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Study of the human skin microbiome associated with acne vulgaris using metagenomic and metatranscriptomic approaches

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A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular & Medical Pharmacology

by

Dezhi Kang

2014

ABSTRACT OF THE DISSERTATION

Study of the human skin microbiome associated with acne vulgaris using metagenomic and metatranscriptomic approaches

by

Dezhi Kang

Doctor of Philosophy in Molecular & Medical Pharmacology

University of California, Los Angeles, 2014

Professor Huiying Li, Chair

Various diseases have been linked to the human microbiota, but the underlying molecular mechanisms of the human microbiota in disease pathogenesis are often poorly understood. Using acne as a disease model, my study aims to understand the functional capability changes and the molecular response of the skin microbiota to host metabolite signaling in disease pathogenesis. By metagenomic analysis, I found that the genes of a pathogenic gene island in the skin bacterium, *Propionibacterium acnes*, were significantly enriched in acne subjects. By metatranscriptomic analysis, I found that the transcriptional profiles of the skin microbiota separated acne patients from healthy individuals. The vitamin B12 biosynthesis pathway in *P. acnes* was significantly down-regulated in acne patients. Through *in-vivo* and *in-vitro*

experiments, I further demonstrated that host vitamin B12 modulates the activities of the skin microbiota and plays a signaling role in acne pathogenesis. To understand the molecular mechanism underlying the transcriptional regulation of *P. acnes* genes by vitamin B12, I analyzed ten putative regulatory elements in multiple *P. acnes* genomes and identified sequence variations among different strains. It suggests that the vitamin B12 modulation of *P. acnes* transcription is individual microbiome-dependent. My findings suggest a novel bacterial pathogenesis pathway in acne. My study also provides evidence that metabolite-mediated interactions between the host and the skin microbiota play essential roles in disease development. To further elucidate how the skin microbiota interacts with the host in disease pathogenesis, I have developed a protocol for enriching bacterial RNA from human skin biopsy samples, which lays a foundation for simultaneously analyzing the interactions between the skin microbiota and host cells at the transcriptional level.

The dissertation of Dezhi Kang is approved.

Jonathan Braun

Jing Huang

Matteo Pellegrini

Huiying Li, Committee Chair

University of California, Los Angeles

2014

DEDICATION

To my supporting parents

Shuxuan Kang and Chunhong Li

and beloved wife

Ying Han

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Vita

Education

2004-2008 Bachelor of Science in Biotechnology, Peking University

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2009-2014 UCLA Molecular and Medical Pharmacology Retreat

2012 Best graduate student's talk

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2012 19th International Meeting on Microbial Genomics

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2012 Keystone Symposia on Molecular and Cellular Biology, the Microbiome

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Publications

Kang D, Shi B, Erfe EC, Craft N, Li H. Vitamin B12 modulates the transcription of the skin microbiota in acne pathogenesis. In revision.

Johnson T, **Kang D**, Barnard E, Li H. Vitamin B12 promotes porphyrin production in acne-associated *Propionibacterium acnes* strains, but not in health-associated strains. In preparation.

Barnard E, Shi B, **Kang D**, Chiu B, Nguyen L, Kim J, Erfe MC, Craft N, Li H. Balance between disease-associated and health-associated metagenomic elements shapes the skin microbiome in acne and healthy skin. In preparation.

Maretti-Mira AC, Bittner J, Oliveira-Neto MP, Liu M, **Kang D**, Li H, Pirmez C, Craft N. (2012) Transcriptome Patterns from Primary Cutaneous *Leishmania braziliensis* Infections Associate with Eventual Development of Mucosal Disease in Humans. PLoS Negl Trop Dis 6 (9): e1816. doi:10.1371/journal.pntd.0001816

Chapter 1: Introduction

Microorganisms inhabit humans and affect the health and disease states of the host (*1*). The ecological community of these microorganisms inhabiting the human body is called human microbiota and the collective genomes of the microorganisms are referred as the human microbiome. Metabolites play important signaling roles in the interactions between the microorganisms and the host. It is well known that the gut microbiota influences the health states of the host via bacterial metabolites (*2-5*). Whether or not the microbial communities at other body sites affect the host health states via bacterial metabolites has not been well characterized. Furthermore, little is known about how host metabolite levels affect the transcriptional and metabolic activities of the microbiota.

Many studies using 16S ribosomal RNA (rRNA) analysis have characterized the taxonomic composition of the human microbiota, including skin microbiota (*6, 7*). Recent technology advancement in metagenomic and metatranscriptomic analyses has enabled us to study the functional potentials and transcriptional activities of the skin microbiota. To date, two metagenomic studies of the skin microbiota have been published (*8, 9*). These studies offered insights into the metagenomic composition of the healthy skin microbiota across different skin sites. But the metagenomic profiles of the skin microbiota in diseases as well as the metatranscriptomic profiles in both health and disease have not been characterized. There are several technical challenges in metatranscriptomic studies of the human skin microbiota. These include low yield of microbial RNA, depletion of rRNA, and the requirement of a large number of samples and high sequencing depth in order to detect differences among individuals with large variations in the microbiota. The amount of available bacterial RNA is particularly limited in

skin samples, thus making it challenging to apply RNA-Seq to understand the transcriptional activities of the skin microbiota at health and disease states.

One of the most common human skin diseases is acne vulgaris (commonly called acne), which is a disease of the pilosebaceous unit (hair follicle). Acne affects more than 80% of adolescents (*10-12*) and young adults globally (*13, 14*). In addition to its high prevalence, acne can be extremely painful and disfiguring in some patients and it can profoundly affect patients' self-esteem and mental health (*15, 16*). Although the etiology of acne is not completely understood, it is believed that skin microorganisms play a role in acne pathogenesis. Bacterial colonization and release of inflammatory mediators into the skin have been proposed as one of the main mechanisms of acne pathogenesis (*17-20*). The skin microbiota in the pilosebaceous unit is a tractable microbial community with a single dominant species, *Propionibacterium acnes*. Both culture-dependent (*21-23*) and culture-independent studies (*24*) have shown that *P. acnes* accounts for nearly 90% of the microbiota. *P. acnes* has long been associated with acne. In fact, antibiotics targeting *P. acnes* have been a mainstay treatment for acne in the past 40 years (*25*). However, the role of *P. acnes* in acne pathogenesis remains undefined.

Bacterial metabolites, including porphyrins and vitamin B12, may play a role in initiating and promoting acne pathogenesis. Porphyrins have been shown as an inflammatory stimulating factor. Produced by bacteria, porphyrins interact with molecular oxygen, generate free radicals to damage adjacent keratinocytes, and stimulate the production of inflammatory mediators in keratinocytes (*26-30*). The biosynthesis of porphyrins in *Propionibacteria* is inversely correlated with the biosynthesis of vitamin B12, an essential vitamin for humans synthesized by host-

associated bacteria. These two biosynthesis pathways share their initial synthetic steps and a common precursor, L-glutamate (*31*). Multiple clinical studies reported that use of vitamin B12 induced acne in a subset of individuals (*32-37*). However, the mechanism underlying vitamin B12-induced acne has not been understood.

Chapter 2: Metatranscriptomic analysis of the skin microbiome associated with acne vulgaris

Abstract

Much progress has been made to characterize the microbial composition of the human microbiota and its link to various diseases, but the underlying mechanisms of the human microbiome in disease pathogenesis are often poorly understood. Using acne as a disease model, we aim to understand the molecular response of the skin microbiota to host metabolite signaling in disease pathogenesis. Using metatranscriptomic analysis, we demonstrated that the transcriptional profiles of the skin microbiota in acne were distinct from those in healthy skin. In particular, the vitamin B12 biosynthesis pathway in the skin bacterium, *Propionibacterium acnes,* was significantly down-regulated in acne patients compared to healthy subjects. To our knowledge, this is the first skin metatranscriptome study. It reveals a novel finding that the *in vivo* transcriptional activities of the microbiota clearly separate the healthy and disease states of the host. This finding also highlights the importance of studying the metatranscriptome of the human microbiota in addition to the microbial composition to better understand the role of the microbial community in human health and disease.

Introduction

Microorganisms live symbiotically with humans and contribute to human health and disease. The taxonomic composition of the human microbiome has been characterized in both healthy and diseased populations (*6, 7, 38-45*), revealing that alterations in the microbiome composition are associated with certain diseases (*1, 46*). However, the underlying molecular mechanisms of the human microbiota in disease pathogenesis are not well understood. At many body sites, including skin, it has not been described whether host metabolites modulate the transcriptional

activities of the microbiota and whether the microbiota responds to the host signals via altered metabolic activities thus impacting on the host health/disease state. Understanding the molecular mechanisms of host-microbiota interactions may lead to more targeted therapy of many microbe-related human diseases. However, the transcriptional and metabolic activities and their regulations of the human microbiome are largely undefined at many body sites including skin. There are several technical challenges in metatranscriptomic studies of the human skin microbiome. These include low yield of microbial RNA, depletion of ribosomal RNA (rRNA), and the requirement of a large number of samples and high sequencing depth in order to detect differences among individuals with large variations in the microbiome. The amount of available bacterial RNA is particularly limited in skin samples, thus making it challenging to apply RNA-Seq to understand the transcriptional activities of the skin microbiota under different health states.

Acne vulgaris (commonly called acne) provides a promising disease model to study the interactions between the host and the skin microbiota in disease pathogenesis, because the microbiota is less complex and the disease has been associated with a single dominant bacterium, *Propionibacterium acnes* (*6, 21-23*). Acne is the most common skin disease, affecting more than 80% of adolescents and young adults globally (*10-13*). Although not fatal, acne can be extremely painful, disfiguring, and scarring, and in many patients can profoundly affect self-esteem and mental health (*15, 16*). It is a disease of the pilosebaceous unit (hair follicle), a unique skin compartment where resident bacteria interact with the host cells. Four main factors are believed to contribute to acne development: increased sebum production, follicular hyperkeratinization, proliferation of skin bacteria, and inflammation (*17-20, 25*). The bacterial pathogenesis mechanism of acne remains elusive. *P. acnes* has been long thought as a pathogenic factor for

acne. However, it is a major skin commensal and dominates the skin microbiota in both acne patients and healthy individuals (*6*). Understanding whether the transcriptional activities of the skin microbiota are different between the two cohorts would provide key insights on the bacterial pathogenesis of acne.

Results

The transcriptional activities of the skin microbiota in acne patients were distinct from those in healthy individuals

To determine whether the transcriptional activities of the skin microbiota contribute to disease development, we compared the metatranscriptome of the skin microbiota in acne patients to the one in healthy individuals (Figure 1-1). We collected the contents inside nose follicles from four acne patients and five healthy individuals (Supplementary Materials). We analyzed the microbial gene expression using RNA-Seq with a high sequencing depth of 44-182 million paired-end reads per sample (3.7G bp-18.1G bp) (Table 1-S1). We found that *P. acnes* was the most transcriptionally abundant bacterium (Figure 1-S1). Additionally, *Staphylococcus*, *Pseudomonas*, and *Shigella* were detected in the metatranscriptome with much lower total transcriptional activities. Given the predominance of *P. acnes* in all samples, we focused our further analysis on the gene expression profile of *P. acnes*.

We first quantified the *P. acnes* gene expression levels. Since each individual harbors various strains of *P. acnes* and the strain population structures of *P. acnes* are different among individuals (*6*), we created a non-redundant *P. acnes* gene set representative of all the genes

encoded in various *P. acnes* strains. This enabled us to compare the gene expression level among different individuals even though they may harbor different strains. We binned the orthologous genes in 71 *P. acnes* genomes, which cover all the major lineages of *P. acnes* found on human skin (*47*), into 5,140 operational gene units (OGUs) as described in Methods. We then determined the expression level of each OGU in each sample. We identified a core set of 3,725 *P. acnes* OGUs (72.5% of all OGUs) expressed in all samples. This core set of OGUs covered most of the metabolic pathways encoded in *P. acnes* genomes, including sugar metabolism, nucleic acid metabolism, amino acid metabolism, lipid metabolism, and the metabolism of cofactors and vitamins (Figure 1-S2).

We next determined whether the gene expression profiles of *P. acnes* were distinct between acne patients and healthy individuals. We grouped the samples based on the expression profiles of *P. acnes* OGUs using an unsupervised hierarchical clustering algorithm as described in Methods. Microbiome samples of the acne patients formed a separate cluster from the samples of the healthy individuals (Figure 1-2A). This result reveals that *P. acnes* transcriptional activities in the skin microbiota of acne patients were distinct from those of healthy individuals. This is a novel finding revealing that the *in vivo* transcriptional activities of the microbiota clearly separate the healthy and disease states of the host. This finding also highlights the importance of studying the metatranscriptome of the human microbiota in addition to the microbial composition to better understand the role of the microbial community in human health and disease.

***P. acnes* vitamin B12 biosynthesis pathway was down-regulated in acne patients**

To determine the molecular mechanism of the skin microbiota in acne pathogenesis, we identified the microbial genes and pathways that were expressed differentially between acne patients and healthy individuals. In acne patients, 109 *P. acnes* OGUs were up-regulated and 27 OGUs were down-regulated (Figure 1-2B). Many of these differentially expressed OGUs encode cellular components that are involved in metabolite and protein transport, including metal transporters (iron, cobalt, and hemin), multi-drug transporters, protein export systems, and type II bacterial secretion systems. Using a functional annotation clustering analysis in DAVID (*48*), we identified several differentially expressed metabolic pathways in acne. They include vitamin B12 biosynthesis, porphyrin metabolism, proteolysis, transport, arginine metabolic process, and glutamine family amino acid metabolism. We further mapped the differentially expressed OGUs to KEGG pathways (Figure 1-3 and Figure 1-S3). It indicated that on acne patients' face, *P. acnes* exhibited increased transcriptional activities of the genes that encode carbohydrate uptake systems, enzymes that catalyze carbohydrate metabolism, and decreased transcriptional activities of the genes that encode enzymes in fatty acid biosynthesis and vitamin B12 biosynthesis.

Among these *P. acnes* transcriptional changes, the down-regulated vitamin B12 biosynthesis pathway drew our attention. Porphyrins and vitamin B12 have been implicated in initiating and promoting acne pathogenesis. Skin bacterial porphyrins have been suggested as inflammatory stimulating factors. They interact with molecular oxygen, generate free radicals to damage adjacent keratinocytes, and stimulate the production of inflammatory mediators in keratinocytes (*27-29*). The biosynthesis of porphyrins in Propionibacteria is inversely correlated with the biosynthesis of vitamin B12 (*31, 49*). Bykhovskii *et al.* has found that inhibiting vitamin B12

biosynthesis in Propionibacteria by vitamin B12 pathway inhibitor, such as hydroxylamine, increases the biosynthesis of porphyrins (*49*). These two biosynthesis pathways share initial synthetic steps and a common precursor, L-glutamate (*50*). Multiple clinical studies report that use of vitamin B12 induced acne in a subset of individuals (*32-35, 51-53*). However, the molecular mechanism of vitamin B12 inducing acne is not understood.

Based on the metatranscriptome data of the skin microbiota described above, we hypothesized that in the microbiota of acne patients the vitamin B12 biosynthesis pathway is down-regulated, resulting in the metabolic flow of the precursor, L-glutamate, being shunted to the porphyrin biosynthesis pathway (Figure 1-3). This results in an over-production of porphyrins by *P. acnes,* which can induce an inflammatory response in the host cells leading to acne. To test this hypothesis, we first verified the down-regulation of the vitamin B12 biosynthesis pathway in the skin microbiota in a new cohort of subjects. We collected skin microbial samples from an additional nine acne patients and 15 healthy individuals. Using qRT-PCR, we quantified the expression levels of three genes in the vitamin B12 biosynthesis pathway. Among the three genes, one encodes cobalamin synthesis protein *CysG+CbiX* (a fusion gene), and one encodes precorrin-2 C20-methyltransferase *CbiL.* These two genes function at the initial steps of corrin ring synthesis controlling the metabolic flow entering the vitamin B12 biosynthesis pathway (*54*). The third gene encodes cob(I)alamin adenosyltransferase *BtuR*, which adenosylates the synthesized corrin ring, and once inactivated, blocks the synthesis of vitamin B12 (*54*). Consistent with the RNA-Seq data obtained from our previous cohort, *CysG+CbiX* fusion gene and *CbiL* were significantly down-regulated in the nine acne patients (both $P < 0.01$, fold changes = 3.34 and 1.94, respectively). *BtuR* also had lower expression levels in the acne patients

compared to the healthy individuals, although the difference was not statistically significant (P = 0.168, fold change = 1.35) (Figure 1-2C). In this new cohort of subjects, we found down-regulated vitamin B12 biosynthesis gene expression in *P. acnes* in acne patients compared to healthy individuals, which is consistent with our previous observation.

Discussion

To our knowledge, the metatranscriptome of the human skin microbiota in health and disease states has not been described prior to this study. While some other human body sites, such as oral cavity, gut, urogenital tract, and upper respiratory tract, can have sufficient biomass for microbiota characterization (*55-58*), the skin presents a challenging organ with low microbial biomass density (*21, 59*). Studies of the skin microbiome have been limited to mostly taxonomic composition analysis (*60*) and some recent metagenomic analysis (Chapter 6) (*9*).Using 16S rRNA and metagenomic approaches (*6*) (Chapter 6), the taxonomic composition and functional capability of the microbiota in pilosebaceous units have been studied. Although these studies were able to identify acne-associated bacterial taxa or metagenomic elements, they could not separate an acne cohort completely from a healthy cohort sorely based on taxonomic compositional differences or functional capability differences. In this study, for the first time, we applied RNA-Seq to quantitatively measure the metatranscriptome of the skin microbiota in pilosebaceous units. We revealed microbial differences at the transcriptional level separating acne patients and healthy individuals. This demonstrates that although the composition and functional capabilities of the skin microbiome in pilosebaceous units may not undergo fundamental changes during disease pathogenesis, the transcriptional activities differed

significantly in different host environments and disease states. This also demonstrates that in addition to the microbial composition and functional capabilities, studying the transcriptional activities of the human microbiome is important to better understand the bacteria-host interactions in health and disease.

Materials and Methods

Subjects

Subjects with acne and subjects with healthy skin were recruited in Southern California. The diagnosis of acne was made by board-certified dermatologists. The presence of acne was graded on a scale of 0 to 5 relating closely to the Global Acne Severity Scale (*61*). Subjects with healthy skin were determined by board-certified dermatologists and were defined as people who had no acneiform lesions on the face, chest, or back. Nine subjects were sampled for RNA-Seq analysis. Among them, four were acne patients (two males and two females) and five were healthy subjects (two males and three females). The average age of the acne patients was 24.8 (19 - 38) and the average age of the healthy subjects was 22.8 (13 - 32). Among the acne patients, two are African American and two are Hispanic. Among the healthy subjects, one is African American, one is Hispanic, two are Caucasians, and one is Asian. There were no significant differences in gender, age and ethnicity between the acne patients and the healthy subjects. The average face acne score of the four acne patients was 2.5 and the average nose acne score was 0.25. Twenty-four additional subjects were recruited for the gene expression analysis of the vitamin B12 biosynthesis pathway. Among them, nine were acne patients (three males and six females) and 15 were healthy subjects (seven males and eight females). The average age of the acne patients

was 23.8 (15 - 42) and the average age of the healthy subjects was 33.8 (21 - 44). Among the acne patients, four are Hispanic, two are Caucasian, two are Asian, and one has more than one race. Their average face acne score was 2.8 and the average nose acne score was 1.6.Among the healthy subjects, three are Hispanic, three are Caucasian, eight are Asian, and one has more than one race. There were no significant differences in gender and ethnicity, but age, between the acne patients and the healthy subjects. Among the 15 healthy subjects, ten were receiving intramuscular injection of vitamin B12 (1 mL of 1,000mcg/mL hydroxocobalamin) for general well-being. Among all the subjects, none of the healthy subjects reported any current or past acne treatment. None of the acne patients were being treated with antibiotics at the time of sampling. Four of the acne patients were currently being treated with other acne therapies, including Tazorac, Tretinoin, Proactive, and Benzoyl peroxide. Seven of the acne patients were treated with acne therapies in the past (at least a week ago or even before), including Benzoyl peroxide, Tretinoin, Proactive, Clindamycin lotion, Minocycline, and Accutane. All subjects provided written informed consent. All protocols and consent forms were approved by both the UCLA and Los Angeles Biomedical Research Institute IRBs.

Sample collection

The follicular contents of the nose skin were sampled from the subjects using Bioré Deep Cleansing Pore Strips (Kao Brands Company, Cincinnati, OH) following the instruction of the manufacturer (*6*). This sampling method is different from the conventional tape-stripping method. It samples mostly the follicular content of the pilosebaceous unit, while the conventional tape stripping method samples the stratum corneum of the epidermis. A comparison of the samples obtained using these two different methods is illustrated in Figure 1-S5. Clean gloves were used

for each sampling. After removal from the nose, the strip was placed into a 50 mL sterile tube and labeled with a coded sample name.

For the ten healthy subjects with vitamin B12 supplementation, the first samples (day 0) were taken from one side of the nose before they received vitamin B12 injection. The second samples (day 2) were taken from the other side of the nose two days after the injection. The third samples (day 14) were taken from the entire nose 14 days after the injection. As a control, three months later, nine of the ten healthy subjects were sampled again using the same sampling protocol over a period of 14 days without vitamin B12 supplementation.

Total RNA extraction

The follicular contents were individually picked from the pore strip using sterile forceps and placed in a 2 mL sterile microcentrifuge tube filled with ATL buffer (Qiagen) and glass beads (0.1mm diameter) (BioSpec Products, Inc., Bartlesville, OK). Cells were lysed using a beadbeater for 3 minutes at 4,800 rpm at room temperature. To avoid overheating the samples, the beating was paused every 1 minute and the samples were placed on ice for at least 1 minute. After centrifugation at 14,000 rpm for 5 minutes, the supernatant was retrieved and used for total RNA extraction. Twenty microliter proteinase K solution (Qiagen) was added, followed by incubation for 10 minutes at 56˚C. After incubation, 1 mL preheated QIAzol (Qiagen) at 65˚C was added and incubated for 5 minutes at room temperature. The supernatant was then separated into two phases by adding 200µL chloroform and centrifuging at 12,000 g for 5 minutes at 4˚C. The total RNA in the aqueous phase was then cleaned up using RNA MinElute Clean-up kit (Qiagen) according to the manufacturer's instruction. After total RNA extraction, each sample

was subjected to DNase digestion to remove residual genomic DNA contamination using TURBO DNA-free kit (Ambion).

Ribosomal RNA (rRNA) depletion, RNA amplification and cDNA synthesis

To enrich messenger RNA (mRNA), rRNA was depleted from the total RNA using MICROBExpress Kit (Ambion). The enriched mRNA was further polyadenylated and amplified by *in vitro* transcription-based mRNA linear amplification using MessageAmp II-Bacteria kit (Ambion). The amplified RNA was then converted to double-stranded cDNA using random hexamer (Promega) and SuperScript Double-Stranded cDNA synthesis kit (Invitrogen) (*62, 63*).

RNA-Seq

A cDNA library with an average 300 bp insert was prepared for each sample. The libraries were sequenced using the Illumina sequencing platform (Illumina, Inc., San Diego, CA). The reads were paired-end with a read length of 82 -100 bp for the nine samples.

