Skin Microbiota composition is determined by host genetics: a genome wide association study

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Abstract

An individual's skin microbiota composition relies on a combination of environmental and genetic factors and can vary dramatically between persons. Skin acts as a protective barrier to the external environment and dysbiosis of its microbiota has already been linked to conditions such as atopic dermatitis. Despite this fact, few studies have investigated the overall impact of the host genome on skin bacterial composition. Here we characterise the skin microbiota from 114 mice representing 30 different strains of an advanced-cross breeding program, The Collaborative Cross. We identify 13 quantitative trait loci, encompassing 294 genes that show significant linkage with specific bacterial lineages. Overall, these findings highlight the importance of host genetics as a determinant of microbial colonization and may help guide strategies to address skin dysbiosis in disease.

Statement of Significance

The host genome has deterministic effects on skin microbiota composition that warrants consideration in both health and disease. These findings highlight key host-microbe interactions that can offer novel pharmacological targets for specifically affecting skin microbial colonisation.

Introduction

All animals are colonised by microbes soon after birth. In recent years, these "microbiomes" have been implicated in driving a wide range of host-responses relevant to homeostasis, or alternatively, its disruption that manifests in a wide range of immune-mediated and/or metabolic related diseases (Catinean, Neag, Mitre, Bocsan, & Buzoianu, 2019; Schoeler & Caesar, 2019). The gut (stool) microbiome has been the most intensively studied and has advanced the concept of "community-based" interactions that trigger conditions including atopic dermatitis, obesity, and mood disorders (Forsythe, Sudo, Dinan, Taylor, & Bienenstock, 2010; Kobyliak, Virchenko, & Falalyeyeva, 2016; Pessi, Sütas, Hurme, & Isolauri, 2000). Although less studied, variations in the skin microbiome have been associated with episodes of atopic dermatitis (Kong et al., 2012), and affecting the healing rate of leg ulcers (e.g.(Min et al., 2020; Wolcott, Gontcharova, Sun, & Dowd, 2009). The skin microbiome has been shown to vary according to patient age and body site (Grice et al., 2009) and environmental cues such as humidity and/or temperature, may explain the inter-individual variation (Grice & Segre, 2011). In that context, diet is also now considered to exert a strong effect on the microbiome, which can be linked to host response and health status (Schoeler & Caesar, 2019).

In contrast, there are relatively few studies that provide a systematic assessment of how host genotype affects microbiome composition. Broader genome-wide studies utilising a BXD inter-cross mouse model have identified many host-specific quantitative trait loci (QTL), with candidate genes involving cytokines and toll-like receptor signalling that associate with specific bacterial compositions (McKnite et al., 2012). In addition, investigation of the gut (stool) microbiota of mice from an advanced inter-cross model found 18 separate QTL associated with the relative abundance of one or more bacterial taxonomies (Benson et al., 2010). Regarding skin, most studies to date have been limited to candidate approaches, such as how matriptase mutations can lead to a shift in the skin microbiota composition (Scharschmidt et al., 2009), or have described the skin microbial populations more generally (Byrd et al., 2017; Grice et al., 2008; Oh et al., 2016).

Specialized cross-breeding programs that make use of recombinant-inbreeding can greatly assist in the analysis of complex genotype-environment interactions and gene pleiotropies (Churchill et al., 2004; Threadgill & Churchill, 2012). The Collaborative Cross (CC) is a breeding program that made use of 8 founding strains of mice to produce many

hundreds of recombinant mouse strains (Churchill et al., 2004). These recombinant strains were inbred over multiple generations, ultimately creating unique strains of mice whose genome's possess > 90% homozygosity (Morgan & Welsh, 2015). This high rate of homozygosity within recombinant-inbred (RI) strains ensures that each region of the genome is defined by the genetic contribution from a single founder strain (haplotype). However, the large heterogeneity in haplotypes between different strains can result in markedly different phenotypes. Greatly reduced costs and complexities associated with the sequencing and modelling of RI strain genotypes can be achieved with CC mice, due to the free availability of previous sequencing results and modelling resources (Churchill et al., 2004). The large genetic scope of the CC RI strains, when combined with the aforementioned positives, provides an excellent resource to investigate murine genetics of complex biological problems.

