The phylogenetic characterization of the *Propionibacterium acnes* by developing new molecular typing methods and determination of the lipase activity of selected strains

Ph.D thesis

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INTRODUCTION AND OBJECTIVES

Human skin is our organism's first line of defense, which protects the organism from harmful environmental effects, for example mechanical injuries and pathogens. There are many microorganisms on the skin, some of these are commensals, others are pathogens, and together, forming a dynamic community, they create the characteristic individual normal flora. A number of hypotheses suggest that a shift in the ratio of this community's populations might increase the chance for acute and chronic illnesses, including acne vulgaris, one of the most frequent skin diseases.

The species that most often colonize healthy human skin are *Staphylococcus epidermidis*, *Malassezia furfur* and *Propionibacterium acnes.* The latter is a Gram-positive, non–spore-forming, anaerobic, opportunistic bacterium. It is most often associated with acne vulgaris, but it also plays a role in a number of other serious diseases, such as corneal ulcer, endocarditis, sarcoidosis, synovitis, hyperostosis and osteitis. However, the pathomechanisms of the listed diseases and the exact role of *P. acnes* is still unknown. Acne vulgaris associated with chronic inflammation occurs most frequently in areas rich in sebaceous glands, e.g. the face, chest and back. There are three clinical forms of this disease: acne comedonica, acne papulopustulosa and acne conglobata. The different forms of acne vulgaris can usually be treated well with antibiotics, but - similarly to many other microorganisms - *P. acnes* has evolved mechanisms to resist antibiotics.

Our largest organ, the skin, can be viewed as an ecosystem with the microorganisms forming a dynamic community. In this ecosystem, niches of different physiology and topography can be identified. In order to distinguish between and learn more about commensal and potentially pathogen strains and strains resistant to antibiotics, it is essential to characterize the skin microbiome.

Based on these facts, my aims were the following:

1. Develop methods for determination and classification that facilitate and promote clinical identification of *Propionibacterium acnes*.

2. Carry out phylogenetic analyses using data gained from the identification and classification methods.

3. Determination of whole genome sequences in different *P. acnes* isolates, which may reveal specific regions and factors responsible for resistance to antibiotics, virulence and morphological differences characteristic to a specific strain.

4. Morphological evaluation and flow cytometry analysis of a recently described *P. acnes* strain belonging to type III phylogenetic group, in order to determine whether cells with atypical phenotype represents group of cells that were unable to split after division, or a single elongated cell.

5. To haracterize *P. acnes* strains isolated from the skin of healthy individuals and the skin of acne patients using the identification methods developed above, and to investigate the colonization dynamics of *P. acnes* on intra- and interpersonal levels.

6. To valuate triacylglycerol lipase activity of all known phylogenetic groups of *P. acnes* by thin-layer chromatography in order to identify exact role of the secreted hydrolytic enzyme in the pathogenity of the bacterium.

7. To develop an effective and reproducible genetic manipulation system and use this system to create lipase mutants from pathogenic strain isolated from acne vulgaris, subsequently characterize the hydrolytic activity of this newly generated strain.

METHODS

Development of *P. acnes* identification and classification methods

Multiplex PCR

As a platform for primer design, we examined a range of housekeeping loci for phylogroup-specific polymorphisms, and also interrogated available whole genome sequences for the presence of unique phylogroup-specific genetic regions. For the development of the multiplex approach, primer sets with identical annealing temperatures were designed. Specific attention was given to the amplicon length: amplicons with characteristic size differences were selected in order to facilitate in-gel visual identification. Polymorphisms in multiple aligned sequences of the *sodA* gene specific for types IA_2 and IB, and *atpD* and *recA* genes specific for types II and III, respectively, were identified as candidate regions for primer development. Primer pairs incorporating phylogroup-specific mismatches at the 3' end and elsewhere in the sequence (where available) were designed. Primers targeting the ATP-binding component (ATPase; GenBank accession no. DQ20821.1) of a previously described ABC-type peptide uptake operon that is present in types IA_1/IA_2/IC, but absent in types IB, II and III strains, were also developed. Type IC strains were identified using a primer pair that targeted the toxin gene (Fic family) located on an approximately 7.3 kb genomic fragment present in the draft genome sequences of the type IC but not in members of other phylogroups. Finally, 16S rDNA primers specific for *P. acnes* were applied in order to facilitate species discrimination.

eMLST (expanded Multilocus Sequence Typing)

The data from the full genome sequences of numerous strains provides a possibility to select genes that could be used to develop the typing scheme. The partial sequences of six housekeeping genes (*aroE, atpD, gmk, guaA, lepA* and *sodA*) and the full sequences of two virulence genes (*tly* and *camp2)* were applied in the final version of the eMLST. After the PCR reaction, the sequences of the amplicons were determined by capillary electroforesis (3500 Series Genetic Analyzer, Life Technologies). The sequences were aligned using Genomic Workbench (CLC Bio) software and compared to the reference database. The sequences of the isolates were uploaded into the MLST database (http://pubmlst.org/pacnes/) which facilitated the e-sequence type (e-ST) determination.

