ORIGINAL ARTICLE

Microbial burden and diversity of commercial airline cabin air during short and long durations of travel

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Total microbial burden and diversity associated with commercial airliner cabin air was assessed by molecular methods in 125 air samples from the business-class sections of 16 domestic and international flights. Viable microbial burden within these cabin air parcels constituted only 1–10% of the total microbial population and ranged from below detection limits to 1.2×10^4 cells m^{-3} as determined with a validated ATP-based technology. Cultivable bacterial diversity was almost entirely limited to Gram-positive bacteria such as *Staphylococcus* and *Bacillus*. In contrast, cloning and sequencing 16S rRNA gene directly from the samples without cultivation indicated a significantly broader diversity, as sequences representing more than 100 species, and encompassing 12 classes of bacteria, were retrieved in varying abundance. Sequences of proteobacterial and Gram-positive lineage were retrieved most frequently (58% and 31% of all clone sequences, respectively), with Gram-positive and α-proteobacterial sequences dominating international flight samples and β- and γ-proteobacterial sequences comprising the largest portion of those retrieved from domestic flights. Significant differences in bacterial load and diversity were noted between samples obtained on domestic and international flights. The disparities observed in microbial abundance and diversity further underscore the immense value of state-of-the art molecular assays in augmenting traditional culture-based techniques.

Introduction

According to the National Research Council (NRC) report (NRC, 2002), the number of air passengers worldwide has nearly quadrupled over the last 30 years: from 383 million in 1970 to 1462 million in 1998. At this rate, this percentage is likely to rise considerably with the number of international travelers expected to increase to several billions by 2020. Although the aircraft cabin is similar to other semi-contained indoor environments, such as homes and offices, in that individuals are exposed to a combination of external and recirculated air, it varies greatly from these environments with respect to occupant density, the inability of occupants to leave at will and the need for pressurization. Furthermore, while in flight, travelers encounter a combination of constantly regulated environmental factors including low humidity, reduced air pressure and potential exposure to chemical contaminants, such as ozone, carbon monoxide, various organic compounds and biological agents.

Although the Federal Aviation Administration adopted a 1986 NRC recommendation to eliminate smoking on most domestic airline flights, several other public health concerns regarding aircraft cabin air quality have yet to be adequately addressed (NRC, 2002). One concern was the microbiological burden in enclosed environments, where passengers breathe continuously recycled air. The air provided to the passengers and crew on commercial jet aircraft is typically a combination of external air brought in through the engines and air that is taken from the cabin, filtered and recirculated. The environmental control system is designed to minimize the introduction of harmful contaminants into the cabin and to control cabin pressure, ventilation, temperature and humidity. An NRC report (NRC, 2002) found that most commercial jet airlines

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provide an ample supply of air to pressurize the cabin, meeting general comfort conditions and diluting or otherwise reducing normally occurring odors, heat and contaminants. The committee noted, however, that the current design standard of a minimum of 0.55 lb of outside air per minute per occupant (Federal Aviation Regulation: 25.831) is less than one-half to two-thirds the ventilation rate recommended in American Society of Heating, Refrigerating, and Air-Conditioning Engineers Standard 62–1999, which was developed for ground structures.

The majority of published investigations of cabin air quality have focused on chemical contaminants, such as cigarette smoke, carbon dioxide, ozone and total organic carbon (Lee et al., 1999; Lindgren and Norback, 2002; Lindgren et al., 2002), rather than microbiological sources. However, recent concerns regarding the transmission of emerging diseases on commercial airliners, SARS and tuberculosis in particular, have brought much attention to the monitoring and control of cabin air quality aboard commercial aircraft (Olsen et al., 2003). Prior to this investigation, knowledge of the microbiological composition in commercial airliner cabin air was limited to a sparse collection of cultivation-based reports (Wick and Irvine, 1995; Dechow et al., 1997; Hines et al., 2003; McKernan et al., 2007). Although these studies have collectively reported a typical microbial burden ranging from 10 to 300 colony-forming units (CFU) per m^3, these results are fraught with bias since a majority of all bacteria are not capable of growing in well-defined conventional media (Pace et al., 1985; Amann et al., 1995; Pace, 1997; La Duc et al., 2007a). At best, plate counts offer only relative comparisons between samples and are incapable of definitive estimations of the size or diversity of the molecular communities present.

Since the presence of viable microorganisms circulating about cabin air is of particular consequence to human health, it is critical to thoroughly and accurately assess the diversity and overall microbial burden associated with commercial cabin air systems. In a previous study, we demonstrated the application of molecular techniques to elucidate the microbial populations aboard four commercial airline flights (La Duc et al., 2007b). The objective of this investigation was to greatly extend the use of these methods over a larger sampling and attempt to identify significant differences in the total microbial burden and composition among individual aircraft, and between domestic and international-bound flights.

Materials and methods

Sampling limitations on commercial airliners
The departure and destination cities, aircraft type, travel time and distance between the cities traveled are given in Table 1. At the request of the carrier that participated in this study, the name of the carrier sampled has not been provided (personal communication). The commercial aviation industry has assured the authors that standard use, positioning and maintenance of HEPA filters apply to all aircraft regardless of carrier. To gain a better understanding of the flow and engineering pertaining to aircraft cabin air systems, the authors requested designs and specifications for aircraft that were sampled, but in the interest of safety and security such information was not made available. Each flight represented a distinct aircraft of that carrier; there were no continuations of the same flight on the same aircraft. All of the flights were at or very near capacity.

Sample collection
The BioCapture BT-550 (Mesosystems Technology Inc., Kennewick, WA, USA) sampler employed in this study collects particles in the size range of 0.5–10 μm (optimum for cells and particles) from ambient air and operates at altitudes in excess of 15 000 m. Previous work has characterized the sampling efficiency of the BioCapture (BT-550) sampler using monodisperse fluorescent oleic acid particles and monodisperse fluorescent polystyrene latex particles (Kesavan et al., 2003). The results showed that the BT-550 sampler had a peak sampling efficiency for 2-μm particles and the average peak sampling efficiencies for the sampler was 38% (Kesavan et al., 2003). The flow rate of this light-weight (4.5 kg), field deployable sampler is 150 l min^{-1} (5.3 cubic feet per min). Each 750 l air parcel (roughly equivalent to the volume of air human lungs exchange every 2 h) was impinged into 5 ml of sterile buffered saline by running the sampler for 5 min. The particular sampler utilized in this investigation was retrofitted to operate on seat-supplied AC power in place of manufacturer standard lead-acid batteries.

