Title

Porphyrin production and regulation in different Propionibacterium acnes lineages contribute to acne vulgaris pathogenesis

Author

Johnson, Tremylla

Publication Date

2017

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Porphyrin production and regulation in different *Propionibacterium acnes* lineages contribute to acne vulgaris pathogenesis

A dissertation submitted in partial satisfaction

of the requirement for the degree

Doctor of Philosophy in Molecular and Medical Pharmacology

by

Tremylla A. Johnson

2017

ABSTRACT OF THE DISSERTATION

Porphyrin production and regulation in
different *Propionibacterium acnes* lineages
contribute to acne vulgaris pathogenesis

by

Tremylla A. Johnson
Doctor of Philosophy in Molecular & Medical Pharmacology
University of California, Los Angeles, 2017
Professor Huiying Li, Chair

Propionibacterium acnes is a dominant human skin commensal. It has been implicated in acne pathogenesis, but its role remains unclear. Recent metagenomic studies have revealed that certain *P. acnes* strains are highly associated with acne, while some others are associated with healthy skin. Little information exists about *P. acnes* strain-level differences beyond the genomic differences. In this study, I revealed that acne-associated type IA *P. acnes* strains produced significantly higher levels of porphyrins (a metabolite important in acne development) than health-associated strains (type II). Strains of type IA-1 and type IA-2 produced similar levels of porphyrins. Moreover, porphyrin production in these *P. acnes* strains is modulated by vitamin B_{12}. On the other hand, health-associated type II strains produced low levels of

porphyrins and did not respond to vitamin B_{12}. Strains from other lineages (type IB and type III) have lower levels of porphyrin production and porphyrin levels were not modulated by vitamin B_{12}. Using small molecule substrates and inhibitors, I demonstrated that porphyrin biosynthesis was modulated at the metabolic level in type IA strains. By comparing the porphyrin operons of different strains, I identified a repressor gene (*deoR*) of porphyrin biosynthesis that was encoded in all health-associated *P. acnes* type II strains, types IB-3, IC, and III strains, but not in acne-associated type IA strains. The expression of *deoR* suggests regulation of porphyrin production at the transcriptional level in health-associated strains. Other skin propionibacteria, *Propionibacterium granulosum, Propionibacterium avidum,* and *Propionibacterium humerusii,* produced little or no porphyrin, and were not regulated by vitamin $B_{12.}$ Additionally, I developed an assay using mass spectrometry to analyze porphyrin levels in skin samples. The findings from my study provide a potential molecular explanation for the different contributions of *P. acnes* strains to skin health and disease, and support the role of vitamin B_{12} in acne pathogenesis in a subset of the population. This study emphasizes the importance of understanding the role of the commensal microbial community in health and disease at the strain level, and suggests potential utility of health-associated *P. acnes* strains in acne treatment.

The dissertation of Tremylla A. Johnson is approved.

Joseph Ambrose Loo

Jing Huang

Desmond Smith

Huiying Li, Committee Chair

University of California, Los Angeles

2017

DEDICATION

To my daughter

Lauryn Winter Johnson

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank the people that supported me throughout this journey. A huge thanks to my committee members Dr. Huiying Li, Dr. Desmond Smith, Dr. Joseph Loo and Dr. Jing Huang for your discussion and suggestions. I would like to give my deepest gratitude to Dr. Michael Phelps, Dr. Harvey Herschman, and Dr. Sam Chow for all of their support. I would like to extend my appreciation to Kym Faull in the UCLA Pasarow Mass Spectrometry Laboratory. I would like to thank Emily Fitch who was always willing to help me with anything. Thank you the Molecular and Medical Pharmacology Department and Crump Institute for Medical Imaging. I want to thank Andy Lin in the UCLA Institute for Digital Research and Education (IDRE) for statistics consultation as well as the UCLA-DOE Biochemistry Instrument Facility for support on experimental instruments. Thank you to my mom Beverly Carroll-Wilson, my friend, my mentor, and an invaluable person in my life. Thank you to my best friend, Melanie McDaniel, who always kept me laughing. Thanks to Dr. Tracy Johnson and Dr. Sherly Mosessian for all of their guidance. Thank you to all my coworkers from the Li Laboratory: Nathaniel Bangayan, Emma Barnard, Baochen Shi, Jared Liu, Dezhi Kang, Brian Chiu, Emily Curd, Sam Ngo, YuLing Chang, Yi-Pei Chen, Alexa Cohen, Mizue Naito, Alison Cheng, Shi Feng, Jonathan Tu. A big thank you to Emily Curd for introducing the R software to me, and just being there when I needed her. Thank you to the members of the UCLA Pasarow Mass Spectrometry Laboratory: Farbod Fazlollahi, Joe Capri, Austin Quach, and Whitaker Cohn. This research was funded by NIH grant R01GM099530 from the National Institute of General Medical Sciences and by Ruth L. Kirschstein National Research Service Award AI007323.

VITA

EDUCATION

2008 MS Chemistry, Southern Illinois University – Edwardsville, Edwardsville IL
2003 BS Chemistry (Minor in Spanish), Butler University – Indianapolis, IN

PROFESSIONAL EXPERIENCE

2011-2012 Staff Research Associate II (UCLA)
2008–2010 R&D Production Biochemist II, Sigma Aldrich Corp. St. Louis, MO
2004–2006 Quality Control Chemist, Elantas PDG**,** St. Louis, MO

TEACHING EXPERIENCE

2007-2008 SIUE Edwardsville, IL
Organic Chemistry
General Chemistry
Biochemistry
Analytical Chemistry

GRANTS, FELLOWSHIPS and AWARDS

2015 – 2016 UCLA Microbial Pathogenesis Training Grant

CONFERENCES

2013-2015 UCLA Molecular and Medical Pharmacology Retreat

PUBLISHED RESEARCH

1) Strain-Level Differences in Porphyrin Production and Regulation in *Propionibacterium acnes* Elucidate Disease Associations. mSphere. 2016 Feb 10;1(1). pii: e00023-15. **Tremylla Johnson**, Dezhi Kang, Emma Barnard, Huiying Li.

2) Conformational States and Kinetics of the Calcium Binding Domain of NADPH Oxidase The Open Biochemistry Journal, 2010, Volume 4, 59-67
Chin-Chuan Wei, Nicole Motl, Kelli Levek, Liu Qi chen, Ya-Ping Yang, **Tremylla Johnson**, Lindsey Hamilton and Dennis J Stuehr

CHAPTER 1:

INTRODUCTION

The human body is a host to trillions of microbes. Most of the microbes are commensal residents, but some are potentially pathogenic. The human microbiota has been implicated in human health and disease. Several bacteria have been demonstrated to be involved in diseases such as periodontitis, irritable bowel syndrome, obesity, foot odor, and acne vulgaris (1-5).

Acne vulgaris (acne) is a multifactorial disease affecting > 80% of adolescents and young adults. It is known for its negative physical and psychological impacts, with 30% of affected teenagers reportedly contemplating suicide (6). While the etiology of acne is unclear, genetic factors, hormones, and skin bacteria have been implicated in the disease pathogenesis (5, 7, 8). In particular, the skin bacterium *Propionibacterium acnes* is thought to play an important role in triggering inflammation in the skin. The current standard of care in acne is through administration of antibiotics or retinoid-based treatments. These treatments are often less effective and can have significantly adverse side effects. There is an urgent need to develop new therapies for acne.

Acne lesions can typically be found on the face, neck, and trunk. These inflammatory lesions can lead to scarring. Acne is often developed during puberty, where the sebaceous glands in pilosebaceous units (hair follicles) become large and produce more sebum. An increase of sebum can clog the hair follicle, making it more anaerobic and preferable for bacteria to grow. The bacteria can produce inflammatory mediators, which can induce inflammation.

P. acnes is a gram-positive anaerobic bacterium that accounts for ~90% of the bacteria that reside in the hair follicle (9). *P. acnes* has been classified into distinct types; types I, II, and III (10-12). McDowell initially classified *P. acnes* strains based on the sequences of housekeeping genes *tly* and *recA* (11). Multilocus sequence typing (MLST) was later used by McDowell (Belfast scheme) and Lomholt and Kilian (Aarhus scheme) to compare multiple gene loci and to classify *P. acnes* strains into lineages IA, IB, IC, II and III (13, 14).

Fitz-Gibbon *et al.* (2013) used the 16S ribosomal RNA (rRNA) gene sequences (ribotyping) to classify *P. acnes* strains into lineages that were highly comparable with the results from the two independent MLST schemes (Belfast and Aarhus MLST scheme) (9, 13, 15). This study also demonstrated *P. acnes* strain-level differences between healthy skin and acne. Ribotypes (RTs) 4, 5, 8, 9 and 10 were highly associated with acne, while RT6 was highly associated with healthy skin. Genomic comparison of acne-associated strains revealed that they encode multiple virulence genes that may contribute to the disease pathogenesis. Health–associated strains, on the other hand, harbor genes that may prevent them from acquiring virulence genes. However, the molecular mechanism for *P. acnes* strain-level differences in health- and disease- associations remains to be elucidated.

A wealth of information exists about genomic variations of different *P. acnes* strains (9, 16, 17). However, less is known about the molecules or metabolites secreted by *P. acnes* that can damage the skin or cause inflammation in the skin. *P. acnes* secretes porphyrins that react with molecular oxygen and produces free radicals, inducing inflammation of the skin. Previous studies have shown reduced levels of porphyrins in healthy skin compared to acne skin, as well as in individuals that respond to acne treatment (18-23).

It has been reported that vitamin B_{12} supplementation induces acne in some individuals (24-28). A recent study from our group revealed that vitamin B_{12} supplementation in *P. acnes* leads to increased porphyrin production (28). Vitamin B_{12} biosynthesis is inversely correlated with porphyrin biosynthesis in *P. acnes*, and vitamin B_{12} can modulate porphyrin production (28, 29). In this study, I compared the porphyrin production and vitamin B_{12} regulation between acne-associated type IA-2 strains and health-associated type II strains to provide a link correlation between porphyrin production and the disease association of *P. acnes* strains and the influence of vitamin B_{12} on the porphyrin production of these strains (30). I compared the porphyrin production and vitamin B_{12} regulation between all major lineages of *P. acnes* as well as other skin *Propionibacterium* species (*P. granulosum*, *P. avidum*, and *P. humerusii*) as a comparison to *P. acnes.* I describe the development of a protocol using high performance liquid chromatography/mass spectrometry (HPLC/MS) to identify and quantify porphyrin production of the skin.

CHAPTER 2:

Strain-level differences in porphyrin production and regulation in *Propionibacterium acnes* elucidate disease associations

INTRODUCTION

Propionibacterium acnes is a major commensal bacterium residing on the human skin. It plays important roles in maintaining skin health, but has also been implicated in the pathogenesis of several diseases and infections, including sarcoidosis, SAPHO syndrome, endodontic lesions, eye infections, prosthetic joint infections, prostate cancer, and acne vulgaris (commonly called acne) (18, 19, 31, 32). Acne is the most common skin disease, affecting more than 80% of adolescents and young adults worldwide (20, 33). Despite the clinical importance of the disease (34, 35), the etiology of acne is not yet clear. *P. acnes* dominates the skin of both acne patients and healthy individuals, and thus its role in acne pathogenesis has not been well understood. Previously, culture-based and 16S ribosomal RNA (rRNA) metagenomic studies identified *P. acnes* strains that were associated with either acne or healthy skin (9, 13-15). Genome comparison of a large number of strains revealed key genetic differences among *P. acnes* lineages (9, 16, 17). Type IA-2 (primarily RT4 and RT5) strains have been associated with acne. They harbor extra genomic elements encoding multiple virulence genes. On the other hand, type II strains, in particular RT6 and some RT2 strains, have been rarely found in acneic skin and thus are defined as health-associated in the context of acne (9). Type II strains encode CRISPR elements, which may prevent these strains from acquiring virulence genes from phage or other foreign DNA. The presence of these genetic elements partly explains how different *P. acnes*

strains may play roles in health or disease, however, the molecular mechanisms underlying *P. acnes* strain-level differences in health- and disease- associations remain to be elucidated.

To date, limited information exists about *P. acnes* strain-level differences beyond the genomic variations. Molecules secreted by *P. acnes*, such as proteases, lipases, hemolysins, and porphyrins, can degrade host tissue and have been suggested as causal factors in acne (28, 36-39). Porphyrins can generate reactive oxygen species and induce inflammation in keratinocytes (40-42). Previous studies have shown correlations between acne severity and the concentrations of bacteria-derived porphyrins in the hair follicle. Increased levels of porphyrins were observed in acne skin compared to healthy skin, as well as in acne lesions compared to non-lesional sites of acne patients (24-26). Consistently, a reduction in porphyrin levels was observed in acne patients who positively responded to acne treatment, while those who did not respond to acne treatment exhibited unchanged or increased levels of bacterial porphyrins on their skin (21-23).

Vitamin B_{12} has been suggested to induce acne (24-28). In propionibacteria, the vitamin B_{12} and porphyrin biosynthesis pathways are inversely correlated (28, 29). Our recent study suggested that vitamin B_{12} supplementation repressed its own biosynthesis in *P. acnes*, resulting in increased porphyrin production, and led to acne development in a subset of individuals (28). In this study, I compared the porphyrin production and regulation between acne-associated type IA-2 strains and health-associated type II strains to investigate a potential molecular link between porphyrin production and disease association of various *P. acnes* strains.

RESULTS

Acne-associated *P. acnes* strains produced significantly more porphyrins than health-associated strains.

To investigate whether different *P. acnes* strains produce the same porphyrin species, I first characterized the types of porphyrins secreted by multiple *P. acnes* strains using mass spectrometry. Four acne-associated type IA-2 strains, HL053PA1 (RT4), HL045PA1 (RT4), HL043PA1 (RT5), and HL043PA2 (RT5), and three health-associated type II strains, HL001PA1 (RT2), HL103PA1 (RT2), and HL042PA3 (RT6), were examined (Table 2-1). I found that there was no difference in the porphyrin species produced by these *P. acnes* strains (Figure 2-S1). Consistent with previous studies (43-45), coproporphyrin III was the dominant porphyrin isomer produced by all strains ($[M+H]^+ = 655.3$). Minimal amounts of coproporphyrin I were also detected.

To determine whether *P. acnes* strains produce different amounts of porphyrins, I next quantified the secreted porphyrins in the seven *P. acnes* strains. The average porphyrin level produced by acne-associated type IA-2 strains was 6.5 µM (5.6 – 8.8 µM), which was significantly greater than that produced by health-associated type II strains, 1.1 µM (0 – 2.1 µM, $P<0.0001$) (Figure 2-1). Notably, the RT2 strain HL001PA1 produced no detectable porphyrins in all experiments. This strain belongs to clonal complex 72 (CC72) based on the Belfast $MLST_8$ scheme (Table 2-1). McDowell *et al.* previously reported that strains from CC72 were isolated from healthy skin (10).

Vitamin B_{12} supplementation significantly increased porphyrin production in acne-associated strains, but not in health-associated strains.

To investigate whether vitamin B_{12} modulates *P. acnes* porphyrin production in a strain-specific manner, I compared the levels of porphyrins produced by different *P. acnes* strains with and without vitamin B_{12} supplementation. I found that vitamin B_{12} supplementation led to increased porphyrin production in all tested acne-associated type IA-2 strains (average porphyrin level increasing from 6.5 μM to 9.2 μM), with statistical significance in three of the four tested strains ($P \leq 0.02$) (Figure 2-2). In contrast, vitamin B_{12} supplementation had no significant effect on porphyrin production in health-associated type II strains (average porphyrin level remaining at 1.1 μM, $P \geq 0.77$). The porphyrin levels in HL001PA1 remained undetectable. To confirm the results, I tested the porphyrin production, with and without the addition of vitamin B_{12}, of three additional type II strains, HL110PA3 (RT6), HL106PA1 (RT2), and HL050PA2 (RT1). I found that these strains produced similarly low levels of porphyrins (1.4 ± 1.2 μM) and the production level was not significantly affected by vitamin B_{12} supplementation. This data suggest that vitamin B_{12} modulates porphyrin production in acne-associated type IA-2 strains, but not in health-associated type II strains, indicating a molecular link between *P. acnes* strain composition of the skin microbiota and the observation that vitamin B_{12} induces acne in a subset of individuals.

Vitamin B_{12} supplementation repressed vitamin B_{12} biosynthesis gene expression.

To determine whether vitamin B_{12} affects porphyrin production via repression of its own biosynthesis in *P. acnes*, I performed qRT-PCR to measure the expression level of *cbiL*, a gene in the vitamin B_{12} biosynthesis pathway. The *cbiL* gene encodes precorrin-2 C20-

methyltransferase, one key enzyme involved in corrin ring formation in the vitamin B_{12} biosynthesis pathway. I found that the addition of vitamin B_{12} to *P. acnes* cultures resulted in the down-regulation of *cbiL* gene expression, with an average fold change of 0.74 in acne-associated type IA-2 strains and 0.24 in health-associated type II strains (Figure 2-3). The down-regulation of *cbiL* gene expression is consistent with the previous finding that vitamin B_{12} supplementation repressed the vitamin B_{12} biosynthesis pathway (18), leading to increased porphyrin production. The expression of *cbiL* was down-regulated in both type IA-2- and type II strains, despite the above observation that the porphyrin production in type II strains was unaffected by vitamin B_{12} supplementation. This suggests that additional mechanisms are involved in inhibiting porphyrin production in health-associated type II strains.