In this study, we propose that the host genome is a major determinant of skin microbiota composition. Using 16S rRNA gene amplicon profiling of the skin microbiota from 30 strains of mice from the Collaborative Cross, we show that the strain of mouse significantly affects overall skin microbiota composition, and identify 13 QTL containing a range of candidate genes that affect innate and adaptive immunity, and are known to influence bacterial colonisation at other body sites. The candidate genes we present here may find use as targets for therapeutic modulation of the microbiota in clinical cases of dysbiosis, such can occur in atopic dermatitis, acne vulgaris or wound healing.

Methods

Mice

All mice were housed in the UQ Centre for Clinical Research Animal Facility. All animal experimentation was conducted in accordance with institutional ethical requirements and approved by the University of Queensland Animal Ethics Committee. Each strain was caged separately and only female mice were used. The number of mice per strain varied due to availability. Two mice from the strains XAV, GIT, LEM and POH were used whereas, all other strains had 3 or more mice.

Collection

Mice were anaesthetized with 2% isoflurane and a sterile rayon swab moistened with TE buffer was used to collect a microbiota sample from the dorsal skin. Samples were stored in 2ml of TE buffer at -80^{o}C for later sequencing.

Microbiota community profiling and data analyses

Microbial DNA was extracted from swab samples of dorsal skin using the Maxwell 16 LEV Buccal Swab DNA kit according to manufacturer's recommendations. The resulting DNA samples were then used to produce bar-coded PCR amplicon libraries of the V6-V8 hypervariable region of the 16s rRNA gene using the universal microbial primers with Illumina primer overhang adapters as follows:

- Forward Primer – 926F:

5’ - TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AAA CTY AAA KGA ATT GRC GG – 3'

- Reverse Primer – 1392R:

5’ - GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAC GGG CGG TGW GTR C – 3'

Sequencing used the Illumina MiSeq sequencing platform and protocols developed by the UQ-Australian Centre for Ecogenomics (www.ecogenomic.org). A sequence Phred quality threshold of 20 was used and sequences checked for chimeras using USEARCH version 6.1.544 (Edgar, 2010). Mapping and clustering of reads into operational taxonomic units (OTUs) with 97% identity threshold against Greengenes core set database 13.8

(DeSantis et al., 2006), was performed using Quantitative insight into Microbial Ecology (QIIME) version 1.9.1 (Caporaso, Kuczynski, et al., 2010) and PyNast (Caporaso, Bittinger, et al., 2010). OTUs were then compiled into an OUT table for further analysis.

Data Analysis

Raw counts for each phylotype were grouped across all mice and plotted using the 'Pareto' package available for R. Shannon and Pielou measures of alpha and beta diversities were calculated using the 'Vegan' package with functions 'diversity' and 'specnumber'. Comparisons of murine skin microbiotas similarities were performed using the ANOSIM function with a Bray-Curtis matrix of dissimilarities. ANOSIM is known to be potentially confound differences in location and dispersions, so we additionally calculated dispersions using the Betadisper function of the 'Vegan' package and tested for significance differences with an Anova model. The median percentage abundance for each bacterial family by mouse strain was calculated and those bacterial families failing a threshold of 0.01% abundance in 50% or more mouse strains were removed, leaving a total of 20 'Core' bacterial families remaining in the dataset.