A total of 125 air samples were collected from business-class seat locations aboard eight domestic and eight international flights on a single commercial carrier. All samples were obtained from Boeing aircraft on either 747, 757 or 777 models (Table 1). In addition to offering access to AC power, business class seats provided the necessary space for obtaining, storing and refrigerating samples. Samples were collected over the entire course of each flight, including preboarding and postlanding whenever possible. In each instance, the sampling device was centrally positioned within the seat row, to ensure uniformity of the aerosols, and three replicate samples were taken at each time point. Immediately following sample collection, two replicates were stored on dry ice and a third was placed in a cooler chilled with ice packs to approximately 4 °C. A total of 110 samples were processed within 24 h for viability assessment (ATP and plate counts). Sample volumes remaining after viability assays were frozen

Table 1 Sample characteristics and summary of analyses performed on air samples collected from commercial cabin air environment

Sample ID	Departing from	Destination	Date	Aircraft[a]	Travel time	Travel distance (km)	Number of samples collected per flight	Estimation of		Bacterial		All samples were pooled and subjected to		
								Total microbial population[b]	Viable microbial population[c]	Enumeration of cultivable population[d]	Identification	DNA extraction	Total bacterial population measurement[e]	Phylogenetic analysis based on 16S rRNA gene sequencing
1-N	Los Angeles, USA	New York-JFK, USA	14/11/2005	757	5 h; 23 min	3960	7	+[f]	+	+	+	+	+	+
1R-N	New York-JFK, USA	Los Angeles, USA	17/11/2005	757	5 h; 55 min	3960	7	+	+	+	+	+	+	+
2-N	Los Angeles, USA	New York-JFK, USA	5/12/2005	757	5 h; 23 min	3960	5	+	+	+	+	+	+	+
2R-N	New York-JFK, USA	Los Angeles, USA	8/12/2005	757	5 h; 55 min	3960	7	+	+	+	NA	+	+	No amplicons[g]
3-L	Los Angeles, USA	London-Heathrow, UK	15/1/2006	777	13 h; 10 min	8730	10	+	+	+	+	+	+	No amplicons[g]
3R-L	London-Heathrow, UK	Los Angeles, USA	20/1/2006	777	11 h; 16 min	8730	8	+	+	+	NA	+	+	No amplicons[g]
4-S	Los Angeles, USA	Sydney, Australia	12/2/2006	747	14 h; 31 min	11 973	18	ND	ND	ND	ND	+	+	+
4R-S	Sydney, Australia	Los Angeles, USA	17/2/2006	747	13 h; 29 min	11 973	10	+	+	+	+	+	+	+
5-N	Los Angeles, USA	New York-JFK, USA	7/3/2006	757	5 h; 23 min	3960	4	+	+	+	+	+	+	+
5R-N	New York-JFK, USA	Los Angeles, USA	10/3/2006	757	5 h; 55 min	3960	5	+	+	+	+	+	+	+
6-J	Los Angeles, USA	Narita-Tokyo, Japan	3/4/2006	747	11 h; 18 min	8722	8	+	+	+	+	+	+	+
6R-J	Narita-Tokyo, Japan	Los Angeles, USA	7/4/2006	747	9 h; 57 min	8722	7	+	+	+	+	+	+	+
7-N	Los Angeles, USA	New York-JFK, USA	24/4/2006	757	5 h; 23 min	3960	6	+	+	+	+	+	+	+
7R-N	New York-JFK, USA	Los Angeles, USA	27/4/2006	757	5 h; 55 min	3960	6	+	+	+	+	+	+	+
8-S	Los Angeles, USA	Sydney, Australia	7/5/2006	747	14 h; 31 min	11 973	10	+	+	+	NA	+	+	+
8R-S	Sydney, Australia	Los Angeles, USA	7/5/2006	747	13 h; 29 min	11 973	10	+	+	+	NA	+	+	+

Abbreviations: NA, not applicable since cultivable bacteria were not isolated; ND, not determined.
[a]Total number of passengers in 747, 757 and 777 aircraft are 524, 243 and 524, respectively.
[b]Measured by total ATP.
[c]Measured by intracellular ATP.
[d]Bacteria were cultured by spread plating onto R2A agar.
[e]Measured by quantitative PCR.
[f]+, analysis performed.
[g]Due to low DNA concentration, PCR did not amplify 1.5-kb fragment of 16S rRNA gene and hence cloning and sequencing analyses were not possible.

for subsequent DNA extraction and phylogenetic analyses. Samples from flight 4-S (Los Angeles to Sydney, Australia) were subjected to the same DNA-based analyses described below, but the portions that would otherwise have been analyzed using viability assays were instead archived for future study.

Microbiological examination
The enumeration of bacterial colonies grown on R2A medium is referred to as 'heterotrophic plate counts' in this study. Since ATP can originate from bacteria, archaea and eukaryotic cells, intracellular-ATP measurements are described as 'total viable microbial population,' while total ATP values are termed 'total microbial population.' Although the ATP assay has a very sensitive lower limit, prior validation studies of this technology have set a threshold of 10^3 relative fluorescence units (RLU) m^{-3} of air to strengthen conclusions drawn from data sets (Venkateswaran *et al.*, 2003; La Duc *et al.*, 2004). The 16S rRNA gene copy numbers measured by the quantitative PCR (Q-PCR) assay are referred to as 'total bacterial population or 16S rRNA gene copies' because bacterial-specific primer-probe sets were used and because these estimates encompass viable and nonviable bacterial cells.

Hetrotrophic plate counts. Duplicate 100 μl aliquots of each sample were spread atop R2A agar (Difco Laboratories, Detroit, MI, USA) plates and CFU were enumerated following 7 days incubation at 25 °C. Each of the plates incubated at the various destination laboratories was enumerated before shipment to the Jet Propulsion Laboratory, where counts were verified and representative colonies were selected as described below. Isolates were aseptically picked, purified and cultured, and stored at −80 °C for further processing and analysis. When fewer than 10 colonies arose on a plate, all colonies were picked. When a greater number of colonies were present, 10 representatives were picked to capture a reasonably diverse collection of isolates. Colony morphology was considered when selecting isolates for further characterization. Identification of purified strains was determined via 16S rDNA sequence analysis as described elsewhere (La Duc *et al.*, 2004).

Total and viable microbial population. A commercially available ATP-assay kit (Checklite HS Plus, Kikkoman, San Francisco, CA, USA) was used according to the manufacturer's protocols, to measure both intracellular and total ATP present in each sampled air parcel. Detailed description of the ATP assay to estimate total and viable microbial population was reported previously (Venkateswaran *et al.*, 2003; La Duc *et al.*, 2007a). It has been documented that for most Gram-negative bacteria, 1 RLU corresponds to approximately 1 CFU, as compared to

Gram-positive bacteria where 1 CFU corresponds to roughly 5 RLU (La Duc *et al.*, 2007a). It should also be assumed that in regularly cleaned, low-humidity, HEPA-filtered environments, such as aircraft cabins, cells exist in a suppressed metabolic state and contain less ATP than actively growing cells (Stanley, 1989). Considering these circumstances, we conservatively estimated that a measurement of 1 RLU was approximately equal to 1 CFU (Venkateswaran *et al.*, 2003; La Duc *et al.*, 2004).

Total bacterial population. Total bacterial population regardless of viability was measured by Q-PCR as described below. All samples collected within an individual flight were pooled to increase the likelihood of obtaining sufficient quantities of nucleic acid for DNA-based analyses. DNA was extracted as described below, and ribosomal RNA gene copy numbers were quantified in triplicate using an MJ Research Chromo4 detection system. Universal bacterial primers targeting the 16S rRNA gene, 1369F (5′-CGGTGAATACGTTCYCGG-3′) and 1492R (5′-GGWTACCTTGTTACGACTT-3′), and the fluorescent-labeled probe TM1389F (5′-FAM-CTTGTA CACACCGCCCGTC-TAMRA-3′) were used in this quantitative analysis (Suzuki *et al.*, 2000, 2001). Each 50 μl reaction mixture consisted of 25 μl of 2X Taqman Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA), 0.8 μM of each oligonucleotide primer, 0.5 μM of oligonucleotide probe and 1 μl of template DNA. Reaction conditions were as follows: 95 °C denaturation for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s and a combined annealing and extension at 60 °C for 1.5 min. Standards were created using known amounts of full-length *Escherichia coli* 16S rRNA genes incorporated into suitable vector plasmids (Invitrogen, Carlsbad, CA, USA) and standard curves were repeated for each Q-PCR reaction.