Addition of 5-aminolevulinic acid (5-ALA) increased porphyrin production, which was further enhanced by vitamin B_{12}.

The porphyrin and vitamin B_{12} biosynthesis pathways in *P. acnes* share the same initial enzymatic steps and a common precursor, 5-ALA, the substrate of the rate-limiting enzyme, porphobilinogen synthase. To investigate whether porphyrin production can be promoted by increasing the availability of 5-ALA, I compared the porphyrin production levels of *P. acnes* strains with and without the addition of the substrate. Additionally, I examined whether vitamin B_{12} has an additive effect on porphyrin production when supplemented in combination with 5-ALA. Upon substrate addition only, porphyrin production was significantly increased by an average of 2.2 fold in acne-associated type IA-2 strains (all $P<0.0001$) and 3.4 fold in health-associated type II strains ($P\leq0.06$, except HL001PA1) compared to untreated controls (Figure 2-4). HL001PA1 consistently produced no detectable porphyrins, even upon addition of 5-ALA.

Supplementation of 5-ALA in combination with vitamin B_{12} further enhanced porphyrin production in acne-associated type IA-2 strains (2.7 fold increase compared to substrate only) with statistical significance in three of the four tested strains ($P \leq 0.04$), but not in health-associated type II strains ($P \geq 0.94$). This is consistent with the earlier finding that type II strains did not respond to vitamin B_{12} in their porphyrin production (Figure 2-2). This data suggest that the metabolic influx of substrates influences porphyrin production in *P. acnes*. The modulation of porphyrin production by vitamin B_{12} supplementation is specific to acne-associated type IA-2 strains, but not health-associated type II strains.

Small molecule inhibitor reduced porphyrin production in *P. acnes* and its inhibition was counteracted by vitamin B_{12} supplementation.

To further demonstrate that porphyrin production can be modulated at the metabolic level, I investigated the effect of a small molecule inhibitor, levulinic acid (LA), on porphyrin biosynthesis in *P. acnes* strains. Additionally, I examined whether vitamin B_{12} counteracts the effect of LA on porphyrin production when supplemented in combination with LA. LA is an analog of 5-ALA and has been shown in *Pseudomonas aeruginosa* to inhibit the enzymatic activity of porphobilinogen synthase (46), blocking the metabolic influx to the porphyrin and vitamin B_{12} biosynthesis pathways. LA molecules can enter through the cell membrane without difficulty (47). I examined a range of concentrations of LA from 0.1 mg/mL to 1.0 mg/mL and found that at the concentration of 0.1 mg/mL, LA did not significantly affect the bacterial growth, thus making the measurements of porphyrin production experimentally feasible. LA (0.1 mg/mL) significantly reduced porphyrin production in all strains except HL001PA1 with an average of 30% reduction compared to untreated cultures ($P \leq 0.0001$) (Figure 2-5). However, in

acne-associated type IA-2 strains, there was no significant reduction of porphyrins when supplemented in combination with 10 µg/mL vitamin B_{12}. Vitamin B_{12} supplementation counteracted the inhibition of porphyrin biosynthesis by LA specifically in acne-associated type IA-2 strains, but not in health-associated type II strains. This is consistent with the above findings (Figure 2-2 and Figure 2-4) and further supports the conclusions that vitamin B_{12} modulates porphyrin production in acne-associated type IA-2 strains and that porphyrin production can be modulated at the metabolic level. These data also suggest that LA is an effective inhibitor of porphyrin biosynthesis in *P. acnes* and that the porphyrin biosynthesis pathway can be a potential therapeutic target for new acne treatments.

Health-associated strains harbor a porphyrin biosynthesis repressor gene, *deoR*.

To identify potential molecular mechanisms that can explain differences in porphyrin levels produced by acne-and health-associated *P. acnes* strains, I compared the porphyrin biosynthesis gene operon (*Hem*) of 82 *P. acnes* strains (16), including the strains tested in this study. I found that all health-associated type II strains and a few type I strains (mainly IB-3 and IC strains) harbor an additional gene in the porphyrin biosynthesis operon, annotated as *deoR* transcriptional repressor (Figure 2-S2 and Figure 2-S3). The *deoR* gene is located 13 bp upstream of the porphyrin biosynthesis gene cluster. This gene is absent in all acne-associated type IA-2 strains (16). To determine whether *deoR* is functional, I performed gene expression analysis and found that it was expressed in two of the three health-associated type II strains, HL001PA1 and HL042PA3, but not in HL103PA1 (Figure 2-6). Characterizations of *deoR*-like repressors in multiple other species have revealed their roles in regulating cellular processes such as sugar phosphotransferase activity, daptomycin production, and fatty acid beta-oxidation (48-

50). The close proximity of *deoR* to the porphyrin biosynthesis genes in the genome and the presence and expression of *deoR* in health-associated type II strains suggest that *deoR* may function as a transcriptional repressor in porphyrin biosynthesis. This may partly explain the low porphyrin levels produced by type II strains and their associations with healthy skin.

Acne-associated type IA-2 strains and health-associated type II strains belong to distinct *P. acnes* lineages (9). To further investigate whether the low levels of porphyrins produced by health-associated type II strains were potentially due to repression by *deoR* and not due to other lineage differences, I examined the porphyrin levels produced by a type I *P. acnes* strain, HL025PA1. HL025PA1 represents a distinct lineage within type I and is phylogenetically more similar to type IA-2 strains than to type II strains (Figure 2-S3). Unique from most other type I strains, HL025PA1 encodes and expresses *deoR* in its porphyrin biosynthesis operon (Figure 2-6). I found that HL025PA1 produced porphyrins at a level similar to that of type II strains (2.7 µM, P=0.31) (Figure 2-S4), significantly lower than type IA-2 strains (P=0.0006). Consistent with the trend observed in health-associated type II strains, vitamin B_{12} supplementation resulted in its own transcriptional repression in HL025PA1 (Figure 2-3), while its porphyrin production was unaffected by vitamin B_{12} supplementation (Figure 2-S4). This finding further supports an inhibitory role for *deoR* in porphyrin biosynthesis and may potentially explain the health association of type II strains.

DISCUSSION

The role of the human microbiota in disease and health is not yet fully understood. In particular, the molecular mechanisms for different strains of the same commensal species with distinct functions in maintaining health or triggering disease have yet to be elucidated. While the dominant skin commensal, *P. acnes,* is thought to provide protection for the skin from

colonization of pathogens such as *Staphylococcus aureus* (51, 52), multiple studies have suggested that *P. acnes* can act as an opportunistic pathogen in various diseases, including sarcoidosis, SAPHO syndrome, endodontic lesions, eye infections, prosthetic joint infections, prostate cancer, acne, and several others (18, 19, 31, 32). In acne, the dominance of *P. acnes* on both acne and healthy skin has long been a concern in defining the role of this bacterium in disease pathogenesis (9, 18, 53). Microbiome studies of the skin follicle have revealed strain-level differences of *P. acnes* in health or disease associations (9, 16, 17). However, the molecular mechanisms explaining the strain differences are not well understood.

In *P. acnes*, strain-level differences have been described at the genomic (16, 17) and proteomic levels (37, 54, 55), however, their molecular link to health and disease remains to be defined. In this study, I investigated the strain-level differences in *P. acnes* at the metabolic level. I discovered that, compared to health-associated type II strains, acne-associated type IA-2 strains produced significantly more porphyrins, a group of bacterial metabolites that induce inflammation in acne (Figure 2-1). This finding is consistent with previous observations that porphyrin levels were higher in acne skin compared to healthy skin (56-58), and provides one potential molecular mechanism for the role of acne-associated type IA-2 strains in the disease pathogenesis.

This study confirmed the earlier finding that vitamin B_{12} modulates the gene expression and metabolic activities of *P. acnes* in acne development (28), and further revealed that acne-associated type IA-2 strains, but not health-associated type II strains, responded to vitamin B_{12} supplementation with increased porphyrin production (Figure 2-2). It has been documented that vitamin B_{12} supplementation leads to acne development in a subset of populations (24-28). However, the determinants in individuals who respond to vitamin B_{12} supplementation and

develop acne have not yet been identified. These data show that vitamin B_{12} modulation of porphyrin production is strain-specific, and suggest that the *P. acnes* strain composition of an individual's skin microbiota contributes to vitamin B_{12}-induced acne. Individuals harboring acne-associated type IA-2 strains are likely to be at increased risk for developing acne in response to high vitamin B_{12} levels due to the ability of their skin bacteria to produce more porphyrins. On the other hand, individuals whose skin is dominated by health-associated type II strains may have lower porphyrin levels produced by the bacterium, leading to a reduced risk for acne development when supplemented with vitamin B_{12}.

This study also revealed a potential transcriptional repression mechanism of porphyrin biosynthesis in health-associated strains through *deoR* regulation (Figure 2-6 and Figure 2-S2). While the function and regulation of *deoR* in *P. acnes* require further investigation, the presence and expression of this gene suggest that *deoR* may play a role in the inhibition of porphyrin production in health-associated type II strains. *P. acnes* strain HL025PA1, although belonging to type I, represents a distinct lineage. It encodes and expresses *deoR.* Lomholt and Kilian previously described that only one of their 13 studied *P. acnes* isolates from this lineage (ST27, as designated by the Aarhus $MLST_9$ scheme) was from severe acne and suggested an association of this lineage with healthy skin (14). This observation supports the theory of a role for *deoR* in skin health. As *deoR* expression was not detected in all tested health-associated type II strains, additional mechanisms regulating porphyrin production likely exist, warranting future investigation. The low levels of porphyrins produced in health-associated strains, especially strain HL001PA1, make these strains candidates for topical probiotics to potentially modify an acne-prone skin microbial community and return the skin to a healthy state. This could be a new strategy in future acne therapeutics.

This current analysis is focused on two major *P. acnes* lineages, types IA-2 and II, which have been associated with acne and healthy skin, respectively. Further investigations of metabolic activities and their regulations including porphyrin production in strains from other lineages will shed light on additional bacterial factors contributing to disease or health.

This study has implications in the development of novel acne therapies. Current acne treatments, such as antibiotics and retinoids, are often ineffective, and can have adverse side effects. Moreover, the use of antibiotics has led to the emergence of antibiotic resistant strains and an increase in treatment failure (59, 60). Although new acne therapeutics has been in demand for a long time, the unclear etiology of the disease has crippled the design of new and effective treatments over the past three decades. I demonstrated that LA at the concentration of 0.1 mg/mL effectively inhibited porphyrin biosynthesis in *P. acnes* strains (Figure 2-5). This inhibition is consistent with the decreased porphobilinogen synthase activity observed in *Pseudomonas aeruginosa* upon LA treatment *in vitro* (46). LA is also known to inhibit bacterial growth, thus a sub-optimal concentration was used in this study to demonstrate the ability of this molecule to effectively reduce the porphyrin levels produced by *P. acnes* without significant inhibition of bacterial growth. In this study, I aimed to model a treatment strategy in which the virulence of acne-associated strains is targeted without disrupting the growth of health-associated strains. Other compounds, such as 4, 6-dioxoheptanoic acid and isonicotinic acid hydrazide, also inhibit porphobilinogen synthase (61, 62) and can potentially be used to inhibit porphyrin biosynthesis. LA and other related small molecules that inhibit porphyrin biosynthesis in *P. acnes* are attractive drug candidates for the treatment of acne.

The benefit of the microbiota to human health is increasingly recognized. There is a need to improve our current approaches in treating microorganism-associated diseases. Most of the

available approaches non-specifically target the microbiota using broad-spectrum antibiotics and antimicrobials, potentially leading to a disruption in the colonization of beneficial microorganisms. This study presents an advance towards a better understanding of the beneficial microbiota and targeted therapeutics. By investigating the molecular mechanisms underlying the differences between health- and disease-associated strains, I suggest that inhibiting disease-associated strains with specific targets while maintaining or supplementing health-associated strains can potentially be a new strategy in the future for treating microorganism-associated diseases. This study highlights the importance of understanding the strain-level differences of the human microbiota in disease pathogenesis. These findings also suggest the porphyrin biosynthesis pathway as a candidate drug target and use of health-associated strains as potential probiotics in novel acne therapeutics.

MATERIALS AND METHODS

P. acnes strains and cultures.

P. acnes strains used in this study (Table 2-1) were isolated previously as described by Fitz-Gibbon *et al.* (9). Briefly, each strain was isolated from the content collected from multiple hair follicles on the nose of an acne patient or healthy individual. The sampled skin site of acne patients may or may not have visible acne lesions, therefore, the strains do not necessarily correspond to the diseased or healthy state. Four RT4 and RT5 *P. acnes* strains, HL053PA1, HL045PA1, HL043PA1, and HL043PA2, were selected to represent type IA-2 strains, which were associated with the disease based on a 16S metagenomic study (9). Common to most of the RT4 and RT5 strains, these strains harbor mutations in their 16S and 23S rRNA genes, which

confer antibiotic resistance. Three RT2 and RT6 strains, HL001PA1, HL103PA1, and HL042PA3, were selected to represent type II strains that were associated with healthy skin (9). To confirm the findings, additional three type II strains, HL110PA3, HL106PA1, and HL050PA2, were also tested. The genome sequences of these ten strains were reported previously (9). For each experiment, 5 mL of reinforced clostridial broth was inoculated with $5x10^5$ *P. acnes* cells per milliliter of culture. Cultures were grown to stationary phase anaerobically at 37^{o}C in a light-protected box. Cultures were supplemented on day 0 with vitamin B_{12} (10 µg/mL), and/or 5-ALA (0.1 mg/mL), and/or LA (0.1 mg/mL). As controls, *P. acnes* strains were also cultured without supplementation. Three to five independent experiments with at least three replicates per experiment were performed for each strain, except strains HL110PA3, HL106PA1, and HL050PA2, which were tested in only one experiment with three replicates.

Extraction, identification, and quantification of extracellular porphyrins.

For each strain, porphyrins were extracted using the method described by Kang *et al.* (28). Briefly, 500 µL of bacterial culture was extracted in ethyl acetate and acetic acid (4:1, v/v), and solubilized in 1.5M HCl. The absorbance at 405 nm was measured from 200 µL of the soluble phase using a Tecan Genios Spectrophotometer M1000 (Tecan US Inc, Morrisville, NC). The standard curve to convert absorbance to concentration was generated using coproporphyrin III standards of known concentration (C654-3, Frontier Sci). I measured the porphyrin level in *P. acnes* strain KPA171202 using the porphyrin extraction method and the quantification method described by Wollenberg *et al.* (44). This measurement, which was 206 pmol/mg, is consistent with the reported value of 220 pmol/mg by Wollenberg *et al*. This indicates that the method is

comparable to previous studies. Porphyrins were extracted from cultures at stationary phase. The results from cultures at mid-log phase had a consistent trend found in cultures at stationary phase. Bacterial culture density was measured at OD_{595} for normalization of porphyrin levels. For mass spectrometry experiments, extracted porphyrins were directly injected onto an Agilent 6460 Triple Quad LC/MS system and each strain's m/z in the negative ion mode was identified.

Statistical analysis for porphyrin production comparisons.

The average amount of porphyrins produced by each strain under each culture condition was calculated based on the data from at least three independent experiments, with at least three replicates for each. The porphyrin levels between strains, groups (acne-associated vs. health-associated), and culture conditions (with treatment vs. without treatment) were estimated in a linear mixed effect model, with random intercepts by trial to account for trial effects. *P*-values for specific comparisons among groups were corrected using Tukey's method. All statistical analysis was performed using R software (version 3.1.3).

Identification of *deoR* transcription repressor.

The sequences of the porphyrin gene clusters from 82 *P. acnes* genomes (13) were aligned using the multiple sequence alignment software (ClustalW2) (63). The *deoR* transcription repressor (PPA0299) was identified as an extra genomic element found in all health-associated type II strains and a few type I strains.

RNA extraction and cDNA synthesis.

For *cbiL* and *deoR* gene expression analysis, cells were lysed with bead beating. Total RNA was extracted using the standard phenol-chloroform method and purified using RNeasy kit (Qiagen). DNA was removed using the Turbo DNA-free kit (Life Technologies). RNA quality was assessed using gel electrophoresis. Single-stranded complementary DNA (cDNA) was synthesized using SuperScript III First-Strand Synthesis SuperMix (Life Technologies).

Analysis of *cbiL* gene expression.

qRT-PCR was performed using the LightCycler 480 High Resolution Melting Master Mix (Roche) on a LightCycler 480 (Roche) with the following primers: *cbiL*-forward, 5′-GCGCGAGGCAGACGTGATCC-3′, and *cbiL*-reverse, 5′-GACACCGGACCTCTCCCGCA-3'. The following qRT-PCR protocol was used: initial denaturation at 95°C for 5 min, followed by 50 cycles of 95°C for 10 sec, 62°C for 30 sec, and 72°C for 30 sec. The fold change in *cbiL* gene expression between cultures with vitamin B_{12} supplementation and cultures without supplementation was calculated. The gene expression level of *cbiL* in each sample was normalized against the 16S rRNA transcript level. Melting curve analysis was performed using the Light Cycler 480 software Version 1.5 (Roche) to verify the specificity of the amplified products based on their melting temperatures. All reactions were run in triplicate.

