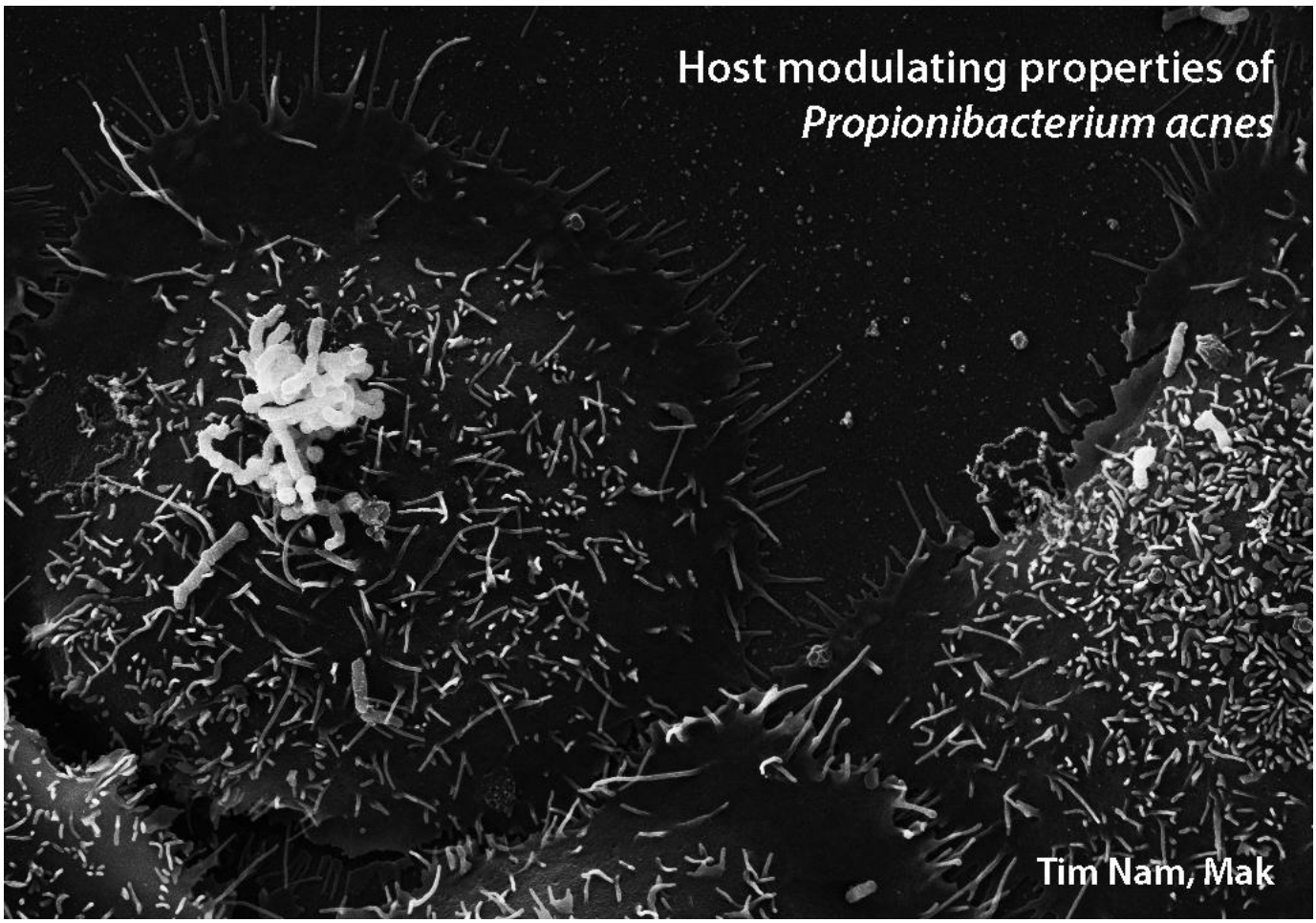
Host modulating properties of
Propionibacterium acnes
Tim Nam, Mak

Host modulating properties of *Propionibacterium acnes*

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Tim Nam, Mak

from Kuala Lumpur, Malaysia

2012

千里之行，始于足下

－ 老子 －

A journey of a thousand miles begins with the first step

- Laozi -

This thesis is based on research conducted from 2008 to 2012 at the Max Planck Institute for Infection Biology in Berlin, Germany, under the supervision of Prof. Dr. Thomas F Meyer and Prof. Dr. Holger Brüggemann

Reviewers

First Reviewer:
Prof. Dr. Rupert Mutzel
Institute für Biologie
Freie Universität Berlin

Second Reviewer:
Prof. Dr. Thomas F Meyer
Max Planck Institute for Infection Biology,
Chariteplatz 1, Berlin

Date of Defense: 15th July 2012

Declaration

I hereby declare that the work presented in this thesis has been conducted independently and without any inappropriate support, and that all sources of content, experimental or intellectual, are suitably referenced and acknowledged.

I further declare that this thesis has not been submitted before, either in the same or a different form, to this or any other university for a degree.

Tim Nam, Mak Berlin, 14 February 2012

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This part of my acknowledgement will be translated to Chinese, dedicated to my parents and my family back home in Malaysia.

我要借此机会感谢父母无私的爱于关怀，你们的细心教诲成就了今天的我。。

Finally, I will write with my broken German since this part is dedicated to Fritz.

Danke fuer alles support, understand *und zeit. Und* of course *du bist spezial und hast sehr wichtige position in mein Leben. Vielen Dank!* No words can describe how thankful I am that you are part of my life.

Summary

Propionibacterium acnes is a Gram-positive bacterium that commonly resides on human skin. It is regarded as a commensal, but recent research has highlighted that it can act as an opportunistic pathogen; the bacterium is associated with multiple inflammatory diseases, in particular acne vulgaris. Its involvement in prostate cancer is currently under investigation. The pathogenic features of *P. acnes* are not well understood. Therefore, the aim of this thesis was to obtain an in-depth understanding of *P. acnes'* pathogenicity; virulence properties of the bacterium were characterised, and subsequently, *P. acnes*-host cell interaction was investigated and its relevance in human diseases was evaluated.

In **paper I**, secreted proteins of a range of skin and clinical *P. acnes* isolates were identified; these isolates covered the four known phylogenetic groups of this species. Several potential virulence factors were discovered; these included Christie-Atkins-Munch-Petersen (CAMP) factors, proteases and proteins possessing host tissue-degrading activities. Genes encoding putative adhesins were found to exhibit phase variation-like sequence signatures, which accounted for the strain-specific expression of these genes. A rich resource for further investigation on *P. acnes* virulence factors and its host modulating properties was provided.

In **paper II**, an insertional mutagenesis protocol to inactivate specific *P. acnes* genes was established; two CAMP knockout-mutants were created. The Δ*camp2* but not the Δ*camp4* mutant showed reduced hemolytic activity in the CAMP reaction with sheep erythrocytes. This mutagenesis approach will aid future investigations on potential virulence factors of *P. acnes*.

In **paper III**, the microarray technology was employed to reveal fundamental differences in the global transcriptional responses of keratinocytes and prostate cells to *P. acnes* infection. An acute, but transient inflammatory response was observed in the infected keratinocyte cell line, HaCaT; by contrast, the prostate cell line, RWPE1, reacted later, but persistently to the infection. In RWPE1 cells, a higher numbers of internalised *P. acnes* were detected by immunofluorescence and electron microscopy, compared to

infected HaCaT cells. The study also showed that the intermediate filament protein, vimentin, is a crucial component in the host cell-interaction and invasion of *P. acnes* as judged from siRNA-mediated vimentin knock-down experiments in RWPE1 cells and vimentin overexpression in HaCaT cells. In summary, it was revealed that host cell tropism is important in *P. acnes* infections; host cell-specific invasion accounts for the persistency of infection, thus continuous inflammation.

In **paper IV**, the prevalence of *P. acnes* in human prostate tissue was investigated; the bacterium was found in 81.7% of cancerous prostate tissue samples, but was absent in healthy prostate tissues and cancerous breast tissue biopsies, as detected from *in situ* immunofluorescence (ISIF) experiments. *In vitro* studies revealed increased cell proliferation and anchorage-independent growth of RWPE1 cells after long-term exposure to *P. acnes*, thus initiating cellular transformation.

In the **additional section**, further investigations on the association of *P. acnes* with prostate pathologies are reported. *P. acnes* were predominantly cultivated from prostate biopsies. By using multi locus sequence typing (MLST), the *P. acnes* prostate isolates were classified as I-2 and II strains. These *P. acnes* subtypes are less frequently found on human skin compared to type I strains, indicative of the presence of a prostate-specific microflora.

Taken together, these data imply a potential association of *P. acnes* and prostate pathologies. Thus, prostate inflammation may be induced or enhanced by virulence factors of *P. acnes*, contributing to either initiation or progression of prostate cancer.

Zusammenfassung

Propionibacterium acnes ist ein Gram-positives Bakterium, das die menschliche Haut besiedelt. Es wird im Allgemeinen als Hautkommensale betrachtet, aber Forschungergebnisse haben gezeigt, dass *P. acnes* auch als opportunistisch-pathogenes Bakterium auftreten kann, welches an diversen entzündlichen Erkrankungen beteiligt ist, vor allem an Acne vulgaris. Seine Beteiligung an Prostatakrebs wird derzeit untersucht. Die krankheitserregenden Mechanismen von *P. acnes* sind bisher nicht gut verstanden. Daher hatte diese Forschungsarbeit zum Ziel, die Pathogenität des Bakteriums besser zu verstehen. Zum einen sollten Virulenzeigenschaften charakterisiert werden, zum anderen die Interaktion des Bakteriums mit humanen Wirtzellen untersucht, sowie die Bedeutung als Krankheitsauslöser bewertet werden.

Veröffentlichung I beschreibt die Identifizierung sekretierter Proteine von *P. acnes*. Dabei wurden klinische Isolate untersucht, welche die phylogenetische Bandbreite der Spezies abdecken. Es wurden verschiedene potenzielle Virulenzfaktoren entdeckt, wie zum Beispiel Christie-Atkins-Munch-Petersen (CAMP) Faktoren, Proteasen, und Enzyme, die Bestandteile des Wirtsgewebes abbauen können. Es wurde zudem entdeckt, dass Gene, die für putative Adhäsine kodieren, Sequenzsignaturen tragen, die eine Phasenvariation ermöglicht; diese führt zur stammspezifischen Expression der Adhäsine. Insgesamt liefern die Daten eine Grundlage für weitere Studien zu Virulenzeigenschaften von *P. acnes*.

Veröffentlichung II beschreibt die Etablierung eines Protokols zur Herstellung von Knock-out-Mutanten in *P. acnes*. Mittels dieser Mutagenese-Strategie wurden zwei CAMP Knock-out-Mutanten hergestellt. Es zeigte sich, dass eine Δ*camp2*, nicht aber eine Δ*camp4* Mutante eine reduzierte hemolytische Aktivität in der CAMP-Reaktion mit Schafserythrozyten aufwies. Die Mutagenese-Strategie wird zukünftig weitere Studien zu Virulenzfaktoren von *P. acnes* ermöglichen.

Veröffentlichung III beschreibt anhand von Experimenten mit der Microarray-Technologie, dass sich das Transkriptionsprofil in infizierten Prostatazellen fundamental von dem in infizierten Keratinozyten unterscheidet. Zu beobachten war eine akute, transiente

Entzündungsantwort in der infizierten Keratinozyten-Zellline HaCaT, wohingegen die Prostatazelllinie RWPE1 zeitlich verschoben, aber lang-anhaltend auf die *P. acnes*-Infektion reagierte. Das Bakterium wurde mittels Immunfluoreszenz- und Elektronenmikroskopie intrazellulär detektiert, wobei infizierte RWPE1-Zellen weitaus mehr Bakterien trugen als HaCaT-Zellen. Zudem konnte gezeigt werden, dass das Wirtsprotein Vimentin, ein Typ 3-Intermediärfilament, eine wichtige Rolle bei der Wirtszellinteraktion und -invasion von *P. acnes* spielt; dieses konnte durch Vimentin-Knockdown (mittels RNA-Interferenz) in RWEP1-Zellen und Vimentin-Überexpression in HaCaT-Zellen belegt werden. Diese Studie zeigte die Bedeutung des Wirtszelltropismus bei *P. acnes*-Infektionen; die wirtszellspezifische Invasion der Bakterien bedingt die Persistenz der Infektion und damit auch die Dauer der inflammatorischen Antwort.

Veröffentlichung IV beschreibt eine Studie über das Vorkommen von *P. acnes* in humanen Prostatagewebeproben. Das Bakterium wurde mit Hilfe der In-Situ-Immunfluoreszenz (ISIF)-Technik in 81.7% aller Prostatakrebsgewebeproben gefunden, aber weder in gesunden Prostatagewebeproben noch in Brustkrebsgewebeproben detektiert. *P. ances*-Langzeitinfektionen in der Prostataepithelzelllinie RWPE1 führten zu einer verstärkten Zellproliferation und zu einem *ankerunabhängigen* Wachstum der Zellen, ein Charakteristikum beginnender Zelltransformation.

Das **zusätzliche Kapitel** beschreibt weitere Untersuchungen bezüglich der Verbindung von *P. acnes* und Erkrankungen der Prostata, wie zum Bespiel die Kultivierung von *P. acnes* aus Prostata-Biopsiematerial. Die klinischen *P. acnes*-Isolate wurden mittels Multi-Locus-Sequence-Typing (MLST) phylogenetisch typisiert. Es handelte sich ausschliesslich um Typ-I-2 und Typ-II Stämme, welche seltener auf der menschlichen Haut anzutreffen sind als Typ-I Stämme, was für das Vorkommen einer prostataspezifischen Mikroflora spricht.

Die gewonnenen Daten sprechen für eine Assoziation von *P. acnes* und Prostataerkrankungen. Möglicherweise wird die Entzündung der Prostata durch Virulenzfaktoren von *P. acnes* ausgelöst oder verstärkt, was die Initiation oder das Fortschreiten von Prostatakrebs unterstützen könnte.