DNA extraction and PCR amplification. Samples collected at all time points (∼5 ml sample per 750 l^{-1} air per time point) of each flight were pooled (4–10; Table 1) and concentrated in Amicon-Ultra 15 centrifugal filters (Millipore, Billerica, MA, USA) using a refrigerated centrifuge at 3000 r.p.m. for 20 min. Each filter unit has a molecular weight cutoff of 50 000 Da allowing isolation of all intact bacterial cells and naked DNA fragments greater than 100 bp. Amicon units were reusable for each sample set, making it possible for all samples from a single flight (∼20–50 ml) to be concentrated to a volume of 200 μl in a single filter tube. A comparable amount of sterile, unused cartridge buffer was concentrated in a separate filter tube to act as a control for each extraction. Total DNA was extracted for each concentrated pooling of flight samples using a standard lysozyme/phenol–chloroform procedure (Johnson, 1981; Ausubel *et al.*, 2001). We have previously reported the percent DNA recovery

using this solvent-based DNA extraction method for low-biomass samples to be $\sim 10\%$ (Bruckner et al., 2005; Bruckner and Venkateswaran, 2007). Briefly, samples were treated with lysozyme (final concentration 10 mg ml^{-1}) to degrade cell walls, followed by proteinase K and RNase treatment to remove unwanted biopolymers and finally phenol–chloroform to clean-up cellular debris. DNA was precipitated with two volumes of ice-cold ethanol, washed briefly with 70% ethanol and resuspended in TE buffer (30 µl) before being stored at $-80\,^{\circ}$C. Bacterial small subunit rRNA genes (~ 1.5 kb) were PCR-amplified (1–5 µl template) with a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA) using eubacterially biased primers 8F and 1492R, as described previously (La Duc et al., 2004).

Sequence analysis and phylogenetics. Sequences of chloroplasts and sequences that exhibited undetermined bases (N's) at a frequency of $>1\%$ were removed from the calculation. An alignment of ca. 20 000 homologous full and partial sequences available in the public ARB database (Ludwig et al., 2004) was used. Novel 16S sequences (ca. 1400 nucleotides) were aligned to their nearest neighbor using automated tools of the ARB software suite (Technische Universitat Munchen, Munich, Germany (http://www.mpi- 180bremen.de/molecol/arb/)). The resulting alignment was checked manually and corrected if necessary. Phylogenetic trees were reconstructed via maximum parsimony and neighbor-joining methods. GenBank accession numbers for the cultivable bacteria are EU379242–EU379312 and clones are EU341129–EU341298.

Statistical analysis. Statistical analyses were carried out using SPSS for Windows (SPSS Inc., Chicago, IL, USA). Comparison of paired sample sets was performed using Student's t-test and Mann–Whitney's U-test. Comparisons of more than two sample sets were carried out using Bonferroni adjusted analysis of variance tests. All statistical tests were carried out at the $\alpha = 0.05$ significance level. In addition, appropriate statistical analyses were performed according to the manufacturers' instructions using software specific for each assay or with the MS Excel software package. For example, an additional measurement was taken from samples when the coefficient of variation exceeded 10% for ATP analyses, and standard deviations were calculated from four individual replicates of each sample. Likewise, standard deviations from three replicates of each Q-PCR-amplified sample were generated and linear regression models were used to determine standard curves for each Q-PCR run. The averages of two measurements were calculated for each sample that underwent plate-count analysis.

Rarefaction analysis (Heck et al., 1975) and coverage calculations (Good, 1953) were applied to estimate the representation of the phylotypes in bacterial libraries. Operational taxonomic units

(OTUs) were defined as clones sharing $>97.5\%$ sequence identity (Stackebrandt and Goebel, 1994; Rossello-Mora and Amann, 2001; Lawley et al., 2004). The rarefaction curve was produced by plotting the number of OTUs observed against the number of clones screened using the Analytic Rarefaction 1.3 software (http://www.uga.edu/~strata/software/index.html). The coverage of clone libraries was calculated according to Good (1953) using the equation $C = (1 - (n1/N))100$, where C is the homologous coverage, $n1$ is the number of OTU's appearing only once in the library and N is the total number of clones examined. For the calculations, bacterial 16S rRNA gene sequences from all samples were combined into one maximum parsimony tree using the ARB software package. Jackknifing (100 permutations) was carried out as described (Ludwig et al., 2004).

Controls and lower detection limits of assays. Appropriate controls as established previously were used whenever necessary (La Duc et al., 2007b). Briefly, unopened Mesosystems cartridges served as negative buffer controls in all molecular assays in addition to water blanks, free of ATP and DNA. To address problems associated with microfluidic components, which transport sample buffer throughout the sampler, a 0.2-µm sterile Millipore disc filter was placed at the mouth of the Mesosystems air sampler and the collecting fluid in the sample-retaining container was assayed for all methodologies adopted in this study. Pure ATP (Sigma, St Louis, MO, USA) was decimally diluted and served as a standard curve for ATP analyses. Purified DNA from *Bacillus pumilus* ATCC 7061 was included in the PCR amplification protocols as a positive control. To prevent false negative results in PCR reactions associated with the presence of inhibitory substances, a known amount of DNA was extracted from *B. pumilus* and spiked (1 pg per reaction mixture) as an internal standard. None of the DNA extracts in this study inhibited the PCR reaction. The lower detection limits were >30 CFU ($= 2.0 \times 10^3$ CFU m^{-3}) for the cultivable plate-count assay, >50 RLU ($= 3.4 \times 10^3$ RLU m^{-3}) for ATP analysis and >100 copies ($= 6.7 \times 10^3$ copies m^{-3}) for Q-PCR analysis. Air sample collection from outside the airliner before take off and after landing was not possible due to security reasons. Sample collection in the gate area will not reflect the true environment and hence such attempts were not made.

Results

Heterotrophic plate counts

Plate counts on R2A media ranged from 0 to 10^3 CFU m^{-3}, and only 1.8% of all samples (2 of 108 samples) showed cultivable counts of 10^3 CFU m^{-3} or higher, none of which exceeded 2.7×10^3 CFU m^{-3} (Figure 1). No significant differences were detected

between plate counts on international flights ($n = 62$ samples) and those on domestic flights ($n = 46$ samples) when compared using Student's t-test and Mann–Whitney's U-test ($P = 0.34$ and $P = 0.46$; Table 2). Approximately 50% of cabin air samples collected during both domestic (25 of 46 samples) and international flights (31 of 62 samples) did not contain any cultivable bacterial counts. Average plate counts on individual flights ranged from below detection limit (2 of 15 flights) to 3.6×10^2 CFU m^{-3} (Figure 1). In general, plate counts fluctuated during

each flight, and no particular trends were noticeable over the course of any single trip.