Analysis of *deoR* gene expression.

deoR was amplified from the cDNA and genomic DNA of several *P. acnes* strains using a C1000 Thermal Cycler (BioRad). The following primers were used in the PCR: *deoR*-forward, 5'-CTGGCACGAGAAGGAACAA-3', and *deoR*-reverse, 5'-

GAATCGAGCAGAACTAGGTCAC-3'. The following PCR protocol was used: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 10 sec, 62°C for 30 sec, 72°C for 30 sec, followed by one cycle at 72°C for 5 min. Amplified *deoR* products were visualized on a 2% agarose gel. The amplification of 16S rRNA was included as a positive control. Acne-associated strain HL045PA1 was used as a negative control for *deoR* expression.

TABLES AND FIGURES

Table 2-1. *P. acnes* strains used in this study

	Lineage[a]	Phylogroup[b]	Strain	Ribotype[c]	Clonal complex (Sequence type by Belfast $MLST_8$)[d]	Clonal complex (Sequence type by Aarhus $MLST_9$)[e]
Acne-associated	IA-2	IA_1	HL053PA1	RT4	CC3 (ST3)	CC3 (ST3)
			HL045PA1	RT4	CC3 (ST17)	CC3 (ST3)
			HL043PA1	RT5	CC3 (ST3)	CC3 (ST58)
			HL043PA2	RT5	CC3 (ST3)	CC3 (ST58)
Health-associated	II	II	HL001PA1	RT2	CC72 (ST30)	CC60 (ST60)
			HL103PA1	RT2	CC6 (ST25)	CC60 (ST60)
			HL042PA3	RT6	CC6 (ST7)	CC60 (ST73)
Type I with *deoR*	I	IA_1	HL025PA1	RT1	CC4 (ST4)	CC28 (ST27)

[a,c] as described by Fitz-Gibbon *et al.* (9)

[b,d] as described by McDowell *et al.* (15)

[e] as described by Lomholt and Kilian (14, 15).

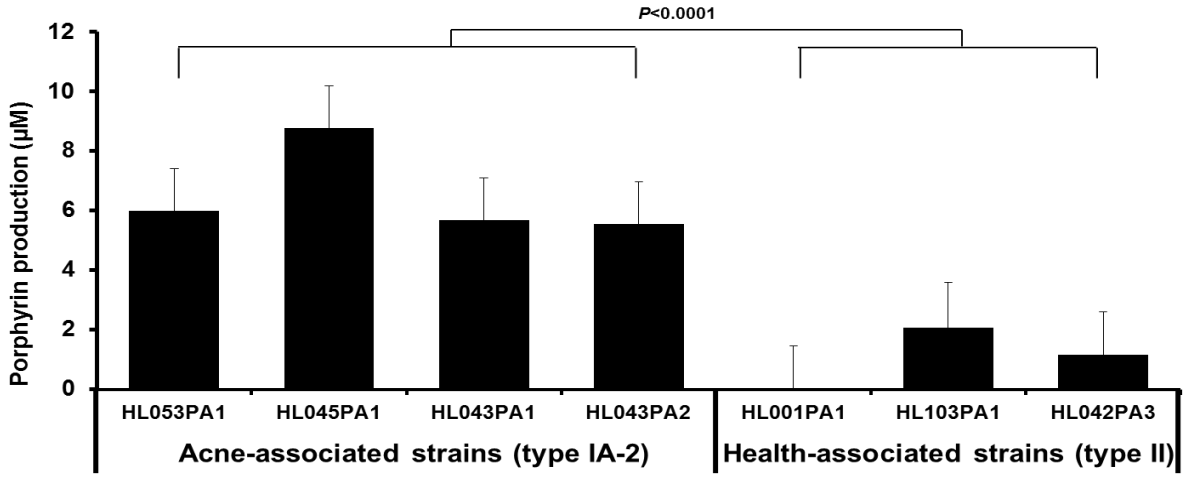

FIGURE 2-1. Acne-associated type IA-2 *P. acnes* strains produced significantly more porphyrins than health-associated type II strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least three independent experiments with at least three replicates each. Error bars represent standard error of the mean.

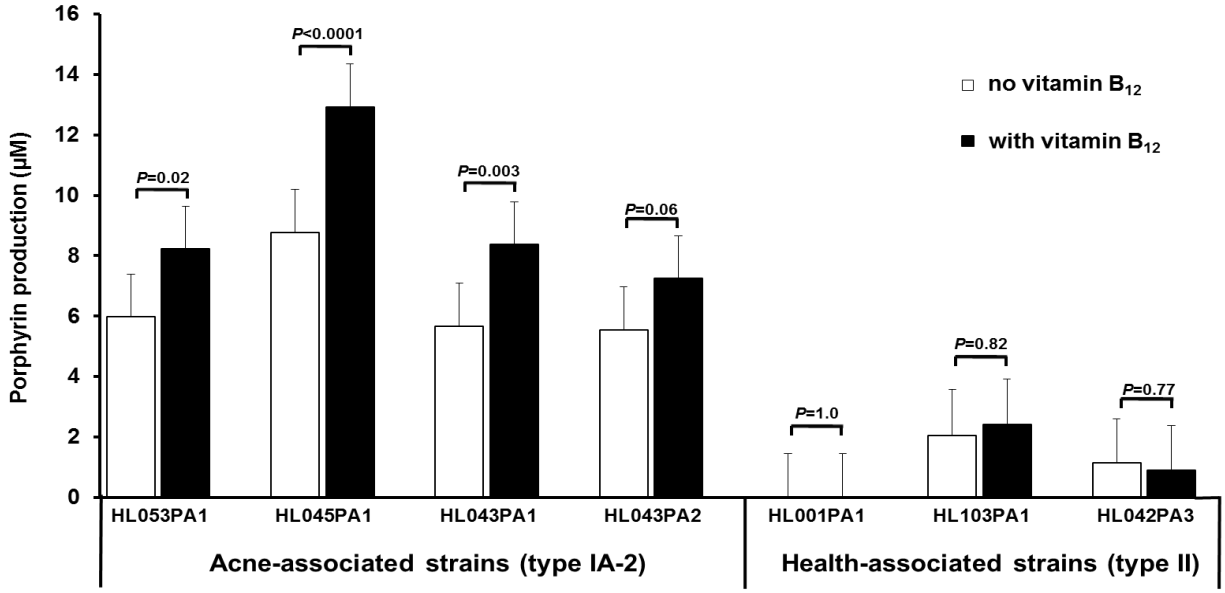

FIGURE 2-2. Vitamin B_{12} supplementation significantly increased porphyrin production in acne-associated type IA-2 strains, but not in health-associated type II strains. *P. acnes* strains were cultured in media with (black bars) or without (white bars) the addition of 10 μg/mL vitamin B_{12}. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least three independent experiments with at least three replicates each. Error bars represent standard error of the mean.

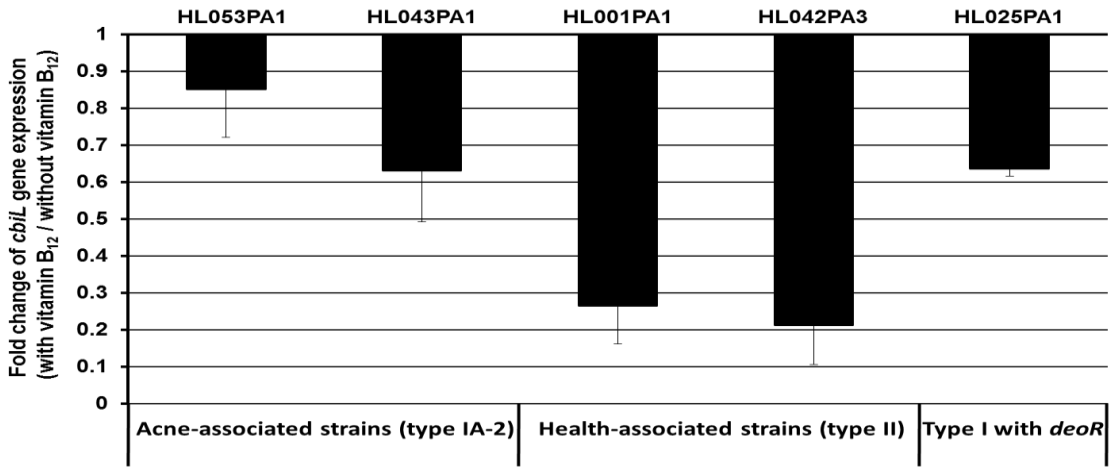

FIGURE 2-3. Vitamin B_{12} supplementation repressed the expression of a vitamin B_{12} biosynthesis gene *cbiL*. The expression level of *cbiL* was quantified by qRT-PCR from *P. acnes* strains cultured with or without the addition of 10 µg/mL vitamin B_{12}. Strains of types IA-2 and II and an RT1 strain encoding *deoR*, HL025PA1, are shown. Each bar represents the fold change in gene expression of *cbiL* in cultures with vitamin B_{12} supplementation compared to cultures without supplementation. Shown is the mean of the data obtained from three independent experiments with three replicates each. Error bars represent standard error of the mean.

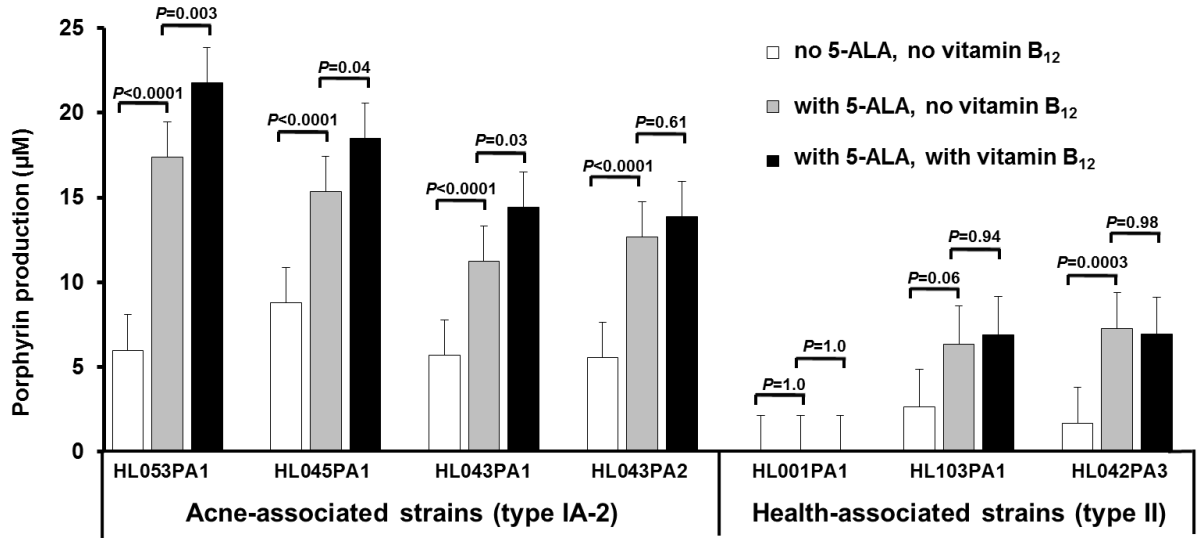

FIGURE 2-4. 5-ALA increased porphyrin production, which was further enhanced by vitamin B_{12} supplementation in acne-associated type IA-2 strains. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) substrate 5-ALA (0.1 mg/mL), or with both 5-ALA and vitamin B_{12} (10 μg/mL) added (black bars). 5-ALA significantly increased porphyrin production in acne-associated type IA-2 strains ($P<0.0001$) and in health-associated type II strains ($P\leq0.06$, except HL001PA1). Vitamin B_{12} supplementation further increased porphyrin production in the presence of 5-ALA in acne-associated type IA-2 strains, but not in health-associated type II strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least three independent experiments with at least three replicates each. Error bars represent standard error of the mean.

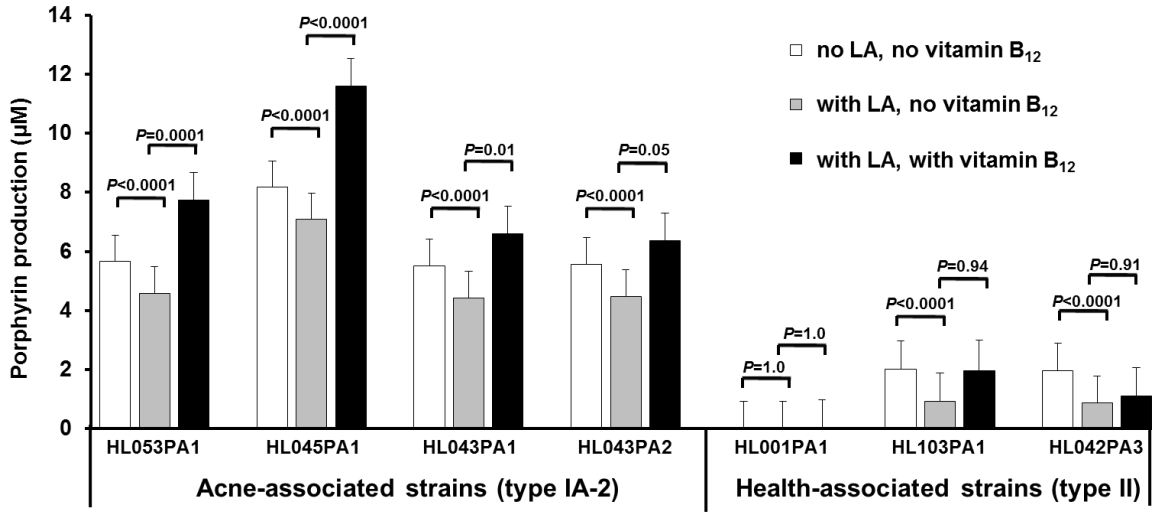

FIGURE 2-5. Small molecule inhibitor reduced porphyrin production in *P. acnes* and its inhibition was counteracted by vitamin B_{12} supplementation in acne-associated type IA-2 strains. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) inhibitor LA (0.1 mg/mL), or with both LA and vitamin B_{12} (10 μg/mL) added (black bars). LA significantly reduced porphyrin biosynthesis in all strains except HL001PA1 ($P \leq 0.0001$). Vitamin B_{12} supplementation counteracted the inhibition of porphyrin biosynthesis by LA in acne-associated type IA-2 strains, but not in health-associated type II strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least three independent experiments with at least three replicates each. Error bars represent standard error of the mean.

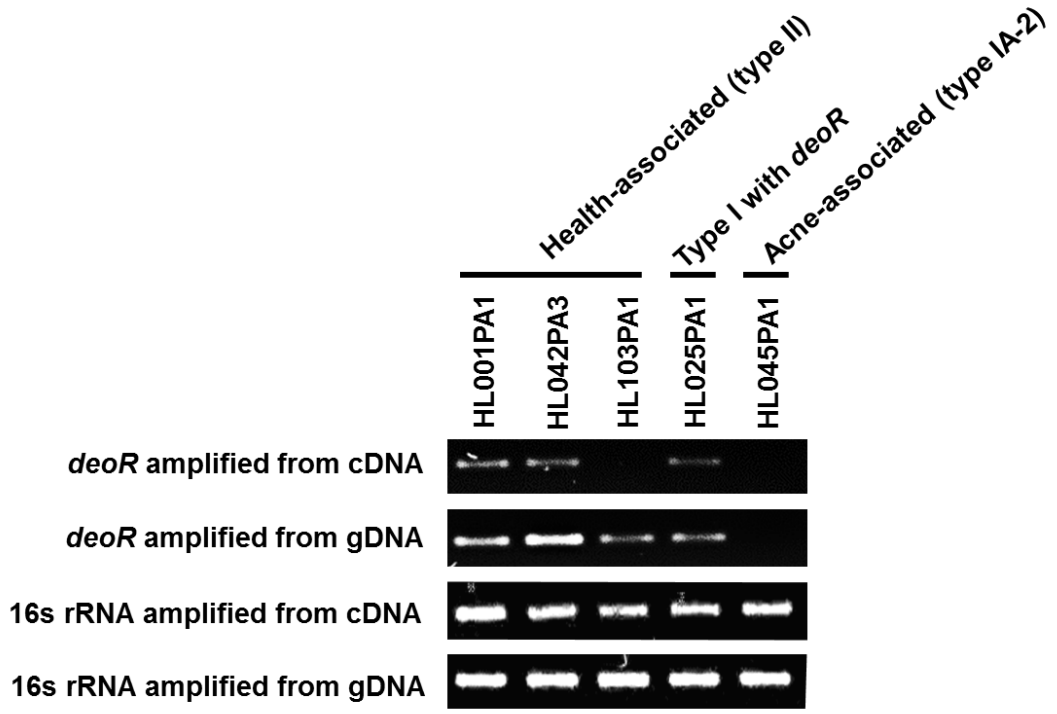

FIGURE 2-6. Health-associated strains encode and expressed *deoR*, a repressor gene in the porphyrin biosynthesis operon. *deoR* amplification from the cDNA and genomic DNA (gDNA) samples of multiple strains is shown in the gel image. 16S rRNA gene was used as a positive control. HL045PA1, which is an acne-associated type IA-2 strain and does not encode *deoR*, is shown as a negative control.