Data cleaning

Sequence reads were trimmed from 3' end to remove low quality bases. Reads containing more than five consecutive A/T at the 3' end or containing more than 90% of A/T within the entire sequence reads, which are artifacts of RNA amplification and rarely occur in *P. acnes* genomes, were removed. After trimming, cleaned sequence pairs, where both reads are longer than 40 bp and have fewer than three ambiguous base pairs, were used for further analysis.

Sequence mapping

Sequence mapping was performed using Bowtie (version 0.12.7) allowing up to three mismatches per read (*64*). Cleaned reads were mapped to *P. acnes* 16S, 23S and 5S rRNA sequences. The rRNA reads from other bacteria and human were identified by aligning them to SILVA database (release 108) (*65*). The remaining reads were aligned to 71 *P. acnes* genomes (*47*). For paired-end mapping, we required that the read pairs were aligned to the same reference genome on different strands with the distance between the two reads shorter than 1 kb. The remaining reads were then searched against the Human Microbiome Project reference genome database (http://www.hmpdacc.org/HMREFG/) and the human genome (hg19). The unaligned reads were further searched against NCBI non-redundant nucleotide database (RefSeq release 48), including microbial, fungal, and viral genomes.

OGU construction

P. acnes OGUs were constructed in a similar way as previously described (*66*) with minor modification. Genes in all 71 *P. acnes* genomes were binned based on their protein sequences using CD-HIT version 4.3 (*67*) and its default parameters (> 90% protein sequence identity).

Quantification of OGU expression level

We assigned a coverage score of 1 to each reference nucleotide aligned by a read with unique alignment. The total coverage score of each reference nucleotide was calculated by summing the scores of the nucleotide from all the aligned reads. The reads with multiple hits were analyzed as previously described (*68*). The coverage score was normalized by the total number of hits (M).

Each nucleotide within the aligned region in the reference genome was assigned a score of 1/M. The expression level of a gene was calculated as the total score for all the nucleotides within the gene region normalized by the gene length. To eliminate sequencing depth differences among different samples, the expression level of each *P. acnes* gene was further normalized by the total number of base pairs aligned to *P. acnes* genomes. Reads aligned to *P. acnes* rRNAs were removed prior to normalization. OGU expression level was calculated by summing the normalized expression level of each gene member of the OGU.

Functional classification of OGUs

The representative protein sequence of each OGU was mapped against the COG database (*69*) and KEGG database (*70*) using BLASTP. The COG identifier or KEGG identifier of the best BLAST hit with an e-value < 0.00001 was assigned to the corresponding OGU. If multiple hits were found from non-overlapping regions of a query sequence, all of them were assigned to the OGU.

Rarefaction curve of the number of OGUs detected

For each sample, a random number of sequence reads aligned to *P. acnes* genomes was sampled. The number of expressed unique OGUs represented by the sampled sequence reads was calculated using the R package Vegan 1.17-0 (*71*). The number of sequence reads randomly sampled was increased from 0 to the sequencing depth of each sample. The rarefaction curve of each sample was plotted using the number of expressed unique OGUs as a function of the number of sampled sequence reads. For all nine samples, rarefaction curves reached plateaus of

detecting expressed *P. acnes* OGUs after sampling 100 million base pairs. Rarefaction analysis showed that our sequencing depth was sufficient for gene expression analysis (Figure 1-S4).

Unsupervised hierarchical clustering analysis

OGUs with detectable expression level (≥ 1 after normalization) in at least five samples and with significant variation across samples (standard deviation ≥ 150) were used in the hierarchical clustering analysis. The clustering was done by R function "hclust" with centered Pearson correlation similarity metric and average linkage clustering method (*72*). A total of 562 OGUs passed filtering criteria and were used for clustering analysis of the nine RNA-Seq samples.

Analysis of differentially expressed OGUs

The expression levels of *P. acnes* OGUs in the RNA-Seq data were compared between samples from acne patients and samples from healthy individuals using the independent samples t-test (unequal variance, two-sided test). Differentially expressed OGUs were identified using a cutoff of p–value < 0.05. These differentially expressed OGUs were further confirmed by a Poisson model based comparison, ShotgunFunctionalizeR (*73*) in R package (http://www.r-project.org, http://shotgun.zool.gu.se), with a cutoff of Akaike's information criterion < 5000 and adjusted p ≤ 0.05. The heat map of differentially expressed OGUs was generated using R package gplots (*74*), heatmap.2 function based on z scores.

KEGG pathway mapping

The global KEGG metabolic pathways were mapped using iPath2 (*75*).

Cob/Cbi gene expression study

Total RNA was extracted from *P. acnes* cell cultures or skin microbial samples collected from the subjects using the protocol described above. The total RNA was converted to single-stranded cDNA using SuperScript III first-strand synthesis supermix (Invitrogen). qRT-PCR was performed on LightCycler 480 (Roche) using the LightCycler 480 High Resolution Melting Master Mix (Roche) and the following primers:

CbiL-forward: 5'-GCGCGAGGCAGACGTGATCC-3', CbiL-reverse: 5'-GACACCGGACCTCTCCCGCA-3', CysG+CbiX-forward: 5'-TGTATTCCGCCCCGCTGTTGC-3', CysG+CbiX-reverse: 5'-GAGCACTGCCGACGTGTCCC-3', BtuR-forward: 5'-GGAAGATGCTCTTCGGGCGCT-3', BtuR-reverse: 5'-GCCTCAGGGTTCTCCGCAGC-3', 16S-forward: 5'-GGGGCTTAACCCTGAGCGTGC-3', 16S-reverse: 5'-TTCGCTCCCCACGCTTTCGC-3'. The qRT-PCR protocol was set as the following: initial denaturation at 95˚C for 5 minutes, followed by 50 cycles of 95˚C for 10 seconds, 62˚C for 30 seconds, and 72˚C for 30 seconds. The expression level of each gene was expressed as the logarithm of its relative expression level to 16S rRNA transcript level.

Statistics

The differentially expressed OGUs in RNA-Seq data were identified by independent sample t-tests. The threshold of statistical significance was set at $P< 0.05$. The differentially expressed OGUs were further confirmed by a Poisson model based comparison, ShotgunFunctionalizeR (*73*) in R package (http://www.r-project.org, http://shotgun.zool.gu.se), with a cutoff of Akaike's information criterion < 5000 and adjusted $P \leq 0.05$. An online functional annotation clustering

analysis, DAVID (*48*), was used to identify the differentially expressed metabolic pathways in acne. Independent sample t-tests (two-tailed) were used to determine the statistical significance of the differences in vitamin B12 gene expression quantified by qPCR.

Supplementary Materials

Nose is a clinically relevant site for acne.

To address this point, we asked two independent medical staff, who are trained in dermatology, to review 61 randomly selected cases of acne vulgaris on the face in an electronic dermatology database where images were only submitted by expert dermatologists. While the two reviewers gave an average acne score on the nose lower than the surrounding face (1.6 vs. 3.4 respectively), 70.5% of the faces examined had obvious acne on the nose. This finding clearly demonstrates that nose is a common site affected by acne and supports that nose is a relevant sampling site for studying acne vulgaris.

Figures and Tables

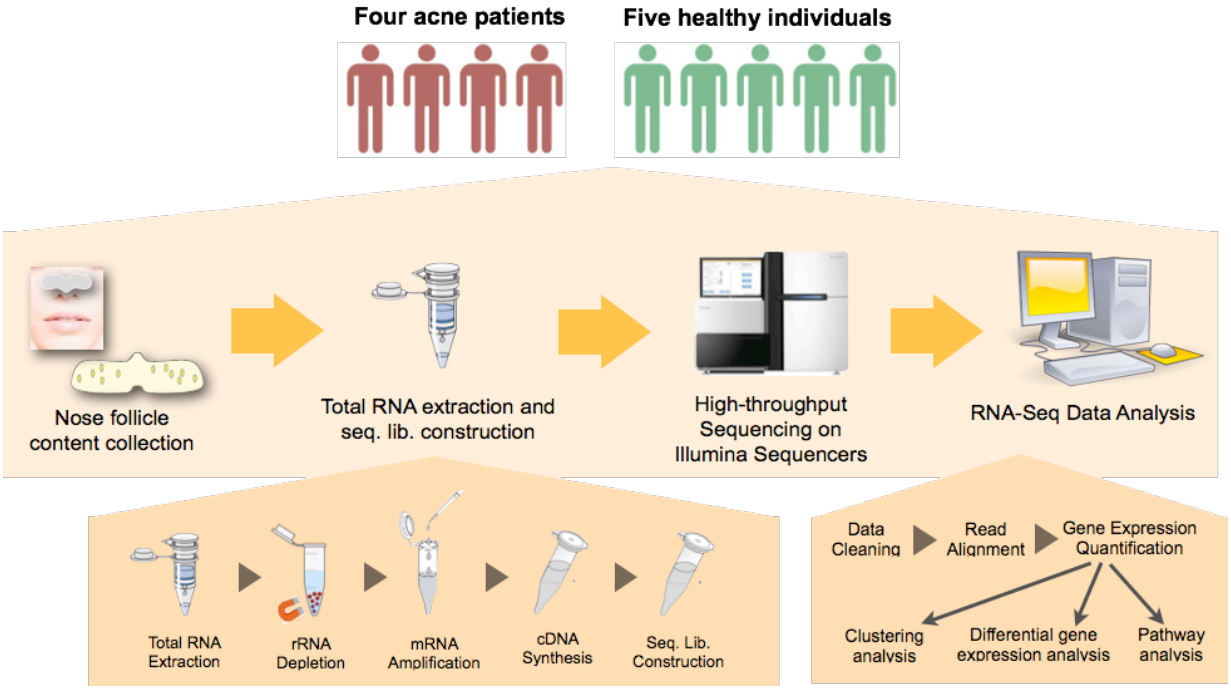

Figure 1-1. Study Design

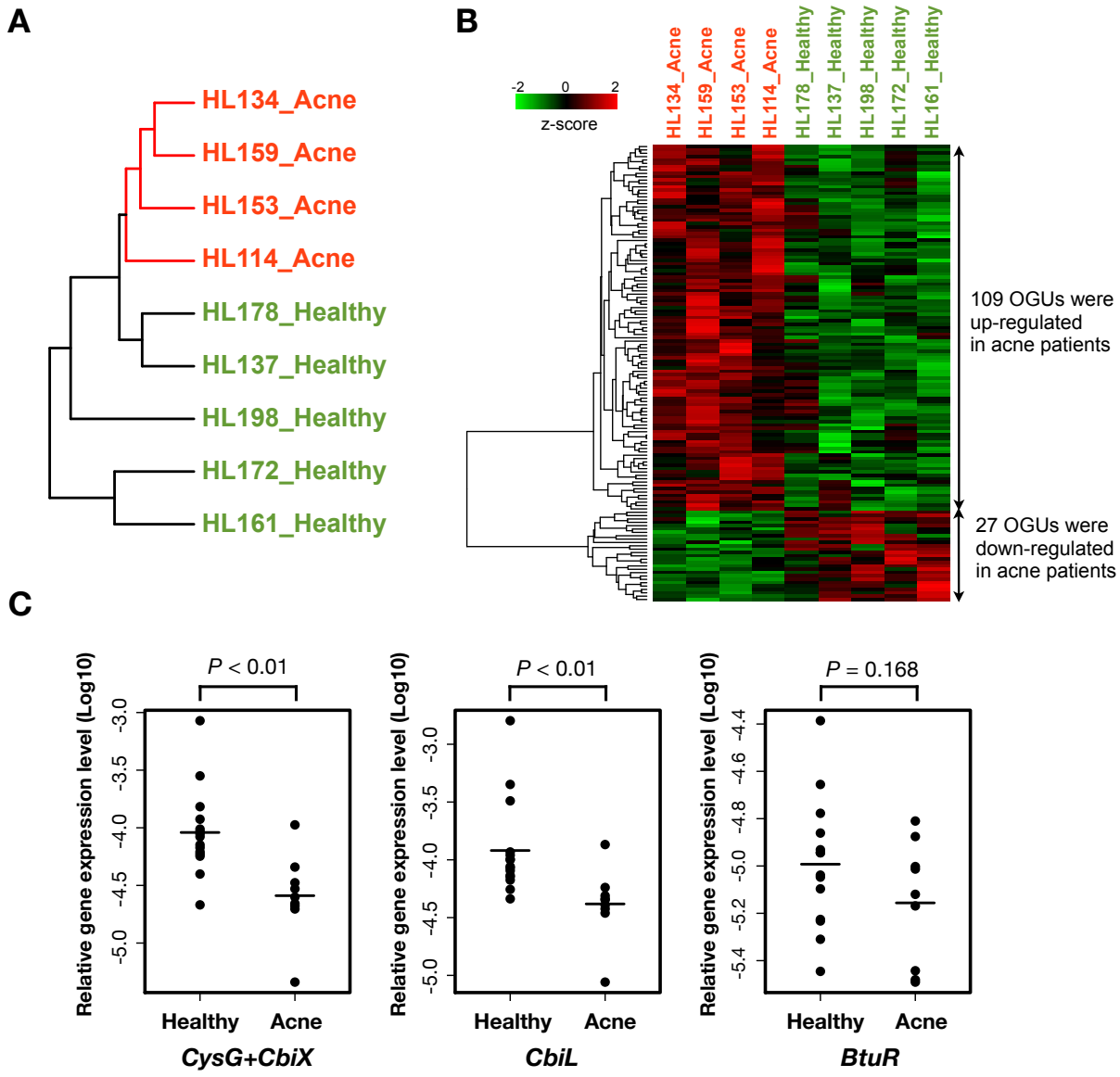

Figure 1-2. The gene expression profiles of *P. acnes* in the skin microbiota were distinct between acne patients and healthy individuals.

(A) Based on the gene expression of *P. acnes* in the skin microbiota, acne patients formed a separate cluster from healthy individuals in an unsupervised hierarchical clustering analysis.

(B) 136 differentially expressed *P. acnes* OGUs were identified between acne patients and healthy individuals. Among them, 109 OGUs were up-regulated (red, positive z score) and 27 OGUs were down-regulated (green, negative z score) in acne patients.

(C) Down-regulation of the genes in *P. acnes* vitamin B12 biosynthesis pathway was validated in a separate cohort of acne patients (n = 9) and healthy individuals (n = 15). Consistent with the RNA-Seq data, *CysG+CbiX* and *CbiL* were significantly down-regulated, and *BtuR* showed a lower average expression level in acne patients compared to healthy individuals. Significance was determined by independent samples t-test. The mean of the expression levels of each gene is indicated by a black bar.

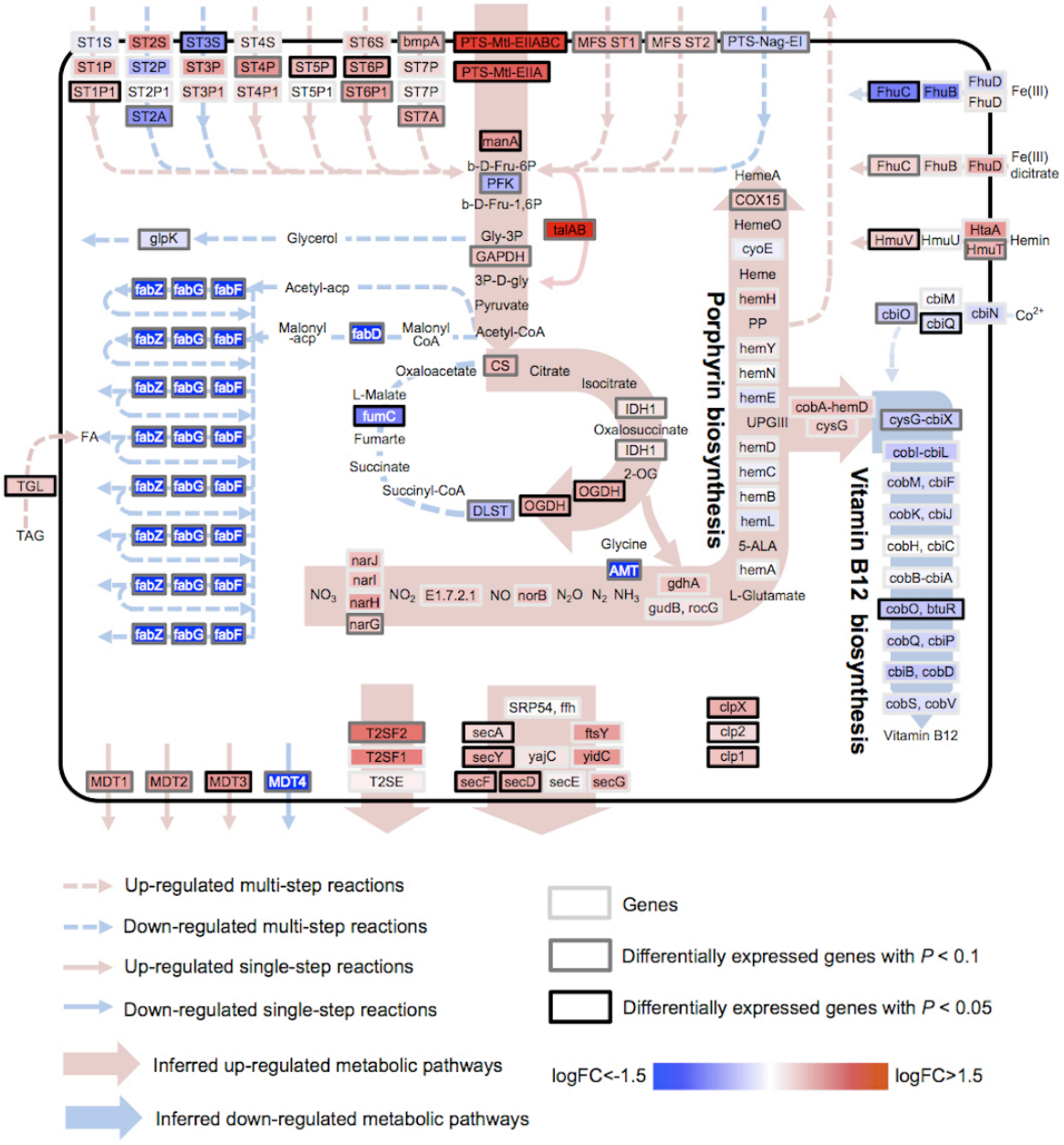

Figure 1-3. A schematic of the metabolic activities in *P. acnes* based on the observed differentially expressed OGUs in acne patients compared to healthy individuals.

Nitrogen assimilation, sugar transport, and sugar metabolism pathways were up-regulated in acne patients. Vitamin B12 biosynthesis and fatty acid biosynthesis pathways were down-

regulated. This finding suggests that increased metabolic activities of sugar metabolism and nitrogen assimilation led to an increased metabolic flow toward porphyrin biosynthesis, while vitamin B12 biosynthesis was repressed in acne. FC: fold change.

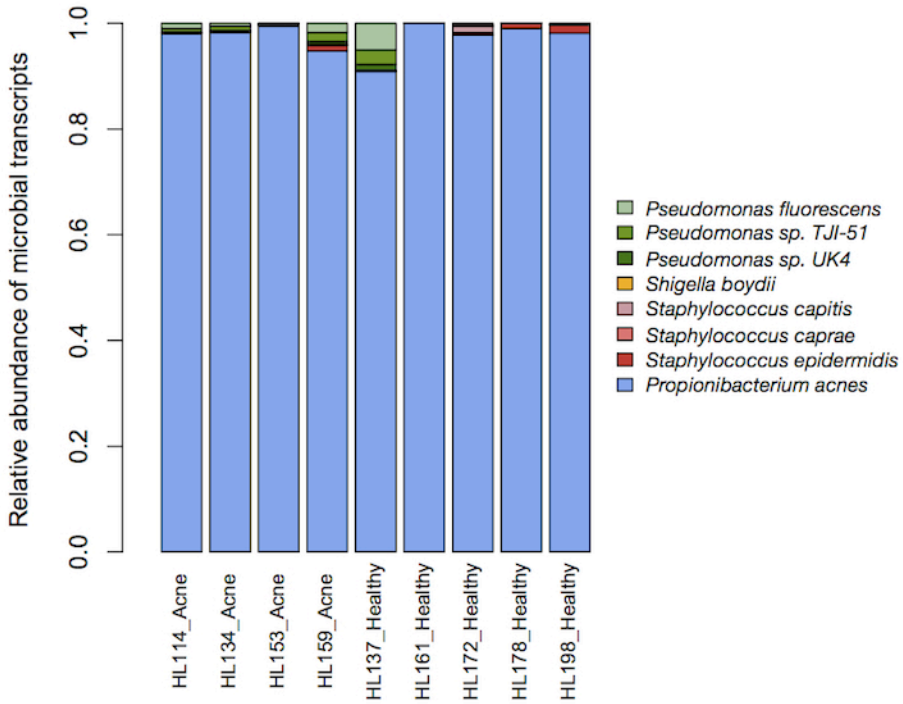

Figure 1-S1. The metatranscriptome composition of the skin microbiota in pilosebaceous units.

P. acnes transcripts are the most abundant microbial transcripts in all samples. Additionally, we found transcripts from several other bacteria, including *Staphylococcus*, *Shigella* and *Pseudomonas*.

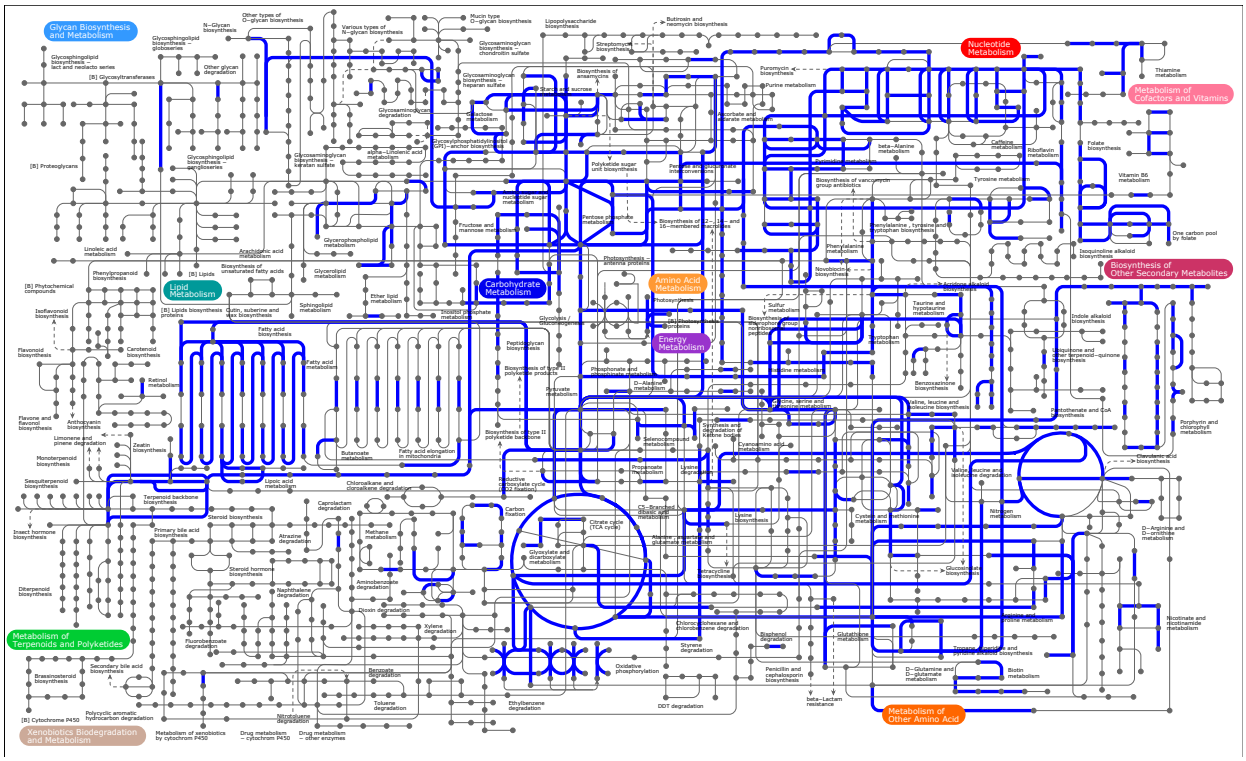

Figure 1-S2. KEGG pathways expressed in all the samples.