Microbial population as measured by ATP. In general, total viable population as measured by the intracellular-ATP assay was in the range of below detection limit to 10^3 RLU m^{-2} (Figure 1) and was consistently 1–2 logs lower than total ATP measurements, suggesting that approximately 1–10% of the microbial load was viable (Supplementary Table 1). No patterns in viable microbial levels were observed either during a single flight or across multiple flights. Comparison of domestic and international samples using both Student's t-test and Mann–Whitney's U-test failed to detect a significant difference in total viable microbial population ($P = 0.81$ and $P = 0.23$; Table 2). On comparing heterotrophic counts with ATP-derived estimates of viable microbes, two flight segments contained no detected CFU (2R-N and 3R-L) and one flight segment showed no viable microbes (5R-N; Figure 1). Approximately 2 logs higher viable but yet to be cultivated microbes were noticed in 7 out of 15 flight segments (Supplementary Table 1) than corresponding plate counts. In addition, ~99% of the viable microbial population was not cultivable in three sets of commercial airline samples, all of which were obtained on domestic segments. Two
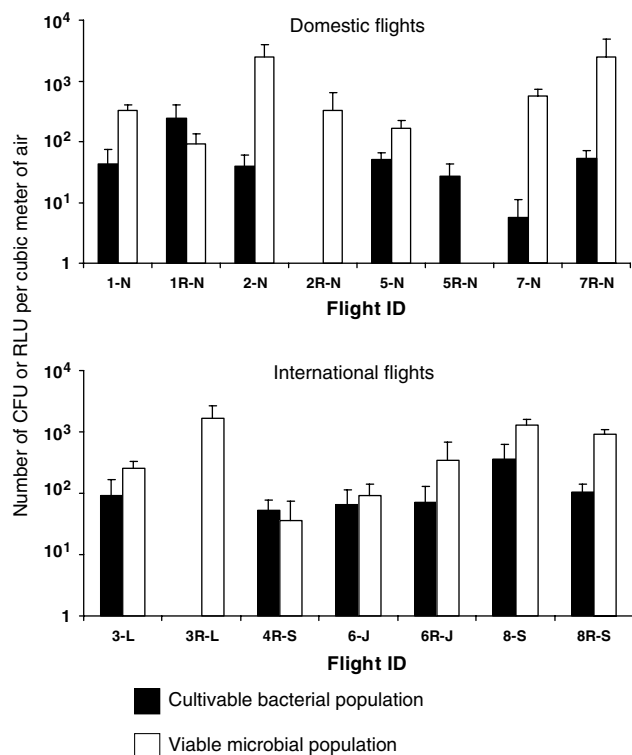

Figure 1 Microbial population of commercial airline cabin air. The solid bar represents cultivable bacterial population measured by R2A medium and viable microbial population estimated by intracellular ATP. Two measurements for cultivable bacterial counts and four replicates were carried out for total microbes. Number of samples for each flight segment is given in Table 1.

Table 2 Statistical analysis to compare microbial populations of cabin air from various domestic and international flights

Microbial population	Number of observations (n)		P-values[a]	
	Domestic	International	Mean[b]	Median[c]
Cultivable heterotrophic bacteria	46	62	0.340	0.460
Viable microbes[d]	46	63	0.810	0.230
Total microbes[e]	46	63	0.001	0.001

[a]$\alpha = 0.05$.
[b]Student's t-test.
[c]Mann–Whitney's test.
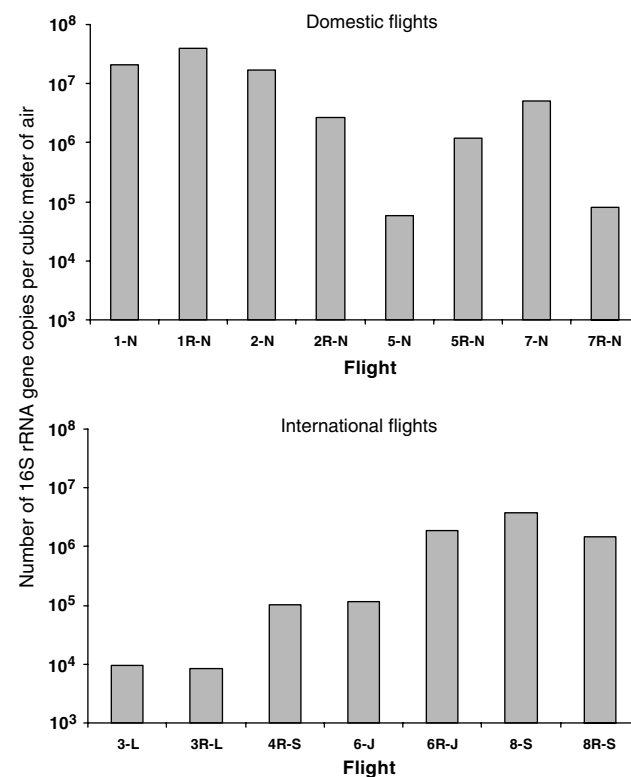[d]Measured by intracellular ATP.
[e]Measured by total ATP.

Figure 2 Total bacterial population as measured by 16S rRNA gene copy numbers of several domestic and international flight cabin air.

flights yielded samples with similar cultivable and viable counts.

Ranges in total microbial population were similar for both domestic (7.3×10^3–1.3×10^5 RLU m^{-3}) and international (8.7×10^3–3.7×10^5 RLU m^{-3}) cabin air samples and mean total microbes were 3.8×10^4 and 6.8×10^4 RLU m^{-3}, respectively (Supplementary Table 1). With the exception of flight 1R-N, all domestic flights (5 h flight time) showed a strong negative correlation ($R^2 = 0.51$–0.84) between total microbes and flight time (Figure 3). Five of seven international flights showed a very similar trend for the initial 5 h of travel ($R^2 = 0.69$–0.88; Figure 3). A less consistent, typically weaker positive correlation between total microbes and sampling time was noted after the 5-h mark on several international flights (Figure 3). Only 0–14% of samples collected aboard domestic segments yielded RLU values above 10^3 RLU m^{-3}, whereas 14–60% of samples collected from international flights exceeded 10^3 RLU m^{-3}.

Total bacterial population as measured by DNA. Bacterial 16S rRNA gene copies, enumerated via Q-PCR, ranged from 5.9×10^4 to 4.0×10^7 *rrn* gene copies per m^3 of air on domestic flights and 9.4×10^3 to 3.8×10^6 *rrn* gene copies per m^3 of air on international flights (Figure 2). Generally, domestic flight samples (6 of 8) typically contained from 10^6 to 10^7 *rrn* gene copies m^{-3}, while most international flight samples (5 of 7) were burdened with 10^5–10^6 *rrn* gene copies m^{-3}. Very low *rrn* gene copy number (10^3) was observed in the London/Los Angeles samples (3N and 3R-N) and coincided with difficulties in amplifying sufficient 16S rRNA gene fragments for subsequent clone library construction. Heterotrophic counts, as well as total and viable microbial population estimates (Figure 1), for these samples were similar to other flights.