SUPPLEMENTAL FIGURES

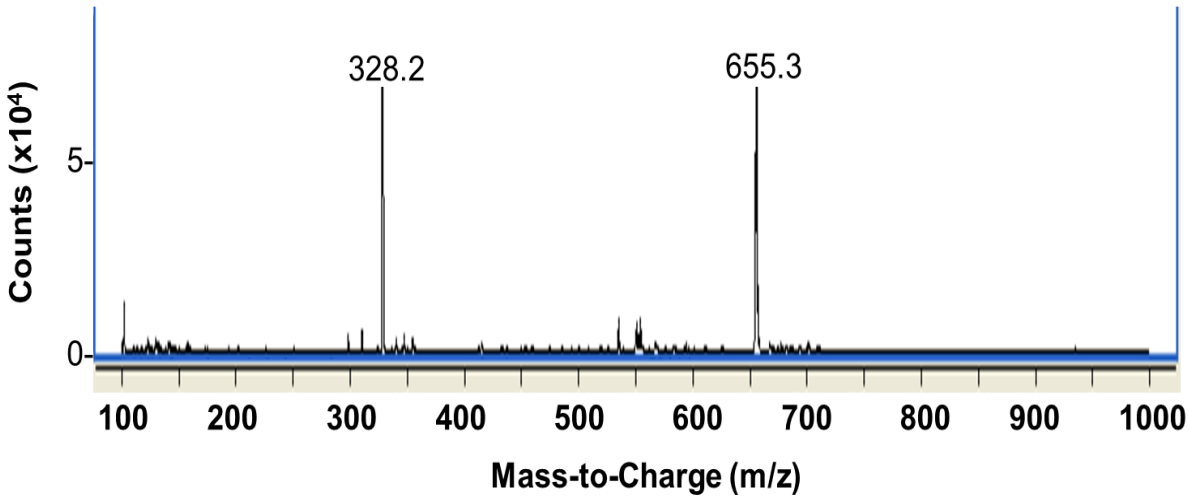

FIGURE 2-S1. Coproporphyrin was the dominant porphyrin isoform produced by *P. acnes*. Porphyrins secreted by *P. acnes* have a mass spectrum characteristic of the monoisotopic coproporphyrin isoform ($[M + H]^{+} = 655.3$). The doubly charged parent ion was also observed ($[M + 2H]^{2+} = 328.2$).

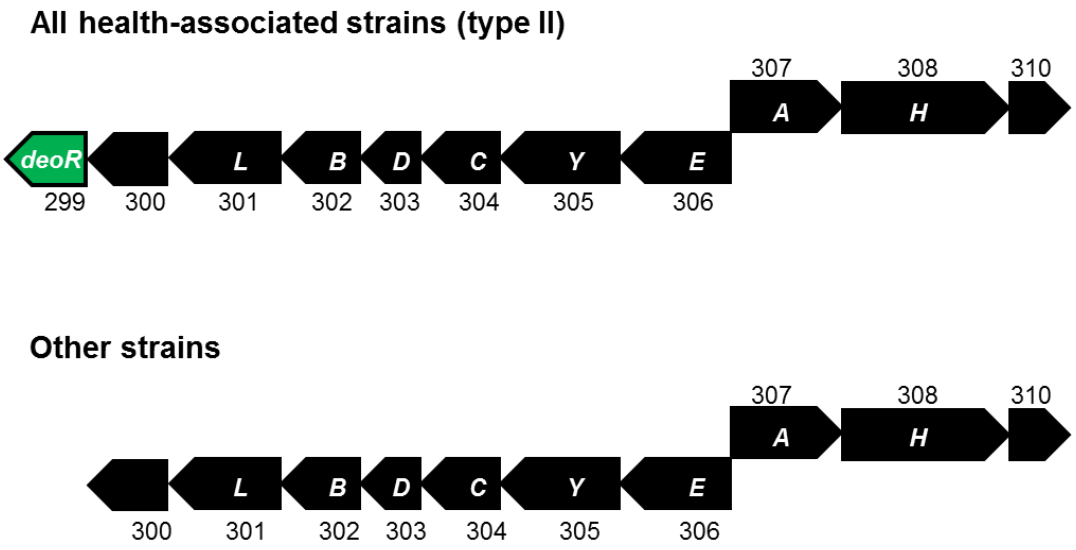

FIGURE 2-S2. Porphyrin (*Hem*) gene cluster in *P. acnes* strains. All health-associated type II strains harbor a *deoR* transcription repressor (PPA0299, in green) in the porphyrin gene cluster. The number below each box represents the gene ID based on the gene annotation in KPA171202. The letters in black boxes indicate the names of the *hem* genes, which are porphyrin biosynthesis genes. PPA0300 and PPA0310 were not assigned a *hem* gene name.

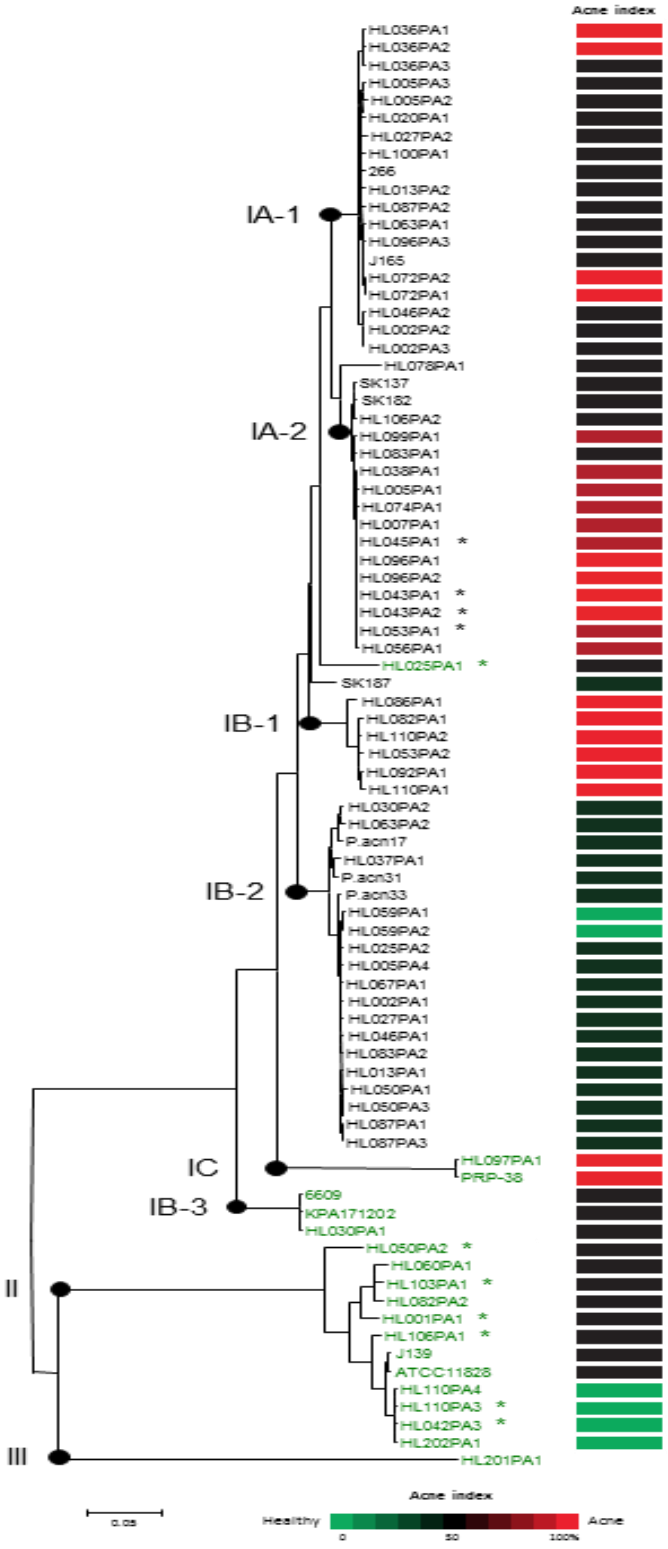
Acne index
HL036PA1
HL036PA2
HL036PA3
HL005PA3
HL005PA2
HL020PA1
HL027PA2
HL100PA1
266
HL013PA2
HL087PA2
HL063PA1
HL096PA3
J165
HL072PA2
HL072PA1
HL046PA2
HL002PA2
HL002PA3
HL078PA1
SK137
SK182
HL106PA2
HL099PA1
HL083PA1
HL038PA1
HL005PA1
HL074PA1
HL007PA1
HL045PA1 *
HL096PA1
HL096PA2
HL043PA1 *
HL043PA2 *
HL053PA1 *
HL056PA1
HL025PA1 *
SK187
HL086PA1
HL082PA1
HL110PA2
HL053PA2
HL092PA1
HL110PA1
HL030PA2
HL063PA2
P.acn17
HL037PA1
P.acn31
P.acn33
HL059PA1
HL059PA2
HL025PA2
HL005PA4
HL067PA1
HL002PA1
HL027PA1
HL046PA1
HL083PA2
HL013PA1
HL050PA1
HL050PA3
HL087PA1
HL087PA3
HL097PA1
PRP-38
6609
KPA171202
HL030PA1
HL050PA2 *
HL060PA1
HL103PA1 *
HL082PA2
HL001PA1 *
HL106PA1 *
J139
ATCC11828
HL110PA4
HL110PA3 *
HL042PA3 *
HL202PA1
HL201PA1
IA-1
IA-2
IB-1
IB-2
IC
IB-3
II
III
0.05
Acne index
Healthy
Acne
0
50
100%

FIGURE 2-S3. Presence of *deoR* in *P. acnes* lineages. *P. acnes* strains encoding *deoR* are colored in green in a phylogenetic tree constructed based on 82 *P. acnes* genomes. All type II strains harbor the *deoR* repressor. Asterisks denote the strains tested in this study. Acne index indicates the strain association with health and disease, as described previously by Fitz-Gibbon *et al*. (9).

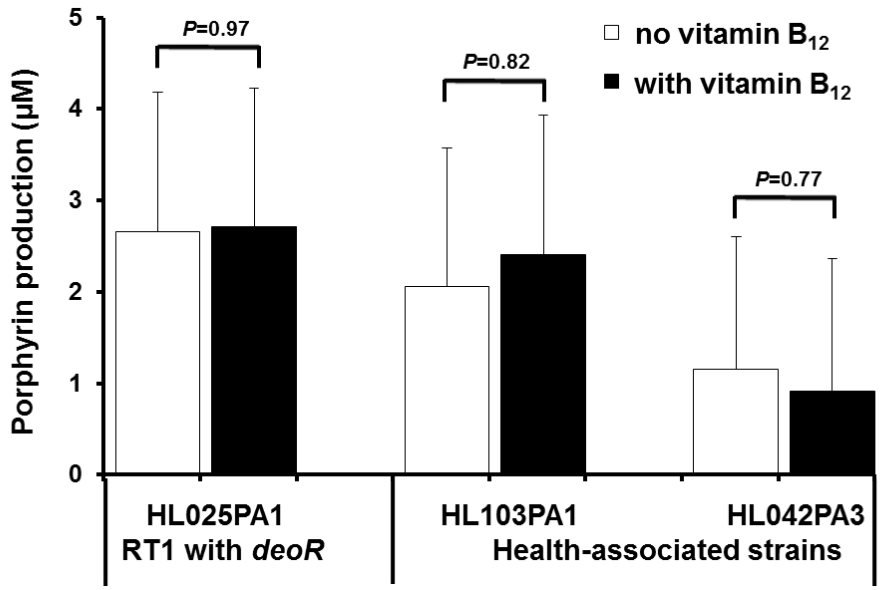

FIGURE 2-S4. Similar to health-associated type II strains, HL025PA1 produced a low level of porphyrins and did not respond to vitamin B_{12} supplementation. HL025PA1 was cultured in the media with (black bars) or without (white bars) vitamin B_{12} (10 μg/mL). Health-associated type II strains, HL103PA1 and HL042PA3, are shown for comparison. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from three independent experiments with three replicates each. Error bars represent standard error of the mean.

CHAPTER 3:

Porphyrin production and regulation in other major lineages of *P. acnes* strains and other *Propionibacterium* species

INTRODUCTION

Personalized medicine potentially would be the most effective treatment for acne. Previous studies have revealed that *P. acnes* strains from different lineages inhabit the hair follicles on the facial skin of the same individual, with an average of two to three dominating ribotypes (9). Typing of the *P. acnes* population on the skin can be useful for personalized medicine. In chapter 2, I describe data demonstrating that porphyrin production in acne-associated type IA-2 strains was enhanced by vitamin B_{12} supplementation. In contrast, porphyrin production in health-associated type II strains was not modulated by vitamin B_{12}. In this chapter, I investigate whether porphyrin production in other *P. acnes* lineages is modulated by vitamin B_{12}.

I recently suggested that a transcriptional repressor, *deoR*, present in health-associated type II strains may play a role in reduced porphyrin production (30). In addition to type II strains, this *deoR* transcriptional repressor is also found in the porphyrin operon of some other *P. acnes* strains, including strains from clades IB-3, IC, and III. These "*deoR* –positive" strains may have reduced porphyrin production compared to clades IA-1, IA-2, IB-1, and IB-2. To investigate the potential application of repressing porphyrin production in *P. acnes* as a novel treatment for acne, I compared the porphyrin levels produced by *deoR*-positive strains from clades IB-3, II and III and from clades IA-1, IB-1, and IB-2. I also determined the porphyrin levels produced by other skin *Propionibacterium* species (*P. granulosum*, *P. avidum*, and *P. humerusii*) as a comparison to *P. acnes*.

RESULTS

Types IA-1 and IA-2 strains produce significantly more porphyrins than types IB-1 and IB-2 strains.

To investigate whether *P. acnes* strains produce different amounts of porphyrins among different lineages, I first measured the porphyrin levels in two type IA-1 strains, HL005PA2 (RT1), HL027PA2 (RT1), two type IB-1 strains, HL110PA2 (RT8), HL053PA2 (RT8), and three type IB-2 strains, HL059PA1 (RT16), HL025PA2 (RT3), and HL046PA1 (RT3) (Table 3-1). The porphyrin levels produced were compared to those produced by the type IA-2 strains described in chapter 2, which produced high levels of porphyrins. The average porphyrin level produced by type IA-1 and IA-2 strains was 6.23 μM, significantly more than the porphyrins produced by type IB-1 and type IB-2 strains (2.39 μM, $P = 0.001$) (Figure 3-1). Type IB-1 and type IB-2 strains had low porphyrin levels similar to the *deoR*-positive strains, suggesting alternative regulations for porphyrin production in these strains. Type IA-1 strains produced similar amounts of porphyrins (6.87 μM) to the type IA-2 strains (6.05 μM, $P = 0.76$). These findings are consistent with the previous observations that type IA strains are associated with acne (14).

The *deoR* –positive strains (type IB-3, II, and III) produce significantly lower levels of porphyrins than the type IA strains.

I measured the porphyrin levels in the *deoR*-positive strains, including one type IB-3 strain, KPA171202 (RT1), three type II strains, HL050PA2 (RT1), HL106PA1 (RT2), and HL110PA3 (RT6), and one type III strain, HL201PA1 (Table 3-1). Porphyrin production in these

strains was compared to type IA and IB strains, as well as the type II strains described in chapter 2. The above mentioned *deoR* –positive strains produced significantly lower levels of porphyrins (average 1.75 μM) than the type IA strains (6.23 μM, $P = 0.0003$), while levels were more comparable to the IB strains ($P = 0.33$) and the type II strains described in chapter 2 ($P = 0.77$) (Figure 3-2). This corroborates our suggestion that the presence of *deoR* gene in these strains may partly explain low porphyrin production.

Vitamin B_{12} supplementation significantly increased porphyrin production in type IA strains, but not the type IB strains.

To determine whether vitamin B_{12} modulates *P. acnes* porphyrin production in the type IA-1 and type IB strains, I compared their levels of porphyrins produced in cultures with and without vitamin B_{12} supplementation. I found that vitamin B_{12} supplementation led to increased porphyrin production in all tested IA-1 strains. The average porphyrin level increased from 6.87 μM to 8.25 μM with statistical significance in one of the two tested strains ($P = 0.034$) (Figure 3-3). This observation of vitamin B_{12} modulation of porphyrin production is similar to the IA-2 strains. In contrast, vitamin B_{12} supplementation had no significant effect on porphyrin production in type IB strains (average porphyrin level 2.26 μM). One type IB-1 strain (HL053PA2) had increased porphyrin production when supplemented with vitamin B_{12}, but it was not significant (average porphyrin level increasing from 3.67 μM to 5.51 μM, $P = 0.098$). These data suggest that vitamin B_{12} modulates porphyrin production in type IA-1 strains and type IA-2 strains, indicating that vitamin B_{12} supplementation may induce acne in individuals dominated by type IA strains.

Vitamin B_{12} supplementation had no effect on porphyrin production in the *deoR* -positive strains.

To determine whether vitamin B_{12} modulates *P. acnes* porphyrin production in the *deoR* -positive **(**type IB-3, II, and III) strains, I compared the levels of porphyrins produced by these *P. acnes* strains with and without vitamin B_{12} supplementation. Vitamin B_{12} supplementation had no effect on porphyrin production in these *deoR* -positive strains (average porphyrin level 1.45μM), compared to without vitamin B_{12} (average 1.75 μM) (Figure 3-4). These results are consistent with the type II strains described in chapter 2.

