.0     | Bacillus sp. (Bacillus M1), Microbacterium sp. (Microbac. M1) |
| 1-Mar-2006  | Nutrient   | 10           | 70.0     | Pseudomonas sp. (Pseudo. M1), Bacillus sp. (Bacillus M1, M5, M7), Staphylococcus sp. (Staph. M3) |
|             | Blood      | 10           | 60.0     | Bacillus sp. (Bacillus M1, M5, M9, M11), Staphylococcus sp. (Staph. M2) |
| 8-Mar-2006  | Nutrient   | 20           | 85.0     | Bacillus sp. (Bacillus M5, M7), Staphylococcus sp. (Staph. M3), Enterococcus sp. (Enterococ. M1) |
|             | Blood      | 20           | 90.0     | Bacillus sp. (Bacillus M1, M11), Staphylococcus sp. (Staph. M2), Moraxella sp. (Morax. M1) |
| 29-Mar-2006 | Nutrient   | 16           | 50.0     | Bacillus sp. (Bacillus M5, M7), Staphylococcus sp. (M1), Enterococcus sp. (Enterococ. M1) |
|             | Blood      | 16           | 50.0     | Bacillus sp. (Bacillus M2, M5a, M11, M13), Staphylococcus sp. (M2) |
| 5-Apr-2006  | Nutrient   | 12           | 41.7     | Bacillus sp. (Bacillus M7, M12), Staphylococcus sp. (Staph. M1, M3), Acinetobacter sp. (Acinet. M1) |
|             | Blood      | 12           | 58.3     | Bacillus sp. (Bacillus M2, M8, M11, M14), Staphylococcus sp. (M2, M3) |
| 12-Apr-2006 | Nutrient   | 16           | 37.5     | Bacillus sp. (Bacillus M7, M12), Brevibacterium sp. (Brevi. M1b), Pseudomonas sp. (Pseudo. M1), Exiguobacterium sp. (Exiguobac. M1) |
|             | Blood      | 16           | 43.8     | Bacillus sp. (Bacillus M1, M2, M8, M11), Staphylococcus sp. (Staph. M2), Brevibacterium sp. (Brevi. M1a), Exiguobacterium sp. (Exiguobac. M1) |
| 19-Apr-2006 | Nutrient   | 16           | 43.8     | Bacillus sp. (Bacillus M10, M12), Acinetobacter sp. (Acinet. M1), Enterococcus sp. (Enterococ. M1) |
|             | Blood      | 16           | 81.3     | Bacillus sp. (Bacillus M1, 2, M15), Brevibacterium sp. (Brevi. M1b), Microbacterium sp. (Microbac. M1a) |

1 Percentage of plates with microbial growth
2 Observed morphologies (see Table 1); N/A = Not Available for that sampling day.
soil dwelling bacteria [38], while Oxalobacter formingenes is found in the human gastrointestinal tract [39]. Unfortunately, our uncultured species appeared to belong to a novel group of Oxalobacteraceae leaving us with little information concerning their source or habitats, apart from a rather basic understanding of their phylogenetic relationships. However, a recent culture-independent study of human-associated microbial communities [40] allowed us to identify the human vaginal epithelium as a likely source of a large number of these sequences (Fig. 1, Results of Bayesian phylogenetic analysis of Pseudomonas-related 16S rRNA gene sequences representing the most common clones found in PCR-amplified libraries from swabs samples of toys and surfaces. Cloned sequences are indicated by “CCTR” (Children’s Center Toddler Room) or “CCIR” (Children’s Center Infant Room) prefixes followed by the date of sampling and whether the sequence was obtained from a toy (T) or a surface (S). The phylogeny includes sequences of closely related cultured and uncultured organisms. GenBank accession numbers are presented next to the names. The values above the branches indicate the Bayesian posterior probabilities (above 0.5) under the specified model of evolution for each node (see Methods for details). Maximum Parsimony and Maximum Likelihood analyses produced highly similar tree topologies. MP bootstrap values were similarly high at nodes well-supported by Bayesian analysis.