MALDI-TOF MS

To investigate the mass spectrometry of the isolates we used tMicroflex LT MALDI-TOF devise (Bruker Daltonik). The spectrums were taken in linear positive ion mode at 60 Hz frequency. The weight range was set between 2000-20.000 Da and 20< spectrums were recorded per isolate. Data analysis was performed by the MALDI Biotyper 3.2.1.1. software and database (Bruker Daltonik).

Follicle-specific sampling

The Dermatology Department of University ofNantes sampled the surface of acne patients focusing on papules, pustules, comedones and proximal healthy skin areas. This sampling strategy facilitated the examination of the dynamics of the *P. acnes* colonization at intra- and interpersonal levels. Each sample from different area was grown in rich liquid media under anaerobic conditions for 24 hours. Next, the cultures were diluted from 10^{-1} to $10^{-4}$and cultivated on semi-selective plates. MPCR and eMLST analysis were subsequently performed in order to determine the exact genotype of the isolates.

Phylogenetic analysis

eBURST (eBased Upon Related Sequence Types)

eMLST allele profiles of the selected strains were further analysed by eBURST v3 algorhythm. To define the diversity of the population, the founder and sub-founder genotypes were identified. For this, the eSTs were classified into clonal complexes (CCs) so that not more than one distinct allele is present; hence, strains belonging to the same CC possess the most common alleles. In contrast to the phylogenetic tree, where the output information is the similarity of the genotypes, by applying eBURST the lineages of the closely related isolates can be determined.

De novo full genome sequencing

Genome sequencings of distinct *P. acnes* strains were performed by combining the cycled ligation sequencing on SOLiD System (Life Technologies) with 454 FLX pyrosequencing (Roche). Assemblies were performed using GS De Novo Assembler and/or Genomics

Workbench. Super-scaffoldings were performed with CodonCode Aligner 4.0.3 (Codon Code Corp.) and gap closings were accomplished using polymerase chain reaction (PCR) followed by Sanger sequencing. Automatic annotations of the genomes were performed by the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

Investigation of the atypical cell morphology

Scanning electron microscopy

The cell culture of the selected strains was dried onto a cover slip surface. Fixation of the cells was carried out with 2.5% glutaraldehyde in 0.05 M Cacodilate buffer (pH 7.5) for 24 hours at 4 ºC. Next, we dehydrated the samples - while keeping them on ice - with an ethanol range of 50-100%. Subsequently, the absolute alcohol was replaced by tert-Butyl alcohol. After lyophilisation and sputtering we examined the cells by HITACHI S-4700 scanning electron microscopy device.

Cell-sorting

The cell fixation was performed in 70% ethanol by overnight incubation. After washing the cells with PBS buffer, we used ultrasonication to avoid cell aggregation (1.5 min, in 0.5 s cycle on 75% amplitude). Next, the cells were labelled with propidium iodide ([10 μg/ml], (λ_{ex} :540 nm; λ_{em}: 608 nm, Sigma). The size of the cells and their DNA content was analysed using FACS Jazz flow cytometer (BD Biosciences).

Determination of the hydrolytic activity of different *P. acnes* isolates

Determination of the lipase activity by thin-layer chromatography (TLC)

The cell cultures were supplemented with 2% of olive oil and grown until exponential phase. Cell cultures were then centrifuged and the supernatants filtered. In order to coagulate the proteins, we treated the supernatants with ammonium sulfate until 85% enrichment. The excess ammonium sulfate was removed by washing the samples with acetone:water (4:1) solution. The precipitated crude extract was dissolved in 20 mM Tris-HCl (pH 8.0) buffer. The samples were then washed three times with n-Hexane to remove the free fatty acid residues. The protein content of the samples was measured using Qubit (Life Technologies).