Addition of 5-ALA increased porphyrin production in all strains, and vitamin B_{12} supplementation further enhanced porphyrin production in acne-associated type IA-2 strains.

To examine whether porphyrin production can be promoted by increasing the substrate (5-ALA), I compared porphyrin levels of type IA and IB *P. acnes* strains with and without the addition of the substrate. In addition, I investigated whether vitamin B_{12} can increase porphyrin production when supplemented in combination with 5-ALA. Upon substrate addition only, porphyrin production was increased by an average of 1.4- fold in type IA strains and 1.3- fold in IB strains (all $P<0.0001$, except one IB-1 strain HL053PA2) (Figure 3-5). Supplementation of 5-ALA in combination with vitamin B_{12} increased porphyrin production in type IA-2 strains as shown in Chapter 2, and in only one type IB-1 strain HL053PA2 ($P = 0.03$).

Upon substrate 5-ALA addition in the *deoR* -positive *P. acnes* strains, porphyrin production was significantly increased by an average of 4.0-fold ($P<0.0001$), expect for three strains HL050PA2 ($P =0.71$), HL106PA1 ($P =0.65$) and HL103PA1 (0.06) (Figure 3-6). Vitamin

B_{12} supplementation in combination with 5-ALA did not modulate porphyrin production in these strains.

A small molecule inhibitor reduced porphyrin production in type IA strains, and its inhibition was counteracted by vitamin B_{12} supplementation, but not in the IB strains.

To demonstrate that porphyrin production can be modulated at the metabolic level in other lineages, I investigated the effect of a small molecule inhibitor, levulinic acid (LA), on porphyrin biosynthesis in *P. acnes* strains. Additionally, I examined whether vitamin B_{12} counteracts the effect of LA on porphyrin production when supplemented in combination with LA. LA (0.1 mg/mL) significantly reduced porphyrin production in type IA strains, with an average reduction of 21% (Figure 3-7), and in *deoR* -positive strains with an average reduction of 31%, compared to untreated cultures ($P \leq 0.0001$) (Figure 3-8). However, LA didn't reduce porphyrin production in the IB strains, except for two strains (HL053PA2 and HL059PA1) when treated with LA ($P \leq 0.05$). Vitamin B_{12} supplementation counteracted the inhibition of porphyrin biosynthesis by LA specifically in type IA strains, and in HL053PA2 (IB-1), but not other strains. These data further support the conclusions that vitamin B_{12} modulates porphyrin production in type IA strains and that porphyrin production can be modulated at the metabolic level.

Other *Propionibacterium* species, *P. granulosum, P. avidum,* and *P. humerusii* produced little or no porphyrins.

Our earlier metagenomic study revealed the presence of other *Propionibacterium* species in the skin (9). I investigated porphyrin production in other skin associated propionibacteria, including two *P. granulosum* strains (HL078PG1 and HL082PG1), three *P. avidum* strains

(HL083PV1, HL307PV1, and HL063PV1) and one *P. humerusii* strain (HL044PA1). Only two of these strains produced porphyrins; *P. granulosum* strain HL078PG1 (0.68 µM) and *P. avidum* strain HL063PV1 (0.28 µM) (Figure 3-9). The porphyrin production in these strains was not modulated by vitamin B_{12}.

Addition of 5-ALA increased porphyrin production in *P. granulosum, P. avidum* and *P. humerusii,* but not vitamin B_{12} supplementation.

To determine whether porphyrin production can be enhanced by increasing the substrate 5-ALA, I compared the porphyrin production of *P. granulosum, P. avidum,* and *P. humerusii* strains with and without the addition of 5-ALA. Additionally, I examined whether vitamin B_{12} has an additive effect on porphyrin production when supplemented in combination with 5-ALA. Upon substrate addition, porphyrin production was significantly increased by an average of greater than 10-fold in all *Propionibacterium* species (all $P<0.0001$) (Figure 3-10). Supplementation of vitamin B_{12} in combination with 5-ALA did not further enhance porphyrin production. These data are consistent with the earlier finding that porphyrin production in other non-*P. acnes* propionibacteria is not modulated by vitamin B_{12}.

LA decreased porphyrin production in *P. granulosum, P. avidum,* and *P. humerusii,* which was not counteracted by vitamin B_{12} supplementation.

I further investigated the effect of inhibitor LA on porphyrin biosynthesis in *P. granulosum, P. avidum, and P. humerusii* strains. I also examined whether vitamin B_{12} counteracts the effect of LA on porphyrin production when supplemented in combination with LA. LA (0.1 mg/mL) significantly reduced porphyrin production of the two stains (*P.*

granulosum strain HL078PG1 and *P. avidum* strain HL063PV1) with an average reduction of 83%, compared to untreated cultures ($P \leq 0.0001$) (Figure 3-11). There was no significant change in porphyrin production when supplemented with LA in combination with vitamin B_{12}, which is consistent with the previous findings.

DISCUSSION

Limited data are available about bacterial metabolites and their link to acne pathogenesis. In this chapter, I investigated porphyrin production of *P. acnes* strains from multiple lineages and other cutaneous *Propionibacterium* species (*P. granulosum, P. avidum,* and *P. humerusii*). I found that *P. acnes* is the prominent porphyrin producing species on the skin. This finding is consistent with previous observations that porphyrin levels in *P. acnes* were higher compared to other propionibacteria (44). Type IA *P. acnes* strains produce significantly more porphyrins than types IB, II, and III strains. This result is consistent with the finding that IA strains were highly correlated with severe acne (14). This suggests one potential molecular mechanism for the role of type IA strains in acne pathogenesis.

Individuals who harbor IA strains, that naturally produce more porphyrins, may benefit from other therapies besides antibiotics treatment. Porphyrins are photosensitizers that react with oxygen and produce free radicals in response to blue light (405- 470nm). The free radicals in photodynamic therapy (PDT) kill the bacteria that produce elevated levels of porphyrins. The strains that produce higher levels of porphyrins may be killed by PDT. On the other hand, the health-associated type II strains, which produce low levels of porphyrins, can survive after PDT.

In Chapter 2, I described the identification of a *deoR* transcriptional repressor in type II strains that may inhibit porphyrin production. Types IB-3, IC, and III are also *deoR*-positive

strains. I confirmed that low levels of porphyrins produced by type IB-3 and type III strains. Type IA-1, IA-2, IB-1, and IB-2 do not harbor *deoR*. While types IA-1 and IA-2 strains produce high levels of porphyrins, the types IB-1 and IB-2 strains produced low levels of porphyrins. These data suggest that additional mechanisms are involved in inhibiting or reducing porphyrin production in types IB-1 and IB-2 strains.

Vitamin B_{12} supplementation can induce acne in a subgroup of people. As described in Chapter 2, acne-associated type IA-2 strains have increased porphyrin production when supplemented with vitamin B_{12}. In this chapter, I found that porphyrin production in type IA-1 strains was also increased when supplemented with vitamin B_{12}. This finding is in contrast to strains from other *P. acnes* lineages (type IB-3, II and III), which did not respond to vitamin B_{12} supplementation in porphyrin production. Individuals harboring acne-associated type IA strains are likely to have higher level of porphyrins on the skin. Vitamin B_{12} modulation of porphyrin production is lineage specific, suggesting that *P. acnes* strain composition in the skin microbiome may contribute to vitamin B_{12}-induced acne. On the other hand, individuals supplemented with vitamin B_{12}, whose skin is dominated by other lineages may have a lower risk for acne. Other propionibacteria species, *P. granulosum*, *P. avidum*, and *P. humerusii*, produced low levels of porphyrins and did not respond to vitamin B_{12} supplementation. These data suggest that increased porphyrin production is characteristic of *P. acnes* type IA strains.

For the type IA strains that produced high levels of porphyrins, I demonstrated that the small molecule inhibitor, LA, effectively reduced porphyrin biosynthesis. The inhibitor also reduced porphyrin biosynthesis in the *deoR* -positive (types IB-3, II, and III) strains that produced low porphyrins. However, LA (0.1 mg/mL) did not significantly reduce porphyrin production in types IB-1 and IB-2 strains, except for two strains (HL053PA2 and HL059PA1).

This may be due to the sub-optimal concentration of the inhibitor used in the experiments. These results indicate that proteins in the porphyrin biosynthesis pathway in the lineages that produced higher porphyrin levels are potential drug targets.

MATERIALS AND METHODS

Propionibacterium strains (*P. acnes, P. granulosum*, *P. avidum,* and *P. humerusii)* and cultures.

Propionibacterium strains used in this study (Table 3-1) were isolated previously as described by Fitz-Gibbon *et al.* (9). Briefly, each strain of *P. acnes, P. granulosum*, *P. avidum,* and *P. humerusii* was isolated from the content collected from multiple hair follicles on the nose of an acne patient or healthy individual. The sampled skin site of acne patients may or may not have visible acne lesions, therefore, the strains do not necessarily correspond to the diseased or healthy state. I tested strains from *P. acnes* of lineages IA-1, IB-1, and IB-2. I cultured two type IA-1 strains, HL005PA2 (RT1), and HL027PA2 (RT1), two type IB-1 strains, HL110PA2 (RT8), and HL053PA2 (RT8), and three type IB-2 strains HL059PA1 (RT16), HL025PA2 (RT3), and HL046PA1 (RT3). I tested *deoR* – positive strains from *P. acnes* lineages type IB-3, II, and III. I cultured one type IB-3 strains KPA171202 (RT1), three type II strains HL050PA2 (RT1), HL106PA1 (RT2), and HL110PA3 (RT6), and one type III strains HL201PA1. For each experiment, 5 mL of reinforced clostridial broth was inoculated with $5x10^5$ *P. acnes* cells per milliliter of culture. Cultures were grown to stationary phase anaerobically at 37^oC in a light-protected box. Cultures were supplemented on day 0 with vitamin B_{12} (10 µg/mL), and/or 5-ALA (0.1 mg/mL), and/or LA (0.1 mg/mL). As controls, *P. acnes* strains were also cultured without supplementation. Two to four independent experiments with at least three replicates per experiment were performed for each strain.

Extraction, identification, and quantification of extracellular porphyrins.

For each strain, porphyrins were extracted using the method described by Kang *et al.* (28). Briefly, 500 µL of bacterial culture was extracted in ethyl acetate and acetic acid (4:1, v/v), and solubilized in 1.5M HCl. The absorbance at 405 nm was measured from 200 µL of the soluble phase using a Tecan Genios Spectrophotometer M1000 (Tecan US Inc, Morrisville, NC). The standard curve to convert absorbance to concentration was generated using coproporphyrin III standards of known concentration (C654-3, Frontier Sci). Porphyrins were extracted from cultures at stationary phase. Bacterial culture density was measured at OD_{595} for normalization of porphyrin levels.

Statistical analysis for porphyrin production comparisons.

The average amount of porphyrins produced by each strain under each culture condition was calculated based on the data from 2-4 independent experiments, and with at least three replicates for each. The porphyrin levels between strains, groups (acne-associated vs. health-associated), and culture conditions (with treatment vs. without treatment) were estimated in a linear mixed effect model, with random intercepts by trial to account for trial effects. *P*-values for specific comparisons among groups were corrected using Tukey's method. All statistical analysis was performed using R software (version 3.1.3).

TABLES AND FIGURES

Table 3-1: *P. acnes* strains and other propionibacteria strains used in this study

Species	Lineage[a]	Strain	Ribotype[b]
P. acnes	IA-1	HL005PA2	RT1
		HL027PA2	RT1
	IA-2	HL083PA1	RT1
	IB-1	HL110PA2	RT8
		HL053PA2	RT8
	IB-2	HL059PA1	RT16
		HL025PA2	RT3
		HL046PA1	RT3
	IB-3	KPA171202	RT1
	II	HL050PA2	RT1
		HL106PA1	RT2
		HL110PA3	RT6
	III	HL201PA1	ND
P. granulosum		HL078PG1	
		HL082PG1	
P. avidum		HL083PV1	
		HL307PV1	
		HL063PV1	
P. humerusii		HL044PA1	

[a,b] as described by Fitz-Gibbon *et al.* 2013

ND: Not Determined

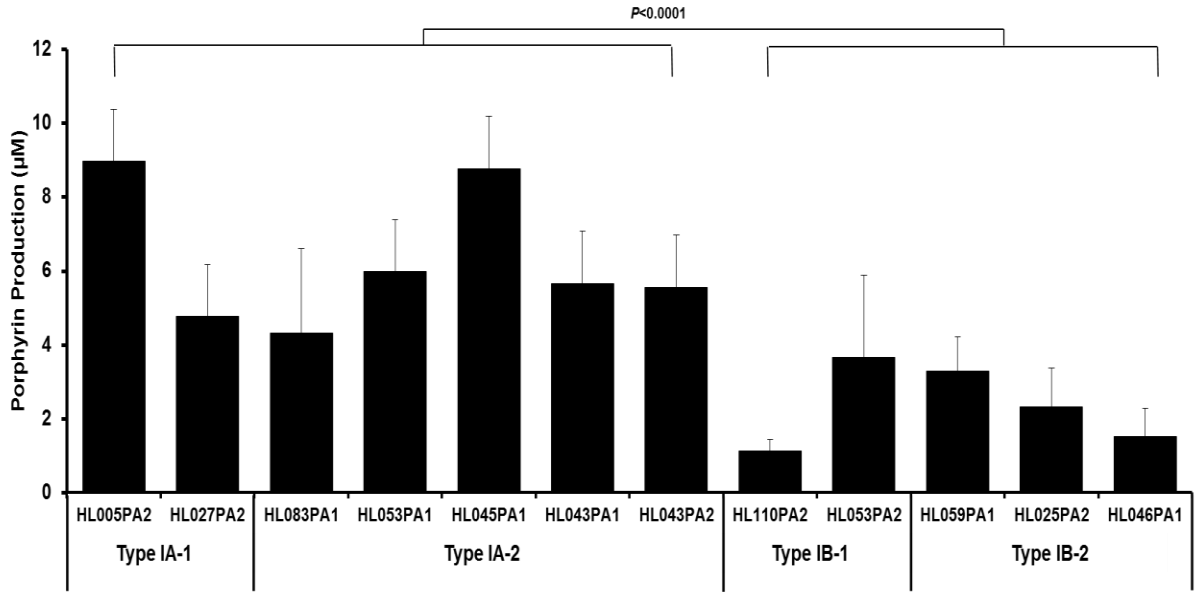

FIGURE 3-1. Type IA (IA-1 and IA-2) strains produce significantly more porphyrins than type IB (IB-1 and IB-2) strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean.

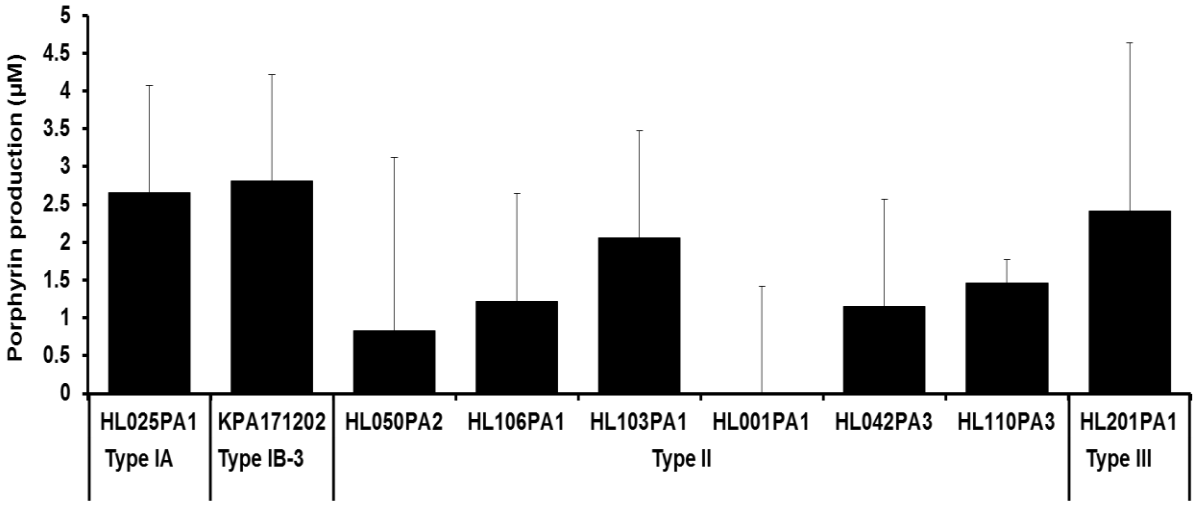

FIGURE 3-2. *deoR* –positive strains (types IB-3, II, III) produce significantly lower levels of porphyrins than type IA strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean.