Cultivable microbial diversity
Several α, β and γ-proteobacteria, as well as sporulating and nonsporulating Gram-positive bacteria, were isolated in varying abundance (Figure 4; Supplementary Table 2). Of the 112 bacterial isolates sequenced, approximately 86% were identified as Gram-positive, of which 60% were of high G/C
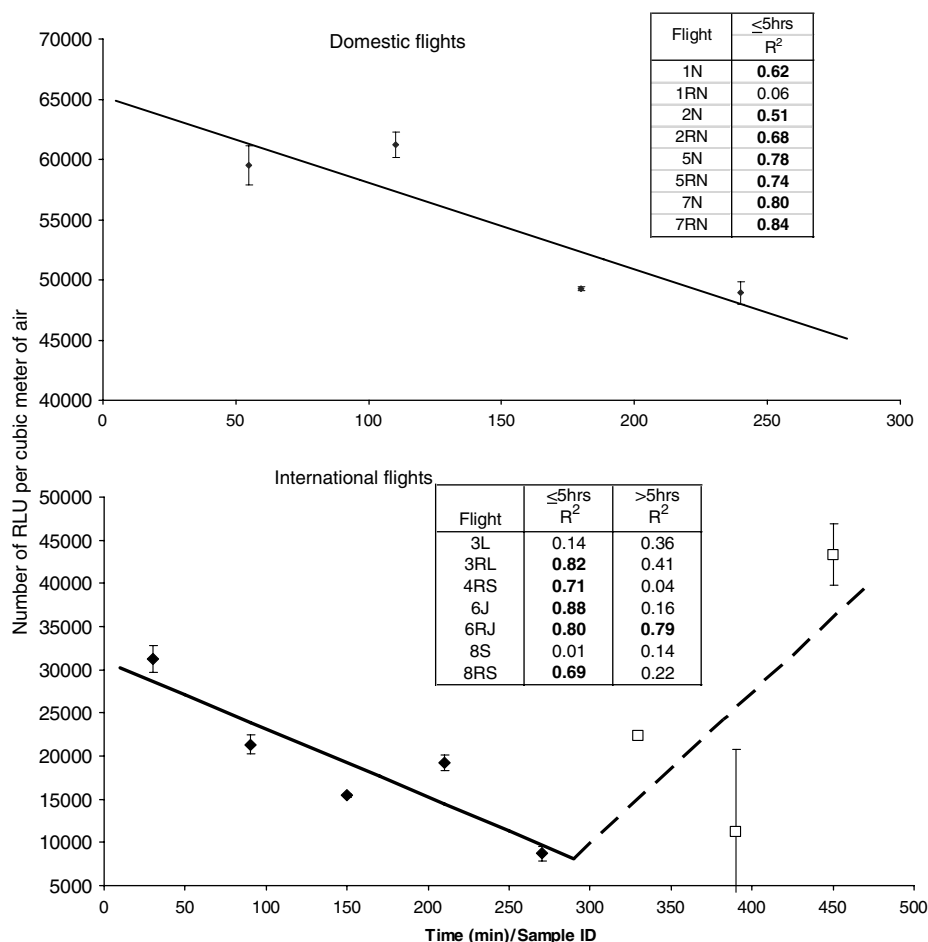

Figure 3 Changes in total microbial population of several domestic and international flight cabin air. Regression analyses of all flight segments for 5 h and more than 5 h are depicted. The letters in bold are significant at 95% confidence level. The changes in total microbial population as measured by total ATP with time for one flight each for domestic (7R-N) and international flights (6R-J) are depicted. Each plot consists of four measurements.
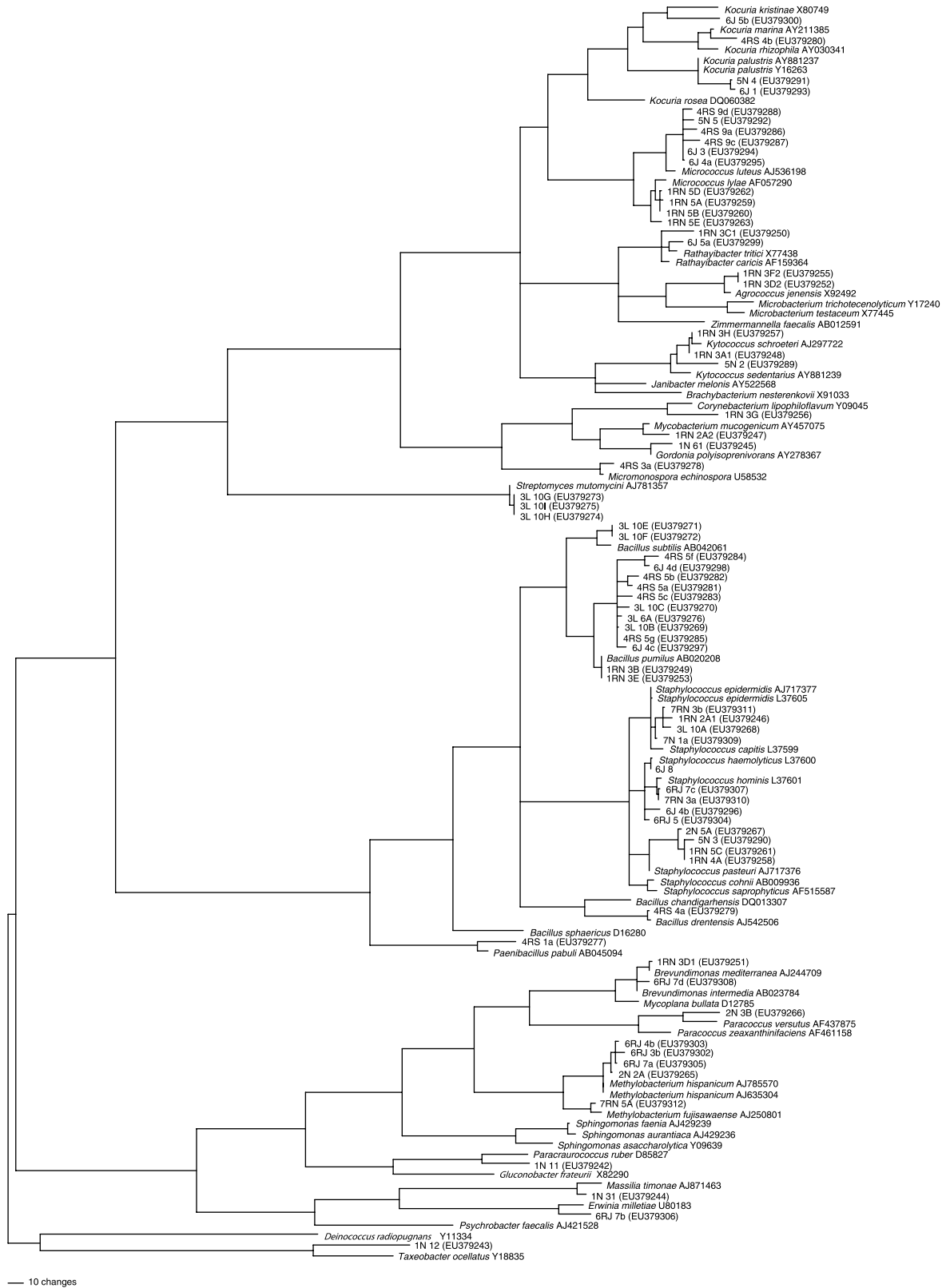
Figure 4 Cultivable bacterial diversity of commercial airline cabin air. Phylogenetic tree (maximum parsimony), showing the cultivable bacterial diversity and the phylogenetic affiliation of the sequences derived from different flights. The GenBank accession number of the closest neighbor of the reference strains is specified after the name and that of the isolated strain is given in parentheses. The strain IDs are flight numbers (1N, 1R-N and so on; refer Table 1) followed by isolate numbers (5b, 4b and so on). The scale bar shows a 10% estimated difference in nucleotide sequence positions.