![Figure 1](http://www.biomedcentral.com/1471-2180/7/27)

| Sequence | Organism Name | Accession Number |
|----------|---------------|------------------|
| CCTR101252006S1BCB EF409263 | Pseudomonas aeruginosa | U38445 |
| CCTR110282005T1BCA EF409259 | Pseudomonas mucidolens | D84014 |
| CCIR103082006S1BCA EF409260 | Shewanella (Alteromonas) hanedi | X82132 |
| CCTR101252006S1BCB EF409265 | Escherichia coli | L10328 |
| CCIR103082006S1BCA EF409266 | Shigella flexneri | X96963 |
| CCTR103012006S1BCA EF409267 | Symbioant S of Acyrthosiphon pisum | M27040 |
| CCIR103012006S1BCA EF409268 | Staphylococcus epidermidis | X75943 |
| CCTR101252006S1BCB EF409269 | Pseudomonas aureofaciens | D84008 |
| CCIR110062005S1BCA EF409270 | Pseudomonas species | Z29622 |
| CCTR101252006S1BCB EF409271 | Pseudomonas fluorescens | D84013 |
| CCTR203012006S1BCA EF409272 | Pseudomonas putida | D84020 |
| CCTR101252006S1BCB EF409273 | Pseudomonas putida | D84020 |
Results of Bayesian phylogenetic analysis of 16S rRNA gene sequences from other phylogenetic groups of bacteria found in PCR-amplified clone libraries from swab samples of toys and surfaces.

Figure 2
Results of Bayesian phylogenetic analysis of 16S rRNA gene sequences from other phylogenetic groups of bacteria found in PCR-amplified clone libraries from swab samples of toys and surfaces. Cloned sequences are indicated by "CCTR" (Children's Center Toddler Room) or "CCIR" (Children's Center Infant Room) prefixes followed by the date of sampling and whether the sequence was obtained from a toy (T) or a surface (S). GenBank accession numbers are presented next to the names. The tree also includes sequences from some of the cultured isolates in Table 1. The values above the branches indicate the Bayesian posterior probabilities (above 0.5) under the specified model of evolution for each node. Maximum Parsimony and Maximum Likelihood analyses produced highly similar tree topologies. MP bootstrap values were similarly high at nodes well-supported by Bayesian analysis.
Graphical representation of common bacterial-type abundance found in the six clone libraries made from furniture surface swabs based on 16S rRNA gene sequence identification. Pseudomonads and uncultured Oxalobacteraceae were consistently found on all surfaces at high levels. Bacillus species were also common, though at lower abundance. One date in particular, Jan 25th (Toddler Room 2) had a particularly high concentration of Streptococcus-related sequences.
3). An intensive sequencing effort by Hyman et al. (2005) revealed a tremendous diversity of uncultured bacteria associated with the vaginal epithelium, and many of the 16S rRNA gene sequences obtained from surface samples, especially within the Oxalobacteraceae, were closely related to these published sequences (Fig. 2). Sequences in our clone libraries from nine other bacterial divisions were also nearly identical to bacterial sequences isolated from the vaginal epithelium (Fig. 2). We also note that we found many sequences of apparently uncultured bacteria related to uncultured bacteria identified from molecular studies of wastewater sludge (Fig. 2; [41,42]).

The predominance of sequences in our libraries related to bacteria found in the vaginal epithelium and in wastewater sludge suggests that a significant proportion of the bacterial contamination in daycares results from frequent diaper changes. This conclusion is supported by the discovery of cultured bacteria known to reside in the human intestine (e.g., E. coli, Enterococcus faecalis; Fig. 2). Since we know so little about the uncultured bacteria, we cannot say whether they pose a particular health threat. However, the significant diversity of so many human-associated bacteria contaminating various toys and surfaces suggests that enteropathogenic bacteria could be easily spread in daycare settings. Given the fact that we did not achieve full sampling coverage of the sequence diversity for any of the samples (average clone library sequence coverage ~54%), we expect that many more bacterial species may be found in daycare settings. These results not only demonstrate how little is known about microbial diversity of indoor environments, but also emphasizes the need for a much more complete understanding of microbes associated with humans, which appeared to be the source of most of the contamination.