The core *P. acnes* transcriptome shared by all the samples covered most of the metabolic pathways (blue lines), including carbohydrate metabolism, nucleic acid metabolism, amino acid metabolism, lipid metabolism, and the metabolism of cofactors and vitamins.

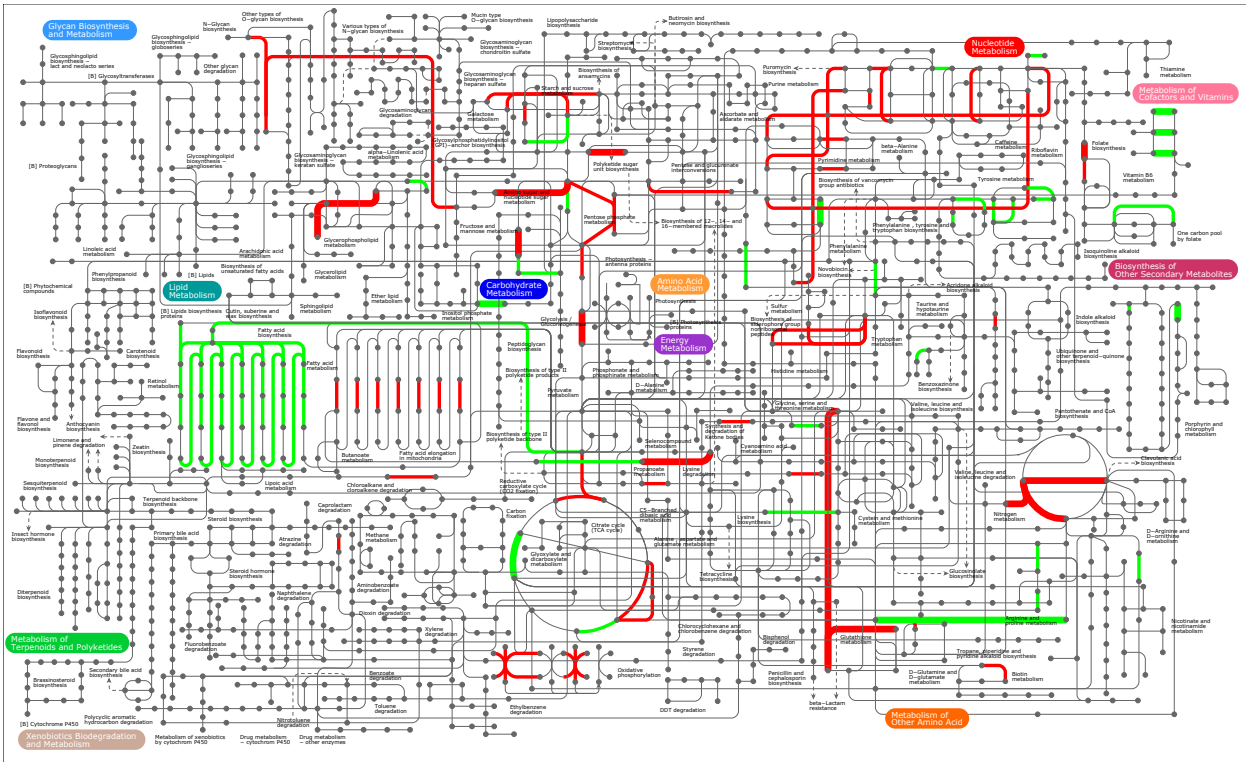

Figure 1-S3. KEGG pathways with differentially expressed *P. acnes* OGUs.

Thick red lines indicate up-regulated OGUs in acne with $P < 0.05$. Thin red lines indicate up-regulated OGUs in acne with $0.05 < P < 0.1$. Thick green lines indicate down-regulated OGUs in acne with $P < 0.05$. Thin green lines indicate down-regulated OGUs in acne with $0.05 < P < 0.1$.

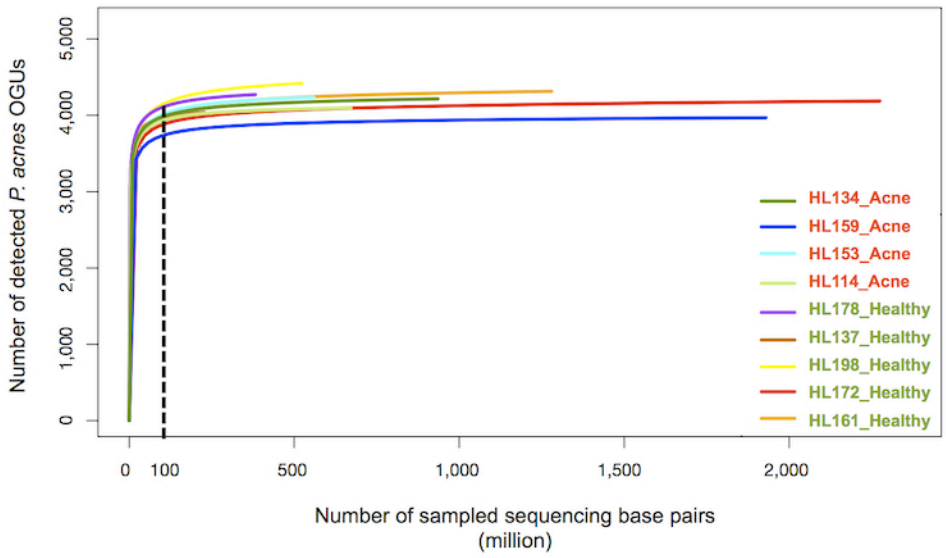

Figure 1-S4. Rarefaction curves indicate sufficient sequencing depths of the samples.

The rarefaction curves of all nine samples reached plateaus in detecting *P. acnes* OGUs with more than 100 million base pairs. This suggests that the sequencing depths of all these samples (≥ 229 million base pairs) were sufficient for the gene expression analysis.

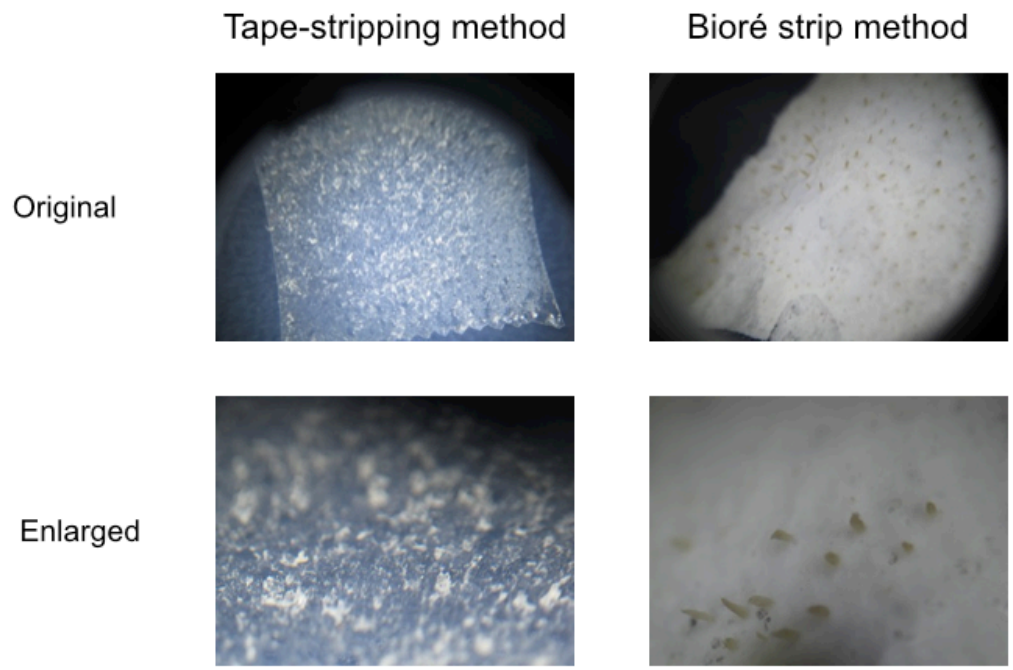

Figure 1-S5. A comparison of the samples obtained using a Biore strip method and a tape-stripping method. These two samples were collected from the opposite sides of the nose of the same individual. As shown, the tape-stripping method removes dead keratinocytes from the outside of the skin, sampling the stratum corneum of the epidermis. In contrast, the Biore strip method removes the whole contents of pilosebaceous units. The height of the extracted spikes ranges up to 2 mm. It samples the microbiota residing inside the follicles, including the anaerobic portion.

Table 1-S1. High sequencing depths of the metatranscriptomic data.

Sample name	Subject type	Read length (bp)	Number of paired-end reads in raw data (million)	Number of paired-end reads in cleaned data (million)
HL114	acne	84	52	42
HL134	acne	100	170	96
HL153	acne	84	66	48
HL159	acne	100	160	136
HL137	healthy	84	44	32
HL161	healthy	84	72	50
HL178	healthy	82	58	46
HL172	healthy	100	182	148
HL198	healthy	84	52	44

Chapter 3:Vitamin B12 modulates the transcriptome of the skin microbiota and induces acne

Abstract

Various diseases have been linked to the human microbiota, but the underlying molecular mechanisms of the human microbiota in disease pathogenesis are often poorly understood. Using acne as a disease model, we aim to understand the molecular response of the skin microbiota to host metabolite signaling in disease pathogenesis. We hypothesized that host vitamin B12 modulates the activities of the skin microbiota and plays a signaling role in acne pathogenesis. To test this hypothesis, we showed that vitamin B12 supplementation in healthy subjects repressed vitamin B12 biosynthesis in *P. acnes* on the skin and induced acne in one of the ten subjects studied. To further understand the molecular mechanism, we revealed that vitamin B12 supplementation in *P. acnes* cultures promoted the production of porphyrins, which are known to induce inflammation in acne. Our findings suggest a novel bacterial pathogenesis pathway in acne and provide a molecular explanation for the long-standing clinical observation that vitamin B12 induces acne. Our study not only discovered that vitamin B12, an essential nutrient in humans, modulates the transcriptional activities of the bacteria on the skin, but also provides evidence that metabolite-mediated interactions between the host and the skin microbiota play essential roles in disease development.

Introduction

Microorganisms coexist with humans and affect the health and disease states of the host (*1*). Metabolites play important signaling roles in the interactions between the various commensal microbes (microbiome) and the host. One well-known example is the gut microbiome, which influences the health states of the host via bacterial metabolites (*2*-*5*). Whether or not the microbial communities at other body sites affect the host health states via bacterial metabolites has not been well characterized. Furthermore, little is known about how host metabolite levels affect the transcriptional and metabolic activities of the microbiome.

Bacterial metabolites, including porphyrins and vitamin B12, may play a role in initiating and promoting acne pathogenesis. Porphyrins have been suggested as an inflammatory stimulating factor. Produced by bacteria, porphyrins interact with molecular oxygen, generate free radicals to damage adjacent keratinocytes, and stimulate the production of inflammatory mediators in keratinocytes (*26*-*30*). The biosynthesis of porphyrins in *Propionibacteria* is inversely correlated with the biosynthesis of vitamin B12, an essential vitamin for humans synthesized by host-associated bacteria. These two biosynthesis pathways share their initial synthetic steps and a common precursor, L-glutamate (*31*). Multiple clinical studies report that use of vitamin B12 induced acne in a subset of individuals (*32*-*37*). However, the mechanism underlying vitamin B12-induced acne has not been understood.

Results

Vitamin B12 repressed its *de novo* biosynthesis in *P. acnes* and promoted porphyrin production

We determined whether vitamin B12 represses its *de novo* biosynthesis in *P. acnes* and whether down-regulation of vitamin B12 biosynthesis promoted porphyrin production. Previous studies in other bacterial species, including *Salmonella typhimurium* (*76*) and *Escherichia coli* (*77*), showed that vitamin B12 repressed the expression of the *Cob/Cbi* operons in vitamin B12 biosynthesis pathway through cobalamin riboswitches. Cobalamin riboswitches are conserved RNA structural elements that regulate the expression of vitamin B12 biosynthesis operons upon vitamin B12 binding (*78*). In *P. acnes*, the upstream regions of *Cob/Cbi* operons also encode cobalamin riboswitches. Consistently, we observed that in *P. acnes* vitamin B12 represses its *de novo* biosynthesis pathway. When we supplemented 10 μg/mL vitamin B12 to *P. acnes* cultures, we found that vitamin B12 persistently repressed the expression of *Cob/Cbi* operons. The above mentioned three genes in the vitamin B12 biosynthesis pathway, *CysG+CbiX, CbiL,* and *BtuR*, were significantly down-regulated by the addition of vitamin B12 from day 2 to day 14 (all $P< 0.05$) in cultures (Figure 2-1A). In the meantime, we compared the amounts of porphyrins produced in the *P. acnes* cultures with and without vitamin B12 supplementation. We found that the addition of vitamin B12 significantly increased porphyrin production by 36% during the exponential phase on day 8 ($P< 0.01$) and nearly 19% at the stationary phase on day 14 ($P< 0.001$) (Figure 2-1B). Our results demonstrated that vitamin B12 supplementation repressed the *de novo* biosynthesis of vitamin B12 in *P. acnes* and increased the biosynthesis of porphyrins, which are known to induce inflammation in acne (*27-29*).

To demonstrate that the increased porphyrin production is directly linked to the reduction in vitamin B12 biosynthesis, we further determined the porphyrin level in *P. acnes* under the condition that vitamin B12 biosynthesis was repressed without down-regulating the gene expression of the vitamin B12 biosynthesis pathway. We repressed vitamin B12 biosynthesis by depleting Co^{2+} ion, which is an essential component required for vitamin B12 biosynthesis. Zaitseva *et al.* showed that without Co^{2+} in culturing medium, *Propionibacteria* was not able to synthesize vitamin B12 (*79*). We observed that the porphyrin production in *P. acnes* cultures was significantly increased compared to the cultures with Co^{2+} added (Figure 2-1C). This result demonstrates that vitamin B12 biosynthesis pathway and porphyrin biosynthesis pathway share the same pool of precursor substrates and are inversely correlated at the metabolic level. It further supports that the down-regulation of vitamin B12 pathway promotes porphyrin production.

Vitamin B12 supplementation in healthy subjects repressed vitamin B12 biosynthesis in *P. acnes* on the skin

To determine whether vitamin B12 represses *P. acnes* vitamin B12 biosynthesis pathway *in vivo* and induces acne in humans, we analyzed the gene expression of the skin microbiota in healthy subjects with vitamin B12 supplementation. We sampled ten healthy subjects with clear skin who were receiving intramuscular injection of vitamin B12 (1 mg hydroxocobalamin) for general well-being. It is documented that a single intramuscular injection of 1 mg hydroxocobalamin leads to a several fold increase of serum vitamin B12 level, which lasts for at least two weeks (*80*). The serum vitamin B12 level usually returns to a normal range in four weeks (*80-82*). It is

also found that the skin vitamin B12 level is positively correlated with the serum vitamin B12 level (*83*). We collected the content in nose follicles from the ten healthy subjects at three time points: immediately before vitamin B12 injection as a baseline control, two days after the injection, and 14 days after the injection. We measured the gene expression levels of *CysG+CbiX, CbiL* and *BtuR* in vitamin B12 biosynthesis pathway using qRT-PCR. Compared to the expression levels before or two days after vitamin B12 injection, these genes were significantly down-regulated 14 days after the vitamin B12 injection (all $P< 0.05$). Moreover, the gene expression levels on day 14 were similar to those observed in the acne patients (Figure 2-2). As a control, we re-sampled nine of the ten subjects with healthy skin three months later using the same sampling procedure without vitamin B12 supplementation. For the healthy subjects without vitamin B12 supplementation, there were no significant changes in the gene expression level of vitamin B12 biosynthesis pathway between day 0 and day 14. There were also no significant differences in the gene expression level between the two sampling periods on day 0 (Figure 2-2). Our study demonstrates that vitamin B12 supplementation in the human host repressed the vitamin B12 biosynthesis pathway in *P. acnes*.

Vitamin B12 supplementation induced acne in one of the ten subjects studied

Multiple clinical studies reported that vitamin B12 supplementation induced acne in subsets of individuals (*32-35, 51-53*). In the current study, one of the ten healthy subjects, HL414, had acne breakouts on the face within the two weeks after vitamin B12 injection (Supplementary Materials). The mechanism of vitamin B12 inducing acne is not understood and it is not known whether vitamin B12 induced acne has a separate pathogenesis mechanism. To determine whether vitamin B12 supplementation in subject HL414 altered the gene expression of the skin

microbiota with a profile similar to those observed in the acne patients, we compared the *P. acnes* gene expression profiles from the samples collected from HL414 as well as the other healthy vitamin B12 supplementation samples and the nine acne-control cohort samples. On day 0, the *P. acnes* gene expression pattern of HL414 was similar to other healthy subjects, but on day 14 it resembled the expression pattern seen in the acne patients and was clustered within the acne group (Figure 2-3). This suggests that the transcriptional changes in the skin microbiota in vitamin B12 induced acne are not different from those in acne patients. Down-regulation of vitamin B12 biosynthesis and increased production of porphyrins in *P. acnes* may be a general downstream pathway in the bacterial pathogenesis of the disease. This result also strongly supports the hypothesis that the host vitamin B12 level modulates the transcriptional and metabolic activities of the skin bacteria, which in turn affect the health or disease state of the host skin.

Discussion

In this study, we revealed a molecular link between vitamin B12 and acne pathogenesis through the skin microbiota. Several lines of evidence suggest that a high level of serum vitamin B12 is associated with acne phenotype. Goldblatt *et al.* found that the serum level of vitamin B12 was significantly higher in acne patients than in healthy individuals (*84*). Moreover, they and Karadag *et al.* reported that in acne patients, the serum level of vitamin B12 was significantly decreased after treatment (*84, 85*). Based on our findings and the evidence mentioned above, we propose a vitamin B12-mediated bacterial mechanism for acne pathogenesis (Figure 2-4). In healthy skin, when the vitamin B12 level is normal, the vitamin B12 biosynthesis pathway in *P.*

acnes is expressed and porphyrins are produced at a low level. When the host vitamin B12 level is increased, the vitamin B12 biosynthesis pathway in *P. acnes* is repressed. The metabolic flow of L-glutamate is shunted toward the porphyrin biosynthesis pathway, leading to an over-production of porphyrins by *P. acnes* in the follicle. The over-produced porphyrins, secreted by *P. acnes*, induce an inflammatory response in the host cells leading to acne development. Supporting this proposed mechanism, clinical improvement after acne treatment has been associated with a lower level of porphyrins produced in follicles (*30, 86-88*). Our proposed mechanism of vitamin B12-mediated interactions between the host and the skin microbiota provides a molecular explanation for the bacterial pathogenesis of acne. It also emphasizes the importance of metabolite-mediated interactions between the host and the microbiota in human health and disease.

Metabolite-mediated interactions between the host and the microbiota play essential roles in human health and disease. This has been recognized in the gut microbiota (*4*). Host nutritional states or interventions, such as diets and use of antibiotics and other small-molecule drugs, have been shown to modulate the composition and transcriptional activities of the gut microbiota (*89-92*). In response, the gut microbiota produces metabolites that are linked to diseases (*4, 5, 93, 94*). Vitamin B12 is an essential nutrient to humans. Its microbial biosynthesis and human absorption mainly take place in the gut. It has been shown that vitamin B12 regulates gut microbial gene expression and affects the selection and competition among microbial species (*95*). Our study presented here, for the first time, showed that supplementing vitamin B12 to the host alters the gene expression in the skin microbiota and leads to acne development. Our study not only provides an explanation for the long-standing clinical observation of vitamin B12 inducing acne,

but also reveals a molecular mechanism for acne pathogenesis. Future studies of larger cohorts based on the findings from this study may potentially lead to the development of new therapeutics for this medically important disease.

Materials and Methods

Subjects

Ten subjects with healthy skin were receiving intramuscular injection of vitamin B12 (1 mL of 1,000mcg/mL hydroxocobalamin) for general well-being. Among all the subjects, none of the healthy subjects reported any current or past acne treatment. We collected the content in nose follicles from the ten healthy subjects at three time points: immediately before vitamin B12 injection as a baseline control, two days after the injection, and 14 days after the injection. As a control, we re-sampled nine of the ten subjects with healthy skin three months later using the same sampling procedure without vitamin B12 supplementation.

Sample collection, Total RNA extraction, Ribosomal RNA (rRNA) depletion, RNA amplification, cDNA synthesis, *and Cob/Cbi* gene expression study

These procedures were the same as described in the methods and materials in the chapter 2.

RNA-Seq

A cDNA library was prepared for each sample from HL414. The libraries were sequenced using the Illumina sequencing platform (Illumina, Inc., San Diego, CA). The reads were single-end or paired-end with a read length of 101 bp.

Data cleaning, Sequence mapping, OGU construction, Quantification of OGU expression level, Functional classification of OGUs

These procedures were the same as described in the methods and materials in the chapter 2.

Unsupervised hierarchical clustering analysis

The clustering analysis was done in the same way as described in the methods and materials in the chapter 2. When the two samples collected from subject HL414 were added in the clustering analysis, 649 OGUs were used.

Porphyrin quantification

P. acnes was cultured in reinforced clostridium broth anaerobically without exposure to light. The culture medium was supplemented with 10 μg/mL vitamin B12 (Sigma, V2876), a concentration similar to the one used in a previous study in *E. coli* (*96*). The controls were cultured without vitamin B12 supplementation. After 14 days, 200 μL bacterial culture was used to measure optical density at 595 nm, and 500 μL bacterial culture was used to extract porphyrins using the method described previously (*97*) with minor modifications. Briefly, bacterial culture was mixed with 250 μL ethyl acetate/acetic acid (4:1) for 10 seconds by

vortexing and then centrifuged for 5 minutes at 12,000rpm. The upper phase was transferred to a new tube and then mixed with 250 μL 1.5M HCl for 10 seconds. After centrifuging for 2 minutes at 12,000rpm, 200 μL extracted porphyrins in HCl lower phase was taken and quantified by measuring its absorbance at 405 nm. All the optical absorbance was quantified on TECAN GENios Microplate Reader. The absorbance at 405 nm was then converted to concentration based on a standard curve measured using coproporphyrin III.

Testing cobalt's effect on the biosynthesis of porphyrins

P. acnes cells were cultured in reinforce clostridium broth anaerobically to stationary phase. The cells were harvested by centrifugation, washed twice with sterile 0.1M sodium phosphate buffer (pH 7.0), and then re-suspended in a synthetic broth without carbon sources for a preliminary starvation. The composition of the synthetic broth is 0.1M sodium phosphate buffer, $(NH_4)_2SO_4$ 3g/L, KH_2PO_4 0.48g/L, K_2HPO_4 0.48g/L, $MgSO_4$ 0.2g/L, NaCl 0.01g/L, and $MnSO_4$ 0.01g/L. After being maintained in this medium for 24 hours at 30˚C anaerobically, the cells were again centrifuged down, washed, and re-suspended in fresh synthetic broth with supplementation of 2% lactose as carbon sources and 5,6-dimethylbenzimidazole (5,6-DMB). Then the suspension were evenly distributed among several experimental conditions, including the condition without additional supplementation (control) and with $CoCl_2 \cdot 6H_2O$ (10mg/L). The suspension was then incubated at 30˚C for 48 hours anaerobically. The porphyrin production in each culture was then measured as described above.

