Published in final edited form as:
J Allergy Clin Immunol. 2011 February ; 127(2): 372–381.e3. doi:10.1016/j.jaci.2010.10.048.

Airway Microbiota and Bronchial Hyperresponsiveness in Patients with Sub-optimally Controlled Asthma

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Abstract

Background—Improvement in lung function following macrolide antibiotic therapy has been attributed to reduction in bronchial infection due to specific bacteria. However, the airway may be populated by a more diverse microbiota, and clinical features of asthma may be associated with characteristics of the airway microbiota present.

Objective—To determine if relationships exist between the composition of the airway bacterial microbiota and clinical features of asthma, using culture-independent tools capable of detecting the presence and relative abundance of most known bacteria.

Methods—In this pilot study, bronchial epithelial brushings were collected from sixty-five adults with sub-optimally controlled asthma participating in a multicenter study of the effects of clarithromycin on asthma control, and ten healthy subjects. A combination of high-density 16S rRNA microarray and parallel clone library-sequencing analysis was used to profile the microbiota and examine relationships with clinical measurements.

Results—Compared to controls, 16S rRNA amplicon concentrations (a proxy for bacterial burden) and bacterial diversity were significantly higher among asthmatic patients. In multivariate analyses, airway microbiota composition and diversity were significantly correlated with bronchial hyperresponsiveness. Specifically, the relative abundance of particular phylotypes, including members of the Comamonadaceae, Sphingomonadaceae, Oxalobacteraceae and other bacterial families, were highly correlated with the degree of bronchial hyperresponsiveness.

Conclusion—The composition of bronchial airway microbiota is associated with the degree of bronchial hyperresponsiveness among patients with sub-optimally controlled asthma. These findings support the need for further functional studies to examine the potential contribution of members of the airway microbiota in asthma pathogenesis.

Keywords

microbiome; bacteria; asthma; 16S rRNA; PhyloChip

Introduction

Interest in the potential role of bacterial colonization or infection of the bronchial mucosa in the pathogenesis of asthma has been raised by several recent reports: associations between bacterial colonization of neonatal airways and subsequent asthma development[1], evidence of bronchial infection by specific intracellular bacteria among adult asthmatics[2], and the efficacy of prolonged macrolide antibiotic treatment in subsets of asthmatic patients[3–5]. While the role of microbial infection in asthma pathogenesis remains unclear, studies to date have primarily applied culture-based or targeted molecular approaches to identify specific bacterial species of interest in the airways. However, recent developments in defining the human microbiome have demonstrated that the composition of bacterial communities colonizing mucosal surfaces, rather than simply the presence of individual species, can be important in defining states of health or disease [6–11].

Culture-independent microbiota profiling based on sequence polymorphisms in the 16S rRNA gene, present in all bacteria, has been widely applied in environmental ecological studies[12–14], and in studies of the human 'superorganism' [9–11]. 16S rRNA-based phylogenetic analysis via sequencing or phylogenetic arrays, permits detection of uncultured or fastidious bacteria, providing information on community composition without *a priori* knowledge of those present[15–17]. One such platform, the 16S rRNA PhyloChip, has been applied in a number of environmental [12, 14, 15, 18] and clinical studies[6,19,20]. This high-density array contains ~500,000 probes that can differentiate ~ 8,500 bacterial taxa (defined

as groups of organisms sharing ≥ 97% 16S rRNA sequence homology)[15, 21] and demonstrates greater resolution of complex bacterial communities than traditional 16S rRNA clone library-sequencing approaches[6, 18, 21]. It provides an ideal tool to examine relationships between microbiota composition, including the presence and relative abundance of members of the 'rare biosphere'[22], and clinical characteristics of disease.

Recognizing the frequency with which the tracheobronchial tree is exposed to the external environment and to secretions from the oropharynx or upper gastrointestinal tract, we hypothesized that complex bacterial communities may colonize asthmatic airway mucosa and exhibit relationships with clinical features of disease. Therefore, in conjunction with a prospective study of the effects of extended clarithromycin therapy in adults with sub-optimally controlled asthma[23], we conducted a pilot study of the airway microbiota in sixty-five adult asthmatic and ten healthy subjects using the PhyloChip and parallel clone library-sequencing. Bronchial airway samples from asthmatics possessed greater bacterial burden and diversity than healthy individuals. Furthermore, the degree of bronchial hyperresponsiveness exhibited by subjects was related to community composition and the relative abundance of specific bacterial families comprising the airway microbiota. Portions of these results have previously been presented in abstract form[24, 25].

Materials and Methods

For supplemental methods, please see the Online Repository.

Subjects

Bronchial epithelial samples for microbial analysis were obtained from a subset of subjects enrolled in the Macrolides in Asthma (MIA) study[23] (NCT00318708, clinicaltrials.gov) conducted by the NHLBI-sponsored Asthma Clinical Research Network. Briefly, adults with clinically stable but sub-optimally controlled asthma, defined as persistent symptoms on Asthma Control Questionnaire[26] after four weeks of standardized treatment with inhaled fluticasone, were studied. Relevant inclusion/exclusion criteria and study procedures are described in the supplemental methods, including exclusion for symptoms of respiratory infection or asthma exacerbation within 6 weeks of the bronchoscopy visit. Bronchoscopy was performed prior to randomization to clarithromycin or placebo therapy. Three protected specimen bronchial brushings for microbiome analysis were obtained and sent to the University of California, San Francisco, where ten healthy, non-smoking, non-atopic adults without asthma were also enrolled. All protocols were approved by the Institutional Review Board at each center.

Sample processing

All samples first underwent screening by PCR for presence of the 16S rRNA gene, using extracted total DNA (100 ng) and the universal 16S rRNA primers, Bact-27F and Bact-1492R[27] in a 40-cycle reaction. Samples were deemed positive or negative for bacteria based on presence or absence of a visible 16S rRNA PCR product. Samples with any evidence of a 16S rRNA PCR product were subsequently subjected to eight-PCR reactions (per sample) using the same primer set across a temperature gradient (48–58 °C) to maximize bacterial diversity captured. Amplicons from each sample were then pooled, purified, and gel-quantified using E-gels® (Invitrogen) prior to hybridization of a standardized quantity to the array for each sample, as previously described[6,15].