List of papers

I. 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 Microbiology* 10:230

II. Sörensen M, **Mak TN**, Hurwitz R, Ogilvie LA, Mollenkopf HJ, Meyer TF, Brüggemann H. 2010 Mutagenesis of *Propionibacterium acnes* and analysis of two CAMP factor knock-out mutants. *Journal of Microbiological Methods* 83 (2): 211-6

III. **Mak TN**, Fischer N, Laube B, Brinkmann V, Metruccio ME, Sfanos KS, Mollenkopf H, Meyer TF, Brüggemann H. 2012 *Propionibacterium acnes* host cell tropism contributes to vimentin-mediated invasion and induction of inflammation. *Cellular Microbiology.* 14(11):1720-33

IV. Fassi Fehri L, **Mak TN**, Laube B, Brinkmann V, Ogilvie LA, Mollenkopf H, Lein M, Schmidt T, Meyer TF, Brüggemann H. 2011 Prevalence of *Propionibacterium acnes* in diseased prostates and its inflammatory and transforming activity on prostate epithelial cells*. International Journal of Medical Microbiology*. 301(1):69-78

Paper not included in this thesis

I. Fassi Fehri L, Rechner C, Janβen S, **Mak TN**, Holland C, Bartfeld S, Brüggemann H, Meyer TF. 2009 *Helicobacter pylori*-induced modification of the histone H3 phosphorylation status in gastric epithelial cells reflects its impact on cell cycle regulation. *Epigenetics* 4(8): 577-86

This is a cumulative dissertation primarily based on the above mentioned publications.

Scientific contributions

Paper I

DNA isolation, PCR, DNA sequencing and analysis

*Part of this paper is included in Carsten Holland's PhD thesis of Humboldt University

Paper II

Co-hemolysis assay (CAMP reaction)

Infection of HaCaT cells

Paper III

Infection of epithelial cells

Western immunoblotting

Immunofluorescence

Antibiotic protection assay

siRNA and plasmid transfection

Paper IV

Infection of prostate epithelial cells

Confocal analysis

PCR typing of prostate clinical isolates

Western Immunoblotting

Wound healing assay

Additional section: *P. acnes* and its potential involvement in prostate cancer

Prostate biopsies handling and bacterial cultivation,

16S rDNA amplification and sequencing, MLST analysis

Abbreviation

2-DE	Two dimensional gel electrophoresis
ADC	Prostate adenocarcinoma
BMK1	Big MAP kinase 1
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CAMP	Christie-Atkins-Munch-Peterson
cfu	Colony forming unit
ECL	Electrochemiluminescence
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
FISH	Fluorescent *in situ* hybridization
GM-CSF	Granulocyte macrophage colony-stimulating factor
h.p.i	Hours post infection
HPV	Human papilloma virus
IF	Immunofluoresence
IκK	Inhibitor κB kinase
ISIF	*In situ* immunofluoresence
JNK	c-Jun amino terminal kinase
LB	Lucia-Bertani
LPS	Lipopolysaccharide
MALDI-MS	Matrix-assisted laser ionization mass spectroscopy

MAPK	Mitogen activated protein kinases
MLST	Multi locus sequence typing
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa B
NLR	NOD-like receptor
NOD	Nucleotide-binding and oligomerisation domain
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular patterns
PCR	Poly chain reaction
PFA	Paraformaldehyde
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PMN	Polymorphonuclear leukocytes
PPR	Pattern recognition receptors
SAPHO	Synovitis, acne, pustulosis, hyperostosis and osteomyelitis
SDS	Sodium dodecyl sulfate
ST	Sequence type
TLR	Toll-like receptor
TMA	Tissue microarray
TNFα	Tumor necrosis factor α
WB	Western blot

Table of Contents

1 Introduction

1.1 *Propionibacterium acnes*

History of *Propionibacterium acnes*

Propionibacterium acnes (*P. acnes*) had been previously classified as *Corynebacterium acnes* (C. acnes) or *Corynebacterium parvum* (*C. parvum*) [1,2]. In 1946, Douglas and Gunter have reported that *C. acnes* shares similar characteristics with the *Propionibacterium* family, thus the bacterium was then reclassified as *P. acnes* [3]. Moss *et al.* (1967) also validated this new classification by reporting that *Propionibacteria* family members and *C. acnes* have comparable fatty acid compositions[4]. The species *C. parvum* was later identified as a heterologous group comprising *P. acnes* and some strains of *Propionibacterium granulosum* (*P. granulosum*) [5].

Phylogeny of *Propionibacterium acnes*

P. acnes is phylogenetically divided into types IA, IB, II and III. Various methods such as fermentation assays, bacteriophage typing and PCR-based identification have been used to characterise the phylogeny of *Propionibacteria*. Johnson and Cummins *et al.* (1972) were the first to describe types I and II of *P. acnes* based on cell wall sugar analysis and agglutination tests [1]. The cell wall of type I strains contains galactose, mannose and glucose, whereas the type II strain is devoid of galactose. In 2005, McDowell *et al*. showed that the differences between type I and II were based on type-specific polymorphisms in *recA* and *tly* genes [6]. Moreover, additional clusters of type IA and IB were identified within type I [7]. McDowell *et al.* (2008) identified a novel *recA* cluster which was assigned to type III [8]. Lomholt and Kilian (2010) have reconstructed the population structure of *P. acnes* based on nine housekeeping genes [9] (Figure 1.1). Later, McDowell *et al.* (2011) also developed another multilocus sequence typing scheme (MLST) based on seven housekeeping genes [10]. Hence, the MLST typing schemes serve as an important platform to identify subsets of *P.acnes* in epidemiological studies.

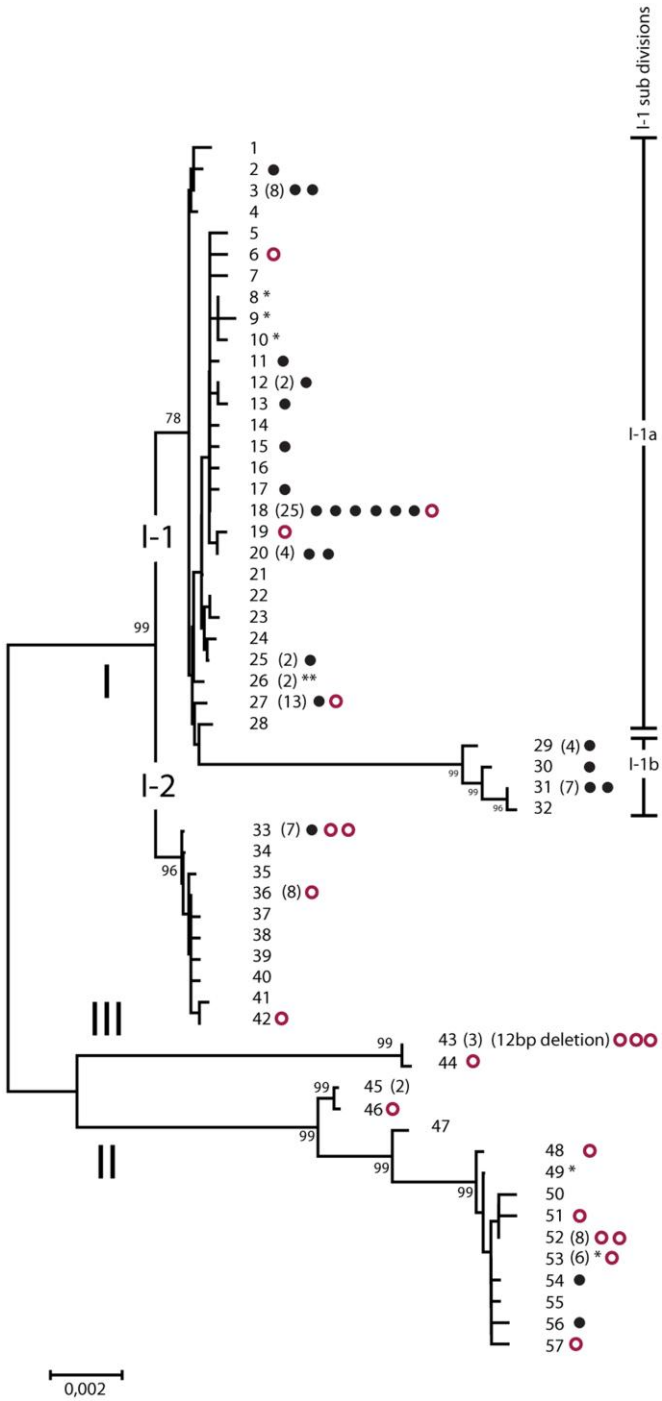

Figure 1.1: Population structure of *P. acnes* based on MLST Aarhus scheme. 57 sequence types (ST) were detected in 210 isolates from skin of healthy individuals and acne patients and from other disease sites. Number of independent isolates assigned to each ST are shown in brackets. Isolates from severe acne patients are indicated by a filled black circle and isolates from infections (e.g. deep tissues and blood) are indicated by an open red circle. The bar represents the genetic distance. (Adapted from [9])

Microbiology of *Propionibacterium acnes*

P. acnes is a Gram-positive bacterium, located in most parts of the skin [11]. They form yellow, circular and opaque colonies on agar plates [12]. Under the microscope, this bacterium exhibits a pleomorphic rod shape as seen in Figure 1.2. It has a distinct peptidoglycan layer outside its cell membrane, which contains glucosamine residues with free amino groups, which are responsible for the resistance to lysozyme [13].

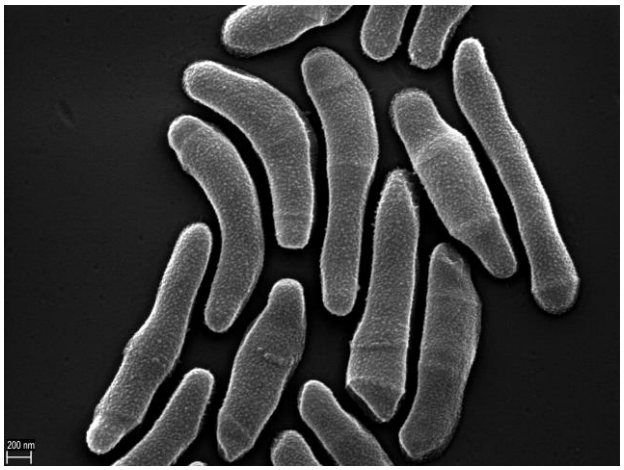

Figure 1.2: Scanning electron microscopy picture of *P.acnes* under scanning electron microscopy (V. Brinkmann, Microscopy Core Facility).

Although *P. acnes* requires an anaerobic or microaerophilic environment for optimal growth, it can tolerate aerobic conditions [14]. The ability of *P. acnes* to tolerate oxygen is due to the production of catalases and superoxide dismutases, which are both expressed during oxidative stress [15,16]. Under anaerobic conditions, *P. acnes* ferments glucose, glycerol, fructose, ribose, mannose and N-acetylglucosamine [16,17]. Moreover, *P. acnes* might utilise anaerobic respiration systems such as nitrate reductase, fumarate reductase and dimethyl sulfoxide reductase [16]. Clinical isolates of *P. acnes* often requires 7 - 10 days of incubation in order to be visualised on plates [12].

Genomes of *Propionibacteriaceae* family members

To date, five *P. acnes* genomes have been completely sequenced. The first *P. acnes* genome sequence of strain KPA171202 was published by Brüggemann *et al.* (2004), followed by genome sequences of strain SK137 and strain 266 [18][19]. Recently, two additional genomes were published: strain 6609 and ATCC11828 [20,21]. All published *P.*

acnes genomes have a similar G+C content of 60% and an average size of 2.51 Mb. Furthermore, 71 more genomes of *P. acnes* have been partially sequenced in the framework of the Human Microbiome Project [22]. The other two completed *Propionibacteria* genomes are from *Propionibacterium freudenreichii* and *Propionibacterium humerusii*. Species identification of the latter strain is provisional. Both genomes have a slightly larger size of about 2.6 Mb and G+C contents of 67% and 60% respectively [23,24]. Even though *P. freudenreichii* is related to *P. acnes*, its genome does not encode many of the putative virulence factors of *P. acnes* [23].

Putative pathogenicity factors of *P. acnes*

Analysis of the *P. acnes* genome revealed genes that encode several enzymes involved in the degradation of eukaryotic cell surface and tissue components. Some examples are lipases, proteases hyaluronidases, chrondroitin sulfatases and gelatinase [19,25]. The *P. acnes*-specific lipase GehA (glycerol ester hydrolase A) has been identified as one of the virulence factors in acne vulgaris pathogenesis and is supposed to degrade sebum triglycerides [26,27]. Lipases are thought to degrade human skin lipids contained in sebum, which could contribute to skin colonisation. *P. acnes* lipase activity releases free fatty acids, which could help bacterial adhesion and colonisation of sebasceous follicles [28]. *P. acnes* also produces a hyaluronate lyase that mediates degradation of hyaluronan, a main polysaccharide component of the host extracellular matrix [29]. These host tissue-degrading enzymes of *P. acnes* could support the disruption of the skin epithelium.

Sialidases/neuraminidases have been shown to cleave sialoglycoconjugates to yield sialic acids, which might supply carbon and energy to *P. acnes* [30]. These authors also showed that sialidase is required for adhesion and cytotoxicity in human sebocytes and proposed this virulence factor as a possible vaccine target to prevent early *P. acnes* infection. Interestingly, sialidases have a role in viral and bacterial invasion of host cells [31].

Five highly similar genes in the *P. acnes* genome encode Christie-Atkins-Munch-Peterson (CAMP) factors. *In vitro*, these factors act as co-hemolysins on erythrocytes; they act together with the sphingomyelinase C of *Staphylococcus aureus* [7]. CAMP factors might

form pores in eukaryotic membranes [32]. Recently, these factors were characterised as cytotoxic to human keratinocytes and macrophages and their contribution to inflammation was proven by mice vaccination experiments with recombinant CAMP factors [33]. **Paper II** employed a mutagenesis approach to knock-out CAMP factor genes; mutants were further characterised to pinpoint the role of CAMP factors.

1.2 Human host cell-*P. acnes* interaction

Ubiquitous presence of *P. acnes*

P. acnes predominantly resides in sebum rich areas of the human skin, mainly on the scalp, forehead, ear and alea nasi [34]. The number of *P. acnes* ranges from 10^1 to 10^5 cfu/cm^2 depending on the location [35,36]. Dry areas like the arms and legs have a low density of *P. acnes* of 10^1 cfu/cm^2, whereas the forehead, which contains massive amounts of pilosebaceous follicles, is highly populated with about 10^5 cfu/cm^2. Besides the skin, the bacterium can also be found as part of the normal flora of the oral cavity and the intestine.