In addition to the sequences from uncultured bacteria, we also identified a number of sequences in the culture-independent molecular analysis with close relatives known to be pathogens or opportunistic pathogens that were not found through culturing. These include sequences related to Streptococcus mutans, S. mitis, Chryseobacterium indologenes [43], Stenotrophomonas maltophilia [44,45], Flavobacterium indologenes [46] and Rothia dentocariosa [47] (Fig. 1, 2). The January 25 sampling date in particular had a particularly high abundance of Streptococcus-related species (Fig. 3). This sampling was right in the middle of the cold and flu season and was around the time a large number of the kids were kept home due to illness (Daycare staff, pers. comm.). The fact that we found so few of these species through standard culturing approaches supports that notion that culture-independent molecular analyses provide a powerful additional means for studying indoor environments and possibly identifying infectious agents.

The results of this study should also be examined from a public health viewpoint, in order to assess which organisms have the greatest potential to cause illness in children. Streptococcus pneumoniae and Staphylococcus epidermidis were identified in several samples, these represent known pathogens that can cause pneumonia and meningitis in children [48,49]. Stenotrophomonas maltophilia was also found, this bacteria has been known to cause infections and bacteremia in pediatric patients who have contracted it as a non-scomial infection during a hospital stay [50,51]. The presence of Stenotrophomonas maltophilia was also somewhat distressing due to the recent emergence of an antibiotic resistant strain [52]. Other pathogenic strains potentially harmful to children that were found included Rothia dentocariosa, known to cause the childhood illness tonsillitis [53], Enterococcus faecalis, a bacteria associated with urinary tract infections [54,55], and Shigella flexneri, which causes the common childhood ailment of acute diarrhea [56,57].

Although culture-independent methods have proven highly useful for uncovering a vast array of new microbes in many environments, including the daycare center, a number of authors have pointed out that methods based on amplifying 16S rRNA gene sequences using "universal" primers may not accurately reflect the true underlying diversity of a given environment [58,59]. Problems such as PCR-bias, ribosomal DNA copy number and the efficiency of DNA extraction procedures all have the potential to significantly skew abundance estimates and there may not be a direct relationship between the number of sequences of a particular type in a clone library and the number of organisms in the environment.

Nevertheless, as demonstrated in this paper, the culture-independent methods do allow for a much more comprehensive assessment of microbial diversity than culturing alone and provide an approximation of the relative diversity in the samples. Given the proper set of growing conditions, many of the uncultured bacteria could be isolated using culture-based methods. For example, by culturing for a longer period of time (five or more days), by culturing at a broader range of temperatures (e.g., 30°C), or by using alternative media (e.g., low-nutrient medium R2A) we might have isolated more types of bacteria. Longer incubation times would have been particularly helpful for growing organisms that tend to live in biofilms. Indeed, the culture-independent methods provide an excellent complement to the culturing approaches. Before the study, we did not expect to find such a diverse array of Pseudomonadaceae and Oxalobacteraceae, but with the knowledge gained from the culture-independent methods we can now adjust our culturing methods to grow these organisms.
Conclusion
The diversity of bacteria in the daycare environment appears to be a rich combination of bacterial species associated with both humans and the outside environment (e.g., *Bacillus* in soils). Given the extremely high bacterial diversity, and the relatively low sequence coverage we achieved in this preliminary study (~54%), the overall diversity is almost certainly higher than we report. Our results suggest that the microbial diversity associated with human environments remains extremely poorly characterized. In terms of public health, we believe greater attention needs to be paid to the microbial contamination of environments (e.g., daycare centers, nursing homes and hospitals) that take care of the most vulnerable members of society. In terms of the child-care facilities per se, our results suggest that diaper changing stations should be moved further away from the play areas, and that more efforts should be focused on removing tough biofilms. Faster and more comprehensive culture-independent methods, such as environmental microarrays and metagenomic approaches, could help better understand the public health risks in these environments.