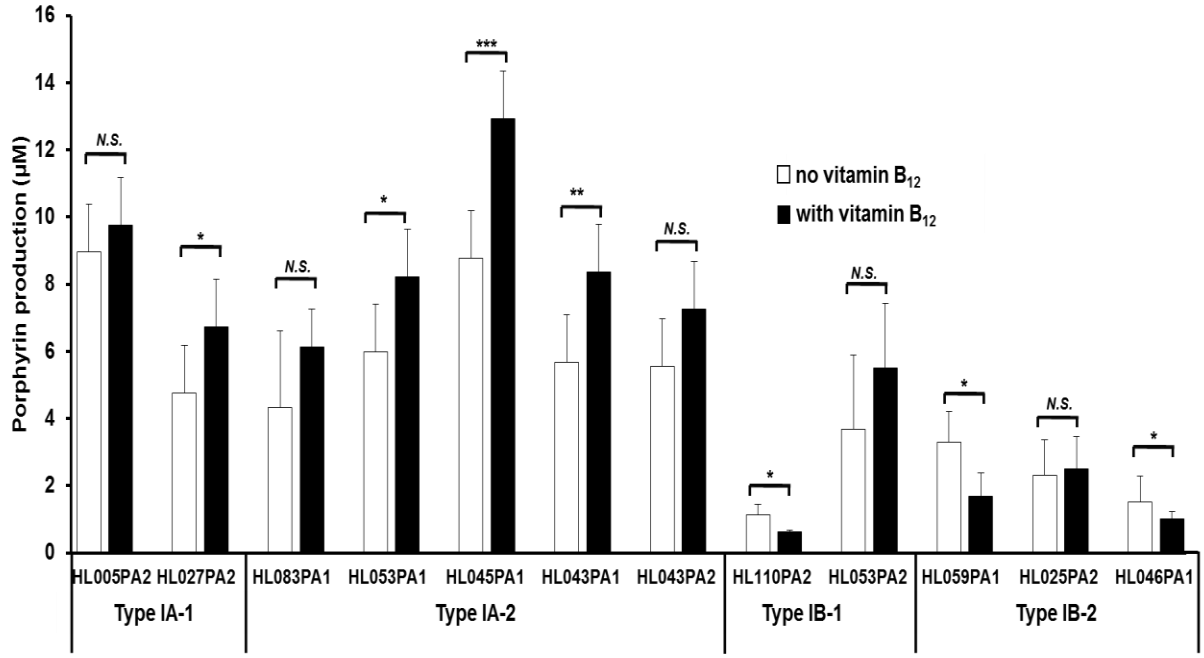

FIGURE 3-3. Vitamin B_{12} supplementation significantly increased porphyrin production in type IA strains, but not type IB strains. *P. acnes* strains were cultured in media with (black bars) or without (white bars) the addition of 10 μg/mL vitamin B_{12}. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ indicate significant differences compared with the corresponding controls.

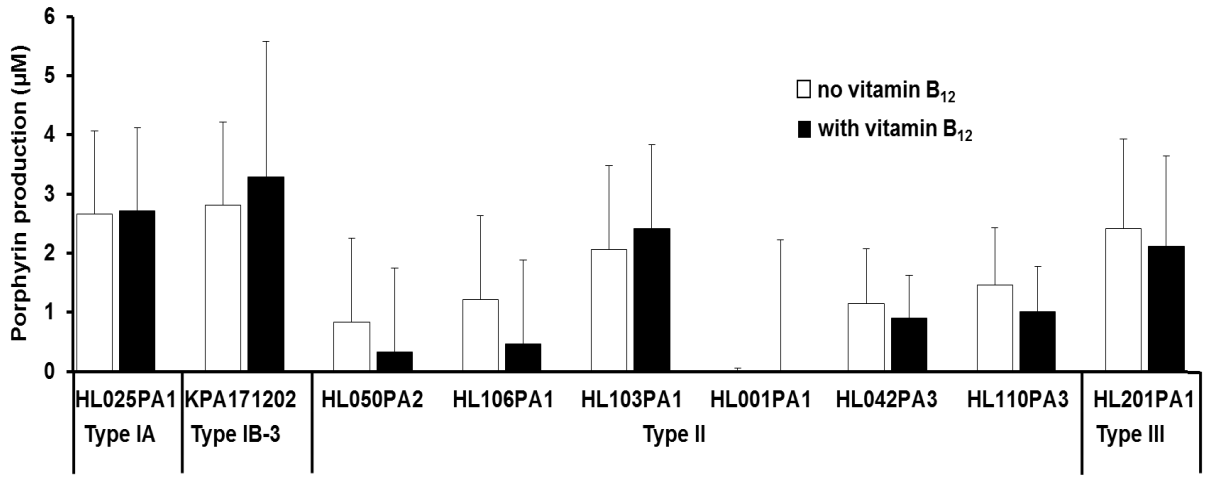

FIGURE 3-4. Vitamin B_{12} supplementation had little effect on porphyrin production in *deoR* -positive strains. *P. acnes* strains were cultured in media with (black bars) or without (white bars) the addition of 10 μg/mL vitamin B_{12}. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. There is no statistically significant difference between the cultures with and without vitamin B_{12} supplementation for all the strains tested here.

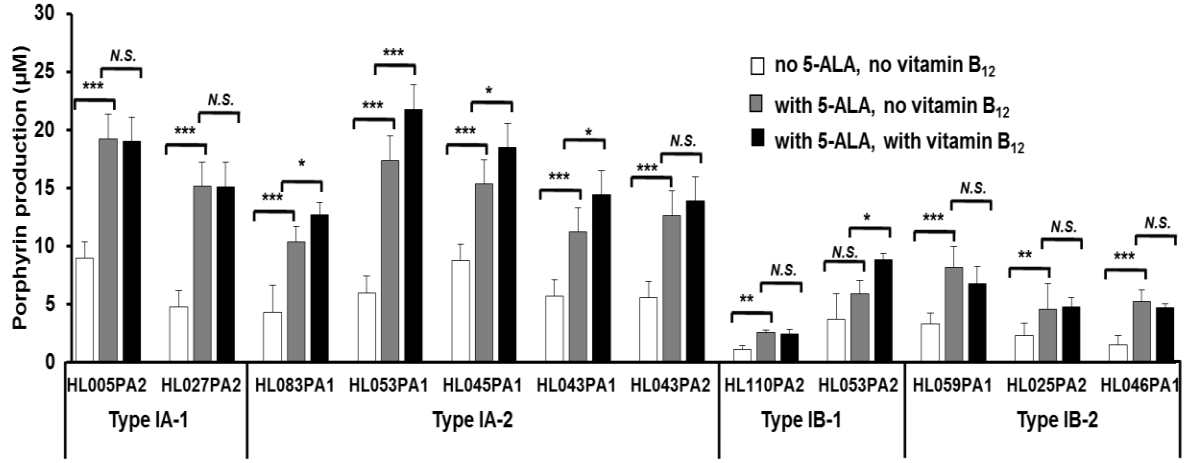

FIGURE 3-5. 5-ALA increased porphyrin production in types IA-1, IA-2, IB-1, and IB-2, which was further enhanced by vitamin B_{12} supplementation in acne-associated type IA-2 strains. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) substrate 5-ALA (0.1 mg/mL), or with both 5-ALA and vitamin B_{12} (10 μg/mL) added (black bars). 5-ALA significantly increased porphyrin production in types IA-1, IA-2, IB-1, and IB-2 strains ($P<0.0001$, except for strain HL053PA2). Vitamin B_{12} supplementation further increased porphyrin production in the presence of 5-ALA in acne-associated type IA strains, but not in type IB-1 or type IB-2 strains (except for type IB-1 strain HL053PA2). Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ indicate significant differences compared with the corresponding controls.

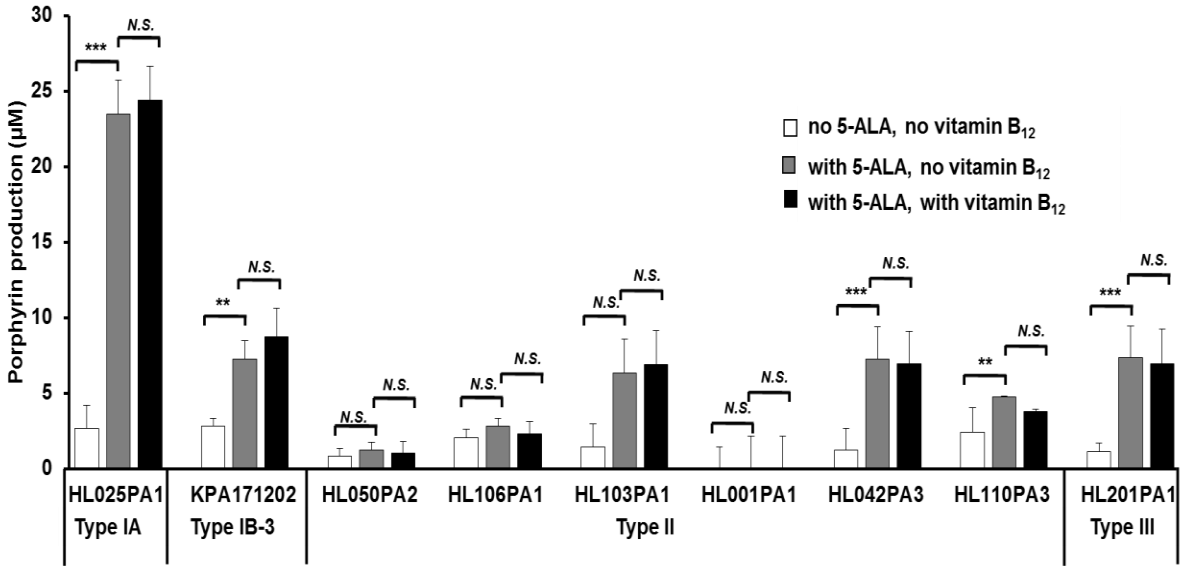

FIGURE 3-6. 5-ALA increased porphyrin production in *deoR* -positive strains (types 1B-3, II, and III), which was not influenced by vitamin B_{12} supplementation. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) substrate 5-ALA (0.1 mg/mL), or with both 5-ALA and vitamin B_{12} (10 μg/mL) added (black bars). 5-ALA significantly increased porphyrin production in *deoR* -positive strains ($P<0.0001$, except for type II strains HL050PA2, HL106PA1 and HL103PA1). Vitamin B_{12} supplementation did not influence porphyrin production in the presence of 5-ALA in *deoR*-positive strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ indicate significant differences compared with the corresponding controls.

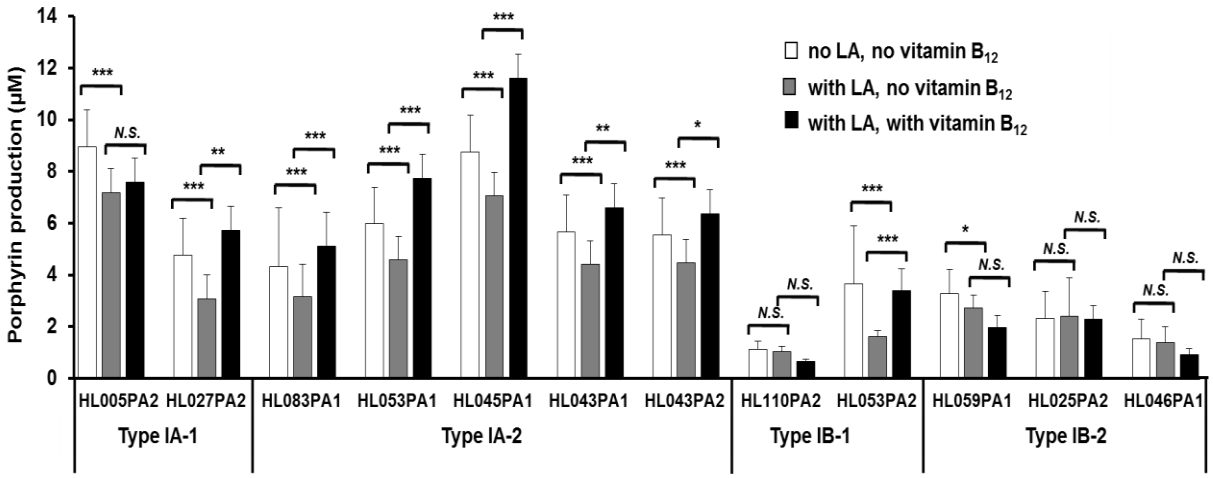

FIGURE 3-7. LA decreased porphyrin production in types IA strains, which was counteracted by vitamin B_{12} supplementation in acne-associated type IA strains. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) inhibitor LA (0.1 mg/mL), or with both LA and vitamin B_{12} (10 μg/mL) added (black bars). LA significantly reduced porphyrin biosynthesis in all type IA strains, and in two of the type IB strains ($P \leq 0.05$). Vitamin B_{12} supplementation counteracted the inhibition of porphyrin biosynthesis by LA in acne-associated type IA strains, but not in other *P. acnes* lineages, (except for type IB-1 strain HL053PA2). Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ indicate significant differences compared with the corresponding controls.

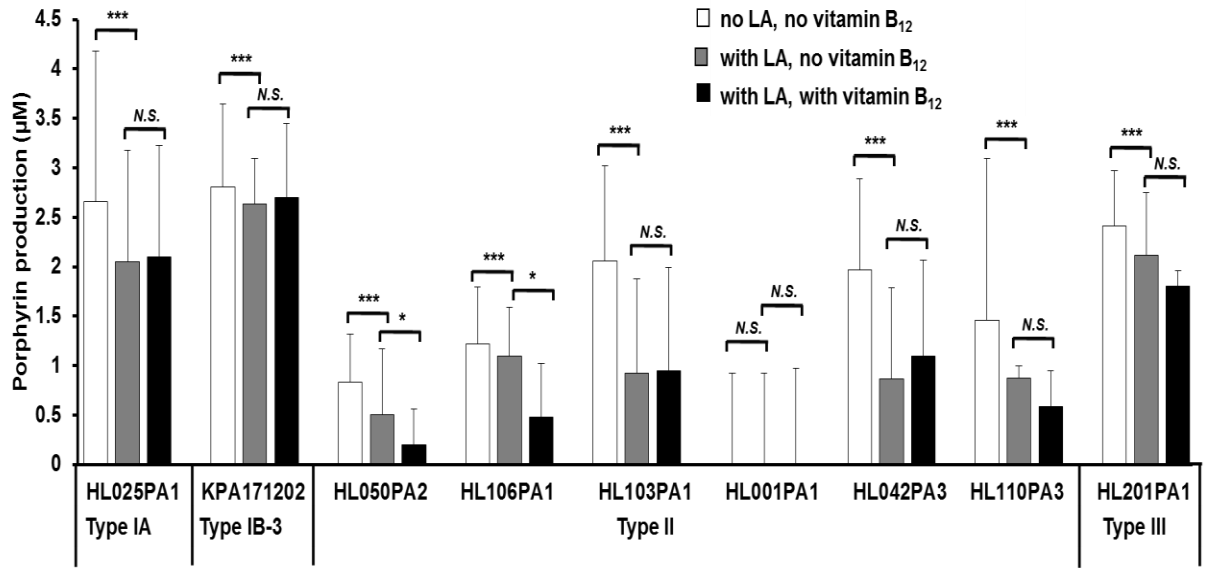

FIGURE 3-8. LA decreased porphyrin production in *deoR* -positive strains (types IB-3, II, and III), which was not counteracted by vitamin B_{12} supplementation. *P. acnes* strains were cultured in media with (grey bars) or without (white bars) inhibitor LA (0.1 mg/mL), or with both LA and vitamin B_{12} (10 μg/mL) added (black bars). LA significantly reduced porphyrin biosynthesis in all strains ($P \leq 0.0001$). Vitamin B_{12} supplementation did not counteract the inhibition of porphyrin biosynthesis by LA in *deoR* –positive strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ indicate significant differences compared with the corresponding controls.

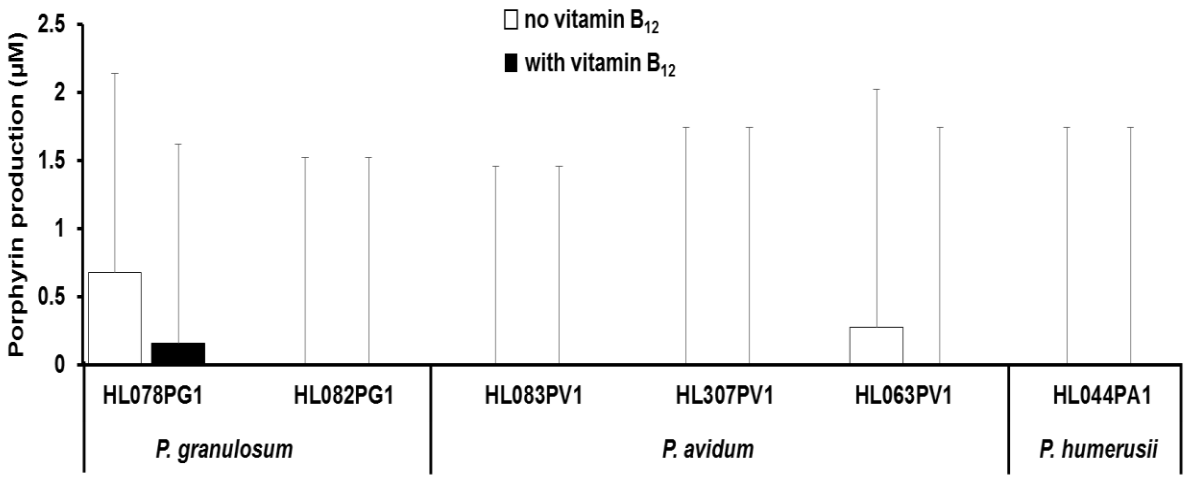

FIGURE 3-9. Other skin *Propionibacterium* species, *P. granulosum, P. avidum,* and *P. humerusii,* produced little or no porphyrins, and their porphyrin production is not modulated by vitamin B_{12}. *P. granulosum, P. avidum, and P. humerusii* strains were cultured in media with (black bars) or without (white bars) the addition of 10 μg/mL vitamin B_{12}. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean.