content, including *Micrococcus*, *Kytococcus* and *Staphylococcus*. Staphylococci and micrococci were detected in high abundance, comprising 38 of 57 high-G/C non-spore-forming isolates. Spore-forming *Bacillus* species were also prevalent, with 19 of the 57 high-G/C Gram-positive strains belonging to this taxon. Members of the α-proteobacteria, such as *Methylobacterium* spp, comprised 10% of all bacterial strains isolated, complementary to results obtained from 16S rDNA-based clone library analyses. Five novel bacterial lineages were uncovered within the cabin air samples analyzed: two members of the α-proteobacteria (*Paracoccus* sp and *Paracraurococcus* sp), one flexibacteraceae (*Taxeobacter* sp) and two Gram-positive bacteria (*Corynebacterium* sp and *Bacillus* sp). The novelty of these strains was determined based on 16S rDNA sequence similarities of less than 97.5% with nearest neighbors in the GenBank public database (Stackebrandt and Goebel, 1994). Several bacteria implicated in human illness were also isolated, including *Janibacter melonis* (responsible for bacteremia), *Microbacterium trichotecenolyticum* –(the causative agent of neutropenia), *Massilia timonae* (a cause of cerebellar lesions), *Staphylococcus saprophyticus* (a cause of urinary tract infections) and *Corynebacterium lipophiloflavum* (the cause of bacterial vaginosis). No spatial or temporal patterns were observed in the isolation of any bacterial species.

Bacterial community analysis
The number of clones analyzed, total number of OTUs present and incidence in clone libraries are presented in Table 3. After critical examination of 2000 clone sequences (13 sample and 7 negative control libraries) to exclude chimeras and non-full-length 16S rRNA ($\sim$1.5-kb) gene sequences, 861 clones were selected for further analysis. Among these 861 quality clone sequences, 507 were removed from the calculations due to their presence in negative control libraries (Supplementary Table 3). Representatives of over 100 species, spanning 12 classes of bacteria, were identified in the remaining 354 clones. The coverage values for the domestic routes ranged from 78–96% as opposed to 57–90% for international segments. The lower coverage index for the international routes indicated that these samples possessed higher diversity than could be resolved with the number of clones sequenced. Two international routes exhibiting high numbers of singularly occurring OTUs were 4S (29 OTUs) and 8R-S (14 OTUs), yielding coverage percentages of only 56.7% and 68.9%, respectively. The two domestic clone libraries with high numbers of OTUs occurring only once were 5N (19 OTUs) and 7R-N (14 OTUs) each with $\sim$78% coverage.

The percent incidence of all clones was determined with respect to bacterial class (Table 4). Among all bacterial classes, sequences arising from proteobacteria were collected in greatest frequency (58% of all clone sequences). The predominance of α- and γ-proteobacteria was apparent on all flights: α-proteobacteria constituted at least 10% of the detected microbial diversity and in some instances comprised 100% of the diversity on an individual flight (Table 4). A similar level of species richness was observed in the Gram-positive bacteria (57 species), which accounted for 31% of all identified clone sequences. Nearly two-thirds of these species were Firmicutes, with the remaining 20 clones representing species of actinobacterial origin.

Table 3 Molecular microbial characterization of various segments of commercial airliner travel

Flight route	Sample ID number	Number of useful clones[a]	Number of OTUs[b]	n1[c]	Coverage (C) (1–((n1/N))100
Domestic routes					
Los Angeles to New York	1N	83	8	3	96.39
New York to Los Angeles	1R-N	68	8	3	95.59
Los Angeles to New York	2N	73	13	7	90.41
Los Angeles to New York	5N	88	25	19	78.41
New York to Los Angeles	5R-N	86	13	7	91.86
Los Angeles to New York	7N	70	13	7	90.00
New York to Los Angeles	7R-N	54	20	11	79.63
International routes					
Los Angeles, USA to Narita, Japan	6J	59	22	11	81.36
Narita, Japan to Los Angeles, USA	6R-J	55	10	6	89.09
Los Angeles, USA to Sydney, Australia	4-S	67	35	29	56.72
Sydney, Australia to Los Angeles, USA	4R-S	84	22	8	90.48
Los Angeles, USA to Sydney, Australia	8S	29	9	3	89.66
Sydney, Australia to Los Angeles, USA	8R-S	45	18	14	68.89

Abbreviation: OTU, operational taxonomic unit.
[a]Number of fully sequenced bacterial clones per sample. Sequences of chloroplasts, chimera and sequences that exhibited undetermined bases (*N*) at a frequency of >1% were removed from calculation.
[b]OTU is defined when the sequence similarity was >97.5% with the type strain sequence Good (1953).
[c]Number of OUT's appearing only once in the library.

Table 4 Bacterial diversity of cabin air samples collected during various domestic and international routes

Identity of the clone	GenBank number	% similarity	Number of clones retrieved from the air samples collected from												
			Domestic routes							International routes					
			1N	1R-N	2N	5N	5R-N	7N	7R-N	4S	4R-S	6J	6R-J	8S	8R-S
Acinetobacter calcoaceticus	Z93434	97.5									1				
Acinetobacter johnsonii	X81663	99.0								1	1				
Acinetobacter junii	X81664	98.7			3	1									
Acetobacter sp	X74066	97.4								1					
Actinomyces sp	X82453	95.0								1					
Aerococcus viridans	M58797	98.6													1
Agrobacterium sp	AB021493	95.6		44								2			1
Arsenicicoccus sp	AJ558133	97.4									1				
Bacillus flexus	AB021185	98.7								2					
Bacillus licheniformis	X68416	97.9								1					
Bacillus muralis	AJ628748	99.2													1
Bacillus pallidus	Z26930	99.5							3						
Bacillus pumilus	AY456263	99.3					1								
Bacillus weihenstephanensis	AB021199	99.2								1					
Bacillus sp	AB021198	95.4									1				
Bacteriovorax sp	M34125	89.6							1						
Bacteroides sp	M58762	94.8											1		
Brevibacillus choshinensis	D78459	99.2								1					
Burkholderia cenocepacia	AF148556	98.5										2			
Burkholderia sp	U96929	94.8	1												
Caulobacter sp	AJ009957	96.9												3	15
Caulobacter sp	AJ227758	97.1												2	
Citrobacter murliniae	AF025369	98.0									1				
Corynebacterium tuberculostearicum	AJ438050	99.9									5				
Curvibacter gracilis	AB109889	99.1						1							
Deinococcus sp	Y13038	86.2													1
Deinococcus sp	Y11329	89.2												2	1
Deinococcus sp	Y13041	89.5												15	12
Diaphorobacter nitroreducens	AB064317	99.7				1									
Erythrobacter sp	AY562220	97.0		3											
Exiguobacterium oxidotolerans	AB105164	98.0									1				
Facklamia sp	Y10772	93.2													1
Fusobacterium canifelinum	AY162220	98.4								1					
Gemella haemolysans	L14326	99.4									1				
Gemmata sp	X56305	89.3							1						
Gemmata sp	AJ231191	90.3													1
Granulicatella adiacens	D50540	99.1				1									
Haemophilus sp	M75045	97.2				1									
Haemophilus sp	M75076	97.4				1									
Herbaspirillum sp	Y10146	94.7													1
Janthinobacterium lividum	Y08846	99.4										1			
Kocuria rhizophila	Y16264	99.9										1			
Lactococcus lactis subsp cremoris	M58837	99.4				1						1			
Leptothrix mobilis	X97071	97.5							1						
Massilia sp	U54470	96.9			2										

Table 4 (Continued)