Quantitative PCR (Q-PCR) analysis

Q-PCR was performed for twenty-one samples to determine total 16S rRNA copy number, normalized to β-actin copy numbers. Further details are provided in the supplemental material.

Data analysis

Relationships between microbiota structure and study variables were analyzed using multivariate statistics in combination with the Multi-response Permutation Procedure and Indicator Species Analysis (MRPP) and non-metric multi-dimensional scaling (NMDS) ordination[28]. Data were log-transformed prior to analysis where appropriate. Sorensen distance-based dissimilarity matrices were calculated and used for subsequent NMDS analysis to examine the relatedness of samples based on their microbiota composition. Three asthmatic samples with mean relative Sorensen distances greater than two deviations from the pooled mean were excluded for this analysis. Pearson's correlations were performed to determine relationships between the relative abundance of all detected phylotypes and study variables with q-value corrections for false discovery[29]. Significance was conservatively based on a false discovery rate of < 3% and a probability of no more than two taxa being falsely identified as significant for each test. Community diversity was estimated using Shannon indices[30], based on the number of taxa present (community "richness") and their relative abundance (community "evenness"). Statistical analyses were conducted using PC-ORD v5[31], JMP v7 (SAS Institute, 2007) and R software v.2.7.1 (http://www.r-project.org).

16S rRNA clone library-sequencing

Clone libraries for a subset of patients were constructed (using the same 16S rRNA amplicon pool hybridized to the array) and sequenced at the Department of Energy, Joint Genome Institute (299–374 clones per library). Quality filtered sequences were aligned and chimera-checked using NAST[32] and Bellerophon[33], respectively, then classified by 'G2-chip' taxonomy (www.greengenes.lbl.gov[34]).

Phylogenetic tree construction

Representative 16S rRNA sequences for taxa of interest were exported from the Greengenes database. A neighbor-joining tree with nearest-neighbor interchange was produced using FastTree[35]. Trees were annotated using the Interactive Tree of Life (http://www.itol.embl.de/)[36].

Results

Airway bacterial burden

Bronchial brushings were obtained from a total of 75 individuals, 65 asthmatic and 10 healthy controls. Subject characteristics are summarized in Table I. All asthmatics fulfilled entry criteria for the parent MIA trial[23], and samples for this pilot study were processed as they were received with no additional selection criteria applied. On initial screening, 54 of 65 (83%) asthmatics and 8 of 10 (80%) controls exhibited a visible 16S rRNA PCR product. However, not all PCR-positive samples subsequently produced sufficient 16S rRNA amplicon for PhyloChip analysis. While very small amplicon concentrations may be hybridized to the array, in our experience, application of 250 ng provides good characterization of the respiratory microbiota[8,19]. A greater proportion of asthmatic (37/65, 56.9%) than healthy subjects (3/10, 30%) produced 250 ng of amplicon, although this difference was not significant ($p = 0.17$). To increase the number of samples for comparative analyses, we analyzed the community composition detected by PhyloChip in six paired samples using both 100 ng and 250 ng of amplicon. Finding no significant difference in the

community composition detected in paired samples ($p_{MRPP} > 0.1$), seven additional subjects (5 asthmatic, 2 healthy) were assayed by PhyloChip using 100 ng of amplicon. Therefore, array data from a total of 47 subjects (42 asthmatic, 5 healthy) was obtained for the subsequent analyses (See Figure E1 in the Online Repository).

To confirm that 16S rRNA amplicon concentrations (ng/μL) reported by gel quantification accurately reflected bacterial burden, we performed total 16S rRNA Q-PCR and regression analysis on these independent measurements, which demonstrated strong concordance ($r = 0.69$; $p < 0.001$). This data also confirmed that samples with sufficient amplicon for array analysis had significantly greater 16S rRNA copy number compared with those unable to be analyzed by PhyloChip ($p = 0.002$). Therefore, gel-quantified 16S rRNA amplicon concentrations were used as a proxy for bacterial burden to examine potential relationships between airway bacterial burden and clinical variables. Of the samples analyzed by PhyloChip, bacterial burden was significantly higher in asthmatic samples compared to controls ($p = 0.008$; Figure 1). Bacterial burden appeared to decline with increasing methacholine PC_{20} measurements albeit this did not reach statistical significance ($r = -0.21$; $p = 0.09$), suggesting that community composition rather than actual bacterial burden may be more related to this clinical feature.

Airway microbiota structure and clinical features of asthma

Phylogenetic analysis identified 1,941 bacterial taxa (detected in at least one subject), representing 161 bacterial families (see Table E1 in the Online Repository). As a form of validation, we compared the phylogenetic distribution of airway microbiota detected by the array with reference trees for airway bacteria reported in the Human Microbiome Project (http://www.hmpdacc.org/reference_genomes.php; accessed May 13, 2010)[37] and observed good agreement in the types of bacteria reported by both (data not shown). Community richness across asthmatic samples was variable and ranged from 48 to 1,240 taxa. Of the five healthy subjects, three exhibited low community richness (200 to 253 taxa) while two demonstrated greater richness (844 and 1,121 taxa), suggesting that variability in airway microbiota composition was not exclusive to asthmatic patients.

We found no association between study center and variation in microbiota composition, indicating that sample collection by different operators in distinct geographic regions was not a major influence on community composition in this study. Multivariate analyses also showed no association between microbiota composition and spirometry measurements (FEV_1 and FVC), sputum eosinophil or neutrophil percentage, history of exacerbations or systemic corticosteroid treatment within the previous two years. However, microbiota structure differed significantly based on asthmatic or healthy group assignment ($p_{MRPP} = 0.028$), and NMDS analysis demonstrated that variability in airway microbiota composition was correlated with both the degree of airway hyperresponsiveness (methacholine PC_{20} concentrations; $r = 0.56$, $p < 0.001$; Figure 2A) and bacterial burden ($r = -0.36$, $p = 0.015$; Figure 2B). This suggests that the types and relative abundance of bacteria present in the airways are associated with these two variables.

Airway bacterial diversity, community composition, and bronchial hyperresponsiveness

Asthmatic subjects possessed greater airway microbiota diversity than controls ($p = 0.012$). Methacholine PC_{20} concentrations were inversely correlated with diversity ($r = -0.46$, $p < 0.003$; Figure 2C), suggesting a strong relationship between increased airway bacterial diversity and greater bronchial hyperresponsiveness.