Supplementary Materials

HL414 was a case for vitamin B12-induced acne.

Similar to many previous studies, which suggest that vitamin B12 induces acne based on the observations that the induced acne occurred within a short period time after vitamin B12 injection and disappeared quickly after the discontinuation of vitamin B12 (*32-35, 52*), we had the same clinical observation on subject HL414. This subject had clear skin prior to vitamin B12 injection. One week after B12 injection, one big erythematous papule developed on the left chin and multiple small erythematous papules appeared on the right chin, left cheek and forehead. After discontinuation of the vitamin B12 injection, the symptoms disappeared quickly. The subject had clear skin when we resampled the face three months later. Throughout this process, board certified dermatologists determined the facial conditions at each time point. Also similar to the observations in the previous studies, vitamin B12 supplementation did not induce acne in all the subjects tested. In our study, we observed one out of ten subjects had acne breakout after vitamin B12 injection. Our observation of vitamin B12 induced acne is highly consistent with the literature, thus we concluded that this acne case was induced by vitamin B12.

Figures and Tables

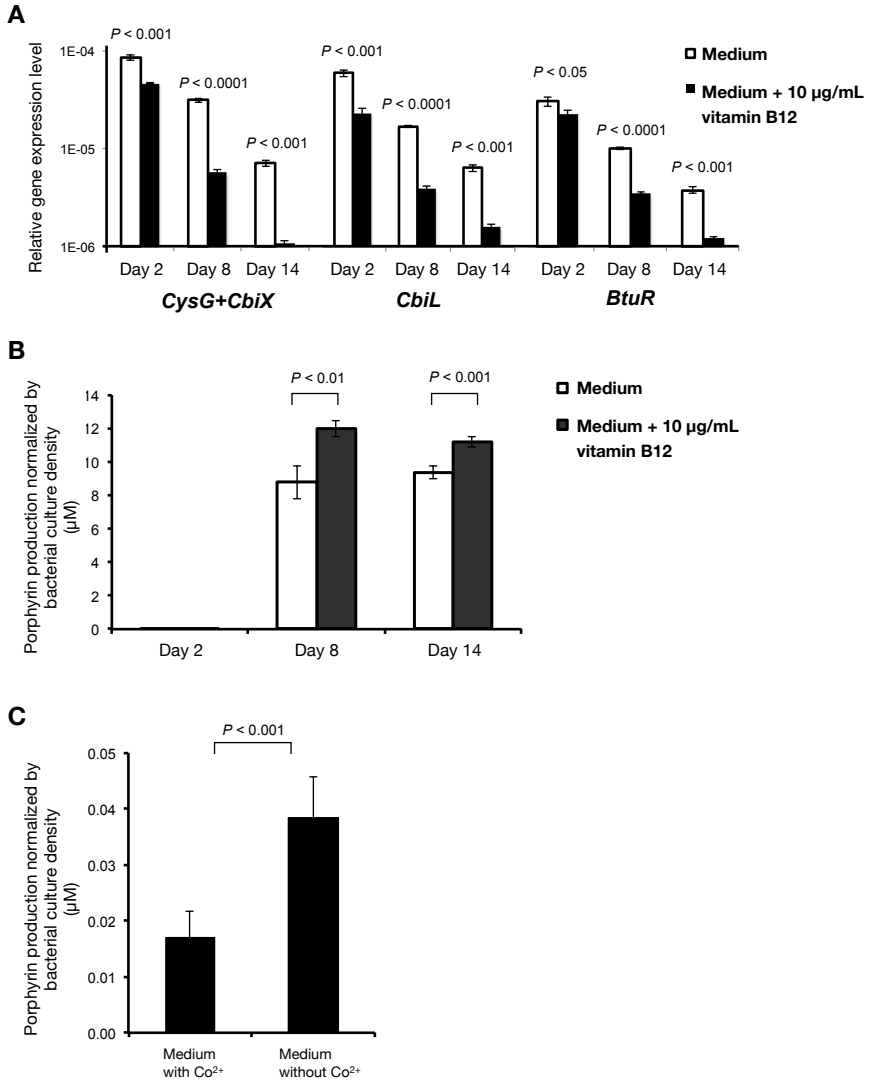

Figure 2-1. Vitamin B12 supplementation repressed the expression of *Cob/Cbi* operons in the vitamin B12 biosynthesis pathway and promoted porphyrin production in *P. acnes* cultures. This shows an inverse relationship between vitamin B12 biosynthesis and porphyrin biosynthesis in *P. acnes*.

(A) The expression levels of *CbiL*, *CysG+CbiX and BtuR* were significantly repressed on day 2, day 8 and day 14 after vitamin B12 supplementation in *P. acnes* cultures. Significance was

determined by independent samples t-test. Data are presented as the mean ±S.D.. The experiments were repeated three times with technical replicates each time.

(B) Porphyrin production in *P. acnes* cultures on day 8 and day 14 after vitamin B12 supplementation was significantly increased. Significance was determined by independent samples t-test. Data are presented as the mean ± S.D.. The experiments were repeated three times with technical replicates each time.

(C) Porphyrin production in *P. acnes* cultures was increased with the depletion of cobalt. Depletion of cobalt, as an alternative way of blocking vitamin B12 biosynthesis, significantly promoted the porphyrin production in *P. acnes*. Significance was determined by independent samples t-test. Data are presented as the mean ± S.D. of five technical replicates.

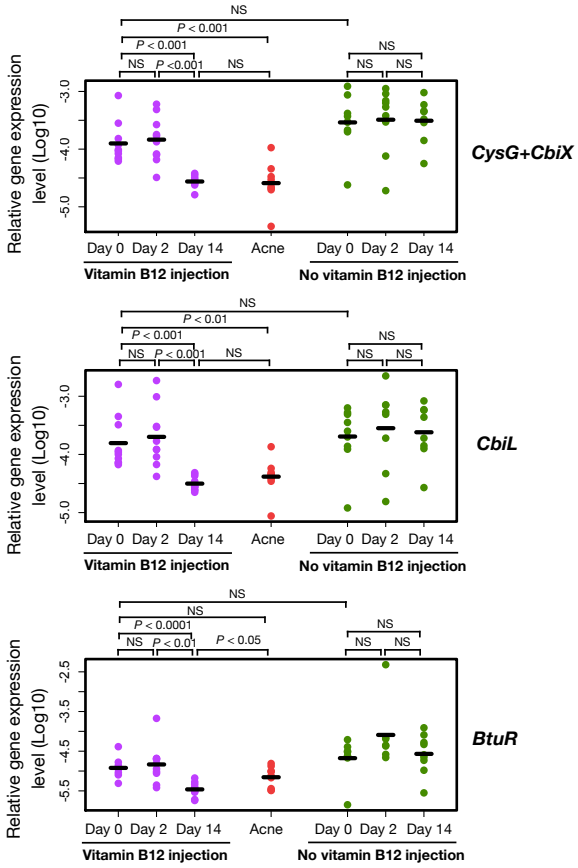

Figure 2-2. Vitamin B12 supplementation in healthy subjects repressed *P. acnes Cob/Cbi* operons and led to a gene expression profile in subject HL414 similar to those observed in the acne patients.

The gene expression levels of *CbiL*, *CysG+CbiX*, and *BtuR* in *P. acnes* vitamin B12 biosynthesis pathway were significantly repressed in the healthy subjects (n = 10) on day 14 after vitamin B12 supplementation, to a level similar to those observed in the acne patients (n = 9). Without vitamin B12 supplementation, the expression levels of these genes did not change significantly in a 14-day period (n = 9). The expression levels, quantified by qRT-PCR, for each gene on day 0, day 2 and day 14 are shown. Data from healthy subjects with vitamin B12 supplementation are shown in purple and without supplementation are shown in green. As a comparison, data from

the acne patients are shown in red. The mean of the expression levels of each gene is indicated by a black bar. Significance was determined by independent samples t-test. NS: not significant.

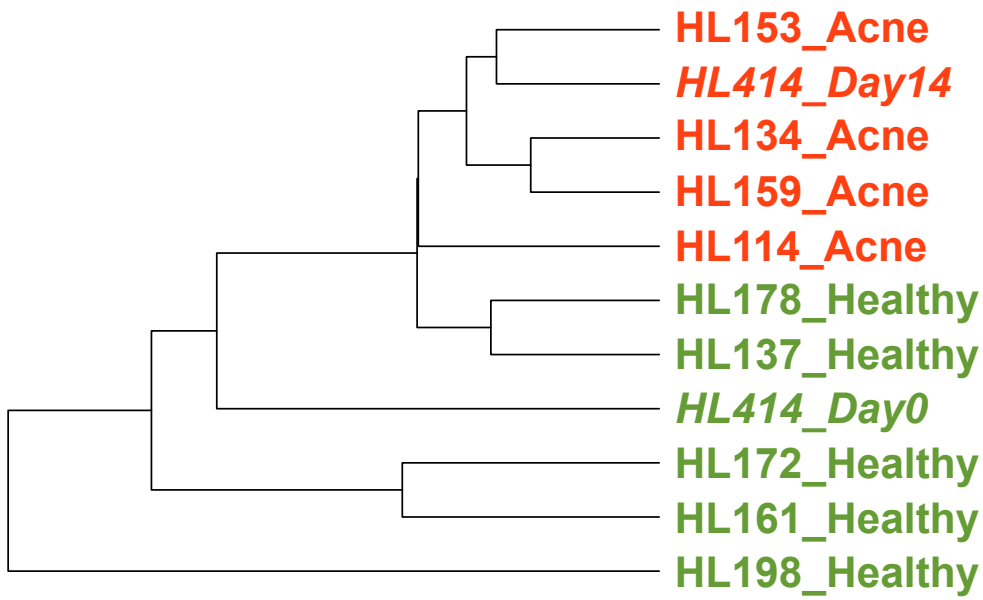

Figure 2-3. Vitamin B12 supplementation in healthy subjects led to a gene expression profile in subject HL414 similar to those observed in the acne patients.

The *P. acnes* gene expression pattern in subject HL414 was converted from a health-associated profile on day 0 to an acne-associated profile on day 14 after vitamin B12 supplementation. Unsupervised hierarchical clustering based on the expression profile of *P. acnes* OGUs was shown. The day 0 sample from subject HL414 ("HL414_Day0", with healthy skin, before vitamin B12 supplementation) was similar to the samples from other healthy individuals. However, the day 14 sample from the same subject ("HL414_Day14", with acne breakouts, after vitamin B12 supplementation) was clustered with samples from the acne patients.

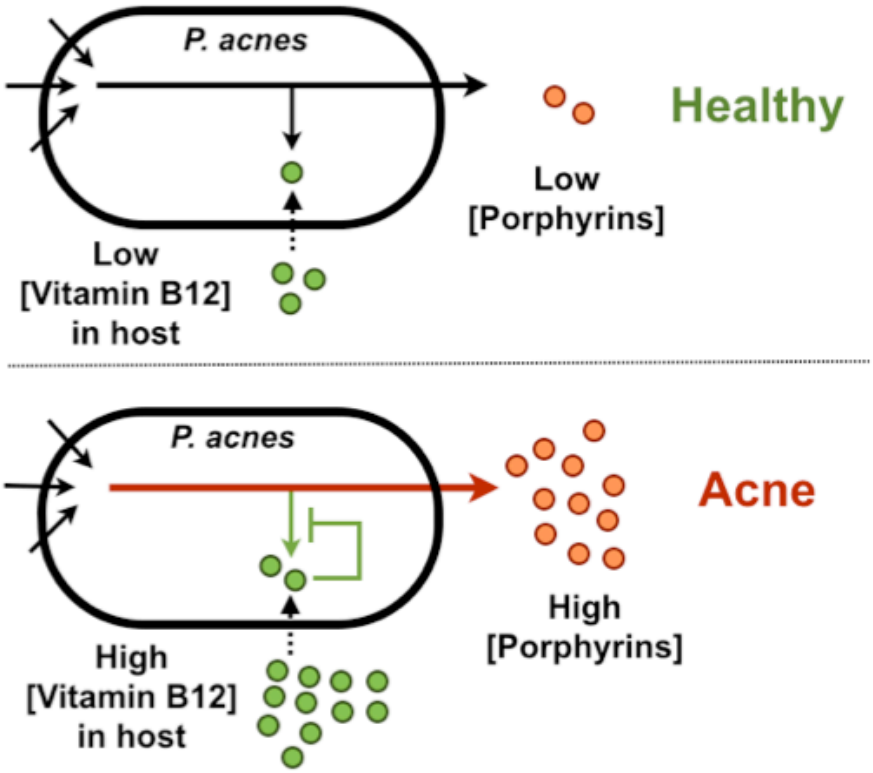

Figure 2-4. A model of vitamin B12 modulating the transcriptional and metabolic activities of the skin bacterium *P. acnes* in acne pathogenesis.

In healthy skin, when the vitamin B12 level is normal, the vitamin B12 biosynthesis pathway in *P. acnes* is expressed and porphyrins are produced at a low level. When the host vitamin B12 level is increased, the vitamin B12 biosynthesis pathway in *P. acnes* is repressed. The metabolic flow of L-glutamate is shunted toward the porphyrin biosynthesis pathway, leading to an over-production of porphyrins by *P. acnes* in the follicle. The over-produced porphyrins, secreted by *P. acnes*, induce an inflammatory response in the host cells leading to acne development in a subgroup of individuals.

Chapter 4: Analysis of *P. acnes* cobalamin riboswitches

Abstract

Cobalamin riboswitches are conserved RNA structural elements that regulate the expression of vitamin B12 biosynthesis operons upon vitamin B12 binding (*78*). In *P. acnes*, the upstream regions of *Cob/Cbi* operons for vitamin B12 biosynthesis also encode cobalamin riboswitches. To understand the molecular mechanism underlying the transcriptional regulation of vitamin B12 biosynthesis genes by vitamin B12, I first identified ten potential cobalamin riboswitch sequences in 72 *P. acnes* genomes. Among them, three cobalamin riboswitches, Cobalamin-3, Cobalamin-5, and Cobalamin-6 are adjacent to the *Cob/Cbi* operons that encode vitamin B12 biosynthesis genes. I further identified a nucleotide deletion in Cobalamin-5 mostly in *P. acnes* strains of ribotypes 4 (RT4) and 5 (RT5), which are acne-associated strains. Based on the sequence of Cobalamin-5, I predicted its secondary structure and found that the sequence variation was in the stem region P5, which maintains the intramolecular kissing loop (KL) structure that controls RNA expression. It suggests that the sequence variation in cobalamin riboswitch may influence the regulation of Cobalamin-5 on the expression of its downstream *Cob/Cbi* operon. Furthermore, this finding suggests that the vitamin B12 modulation of the transcriptional activities may vary among different *P. acnes* strains and the effect of vitamin B12 level on the metatranscriptome of the skin microbiota may be dependent on the strain composition. This finding may potentially link the previous observations that RT4 and RT5 strains were highly associated with acne and my finding that vitamin B12 modulates the transcriptional activities of the skin microbiota and induces acne.

Introduction

Riboswitches are non-coding regulatory mRNA elements. After being transcribed, they bind small molecules and change the gene expression (*98*). Many types of riboswitches have been found and experimentally validated, for example, cobalamin riboswitch (*99*), FMN riboswitch (*100*), and S-adenosyl methionine riboswitch (*101*). Cobalamin riboswitch is the first validated riboswitch that was found to be able to directly bind vitamin B12 coenzyme (AdoCbl) and regulate the expression of cobalamin transport gene *BtuB* in *E. coli* (*102*). Cobalamin riboswitches have also been found in a wide range of bacteria (*99*). Previous studies in some bacterial species, including *Salmonella typhimurium* (*76*) and *Escherichia coli* (*77*), showed that vitamin B12 repressed the expression of the *Cob/Cbi* operons in vitamin B12 biosynthesis pathway through cobalamin riboswitches. In 2012, the first crystal structure of cobalamin riboswitch was described by Johnson *et al*. (*103*). It shows that cobalamin-RNA recognition is accomplished through shape complementarity. A composite cobalamin-RNA scaffold stabilizes a long-range intramolecular kissing-loop interaction that controls mRNA expression (*103*).

Cobalamin-riboswitch-like sequences have been found in *Propionibacterium acnes* (*104, 105*). They are located upstream of cobalamin biosynthesis *Cob/Cbi* operons and operons that encode other functions, including ribonucleotide reductase, methylmalonyl-CoA mutase, and iron transporters. My previous study has shown that the vitamin B12 biosynthesis genes in *P. acnes* were regulated by the vitamin B12 level. It suggests that in *P. acnes*, the cobalamin riboswitches are functional. Given that vitamin B12 biosynthesis pathway is inversely correlated with the production of porphyrins, a group of virulent factors in acne pathogenesis, it is important to understand the molecular mechanism of *P. acnes* cobalamin riboswitches regulating the expression of vitamin B12 biosynthesis genes. It is also important to investigate whether

sequence variations in cobalamin riboswitch exist among different *P. acnes* strains and whether the sequence variations can explain the different associations of the strains with the disease or health.

Results

To understand the distribution of cobalamin riboswitches in *P. acnes* genomes, I first used the cobalamin riboswitch sequences predicted in the genome of KPA171202, a reference *P. acnes* strain. There are ten cobalamin riboswitches in KPA171202 predicted by Rfam (*104*) (Table 4-1). Based on their genomic locations in the KPA171202 genome, I named them sequentially from Cobalamin-1 to Cobalamin-10. These ten sequences were aligned against 71 other *P. acnes* genomes by Blastn. The significant hits (e-value < 1E-20) were defined as homologous sequences of cobalamin riboswitches in other *P. acnes* genomes. All the ten cobalamin riboswitches in KPA171202 are found in all the 72 genomes analyzed, with > 97% nucleotide sequence identity, suggesting that these cobalamin riboswitches are highly conserved regulatory elements in *P. acnes* strains.

The ten cobalamin riboswitches are adjacent to ten operons, respectively. The ten operons encode ribonucleotide reductase, methylmalonyl-CoA mutase, iron transporters and several genes in the vitamin B12 biosynthesis pathway (*Cob/Cbi* operons). To understand the molecular mechanism underlying the transcriptional regulation of vitamin B12 biosynthesis genes by vitamin B12, I further examined the sequence variations in the cobalamin riboswitches that are adjacent to the *Cob/Cbi* operons, Cobalamin-3, Cobalamin-5, and Cobalamin-6. A phylogenetic analysis of these three cobalamin riboswitches indicated that the major sequence differences are

between type I strains (ribotypes 1, 3, 4, 5 and 8) and type II strains (ribotypes 2 and 6) (Figure 4-1, 4-2, and 4-3). Six strains of ribotypes 1 and 532 have additional sequence variations in Cobalamin-3, which separate them as a sub-group from other type I strains (Figure 4-1).

To identify sequence variations among different *P. acnes* strains, I performed multiple sequence alignments of the three cobalamin riboswitches. For Cobalamin-3, strains of ribotypes 2 and 6 have a G->A mutation at the 15th position, a C insertion at the 88th position, a G->A mutation at the 120th position, and an A->G mutation at the 134th position. The six strains of ribotypes 1 and 532 have an A->G mutation at the 106th position. Additionally, strain HL086PA1 has a C->T mutation at the 127th position and strain J139 has a C->T mutation at the 164th position (Figure 4-4). For Cobalamin-5, strains of clade IA-2 (mostly ribotypes 4 and 5) have a C deletion at the 53rd position. Strains of ribotypes 2 and 6 have a T->C mutation at the 91st position and an A->C mutation at the 122nd position. Strain HL086PA1 has a C->T mutation at the 126th position and strain HL025PA1 has a G->A mutation at the 104th position (Figure 4-5). For Cobalamin-6, strains of ribotypes 2 and 6 have a T->C mutation at the 54th position, an A->T mutation at the 81st position, a T->C mutation at the 89th position, a T->C mutation at the 119th position, and a G->A mutation at the 184th position. Additionally, strain HL042PA3 has a C->T mutation at the 87th position (Figure 4-6). Notably, strain HL050PA2 has a truncated Cobalamin-6 sequence, which is likely due to the incomplete genome assembly.

Among all these sequence variations, the C deletion at the 53rd position of Cobalamin-5 in strains of clade IA-2 (mostly ribotypes 4 and 5 strains) is in a conserved P5 stem domain of a cobalamin riboswitch census. To visualize the location of this C deletion in Cobalamin-5 and to understand the impact of this nucleotide deletion on the structure and the function of Cobalamin-5, I predicted the secondary structure of Cobalamin-5 based on the consensus secondary structure of

cobalamin riboswitches (Figure 4-7) and two RNA structure prediction programs, mFold and PknotsRG (*106, 107*). In a cobalamin riboswitch, P1, P3, P4, P5, P6, P7 are the important stems that maintain the basic secondary structure. P3, P4, P5, P6 form a central four-way junction, the core domain for cobalamin binding. A cobalamin riboswitch regulates mRNA expression upon binding cobalamin, through the kissing loop interaction between loop L5 and a remote cis-element in the regulatory domain with a start codon and a ribosomal binding site (*103*).The C deletion in Cobalamin-5 was found in the P5 stem region. With a C deletion, the P5 stem structure may be altered, which may affect its affinity to cobalamin molecule and the maintenance of the loop L5. This suggests that the C deletion at the 53rd position of the Cobalamin-5 riboswitch may lead to change in expression regulation. On the other hand, the two sequence variations found in ribotypes 2 and 6 are unlikely to cause functional changes. The T->C mutation was found in P1 stem region, but it only changes the original GT pairing to a GC pairing, which maintains the original RNA secondary structure. The A->C mutation was found in an unmatched region, which was not in known functional elements of a cobalamin riboswitch.

Discussion

Cobalamin riboswitches are important regulatory elements in many prokaryotic cells (*99*). In *P. acnes*, I identified ten conserved cobalamin riboswitches. Although each of these cobalamin riboswitches has a high sequence identity (>97%) among all the sequenced *P. acnes* genomes, sequence variations among different strain lineages were observed. Based on sequence alignment and secondary structure analysis, the nucleotide deletion at the 53rd position of the Cobalamin-5 riboswitch that is adjacent to one *Cob/Cbi* operon was found to be unique to the strains of clade IA-2. The deletion potentially can affect the affinity of the cobalamin riboswitch to cobalamin

molecules and may affect the stability of the loop L5. Thus, it may change the interaction between L5 and the remote cis-element that regulates mRNA expression. Although this bioinformatic prediction needs to be confirmed experimentally, it suggests that the regulation of vitamin B12 biosynthesis gene expression by vitamin B12 may differ between these clade IA-2 strains (mostly acne-associated strains) and other *P. acnes* strains. It is attempting to speculate that, the effect of vitamin B12 on the transcriptional regulations of the skin microbiota may be dependent on the microbial composition.

Materials and Methods

Cobalamin riboswitch sequence identification

The search for homologous cobalamin riboswitch sequences was performed using blastn, requiring e value < 1E-20. The 72 *P. acnes* genomes were from an in-house database of *P. acnes* reference genomes and incomplete draft genomes. The reference cobalamin riboswitch sequence information was downloaded from the UCSC genome browser Rfam track for KPA171202 (*104, 108*).