QTL Analysis

The (-)log transform of each mouse strains' median percentage abundance for a single bacterial family were imputed to the GeneMiner software (www.sysgen.org/GeneMiner) and GWAS analysis using a model based on SNP segregation was performed. This was repeated for every bacterial family in the study to produce a collection of 20 separate GWAS style trials that represent the 'Core' microbiota. The results from all trials were combined into a single analysis with an adjusted Bonferroni significance level. All SNPs whose p-values were classed as significant at this level were recorded. SNPs within 2 Mbps of one another were assigned to the same region with distances greater than 2 Mbps representing different regions.

The identified regions were then cross-validated with a second analysis method that makes use of a hidden markov model (HMM) to create vectors of founder haplotype probabilities at each genomic marker and then regresses phenotype values onto these probabilities (Ram, Mehta, Balmer, Gatti, & Morahan, 2014), *p*-values are calculated using ANOVA and a Benjamini-Hochberg correction (FDR). Any regions from the first analysis that contained locations, or were contained within locations, of significance or suggested significance (95^{th} and 90^{th} percentiles respectively) in the second analysis were shortlisted (Sup).

Candidate Gene Shortlisting

We used haplotype diagrams for the shortlisted regions to identify the founder strains' which contributed most to the abundances of significantly linked bacteria. We searched Sanger UK mouse genomes project for SNPs specific to the previously identified haplotypes that result in stop lost/gained, missense variants, and frameshift variants. From these results we created a list of candidate genes.

Results

Microbiota colonisation and diversity varies between strains

The Firmicutes represented the largest phylum followed by Actinobacteria, Proteobacteria, Cyanobacteria and Bacteroidetes, largely agreeing with previous studies of mouse skin microbiota ((Srinivas et al., 2013), Figure 1a). Although the Shannon and Pielou metrics of alpha (within sample) diversity for the majority of strains were greater than 1.5 and 0.5, respectively, and overall there was a positive correlation between these two diversity metrics (Figure 1b), some strains showed a considerable degree of variation in both scores and (Figure 1c, and Supplementary Figure 1). In contrast, no correlation was observed between the raw OTU counts produced from each mouse strains and the calculated Shannon index (Supplementary Figure 2) suggesting the observed differences in microbial diversity were directly attributable to host strain-based variations rather than sequencing artefacts.

Deeper comparisons of the skin microbiota profiles at the family level of classification confirmed there was a significant difference between one or more mouse strains' (ANOSIM, p-value = 0.001), but with no significant differences in dispersion (p-value = 0.52) further suggesting the communities are primarily host-specific. Bacterial families Staphylococcaceae and Corynebacteraceae predominated most biotas with 26.8% and 1.3% median abundances, respectively, across all mice, though there remained considerable variability between mice with ranges 3.4-77.2% and 0-45.4%, respectively (Supplemental Figure 3a). Differences in abundance of rarer bacterial families after log transformation highlights the presence of heterogeneity in bacterial abundances at all levels of rarity (Figure 1d).

Overall, these findings clearly indicated that despite maintenance in a similar environment for long periods, murine strains significantly vary in their skin microbial composition.

GWAS-linkage studies identifies multiple key bacterial taxa-QTL relationships

To account for high variability in low abundance bacterial families, we used the log transformed median percentage abundances of each bacterial family as a quantitative trait across mouse strains for performing GWAS analysis using SNP segregation (GeneMiner, as exemplified in Figure 2a for abundance of *Corynebacteriaceae*). We performed a total of 20 separate GWAS trials, one for each bacterial family present in the ‘Core’ biota. Using a