To determine which of the airway microbiota were most associated with bronchial hyperresponsiveness, relationships between PC_{20} and relative abundance of all taxa detected

were analyzed. After false discovery correction, ~100 taxa demonstrated a significant ($p < 0.01$, $q \leq 0.015$) linear relationship between increasing relative abundance and greater bronchial hyperresponsiveness (Figure 3. See also Table E2 in the Online Repository). These taxa, primarily belonging to the Proteobacteria, represented 31 bacterial families such as the Comamonadaceae, Sphingomonadaceae, Nitrosomonadaceae, Oxalobacteraceae, and Pseudomonadaceae, and included a number of potentially interesting organisms. While samples were collected in a manner to minimize oral secretion contamination (e.g. use of triple-lumen protected specimen brushes, sample collection early in the procedure preferentially from upper lobes), we recognized the potential for organisms associated with the oropharynx to reside in the lower airways. Thus we investigated whether these ~100 phylotypes have previously been identified in the oral cavity by interrogating both the extensive human oral microbiome sequence database[38] and PUBMED, using species or genus names and the search terms “oral”, “oropharynx” “mouth”, or “tongue”. The majority (87.5%) of these phylotypes were not found referenced to the oral cavity in either database.

Post-treatment bronchoscopy was not performed in the parent study, therefore examination of relationships between clarithromycin-induced changes in airway microbiota composition and treatment outcomes was not possible. Instead, we explored whether relationships existed between pre-treatment airway microbiota and post-treatment clinical outcomes. Airway bacterial diversity was significantly greater in asthmatic subjects who demonstrated a significant reduction in bronchial reactivity in response to clarithromycin treatment (defined as at least a doubling in methacholine PC_{20} dose before and after treatment) than those with a smaller reduction ($p = 0.03$, Figure 4). This pattern was not evident in patients who received placebo intervention ($p = 0.64$).

16S rRNA clone library-sequencing validation

The PhyloChip, like other array technologies, is potentially susceptible to cross-hybridization, despite features of the array design intended to minimize this. To validate array-based findings, 16S rRNA clone libraries were constructed utilizing the same amplicon pool hybridized to the array for a subset of asthmatics (n=6). An average of 333 clones per sample were analyzed yielding ~2,000 non-chimeric sequences representing 197 taxa, the majority of which were present in more than one sample. The majority of bacterial sub-families identified by sequencing were also present in the corresponding array dataset, validating large numbers of array-based positive hits (Table II). Two sub-families (Fusobacteriaceae sub-family 3 and Prevotellaceae sub-family 1) were detected by sequencing only in two samples (one sub-family per sample). However, taxa within these sub-families were very close (pf =0.84 and 0.75, respectively) to the conservative arbitrary cut-off ($pf \geq 0.9$) used to define array-based presence or absence[15]. Clone library analysis also indicated that two samples (AB01 and AB04) were each dominated by a specific bacterial family. The majority of sequences for sample AB01 were classified as Pasteurellaceae sub-family 1 [this sample also exhibited the lowest array-reported community richness (48 taxa)], while for subject AB04, a majority of the sequences belonged to the Cellulomonadaceae.

Discussion

To our knowledge this is the first study to examine whether specific aspects of the airway microbiota are related to relevant clinical or physiologic features of asthma. Our findings suggest that bacterial diversity, variations in community composition, and the relative abundance of specific phylotypes, are associated with the degree of bronchial hyperresponsiveness in asthmatics administered inhaled corticosterids. This culture-independent study also expands the repertoire of organisms of potential relevance to asthma pathogenesis within this cohort beyond those previously implicated[1,2]. These data, together

with recent studies in other asthmatic patients[7], suggest that the microbiome of the airways, as with other discrete host niches, may be an important contributor to asthma pathophysiology and to the heterogeneity of disease[39–41].

Overall, these findings highlight two key aspects in defining airway health. The existence of a bronchial microbiota suggests that the bronchial tree is unlikely to be completely sterile, and that the composition of its microbiome may directly or indirectly modulate airway function. The role of airway microbiota in airway disease is under-investigated compared with other human niches such as the intestinal microbiota, which has a demonstrated role in chronic inflammatory gastrointestinal diseases[10, 42]. That a bronchial epithelia-associated microbiota exists is not entirely unexpected, particularly in the setting of airway disease and/or inhaled corticosteroid (ICS) therapy. Specific bacterial communities persist in the airways despite antimicrobial treatment in patients with COPD and cystic fibrosis[8,19]. Recently, Hilty and colleagues also described airway microbiota in patients with mild to moderate asthma (all on ICS therapy), COPD, and healthy subjects[7]. Those with airway disease had an increased prevalence of pathogenic Proteobacteria compared to controls, although relationships to clinical measurements were not examined in their study.

Complex microbial communities are now recognized to reside in various host mucosal sites[43], where perturbations of community structure are associated with disease[6, 8, 10, 42]. Moreover, the airway epithelium is increasingly recognized as important in immunologic responses to environmental and microbial exposures[44]. Thus, the airway microbiome could potentially influence presentations of asthma. As such, our findings of specific community relationships with bronchial hyperresponsiveness, in the context of a well-characterized asthmatic cohort receiving standardized baseline treatment, implicate additional bacterial groups in asthma. These include a *Nitrosomonas* spp. possessing a functional nitric oxide reductase[45]. Their relative abundance may be a microbial indicator of airway concentrations of nitric oxide which, though not assessed in this study, reflect airway inflammation[46] and correlate with measures of airway hyperresponsiveness[47]. Also notable is *Oxalobacter formigenes* (Oxalobacteraceae), an anaerobic bacterium known to be susceptible to macrolide antibiotics[48]. Other bacteria of interest include the Comamonadaceae and Sphingomonadaceae. Members of the Comamonadaceae, previously identified in cystic fibrosis patients[49], possess steroid-responsive degradation pathways[50, 51], and their presence could plausibly be related to the selective pressure of ICS therapy. This raises the intriguing possibility that steroid non-responsiveness, as observed in some asthmatics despite adherence to therapy[52], may be due in part to the presence of airway bacteria with steroid-degrading capacity.