Sequence alignment and Phylogenetic analysis

Sequence alignment was done using the built-in muscle program (*109*) in Seaview (*110*). The phylogenetic analysis was done using the built-in PhyML program (*111*) in Seaview.

Secondary structure prediction

The sequence of Cobalamin-5 is composed of two portions, the portion of the riboswitch, and the

portion of the remote cis-element that controls RNA expression. To predict the secondary structure of the riboswitch, the consensus secondary structure of cobalamin riboswitches was obtained from the research article of Barrick *et al.* (*112*).The cobalamin riboswitch 5 sequences in *P. acnes* were then manually compared to the consensus sequence to predict the positions of nucleotide matching. For the cis-element portion, its secondary structure was predicted separately using both mFold and PknotsRG (*106, 107*). Both RNA secondary structure prediction methods gave the same results. The predicted secondary structure of the full sequence was then visualized by VARNA, a JAVA applet for drawing RNA secondary structure (*113*).

Figures and Tables

Table 4-1: The distribution of cobalamin riboswitches in *P. acnes*

Cobalamin Riboswitch ID	Start position in KPA 171202 genome	End position in KPA 171202 genome	Strand	The operons that follow the cobalamin riboswitches (KPA171202_ID)	Operon Functions	The number of *P. acnes* genomes that have the cobalamin riboswitches among the 72 genomes analyzed
Cobalamin-1	129916	130161	-	PPA0107-PPA0098	Iron transport/Vitamin B12 biosynthesis/Magnesium chelatase/Radical SAM superfamily protein	72
Cobalamin-2	387563	387723	-	PPA0336-PPA0334	Iron complex transport	72
Cobalamin-3	463681	463854	+	PPA0420-PPA0423	Vitamin B12 biosynthesis	72
Cobalamin-4	470739	470914	+	PPA0425-PPA0427	Iron transport	72
Cobalamin-5	480469	480643	+	PPA0437-PPA0439	Vitamin B12 biosynthesis	72
Cobalamin-6	485217	485420	+	PPA0441-PPA0443	Vitamin B12 biosynthesis	72
Cobalamin-7	656896	657117	+	PPA0595-PPA0597	Methylmalonyl-CoA mutase	72
Cobalamin-8	720173	720358	+	PPA0656-PPA0659	Iron transport	72
Cobalamin-9	2294566	2294765	+	PPA2121-PPA2122	Ribonucleotide reductase	72
Cobalamin-10	2317476	2317662	-	PPA2137-PPA2136	Ribonucleotide reductase	72

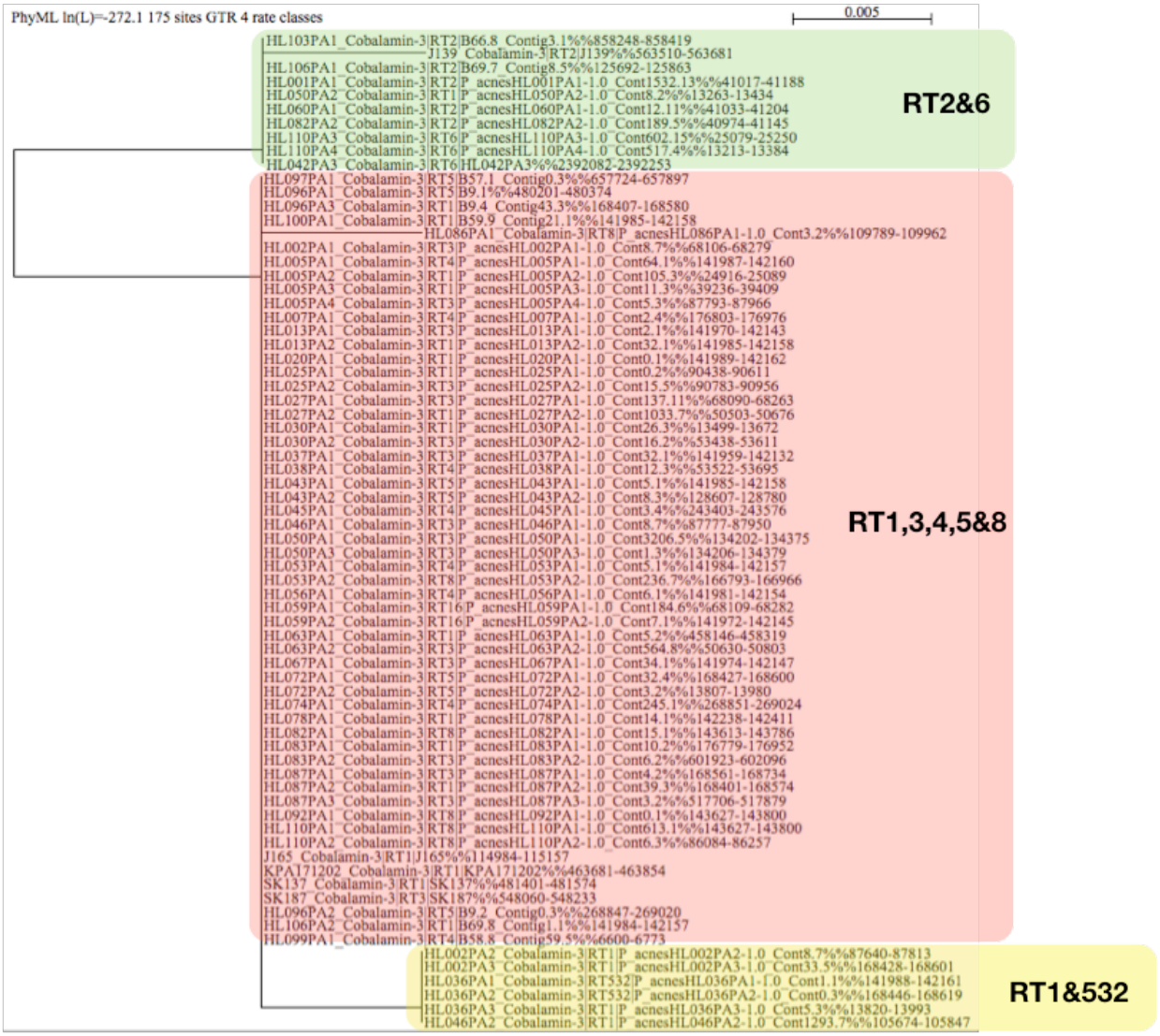

Figure 4-1: The phylogenetic tree of cobalamin riboswitch 3 (Cobalamin-3)

The tree is constructed based on the cobalamin riboswitch 3 (Cobalamin-3) sequences from 72 *P. acnes* genomes by Seaview PhyML.

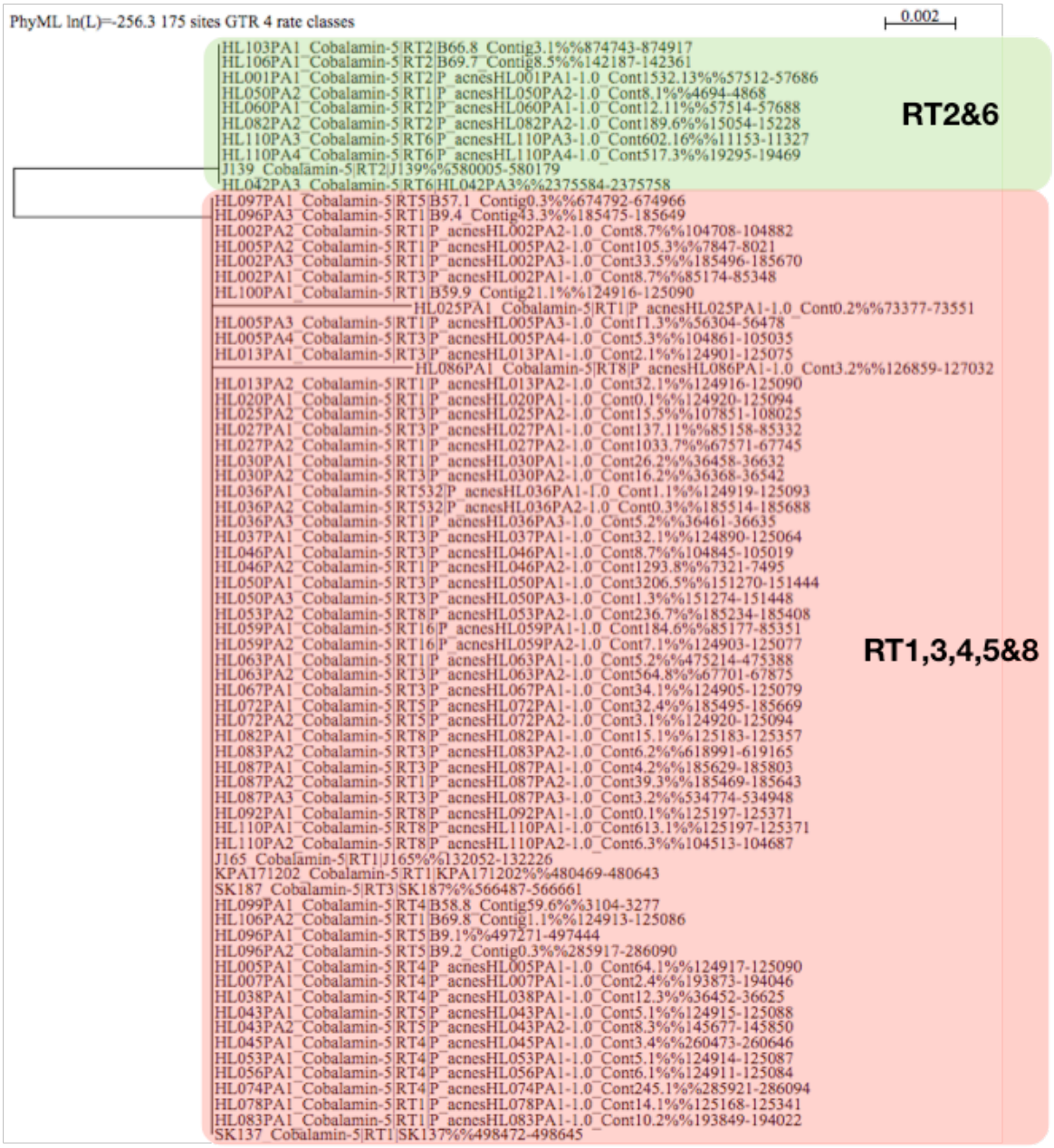

Figure 4-2: The phylogenetic tree of cobalamin riboswitch 5 (Cobalamin-5)

The tree is constructed based on the cobalamin riboswitch 5 (Cobalamin-5) sequences from 72 *P. acnes* genomes by Seaview PhyML.

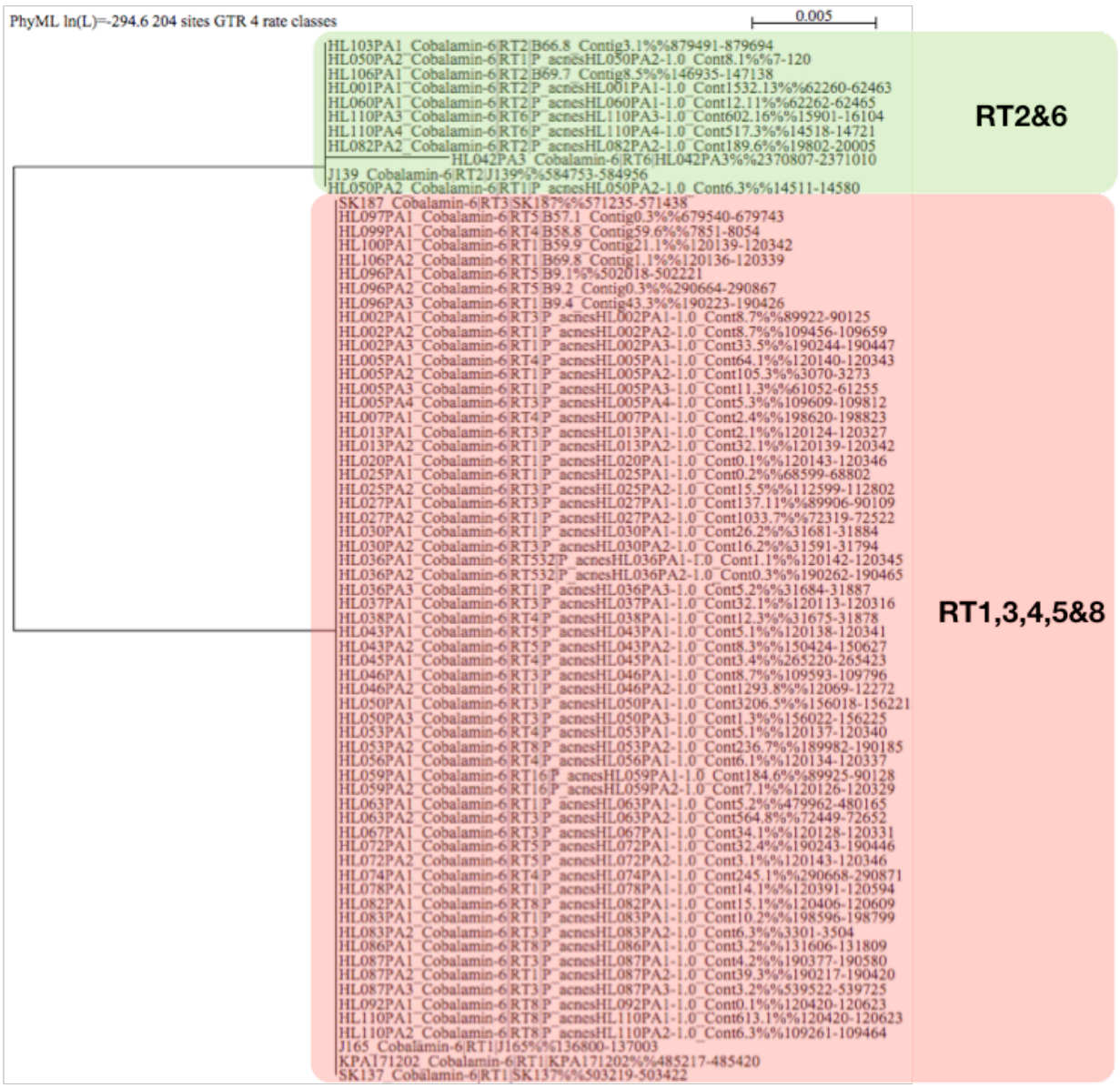

Figure 4-3: The phylogenetic tree of cobalamin riboswitch 6 (Cobalamin-6)

The tree is constructed based on the cobalamin riboswitch 6 (Cobalamin-6) sequences from 72 *P. acnes* genomes by Seaview PhyML.

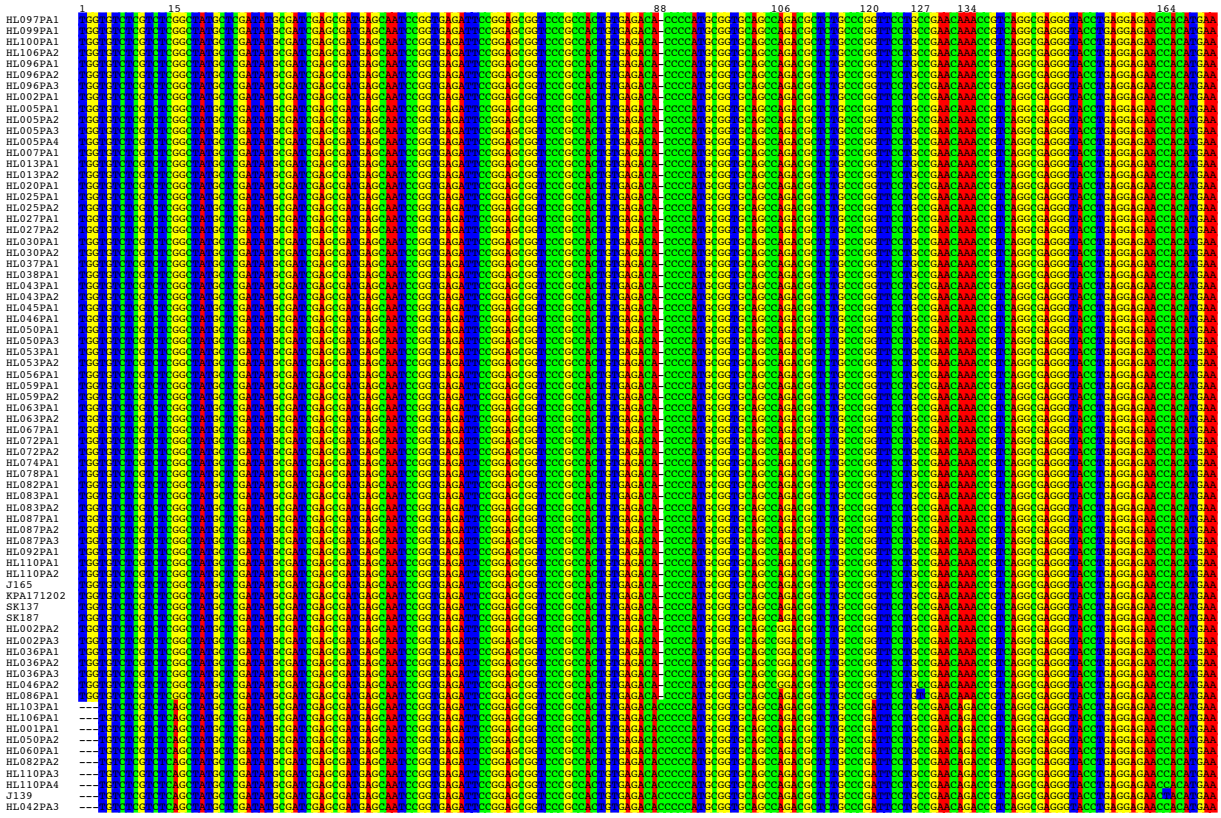

Figure 4-4: The sequence alignment for cobalamin riboswitch 3 (Cobalamin-3)

Only the sequence of 1-171 nucleotides in cobalamin riboswitch 3 is shown. The rest nucleotides have no differences among all the 72 P. acnes genomes analyzed.

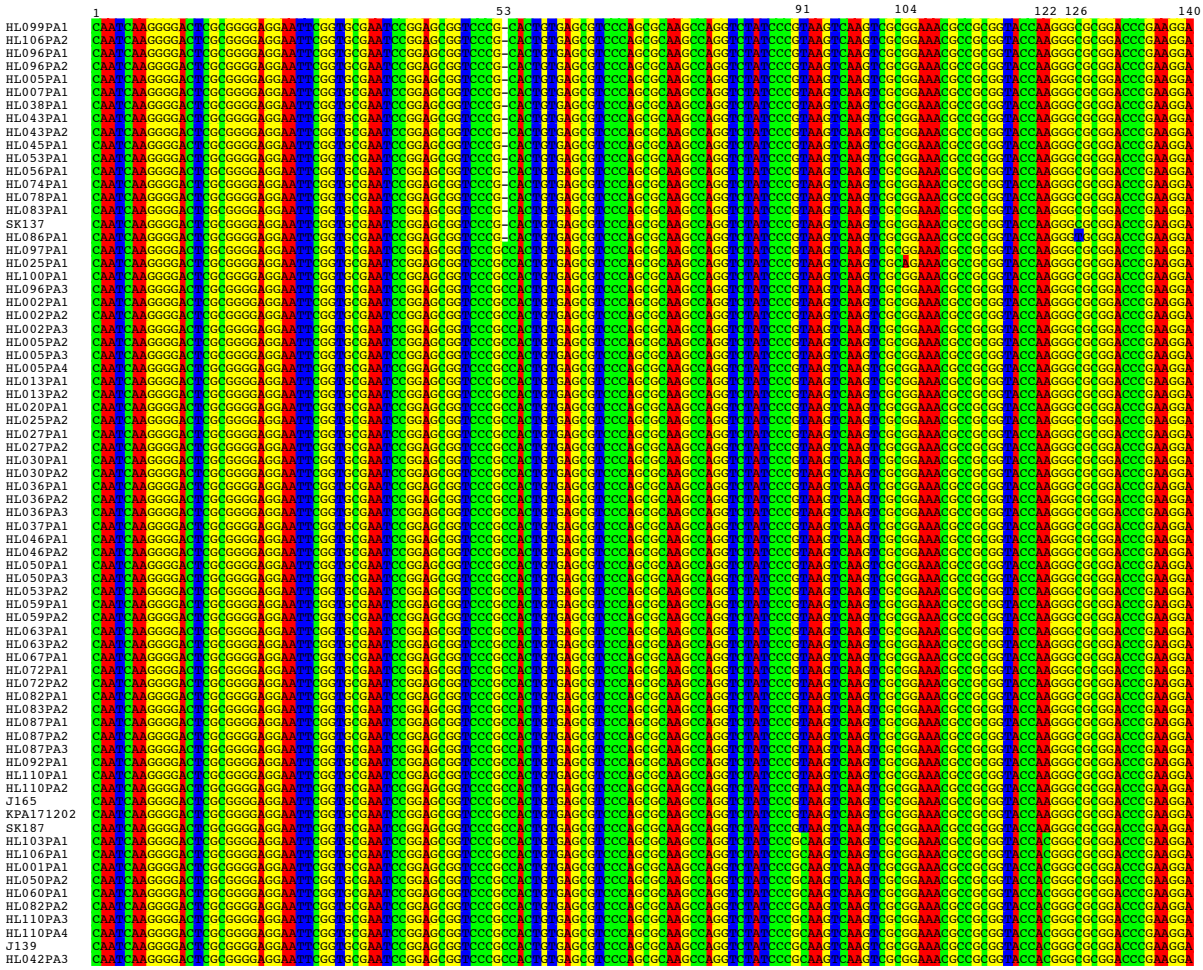

Figure 4-5: The sequence alignment for cobalamin riboswitch 5 (Cobalamin-5)

Only the sequence of 1-140 nucleotides in cobalamin riboswitch 5 is shown. The rest nucleotides have no differences among all the 72 P. acnes genomes analyzed.