Identity of the clone	GenBank number	% similarity	Number of clones retrieved from the air samples collected from												
			Domestic routes							International routes					
			1N	1R-N	2N	5N	5R-N	7N	7R-N	4S	4R-S	6J	6R-J	8S	8R-S
Meiothermus sp	X84211	84.5						1							
Methylobacterium hispanicum	AJ635304	98.9											29	2	
Methylobacterium organophilum	D32226	99.3								1					
Methylobacterium sp	AJ635304	96.8											2		
Microbacterium kitamiense	AB013907	97.9										1			
Microcoleus sp	X70770	86.9						1							
Mycobacterium chitae	M29560	98.1			2					2					
Neisseria flavescens	L06168	97.5				1									
Neisseria sp.	L10738	96.6										1			
Novosphingobium sp	X94102	91.7							1						
Novosphingobium sp	AJ416411	95.1				1									
Novosphingobium sp	AF502400	95.8							2	1					
Pantoea agglomerans	U80202	99.0													1
Paracoccus carotinifaciens	AB006899	99.5								1					
Paracoccus sp	D32238	96.5								1					
Propionibacterium sp	AJ003058	93.3				1				1					
Pseudomonas hibiscicola	AB021405	98.8				2			1		1				
Pseudomonas putida	Z76667	99.7			1	1									
Pseudomonas saccharophila	AB021407	98.6						3							
Pseudomonas trivialis	AJ492831	98.9							1						
Pseudomonas sp	AB021390	95.1								1					
Psychrobacter sp	AJ313425	93.5	64												
Psychrobacter sp	AB016057	87.9	8												
Rhizobium leguminosarum	AY509899	97.6												2	2
Rhizobium sp	AF025852	92.3			1										
Rubrobacter sp	U65647	92.7										1			
Salmonella typhi	Z47544	99.1				1		1							
Serratia entomophila	AJ233427	98.8			1										
Sphingomonas sp	AJ429239	96.9										1			
Sporanaerobacter acetigenes	AF358114	100.0		1						1					
Staphylococcus arlettae	AB009933	99.5								1					
Staphylococcus caprae	AB009935	99.6								1	2				
Staphylococcus cohnii	AB009936	99.9				1									
Staphylococcus epidermidis	D83363	99.5				1	1					3			
Staphylococcus haemolyticus	X66100	99.0									2				
Staphylococcus hominis	X66101	98.7				2	1			1		2	4		
Staphylococcus pasteuri	AB009944	98.9												1	
Staphylococcus warneri	L37603	99.4								1	1				
Stella sp	AJ535711	90.7				1									
Streptococcus australis	AY485604	98.8										2			
Streptococcus mitis	AF003929	98.9							1	1	2	1			
Streptococcus parasanguinis	AF003933	98.9								1					
Streptococcus pseudopneumoniae	AY612844	98.4													1
Streptococcus sanguinis	AF003928	99.6												1	
Streptococcus thermophilus	X68418	98.1				1				1	2				1
Streptococcus sp	AF003930	96.1							2						

Table 4 (Continued)

Identity of the clone	GenBank number	% similarity	Number of clones retrieved from the air samples collected from												
			Domestic routes							International routes					
			1N	1R-N	2N	5N	5R-N	7N	7R-N	4S	4R-S	6J	6R-J	8S	8R-S
Streptococcus sp	AF003933	97.3								1					
Wautersia paucula	AF085226	98.9						3							
Williamsia maris	AB010909	96.9					5								
Williamsia sp	AB010909	96.3							1						
Total number of sequences			73	48	11	18	8	10	15	26	23	18	36	28	40

When compared with clone libraries from domestic flights, international flight clone libraries exhibited greater biodiversity. Gram-positive and α-proteobacterial sequences were retrieved more from the international flights (Table 4), whereas β- and γ-proteobacteria are far more common in domestic cabin air parcels (Table 4). A total of 183 full-length 16S gene sequences were obtained from 7 clone libraries of domestic flights while 171 sequences were obtained from the 6 clone libraries constructed from international flight samples. Of the 95 unique OTUs obtained from these 354 clones, 51 OTUs were found only on international flights, while 31 OTUs were confined exclusively to domestic flights (Figure 5). No sequences were so ubiquitous as to appear in all sampled flights of either domestic or the international-bound aircraft. Only 11 OTUs were detected on both domestic and international flights (Figure 5). The most widely observed bacteria in the domestic clone libraries were *Acinetobacter junii*, *Pseudomonas hibiscicola*, *Pseudomonas putida*, *Salmonella typhi*, *Staphylococcus epidermidis* and *Staphylococcus hominis*, each detected in two of the seven domestic clone libraries, whereas *S. hominis*, *Streptococcus mitis* and *Streptococcus thermophilus* sequences were retrieved in high abundance in the clone libraries of international flights.

With regard to the presence of potentially pathogenic bacterial species, it should be noted that pathogenicity is often strain specific, and the phylogenetic analyses employed may only detect OTUs with known disease-causing variants. Two species of disease-associated β-proteobacteria (*Burkholderia cepacia* and *Massilia timonae*) were retrieved at frequencies greater than 10% from at least one clone library, and 50% of all γ-proteobacterial sequences (20 species) originated in organisms with some connection to human illness (Supplementary Table 3). Six of the actinobacterial species had pathogenic activity in humans, and three of these composed over 10% of at least one clone library (Supplementary Table 3). Clones indicative of the presence of numerous firmicute pathogens (six clones) were retrieved in varying abundance, including *Staphylococcus aureus* and several streptococci.

Discussion

The efficiency of HEPA filters in retaining bacterial-sized particles and aerosols has been well documented and while specifications for modern filters vary, they typically exceed 99.97% for 0.3 μm particles and 98% for the most penetrating particle size of 0.1 μm. Viruses (0.01–0.02 μm) and larger bacteria/fungi (0.1–1.0 μm) are retained even more effectively (Aviation-Safety, 2004). Cabin air that has passed through an HEPA filter is considered comparable to the recirculated air used in operating
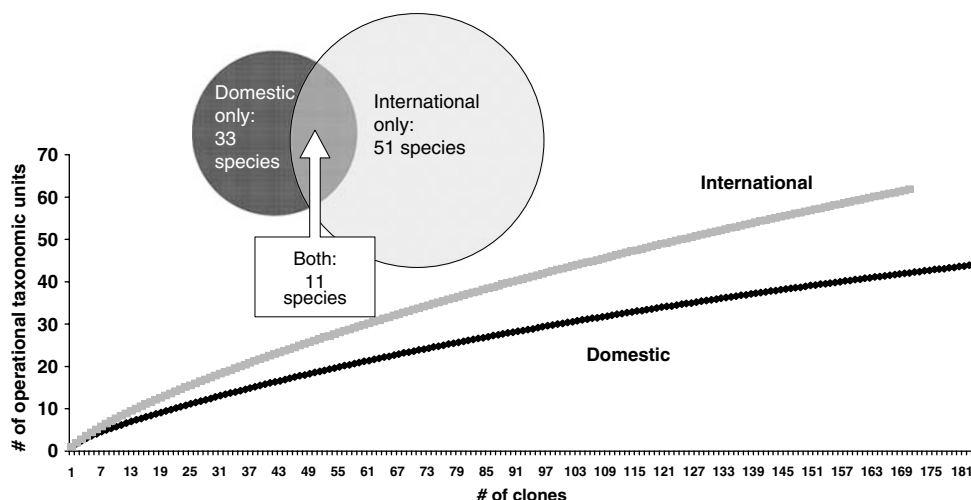
Figure 5 Rarefaction curves constructed for bacterial clone libraries from several international and domestic flight cabin air. Clones were grouped into OTUs at a level of sequence similarity of >97.5%. The overlapping OTUs between domestic and international flights are given in the inset.