Methods
Sample Collection
Samples were collected from 4 different classrooms where children ages 0–4 years were taught daily. Environmental samples were taken with dual tip sterile cotton swabs (BBL CultureSwab™, catalog # 220135, Becton Dickinson, Sparks, MD) and these were stored in sterile-labeled tubes for immediate transport back to the lab. Toys and surfaces were sampled on a fixed surface area of approximately 13 cm². During the course of the study the same furniture surfaces were sampled repeatedly, while the toys varied between samplings.

Bacterial Culturing Methods
Immediately upon return to the lab, one tip of the dual-tip swab samples was placed into 7 mL of nutrient broth (Difco™, Becton Dickinson, Sparks, MD) and allowed to incubate overnight at 37°C. The second tip of each dual-tip swab was labeled and placed at -80°C for later analysis. Overnight cultures of nutrient broth (Difco™) were used to streak 5% blood (Blood Agar Contact Plate, Hardy Diagnostics, Santa Maria, CA) and nutrient agar plates (Difco™, Becton Dickinson, Sparks, MD), which were also incubated overnight at 37°C. To minimize outside contamination, all culturing was performed in a biological hood using sterile instruments. The following day, plate growth and colony morphology were evaluated and recorded.

DNA Extraction and PCR Amplification of Colonies and Swabs
DNA was extracted using a lysozyme-extraction protocol [60] directly from the bacterial colonies picked off plates using a sterile toothpick. One colony was selected from each observed morphology type. We used the same protocol to isolate DNA directly from the swabs (the environmental samples) for culture-independent analysis.

For the environmental extractions, cotton from the swab samples was removed using a sterile razor blade and placed into the lysozyme reaction mixture. The reaction mixture had a total volume of 200 μl and included the following final concentration: 20 M Tris, 2 mM EDTA (pH 8.0), 1.2% P40 detergent, 20 mg ml⁻¹ lysozyme, and 0.2 μm filtered sterile water (Sigma Chemical Co., St. Louis, MO). We used the same reaction buffer and extraction method for isolating DNA from the cultured organisms (Table 1). For the cultured bacteria, we used a sterile toothpick to pick a single colony from the agar plates, which was then swirled into the reaction mixture. Samples were incubated in a 37°C water bath for thirty minutes. Next, Proteinase K (DNeasy Tissue Kit, Qiagen Corporation, Valencia, CA) and AL Buffer (DNeasy Tissue Kit, Qiagen Corporation, Valencia, CA) were added to the tubes and gently mixed. Samples were incubated in a 70°C water bath for 10 min. All samples were subjected to purification using the DNeasy Tissue Kit.

Following extraction, the DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Willmington, DE). We created PCR-based clone libraries from nine of the swabs collected over the course of the study. With one exception, swabs we chose came from the same surface type (the toy shelf). Six of the swabs were selected to represent each month from October 2005 through April 2006, while the other three were selected as duplicates for three of the sampling days to determine the consistency of contamination across surfaces. This strategy allowed us to find the most consistently abundant types of bacteria and to detect any significant changes in diversity that might occur during the "cold and flu" season.

The PCR reactions utilized published bacterium specific primers primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 805R (5'-GACTACGGGTATCTAATCC-3') to amplify the 16S rRNA gene. The ~800 bp PCR products from amplification using these primers includes a portion of the 16S rRNA gene that has been shown to be particularly useful for database analysis and identification of bacterial sequences [13]. PCR was carried out in a total reaction volume of 50 μl including 1 μl (approx. 10 ng μl⁻¹) of sample DNA as template, each deoxynucleoside triphosphate at 200 μM, 1.5 mM MgCl₂ in 10× buffer (10×...
μ(C) was calculated using the following equation,

\[ C = 1 - \frac{n}{N} \]

, where \( n \) is the number of unique OTU sequences observed and \( N \) is the total number of OTUs (i.e., sum of unique OTUs plus OTUs observed more than once) [63].