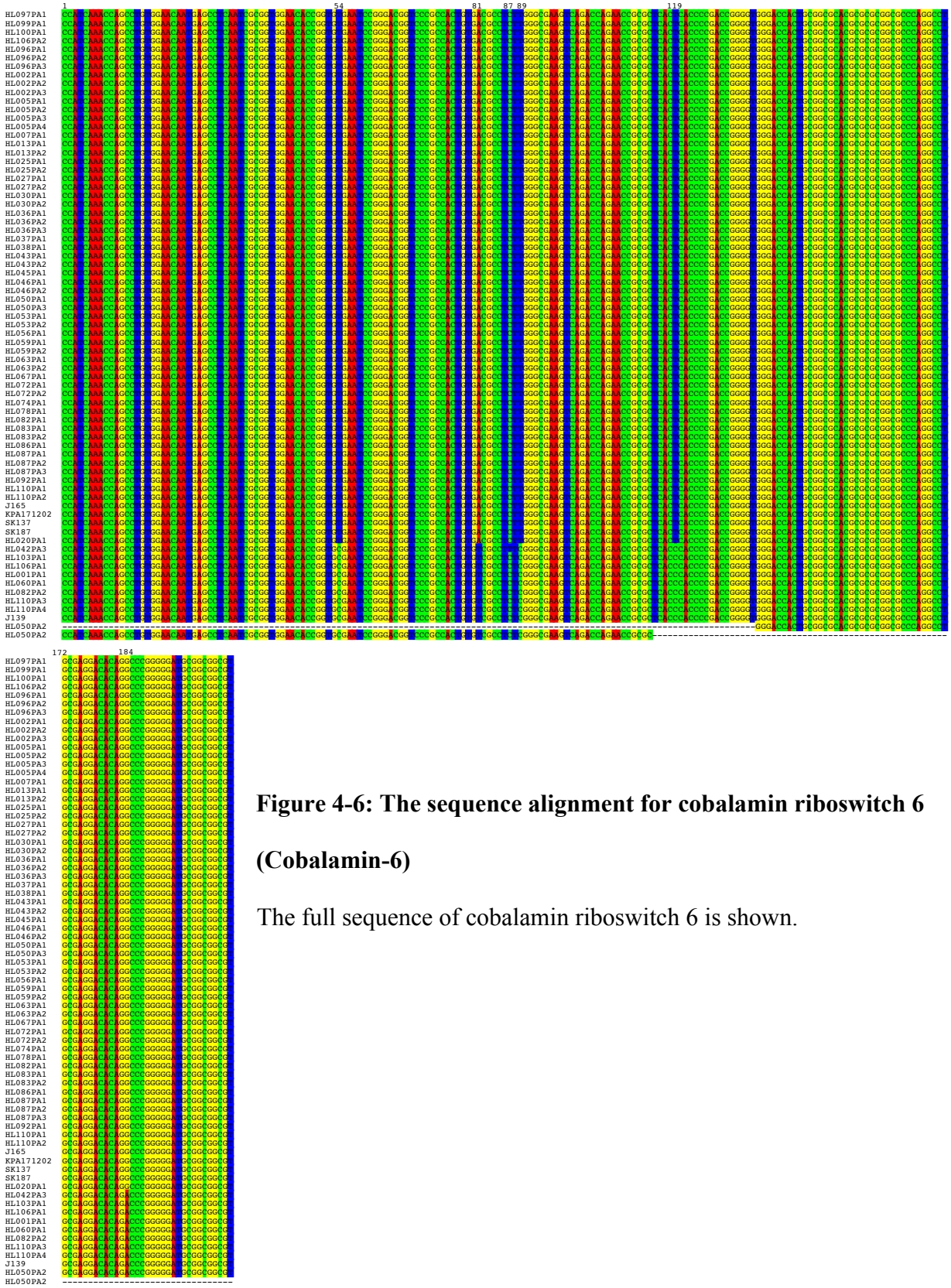

Figure 4-6: The sequence alignment for cobalamin riboswitch 6 (Cobalamin-6)

The full sequence of cobalamin riboswitch 6 is shown.

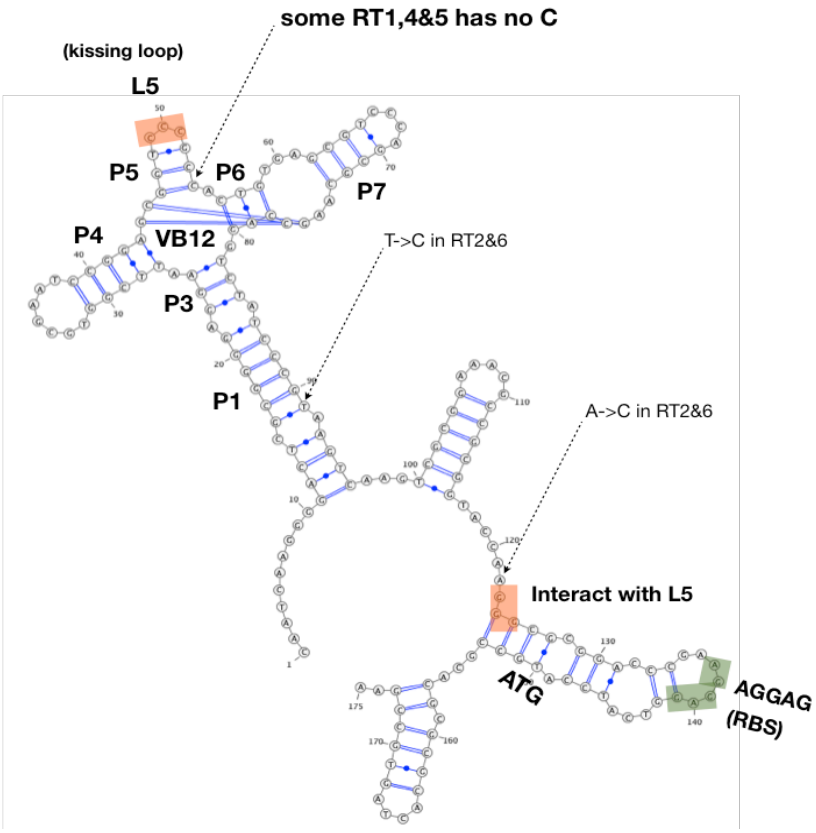

Figure 4-7: The predicted secondary structure of *P. acnes* cobalamin riboswitch 5 (Cobalamin-5)

The secondary structure of *P. acnes* cobalamin riboswitch 5 (Cobalamin-5) was predicted based on the consensus secondary structure of cobalamin riboswitches. P1, P3, P4, P5, P6, P7 are the important stems that maintain the basic secondary structure of the cobalamin riboswitch. P3, P4, P5, P6 form a central 4-way junction, the core domain for cobalamin binding. The red boxes highlight the kissing loop interaction between loop L5 and a remote cis-element in the regulatory domain with a start codon and a ribosomal binding site (highlighted in green), which regulates the expression machinery. Dashed arrows point three variable sites among *P. acnes* strains.

Chapter 5: Protocol development for studying the interactions between the skin microbiota and host cells at the transcriptional level

Abstract

A front of the current human microbiome field is to understand the host-bacterial interactions. Simultaneously analyzing the gene expression of both the host cells and the microbiota provides insights on the interactions between the host and the microbes *in vivo*. However, the massive amount of human RNA isolated from human tissues often makes the bacterial RNA undetectable. This is especially true for the samples taken from the body sites that have a low ratio of bacterial cells versus human cells, such as skin biopsies. To enable a simultaneous gene expression analysis on skin cells and skin bacteria, I have developed a protocol enriching bacterial RNAs from skin samples, using which I was able to successfully prepare both bacteria cDNA libraries and human cDNA libraries from the same biopsy samples. After preparing the cDNA libraries using this protocol, RNA-Seq can be conducted to profile the gene expression in both bacterial cells and the human cells. It will empower researchers to reveal the complex interaction between the host and the residing skin bacteria at the transcriptional level.

Introduction

The human microbiome studies in the past several years greatly advanced our understanding of the composition of the human microbiota at different body sites and their links to various diseases (*1, 42, 43*). To further investigate the microbial pathogenesis mechanisms of diseases, many researches focus on understanding the interactions between the host and the microbiota (*5, 93*). *In vivo* transcriptional profiling of the host cells and the microbial cells provides valuable insights into what microbial factors are associated with disease pathogenesis and how host cells respond to microbial colonization and infection (*114-117*). However, most of the current studies

focus on either the host side or the bacterial side, but not both, partly due to the lack of an effective method for quantifying gene expression for both host and microbiota simultaneously.

Skin is the largest organ of human body. As the interface between the environment and the internal body, skin has evolved to withstand large mechanical force and to shield the internal tissues and organs from invading pathogens. Skin is also colonized by a wide range of microorganisms, including bacteria, fungi, viruses, and mites (*60*). To study the interactions between skin cells and the skin microbiota, skin biopsies are often used in analyses. The success of these studies largely relies on the successful preparation of DNA or RNA libraries from the skin biopsy samples. Skin is usually considered as a "tough tissue" for RNA extraction, because its fibrous nature made it difficult lyse the cells and also because it contains high levels of RNase that degrade RNA during extraction process. Furthermore, to capture the interactions between the host and bacteria in situ, the RNAs from the host and the bacteria need to be extracted from the same sample. However, most of the skin biopsy samples consist of predominantly human cells. This makes it difficult to detect and extract the bacterial RNA.

In this study, I have developed a protocol that effectively extracts both human RNA and bacterial RNA from human skin biopsy samples. With a bacterial RNA enrichment procedure, my protocol allows to enrich the bacterial RNA of limited amounts from skin samples. This developed protocol can be used to extract human RNA and bacterial RNA from the same skin biopsy sample and convert them to cDNA libraries for RNA-Seq-based gene expression analysis.

Results and Discussion

I developed a protocol to prepare bacterial cDNAs and human cDNAs from the same skin biopsy

sample. The protocol consists of RNA extraction, DNase treatment, microbial RNA enrichment, bacterial rRNA depletion, RNA amplification, and bacterial double-stranded cDNA synthesis, human mRNA selection, fragmentation and human double-stranded cDNA synthesis.

The protocol was tested on skin biopsy samples, *P. acnes* bacterial cells, and human 293 cell lines. After total RNA extraction, the quality of the total RNA from one biopsy sample and bacterial cells were examined using 0.8% agarose gel electrophoresis. Both samples yielded total RNAs with good quality, showing two clear ribosomal RNA bands on the gel (Figure 5-2). The bacterial RNA was enriched from an aliquot of the total RNA of the biopsy sample. After enriched twice, the bacterial RNA was subjected to bacterial rRNA depletion immediately without purification to avoid unnecessary loss. Compared to the biopsy total RNA, the biopsy RNA after bacterial RNA enrichment and bacterial rRNA depletion did not show any bands. This is largely due to the limited amount of bacterial RNA in skin biopsies (Figure 5-3). The bacterial RNA was further linearly amplified to obtain sufficient amount of RNA for down-stream applications (Figure 5-4). The amplified RNA was then converted to double-stranded cDNAs (Figure 5-5). At the end, the bacterial cDNA library was composed of cDNA molecules with a range of molecular weights representing the mRNAs of different transcript lengths. For human cDNA preparation, a second aliquot of the biopsy total RNA was enriched for human mRNA based on poly-A selection. After the human mRNA selection, the two strong human ribosomal RNA bands were disappeared (Figure 5-6). Similarly, a control total RNA from human 293 cell lines was also enriched for human mRNA using the same procedure. The strong human ribosomal RNA bands in the control total RNA were also largely reduced. The human mRNAs were further fragmented using RNA fragmentation reagent. Although the fragmented biopsy RNA could not be visualized on a gel due to the limited amount of mRNAs, the

fragmented control human RNAs were visualized on a gel showing that the length of the fragmented RNAs ranged from 100 bp to 400 bp (Figure 5-7). Lastly, the fragmented human RNAs were converted to human double-stranded cDNAs with a length ranging from 100bp to 400bp as well (Figure 5-8).

To determine the quality of the cDNAs prepared using the protocol described above, I amplified the cDNAs of two genes, *GAPDH*, a human house-keeping gene, and *RecA*, a *P. acnes* house-keeping gene, using the bacterial cDNA library and human cDNA library as templates (Figure 5-9). These PCRs revealed three findings. First, both the cDNAs from the control human RNA and from the biopsy samples without bacterial enrichment have substantial human transcripts, indicated by GAPDH, but not any detectable bacterial transcripts, indicated by *P. acnes RecA*. Second, after bacterial RNA enrichment, the abundance of human transcripts in biopsy cDNAs was decreased, whereas the abundance of bacterial transcripts was increased. Third, performing two sequential bacterial RNA enrichment procedures further improved the bacterial enrichment efficiency, which is reflected by the increased intensity of the amplification products of *P. acnes RecA*. I also demonstrated that the two enrichment procedures should be performed sequentially without purifying the bacterial RNA in order to avoid unnecessary loss of RNA due to purification procedure.

Figures and Tables

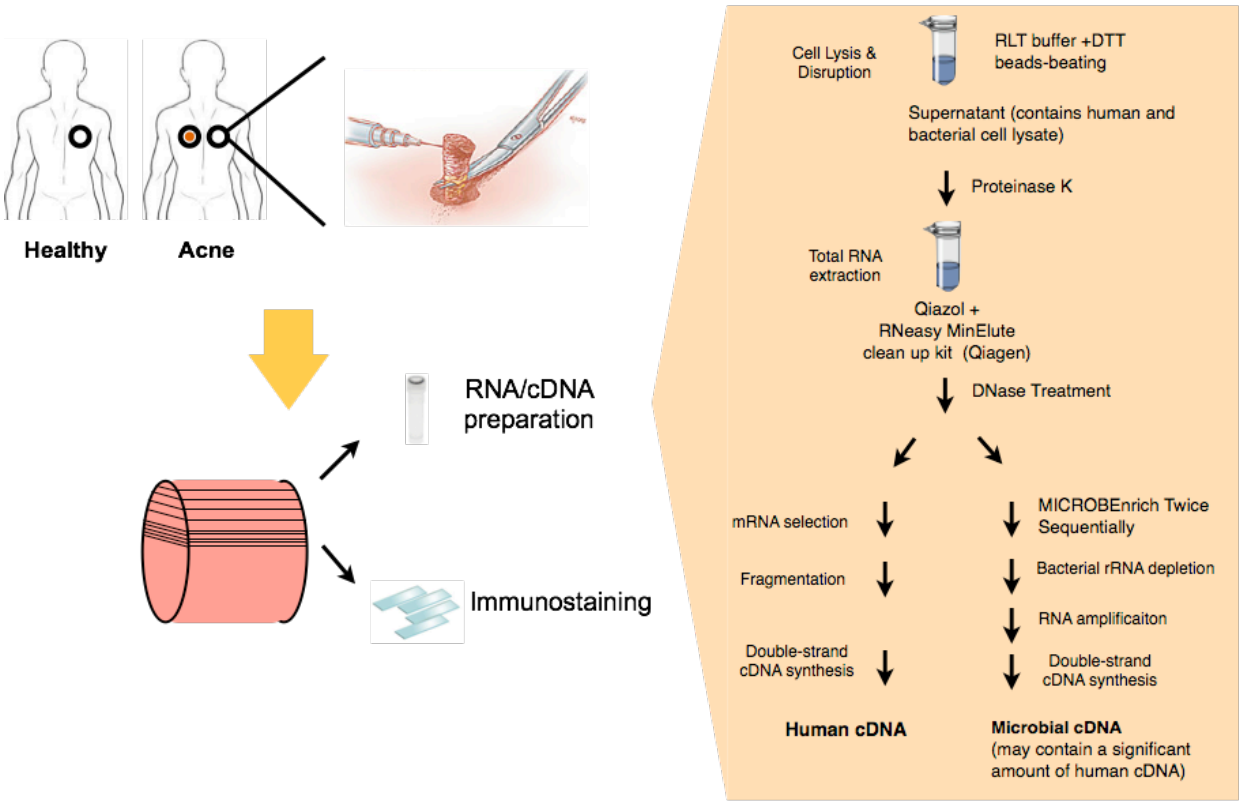

Figure 5-1. Protocol summary

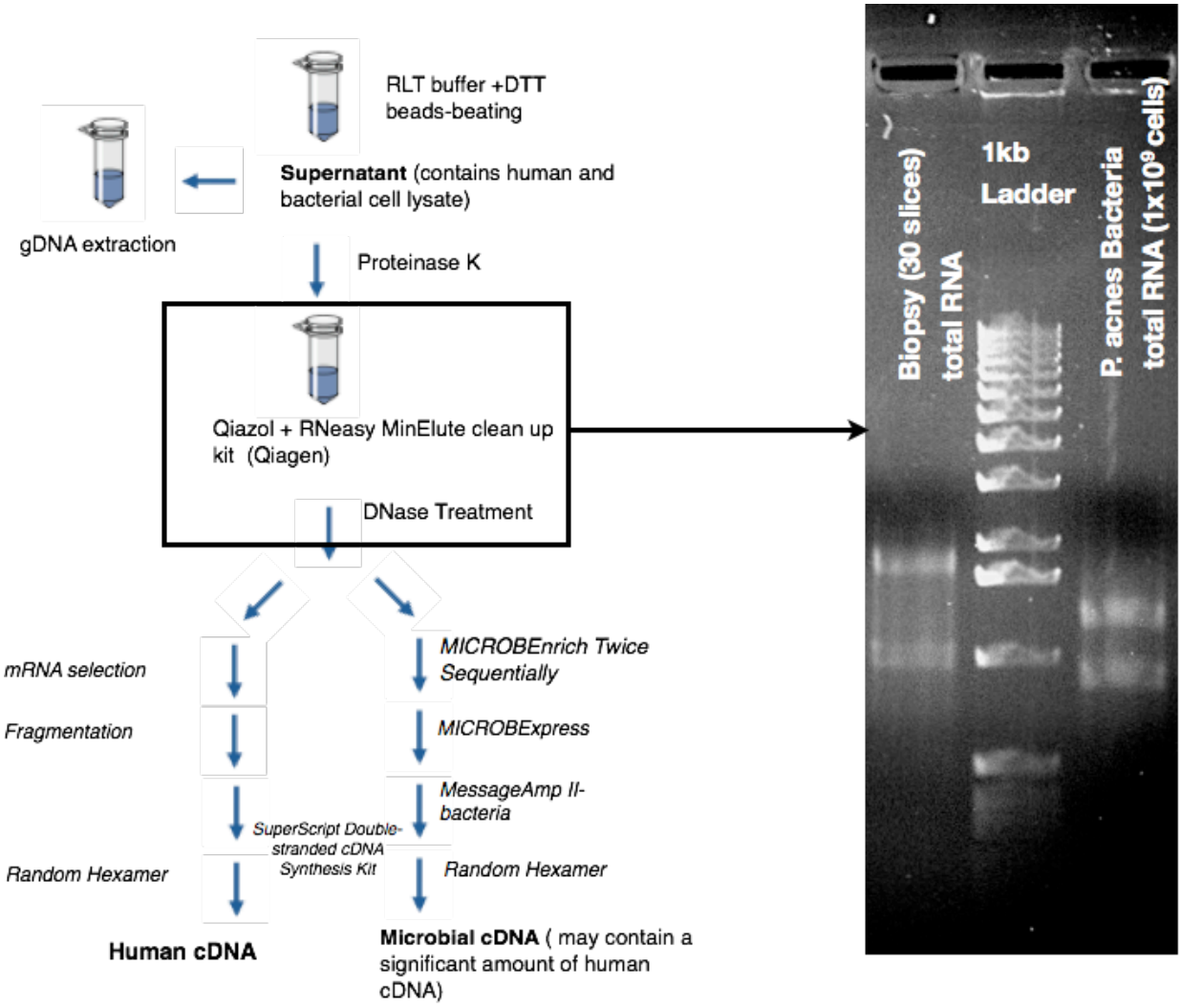

Figure 5-2. Total RNA quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder.

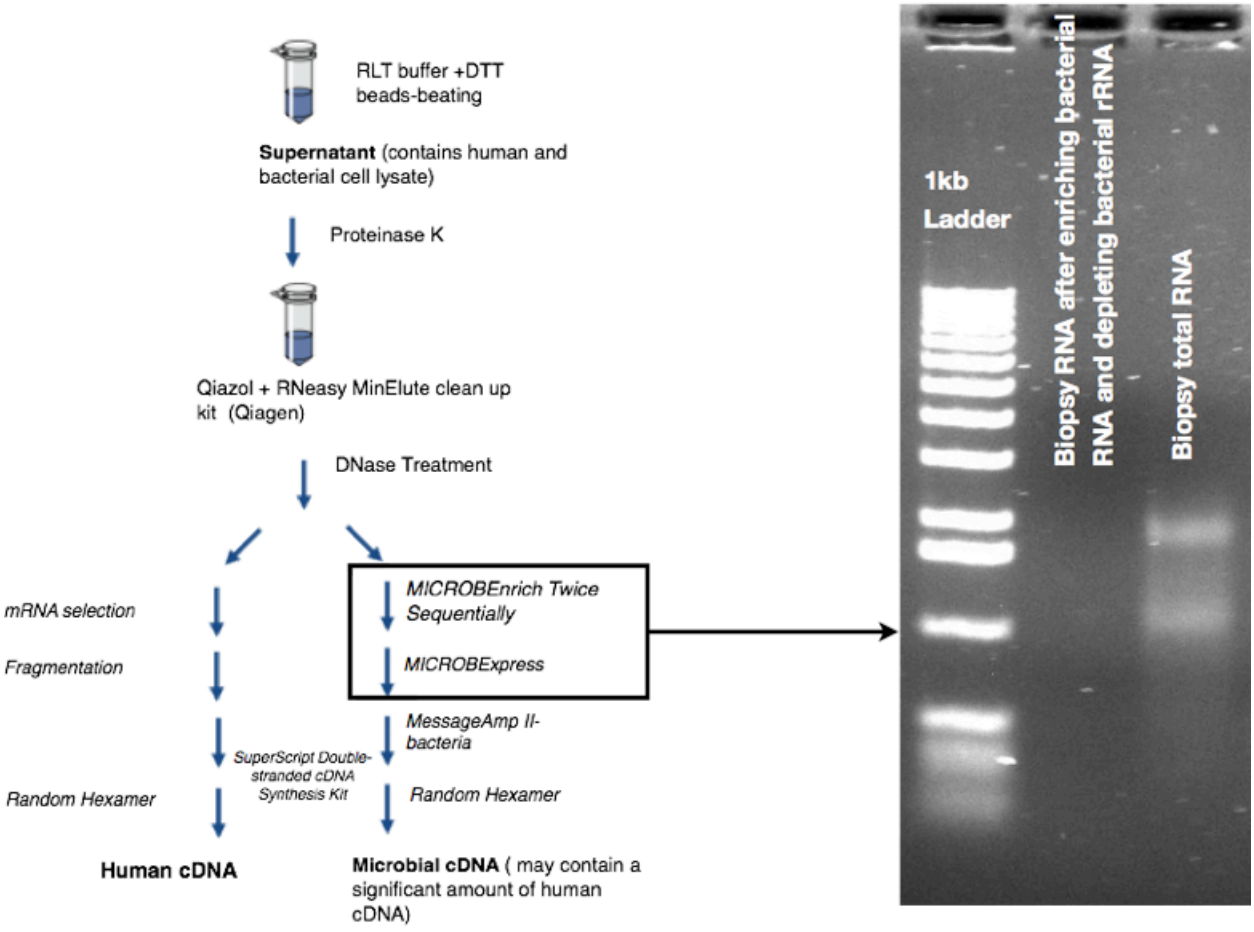

Figure 5-3. Bacterial rRNA subtraction quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder.