rooms and infectious-disease containment facilities (WHO, 1998). When compared to standard office buildings, the air in a commercial aircraft is exchanged more frequently per hour and has even been described as 'sterile' by investigations carried out using conventional plate-count assays (FSF, 1998). According to the limited reports available, it has been documented that airborne microbial levels within airline cabins are much lower than public areas on the ground (CDC, 1995; Wick and Irvine, 1995). Unfortunately, these culture-based assessments of environmental samples grossly underestimate microbial levels and are ill suited for describing the community structures of the microbial populations that are present (Pace et al., 1985; Pace, 1997). Indeed, biomolecule-based examinations of these 'sterile' air samples collected directly from blowing sources, such as overhead gaspers, revealed high microbial levels and biodiversity, suggestive of biofilm formation in air ducts (La Duc et al., 2007b).

The power of molecular techniques in describing the microbial burden and diversity of the HEPA-filtered airline cabins examined was apparent, as heterotrophic plate counts typically underestimated total viable microbes by at least an order of magnitude (Figure 1). Statistical analyses failed to detect significant differences between domestic and international flights with respect to both heterotrophic plate counts and total viable microbes, most likely due to constant HEPA filtration, maintaining consistently low levels of airborne microorganisms. Measurements of 16S rRNA gene copy numbers (Figure 2) and total ATP (Figure 3), however, showed large overall variation between domestic and international-bound flights. Linear regression analysis revealed repeated patterns in the levels of total ATP levels present over the course of each

flight. Domestic flights (approximately 5 h in duration) and the initial 5 h of international flights typically demonstrated strong downward trends in measures of total ATP, which may be indicative of the progressive elimination of biological agents due to air exchange and continuous HEPA filtration. International flights showed a weaker upward trend in total ATP as flight time progressed beyond the five-hour point, possibly due to increase crew and passenger activity as the destination approached, or differences in air-handling protocols on longer flights. This much weaker association in the later portions of international flights indicates that a more complex set of factors may contribute to cabin cleanliness than can be correlated with the passage of time alone. Because exogenous sources (for example, food particles in the aerosols) could contribute to measurements of total ATP, the results of this assay cannot specifically be attributed to the microbial populations alone. They do, however, suggest that the length of international flight may have an impact on the aggregation of biological material that could, in the absence of HEPA filtration and air exchange, contribute to microbial persistence and proliferation.

Environmental cluster UniFrac analysis (Supplementary Figure 1; Lozupone and Knight, 2005; Lozupone et al., 2006) showed that the clone library composition of different cabin air samples did not cluster by any flight segment and was confirmed by Jackknife analysis. The only exception was the clone libraries from Sydney (8S and 8R-S), which clustered together with high confidence level (91% Jackknife analysis). When four samples collected from two separate trips to Sydney (segments 4 and 8) were subjected to UniFrac analyses, these clone libraries did not form any cluster. Analysis of biodiversity for domestic and international flights

indicated vastly different population structures. Figure 5 presents rarefaction curves and the population overlap for domestic and international clone libraries. The rarefaction curve for the domestic aircraft clones ($n = 522$) suggested better coverage of the biodiversity present aboard these aircraft. Despite a smaller number ($n = 339$), clone libraries of international-bound aircraft had a steeper rarefaction curve and demonstrated much greater biodiversity, being distributed among 62 bacterial species, while clones from domestic flights comprised only 42 species. Only 11 species were detected in both sets of clones, representing 26% and 18% of species detected on domestic flights and international flights, respectively. The cultivable bacterial diversity also indicated clustering of bacterial species with flight duration or destination. The isolation of staphylococci and micrococci on both domestic and international samples was expected, since cells in these genera are found in tight association with human skin cells, which are constantly shed. Interestingly, despite expectations that the artificially dry conditions aboard these flights would promote their prevalence, *Bacillus* isolates were not widely distributed across all flights and were limited almost entirely to longer international flights. It is important to note that while differences in microbial population structures correlated with flight duration, they may also be attributed to varying cabin design and air filtration on domestic and international flights. Internationally operated 747 and 777 airplanes have much larger passenger capacity (approximately two- to threefold) than that of domestically utilized 757 equipment (http://www.boeing.com/commercial). Passenger density as well as potential differences in air handling on larger planes may have significant impact on the biodiversity of air parcels obtained from these flights.

Although distinctions were observed by clustering clones from cabin air samples by destination (international vs domestic), taxonomic groupings were also generated on an individual, flight-by-flight basis (Figure 4; Table 4). Phylogenetic analysis revealed multiple monophyletic groups containing clones from a single flight, suggesting that each plane may harbor its own unique microbial consortium. Numerous DNA-based studies have noted that microbial communities vary significantly based on the location of sampling (Brodie et al., 2007; Moissl et al., 2007a, b). Microbial populations aboard aircraft could easily reflect this phenomenon, particularly if planes are dedicated to particular routes of travel. Unfortunately, confirmation of this hypothesis would require disclosure from airlines on the regular deployment schedules of their aircraft.

The empirical results of this study indicated the presence of a wide range of opportunistic human-related pathogens, including causative agents of pneumonia, bacteremia, neutropenia and cerebellar lesions (US-HHS-Department, 2003), and passengers and crewmembers are thought to be their principal source (Wick and Irvine, 1995; Dechow et al., 1997). It should be stressed that the retrieval of 16S rRNA gene sequences from these pathogens is not in itself evidence for public health concerns over commercial air travel. The detection of DNA arising from these pathogens does not imply viability, and the human pathogens detected in plate-count assay were overwhelmingly opportunistic, posing little threat to healthy individuals. Furthermore, the combination of engineering controls built into commercial aircraft, including frequent air exchange, HEPA filtration and directed cabin airflow (Aviation-Safety, 2004), as well as our own cultivation and viable microbial measurements (Figure 1) suggests that exposure to disease-causing microbes in doses sufficient for infection is unlikely during normal airline travel. Although high prevalence of health issues among travelers and aircraft crew members has been reported (Low and Chan, 2002; Whelan et al., 2003), these reports did not account for other factors that could be responsible for this correlation, such as travel-related stress on the immune system, or transient responses to environmental conditions within the air cabin. Recent studies have shown, for example, that symptoms experienced by air travelers, especially on flights greater than 3 h, may stem from low humidity (Nagda and Hodgson, 2001; Leder and Newman, 2005), and that increasing the humidity of the air cabin by as little as 3% is sufficient to significantly alleviate physical symptoms commonly reported by air crew (Norback et al., 2006).

The results of this study indicate that a much broader microbial diversity exists aboard commercial airplanes than was previously suspected. The advantages of the molecular-based approaches presented here rest in their potential for furthering the development of environmental-monitoring technologies capable of detecting premeditated bioterrorism (Hartley and Baeumner, 2003) and natural outbreaks of pandemic-causing agents (Schafer, 1999; US-HHS-Department, 2003; Sampathkumar, 2007). Biological sensors will rely on accurate and precise measurements of microbial levels and composition in public spaces, not only for equipment design, but also for validation and ongoing calibration.

Acknowledgements

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