100µg protein extract of each sample was incubated for 3 or 24 hours with the TLC reaction mix (20 mM Tris-HCl (pH 6.0), 5% triton-X100 and 0.2% triolein) at 37 °C. HCl was used to stop the reaction; the samples were dried in SpeedVac and dissolved in toluol. 5µl of the upper phase of the supernatant was then sprayed onto silica gel (Linomat 5, Camag) in 3 mm wide bands. A solvent of petroleum-ether:diethyl-ether:formate (80:30:1) was used to separate the decomposition products into discrete components. After the separation of the products, the silica gel was air dried and sprayed with the developer solvent (10% copper-sulfate, 8% phosphoric-acid in aqueous solution) incubated for 10 min at 100ºC. All experiments were carried out in triplicates; positive controls consisted of serial dilutions (10^{-1} - 10^{-7}) of the 76g/l (w/v) oleic acid analytical standard and negative controls were prepared by boiling an aliquot of each sample for 30 min.

The discrete bands were analysed with the CP-Atlas gel analyser software (lazarsoftware.com). The free fatty acid content of the samples was calculated based on the ratio of the area and density of the bands. Linear curves were generated using the data of the standards and these curves were used for the gradation of the equation for defining the unit of the hydrolysis of the oleic acid. OneUnit was defined as the amount lipase which releases 350µg oleic acid per hour.

Determination of the lipase activity by fluorimetry

The bacterial growth conditions and sample preparation were the same as for the TLC experiments. After the cells were harvested, the supernatants were concentrated using Amicon 10K NMWL tubes. In order to investigate the exact substrate specificity of the isolates 4-methyl-umbelliferil-oleate fluorogenic substrate was used (λ_{ex}327nm; λ_{em}449nm, 0.1 M phosphate buffer, pH 7.0). For the calibration we utilized 4-methylumbelliferil (4-MU) in serial dilutions ([15 µm/ml] 10^{-1} to 10^{-6} dilution). Lipase activity was determined by end point measurements (FLUOStar Optima).

Mutagenesis

Since the only available mutagenesis strategy for *P. acnes* is not reliable, therefore we developed a novel strategy and applied it on a pathogenic isolate originating from a patient with acne vulgaris. We carried out the manipulation based on the double homologous recombination.

By utilizing the 2 step joining PCR we created the 2 members of the deletion cassette. In the first PCR we designed the 500 bp long A and C elements of the cassette which are located up- and downstream from the targeted gene. In the next PCR these amplicons were used as a template. In order to avoid transcription errors, a polymerase with proofreading activity was applied. The purified cassette was then ligated into a pK19 vector. The insert-containing kanamycin (50 μg/ml) resistant clones were selected and a colony PCR was carried out using the universal primers (univ and opseq). The direction of the insert was determined with Sanger sequencing. Plasmid was purified from the suitable insert containing colonies and was transformed into wild type *P. acnes* by protoplast formation (10 mg/ml lysozyme at 37ºC, for 30 min). After one week of anaerobic incubation, the developing kanamycin resistant clones were again investigated by colony PCR with the plasmid specific primers. One positive colony was selected and enriched in BHI supplemented with kanamycin and replicates were prepared from the culture in a 96-well plate. After they were grown, we again replicated them at least 10 times onto anaerobic agar plates with or without kanamycin. The colony which grew on the plate that did not contain the antibiotic and did not grow on the plate containing kanamycin was selected. We subsequently validated the deletion by PCR. The hydrolitic activity of the created mutant was analysed by TLC and fluorimetry.

RESULTS

P. acnes is a Gram-positive, coryneform, non-spore-forming, anaerobic, opportunistic bacterium that inhabits the skin. Although it is predominantly commensal organism, pathogenic strains have also been identified and correlated with several diseases. Most often it plays a role in acne vulgaris, a multifactorial disease characterized by the chronic inflammation of the pilosebaceous follicles. It is primarily prevalent in teenagers and young adults, typically on skin surfaces with many sebaceous glands, e.g. on the face, neck, chest, back and shoulders. The pathogenesis of acne vulgaris and the exact role *P. acnes* plays are currently unknown.

In the course of this project we developed methods, such as touchdown multiplex PCR and eMLST, that allowed us to perform fast, efficient and reliable identification and classification of various *P. acnes* isolates. Therefore these methods provide indispensable tools not only for microbiological diagnostics, but also for the design of personalized therapy.

Results derived from eBURST and the eMLST typing method allowed us to classify isolates originating from healthy and diseased skin into clonal complexes, using phylogenetic analysis based on eSTs. We determined that isolates from diseased skin mainly belong to clonal complexes CC1, CC3 and CC4 (type IA_1 isolates), while isolates from healthy skin belong to CC5 (type IB), CC72 (type II) and CC77 (type III) clonal complexes.