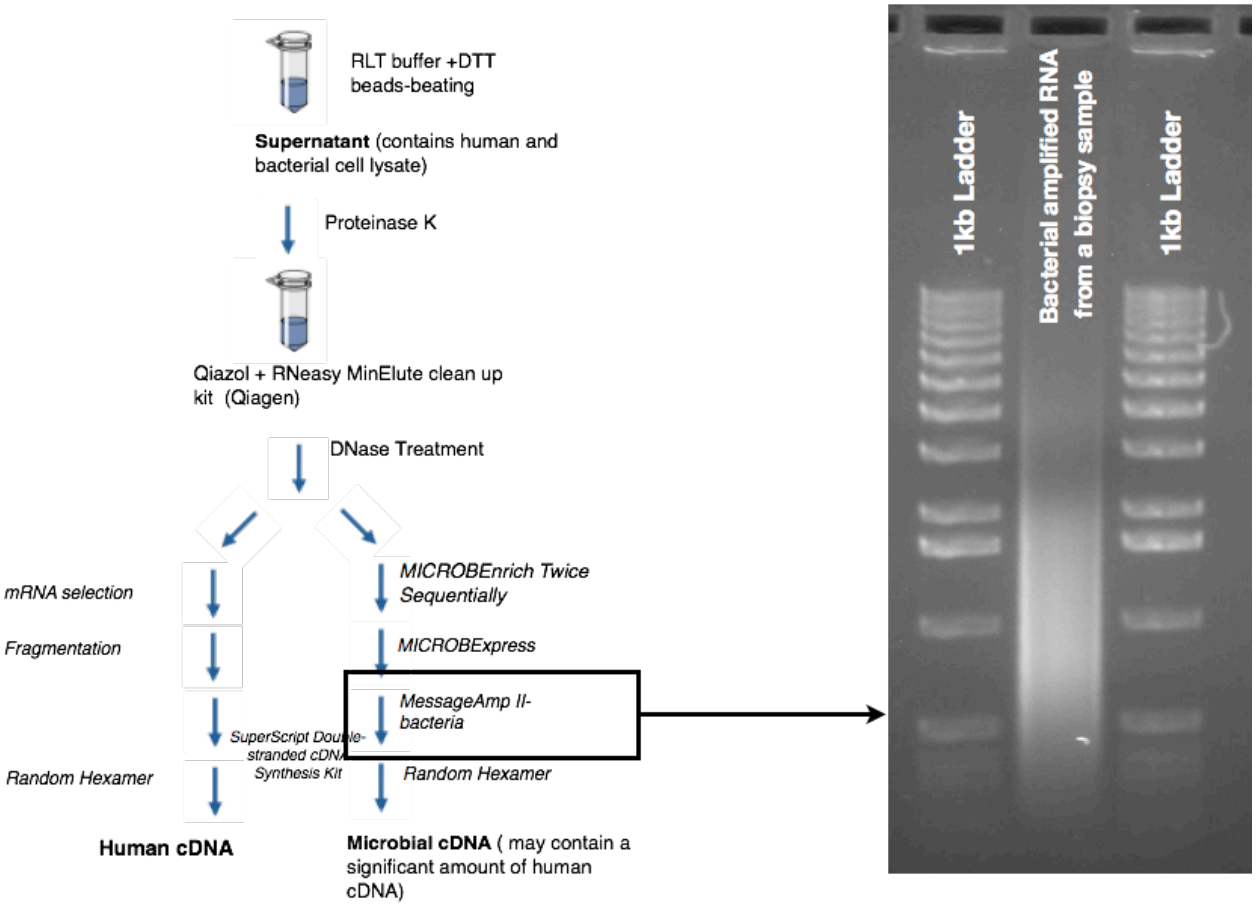

Figure 5-4. Bacterial RNA amplification quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder.

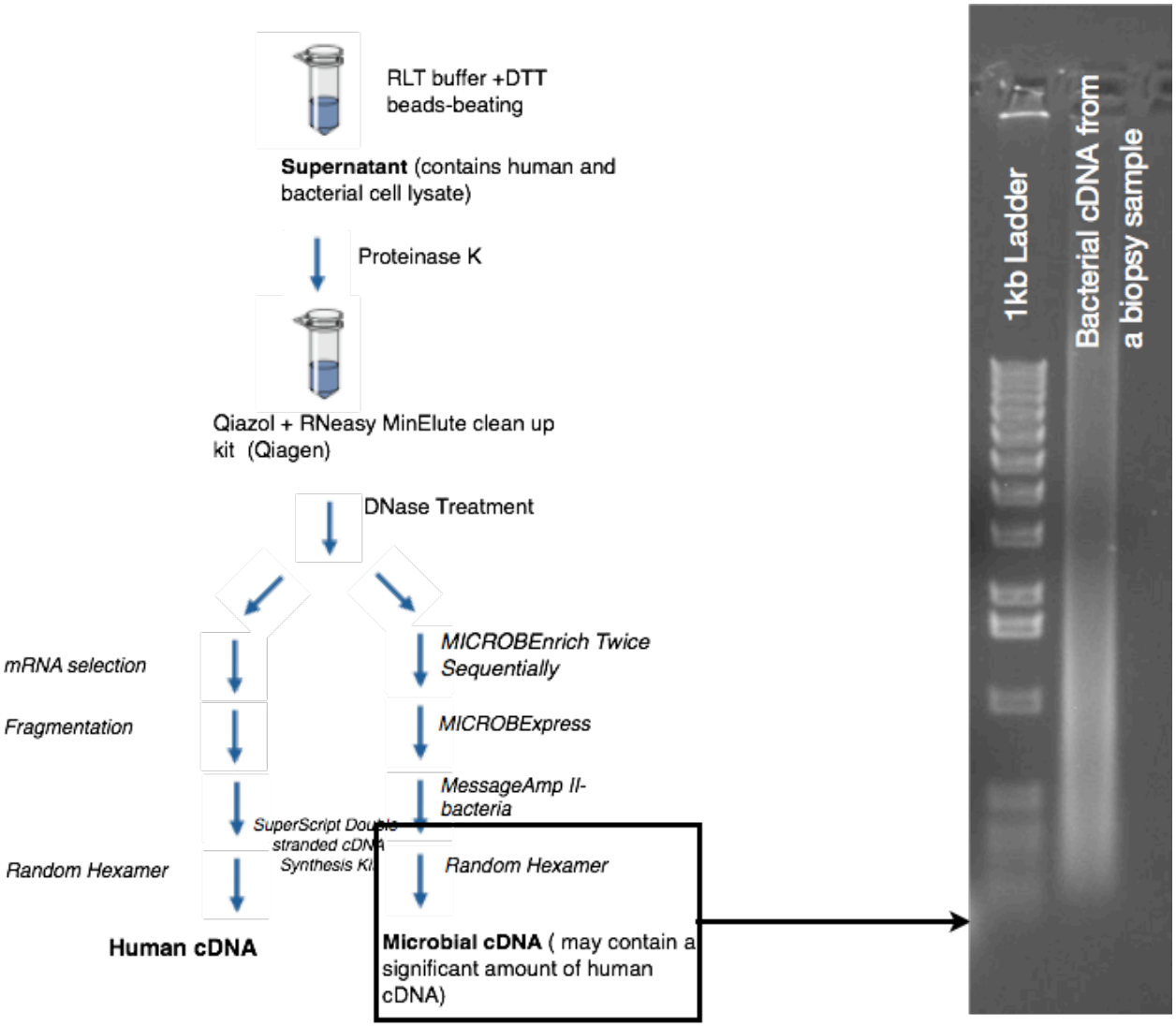

Figure 5-5. Bacterial cDNA synthesis quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder.

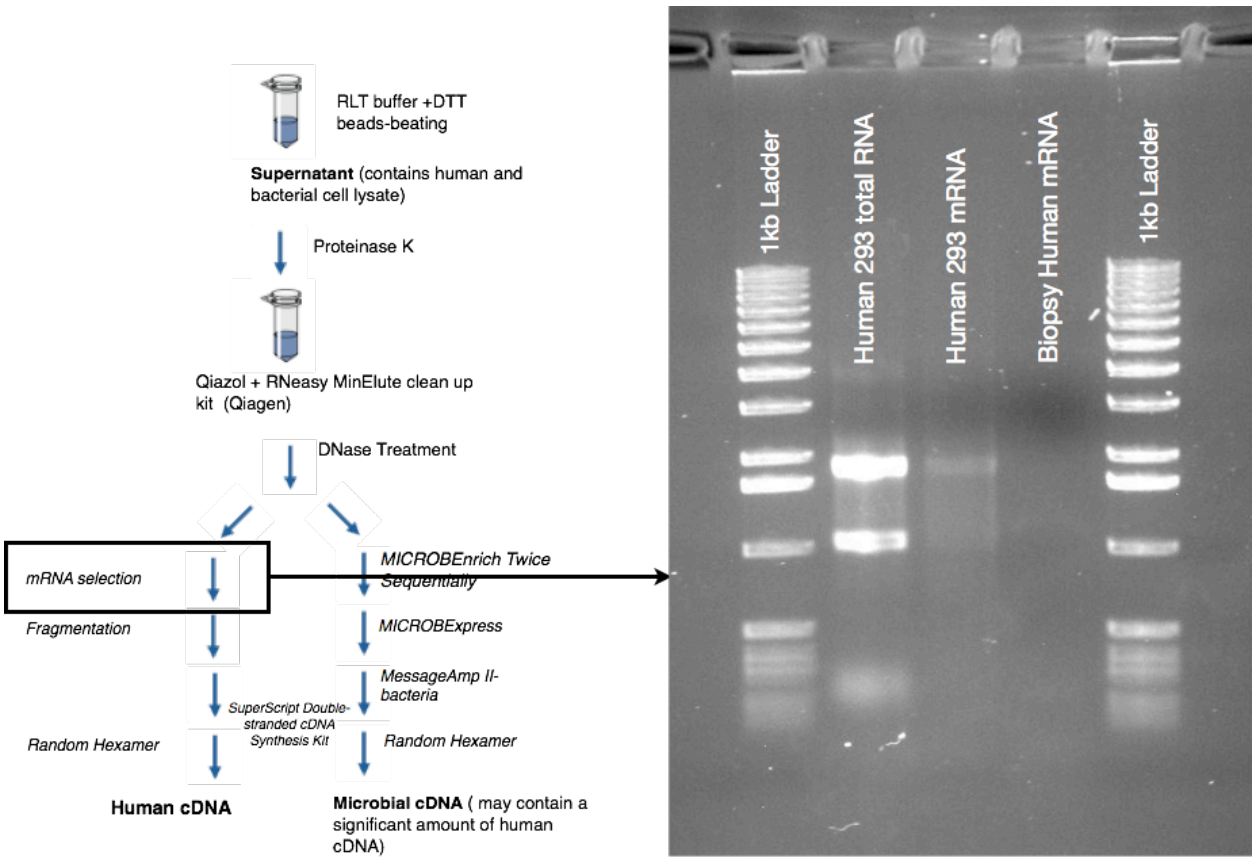

Figure 5-6. Human mRNA selection quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder.

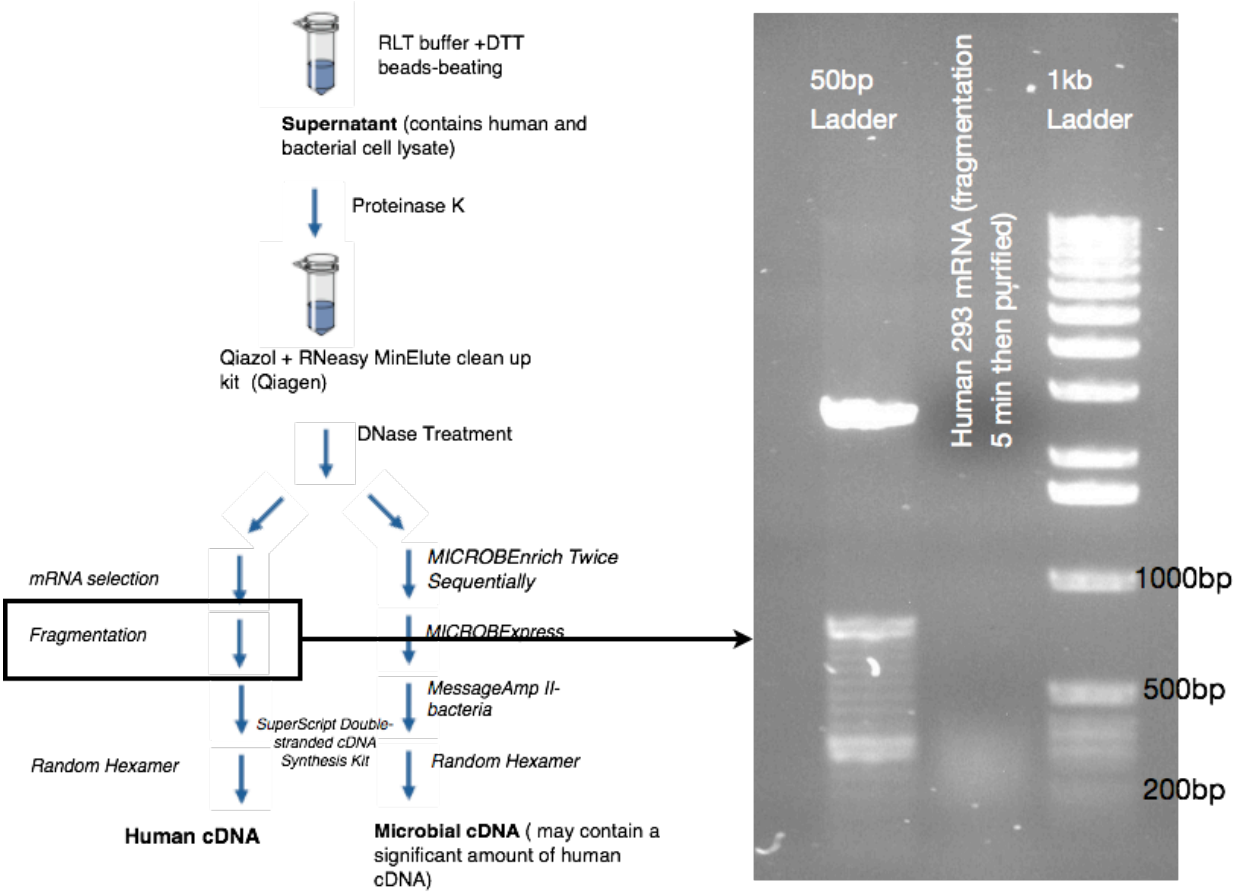

Figure 5-7. Human RNA fragmentation quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min. 1kb ladder: Invitrogen 1kb ladder. 50bp ladder: Invitrogen 50bp ladder

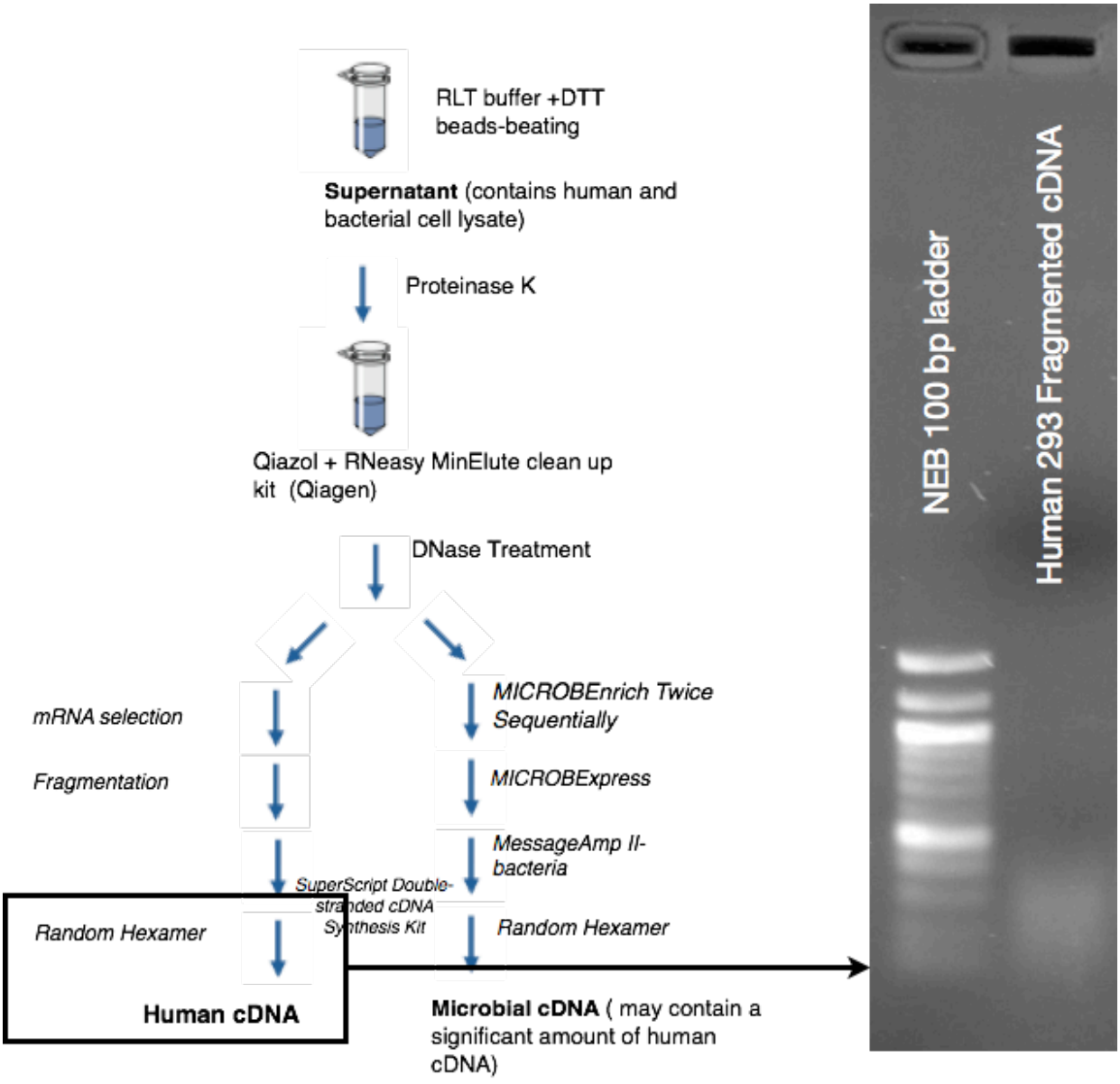

Figure 5-8. Human cDNA synthesis quality control

The gel was visualized after 0.8% agarose electrophoresis for 25 min.

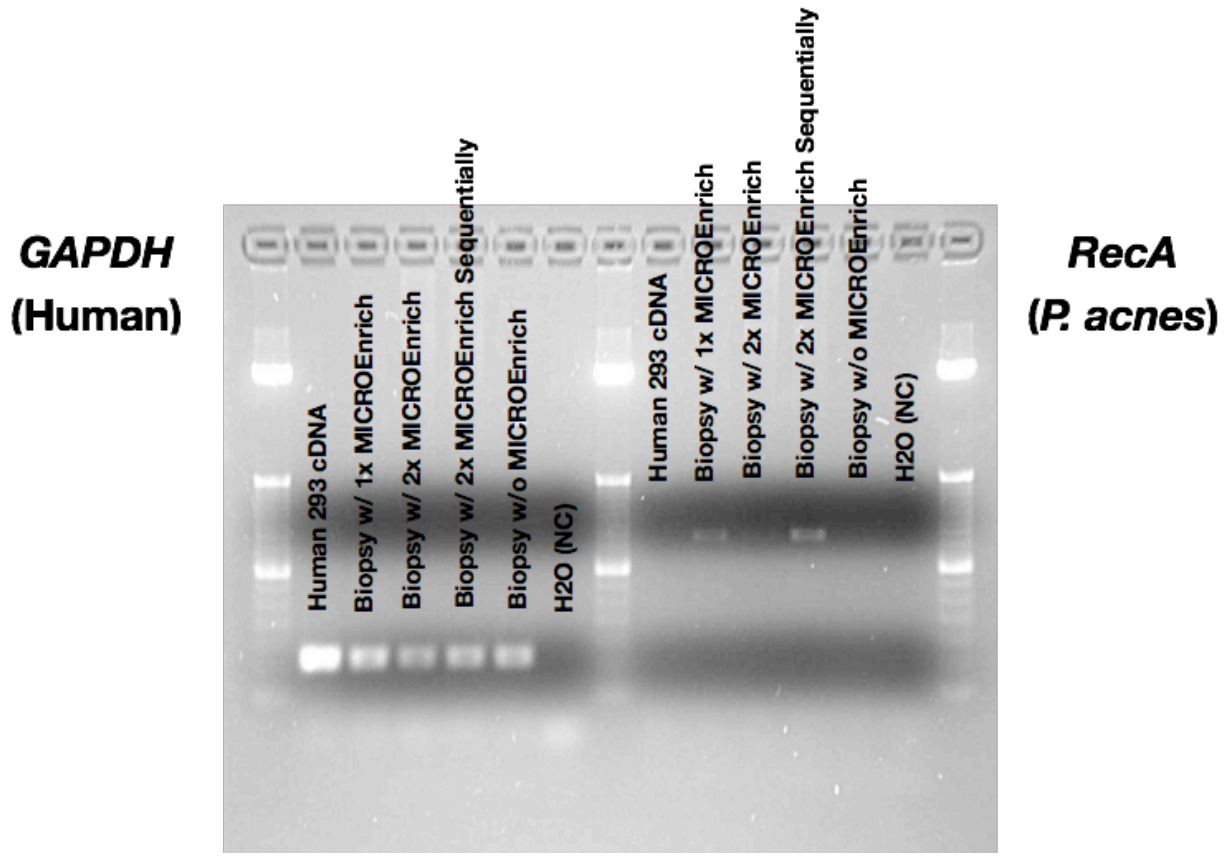

Figure 5-9. cDNA quality control by amplifying a human house-keeping gene GAPDH and a bacterial house-keeping gene RecA

The gel was visualized after 0.8% agarose electrophoresis for 25 min.

Chapter 6: Metagenomic analysis of the skin microbiome associated with acne vulgaris

Abstract

Altered skin microbial community composition has been associated with skin diseases (*6*). To understand the role of the skin bacteria in acne, it is important to determine the changes in the genetic potential of the skin microbial communities in acne pathogenesis. In collaboration with Dr. Emma Barnard and Dr. Baochen Shi, we performed a metagenomic shotgun sequencing analysis of the pilosebaceous microbiota and compared the differences in the metagenome of the microbial communities between acne patients and healthy individuals. We found that subsets of the acne patients and healthy individuals harbored unique genetic elements of the dominant skin bacteria, *P. acnes.* These genetic elements separated them from the rest of the acne patients and healthy individuals on a principal component analysis. Consistent with our previous finding (*47*), locus 2, a genomic island of 20-kilo base pairs, was found to be highly associated with acne, and was rarely found in individuals with naïve healthy skin. Our findings identified potential pathogenic factors in acne, which may provide new therapeutic targets for acne treatment.

Introduction

Bacterial factors have been implicated in the pathogenesis of acne vulgaris (*19*), the most common skin disease affecting more than 80% of the population (*10-13*). The microbial community in the pilosebaceous units, where acne arises, is a tractable community with one single dominant bacterial species, *P. acnes* (*6, 21-23*). *P. acnes* has been long thought as a pathogenic factor for acne. Our previous 16S rRNA study revealed that specific *P. acnes* strains are associated with acne, suggesting their potential roles in acne pathogenesis (*6*). However, the

bacterial pathogenesis mechanism of acne remains elusive.

To identify the bacterial factors in acne pathogenesis, we investigated the metagenome and the functional potentials of the skin microbiome during disease. The technical advancement in metagenomic sequencing has made it possible to study the skin microbiome using a metagenomic shotgun sequencing approach. To date, two metagenomic studies of the superficial skin microbiome from 2 or 15 subjects have been published (*8, 9*). These studies offered insights into the metagenomic composition of healthy skin microbiomes across different body sites. In this study, for the first time, we compared the metagenomic composition of the skin microbiome in disease to the healthy skin microbiome. The skin samples from 39 acne patients and 37 healthy individuals were analyzed to identify the functional changes in the skin microbiome during acne pathogenesis.

I performed part of the data analysis in this project. Only those results will be described in this dissertation.