Given the existence of airway microbiota, it is conceivable that coincident presence of multiple potentially pathogenic bacteria contribute to persistent airway inflammation in asthma. This possibility is supported by the finding of neutrophilia in bronchial airway samples of some patients with severe asthma despite regular use of high-dose inhaled corticosteroids[53]. Such inflammation could represent an appropriate response by the host to inappropriate airway microbial colonization. For example, Sphingomonadaceae, members of which were significantly correlated with bronchial hyperresponsiveness, are characterized by the presence of cell membrane glycosphingolipids, which are recognized by and can activate invariant natural killer T (iNKT) cells resulting in induction of IL-4 and IL-13[54, 55]. Glycolipid activation of iNKT cells can also induce airway hyperreactivity independent of conventional $CD4^+$ T cells[56]. While the presence of iNKT cells in asthmatic airways remains controversial[57, 58], our findings suggest a possible role for Sphingomonadaceae in airway pathophysiology and the possibility that the observed variability of iNKT cell populations in asthmatics may depend in part on the relative abundance of these species.

We recognize that this pilot study is limited by the relatively small number of subjects, especially of healthy controls, and by the absence of asthmatics not taking an ICS. Nonetheless, the data indicate that airway bacterial burden in individuals without airway disease is much lower than in asthma patients requiring ICS therapy. As all asthmatics were required to be on standardized ICS for the parent study[23], we are unable to infer whether the higher bacterial burden among asthmatics is a function of the disease itself or ICS treatment, although the relationships of bacterial burden, and the composition and diversity of the microbiota, to bronchial hyperresponsiveness, are robust. Determining the effects of ICS use on the airway microbiome will require further investigation and is an important question, given the wide use of ICS therapies in airway diseases, which, among patients with chronic obstructive pulmonary disease, has been associated with an increased risk of pneumonia[59].

The array-based analysis in this study identified a greater diversity of airway microbiota than has previously been described, although to our knowledge there has been only one other study involving asthmatics that applied 16S rRNA phylogenetic analysis[7]. In addition to clone library-sequencing validations, our results also agreed with those of Hilty *et al*., in that we detected all bacterial phyla and genera identified in their study of the lower airways of eleven adult and thirteen pediatric asthma patients on ICS therapy. The PhyloChip platform permits in-depth analysis of relatively large numbers of samples using a standardized assay that applies several levels of stringent criteria for determining the microbiota profile in a given sample. It is impractical to perform sequence corroboration for every array-based positive hit. Moreover, the extent of microbial diversity detected is dependent on the community structure and the depth of sequencing performed[60]. Our study included the largest number of clones sequenced to date for asthmatic airway samples and confirmed the presence of many array-detected phylotypes, indicating that complex microbiota do exist in this niche.

Finally, we observed that asthmatics exhibiting a significant decrease in bronchial hyperresponsiveness post-clarithromycin treatment in the parent study, possessed greater pre-treatment airway bacterial diversity than did non-responders. Despite the small number of subjects in this exploratory analysis, the trend is interesting given that prior studies have also observed decreased bronchial responsiveness following prolonged macrolide therapy[4, 61].

Furthermore, while the antimicrobial susceptibility of the entire airway microbiota is unknown, conceivably a multitude of community members may be sensitive to macrolides, resulting in a reduction of bacterial diversity and/or burden upon treatment and manifesting clinically in a reduction in bronchial responsiveness. Thus, while the efficacy of macrolides in airway disease is often attributed to their anti-inflammatory properties, their effectiveness could also reflect an extended spectrum of antibacterial activity against members of the airway microbiota, whose composition, as in the setting of ICS, may influence outcomes.

In summary, several features of the airway microbial community are significantly associated with the degree of bronchial hyperresponsiveness among patients with sub-optimally controlled asthma. Despite limitations, these study findings are notable in providing the first evidence for the potential functional, physiologic and clinical relevance of the airway microbiome in asthma. Our results suggest that variations in airway microbiome structure and function may exert distinct effects that contribute to asthma heterogeneity and provide novel targets and hypotheses for future studies on disease mechanisms.

Clinical implications

Relationships between airway microbiota and clinical features of asthma were investigated using culture-independent approaches. Among sub-optimally controlled

adult asthmatics, bronchial hyperresponsiveness correlated strongly with airway microbiota composition and diversity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NHLBI (U10 HL 074204), and by the Strategic Asthma Basic Research Center at UCSF, supported by the Sandler Family Foundation. Y.J.H. was funded by NIH/NHLBI T32 HL007185 and by University of California Tobacco-related Disease Research Program award (17FT-0040). C.E.N. is funded by NSF 0709975 (to C.E.N. and J.M. Melack). S.V.L. is funded by NIH/NIAID U01 AI075410. E.L.B. and T.Z.D. are funded under the auspices of the University of California under contract number DOE DE-AC02-05CH11231.

The authors wish to express their thanks to the Steering Committee of the Asthma Clinical Research Network, which permitted, indeed encouraged, our use of clinical data from the Macrolides in Asthma trial for our analysis of relationships with bronchial microbial community composition, and also to the ACRN investigators* and coordinators who obtained and provided the protected bronchial brush samples from asthmatic subjects. The authors also thank Yvette Piceno and Gary Andersen at Lawrence Berkeley National Lab for their PhyloChip expertise and advice.

Abbreviations

16S rRNA	16S ribosomal RNA
ACRN	Asthma Clinical Research Network
ACQ	Asthma Control Questionnaire
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
ICS	inhaled corticosteroids
IgE	immunoglobulin E
MRPP	Multi-response Permutation Procedure and Indicator Species Analysis
NHLBI	National Heart, Lung and Blood Institute
NMDS	non-metric multidimensional scaling
PC_{20}	methacholine concentration inducing a 20% decrease in FEV_1

References

1. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. N Engl J Med 2007;357:1487–95. [PubMed: 17928596]
2. Martin RJ, Kraft M, Chu HW, Berns EA, Cassell GH. A link between chronic asthma and chronic infection. J Allergy Clin Immunol 2001;107:595–601. [PubMed: 11295645]
3. Black PN, Blasi F, Jenkins CR, Scicchitano R, Mills GD, Rubinfeld AR, et al. Trial of roxithromycin in subjects with asthma and serological evidence of infection with Chlamydia pneumoniae. Am J Respir Crit Care Med 2001;164:536–41. [PubMed: 11520711]