Bonferroni adjustment, we pooled and simultaneously analysed all the bacterial families' GWAS results for significant SNPs. We identified 1103 SNPs across the murine genome at or above the adjusted significance level (p-value=$2.5x10^{-9}$), which determined 53 separate regions of the genome when grouping all significant SNPs within 2Mbps of each other (Figure 2b). Novel associations include regions on Chr18 for *Moraxellaceae* and *Bacteroidaceae*, as well as on Chr2 for *Propionibacteriaceae*. A number of these regions were also significant for more than one bacterial family, such as the region on chromosome 4, between positions 132.4-134.7 Mbps (Figure 2b) which was significant for both *Moraxellaceae* and *Comamonadaceae*. We also identified a significant association between a region on Chr10 associated with *Bacteroidaceae* that falls within the 95% confidence interval of the QTL identified as significant or suggestive with the relative abundance *Lactobacillus* spp. in the murine gut (Benson et al., 2010; McKnite et al., 2012), respectively. Interestingly we also found a significant association for a region on Chr15 with the relative abundance of *Comamonadaceae* that crosses over with a suggested QTL identified from an autoimmune skin blistering mouse model (Srinivas et al., 2013).

We next sought to cross-validate the regions identified from the GWAS using a second method that consisted in linear regression of phenotype values onto a matrix of haplotypes with ANOVA testing and adjustment for false discovery rate. Each of the 20 families of bacteria was again considered using this method (Figure 3). These latter analyses showed 13/53 of the GWAS outputs as being significant or suggestive (at the 95^{th} and 90^{th} percentiles, respectively, compare Figures 2a and 3a), while many of the other 40 regions detected by GWAS were only just excluded based on failing to reach the minimum 90^{th} percentile threshold of the second test, possibly due to lack of overall power. Taken together, these independently-derived and stringent methods of analysis validate that host genetics play a key deterministic role in constructing the bacterial composition of the skin microbiota, via no less than 13 genomic regions identified and predicted to encode almost 300 genes.

Candidate genes possessing haplotype-specific SNPs include functionally relevant targets such as platelet-activating factor receptor (ptafr) and IL-17RB

We next examined specific loci identified by both methods of analysis for evidence of potential mechanisms by which bacterial composition was controlled, and the haplotype coefficients and diagrams (Figure 3a & 3b) were also used to identify the founder strain haplotype most likely responsible for each of these. We first examined the well-defined peak

of association identified on Chr4 for *Moraxellaceae* (LOD score >15, Figure 3a). This QTL possessed a large coefficient for the CAST founder strain haplotype between 132.4-134.7 Mbps. This QTL is present in the LOT mouse strain and encodes 26 genes and, according to the Sanger UK mouse SNP database, missense SNPs specific to the CAST strain not present in any of the other 7 founder strains. As such, one or more these genes may be responsible for the high linkage in this region. Among the candidate genes, *Ptafr* whose expression is known to affect bacterial colonisation seemed of particular interest (Iovino, Brouwer, van de Beek, Molema, & Bijlsma, 2013; Smani et al., 2012). Another QTL identified on chromosome 14 shows high association of the CAST haplotype with reduced colonisation by *Propionibacteriaceae*, a family of bacteria often present in sebaceous areas of human skin (Figure 3b). One gene of interest from this region, between 29-31Mbps, is *IL-17rb*, which may have a role in skin inflammation (Supplemental Table1 – region 38). Furthermore, we identified another QTL associated with reduced *Propionibacteriaceae* abundance on Chr2. which included the gene *MAVS* which has been previously implicated in gut dysbiosis (Plantamura et al., 2018). Repeating this same process for the remaining 11 QTL yielded a total of 294 candidate genes (Supplemental Table1) that possess SNPs specific to QTL respective haplotypes.

Discussion

While host genetics have been shown to clearly affect the composition of microbiota in the gut, the genetic determinants of the skin microbiota have remained less explored with most investigators relying on candidate gene approaches. Here we combined 16S rRNA gene amplicon sequencing of the dorsal skin microbiota in 30 different mouse strains representing the Collaborative Cross, and show that the skin microbiota varies between different mouse strains. The use of skin swabs for sampling the skin microbiota for 16S rRNA gene profiling is a robust method for comparative analyses (Grice et al., 2008; Wang et al., 2014). The Collaborative Cross also provides a powerful tool for GWAS-style analyses through the combinatorial recombination of genomic information from 8 different founder strains (Churchill et al., 2004; Threadgill & Churchill, 2012). The Collaborative Cross strains affords the inclusion of a greater variety of genetic material than that inherent to the mouse strains used in the small number of other studies investigating host-genetics and stool microbiota interactions (Benson et al., 2010; McKnite et al., 2012). Here, we have used the family-level