The classification of strains into phylogenetic groups and sequence types enabled us to distinguish between commensal and pathogenic *P. acnes* isolates. By comparing whole genome sequences of the representatives of main phylogenetic clusters, we identified many differences: these factors are putative factors responsible for morphological differences, pathogenicity, antibiotic resistance and virulence.

Using data derived from whole genome sequencing, we examined the genetic background of the atypical phenotype of type III isolates: we identified a mutation causing amino-acid change in a GroEL, a chaperone protein responsible for protein folding. By using electron and immunofluorescent microscopy and FACS analysis, we determined that the elongated cells are indeed single cells.

A MALDI-TOF MS analysis was completed on 61 *P. acnes* clinical isolates: we determined that, by using this approach, five out of six major phylogenetic groups can be distinguished. Thus, this approach may be a helpful tool for the automated microbiological diagnostics and may bring us closer to revealing the prevalence of pathogenic strains colonizing single individuals.

Furthermore, we established a novel sampling and characterization workflow for studying *P. acnes* strains from single individuals: follicle-specific sampling and rapid characterization of cultivated strains by multiplex PCR and/or eMLST readily identifies the exact phylotype of *P. acnes* strains providing fast and valuable information about the pathogenic potential of the colonizing strains.

A potential pathogenic factor in the development of acne vulgaris is the overproduction of sebum. The produced sebum is rich in lipids and fatty acids, serving as an excellent energy source for bacteria such as *P. acnes*. We investigated the capacity to hydrolyse fatty acids of

representatives of all major phylogenetic groups and have determined that potentially pathogenic strains exhibit higher hydrolytic activity. This is particularly important as intermediates of hydrolysis exhibit inflammatory features, hence, by producing higher amounts of intermediates pathogenic strains evoke inflammation.

In order to further explore the molecular background underlying the pathogenesis of *P. acnes*, an efficient gene manipulation is essential. Here we developed a strategy for *P. acnes* mutagenesis in which the insertion of an antibiotic resistance gene is not necessary, and applied this method for the deletion of the *gehA* gene. After measuring the lipase activity of the generated mutant, we determined that the activity has been reduced to two thirds compared to the ancestor pathogenic strain. We intend to further utilize the mutagenesis scheme for the analysis of other putative virulence factors in the near future.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS

Hunyadkürti J., Feltóti Zs., Horváth B., Nagymihály M., Vörös A., McDowell A., Patrick S., Urbán E. and Nagy I. (2011) Complete Genome Sequence of *Propionibacterium acnes* Type IB strain 6609. Journal of Bacteriology vol: 193, issue 17; 4561-4562.
IF=3.825

Horváth B., **Hunyadkürti J.**, Vörös A., Fekete C., Urbán E., Kemény L. and Nagy I. (2012) Genome Sequence of *Propionibacterium acnes* Type II strain ATCC 11828. Journal of Bacteriology vol: 194, issue 1; 202-203.
IF=3.194

Vörös A., Horváth B., **Hunyadkürti J.**, McDowell A., Barnard E., Patrick S. and Nagy I. (2012) Complete Genome Sequences of three *Propionibacterium acnes* Isolates from the Type IA2 Cluster. Journal of Bacteriology vol: 194, issue 6; 1621-1622.
IF=3.194

McDowell A., **Hunyadkürti J.**, Horváth B., Vörös A., Barnard E., Patrick S. and Nagy I. (2012) Draft Genome Sequence of an Antibiotic Resistant *Propionibacterium acnes* strain, PRP-38, from the Novel Type IC Cluster. Journal of Bacteriology vol: 194, issue 12; 3260-3261.
IF=3.194

Nagy E., Urbán E., Becker S., Kostrzewa M., Vörös A., **Hunyadkürti J.** and Nagy I. (2013) MALDI-TOF MS fingerprinting facilitates rapid discrimination of phylotypes I, II and III of *Propionibacterium acnes.* Anaerobe vol: 20:20-26.
IF=2. 022

RELATED PUBLICATION

Ördögh L., **Hunyadkürti J.**, Vörös A., Horváth B., Szűcs A., Urbán E., KeresztA., Kondorosi E. and Nagy I. (2013) Complete Genome Sequence of *Propionibacterium avidum* isolated from human skin abscess. Genome Announcements 1(3):e00337-13.