After Fastgroup analysis, the sequences were aligned using the NAST alignment software [64]. This program aligns rRNA gene sequences to a diverse set of full-length rRNA gene sequences that have been rigorously aligned using the RNA secondary structure. From here the aligned sequences were imported into the ARB application [65]. Bacterial colony species identifications were completed using a combination of BLAST results and phylogenetic analysis using the ARB program. Clone library sequences and other sequences identified in GenBank were aligned using ARB and exported as Nexus files for phylogenetic analysis using PAUP* [66] and MrBayes version 3.1.2 [67].

Phylogenetic analyses were performed with two different data sets (see Fig. 1 and Fig. 2). For each of the data sets, trees were constructed using three different methods: Bayesian, Maximum Parsimony (MP) and Maximum Likelihood (ML). The MODELTEST program [68] was used to choose the DNA substitution model that best fit our particular dataset. Bayesian analyses were performed using the General Time Reversible model [69] with a gamma-distributed among-site substitution rate heterogeneity and a fraction of sites constrained to be invariable (GTR+I+G).

All Bayesian analyses were done with four independent Markov chains run for 3,000,000 MCMC generations. Trees were sampled every 200 generations with a burn-in of 2000 trees. The best Maximum Parsimony (MP) tree, or set of trees, was found through a random addition heuristic search strategy with 100 replicates. The maximum number of trees kept during each search was capped at 1000. For the MP bootstrap analyses, we performed MP searches on 100 bootstrap replicated datasets using the same heuristic search strategy except with 10, rather than 100, search replicates. We also performed a Maximum Likelihood (ML) analysis using the GTR+I+G model of evolution and a random addition sequence heuristic search strategy with 10 replicates to find the highest likelihood tree.

Authors’ contributions
LL collected half of the samples; performed most of the culturing; and identified isolates based on 16S rRNA gene sequencing. LL also created 3 of the 9 clone libraries; cleaned, edited and aligned all the sequence data; performed much of the Bioinformatics analyses and wrote early drafts of the manuscript. ST collected the other half...
of the samples, created 6 of the 9 clone libraries and helped with the sequence analyses. STK designed the study, wrote and edited the manuscript, completed the phylogenetic analyses and created the figures. All authors read and approved the final manuscript.