community profiles for GWAS based on SNP segregation, and then validated our significant findings via a second independent and stringent analysis, based on founder haplotype reconstructions. These combined analyses have identified 13 regions of the mouse genome as being significantly associated with skin microbiota composition, and further identified SNPs in 294 genes spanning these regions. The veracity of the novel findings from this study is further established by previous candidate gene approaches establishing relationships with bacterial colonization (e.g. (Srinivas et al., 2013)).

Host immune regulation represents an obvious method for mediating skin bacterial colonisation, and the identification of QTLs spanning genes such as *IL-17rb* (Chr14, LOD), *Ptafr* (Chr4, LOD) and *MAVS* (Chr2, LOD) highlights potential mechanisms of host-microbiota regulation. Here, those mouse strains bearing the *IL-17rb* SNP possessed a lower median percentage abundance of *Propionibacteriaceae*, which is common member of the skin microbiome due to the association of *Propionibacterium acnes* with acne (Beylot et al., 2014). Interestingly different P.acnes phylotypes taken from healthy, neutral and acne prone subjects result in different levels of IL-17 induction in mononuclear cells (Yu et al., 2016). Additionally, IL-17 signalling has been implicated in inflammatory skin conditions such as psoriasis (Xu et al., 2018). Furthermore, MAVS deficiency has previously been associated with gut dysbiosis and allergenic skin responses in mice (Plantamura et al., 2018). Both *MAVS* and *IL-17rb* genes were associated with a reduction in *Propionibacteriaceae* abundances and interestingly previous authors have noted a reduction in overall *Propionibacteriaceae* is associated with more severe acne in humans. Our findings lend more support to previous suggestions that skin microbiota possesses a potential covariant relationship with skin disorders and host polymorphisms (Srinivas et al., 2013).

While single mutations can be responsible for disease phenotypes, in many cases the causes of disease tend to be complex and multi-factorial. Our findings of multiple regions of the mouse genome with significant linkage to bacterial families indicates that the host genome and skin microbiota possess a complex interaction pattern. While the model we used is ultimately limited in genetic material to just 8 founding strains of mice, it none the less builds on previous work in murine gut and on murine skin disorders to demonstrate a level of genetic determinism for host microbiota. Many of the candidate genes identified here may present novel targets for therapeutic manipulation of skin microbiota composition.

Acknowledgements

Figure 1 – Skin microbiota composition and diversity across 30 different Collaborative Cross mouse strains a) Total OTU counts from 16s rRNA sequencing of mouse dorsal skin swabs, stratified by Phyla taxonomic group. The top 5 phyla by OTU counts include Firmicutes, Actinobacteria, Proteobacteria, Cyanobacteria and Bacteroidetes, with Firmicutes accounting for >90% of all OTU counts across all samples. **b)** Scatter plot of Pielou's Evenness against Shannon's diversity for all samples. The fitted regression line clearly indicates a positive correlation between the 2 indices, as expected, though a few samples appear to drift more leftward from the line, indicating fewer total species present whilst maintaining similar levels of species evenness. **c)** Trellis plot of Pielou's evenness against Shannon's diversity by mouse strain. While all samples generally follow the trend of diversity being proportional to evenness, some strains show greater within strain variation than others, such as DAVIS and TOP compared to LUG, PUB or LAM respectively. **d, e)** Hierarchical clustering heatmaps of the 20 'Core' skin bacterial families, as defined by a presence of at least 0.01% abundance in 50% or more mouse strains. Clustering is performed using Ward's method on a Bray-Curtis dissimilarity matrix obtained from the non-transformed median OTU counts. Heatmap (d) uses non-log transformed median percentage abundances while heatmap (e) shows the -log2 transform of median OTU counts within each mouse strain for each bacterial family. Mice can be broadly clustered into 4 groups whilst bacterial families largely cluster into 3 groups. Due to the dominance of certain bacterial families, such as Staphylococcaceae, the variability in lower abundant families is more easily observed in the log2 transformed data.