Results

To determine whether the relative abundances of *P. acnes* OGUs are different between acne and healthy subjects, I conducted principal component analysis based on all the *P. acnes* OGUs (Figure 6-1). A subset of 12 acne samples was separated along the axis of the first principal component, whereas a subset of five healthy samples was separated along the axis of the second principal component. The rest of the acne and healthy samples were not separated based on the first two principal components. This finding suggested that subsets of the acne patients and

healthy individuals harbored unique metagenomic elements that were associated with either acne or healthy skin.

To determine the metagenomic differences between acne patients and healthy individuals, I plotted the relative abundances of all the *P. acnes* OGUs for all the samples (Figure 6-2). Although most of the *P. acnes* OGUs were prevalent in almost all the samples, the OGUs in the three previously identified disease-associated genetic loci, loci 1, 2, and 3, had varied relative abundance in samples. Additionally, OGUs that are not in the core genome showed variable abundances across samples. Specifically,, the OGUs in locus 2 were highly correlated with acne status (Figure 6-3). Based on 82 sequenced *P. acnes* genomes, locus 2 is a genomic island that is found mostly in *P. acnes* ribotypes 4 and 5 strains (*47*). Consistently, in our metagenomic data, we found that the acne samples with ribotypes 4 and 5 strains also had abundant locus 2 OGUs. Additionally, some of the acne samples with no detectable ribotypes 4 and 5 had abundant locus 2 OGUs, suggesting that some other ribotypes may also harbor this disease-associated island.

Ribotype 1 is a ubiquitous *P. acnes* strain in the skin microbiota with similar prevalence found in acne patients and healthy individuals (*6*).The genome sequences of ribotype 1 strains show that some ribotype 1 strains harbor the three disease-associated loci. With only the ribotyping data, it is difficult to distinguish the metagenomic and functional differences between acne patients and healthy individuals that are both dominated by ribotype 1. Among the 76 samples, 16 samples from acne patients and 15 samples from healthy individuals were dominated by ribotype 1 (Microbiome-type 1, MT1). We found that the OGUs from locus 2 were abundant in the MT1 samples from acne patients, but rarely found in the MT1 samples of healthy individuals (Figure

6-4). This finding suggests that the ribotype 1 strains in acne patients often harbor the disease - associated locus 2, different from the ribotype 1 strains found in healthy individuals. This also suggests that metagenomic sequencing provides a higher resolution of the distributions of potential pathogenic elements.

Discussion

In this study, we identified that the OGUs in the disease-associated locus 2 were highly enriched in acne patients and rarely found in healthy individuals. The abundances of the OGUs in locus 2 were correlated with the presence of ribotypes 4 and 5, which are known to be antibiotic resistant *P. acnes* strains. However, it is unlikely that the enrichment of locus 2 in acne patients is a result of antibiotic treatment because of two reasons. First, a significant portion of the acne patients with abundant locus 2 has no history of antibiotic treatment. Second, a significant portion of the acne patients who were treated with antibiotics had no detectable locus 2 OGUs. These suggest that the enrichment of locus 2 in acne patients is a characteristic of the disease rather than a result of the treatment. Our previous genomic sequencing study revealed that the acne-associated *P. acnes* strains of clade IA-2 harbor this genomic island as well as locus 1 and locus 3 (*6*). Intriguingly, although some OGUs in locus 1 and locus 3 were also enriched in acne patients, the enrichment of the OGUs in these two loci is less significant compared to the enrichment of the OGUs in locus 2. The genes in the locus 2 include homologs of the Streptolysin S-Associated Gene cluster (Sag), suggesting their potential involvement in the biosynthesis and transport of toxins. The roles of the genes in these loci in acne pathogenesis need to be further investigated.

Materials and Methods

Data Collection

Genomic DNAs of the microcomedone samples from subjects were extracted and constructed into sequencing libraries using Nextera XT kit (Illumina). The shotgun genomic libraries were then sequenced by MiSeq and HiSeq platforms. After data cleaning, reads of each sample were aligned against bacterial genomes from the Human Microbiome Project reference genome database (http://www.hmpdacc.org/HMREFG/) and the *Propionibacterium* genomes of *P. avidum*, *P. granulosum*, *P. humerusii*, *P. acnes* as well as *P. acnes* phage. The *P. acnes* gene abundances were counted using a RPKM method based on *P. acnes* operational gene unit (OGU). The OGUs used in this metagenomic study were constructed based on the clustering of genes in 80 *P. acnes* genomes with 90% identity in nucleic acid sequences using CD-HIT version 4.3. The ribotype composition of each sample was inferred based on the reads mapped on the *P. acnes* 16S rRNA gene.

Principal Component Analysis

The principal component analysis was conducted on the correlation matrix of the *P. acnes* OGUs. The analysis was done using the "princomp" function in R (*72*).

Heatmap plotting

The *P. acnes* OGU abundances were plotted as white-black heatmaps using the heatmap.2 function in gplots package in R (*74*).

Figures and Tables

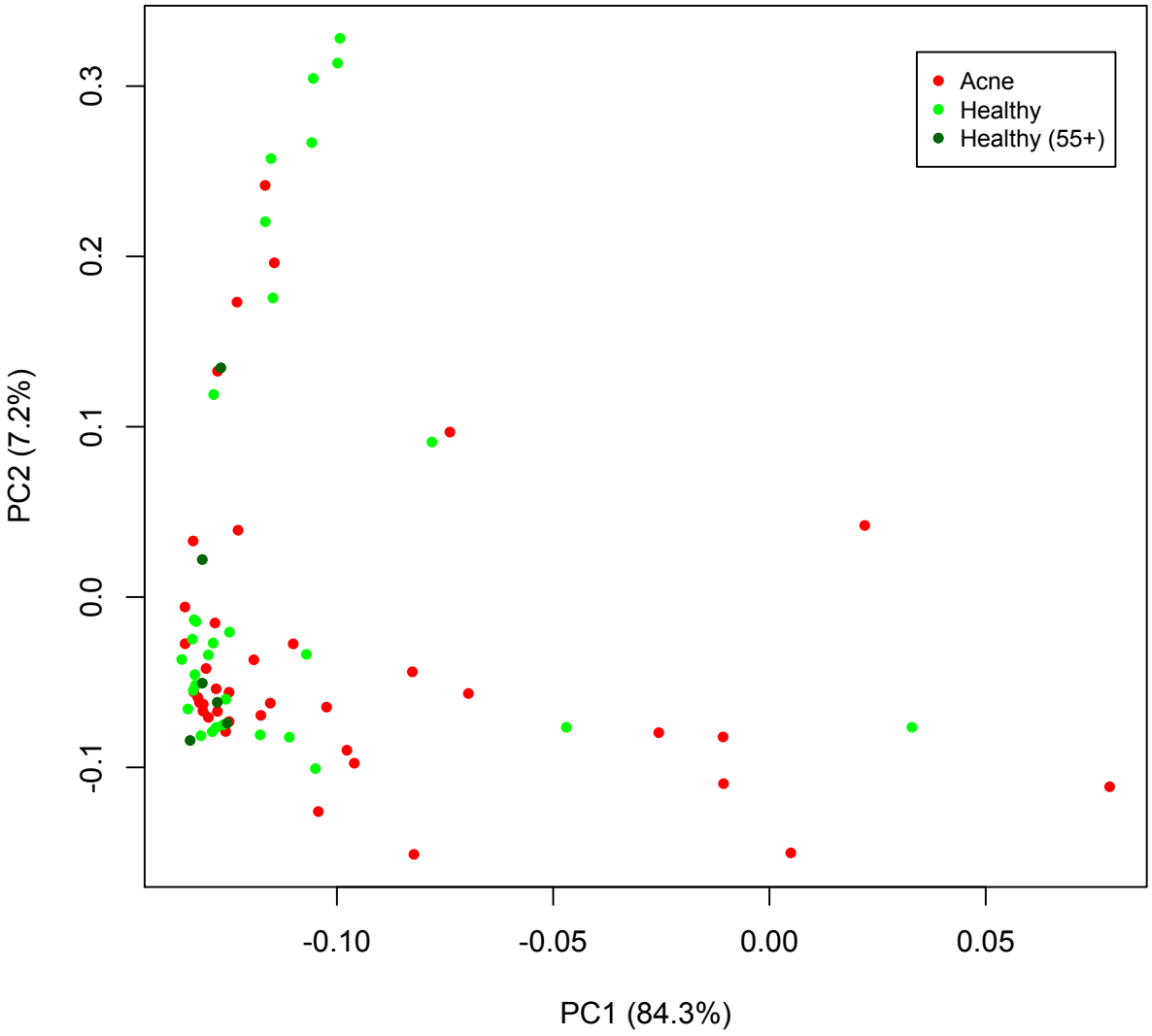

Figure 6-1. Principal component analysis based on all the *P. acnes* OGUs.

The principal component analysis was performed based on metagenomic composition of all the *P. acnes* OGU. The first two principal components were plotted to show the relationship between acne samples (n=39; red circles), healthy samples (n=31; green circles), and senior healthy samples with more than 55 years old (n=6; dark green circles). PC1 explains 84.3% of the variation and PC2 explains 7.2% of the variation.

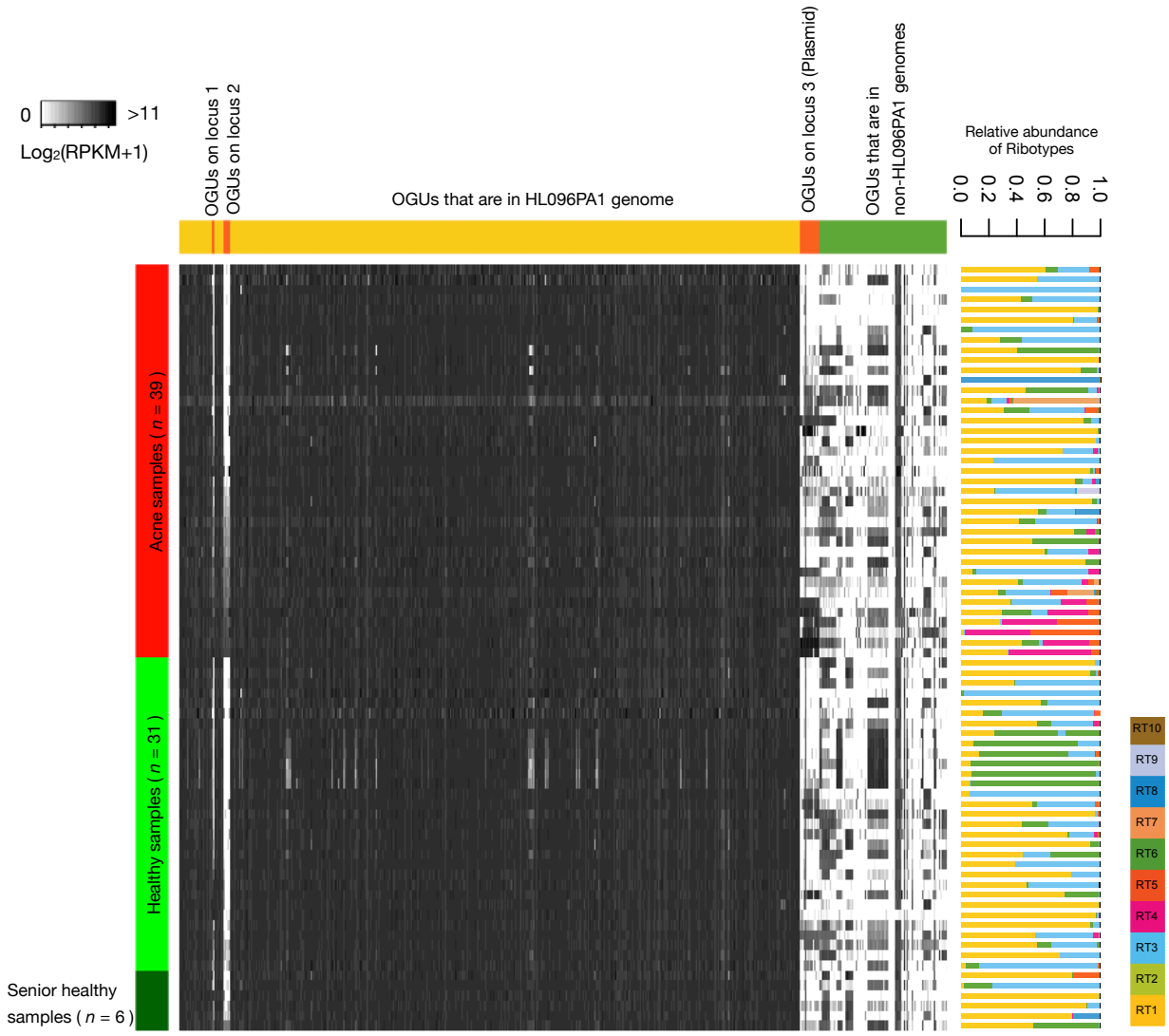

Figure 6-2. *P. acnes* OGU prevalence and abundance in acne and healthy samples

A heatmap was constructed to show the prevalence and abundance of *P. acnes* OGUs for acne samples (n=39), and healthy samples (n=37; including six senior individuals with more than 55 years old). Samples were clustered based on locus 2 abundance. OGU abundances were plotted in their order on the pan-genome.

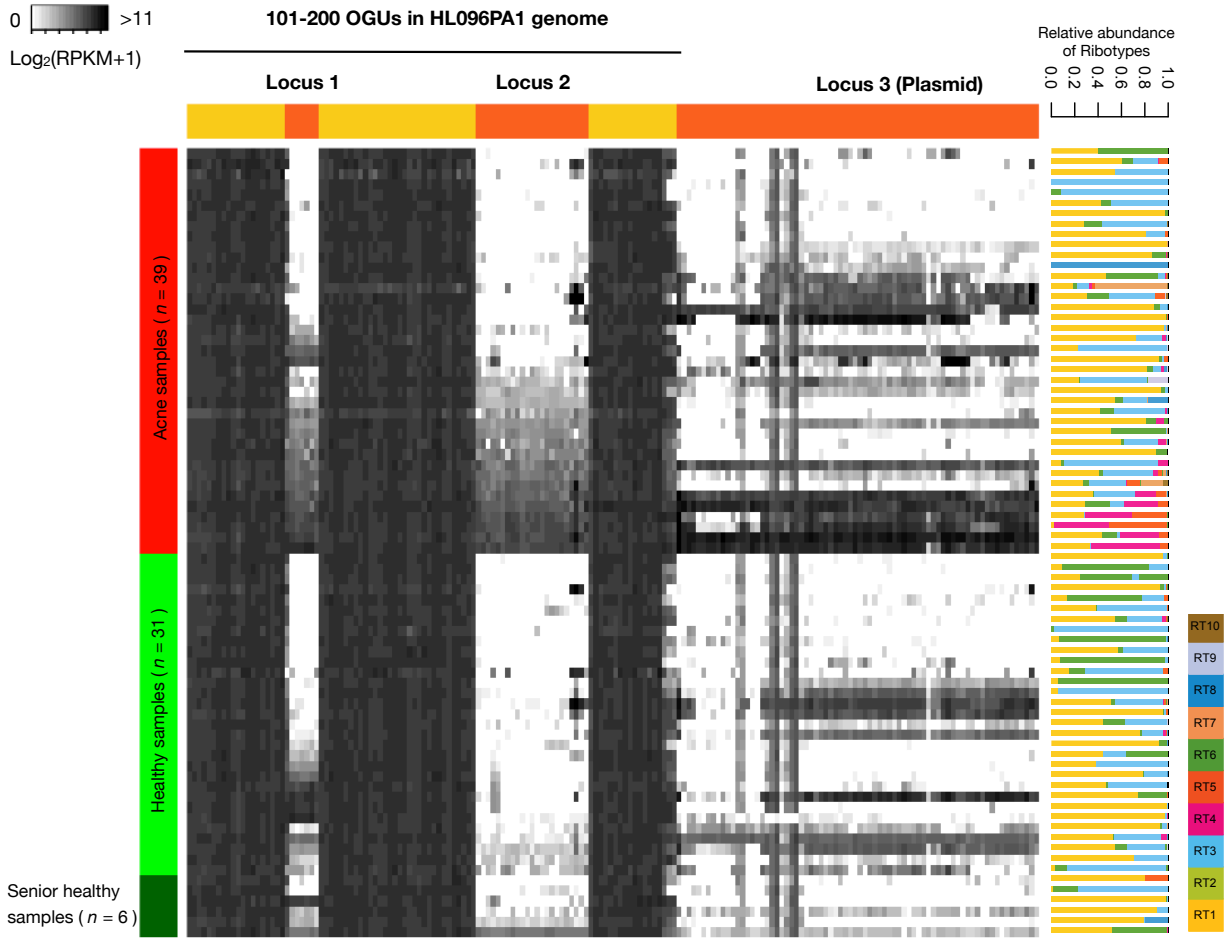

Figure 6-3. *P. acnes* OGUs in locus 2 were enriched in acne samples

A heatmap was constructed to show the abundance of *P. acnes* OGUs from the two chromosomal loci (locus 1 and 2) and *P. acnes* plasmid (locus 3) for acne samples (n=39), and healthy samples (n=37; including six senior individuals with more than 55 years old). Samples were clustered based on locus 2 abundance. OGU abundances were plotted in order of appearance from 101-200 on the pan-genome (flanking locus 1 and 2) and from locus 3 (*P. acnes* plasmid).

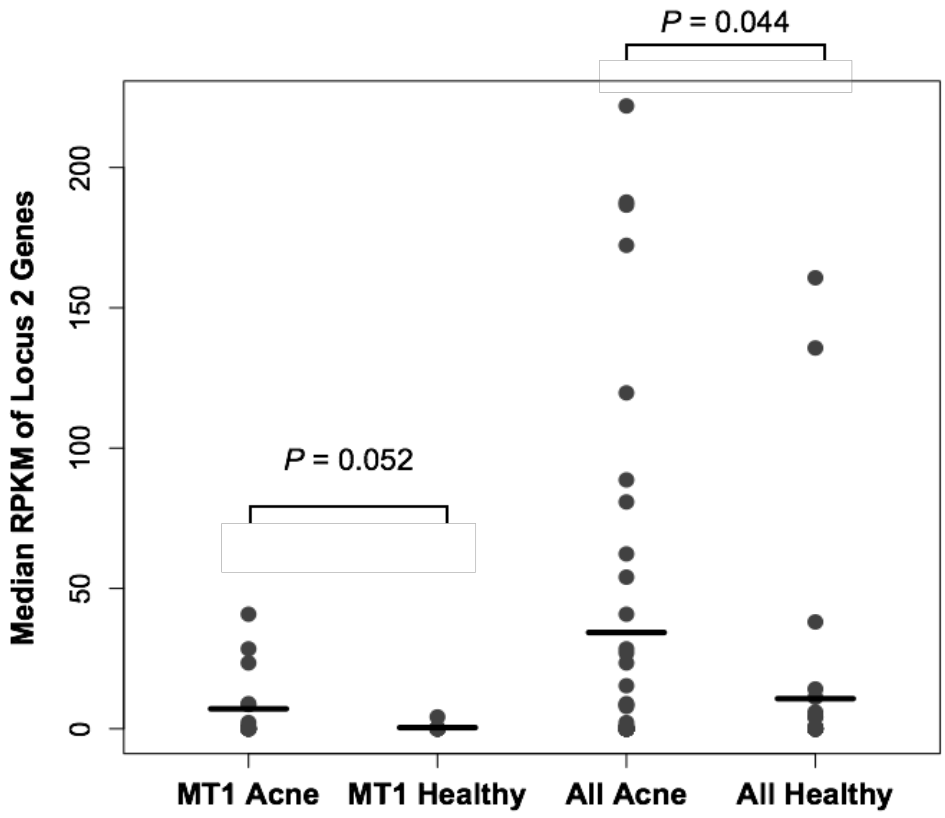

Figure 6-4. Microbiome-type 1 samples from acne patients displayed higher abundances of locus 2-associated OGUs than those of the microbiome-type 1 samples from healthy individuals.

The median RPKM value of the locus 2 genes in each sample was plotted as one dot. Significance was determined by independent t-test. MT1: microbiome type 1.

Chapter 7: Concluding remarks

In this dissertation study, I investigated the role of the skin microbiota in the pathogenesis of acne vulgaris. I employed a number of "omics" approaches based on the next-generation sequencing technologies to make new discoveries. I combined computational analysis of large sequencing datasets and microbiology experiments to test several hypotheses. The findings from this dissertation shed light on a novel molecular mechanism of metabolite-mediated interactions between host skin cells and the skin microbiota in acne pathogenesis.

The first part of the dissertation (Chapter 2) described a metatranscriptomic study of the skin microbiota from acne patients and healthy individuals. It demonstrated that the transcriptional profiles of the skin microbiota in acne patients were distinct from those in healthy individuals. It highlighted that the vitamin B12 biosynthesis pathway in the dominant skin bacterium, *P. acnes*, was down-regulated in acne patients compared to healthy subjects. This finding also revealed that *in vivo* transcriptional activities of the microbiota could separate the healthy and disease states of the host.

The second part of the dissertation (Chapter 3) focused on assessing the hypothesis that host vitamin B12 modulates the transcriptional and metabolic activities of the skin microbiota and plays a signaling role in acne pathogenesis. First, the study showed that vitamin B12 supplementation in healthy subjects repressed *P. acnes* vitamin B12 biosynthesis and induced acne in a subset of the subjects studied. Second, the study demonstrated that vitamin B12 supplementation in *P. acnes* cultures promoted the production of porphyrins, which are known to induce inflammation in acne. Our findings suggest a novel vitamin B12-mediated bacterial pathogenesis pathway in acne and provide a molecular explanation for the long-standing clinical

observation that vitamin B12 induces acne. Our study not only discovered that vitamin B12, an essential nutrient in humans, modulates the transcriptional activities of the bacteria on the skin, but also provided evidence that metabolite-mediated interactions between the host and the skin microbiota play essential roles in disease development.

The third part of the dissertation (Chapter 4) aimed to understand the molecular mechanism of *P. acnes* cobalamin riboswitches regulating the expression of vitamin B12 biosynthesis genes. It also aimed to determine whether strain-variations in cobalamin riboswitches affect the gene regulation differently in strains of different lineages. A nucleotide deletion at the 53rd position of the Cobalamin-5 riboswitch unique to the clade IA-2 strains was identified. This deletion is in the stem region P5, which maintains the intramolecular kissing loop (KL) structure that controls RNA expression. This suggests that the sequence variation in cobalamin riboswitch may affect the regulation of Cobalamin-5 on the expression of its downstream Cob/Cbi operon. This study also indicates that vitamin B12 modulation of the transcriptional activities may vary among different *P. acnes* strains and the effect of vitamin B12 on the metatranscriptome of the skin microbiota may be dependent on the microbial community composition.

The fourth part of the dissertation (Chapter 5) developed a protocol that prepares enriched bacterial cDNA and human cDNA libraries from skin biopsy samples. This protocol enables simultaneous gene expression analysis of the skill cells and skin bacterial cells. It laid a foundation for studying the transcriptional interactions between the host and skin bacteria.

The fifth part of the dissertation (Chapter 6) described a metagenomic analysis of the skin microbiome associated with acne vulgaris. I made two important discoveries from the analysis. First, a subset of acne patients and healthy individuals harbored their respectively unique metagenomic elements, which separated them from the rest of the acne and healthy individuals on a principal component analysis. Second, a 20 kb *P. acnes* genomic island, locus 2, was found to be enriched in acne patients, but rarely found in healthy individuals. Understanding the roles of these disease- or health-associated genetic factors potentially can lead to new therapeutics for acne.

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