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References

1. Dales RE, Calmaks S, Brand K, Judek S: Respiratory illness in children attending daycare. Pediatr Pulmonol 2004, 38(1):64-69.
2. Amorim KS, Ross eti-Ferreira MC: [Critical analysis of respiratory infectious disease investigations related to children attending day care centers]. J Pediatr (Rio J) 1999, 75(5):313-320.
3. Slack-Smith LM, Read AW, Stanley FJ: Experience of respiratory and allergic illness in children attending childcare. Child Care Health Dev 2002, 28(2):171-177.
4. Kampfer P, Andersson MA, Rainey FA, Kroppenstedt RM, Salkinoja-Salonen MS: Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci U S A 2005, 102(13):4859-4865.
5. Kelley ST, Theisen U, Angenent LT, St Amand A, Pace NR: Molecular analysis of shower curtain biofilm microbes. Appl Environ Microbiol 2004, 70(7):4187-4192.
6. McManus CJ, Kelley ST: Molecular survey of aeroplane bacterial contamination. J Appl Microbiol 2005, 99(3):502-508.
7. Angenent LT, Kellett A, St Amand A, Pace NR: Molecular analysis of shower curtain biofilm microbes. Appl Environ Microbiol 2004, 70(7):4187-4192.
8. Johansen C, Falholt P, Gram L: Transmission of Escherichia coli O157:H7 infection in Minnesota child day-care facilities. JAMA 1993, 269(7):883-888.
9. Liu LJ, Krahmer M, Fox A, Feigley CE, Featherstone A, Saraf A, Larsen L: Investigation of the concentration of bacteria and their cell envelope components in indoor air in two elementary schools. J Air Waste Manag Assoc 2000, 50(11):1957-1967.
10. Machter JM: Evaluation of a procedure to isolate culturable microorganisms from carpet dust. Indoor Air 2001, 11(1):134-140.
11. Kappner P, Andersson MA, Rainey FA, Kroppenstedt RM, Salkinoja-Salonen M: Williamsia muralis gen. nov., sp. nov., isolated from the indoor environment of a children's day care centre. Int J Syst Bacterial 1999, 49 Pt 2:681-687.
12. Tsai FC, Machter JM: Concentrations of airborne culturable bacteria in 100 large US office buildings from the BASE study. Indoor Air 2005, 15 Suppl 9:71-81.
13. Tannen MA, Shoskes D, Shahed A, Pace NR: Prevalence of corynebacterial 16S rRNA sequences in patients with bacterial and “nonbacterial” prostatitis. J Clin Microbiol 1999, 37(6):1863-1870.
14. Amann RI, Ludwig W, Schleifer KH: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 1995, 59(1):143-169.
15. Pace NR: A molecular view of microbial diversity and the biosphere. Science 1997, 276(5313):734-740.
16. Hugenholtz P, Goebel BM, Pace NR: Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 1998, 180(18):4785-4774.
17. Hugenholtz P, Grimet P, Kuske CR, Connerton IF, St John J, Pace NR: Nucleotide sequence submission of new bacterial 16S rRNA gene sequences. J Bacteriol 1996, 178(14):4095-4105.
18. Scanlan PD, Shanahan F, O’Mahony C, Marchesi JR: Culture-independent analyses of the temporal variation of the dominant faecal microbiota and targeted bacterial sub-groups in Cohn’s disease. J Clin Microbiol 2006.
19. Siqueira JF Jr., Rocsin IN: Catonella morbi and Granulicatella adiacens: new species in endodontic infections. Oral Surg Oral Pathol Oral Radiol Endod 2006, 102(2):259-264.
20. Tsuineishi M, Yamamoto T, Koguchi S, Maki N, Fukui K, Watanabe T: Composition of the bacterial flora in tonsilloliths. Microbes Infect 2006.
21. Zoletti GO, Siqueira JF Jr., Santos KR: Identification of Enterococcus faecalis in root-filled teeth with or without periradicular lesions by culture-dependent and independent approaches. J Endod 2006, 32(8):722-726.
22. R. E. Ley, Backhed F, Turnbaugh P, Lezpunza CA, Knight RD, Gordon JJ: Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A 2005, 102(31):11070-11075.
23. Kuske CR, Banton KL, Adarada DL, Stark PC, Hill KK, Jackson PJ: Small-Scale DNA Sample Preparation Method for Field PCR Detection of Microbial Cells and Spores in Soil. Appl Environ Microbiol 1998, 64(2):2463-2472.
24. Nicholson WL: Roles of Bacillus endospores in the environment. Cell Mol Life Sci 2002, 59(3):410-416.
25. Vitain S, Luo Y, Hildreth PH, Brozel VS: Analysis of the life cycle of the soil saprophyte Bacillus cereus in liquid soil extract and in soil. Appl Environ Microbiol 2006, 72(7):4970-4977.
26. Murray BE: The life and times of the Enterococcus. Clin Microbiol Rev 1990, 3(1):46-65.
27. Han XY, Tarrand JJ: Moraxella osloensis blood and catheter infections during anticancer chemotherapy: clinical and microbiologic studies of 10 cases. Am J Clin Pathol 2004, 121(4):581-587.
28. Foggatt JW, Johnston JL, Galetto DW, Archer GL: Antimicrobial resistance in nosocomial isolates of Staphylococcus haemolyticus. Antimicrob Agents Chemother 1989, 33(4):460-466.
29. Tancred C: Role of human microflora in health and disease. Eur J Clin Microbiol Infect Dis 1992, 11(11):1012-1015.
30. Lessie TG, Phibbs PV Jr.: Alternative pathways of carbohydrate utilization in pseudomonads. Ann Rev Microbiol 1984, 38:339-388.
31. Drenkard E, Ausubel FM: Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 2002, 418(6882):740-743.
32. Parchmann C, Falholt P, Grem L: Enzymatic removal and disinfection of bacterial biofilms. Appl Environ Microbiol 1997, 63(9):3724-3728.
33. Lalucat J, Bennasar A, Bosch R, Garcia-Valdes E, Pullinger NJ: Biology of Pseudomonas stutzeri. Microbiol Mol Biol Rev 2006, 70(2):510-547.
34. Yee-Guardino S, Danziger-Isakov L, Knouse M, Bingman W, Sabela C, Goldfarb J: Nosocomially acquired Pseudomonas stutzeri brain abscess in a child: case report and review. Infect Control Hosp Epidemiol 2006, 27(6):539-532.
35. Idles A, Maclusky I, Coney M, Gold R, Prober C, Fleming P, Vehnin J: Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J Pediatr 1984, 104(2):206-210.
36. Costerton JW, Stewart PS, Greenberg EP: Bacterial biofilms: a common cause of persistent infections. Science 1999, 284(5418):1318-1322.
37. Berg G, Eberl L, Hartmann A: The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ Microbiol 2005, 7(1):1673-1685.
38. Mital RD, Kumar R, Bid HK, Mital B: Effect of antibiotics on Oxalobacter formigenes colonization of human gastrointestinal tract. J Endourol 2005, 19(1):102-106.
39. Hyman RW, Fukushima M, Diamond L, Kumm J, Giudice LC, Davis RW: Microbes in the human vaginal epithelium. Proc Natl Acad Sci U S A 2005, 102(23):8050-8055.
40. Bond PL, Hugenholtz P, Keller J, Blackall LL: Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Appl Environ Microbiol 1993, 61(5):1910-1916.
41. Layton AC, Karanth PN, Lajie CA, Meyers AJ, Gregory IR, Stapleton RD, Taylor DE, Sayer GS: Quantification of Hyphomicrobium populations in activated sludge from an industrial wastewater treatment system as determined by 16S rRNA analysis. Appl Environ Microbiol 2000, 66(3):1167-1174.
43. Hsueh PR, Teng LJ, Yang PC, Ho SW, Hsieh WC, Luh KT: Increasing incidence of nosocomial Chryseobacterium indolgenes infections in Taiwan. Eur J Clin Microbiol Infect Dis 1997, 16(8):568-574.