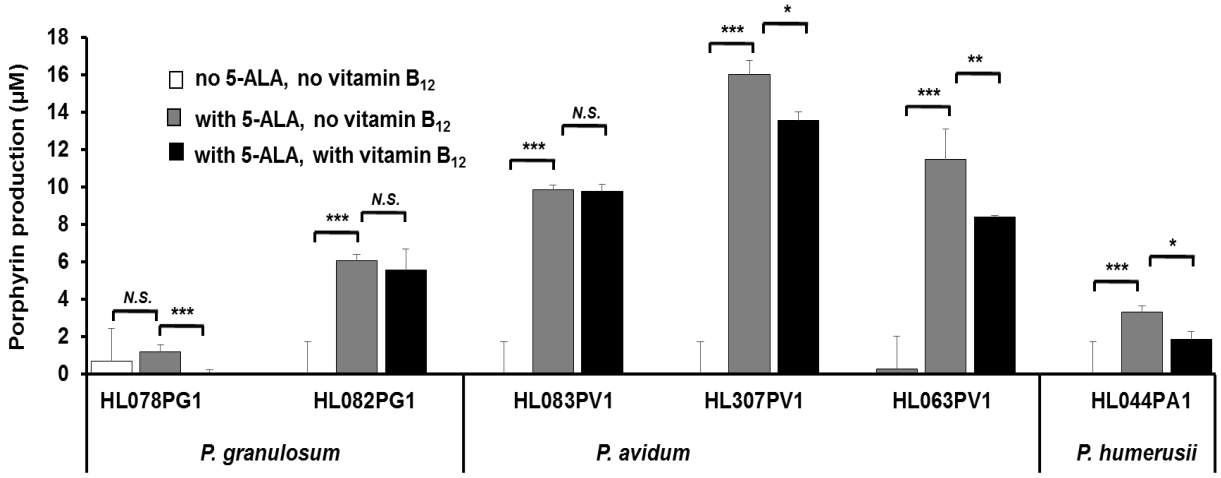

FIGURE 3-10. 5-ALA increased porphyrin production in *P. granulosum, P. avidum,* and *P. humerusii* which was not further enhanced by vitamin B_{12} supplementation.

P. granulosum, P. avidum, and P. humerusii strains were cultured in media with (grey bars) or without (white bars) substrate 5-ALA (0.1 mg/mL), or with both 5-ALA and vitamin B_{12} (10 μg/mL) added (black bars). 5-ALA significantly increased porphyrin production in these *Propionibacterium* strains (all $P<0.0001$). Vitamin B_{12} supplementation did not influence porphyrin production in the presence of 5-ALA. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ indicate significant differences compared with the corresponding controls.

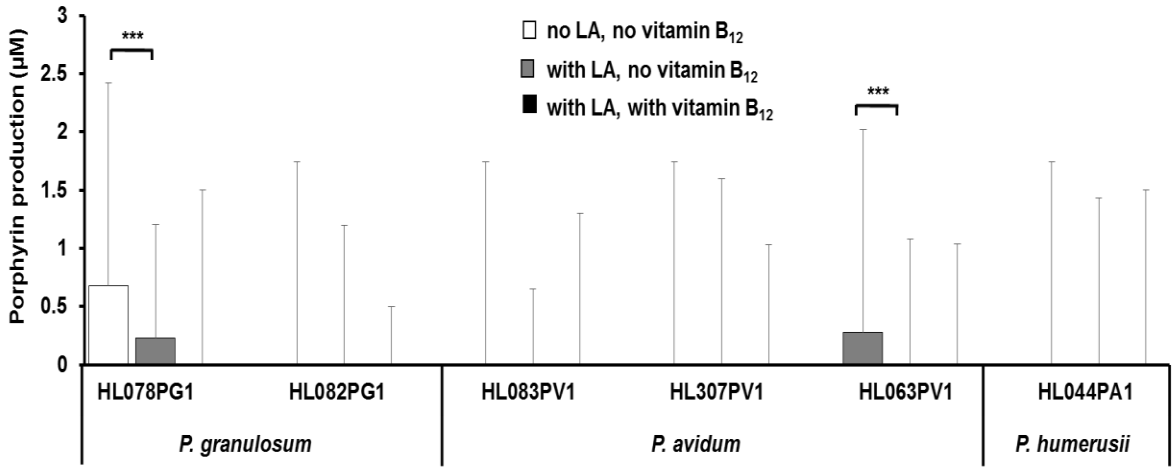

FIGURE 3-11. LA decreased porphyrin production in *P. granulosum, P. avidum* and *P. humerusii*, which was not counteracted by vitamin B_{12} supplementation.

P. granulosum, P. avidum, and P. humerusii strains were cultured in media with (grey bars) or without (white bars) inhibitor LA (0.1 mg/mL), or with both LA and vitamin B_{12} (10 μg/mL) added (black bars). LA significantly reduced porphyrin biosynthesis in two strains (*P. granulosum* strain HL078PG1 and *P. avidum* strain HL063PV1) ($P \leq 0.0001$). Vitamin B_{12} supplementation did not counteract the inhibition of porphyrin biosynthesis by LA in these strains. Each bar represents the porphyrins produced by each strain normalized by the bacterial culture density. Shown is the mean of the data obtained from at least two independent experiments with at least three replicates each. Error bars represent standard error of the mean. ***$P \leq 0.001$ indicate significant differences compared with the corresponding controls.

CHAPTER 4:

Mass spectrometry protocol for identifying and quantifying porphyrins

INTRODUCTION

An appropriate *in vivo* acne-disease model has been hampered by the lack of mouse strains that colonize bacteria on the skin. Mice and other animal models used in many other disease models fail to recapitulate acne vulgaris phenotypes. The most attractive animal model for acne is the hairless mouse strain (rhino-mouse RHJ/LeJ), an experimental model for non-inflammatory acne (64). However, these mice lack the commonly associated hyper-keratinization and increased sebum production seen in human acne skin. Most of the current acne research is based on keratinocytes and sebocytes in cell culture, and the most commonly used *in vivo* system is derived from human skin surface swabs (65-67).

A useful method for characterizing human skin bacteria associated with acne is through the extraction of the contents inside the pilosebaceous unit. This largely represents the *in vivo* condition of the skin. This extraction method can be used to identify and quantify the porphyrin species in the hair follicles. Porphyrins are aromatic molecules that are fluorescent and absorb light of approximately 400nm (Figure 4-1). These porphyrin extraction and quantification methods are not sensitive for clinical samples with low quantities. High performance liquid chromatography (HPLC) has been shown as a sensitive method to separate porphyrin isoforms in a complex sample (68). Quantifying porphyrins by mass spectrometry using selected-reaction monitoring (SRM) fragments the parent ions and can limit the sensitivity of this method (69). In this chapter, I describe the development of a protocol using high performance liquid chromatography/mass spectrometry (HPLC/MS) using single ion monitoring (SIM) to identify and quantify porphyrins from small clinical samples.

RESULTS

HPLC/MS of the porphyrin mixture.

The components of a mixture of three porphyrin species (coproporphyrin III, mesoporphyrin IX and protoporphyrin IX) were separated by HPLC (Figure 4-2). Mesoporphyrin was used as an internal standard to measure variance of the HPLC runs. The elution order was coproporphyrin III (21 minutes), mesoporphyrin IX (36 minutes) and protoporphyrin IX (39 minutes) corresponding to the increasing hydrophobicity of the molecules. Using the Selected-Ion Monitoring (SIM) mode of mass spectrometry, the limit of detection for coproporphyrin III was 125 femtomoles (Table 4-1). The detection limit for both mesoporphyrin IX and protoporphyrin IX was 1.25 picomoles.

The standard curve for coproporphyrin III was linear. The coefficient of determination (R^2) was greater than 0.99. The linear graphs can be used to quantify porphyrin level inside the pilosebaceous unit from clinical samples, although calibration curves should be repeated in other experiments (Figure 4-3).

DISCUSSION

A sensitive method for identifying and quantifying porphyrin production by HPLC/MS has been developed. The HPLC separated coproporphyrin III, mesoporphyrin IX and protoporphyrin IX from their mixture (Figure 4-2). Porphyrin species cannot be identified when measuring the absorbance or fluorescence from a mixture of multiple species; these methods are non-selective. Identification of porphyrin species can be accomplished readily by mass

spectrometry (69, 70). This HPLC/MS protocol can identify and quantify porphyrin species for future study of *P. acnes* cultures and clinical samples.

Porphyrins can be extracted from clinical skin samples and quantified by HPLC/MS. Coproporphyrin III was the dominant porphyrin species produced by *P. acnes.* This HPLC/MS method can potentially quantify coproporphyrin III secreted by *P. acnes* in pilosebaceous units (Figure 4-3). This method requires the optimal separation efficiency of the C18 HPLC column and the porphyrin standard curves should be reconstructed in future clinical experiments. Furthermore, the utility and efficiency of this protocol for the analysis of clinical samples, which are much more complex than porphyrin standards, still need to be tested.

MATERIALS AND METHODS

Porphyrin Standards

Protoporphyrin IX and Mesoporphyrin IX were purchased from Sigma-Aldrich (St. Louis, MO). Coproporphyrin III was purchased from Frontier Scientific (Logan, Utah). Stock solutions were prepared in H_2O/acetonitrile/formic acid (50/50/0.1 v/v/v) at 2.5 pmol/µL concentration.

High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS)

Direct injection of each porphyrin species onto the HPLC/MS system identified the expected molecular ions (monoisotopic isoform of coproporphyrin, $[M + H]^+ = 655.3$; mesoporphyrin, $[M + H]^+ = 567.3$; protoporphyrin, $[M + H]^+ = 563.3$). For enhanced limit of detection, Selected-Ion Monitoring (SIM) mass spectrometry was carried out. In the negative ion mode, the optimal instrument conditions for maximal signal intensity for the precursor ion were as follows: Frequency 300, and 0 collision energy. The porphyrin standards were mixed and 5µL was injected (in triplicate) onto a reversed phase HPLC column (Phenomenex Kinetex C18, 100 x 2.1

mm, 1.7 μm particle size, 100 Å pore diameter). The column was equilibrated in solvent A (H_2O/formic acid (100/0.1 v/v)) and eluted with a linear gradient of solvent B (acetonitrile/formic acid (100/0.1 v/v)) for 60 minutes. The HPLC eluents were detected by a triple quadrupole mass spectrometer (Agilent 6460) via an electrospray ionization source. The mass spectrometry parameters are as follows: Gas temperature 300^{o}C, Gas flow 6 L/min, nebulizer gas 45psi, capillary voltage 4.5kV, Sheath temperature 375, Sheath flow 10L/min. Porphyrin quantification data was collected (in triplicate) and analyzed by the software Mass Hunter - (Agilent).

Porphyrin standard curves

A mixture of porphyrin species (coproporphyrin III, mesoporphyrin IX and protoporphyrin IX) was injected onto the HPLC/MS system at the following concentrations: 12.5 picomoles, 1.25 picomoles, 125 femtomoles, and 12.5 femtomoles). Internal standard peak areas were used to construct the calibration curve.

TABLES AND FIGURES

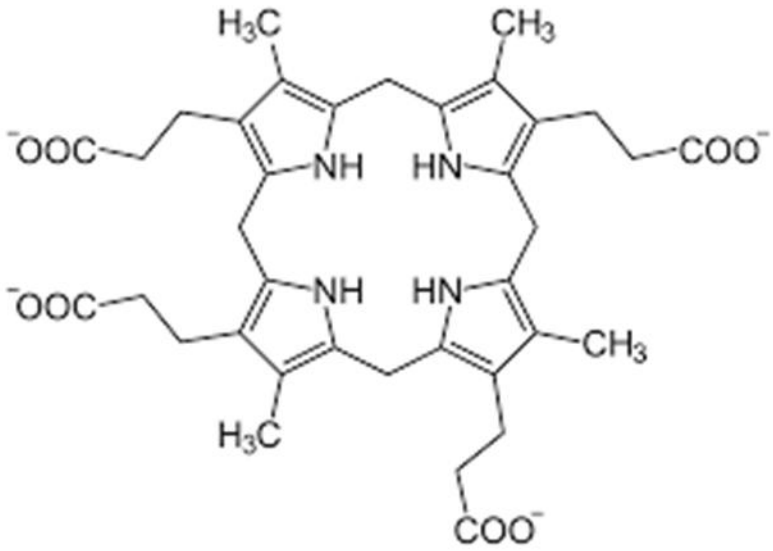

FIGURE 4-1: Coproporphyrin III. Coproporphyrin III is the major porphyrin species produced by *P. acnes*.

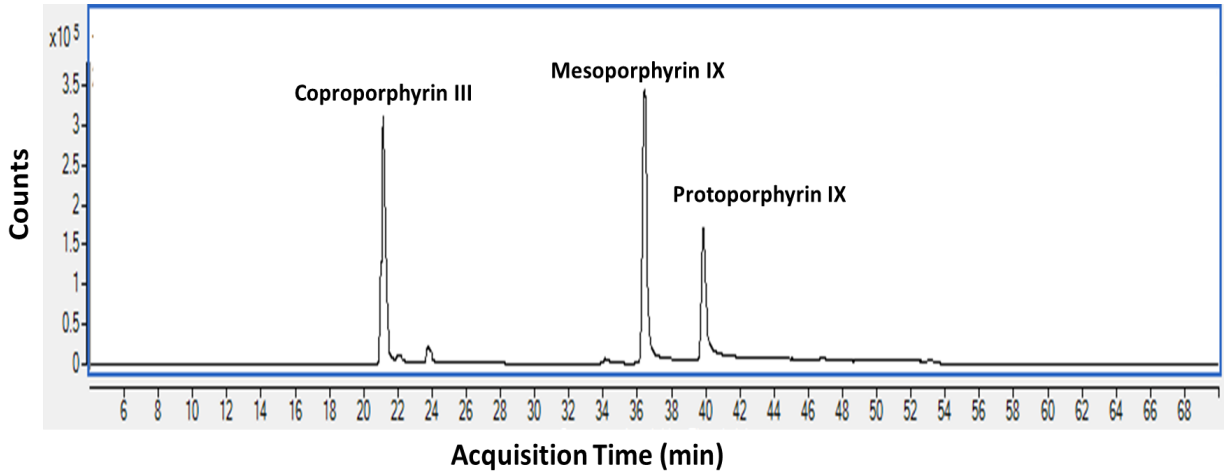

FIGURE 4-2: HPLC/MS chromatogram for the porphyrin standard mixture. The elution order was as follows: Coproporphyrin III (21 minutes), mesoporphyrin IX (36 minutes), and protoporphyrin IX (39 minutes).

PORPHYRIN	Limit Of Detection
COPROPORPHYRIN	125 fmol
MESOPORPHYRIN	1.25 pmol
PROTOPORPHYRIN	1.25 pmol

TABLE 4-1: Limit of detection of porphryins. Coproporphyrin limit of detection is 125 femtomoles. The limit of detection for both mesoporphyrin and protoporphyrin is 1.25 picomoles.

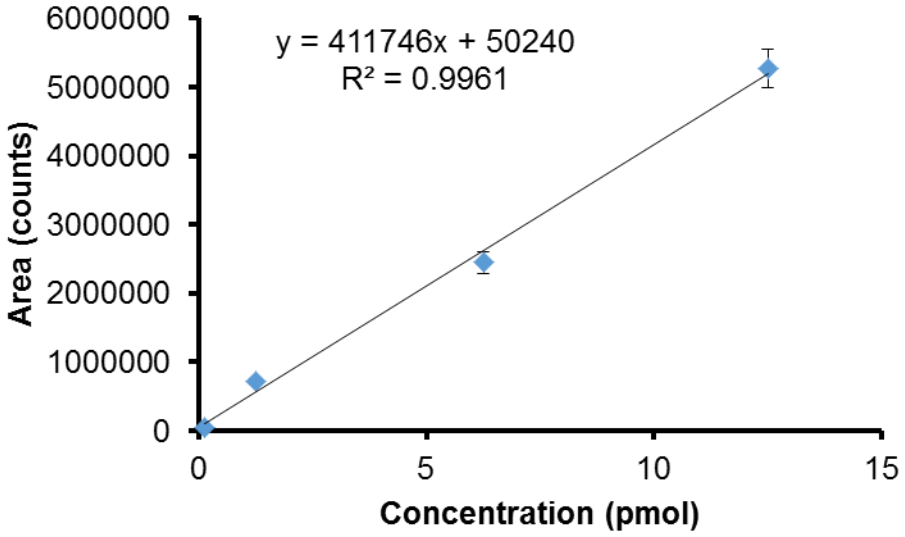

FIGURE 4-3: Standard curve of coproporphyrin III.