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4. Kostadima E, Tsiodras S, Alexopoulos EI, Kaditis AG, Mavrou I, Georgatou N, et al. Clarithromycin reduces the severity of bronchial hyperresponsiveness in patients with asthma. Eur Respir J 2004;23:714–7. [PubMed: 15176685]
5. Kraft M, Cassell GH, Pak J, Martin RJ. Mycoplasma pneumoniae and Chlamydia pneumoniae in asthma: effect of clarithromycin. Chest 2002;121:1782–8. [PubMed: 12065339]
6. Flanagan JL, Brodie EL, Weng L, Lynch SV, Garcia O, Brown R, et al. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with Pseudomonas aeruginosa. J Clin Microbiol 2007;45:1954–62. [PubMed: 17409203]
7. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. PLoS One 2010;5:e8578. [PubMed: 20052417]
8. Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, et al. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and Pseudomonas aeruginosa. Environ Microbiol 2010;12:1293–303. [PubMed: 20192960]
9. Ledder RG, Gilbert P, Huws SA, Aarons L, Ashley MP, Hull PS, et al. Molecular analysis of the subgingival microbiota in health and disease. Appl Environ Microbiol 2007;73:516–23. [PubMed: 17085691]
10. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4. [PubMed: 19043404]
11. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 2008;455:1109–13. [PubMed: 18806780]
12. Brodie EL, DeSantis TZ, Parker JP, Zubietta IX, Piceno YM, Andersen GL. Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A 2007;104:299–304. [PubMed: 17182744]
13. Green BD, Keller M. Capturing the uncultivated majority. Curr Opin Biotechnol 2006;17:236–40. [PubMed: 16701994]
14. Lin LH, Wang PL, Rumble D, Lippmann-Pipke J, Boice E, Pratt LM, et al. Long-term sustainability of a high-energy, low-diversity crustal biome. Science 2006;314:479–82. [PubMed: 17053150]
15. Brodie EL, Desantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, et al. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 2006;72:6288–98. [PubMed: 16957256]
16. Sagaram US, DeAngelis KM, Trivedi P, Andersen GL, Lu SE, Wang N. Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. Appl Environ Microbiol 2009;75:1566–74. [PubMed: 19151177]
17. Schonmann S, Loy A, Wimmersberger C, Sobek J, Aquino C, Vandamme P, et al. 16S rRNA gene-based phylogenetic microarray for simultaneous identification of members of the genus Burkholderia. Environ Microbiol 2009;11:779–800. [PubMed: 19396938]
18. La Duc MT, Osman S, Vaishampayan P, Piceno Y, Andersen G, Spry JA, et al. Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. Appl Environ Microbiol 2009;75:6559–67. [PubMed: 19700540]
19. Huang YJ, Kim E, Cox MJ, Brodie EL, Brown R, Wiener-Kronish JP, et al. A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. OMICS: A Journal of Integrative Biology 2010;14:9–59. [PubMed: 20141328]
20. Cox MJ, Huang YJ, Fujimura KE, Liu JT, McKean M, Boushey HA, et al. Lactobacillus casei abundance is associated with profound shifts in the infant gut microbiome. PLoS One 2010;5:e8745. [PubMed: 20090909]
21. DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol 2007;53:371–83. [PubMed: 17334858]
22. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 2008;6:e280. [PubMed: 19018661]

23. Sutherland ER, King TS, Icitovic N, Ameredes BT, Bleecker E, Boushey HA, et al. A trial of clarithromycin for the treatment of suboptimally controlled asthma. J Allergy Clin Immunol 2010;126(4):747–53. [PubMed: 20920764]
24. Huang YJ, Lynch SV, Brodie EL, Wiener-Kronish JP, Sutherland E, Investigators A, et al. Detection of diverse bacterial populations in asthmatic airways by 16S rRNA PhyloChip. American Journal of Respiratory and Critical Care Medicine 2008;177:A335.
25. Lynch SV, Huang YJ, Nelson CE, Brodie EL, DeSantis TZ, Baek MS, et al. Diverse bacterial communities in the airways of patients with sub-optimally controlled asthma. American Journal of Respiratory and Critical Care Medicine 2010;181:A5093.
26. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. Eur Respir J 1999;14:902–7. [PubMed: 10573240]
27. Lane, DJ. 16S/23S rRNA sequencing. Chichester, United Kingdom: Wiley; 1991.
28. Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 2007;62:142–60. [PubMed: 17892477]
29. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 2003;100:9440–5. [PubMed: 12883005]
30. Hill MO. Diversity and evenness: a unifying notation and its consequences. Ecology 1973;54:428–32.
31. McCune, BMM. Multivariate Analysis of Ecological Data. Gleneden Beach, OR: MJM Software Design; 2006. PC-ORD v5.
32. DeSantis TZ Jr, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, et al. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res 2006;34:W394–9. [PubMed: 16845035]
33. Huber T, Faulkner G, Hugenholtz P. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 2004;20:2317–9. [PubMed: 15073015]
34. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72:5069–72. [PubMed: 16820507]
35. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 2009;26:1641–50. [PubMed: 19377059]
36. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 2007;23:127–8. [PubMed: 17050570]
37. Nelson KE, Weinstock GM, Highlander SK, Worley KC, Creasy HH, Wortman JR, et al. A catalog of reference genomes from the human microbiome. Science 2010;328:994–9. [PubMed: 20489017]
38. Chen T, Yu W-Han, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database 2010 Article ID baq013. 10.1093/database/baq013
39. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. Am J Respir Crit Care Med 2008;178:218–24. [PubMed: 18480428]
40. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the severe asthma research program. Am J Respir Crit Care Med 2010;181:315–23. [PubMed: 19892860]
41. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388–95. [PubMed: 19483109]
42. Willing B, Halfvarson J, Dicksved J, Rosenquist M, Jarnerot G, Engstrand L, et al. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. Inflamm Bowel Dis 2009;15:653–60. [PubMed: 19023901]
43. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 2007;449:811–8. [PubMed: 17943117]
44. Lloyd CM, Saglani S. Asthma and allergy: the emerging epithelium. Nat Med 2010;16:273–4. [PubMed: 20208514]