44. Gopalakrishnan R, Hawley HB, Czachor JS, Markert RJ, Bernstein JM: Stenotrophomonas maltophilia infection and colonization in the intensive care units of two teaching hospitals: A study of 143 patients. Heart Lung 1999, 28(2):134-141.

45. Lanotte P, Cantagrel S, Mereghetti L, Marchand S, Van der Mee N, Besnier JM, Laugier J, Quentin R: Spread of Stenotrophomonas maltophilia colonization in a pediatric intensive care unit detected by monitoring tracheal bacterial carriage and sputum typing. Clin Microbiol Infect 2003, 9(11):1142-1147.

46. Hsueh PR, Teng LJ, Ho SW, Hsieh WC, Luh KT: Clinical and microbiological characteristics of Flavobacterium indolgenes infections associated with indwelling devices. J Clin Microbiol 1996, 34(8):1908-1913.

47. Noda M, Yasuda KA, Krajewski LP, Washington JA: Infection of an arteriovenous fistula with Rothia dentocariosa. Diagn Microbiol Infect Dis 1991, 14(2):167-169.

48. Tajima T, Nakayama E, Kondo Y, Hirai F, Ito H, Itsuka T, Momomura M, Kusumoto H, Kodaka Y, Funaki N, Tanagawa Y, Yubukata K: Epidemiology and clinical study of community-acquired pneumonia in 157 hospitalized children. J Infect Chemother 2006, 12(6):372-379.

49. Tan L, Sun X, Zhu X, Zhang Z, Li J, Shu Q: Epidemiology of nosocomial pneumonia in infants after cardiac surgery. Chest 2004, 125(2):410-417.