Figure 2 – GWAS based on SNP segregation identifies 53 QTL affecting skin microbiota composition. a) Manhattan plot of Corynebacteriaceae GWAS. Median percentage abundances of Corynebacteriaceae for each mouse strain were -log2 transformed and analysed by GeneMiner. A model based on SNP segregation was then used to assess linkage with a Bonferroni correction for multiple comparisons across all of the 'Core' bacterial families. The adjusted significance threshold is shown in red (*p-value* < 2.5*10^-9) indicating significant linkage for SNPs on chromosome 15 with -log2 Corynebacteriaceae percentage abundance on murine dorsal skin. Genes in this region include *Cdh9*, *Acot10*, and *Basp1*. **b)** A map of significant SNP linkage across the mouse genome for the aggregated GWAS data from all 20 'Core' bacterial families identifying 7 of the 20 families as reaching significance in at least one SNP at the adjusted significance threshold. Significant SNPs were grouped into the same region if within 2Mbps or one another. Chromosome 4 possesses a region significantly associated with both Moraxellaceae and Commonadaceae abundances, while Propionibactereaceae and Enterobacteraceae in particular show multiple areas of significance across multiple chromosomes.

Figure 3 – GWAS based on haplotype reconstructions further shortlists 13 QTL affecting skin microbiota composition. **a)** Linkage maps for regions passing at least the 90% significance threshold for haplotype reconstruction GWAS in addition to passing the Bonferroni adjusted significance level of the SNP segregation GWAS. Moraxellaceae percentage abundance shows a significant linkage across both methods for Chromosome 4 at 132.4-134.7 Mbps, achieving the 99% significance threshold. From the haplotype maps the peak of significance seems due to the founder strains CAST and/or PWK (higher positioning

the map indicates higher percentage abundance). Putative genes include *Ptafr*. **b)** Propionibacteraceae shows a peak on Chromosome 2 again likely due to the founder strain CAST, though this time the CAST strain is associated with lower percentage abundance. Putative genes include *IL-17Rb* (for a full list of possible candidates see supplemental).

Supplemental 1. Dot plots of Pielou's evenness and Shannon's diversity indices across all mouse strains, in descending order. Both measures show the same rough trend, which is to be expected, with most mice possessing similar levels of diversity and evenness but with a number of more extreme cases at the low end of the scales, such as LOT.

Supplemental 2. Trellis plots of Shannon's diversity index against OTU count by mouse strain. **a)** Total OTU counts vary across the mouse strains with a few very large OTU counts obscuring any possible patterns. **b)** Only individual mouse samples with OTU counts < 1500, we can now see relative differences between mice. Qualitative interpretation of the graphs cannot identify a particular pattern, with multiple examples of positive/negative/no correlations (not tested for statistically).

Supplemental 3. Distribution of the different mouse strain's median OTU counts by bacterial family, before (a) and after (b) -log2 transformation. The log transformation clearly produces more normally distributed data, though a number of mice still possess 0 counts of certain families, hence the long tails to ~-33. Since GeneMiner uses a linear model as its basis, we opted to use the transformed data for inputs.

Supplemental 4. Rarefaction curves for the different CC mice, top each individual sample, bottom samples aggregated by Strain. Some mice strains clearly show a higher richness than others (such as YID and POH). It is possible that these mice would have more varied skin biota's than other strains that may not be fully captured by the use of a 'Core' biota definition.

Supplemental 5. Haplotype maps and linkage diagrams for all of the remaining cross-validated significant regions.

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