45. Beaumont HJ, van Schooten B, Lens SI, Westerhoff HV, van Spanning RJ. Nitrosomonas europaea expresses a nitric oxide reductase during nitrification. J Bacteriol 2004;186:4417–21. [PubMed: 15205449]
46. Payne DN, Adcock IM, Wilson NM, Oates T, Scallan M, Bush A. Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma, after treatment with oral prednisolone. Am J Respir Crit Care Med 2001;164:1376–81. [PubMed: 11704581]
47. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. Thorax 1998;53:91–5. [PubMed: 9624291]
48. Duncan SH, Richardson AJ, Kaul P, Holmes RP, Allison MJ, Stewart CS. Oxalobacter formigenes and its potential role in human health. Appl Environ Microbiol 2002;68:3841–7. [PubMed: 12147479]
49. Coenye T, Goris J, Spilker T, Vandamme P, LiPuma JJ. Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of Inquilinus limosus gen. nov., sp. nov. J Clin Microbiol 2002;40:2062–9. [PubMed: 12037065]
50. Gohler A, Xiong G, Paulsen S, Trentmann G, Maser E. Testosterone-inducible regulator is a kinase that drives steroid sensing and metabolism in Comamonas testosteroni. J Biol Chem 2008;283:17380–90. [PubMed: 18424443]
51. Horinouchi M, Kurita T, Yamamoto T, Hatori E, Hayashi T, Kudo T. Steroid degradation gene cluster of Comamonas testosteroni consisting of 18 putative genes from meta-cleavage enzyme gene tesB to regulator gene tesR. Biochem Biophys Res Commun 2004;324:597–604. [PubMed: 15474469]
52. Campbell JD, Borish L, Haselkorn T, Rasouliyan L, Lee JH, Wenzel SE, et al. The response to combination therapy treatment regiments in severe/difficult-to-treat asthma. Eur Respir J 2008;32:1237–42. [PubMed: 18684845]
53. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am J Respir Crit Care Med 1997;156:737–43. [PubMed: 9309987]
54. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat Med 2003;9:582–8. [PubMed: 12669034]
55. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 2005;434:520–5. [PubMed: 15791257]
56. Meyer EH, Goya S, Akbari O, Berry GJ, Savage PB, Kronenberg M, et al. Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells. Proc Natl Acad Sci U S A 2006;103:2782–7. [PubMed: 16478801]
57. Akbari O, Faul JL, Hoyte EG, Berry GJ, Wahlstrom J, Kronenberg M, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. N Engl J Med 2006;354:1117–29. [PubMed: 16540612]
58. Vijayanand P, Seumois G, Pickard C, Powell RM, Angco G, Sammut D, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. N Engl J Med 2007;356:1410–22. [PubMed: 17409322]
59. Drummond MB, Dasenbrook EC, Pitz MW, Murphy DJ, Fan E. Inhaled corticosteroids in patients with stable chronic obstructive pulmonary disease: a systematic review and meta-analysis. JAMA 2008;300:2407–16. [PubMed: 19033591]
60. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59–65. [PubMed: 20203603]
61. Amayasu H, Yoshida S, Ebana S, Yamamoto Y, Nishikawa T, Shoji T, et al. Clarithromycin suppresses bronchial hyperresponsiveness associated with eosinophilic inflammation in patients with asthma. Ann Allergy Asthma Immunol 2000;84:594–8. [PubMed: 10875487]

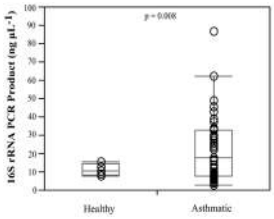

Figure 1. 16S rRNA bacterial burden in bronchial brushings from asthmatic and healthy subjects

Of subjects analyzed by PhyloChip (total n = 47), asthmatic subjects exhibited greater bacterial burden (measured concentration of 16S rRNA PCR product) than healthy subjects ($p = 0.008$, Welch's t-test with log-transformed data). Median and interquartile ranges are noted.

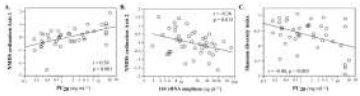

Figure 2. Relationships between variability in airway microbiota composition and community diversity with bronchial hyperresponsiveness

Each circle represents the microbiota present in a single sample (n=44). 2A, 2B. NMDS ordination, based on Sorensen dissimilarity matrices, demonstrates that variability in community composition is most strongly related to bronchial hyperresponsiveness and bacterial burden. Axes have no inherent units and indicate spatial relationships of samples based on their phylogenetic (dis)similarity. Three asthmatic samples were excluded due to extreme mean relative Sorensen distances from the pooled mean. 2C. Increased diversity (higher Shannon indices) is correlated with greater bronchial hyperresponsiveness. (Pearson correlations with log-transformed data.)