Life as a commensal bacterium

The omnipresence of *P. acnes* on human skin might indicate that the host benefits from this bacterium. A few studies suggested that *P. acnes* could indeed contribute to healthy skin [11]. *P. acnes* uses lipids like sebum as an energy source to produce propionic acid by fermentation [37]. The acid production lowers the skin pH, potentially preventing colonisation of other microbial pathogens. Other studies highlighted the anti-microbial effects of *Propionibacteria*. For example, *Propionibacterium jensenii* and *Propionibacterium thoenii* secrete bacteriocins [38,39] and *P. acnes* also secretes a bacteriocin-like substance called acnecin [40]. These bacteriocins could work together with propionic acid to create a special environment for successful competition, which might protect the skin.

P. acnes and acne vulgaris

Acne vulgaris is a skin disease of the pilosebaceous units which consists of hair follicles and the sebaceous gland [41](Figure 1.3A). The main function of this gland is to excrete sebum. Increased sebum production contributes to the development of acne lesion

[42]. This pilosebaceous unit is an immunocompetent organ in which immune cells like keratinocytes and sebocytes are able to recognise pathogens [43].

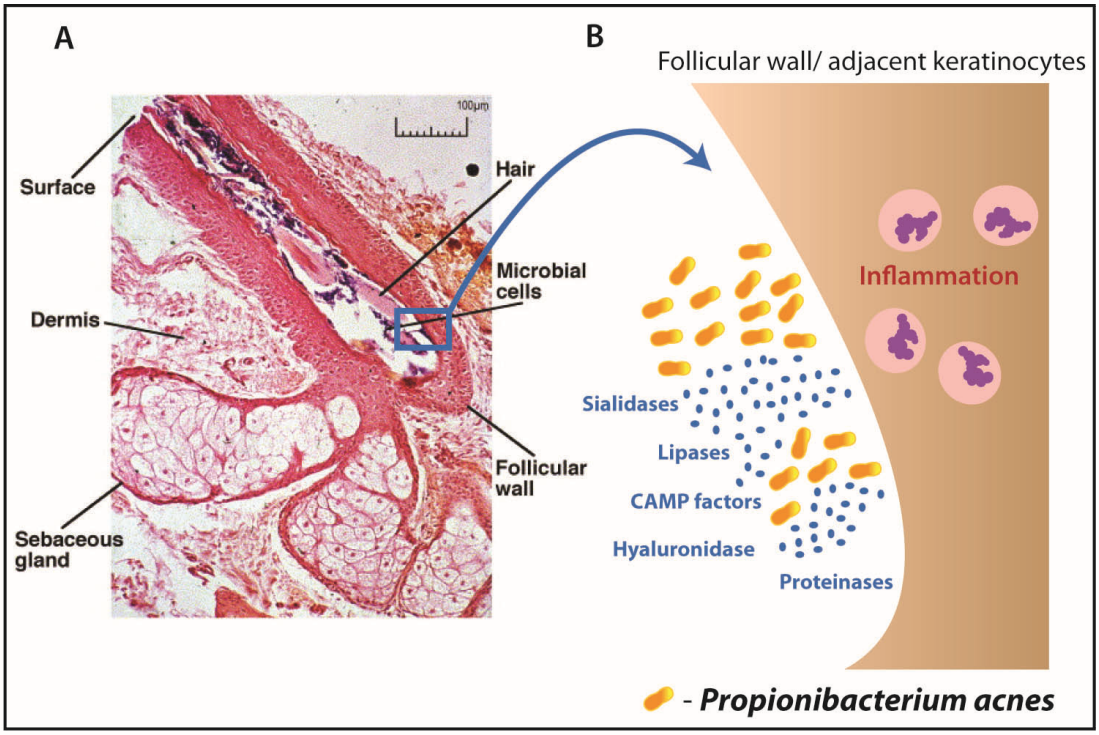

Figure 1.3: Involvement of *P. acnes* in acne vulgaris. (A) Gram-stained section of a single pilosebaceous follicle. The pilosebaceous duct of a single pilosebaceous follicle is colonised with Gram-positive bacteria. Scale bar = 100µm. (B) A possible scenario of *P. acnes* and its secreted factors to induce an inflammatory response in skin. (Adapted and modified from [44])

There are four main mechanisms to develop acne lesions: hyperconification, increased sebum production, inflammation and follicular colonisation by *P. acnes* [41]. The succession of events during pathogenesis and the mechanisms involved are still a matter of controversial debate. Hyperconification is believed to be an early stage in acne. Keratinocytes undergo hyperproliferation, hyperkeratinisation and reduced desquamation at the same time, leading to the formation of comedones. A comedo is formed after accumulation of scales in the pilosebaceous duct. Several factors are thought to be responsible for the hyperconification, such as androgens, IL-1α and the production of fatty acids by *P. acnes* [45]. In addition, Jeremy *et al.* (2003) also found accumulation of inflammatory cells like CD4+ lymphocytes and macrophages at the periphery of comedones,

suggesting a link between inflammation and comedones formation [46]. Another factor is sebum overproduction, which is usually a result of androgen-stimulated activity of the sebaceous gland. The excessive sebum is then trapped in the microcomedones forming a keratin plug, which results in enlarged follicles. The presence of *P. acnes* contributes to the pathogenesis by forming biofilms that act together with sebum as a kind of biological glue in the microcomedone to block the follicle in the fundibulum [47]. The blockage and proliferating keratinocytes create a pressure on the duct wall leading to rupture of the epithelium. Moreover, the blockage of the follicle leads to an anoxic environment, a favorable growth condition of *P. acnes*. Subsequently, the massive presence of *P. acnes* provokes an inflammatory responses [48](Figure 1.3B).

The involvement of *P. acnes* in acne was first suggested as early as 1896 when Unna isolated *P.acnes* from an acne lesion [49]. Besides *P. acnes*, *Staphylococcus spp* and *Malassezia spp* were also frequently isolated from these lesions [50]. Interestingly, Bek-Thomsen *et al.* (2008) reported that only *P. acnes* colonises follicles of normal skin, while *Staphylococcus epidermidis* (*S. epidermidis*), and to a lesser extent other species were found together with *P. acnes* in acne follicles, suggesting an interplay of different species in the formation and progression of acne [11].

Successful treatment of acne with antibiotics like erythromycin and clindamycin has supported the hypothesis of bacterial involvement in acne. Furthermore, Eady *et al.* (1989) reported treatment failure was due to antibiotic resistant strains of *P. acnes* [51]. The decipherment of the *P. acnes* genome further supported the link between *P. acnes* and acne; it revealed the potential pathogenic side of this bacterium [16]. Interestingly, Höffler *et al.* (1977) reported that *P. acnes* isolates from acne patients secrete more sialidases compared to an isolate from normal individuals [25]. Recently, Lomholt and Kilian (2010) investigated the population structure of *P. acnes* and showed that distinct *P. acnes* phylotypes exist which are associated with acne, while other phylotypes are preferentially associated with healthy skin [9].

Other diseases associated with *P. acnes*

P. acnes' contribution to the development of acne vulgaris has been generally accepted, mainly due to its ability to induce an inflammatory response. Besides that, there are a number of other diseases like endophthalmitis, osteomyelitis, sarcoidosis, SAPHO syndrome and shunt associated central nervous system, which have been reported to be associated with this bacterium.

Endophthalmitis describes an inflammation in the intraocular region of the eyes due to an infectious process. It commonly occurs as a late post-surgery infection. In 1978, Friedman isolated *P. acnes* from the conjuctiva of patients with endophthalmitis; the authors suggested that the bacterium is a normal inhabitant of the conjunctiva [52]. However, several other studies have indicated that *P. acnes* can actually causes endophthalmitis; antibiotic treatment is usually an efficient treatment [53,54].

Sarcoidosis is an abnormal accumulation of inflammatory cells forming nodules in multiple organs. Two different studies were able to detect *P. acnes* in bronchoalveolar lavage and lymph nodes from sarcoidosis patients [55,56]. The role of *P. acnes* in sarcoidosis is challenged by Ishige *et al.* (2005) [57]. They have shown that *P. acnes* is present in both normal peripheral lung tissue and mediastinal lymph nodes, indicating that the bacterium is not specific to sarcoidosis. Hence, the causative role of *P. acnes* in sarcoidosis remains questionable.

SAPHO syndrome consists of synovitis, acne, pustulosis, hyperostosis and osteomyelitis. *P. acnes* has been isolated from biopsies of SAPHO patients and treatment with antibiotics has controlled the disease [58][59]. This syndrome has been suggested as chronic, relapsing and immune mediated and it still remains unclear whether it is caused by infection, an autoimmune reaction or perhaps by both factors [60][61].

P. acnes plays a role to device-related infections such as joint prostheses, shunts and prosthetic heart valves. By using 16S rRNA PCR amplification, it was shown that *P. acnes,* together with *Staphylococcus spp,* is present in prosthetic joint infection sites [62]. *P. acnes* has been suggested to be the causative agent of shunt-associated central nervous system

infections. In 1976, Beeler *et al.* reported that shunt infected patients had antibodies against *P. acnes* while normal control patients had not [63]. *P. acnes* has also been reported to cause a unique sign of massive ascites during shunt infections [64]. Furthermore, *P. acnes* infections are predominately linked to surgery, trauma or the presence of foreign devices like prosthetic aortic valves and shoulder implants[65,66].

It is difficult to attribute *P. acnes* as an etiological agent for the mentioned diseases, since there is a high risk of skin-derived contamination. Some studies showed that there are different subtypes of *P. acnes* depending on the isolation sites; currently, it is being evaluated if some subtypes could be regarded as pathogenic strains that are distinct from commensal strains [9]

1.3 Immuno-stimulatory properties upon microbial encounter

Innate Immunity

Our immune systems protect us from attack from pathogens like bacteria, virus and fungi. Pathogens have evolved strategies to manipulate the host response in order to persist and multiply. In response to these microbial threats, both the innate and adaptive arms of an immune-competent host mobilise to clear the infection. The innate immune system not only is the first line of defense against infection, but also activates the adaptive arms, thereby leading to an efficient effector response and the development of memory T and B cells against the microbial pathogen [67].

The innate immune system depends on pattern recognition receptors (PPR) that are responsive towards pathogen associated molecular patterns (PAMP) like LPS and bacterial CpG DNA[68]. Well-known PRRs are Toll-like receptors (TLRs), which were first discovered in *Drosophila melanogaster* [69]. Other PRRs include receptors responsible for intracellular pattern recognition such as nucleotide-binding and oligomerisation domain (NOD)-like receptors (NLRs) [70]. Both TLRs and NLRs induce an immune response by activating the nuclear factor (NF-κB) and mitogen activated protein kinases (MAPK) signalling pathways [71,72]. These pathways induce the production and secretion of a wide range of inflammatory molecules like cytokines and chemokines, which in turn activate other

immune cells. Moreover, these pathways are known to regulate cell division, cell differentiation, proliferation, apoptosis and survival [73,74].

MAPK pathway and infection

There are four distinct kinase members of MAPK cascades: extracellular signal-regulated kinases (ERKs), p38 MAPKs, c-Jun amino terminal kinases (JNKs) and extracellular signal-regulated kinase-5 (ERK5) or Big MAP kinase 1 (BMK1). MAPK cascades respond rapidly to physical and environmental stimuli and are activated through phosphorylation of target protein substrates. MAPK signalling pathways also have a profound role in the response to a wide range of bacterial and viral pathogens [75]. For example, *Helicobacter pylori*, *Shigella spp*, *Salmonella spp* and the influenza virus [76–79].

NF-κB: a brief introduction

NF-κB was first described as a transcription factor in B cells, binding to the κ light chain enhancers and immunoglobulin heavy chain [80]. The NF-κB pathway governs the transcription of many inflammation-associated genes in a wide range of cell types. NF-κB can be activated by two pathways: the classical pathway and the alternative pathway (Figure 1.4). The classical pathway is induced by pro-inflammatory cytokines like IL-1β and TNFα with the aid of TLRs. The alternative (or non-classical) NF-κB pathway is only activated by certain mediators such as TNF family members [81]. It was suggested that this pathway governs genes expression in the development and maintenance of secondary lymphoid organs [80].

Components of NF-κB and its basic signalling principle

The family of NF-κB transcription factors consists of five subunits: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB 1) and p52/p100 (NF-κB 2). They can form any combination of homo- or heterodimers, resulting in different functions. One example is the p65:p50 dimer, which is commonly involved in inflammation [82]. For its activation, NF-κB also requires the dissociation from an inhibitor named inhibitor of κB (IκB). To date, there are five known inhibitors: IκBα, IκBβ, IκBε, IκBγ and IκBδ [82]. Degradation of inhibitors depends on phosphorylation of the inhibitor proteins. This process is catalysed by kinases named

inhibitor κB kinase (IκK) α and β [83].

The NF-κB complex (usually the p65:p50 dimer) is predominantly present in the cytoplasm in non-stimulated cells, sequestered by its inhibitors [84]. After stimulation, the IκK complex phosphorylates IkB on two conserved serine residues at S32 and S36 [85]. The phosphorylated complex will be recognised by the ubiquitin-protein ligase complex, which polyubiquitinylates the inhibitors [86]. This will lead to IκBα degradation by the proteasome; subsequently, free p65:p50 dimers can enter the nucleus and trigger gene transcription by binding to NF-κB sites within the promoter of responsive genes[87] (Figure 1.4).