CHAPTER 5:

SUMMARY

In this dissertation, I demonstrated that high levels of porphyrins (pro-inflammatory factors in the skin) are produced by acne-associated type IA *P. acnes* strains. This provides one potential molecular mechanism for the role of acne-associated type IA strains in the pathogenesis of acne. Vitamin B_{12} modulates the porphyrin biosynthesis in acne-associated type IA strains. This suggests a molecular link between porphyrin production by *P. acnes* strains of the skin and the observation that vitamin B_{12} induces acne in a subset of individuals.

I demonstrated that porphyrin production was regulated at the metabolic level in type IA strains by supplementing the cultures with substrates and inhibitors. All other *P. acnes* strains produced low levels of porphyrins and did not respond to vitamin B_{12}. A *deoR* transcriptional repressor of porphyrin biosynthesis was identified in *P. acnes* type II, IB-3, IC, and III strains. The presence of this transcriptional repressor could partly explain low levels of porphyrin production in these strains. *P. granulosum, P. avidum,* and *P. humerusii* produced little or no porphyrins. This study highlights the importance of understanding strain-level differences of the human microbiota in disease pathogenesis.

For future directions, being able to quantify the levels of porphyrins and identify *P. acnes* strain composition on the skin could advance personalized treatment for acne. I developed a sensitive HPLC/MS-based method for identifying and quantifying porphyrin levels in clinical skin samples. Type IA strains produce more porphyrins when supplemented with vitamin B_{12}. Individuals taking vitamin B_{12} supplements, who carry type IA strains, are at risk for developing acne and could benefit from potential new drugs targeting the porphyrin biosynthesis pathway.

This study suggests that the porphyrin biosynthesis pathway could be a viable candidate drug target for novel acne therapeutics.

REFERENCES

1. Shi B, Chang M, Martin J, Mitreva M, Lux R, Klokkevold P, Sodergren E, Weinstock GM, Haake SK, Li H. 2015. Dynamic changes in the subgingival microbiome and their potential for diagnosis and prognosis of periodontitis. MBio 6:e01926-14.

2. Tap J, Derrien M, Törnblom H, Brazeilles R, Cools-Portier S, Doré J, Störsrud S, Le Nevé B, Öhman L, Simrén M. 2016. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. Gastroenterology.

3. Angelakis E. 2016. Weight gain by gut microbiota manipulation in productive animals. Microb Pathog.

4. Stevens D, Cornmell R, Taylor D, Grimshaw SG, Riazanskaia S, Arnold DS, Fernstad SJ, Smith AM, Heaney LM, Reynolds JC, Thomas CL, Harker M. 2015. Spatial variations in the microbial community structure and diversity of the human foot is associated with the production of odorous volatiles. FEMS Microbiol Ecol 91:1-11.

5. Webster GF. 1998. Inflammatory acne represents hypersensitivity to Propionibacterium acnes. Dermatology 196:80-1.

6. Purvis D, Robinson E, Merry S, Watson P. 2006. Acne, anxiety, depression and suicide in teenagers: a cross-sectional survey of New Zealand secondary school students. J Paediatr Child Health 42:793-6.

7. Bhate K, Williams HC. 2013. Epidemiology of acne vulgaris. Br J Dermatol 168:474-85.

8. James WD. 2005. Clinical practice. Acne. N Engl J Med 352:1463-72.

9. Fitz-Gibbon S, Tomida S, Chiu BH, Nguyen L, Du C, Liu M, Elashoff D, Erfe MC, Loncaric A, Kim J, Modlin RL, Miller JF, Sodergren E, Craft N, Weinstock GM, Li H. 2013.

Propionibacterium acnes strain populations in the human skin microbiome associated with acne. J Invest Dermatol 133:2152-60.

10. Johnson JL, Cummins CS. 1972. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of Arachnia propionica. J Bacteriol 109:1047-66.

11. McDowell A, Valanne S, Ramage G, Tunney MM, Glenn JV, McLorinan GC, Bhatia A, Maisonneuve JF, Lodes M, Persing DH, Patrick S. 2005. Propionibacterium acnes types I and II represent phylogenetically distinct groups. J Clin Microbiol 43:326-34.

12. McDowell A, Perry AL, Lambert PA, Patrick S. 2008. A new phylogenetic group of Propionibacterium acnes. J Med Microbiol 57:218-24.

13. McDowell A, Gao A, Barnard E, Fink C, Murray PI, Dowson CG, Nagy I, Lambert PA, Patrick S. 2011. A novel multilocus sequence typing scheme for the opportunistic pathogen Propionibacterium acnes and characterization of type I cell surface-associated antigens. Microbiology 157:1990-2003.

14. Lomholt HB, Kilian M. 2010. Population genetic analysis of Propionibacterium acnes identifies a subpopulation and epidemic clones associated with acne. PLoS One 5:e12277.

15. McDowell A, Barnard E, Nagy I, Gao A, Tomida S, Li H, Eady A, Cove J, Nord CE, Patrick S. 2012. An expanded multilocus sequence typing scheme for propionibacterium acnes: investigation of 'pathogenic', 'commensal' and antibiotic resistant strains. PLoS One 7:e41480.

16. Tomida S, Nguyen L, Chiu BH, Liu J, Sodergren E, Weinstock GM, Li H. 2013. Pan-genome and comparative genome analyses of propionibacterium acnes reveal its genomic diversity in the healthy and diseased human skin microbiome. MBio 4:e00003-13.

17. Kasimatis G, Fitz-Gibbon S, Tomida S, Wong M, Li H. 2013. Analysis of complete genomes of Propionibacterium acnes reveals a novel plasmid and increased pseudogenes in an acne associated strain. Biomed Res Int 2013:918320.

18. Leeming JP, Holland KT, Cuncliffe WJ. 1988. The microbial colonization of inflamed acne vulgaris lesions. Br J Dermatol 118:203-8.

19. Perry A, Lambert P. 2011. Propionibacterium acnes: infection beyond the skin. Expert Rev Anti Infect Ther 9:1149-56.

20. Cunliffe WJ, Gould DJ. 1979. Prevalence of facial acne vulgaris in late adolescence and in adults. Br Med J 1:1109-10.

21. Martin R, Kahn G, Gooding J, Brown G. 1973. Cutaneous porphyrin fluorescence as an indicator of antibiotic absorption and effectiveness. Cutis 12:758-764.

22. Mills OH, Kligman AM, Pochi P, Comite H. 1986. Comparing 2.5%, 5%, and 10% benzoyl peroxide on inflammatory acne vulgaris. Int J Dermatol 25:664-7.

23. Borelli C, Merk K, Schaller M, Jacob K, Vogeser M, Weindl G, Berger U, Plewig G. 2006. In vivo porphyrin production by P. acnes in untreated acne patients and its modulation by acne treatment. Acta Derm Venereol 86:316-9.

24. Jadassohn W, Paillard R, Hofer R, Golaz M. 1958. Vitamine B12 et poussée aenéïforme. Dermatologica 116:349.

25. Puissant A, Vanbremeersch F, Monfort J, Lamberton JN. 1967. [A new iatrogenic dermatosis: acne caused by vitamin B 12]. Bull Soc Fr Dermatol Syphiligr 74:813-5.

26. Dugois P, Amblard P, Imbert R, de Bignicourt B. 1969. [Acne due to vitamin B 12]. Bull Soc Fr Dermatol Syphiligr 76:382-3.

27. Balta I, Ozuguz P. 2014. Vitamin B12-induced acneiform eruption. Cutan Ocul Toxicol 33:94-5.

28. Kang D, Shi B, Erfe MC, Craft N, Li H. 2015. Vitamin B12 modulates the transcriptome of the skin microbiota in acne pathogenesis. Sci Transl Med 7:293ra103.

29. Bykhovskii VY, Zaitseva N, Bukin V. 1967. Possibility of competition for utilization of 5-aminiolevulinic acid for the biosynthesis of VB12 and porphyrins in resting suspensions of Propionibacterium shermanii. Dokl Akad Nauk SSSR 180:232-234.

30. Johnson T, Kang D, Barnard E, Li H. 2016. Strain-Level Differences in Porphyrin Production and Regulation in Propionibacterium acnes Elucidate Disease Associations. mSphere 1.

31. Nagy I, Pivarcsi A, Kis K, Koreck A, Bodai L, McDowell A, Seltmann H, Patrick S, Zouboulis CC, Kemény L. 2006. Propionibacterium acnes and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. Microbes Infect 8:2195-205.

32. Bojar RA, Holland KT. 2004. Acne and Propionibacterium acnes. Clin Dermatol 22:375-9.

33. White GM. 1998. Recent findings in the epidemiologic evidence, classification, and subtypes of acne vulgaris. J Am Acad Dermatol 39:S34-7.

34. Gupta MA, Gupta AK. 1998. Depression and suicidal ideation in dermatology patients with acne, alopecia areata, atopic dermatitis and psoriasis. Br J Dermatol 139:846-50.

35. Mallon E, Newton JN, Klassen A, Stewart-Brown SL, Ryan TJ, Finlay AY. 1999. The quality of life in acne: a comparison with general medical conditions using generic questionnaires. Br J Dermatol 140:672-6.

36. Brüggemann H, Henne A, Hoster F, Liesegang H, Wiezer A, Strittmatter A, Hujer S, Dürre P, Gottschalk G. 2004. The complete genome sequence of Propionibacterium acnes, a commensal of human skin. Science 305:671-3.

37. Holland C, Mak TN, Zimny-Arndt U, Schmid M, Meyer TF, Jungblut PR, Brüggemann H. 2010. Proteomic identification of secreted proteins of Propionibacterium acnes. BMC Microbiol 10:230.

38. Valanne S, McDowell A, Ramage G, Tunney MM, Einarsson GG, O'Hagan S, Wisdom GB, Fairley D, Bhatia A, Maisonneuve JF, Lodes M, Persing DH, Patrick S. 2005. CAMP factor homologues in Propionibacterium acnes: a new protein family differentially expressed by types I and II. Microbiology 151:1369-79.

39. Lodes MJ, Secrist H, Benson DR, Jen S, Shanebeck KD, Guderian J, Maisonneuve JF, Bhatia A, Persing D, Patrick S, Skeiky YA. 2006. Variable expression of immunoreactive surface proteins of Propionibacterium acnes. Microbiology 152:3667-81.

40. Fanta D, Formanek I, Poitschek C, Thurner J. 1978. [Porphyrinsynthesis of Propionibacterium acnes in acne and seborrhea (author's transl)]. Arch Dermatol Res 261:175-9.

41. Schaller M, Loewenstein M, Borelli C, Jacob K, Vogeser M, Burgdorf WH, Plewig G. 2005. Induction of a chemoattractive proinflammatory cytokine response after stimulation of keratinocytes with Propionibacterium acnes and coproporphyrin III. Br J Dermatol 153:66-71.

42. Saint-Leger D, Bague A, Cohen E, Chivot M. 1986. A possible role for squalene in the pathogenesis of acne. I. In vitro study of squalene oxidation. Br J Dermatol 114:535-42.

43. Lee WL, Shalita AR, Poh-Fitzpatrick MB. 1978. Comparative studies of porphyrin production in Propionibacterium acnes and Propionibacterium granulosum. J Bacteriol 133:811-5.

44. Wollenberg MS, Claesen J, Escapa IF, Aldridge KL, Fischbach MA, Lemon KP. 2014. Propionibacterium-produced coproporphyrin III induces Staphylococcus aureus aggregation and biofilm formation. MBio 5:e01286-14.

45. Cornelius CE, Ludwig GD. 1967. Red fluorescence of comedones: production of porphyrins by Corynebacterium acnes. J Invest Dermatol 49:368-70.

46. Frère F, Schubert WD, Stauffer F, Frankenberg N, Neier R, Jahn D, Heinz DW. 2002. Structure of porphobilinogen synthase from Pseudomonas aeruginosa in complex with 5-fluorolevulinic acid suggests a double Schiff base mechanism. J Mol Biol 320:237-47.

47. Kim JK, Cho JH, Lee JS, Hahm KS, Park DH, Kim SW. 2002. Mass production of methane from food wastes with concomitant wastewater treatment. Appl Biochem Biotechnol 98-100:753-64.

48. Gaigalat L, Schlüter JP, Hartmann M, Mormann S, Tauch A, Pühler A, Kalinowski J. 2007. The DeoR-type transcriptional regulator SugR acts as a repressor for genes encoding the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in Corynebacterium glutamicum. BMC Mol Biol 8:104.

49. Wang F, Ren NN, Luo S, Chen XX, Mao XM, Li YQ. 2014. DptR2, a DeoR-type auto-regulator, is required for daptomycin production in Streptomyces roseosporus. Gene 544:208-15.

50. Elgrably-Weiss M, Schlosser-Silverman E, Rosenshine I, Altuvia S. 2006. DeoT, a DeoR-type transcriptional regulator of multiple target genes. FEMS Microbiol Lett 254:141-8.

51. Shu M, Wang Y, Yu J, Kuo S, Coda A, Jiang Y, Gallo RL, Huang CM. 2013. Fermentation of Propionibacterium acnes, a commensal bacterium in the human skin microbiome, as skin probiotics against methicillin-resistant Staphylococcus aureus. PLoS One 8:e55380.

52. Sikorska H, Smoragiewicz W. 2013. Role of probiotics in the prevention and treatment of meticillin-resistant Staphylococcus aureus infections. Int J Antimicrob Agents 42:475-81.

53. Jahns AC, Lundskog B, Ganceviciene R, Palmer RH, Golovleva I, Zouboulis CC, McDowell A, Patrick S, Alexeyev OA. 2012. An increased incidence of Propionibacterium acnes biofilms in acne vulgaris: a case-control study. Br J Dermatol 167:50-8.

54. Dekio I, Culak R, Fang M, Ball G, Gharbia S, Shah HN. 2013. Correlation between phylogroups and intracellular proteomes of Propionibacterium acnes and differences in the protein expression profiles between anaerobically and aerobically grown cells. Biomed Res Int 2013:151797.

55. Nagy E, Urbán E, Becker S, Kostrzewa M, Vörös A, Hunyadkürti J, Nagy I. 2013. MALDI-TOF MS fingerprinting facilitates rapid discrimination of phylotypes I, II and III of Propionibacterium acnes. Anaerobe 20:20-6.

56. Nacht S, Gans EH, McGinley KJ, Kligman AM. 1983. Comparative activity of benzoyl peroxide and hexachlorophene. In vivo studies against propionibacterium acnes in humans. Arch Dermatol 119:577-9.

57. Meffert H, Gaunitz K, Gutewort T, Amlong UJ. 1990. [Therapy of acne with visible light. Decreased irradiation time by using a blue-light high-energy lamp]. Dermatol Monatsschr 176:597-603.

58. Meyer K, Pappas A, Dunn K, Cula GO, Seo I, Ruvolo E, Batchvarova N. 2015. Evaluation of Seasonal Changes in Facial Skin With and Without Acne. J Drugs Dermatol 14:593-601.

59. Leyden JJ, McGinley KJ, Cavalieri S, Webster GF, Mills OH, Kligman AM. 1983. Propionibacterium acnes resistance to antibiotics in acne patients. J Am Acad Dermatol 8:41-5.

60. Eady EA, Cove JH, Holland KT, Cunliffe WJ. 1989. Erythromycin resistant propionibacteria in antibiotic treated acne patients: association with therapeutic failure. Br J Dermatol 121:51-7.

61. Schuster A, Harel E. 1985. A Low Molecular Weight Polypeptide Which Accumulates upon Inhibition of Porphyrin Biosynthesis in Maize. Plant Physiol 77:648-52.

62. Kotal P, Klepácek I, Jirsa M, Kordac V. 1989. [Inhibition of porphyrin synthesis by isonicotinic acid hydrazide in models simulating porphyria]. Sb Lek 91:161-8.

63. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-80.

64. Mirshahpanah P, Maibach HI. 2007. Models in acnegenesis. Cutan Ocul Toxicol 26:195-202.

65. Kurokawa I, Danby FW, Ju Q, Wang X, Xiang LF, Xia L, Chen W, Nagy I, Picardo M, Suh DH, Ganceviciene R, Schagen S, Tsatsou F, Zouboulis CC. 2009. New developments in our understanding of acne pathogenesis and treatment. Exp Dermatol 18:821-32.

66. Dick GF, Ashe BM, Rodgers EG, Diercks RC, Goltz RW. 1976. Study of elastolytic activity Propionibacterium acnes and Staphylococcus epidermis in acne vulgaris and in normal skin. Acta Derm Venereol 56:279-82.

67. Schafer F, Fich F, Lam M, Gárate C, Wozniak A, Garcia P. 2013. Antimicrobial susceptibility and genetic characteristics of Propionibacterium acnes isolated from patients with acne. Int J Dermatol 52:418-25.

68. Macours P, Cotton F. 2006. Improvement in HPLC separation of porphyrin isomers and application to biochemical diagnosis of porphyrias. Clin Chem Lab Med 44:1433-40.

69. Fyrestam J, Bjurshammar N, Paulsson E, Johannsen A, Östman C. 2015. Determination of porphyrins in oral bacteria by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Bioanal Chem 407:7013-23.

70. Mancini S, Imlay JA. 2015. Bacterial Porphyrin Extraction and Quantification by LC/MS/MS Analysis. Bio Protoc 5.