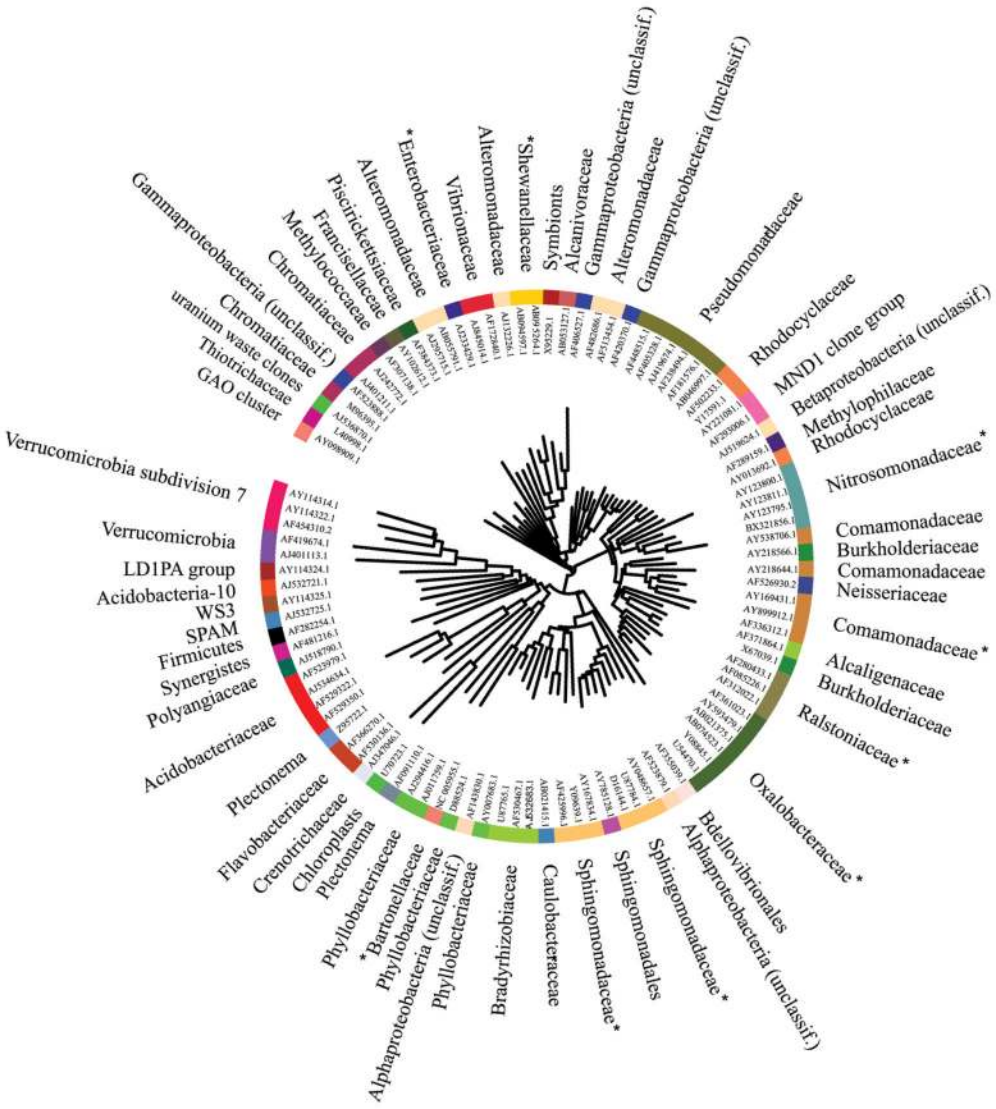

Figure 3. Phylogenetic tree based on 16S rRNA representative gene sequences (GenBank accession numbers) of the ~100 bacterial taxa highly correlated with greater bronchial hyperresponsiveness

($p < 0.01$, $q \leq 0.015$). Colors represent different bacterial families. Asterisks denote taxa with member species previously associated with clinical disease or possessing notable functional features (See also Table E2 in the Online Repository).

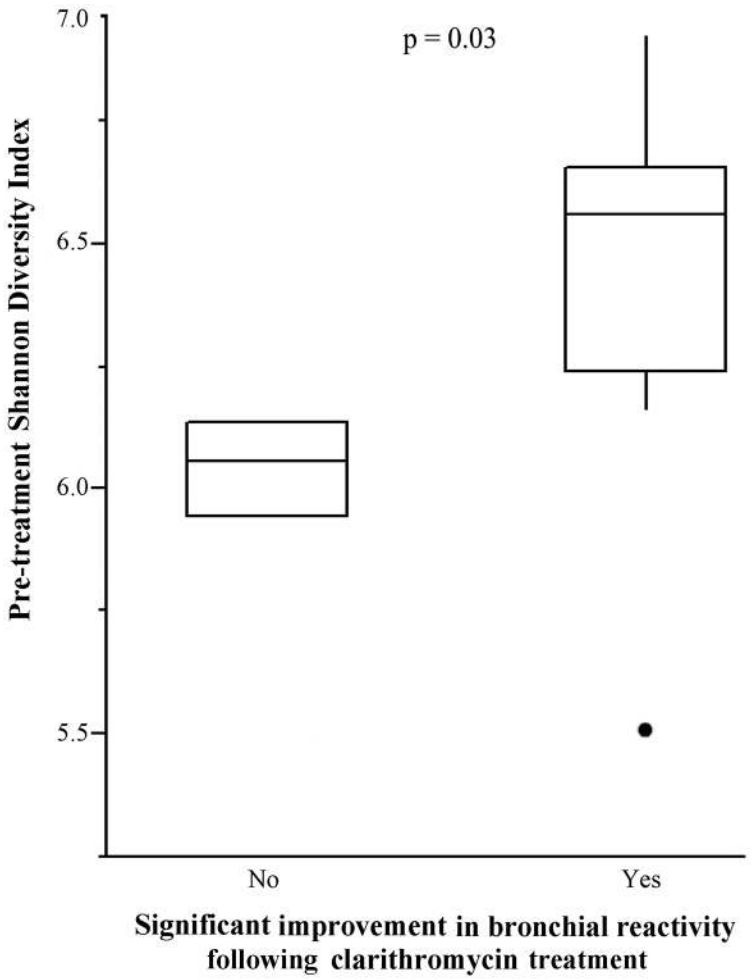

Figure 4. Baseline airway microbiota diversity and change in bronchial hyperresponsiveness with clarithromycin treatment

Sixteen asthmatic subjects with pre-treatment samples analyzed by microarray received clarithromycin therapy. Subjects with significant improvement in bronchial hyperresponsiveness post-clarithromycin (n=9) possessed higher pre-treatment airway bacterial diversity than those with no significant response (n=3). Four subjects with incomplete data due to study discontinuation were not included.

Table I

Baseline characteristics of subjects

	Asthmatic (n=65)	Healthy (n=10)	p-value
Age (years)	39.3 ± 11.2	35.5 ± 12.9	NS
Gender (% Female:Male)	52:48	60:40	NS
FEV_1 (% predicted)	74.9 ± 15.6	94.1 ± 12.2	< 0.001
PC_{20} (mg ml^{-1})	1.3 (2.9)^	>16	
Sputum eosinophil (%)	2.0 ± 5.2	N/A	
Sputum neutrophil (%)	33.6 ± 23.7	N/A	
ACQ Score	1.8 ± 0.7	N/A	
Total IgE (IU ml^{-1})	128.4 (0.03)^	N/A	
	Asthmatic group		
	Analyzed by PhyloChip* (n=42)	Unable to analyze by PhyloChip* (n=23)	p-value
Age	40.4 ± 10.7	37.3 ±12.1	0.3
Gender (% Female:Male)	45:55	52:48	0.4
FEV1 (% predicted)	75.5 ± 13.6	77 ± 14.8	0.7
PC_{20} (mg ml^{-1})^	1.2 (3.2)	1.8 (2.2)	0.2
Sputum eosinophil (%)	2.2 ± 5.9	1.5 ± 3.3	0.5
Sputum neutrophil (%)	30.0 ± 24.0	40.3 ± 22.3	0.1
ACQ Score	1.8 ± 0.8	1.8 ± 0.5	0.6
Positive allergen skin test result (reaction to at least one allergen)	79% (33/42)	74% (17/23)	
Total IgE (IU ml^{-1})^	114.6 (0.03)	158.3 (0.03)	0.4
History of last oral corticosteroid use (# subjects)			
None since age 12	15	5	
Age 12 – up to two years prior to study enrollment	13	7	0.40†
Within last two years prior to study enrollment	14	11	
Exacerbation events during study (# subjects)			
Within 1 week of bronchoscopy	5	2	
Greater than 1 week after bronchoscopy	3	2	0.90†
No exacerbations during study	34	19	

Values are mean ± SD, except where noted;

^Geometric mean (CV) reported.