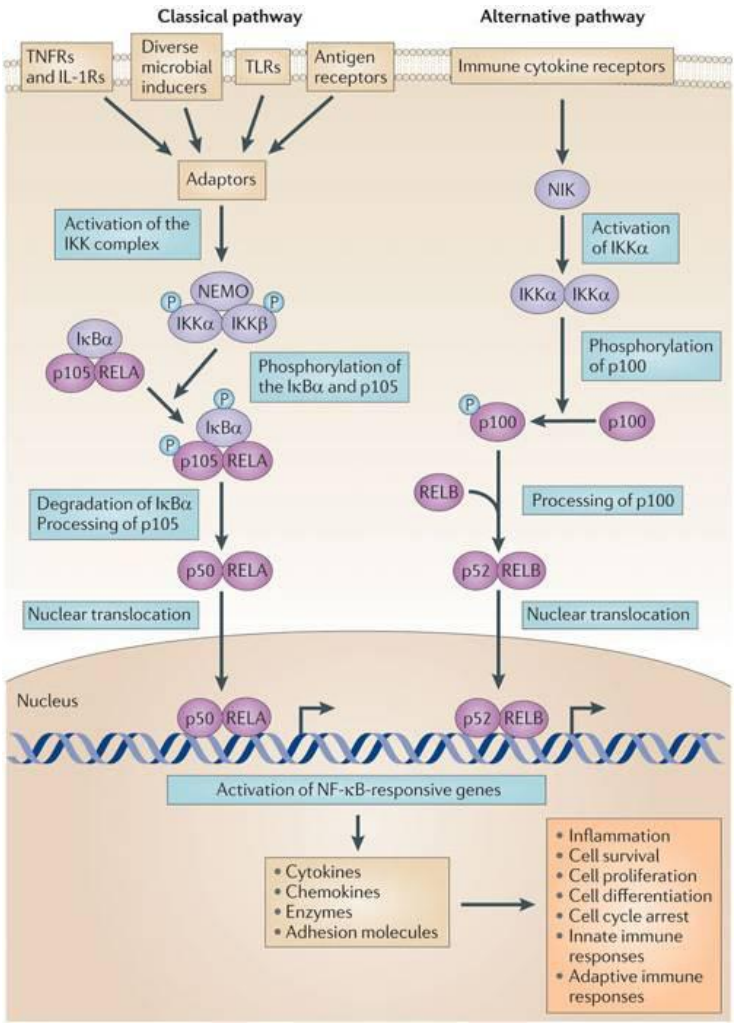

Figure 1.4: The classical and alternative NF-κB signalling pathways. NF-κB activation results in genes transcription that encode pro-inflammatory factors and factors involved in cell proliferation. Degradation of IκBα leads to free p65(RelA):p50 dimers and enter into nucleus for genes transcription. (Adapted from [88]).

NF-κB pathway and infection

Upon infection, NF-κB has an important role for the immediate antimicrobial responses of the immune system. It regulates the production of antimicrobial nitrogen and oxygen species, and defensins following exposure to infection[89]. Moreover, NF-κB signalling responds to infection by activating the innate and acquired host immune responses. The NF-κB activation leads to the recruitment of T- and B-cells to slow down the infection. It forms a protective barrier against pathogens such as *Streptococcuss pneumoniae*, *Leishmania major* and parasite *Taxoplasma gondii* [88].

On the other hand, some pathogens modulate the NF-κB signalling for their own benefits. Medically important pathogens like *H. pylori* and *Legionella pneumophila* are able to hijack host signalling molecules by upregulating anti-apoptotic factors [90,91]. Viruses (e.g. Epsten-Barr virus and human papillomavirus) are known to manipulate NF-κB signalling by inhibiting the phosphorylation of NF-κB inhibitor to prevent transcriptional activation, thus increasing host cells survival for prolonged infection [92,93].

***P. acnes* and its interaction with the immune system**

Inflammation in acne vulgaris has been strongly linked to the presence of *P. acnes*. Pro-inflammatory cytokines (e.g. tumor necrosis factor TNFα, IL-1α, IL-1β, IL-12 and IL-8) are induced by *P. acnes*. All these cytokines could contribute to the migration of neutrophils, monocytes and lymphocytes to infected tissues [43,94].

Some TLR family members can recognise PAMPs of *P. acnes*. PAMPs include heat shock proteins (e.g. GroEL and DnaK) as well as lipoglycans of *P. acnes* [95,96]. GroEL of *P. acnes* upregulates proinflammatory cytokine production in keratinocytes in a dose-dependent manner [95]. *P. acnes*-responsive TLRs are TLR2 and TLR4 as extracellular receptors and TLR9 and NOD1 as intracellular receptors [97–99]. Activated receptors then trigger the NF-κB signalling pathway. TLR2 is a major receptor for *P. acnes.* Through this receptor, *P. acnes* can activate MAPK (mainly JNK and ERK) to increase IL-8 production and reactive oxygen species (ROS) production in both phagocytes and keratinocytes, possibly contributing to inflammatory acne [100][101]. A study by Kim *et al.* (2002) showed that TLR2

was sufficient for NF-κB activation upon *P. acnes* infection to induce pro-inflammatory cytokines [102]. In addition, human β-defensin 2 and IL-8 are induced in TLR2- and TLR4-dependent manners by *P. acnes*. It was also implicated that antimicrobial peptides and cytokines produced in keratinocytes may attract inflammatory cells to the site of infection [103]. Besides in keratinocytes, *P. acnes* can induce human β-defensin 2, TNFα and IL-8 production in sebocytes, possibly via TLR2 and TLR4 [103]. This research work indicated that inflammatory acne involves both sebocytes and keratinocytes that recognise *P. acnes*.

P. acnes were shown to activate both classical and alternative pathways of complement system [104]. The activation leads to production of C5-derived neutrophil chemotactic activity that attracts polymorphonuclear leukocytes (PMN) in the region with the acne lesion [105,106]. On the other hand, *P. acnes* also secretes low molecular weight PMN chemotactic factors like lipases [105,107].

1.4 Infection and cancer

The history of research investigating a link between infection and cancer dates back to 1911 when Rous (1983) studied cell filtrates from a chicken sarcoma; these cause cancer in other healthy animals. It was later identified that there was an avian virus in these filtrates, which was subsequently named after him, Rous sarcoma virus [108]. This revealed for the first time a potential infectious origin of cancer. This led to the recognition of a new paradigm in cancer research - infection and cancer. Later on, other studies confirmed and extended findings of infectious agents (virus and bacteria) as carcinogenic agents.

Currently, it is estimated that about 20% of all human cancers are caused by viral, bacterial or trematode infections [109]. Viruses have long been suspected to be carcinogenic agents; indeed Hepatitis B and Epstein-Barr viruses were discovered as oncoviruses for hepatocellular cancer and Burkitt lymphoma respectively [110]. Similar to Hepatitis B virus, the Hepatitis C virus is also associated to hepatocellular cancer, although the exact carcinogenic mechanism of viruses remains unclear [111]. One of the most well-known discoveries in the field of oncoviruses was by Harald zur Hausen, earning him the prestigious Nobel Prize in Physiology or Medicine in 2008. He discovered that human

papilloma virus (HPV) is an infectious agent contributing to cervical cancer [112]. This led to a vaccine development protecting against cervical cancer and, which appears to be a highly efficient method to prevent HPV infection in young women [113].

In the bacterial field, the so far accepted connection between bacteria and cancer is the discovery of *Helicobacter pylori* in gastric cancer in 1982. This finding by Barry Marshall and Robin Warren was rewarded a Nobel Prize in Physiology or Medicine in 2005 [114]. However, other bacterial infections are linked to cancer. For example *Streptococcus bovis* in colorectal cancer and *Salmonella typhii* in gallbladder carcinoma [115–117]. With the emergence of metagenomic methods as a powerful approach to identify microbes at infection sites, the pathogen *Fusobacterium nucleatum* was recently detected in colorectal cancer tissue by two independent research groups[118,119]. Another bacterium which was detected in tumours is *Propionibacterium acnes*; this link will be introduced in the next section.

One possible mechanism of how these viruses and bacteria might be linked to cancer development and progression is their role in causing chronic inflammation which could lead to severe tissue damage and aberrant cell proliferation [120]. The association of inflammation and cancer is well accepted in some types of cancer. The inflammatory response triggered by infectious agents controls host defenses against infection and plays a crucial role in tissue repair and regeneration. This might act as an initial gate opener to a cascade of events leading to carcinogenesis [121]. The best example for the relationship between chronic inflammation and cancer is *H. pylori*, which has been classified as a class I carcinogen for gastric cancer. This Gram-negative bacterium causes DNA damage resulting from chronic inflammation [122][123]. DNA damage could lead to the accumulation of potential oncogenic mutations which might incite gastric cancer progression [120].

Prostate cancer

Prostate cancer is the most common cancer in men in the Western world. In United States alone, there are about 250 000 cases of prostate cancer per year, leading to 30 000 death [124]. The risk of getting prostate cancer is higher with increasing age. Intriguingly,

several studies based on autopsy experiments have reported the presence of adenocarcinomas in prostate glands of one third of men between the age of 30-40, suggesting that the initiation of prostate cancer occurs earlier in life [125]. In addition to the risk factor of age, a growing body of evidence links chronic or recurrent inflammation to prostate carcinogenesis [126,127].

In a diseased prostate, most lesions harbor either acute or chronic inflammatory infiltrates in areas of the atrophic epithelium. This fraction of epithelial cells proliferate more in focal atrophy lesions compared to normal epithelium and thus, the term proliferative inflammatory atrophy (PIA) was assigned [128]. Another form of prostate inflammation is benign prostatic hyperplasia (BPH) in which the prostate gland increases in size due to the infiltration of inflammatory cells [129]. Although several factors such as urine reflux, diet and hormones influence the risk of prostatic inflammation, the exact cause of prostatic inflammation remains unclear [127](Figure 1.4).

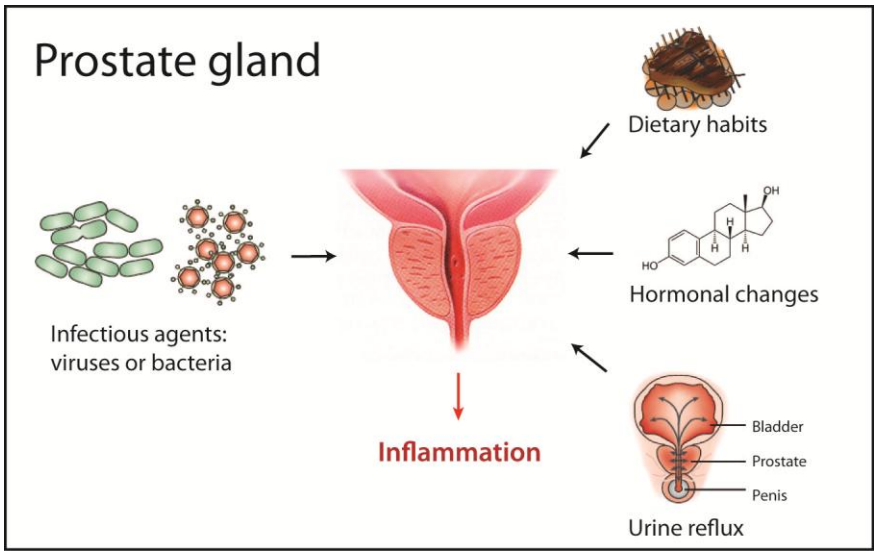

Figure 1.5: Possible factors in prostate inflammation. One of the possible inflammatory stimulation sources is infection by viruses or bacteria. Others include hormonal alteration, urine reflux and compounds from charred meat can activate inflammatory cells (Adapted and modified from [127])

Some studies investigated the possibility that infections might play a causative role in prostate cancer. Sexually transmitted pathogens like *C. trachomatis*, *Trichomonas vaginalis* and *Treponema pallidum* have been reported to be linked with prostatitis [130–

132]. Other pathogens include non-sexually transmitted bacteria such as *E. coli* and *P. acnes* [133,134]. Moreover, bacterial cultivation in 21 out of 59 (36%) prostate biopsy samples consisted bacteria such as *Staphylococci*, diptheroids and anaerobic bacteria [135]. Although these pathogens could be identified in diseased prostate glands and known to cause prostatitis, it is unknown if they have an etiological role in prostate cancer.

P. acnes and prostate cancer

The role of *P. acnes* in prostate cancer remains controversial. A substantial number of studies suggest a possible causative role of *P. acnes* in prostate cancer. For example, a study by Cohen *et al.* reported that 35% of malignant prostate tissue samples are positive for *P. acnes* [136]. Moreover, they were able to demonstrate an association of *P. acnes* with inflammation in the prostatectomy samples. Furthermore, they reported that prostate isolates (types IB and II) differ genetically from skin isolates (mainly type IA). The prostate isolates were found to be phylogenetically similar to *P. acnes* isolates from the urinary tract. Next, Alexeyev *et al.* (2007) detected *P. acnes* in 5 out of 10 samples from prostate cancer patients by fluorescent *in situ* hybridization (FISH) using *P. acnes* specific DNA probes [133]. The bacteria were reported to be present in two visible forms; as intracellular bacteria and as biofilm-like aggregates. Using 16S rDNA PCR analysis, Alexeyev *et al.* (2006) found that *P. acnes* is a predominant microorganism, present in 23% of BPH cases, followed by *E. coli* and other bacteria [137].

It was proposed that *P. acnes*-triggered inflammatory events in the prostate might be similar to the pathogenesis of acne vulgaris [138]. *P. acnes* could colonise the acinar lumens that might result in attraction and stimulation of nearby immune cells, similar to processes in inflammatory acne. Interestingly, people with a history of acne tend to have a higher risk of prostate cancer compared to healthy individuals [139]. However, there are contradictory studies; one study revealed an inverse correlation between acne and prostate cancer [140]. Recently, a study by Severi *et al.* (2010) showed that high titres of *P. acnes* antibodies correlate with a decreased risk of prostate cancer [141]. The authors suggested that the increased antibody concentration could be an indirect sign of *P. acnes* induced inflammation response and protects against prostate cancer.

In vitro studies using prostate epithelial cell culture infection models with *P. acnes* have been performed by Drott *et al.* (2010) and us (**Paper IV**)[142]. Induction of the inflammatory response by *P. acnes* is similar to the skin cell culture model (keratinocytes), where the secretion of IL-6, IL-8 and granulocyte macrophage colony-stimulating factor (GM-CSF) signals via TLR2 and NF-κB dependent pathways [142]. This *in vitro* study showed a potential strong inflammatory response towards *P. acnes* that might contribute to carcinogenesis. **Paper IV** describes this infection model, and the cancer-like signature of the responses towards this bacterium.