50. Wu PS, Lu CY, Chang LY, Hsueh PR, Lee PI, Chen JM, Lee CY, Chan PC, Chang PY, Yang TT, Huang LM: Stenotrophomonas maltophilia bacteremia in pediatric patients--a 10-year analysis. J Microbial Infect Dis 2006, 39(2):1-19.

51. Kosoglu O, Sener B, Gulmez D, Altun B, Gur D: Stenotrophomonas maltophilia as a nosocomial pathogen. New Microbiol 2004, 27(3):273-279.

52. Gulmez D, Hascelik G: Stenotrophomonas maltophilia: antimicrobial resistance and molecular typing of an emerging pathogen in a Turkish university hospital. Clin Microbiol Infect 2003, 11(1):80-89.

53. Ohashi M, Yoshikawa T, Akimoto S, Fujita A, Hayakawa S, Takahashi M, Arakawa T, Asano Y: Severe acute tonsillitis caused by Rothia dentocariosa in a healthy child. Pediatr Infect Dis J 2005, 24(5):466-467.

54. Katsova LK, Zorkin SN, Alekhina VM, Chashchina IL, Abramov KS: [Resistance of urinary tract infection pathogens and choice of antibacterial therapy in pediatric urologic practice]. Anti-biot Khimioter 2004, 49(1):34-39.

55. Pape L, Gunzer F, Ziesing S, Pape A, Offner G, Ehrich JH: Bacterial pathogens, resistance patterns and treatment options in community acquired pediatric urinary tract infection. Klin Padiat 2004, 216(3):83-86.

56. Denno DM, Staap JR, Boster DR, Qin X, Clausen CR, Del Beccaro KH, Swerdlow DL, Braden CR, Tarr PI: Etiology of diarrhea in pediatric outpatient settings. Pediatr Infect Dis J 2005, 24(2):142-148.

57. Flores A, Araque M, Viczay L: Multiresistant Shigella species isolated from pediatric patients with acute diarrheal disease. Am J Med Sci 1998, 316(6):379-384.

58. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG: Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rDNA. Appl Environ Microbiol 1998, 64(6):2233.

59. Suzuki MT, Giovannoni SJ: Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 1996, 62(2):625-630.

60. Birdsell DC, Costa-Robles EH: Production and ultrastructure of lysozyme and ethylenediaminetetraacetae-lysozyme spheroplasts of Escherichia coli. J Bacteriol 1997, 169(1):427-437.

61. Ewing B, Hillier L, Wendl MC, Green P: Base-calling of automated sequencer traces using phred. 1. Accuracy assessment. Genome Res 1998, 8(3):175-185.

62. Yu Y, Breibart M, McNairne P, Rohwer F: FastGroup II: a web-based bioinformatics platform for analyses of large 16S rDNA libraries. BMC Bioinformatics 2006, 7:57.

63. Stach JE, Maldonado LA, Masson DG, Ward AC, Goodfellow M, Bull AT: Statistical approaches for estimating actinobacterial diversity in marine sediments. Appl Environ Microbiol 2003, 69(10):6189-6200.

64. DeSantis TZ Jr., Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL: NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 2006, 34(Web Server issue):W394-9.

65. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grummann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Srethlow R, Stamatakis A, Stockmann N, Vilig B, Lenke M, Ludwig T, Bode A, Schleifer KH: ARB: a software environment for sequence data. Nucleic Acids Res 2004, 32(4):1363-1371.

66. Swofford D: PAUP*: phylogenetic analysis using parsimony (* and other methods). 4th edition. Sunderland, Massachusetts, Sinauer Associates; 1998.

67. Huelsenbeck JP, Ronquist F: MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 2001, 17(8):754-755.

68. Posada D, Crandall KA: MODELTEST: testing the model of DNA substitution. Bioinformatics 1998, 14(9):817-818.

69. Yang Z: Estimating the pattern of nucleotide substitution. J Mol Evol 1994, 39(1):105-111.