†Fisher's exact p-value. NS, not significant.

*Samples with a negative 16S rRNA PCR screen or < 100 ng of total 16S rRNA PCR product for hybridization were not analyzed by PhyloChip.

Table II

Bacterial families identified by both 16S rRNA clone library-sequencing and 16S rRNA PhyloChip

Phylotypes reported at sub-family level classified by Greengenes G2 chip taxonomy (number of clones sequenced per library indicated in parentheses). Highlighted sub-families were undetected by array based on conservative criteria for determining taxon presence.

AB01 (374)			**AB02 (301)**			**AB03 (299)**		
Phylum	***Family***	[a]***SF***	***Phylum***	***Family***	***SF***	***Phylum***	***Family***	***SF***
Firmicutes	Streptococcaceae	sf-1	Actinobacteria	Cellulomonadaceae	sf-1	Actinobacteria	Cellulomonadaceae	sf-1
Proteobacteria	Pasteurellaceae	sf-1	Bacteroidetes	Flavobacteriaceae	sf-1	Bacteroidetes	Prevotellaceae	sf-1
Bacteroidetes	Prevotellaceae	sf-1	Bacteroidetes	Porphyromonadaceae	sf-1	Firmicutes	Enterococcaceae	sf-1
			Bacteroidetes	Prevotellaceae	sf-1	Firmicutes	Peptococc/Acidaminococc	sf-11
			Firmicutes	Lachnospiraceae	sf-5	Firmicutes	Peptostreptococcaceae	sf-5
			Firmicutes	Mycoplasmataceae	sf-1	Firmicutes	Staphylococcaceae	sf-1
			Firmicutes	Peptococc/Acidaminococc	sf-11	Firmicutes	Streptococcaceae	sf-1
			Firmicutes	Peptostreptococcaceae	sf-5	Fusobacteria	Fusobacteriaceae	sf-1
			Firmicutes	Staphylococcaceae	sf-1	Fusobacteria	Fusobacteriaceae	sf-3
			Firmicutes	Streptococcaceae	sf-1	Proteobacteria	Campylobacteraceae	sf-3
			Fusobacteria	Fusobacteriaceae	sf-1	Proteobacteria	Pasteurellaceae	sf-1
			Fusobacteria	Fusobacteriaceae	sf-3	TM7	Unclassified	sf-1
			Proteobacteria	Campylobacteraceae	sf-3			
			Proteobacteria	Pasteurellaceae	sf-1			
			Spirochaetes	Spirochaetaceae	sf-1			
			TM7	Unclassified	sf-1			
AB04 (344)			**AB05** (332)			**AB32** (349)		
Phylum	*Family*	*SF*	*Phylum*	*Family*	*SF*	*Phylum*	*Family*	*SF*
Actinobacteria	Cellulomonadaceae	sf-1	Actinobacteria	Micrococcaceae	sf-1	Actinobacteria	Corynebacteriaceae	sf-1
Bacteroidetes	Porphyromonadaceae	sf-1	Bacteroidetes	Flavobacteriaceae	sf-1	Bacteroidetes	Porphyromonadaceae	sf-1
Bacteroidetes	Prevotellaceae	sf-1	Bacteroidetes	Porphyromonadaceae	sf-1	Bacteroidetes	Prevotellaceae	sf-1
Firmicutes	Aerococcaceae	sf-1	Bacteroidetes	Prevotellaceae	sf-1	Firmicutes	Aerococcaceae	sf-1
Firmicutes	Lachnospiraceae	sf-5	Firmicutes	Aerococcaceae	sf-1	Firmicutes	Enterococcaceae	sf-1
Firmicutes	Peptococc/Acidaminococc	sf-11	Firmicutes	Enterococcaceae	sf-1	Firmicutes	Lachnospiraceae	sf-5

AB01 (374)			AB02 (301)			AB03 (299)		
Phylum	***Family***	***[a]SF***	***Phylum***	***Family***	***SF***	***Phylum***	***Family***	***SF***
Firmicutes	Peptostreptococcaceae	sf-5	Firmicutes	Erysipelotrichaceae	sf-3	Firmicutes	Mycoplasmataceae	sf-1
Firmicutes	Streptococcaceae	sf-1	Firmicutes	Lachnospiraceae	sf-5	Firmicutes	Peptococc/Acidaminococc	sf-11
Fusobacteria	Fusobacteriaceae	sf-3	Firmicutes	Peptococc/Acidaminococc	sf-11	Firmicutes	Peptostreptococcaceae	sf-5
Proteobacteria	Campylobacteraceae	sf-3	Firmicutes	Peptostreptococcaceae	sf-5	Firmicutes	Staphylococcaceae	sf-1
Proteobacteria	Pasteurellaceae	sf-1	Firmicutes	Staphylococcaceae	sf-1	Firmicutes	Streptococcaceae	sf-1
Proteobacteria	Pseudomonadaceae	sf-1	Firmicutes	Streptococcaceae	sf-1	Fusobacteria	Fusobacteriaceae	sf-1
			Fusobacteria	Fusobacteriaceae	sf-1	Fusobacteria	Fusobacteriaceae	sf-3
			Proteobacteria	Campylobacteraceae	sf-3	Proteobacteria	Campylobacteraceae	sf-3
			Proteobacteria	Pasteurellaceae	sf-1	Proteobacteria	Moraxellaceae	sf-3
			Proteobacteria	Pseudomonadaceae	sf-1	Proteobacteria	Pasteurellaceae	sf-1
			Spirochaetes	Spirochaetaceae	sf-1	Proteobacteria	Pseudomonadaceae	sf-1
			TM7	Unclassified	sf-1	TM7	Unclassified	sf-1

[a]SF, sub-family