Prostate cancer has been a long battled disease and the causative factors are still not identified. A chronic bacterial or viral infection could certainly lead to an inflammatory microenvironment that could initiate or support carcinogenesis. This thesis aims at the characterisation of *P. acnes* as a possible contributing factor to prostate carcinogenesis.

1.5 Collective aims of thesis work

The **general aim** of this research work was to investigate the pathogenic potential of *P. acnes*. The first focus was directed at the bacterium itself by identifying its secretome and by genetic manipulation approaches (**Paper I and II**). The second focus was directed at the host cell responses towards *P. acnes* (**Paper III**). Finally, the clinical relevance of *P. acnes* regarding prostate pathologies was investigated (**Paper IV**) (**Additional section**).

Paper I

Secreted proteins of *P. acnes* strains, representing all four phylotypes, were identified using a combination of 2-DE PAGE and MALDI-MS. This provided a basis for further in-depth studies on individual proteins such as CAMP factors.

Paper II

A mutagenesis approach was established to study the biological function of single gene products of *P. acnes*.

Paper III

Host cell interaction of skin and prostate epithelial cells with *P. acnes* was investigated, including inflammatory responses, modulation of MAPK and NF-κB pathways, host cell specific invasion and the intracellular persistence of *P. acnes*.

Paper IV

An *in situ* immunofluoresence (ISIF) technique was established to detect the presence of *P. acnes* in prostate tissue samples of cancer patients and healthy men. This paper also reports the long-term effects of *P. acnes* infection of prostate epithelial cells.

Additional section: *P. acnes* and its potential involvement in prostate cancer

Bacteria were cultivated from human prostate biopsies and identified by 16S rDNA sequence analysis. Further subtypes were identified using MLST genotyping.

2 Material and Methods

This section contains information on some general methods used for the work reported in paper I to IV. For further detailed information and other methods, please see attached papers. In the **additional section**, the methods were performed in Johns Hopkins School of Medicine, Baltimore, USA.

2.1 Paper I - IV

Bacteria and cell cultures

Propionibacterium acnes were cultured on Brucella agar plates for 3 days at 37^{o}C under anaerobic condition. For liquid cultures, brain heart infusion broth (BHI, Sigma-Aldrich) was used.

Table 2.1: *P. acnes* wildtype and mutant strains

Strain	Isolation site	Stock collection #	Type
329	Acne	X522	II
487	Post operative prosthetic joint infection	X523	III
266	Pleuropulmonary infection	X521	IA
P6	Cancerous prostate	X524	IB
KPA	Skin	X520	IB
	Mutants		**Gene**
KPA Δ*camp2*	CAMP factor 2	H3919	PPA0687
KPA Δ*camp4*	CAMP factor 4	H3920	PPA1231

The human keratinocyte cell line HACAT (CLS) was cultured in DMEM medium (Gibco) supplemented with 10% heat-inactivated FCS. The human prostate epithelial cell line RWPE1 (ATCC CRL-11609) was cultured in keratinocyte medium supplemented with 50 µg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Gibco).

Infection of epithelial cells

Individual *P. acnes* strain at various multiplicity of infection (MOI) were co-incubated with the cells in a humidified 5% CO_2 atmosphere at 37 °C. Infections were stopped at various times, i.e. after 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h and 72 h. For long-term infections (7 - 21 days), media were changed every third day and cells were split once a week.

Immunofluorescence

RWPE1 and HaCaT cells were grown on 12-mm coverslips. After infection, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Cells were then stained by permeabilization with 0.2% Triton X-100 for 15 min and blocking with 0.2% bovine serum albumin (BSA, Biomol) in PBS for 10 min at room temperature. Primary antibodies were incubated for 1 h, followed by a detection step using Cy2- or Cy3-conjugated anti-rabbit/anti-mouse IgG secondary antibodies (1:150, 1 h; Jackson Immunosearch). Actin was stained with Phallodin (Invitrogen). Nuclei were stained with Draq5 (Cell Signaling). Coverslips were mounted in Mowiol and analysed by confocal laser scanning microscopy using a Leica TCS SP and an Epi-fluorescence microscope. Images were taken using appropriate excitation and emission filters for the fluorescent dyes used. Overlay images of the single channels were obtained using Adobe Photoshop.

Western Immunoblotting

Cells samples were lysed in SDS-Laemmli buffer and denatured at 95^{o}C. Resulting protein extracts were separated for about 1 h at 120 V using a Mini Protean III system (Biorad). PVDF membranes were activated with methanol. Gels were semi-dry blotted and then blocked for 1 h in BSA blocking buffer. Blots were probed with primary antibodies overnight. Blots were then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham). Subsequently, ECL reagents (ICN) were added on blots and developed with sensitive photographic films. Blots were scanned and quantified using ImageJ software.

Table 2.2: Primary and secondary antibodies

Antibody	Origin	Working dilution	Supplier
Primary antibodies			
Anti *P. acnes*	Mouse	1:2000 (IF)	In-house
Phospho-JNK	Rabbit	1:1000 (WB)	Cell Signaling
Phopho-p38	Rabbit	1:1000 (WB)	Cell Signaling
Phosphor-ERK1/2	Mouse	1:1000 (WB)	Sigma-Aldrich
Ικβα	Rabbit	1:1000 (WB)	Cell Signaling
Vimentin H84	Rabbit	1:1000 (WB)	Santa Cruz
Vimentin V9	Mouse	1:200 (IF)	Invitrogen
Anti-GFP	Mouse	1:2000 (WB)	Roche
p65	Rabbit	1:50 (IF)	Santa Cruz
β–actin	Mouse	1:5000 (WB)	Sigma-Aldrich
Secondary antibodies			
Cy™ 2 conjugated anti-mouse IgG	donkey	1:150 (IF)	Jackson ImmunoResearch
Cy™ 2 conjugated anti-mouse IgG	goat	1:150 (IF)	Jackson ImmunoResearch
Cy™ 3 conjugated anti-mouse IgG	donkey	1:150 (IF)	Jackson ImmunoResearch
Cy™ 3 conjugated anti-mouse IgG	goat	1:150 (IF)	Jackson ImmunoResearch
ECL™ anti-mouse IgG, HRP-linked	sheep	1:2000 (WB)	GE Healthcare
ECL™ anti-rabbit IgG, HRP-linked	donkey	1:2000 (WB)	GE Healthcare

Antibiotic Protection Assay

In order to quantify bacterial entry into host cells, an antibiotic protection assay was performed. Both HaCaT and RWPE1 cells were seeded onto 24-well plates prior to infection. At 24 h p.i., 30 µg/ml of streptomycin/penicilin (Invitrogen) was added for 2 h to kill extracellular bacteria. Cells were washed and 1% saponin (Sigma) was added to permeabilise cells, followed by plating out appropriate dilutions of lysate on Brucella agar. Assays were performed in duplicates. Each experiment was repeated at least three times. Data were tested for significance using unpaired t test (GraphPad Prism).

Core Facilities

Several experiments were performed in collaboration with the Core Facilities at the Max Planck Institute for Infection Biology, Berlin, Germany. 2D-Gel Electrophoresis and MALDI-MS experiments were performed together with the Proteomic Core Facility. Microarray experiments were performed together with the Microarray Core Facility. Tissue microarray staining experiments was performed together with the Microscopy Core Facility. For all detailed experiments, please refer to the attached papers.

2.2 Additional section: *P. acnes* and its potential involvement in prostate cancer

Growth of bacteria from prostate biopsies

A total of 20 prostate cancer cases were selected in this study. Biopsy samples were collected using a Biopty gun (Bard Peripheral Vascular) and transfered into 2ml of PBS. The prostate biopsies were then minced into small pieces using a sterile razor. They were equally distributed for aerobic and anaerobic culturing. For aerobic culturing, biopsies were incubated in Lucia Bertani (LB) broth (BD) at 37^{o}C in a shaking incubator (200 rpm). For anaerobic culturing, samples were incubated in BHI in an anaerobic pouch (GasPak EZ Gas from BD) at 37^{o}C for 5-10 days. The study protocol was developed under a Johns Hopkins Internal Review Board approved protocol.

Identification of bacteria from prostate tissue biopsies

Bacterial genomic DNA was isolated using a modified protocol for DNA extraction for Gram-positive bacteria (QIAamp DNA mini kit). 16S primer Eco9R and Loop27 were used for PCR amplication. PCR conditions were 94^{o}C for 2 min, 35x cycle of 94^{o}C for 30s, 53^{o}C for 30s and 72^{o}C for 1 min and 72^{o}C for 5 min. All PCR products were analysed by electrophoresis on a 1% (w/v) agarose gels. Purified PCR products using Qiagen PCR purification kit were sent for sequencing to the BioCore Store (Johns Hopkins School of Medicine). Gene sequences were compared to sequences of the NCBI database using BLAST.

Multi Locus Sequence Typing (MLST) scheme of *Propionibacterium acnes*

MLST scheme was developed by Lomholt and Kilian (2010), relying on 9 housekeeping genes [9]. In short, PCR conditions for amplifying all the housekeeping genes were: 96^{o}C for 40s, 35x cycle of 94^{o}C for 35s, 55^{o}C for 40s and 72^{o}C for 40s and 72^{o}C for 7 min. PCR condition for *recA* was different: 95^{o}C for 3min, 35x cycle of 95^{o}C for 1min, 55^{o}C for 30s and 72^{o}C for 90s and 72^{o}C for 10 min. PCR products were purified using the Qiagen PCR purification kit prior to sequencing. The MLST analysis of the sequence was performed using the following website: http://pacnes.mlst.net/#.

Table 2.3: Primers used for MLST analysis and 16S rDNA amplifcation

Locus	Primer sequences
cel	F-5’ GCC GAC GTT TTC TAC AGT GAG C 3’
	R-5’ GGC GGT GAG GGT CCA TTC A 3’
coa	F- 5’ GCG GGA ATC GAG GGT GCT A 3’
	R-5’ AGG GCC GCC GCT AGA TAA GTA 3’
fba	F-5’ AGG ACC CGC TAT TTC AAC TCT CA 3’
	R-5’ ACG CGG GTC GTA CAT CTT CTT 3’
gms	F-5’ CCG CCT CAC CGT CCA GCA 3’
	R-5’ CAC ATC GAG AAC CGC ATC ACTC 3’
lac	F-5’ GCC GCA GCC TTG GGA CTC T 3’
	R-5’ GAA ATG CTG TCG CCC CGT G 3’
oxc	F-5’ GTG CTG CCG GAA AAG TCG 3’
	R-5’ CAC CGG CGT CAG GAT TGT 3’
pak	F-5’ CGACGC CTC CAA TAA CTT CC 3’
	R-5’ GTC GGC CTC CTC AGC ATC 3’
recA	F-5´AGCTCGGTGGGGTTCTCTCATC 3´
	R-5´GCTTCCTCATACCACTGGTCATC 3´
zno	F-5’ CGC CGG CAT CAC CAC CTA TT 3’
	R-5’ TCT CAC ATC GCC CGC AAC C 3’
16S	Eco9 F-5’GAG TTT GAT CCT GGC TC AG 3’
	Loop27 R-5’ GAC TAC CAG GGT ATC TAA TC 3’

3 Results and Discussion

The thesis work aimed to investigate the human host cell modulating properties of *P. acnes*. In **paper I**, we identified secreted factors of *P. acnes* in four different phylotypes; identified proteins include Christie-Atkins-Munch-Petersen (CAMP) factors and human tissue-degrading enzymes. In **paper II**, we successfully established an insertional mutagenesis approach for *P. acnes* to create knock-out mutants. In **paper III**, we reported fundamental differences in inflammatory response of skin and prostate epithelial cells upon *P. acnes* infection. In **paper IV**, we have detected *P. acnes* in cancerous prostate tissue samples and showed that *P. acnes* has the potential to partially transform prostate epithelial cells. In the **additional section**, we isolated bacteria from human prostate biopsies and employed MLST genotypic scheme to identify the isolated *P. acnes*.

3.1 Paper I

In **paper I**, five *P. acnes* strains were chosen for secretome analysis. Each strain represents a different subtype: IA, IB, II and III. All identified secreted proteins were assigned to the reference KPA genome (a type IB strain).

A group of 20 proteins was identified to be secreted in at least three out of the five strains. These include eight proteins secreted by all strains (Figure 2 in **paper I**). The P6 strain (type IB) secreted all 20 proteins, whereas KPA, 266, 329 and 487 strains secrete 19 (95%), 15 (75%), 15 (75%) and 12 (60%) of these proteins respectively.

Five highly similar Christie-Atkins-Munch-Petersen (CAMP) factors (PPA687, PPA1198, PPA1231, PPA1340 and PPA2108) are encoded in the *P. acnes* KPA genome. CAMP factors are mainly found in *Streptococcal* species as putative pore forming toxins, which have co-hemolytic activity [32][7]. The CAMP reaction describes sheep erythrocytes hemolysis by the CAMP factor of *Streptococcus agalactiae* and sphingomyelinase (SMase) from *S. aureus* [143]. The SMase hydrolyses sphingomyelin on the erythrocyte membranes, causing the cells to be more susceptible to the hemolytic activity of the CAMP factors [32].

P. acnes synergistically enhances hemolysis in the presence of β-hemolytic microorganisms (e.g. *S. aureus* and *Clostridium perfringens*), in accordance with the

demonstrated classical CAMP reaction [144][145]. The CAMP factors were further characterised as pathogenic determinants with lethal effects when administered to rabbits and mice [33]. In our secretome data, all *P. acnes* secreted CAMP2 (PPA0687) while the KPA strain exclusively secreted CAMP4 (PPA1231). The other three CAMPs were not detected in any strain, which contradicted a previous report that showed an abundance of CAMP1 by western blotting using different anti-CAMP sera [7]. This discrepancy could be explained due to fact that immunoblotting is a more sensitive approach, whereas our 2-DE-PAGE approach only detected the most prominently secreted factors.

Two different type IB strains (KPA and P6) were used for this secretome analysis. The KPA strain is derived from skin while P6 was isolated from cancerous prostate tissue. As expected, both strains share a higher degree of similarity with each other in comparison to the other three strains. Nevertheless, we identified a few prominent differences between KPA and P6. KPA secreted both CAMP4 and CAMP2 while P6 exclusively secreted CAMP2. Another distinct difference is the protein of PPA1880; it was secreted exclusively by P6. It has no homology to the other proteins. By PCR analysis, we observed that PPA1880 possesses a phase variation-like signature: a stretch of guanine residues located within the putative promoter region of PPA1880. Variable numbers of guanine residues existed in these strains (11, 13 and 15 nucleotides in P6, KPA and 266 strains respectively) (Figure 3 in **paper 1**). The length of the guanine stretch determines the length of the spacer region of the putative promoter; this might explain the P6 specific expression of PPA1880.

Various studies suggested the opportunistic nature of *P. acnes*. The identification of secreted factors is important to further investigate *P. acnes*' pathogenicity. Thus, our study has highlighted different secreted factors such as human tissue-degrading proteins, adhesins, hemolysins and others that might contribute to the virulence of *P. acnes.* This secretome analysis has also revealed phylotype differences and even between strains from the same phylotype. In the next step, we employed a mutagenesis approach to knock-out abundant secreted factors (especially CAMP factors) in order to study their properties (**Paper II**).

3.2 Paper II

To date, *P. freudenreichii* and *P. acidipropioni* are the only bacteria in the *Propionibacteria* genus that have been successful transformed. **Paper II** employed and modified two published transformation protocols [146,147]. Our method resulted in the generation of two knock-out mutants of *P. acnes,* targeting the CAMP factors, CAMP2 and CAMP4. In **paper I**, we have shown that CAMP2 and CAMP4 are abundantly secreted factors from *P. acnes* KPA strain. Thus, we asked whether the co-hemolytic reaction of *P. acnes* differed between wildtype and mutant strains

All phylotypes of *P. acnes* strains were tested positive for a co-hemolytic reaction on sheep blood agar [7]. It might be possible that this reaction is a result of the activity of all five CAMP factors of *P. acnes*. Since both CAMP2 and CAMP4 are abundant in KPA, we raised the question whether deletion of both genes would abrogate co-hemolytic properties of *P. acnes*. Supernatants of wild-type and mutant *P. acnes* strains were harvested and co-incubated with sphingomyelinase-pretreated sheep red blood cells. In comparison to the wild-type strain, a reduced co-hemolytic activity could be observed for the Δ*camp2* but not for the Δ*camp4* mutant (Figure 4 in **paper II**). However, there was still co-hemolytic activity in Δ*camp2*, suggesting that other CAMP factors also contribute to the CAMP reaction. In addition, *camp2* and *camp4* were recombinantly expressed: GST-CAMP2/4 fusion proteins. GST-CAMP2 showed a stronger co-hemolytic activity than GST-CAMP4, indicating again a more profound role of CAMP2 as co-hemolysin.

For the first time, an insertional mutagenesis strategy was developed for *P. acnes* to inactivate specific genes. This method will facilitate the identification and characterisation of *P. acnes'* virulence factors and host interacting factors and at the same time will contribute to future understanding of the interaction of this opportunistic bacterium with human host cells.

3.3 Paper III

P. acnes is ubiquitously present on human skin but yet the biological role of *P. acnes* has not been well-studied. The association of *P. acnes* with acne vulgaris has been well accepted. However, *P. acnes* involvement in diseased prostates is still obscure. Several reports confirmed the association of this bacterium with prostatic tissue by a variety of detection methods, including bacterial cultivation, PCR-based approaches and fluorescence *in situ* hybridisation (FISH) [133,136,148]. In order to gain a better understanding of host cell responses to this bacterium, two infection models using skin-derived keratinocytes (HaCaT cells) and prostate-derived epithelial cells (RWPE1 cells) were established. These cell lines exhibit immortalized but non-tumorigenic characteristics. The infection of HaCaT cells would mimics the situation on the skin, while infected RWPE1 cells imitate the possible scenario of *P. acnes* in prostatic glandular tissues.

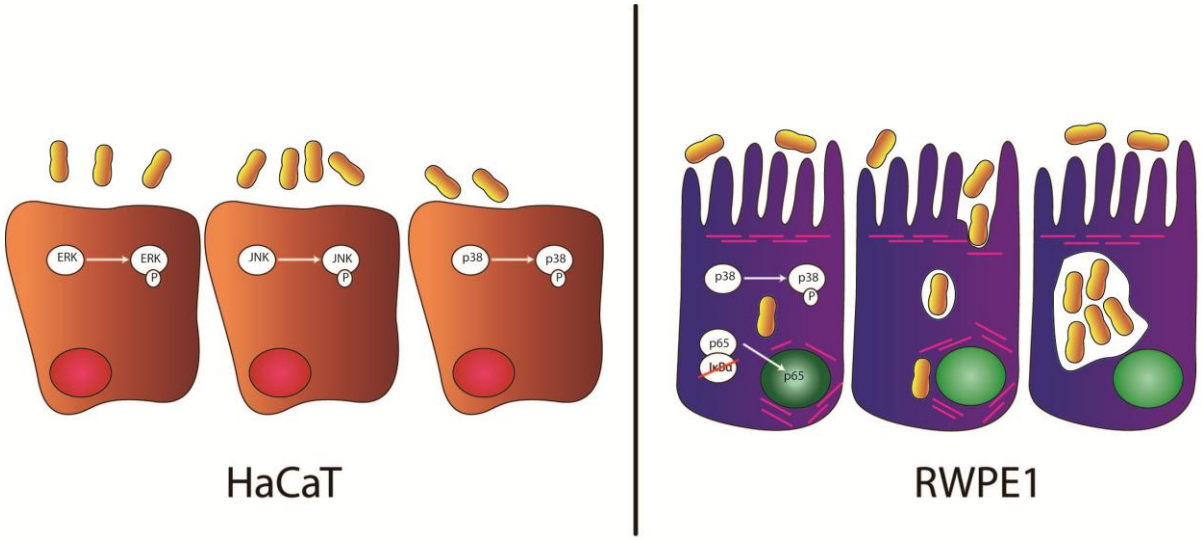

Figure 3.1: Model of *P. acnes*-infected skin and prostate cells, summarising the main results of Paper III. In HaCaT cells, MAPK pathways were activated by extracellular *P. acnes*. In contrast, both NFKB and MAPK pathways were incited by extra- and intracellular *P. acnes* in RWPE1 cells. Vimentin (as indicated as pink bars) is a crucial component of the cytoskeleton which facilitates the invasion process.

Two distinct cell lines (HaCaT and RWPE1) showed fundamental differences regarding the host cell interaction with *P. acnes*. Figure 3.1 shows a model summarising the major findings presented in **paper III** on host cell responses to *P. acnes*. We observed a

stronger upregulation of many inflammation-associated genes in HaCaT in the first 24 h of infection compared to RWPE1. A possible explanation is the higher responsiveness of *P. acnes* in HaCaT cells mediated by TLRs which led to the activation of MAP kinases ERK, JNK and p38 (Figure 2 in **paper III**). This activation of MAPK is in agreement with previous findings [100].

A persistent infection scenario was imitated by the long-term infection of cell cultures (up to 7 days). The consequences of long-term infection differed strongly: RWPE1 cells could be persistently infected with *P. acnes,* as the bacterium was able to invade into the prostate epithelial cells resulting in an inflammatory response compared to HaCaT. This was most likely due to continuous activation of NF-κB, as judged from NF-κB inhibitor (Iκβα) degradation and p65 translocation into the nucleus (Figure 3 in **paper III**).

The existence of *P. acnes* as intracellular bacteria is not well characterised. Several studies have shown the presence of intracellular *P. acnes* in various cell lines [99,149]. In our study, *P. acnes* was still detected in RWPE1 at 21 days p.i. These bacteria are able to survive intracellularly in these host cells suggesting that it can be responsible for persistent infection *in vivo*. However, the mechanism of *P. acnes* invasion into cells remains unclear.

Intracellular pathogens (e.g. *Shigella flexneri*, *Porphyromonas gingivalis* and *Toxoplasma gondii*) can harness the host cell's cytoskeleton like actin and microtubule to facilitate the invasion process [150–152]. Using actin inhibitors (cytochalasin B and D) and microtubules inhibitors (nocodazole and colcemide), we could show that *P. acnes'* invasion into RWPE1 was partially dependent on a functional cytoskeleton (Supplementary Figure S5 in **paper III**). Since actin and microtubules are both present in HaCaT and RWPE1 cells, we hypothesised that another cytoskeleton component could set this major difference of bacterial invasion. We found that vimentin is strongly expressed in RWPE1 but little or not at all in HaCaT (Supplementary Table S1 in **paper III**).

The intermediate filament, vimentin, is part of the cell cytoskeleton that provides the cell integrity in mesenchymal cells. It is expressed in a wide range of cell types such as

fibroblasts, endothelial cells, macrophages, neutrophils, leukocytes and renal stromal cells [153]. In addition, vimentin is commonly used as a marker of the epithelial mesenchymal transition (EMT). Thus, vimentin overexpression was reported in various epithelial cancers including prostate cancer [154]. Vimentin is absent from keratinocytes, which are of ectodermal origin; this is in accordance with the present study [155]. The prostate epithelial cells, RWPE1 are derived from the endoderm; although their lineage dependence has not been unequivocally determined, it was excluded that they were derived from endothelial cells [156]. A recent study reported that mesenchymal (vimentin, CDH2) and epithelial cadherins (CDH1) were co-expressed in RWPE1 cells, indicating that these cells might have undergone a partial EMT [157]. In addition to RWPE1 cells, other cell lines are vimentin-positive, including A549(adenocarcinomic human alveolar basal epithelial cells) and HEK293T(human embryonic kidney cells). Interestingly, these cell lines were reported to be invaded by *P. acnes*, supporting our findings that vimentin is a crucial component for bacterial invasion [158,159].

Viruses and bacteria can modulate vimentin to establish an intracellular niche and for the optimal positioning of bacteria-containing vacuoles. The African swine fever virus initiated vimentin re-arrangement to form a cage surrounding viral factories [160]. Similarly, *Chlamydia trachomatis* also recruited several cytoskeleton components of the host cells, including vimentin to form a dynamic scaffold that provided structural stability to the inclusion [161]. Vimentin has been found on the extracellular surface of various cells. Bacteria like *E. coli* interact with the surface exposed vimentin to gain entry into human brain microvascular endothelial cells [162]. A vimentin antibody neutralising assay points towards a role of vimentin as cell surface associated mediator of *P. acnes* invasion.

To further investigate the presence of vimentin in human tissue, staining was performed on human skin and prostate tissue samples. Vimentin was absent on skin epithelial cells (Figure 3.2A). Interestingly, vimentin was present on the prostate epithelium, at least in some cases, strong vimentin staining was observed surrounding the prostate epithelium of the lumen in a BPH case (Figure 3.2C).

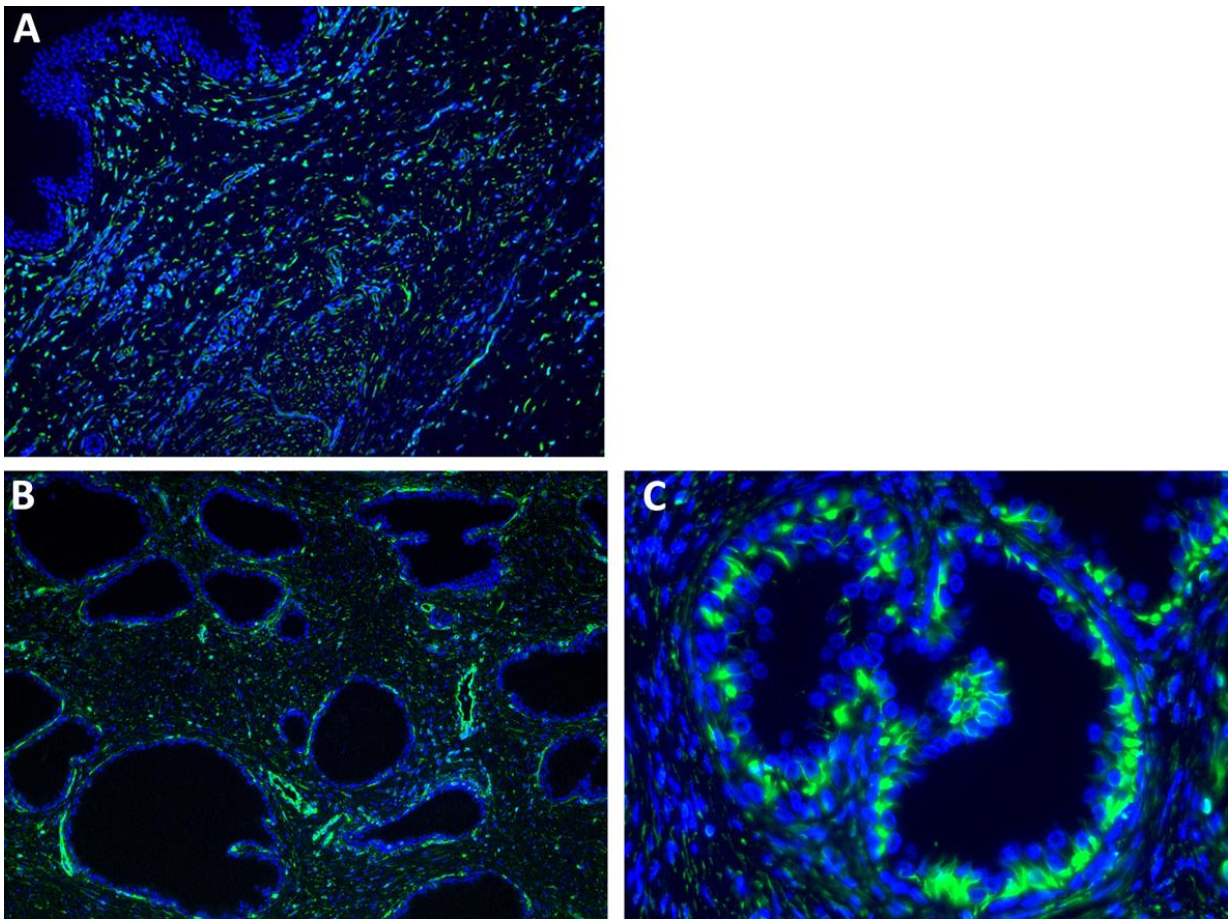

Figure 3.2: Vimentin expression in human tissue. Presence of vimentin exclusively on stroma cells of skin tissue (A). Only stroma cells and blood vessels express vimentin in this PCa tissue sample (B). Strong vimentin expression in prostate epithelium surrounding the gland in this BPH tissue sample (C).

Our data in **paper III** contributed to the growing body of evidence that *P. acnes* can lead to persistent deep tissue infections. Here, we have demonstrated fundamental differences in host cells responses to *P. acnes* in two distinct cell types; a sustained inflammatory response was observed in the prostate-derived cells, whereas an acute but transient response was observed in HaCaT cells. Since vimentin is strongly expressed in prostate cancer, our findings suggest that *P. acnes* could act as an opportunistic invader in cancerous prostate tissue and could contribute to disease progression due to its inflammation stimulating properties.

3.4 Paper IV

In **paper IV**, we developed an *in situ* Immunofluoresence (ISIF) method to detect *P. acnes* in prostate tissue samples. Tissue microarrays (TMAs) were used, which contained samples of normal and diseased prostate. The diseased samples include prostate adenocarcinoma (ADC), prostatic intraepithelial neoplasia (PIN) and benign prostatic hyperplasia (BPH) from patients aged 51 to 77 years. We detected *P. acnes* in all 12 PIN samples, in 46 out of 59 ADC samples and in 6 out of 9 BPH samples. In total, 82% (58 out of 71) prostate tissue samples were positive for *P. acnes*. The bacteria were usually found almost exclusively in the secretory epithelium of the glandular lumen. In contrast, no bacteria were detected in 10 normal prostate samples and 10 tissue samples originating from sites adjacent to prostate tumours (Table 1 in **paper IV**). Intriguingly, our ISIF approach detected *P. acnes* in diseased prostates with a higher frequency compared to other studies [133,136]. The varying frequencies can be explained with different detection methods used.

De Marzo *et al.* (2007) published a review about the link between inflammation and prostate cancer; it was suggested that inflammation triggered by infectious agents could cause or support cancerous transformation in the prostate [127]. In our study, we showed that *P. acnes* infection in vitro could induce a strong inflammatory response in prostate epithelial cells; this response had many molecular similarities with the signature of prostate cancer, i.e. the modulation of NF-κB, IL-6 Stat4 and COX2-PGE2 pathways. These pathways have been heavily discussed as hallmarks of tumorigenesis in inflammation-associated cancer [163].

Cell transformation is a process characterised by increased invasion, migration and anchorage independent growth of cells, usually resulting in their immortalisation. Transformation of non-tumorigenic RWPE1 cells has been reported by Achanzar *et al.* (2001); cells transformed upon treatment with cadmium, a carcinogenic substance for 8 weeks [164]. In our study, a wound healing assay showed 3 weeks infected RWPE1 cells closed the wound faster than non-infected cells. This effect was abrogated when cells were pre-treated mitomycin C, a cell division inhibitor, indicating that increased proliferation was responsible for this effect rather than cell migration. The second experiment employing soft

agar assays revealed anchorage independent growth of 3 weeks infected cells (Figure 5 in **paper IV**). Taken together, increased proliferation and anchorage independent growth of prostate epithelial cells upon long term infection indicate the initiation of cellular transformation.

Paper III reported intracellular presence and persistency of *P. acnes* in RWPE1 cells. Future experiments should aim to determine whether certain subtypes of *P. acnes* are predominantly found in the prostate gland and/or initiate cell transformation in prostate cells. It is possible that the severity of prostate disease could depend on presence of certain *P. acnes* types, as distinct strains exhibit differential ability in secreting putative virulence factors which might elicit pro-inflammatory responses from the host cells(**Paper I**)[103].

3.5 Additional section: *P. acnes* and its potential involvement in prostate cancer

The following section focuses on the potential link between *P. acnes* and prostate cancer. Part of the work reported here was performed during a research stay in the research laboratory of Prof. Dr. Angelo De Marzo at the Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America.

Due to its ubiquitous presence on human skin, the identification of *P. acnes* in human tissue samples has often been regarded as a skin-derived contamination. However, a recent study has shown that prostate isolates of *P. acnes* are phylogenetically distinct from skin types. Skin isolates are usually type IA, while prostate isolates were identified as types IB and II. Shannon *et al.* (2006) suggested that these prostate isolates originated from the urinary tract since similar *P. acnes* types can be found there [148].

Prostate biopsies were taken from 20 prostate cancer patients, processed and further incubated in both aerobic and anaerobic conditions. From 10 out of 20 cases bacteria could be cultivated; *P. acnes* was the predominant cultivated microorganisms (6 samples) followed by *P. avidum*, *P. granulosum* and *S. epidermis*. This indicates that *Propionibacteria* are often present in human prostates. We did not manage to culture any aerobic bacteria (except for *S. epidermidis*) mentioned in a previous study [165]. The

possible explanation is that prior to radical prostatectomy, the patients were treated with antibiotic to prevent post surgery infections. Those bacteria, which developed resistance towards antibiotics, might have survived the treatment. Several studies identified antibiotic resistant strains of *P. acnes* [51,166,167]..

Table 3.1: Bacteria isolated from prostatic tissue of 20 patients with prostate cancer. In a single case, 2 different bacteria (*P. acnes* and *P. avidum*) were isolated from a single sample.

Bacteria	Number of cases	Percentage %
Propionibacterium acnes	6	30
Propionibacterium avidum	2	10
Propionibacterium granulosum	1	5
Staphylococcus epidermidis	1	5
Total	**10**	**50**

We employed the MLST scheme of Lomholt and Kilian (2010) to type six *P. acnes* isolates. Two of the isolates were the sequence types (ST) 36 and 38, which belong to type IB, while the others were typed II strains (ST 51, 52 and 53) (Figure 3.3). As reported in Lomholt and Kilian (2010), most *P. acnes* isolates from healthy and acne patients were type IA strains and isolates from deep tissue infections (e.g. tissues or blood) were types IB, II and III [9]. Our data is in accordance with a previous study showing identical subtypes, which are distinct from skin isolates. These findings oppose the argument of skin-derived contamination during or after radical prostatectomy or biopsy handling.

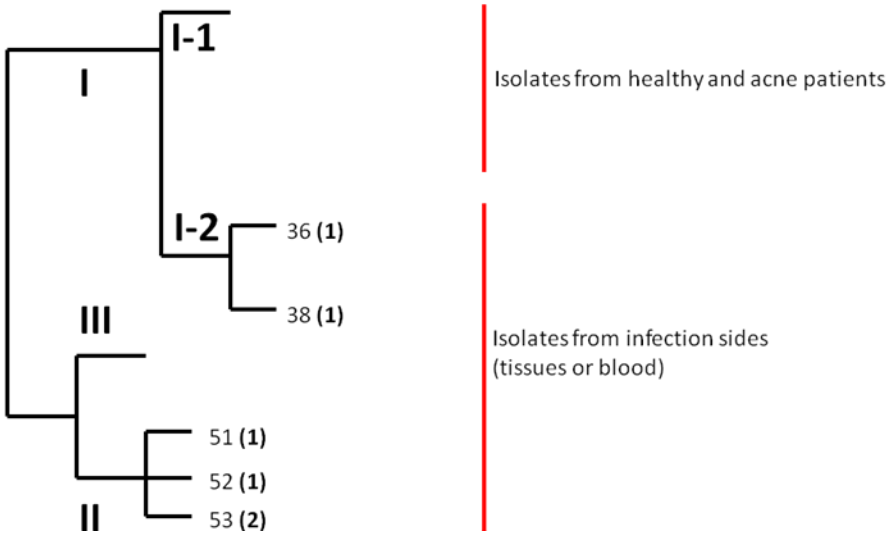

Figure 3.3: Population structure of isolated *P. acnes* based on MLST Aarhus Scheme. Five different sequence types (36, 38, 51, 52 and 53) detected among six *P. acnes* isolate from prostate tissue. The number in bracket indicates number of *P. acnes* isolate. The right panel depicts *P. acnes* isolates from Lomholt and Kilian (2010). Majority of cutaneous isolates are typed I-1/IA with the exception of typed II, which can be isolated from the skin but in a smaller proportion.

An interesting question is how *P. acnes* enters the prostate gland. There are several possibilities employing both iatrogenic and biological ways. During surgery or preceding bladder catherisation, *P. acnes* could be introduced to the prostate gland[168]. Another possible route is from the urethra where *P. acnes* is a frequent isolate [148]. Finally, it could invade macrophages at some parts of human body and hitch a ride to the prostate gland. Interestingly, persistency of *P. acnes* was reported in murine macrophages [169]. Further epidemiological experiments should be performed to strengthen the association of *P. acnes* and prostate inflammation.

4 Conclusions and Perspectives

The human skin microbiome revealed that *P. acnes* is the dominant bacterium in sebaceous rich areas, which is in accordance with classical microbiological studies that isolated *P. acnes* from the pilosebaceous unit [170]. Several lines of evidence show that *P. acnes* can act as an opportunistic pathogen and studies have suggested that this bacterium has an etiological role in a number of inflammatory diseases due to its immuno-stimulatory activity. Recently, *P. acnes* has been detected in human prostate tissues, indicating a potential contribution of this bacterium to prostate carcinogenesis. The involvement of *P. acnes* in acne is generally well accepted, but the role of *P. acnes* as a pathogen in other diseases remains elusive.

The overall aim of this thesis was to investigate the pathogenicity of *P. acnes*. In **paper I**, the secretome of *P. acnes* was identified. In **paper II**, an insertional mutagenesis technique to knock-out putative virulence genes was established; Δcamp2 and Δcamp4 mutants were generated and then used for virulence studies such as the hemolysis assay. In **paper III**, *in vitro* studies were performed to investigate the host cell interaction of *P. acnes* with skin or prostate epithelial cells. We observed that the immune response related pathways MAPK and NF-κB were differently activated in the two cell lines; continuous NF-κB activation in prostate epithelial cells might facilitate the survival of intracellular *P. acnes*. Future studies should address the specific contribution of potential virulence factors. Our study showed that *P. acnes* is a facultative intracellular bacterium which requires the host protein vimentin to facilitate the bacterial entry into host cells. Intracellular bacteria probably interfere with many other aspects of host cell signalling in order to promote their survival. Further work will define vimentin-mediated uptake of *P. acnes* and to which extent *P. acnes* can manipulate host cell signalling. Dissecting the roles of host proteins such as vimentin and *P. acnes* virulence factors in the invasion process will allow a better understanding of the intracellular lifestyle of *P. acnes*.

We have shown that *P. acnes* can be detected in clinical human prostate tissue samples by immunostaining. Moreover, transforming activity of *P. acnes* was observed in prostate epithelial cells (**Paper IV**). It is interesting to determine the mechanisms involved in

host cell transformation by *P. acnes*. Specifically, the use of a xenograft approach might be helpful to confirm malignant transformation induced by *P. acnes*. Long-term infected cells have shown increased migration and anchorage independent growth *in vitro*. *P. acnes* prostate isolates have been found to be genotypically distinct from skin isolates based on MLST typing (**Additional section**). Whole genome sequencing of *P. acnes* prostate isolates should give a clearer picture of the specific genetic repertoire of the prostate-specific subtypes. The decoded genomes could reveal putative virulence genes specific to prostate isolate subtypes. Another question is whether *P. acnes* could be an initial cause of malignancy or contribute to disease progression. To address this question, transgenic adenocarcinoma of the mouse prostate (TRAMP) model could be used [171].

Taken together, our findings suggest that *P. acnes* could contribute to prostate carcinogenesis. Future studies need to confirm the presence of specific *P. acnes* subtypes in a wider range of prostate samples. As we continue to pursue this research topic of infection and cancer, these questions and factors will guide future research work dedicated to identify the etiology of prostate cancer.

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Publications: Paper I - IV

Paper I

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Proteomic identification of secreted proteins of *Propionibacterium acnes*

Carsten Holland[1], Tim N Mak[1], Ursula Zimny-Arndt[2], Monika Schmid[2], Thomas F Meyer[1], Peter R Jungblut[2], Holger Brüggemann[1*]

Abstract

Background: The anaerobic Gram-positive bacterium *Propionibacterium acnes* is a human skin commensal that resides preferentially within sebaceous follicles; however, it also exhibits many traits of an opportunistic pathogen, playing roles in a variety of inflammatory diseases such as *acne vulgaris*. To date, the underlying disease-causing mechanisms remain ill-defined and knowledge of *P. acnes* virulence factors remains scarce. Here, we identified proteins secreted during anaerobic cultivation of a range of skin and clinical *P. acnes* isolates, spanning the four known phylogenetic groups.

Results: Culture supernatant proteins of *P. acnes* were separated by two-dimensional electrophoresis (2-DE) and all Coomassie-stained spots were subsequently identified by MALDI mass spectrometry (MALDI-MS). A set of 20 proteins was secreted in the mid-exponential growth phase by the majority of strains tested. Functional annotation revealed that many of these common proteins possess degrading activities, including glycoside hydrolases with similarities to endoglycoceramidase, β-N-acetylglucosaminidase and muramidase; esterases such as lysophospholipase and triacylglycerol lipase; and several proteases. Other secreted factors included Christie-Atkins-Munch-Petersen (CAMP) factors, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and several hypothetical proteins, a few of which are unique to *P. acnes*. Strain-specific differences were apparent, mostly in the secretion of putative adhesins, whose genes exhibit variable phase variation-like sequence signatures.

Conclusions: Our proteomic investigations have revealed that the *P. acnes* secretome harbors several proteins likely to play a role in host-tissue degradation and inflammation. Despite a large overlap between the secretomes of all four *P. acnes* phylotypes, distinct differences between predicted host-tissue interacting proteins were identified, providing potential insight into the differential virulence properties of *P. acnes* isolates. Thus, our data presents a rich resource for guiding much-needed investigations on *P. acnes* virulence factors and host interacting properties.

Background

The Gram-positive skin commensal *Propionibacterium acnes* is ubiquitously present on human skin. It has been speculated that this bacterium contributes to healthy skin by deterring the colonization of severe pathogens [1,2]; however, it is most well known for its role in skin disorders such as *acne vulgaris* [3,4]. Acne, a multifactorial disorder related to the formation of comedones, hormonal stimulation, bacterial colonization and the host inflammatory response, is an extremely common condition affecting approximately 80% of adolescents. Despite intense research effort, the precise role of *P. acnes* in acne formation is still unclear [5-7].

In addition to acne, *P. acnes* has been frequently associated with a variety of inflammatory diseases, including prosthetic joint infections, shunt-associated central nervous system infections, endocarditis, sarcoidosis, endophthalmitis, osteomyelitis, allergic alveolitis, pulmonary angitis, acne inversa (alias hidradenitis suppurativa), and the SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome [8-10]. This bacterium is also a common isolate of prostatic glands from patients with prostate inflammation [11,12]. Interestingly, the role of *P. acnes* in the development of prostate

* Correspondence: brueggemann@mpiib-berlin.mpg.de
[1]Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany
Full list of author information is available at the end of the article

cancer through an inflammatory mechanism is currently a subject of much speculation [12-14].

The prevalence of *P. acnes* in the above-mentioned conditions suggests that this bacterium is an etiological agent of infection and that it possesses an elevated pathogenic potential. *P. acnes* has been shown to exhibit haemolytic and cytotoxic activities [15,16] as well as extensive immunostimulatory activity and complement activation [6,17-20]. *P. acnes* isolates differ in their virulence properties, such as in their ability to trigger production of proinflammatory cytokines/chemokines in infected keratinocytes [21,22]. The genetic basis for this has not yet been studied in detail. To date four phylogenetic groups of *P. acnes* have been described, designated types IA, IB, II and III, based on sequence differences in two genes, namely *recA* and *tly* [23,24].

Despite the apparent role of *P. acnes* in disease formation, information on putative pathogenic traits and antigenic substances of this bacterium is scarce. The complete genome sequence of a cutaneous type IB isolate of *P. acnes* (strain KPA171202) provided insights into the pathogenic potential of *P. acnes*, revealing numerous gene products with putative host tissue-degrading activities as well as predicted cell wall-associated and secreted proteins, the presence or activity of which might be involved in triggering host tissue inflammation [25]. Some of these proteins are differentially expressed among *P. acnes* isolates and were shown to be immunoreactive [26].

To shed light on the biological relevance of predicted genes from the genome sequence, we used a combination of two-dimensional electrophoresis (2-DE) and matrix-assisted-laser-desorption/ionization mass spectrometry (MALDI-MS) to identify proteins secreted by *P. acnes*. Isolates representing all four phylotypes were investigated. Several hydrolases and putative virulence factors were secreted by all strains tested. These factors are potential host-interacting factors, likely important in inflammatory responses to *P. acnes*, as observed in *acne vulgaris*. Thus, our data provide a basis to guide further in-depth studies on individual factors.

Results and Discussion

Choice of *P. acnes* strains

We selected five strains of *P. acnes* for analysis of their secreted proteins. These strains, representing all known *P. acnes* phylotypes, i.e. types IA, IB, II and III, were isolated from a range of tissue sites: a type II skin acne isolate (strain 329); a type III strain isolated from a postoperative prosthetic joint infection (strain 487); a type IA strain isolated from a pleuropulmonary infection (strain 266); and two type IB strains: a skin isolate for which the genome sequence is available (strain KPA171202; KPA); and an isolate from a cancerous prostate (strain P6).

2-DE-MALDI-MS analysis of *P. acnes* culture supernatants

To identify proteins secreted by *P. acnes* using proteome analysis, we cultured each strain under anaerobic conditions in brain heart infusion (BHI) broth, previously used for secretome analyses [27]. Growth curves were generated (data not shown) and culture supernatants were harvested in the mid-exponential phase. Precipitated proteins from supernatants were separated by 2-DE and Coomassie stained, generating reproducible secretomes of all five strains tested (Fig. 1A-E and additional file 1). All visible protein spots were analyzed by MALDI-MS and searched against the NCBI non-redundant database, which included (at the time of analysis) the genome sequence of the type IB strain KPA and the partial genome sequence of the type IA strain SK-137.

The identified proteins for each strain, with molecular weights, isoelectric points, Mascot scores and sequence coverage are listed in additional file 2. In total, 64, 63, 54, 30, and 28 protein spots for *P. acnes* strains 266, KPA, P6, 329 and 487, respectively, were unambiguously identified and assigned to database entries. Several proteins occurred in spot series, representing different protein species of the same protein. Post-translational modifications are a likely explanation, resulting in altered molecular masses and/or isoelectric points [28]. A few MS spectra originating from secreted proteins of strain 329 could not be assigned to any database entry (Fig. 1D, spots 39-41), indicating that these proteins are strain-specific. The inability to identify these proteins also reflects the absence of genome sequence data from type II and type III strains; only genome sequences from type I strains are currently available.

Twenty commonly secreted proteins of *P. acnes*

The identified proteins secreted by the five strains tested were assigned to the reference KPA genome (Fig. 2, additional file 2). A set of 20 proteins was secreted by at least three of the five strains, including eight proteins secreted by all strains (Table 1). All 20 proteins were secreted by the P6 strain, whereas 19 (95%), 15 (75%), 15 (75%) and 12 (60%) of these proteins were secreted by the KPA, 266, 329 and 487 strains, respectively. We cannot exclude, however, that proteins secreted at lower levels were missed by our approach, as the amount of secretion varied between the strains and the sensitivity of the Coomassie stain is limited to the 100 ng range.

All 20 proteins except one, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), carried a secretion signal in its N-terminus. *P. acnes* has a general secretion (Sec)/signal recognition particle (SRP) and a twin-arginine translocation (TAT) secretion system. Other secretion systems have not been reported for *P. acnes*, and rescanning of the genome sequence gave no indication that additional ones exist.

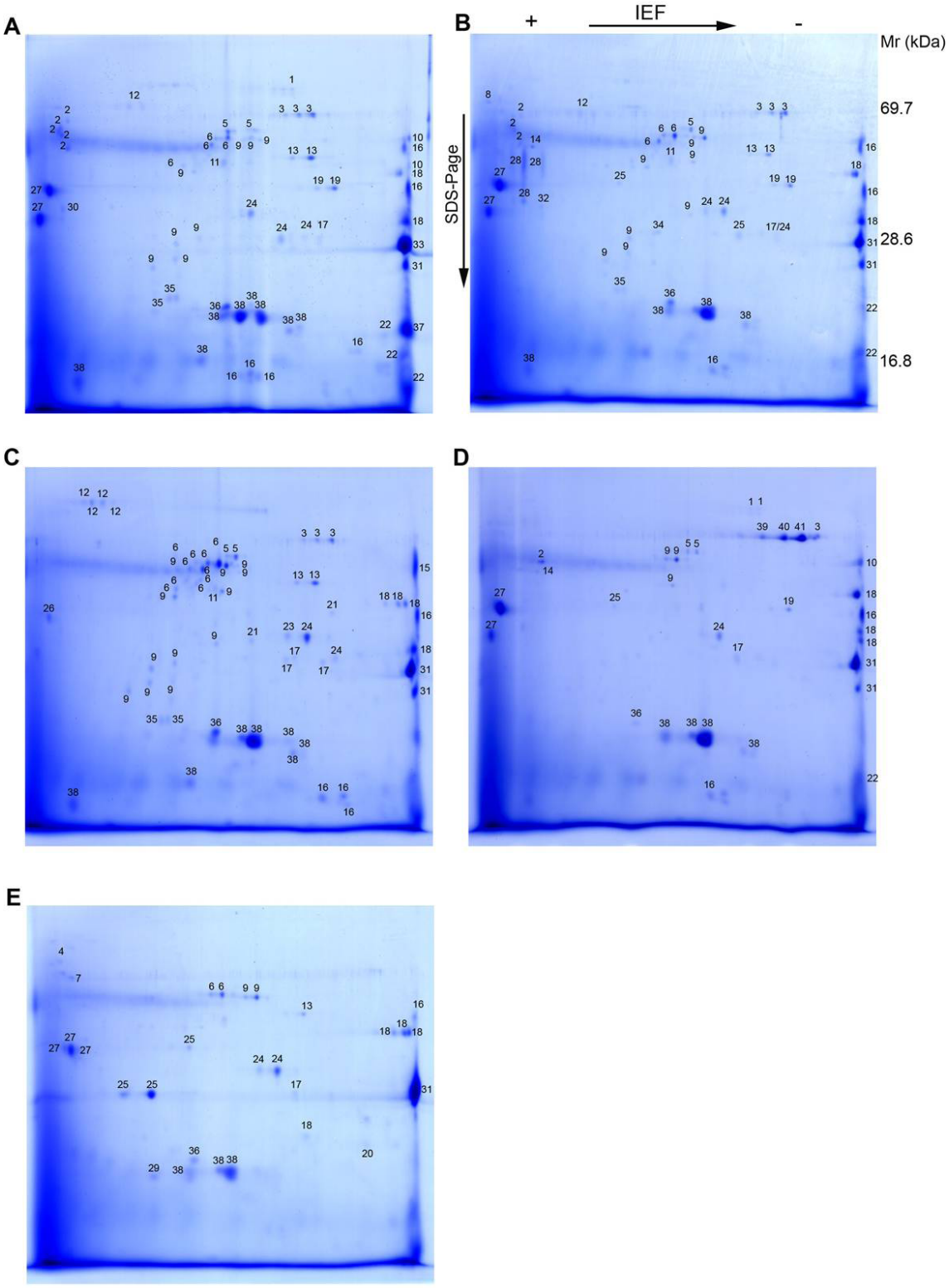

Figure 1 2-DE of *P. acnes* culture supernatants. Bacteria were grown in BHI medium to an OD_{600} of 0.6. Supernatants were harvested and precipitated. Protein samples (200 μg) from each strain were separated on 2-DE gels and visualized by staining with Coomassie Brilliant Blue G-250. The following strains were used: (a) KPA171202 (type IB); (b) P6 (type IB); (c) 266 (type IA); (d) 329 (type II); (e) 487 (type III). Information about the identified protein spots is provided in additional file 2.

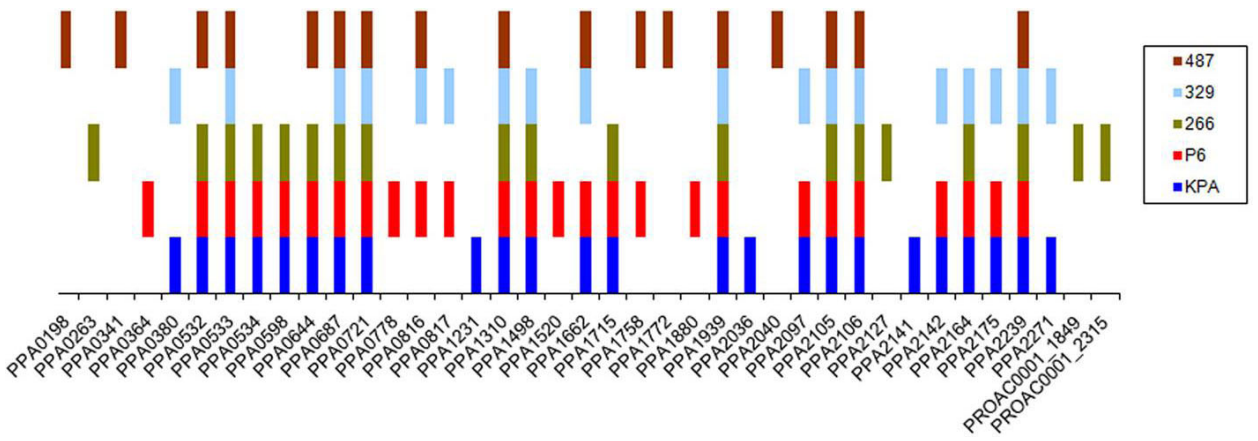

Figure 2 Distribution of secreted proteins in five *P. acnes* strains. The identified proteins in each strain were assigned to the gene nomenclature of the KPA genome (PPA numbers) and of the partial genome of SK137 (PROAC numbers).

Hydrolytic enzymes are secreted by *P. acnes*

To gain insight into the biological functions of the 20 commonly secreted proteins, re-annotation was performed based on similarity searches against protein sequence and protein-domain/-family databases (Table 1). Many of the secreted proteins were found to have predicted hydrolytic activities: two genes (PPA0644 and PPA2106) are predicted endo-glycoceramidases, sharing 42% identity on the protein level. Although their substrate specificities are unknown, PPA0644 and PPA2106 share 27% and 30% protein identity, respectively, with the characterized and structurally analyzed endo-glycoceramidase II from *Rhodococcus* sp., which hydrolyzes glycosidic linkages between the oligosaccharide and ceramide moieties of gangliosides [29]. Another secreted protein, PPA2164, a glycoside hydrolase family 3 protein, shares 31% identity on the protein level with NagZ (formerly YbbD) of *B. subtilis*. NagZ is a β-N-acetylglucosaminidase involved in the peptidoglycan recycling pathway; it cleaves the terminal non-reducing N-acetylglucosamine of muropeptides [30]. *P. acnes* also secreted a putative lysozyme (PPA1662) which is 47% identical on the protein level to the muramidase from *Streptomyces coelicolor*. This muramidase not only cleaves the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine units, but also exhibits β-1,4-N,6-O-diacetylmuramidase activity, enabling this enzyme to degrade *Staphylococcus aureus* cell walls [31]. Whether PPA1662 is an autolytic lysozyme involved in cell wall turnover has still to be elucidated. However, the peptidoglycan of *P. acnes* contains non-N-acetylated glucosamine residues and is therefore resistant to lysozyme [32]. We speculate that PPA1662 has a different substrate specificity, acting on non-N-acetylated peptidoglycan, or, alternatively, it acts as a defense system against competing bacteria on the skin.

Two strains, KPA and 329, secreted a hyalorunate lyase (PPA0380), confirming previous investigations on a *P. acnes* protein with hyalorunate lyase activity [33,34]. Preliminary functional characterization revealed that the enzyme exerted activity against chondroitin 4- and 6-sulphates but not against dermatan sulphate [33]. In accordance, the closest characterized homolog, the chondroitin lyase of *Arthrobacter aurescens* (37% protein identity to PPA0380) acts on chondroitin sulfate but not on dermatan sulfate [35]. Similar to other chondroitin lyases, it is capable of cleaving hyaluronan, a non-sulfated glycosaminoglycan and a major component of the extracellular matrix of connective tissues.

Consistent with the known lipolytic activity of *P. acnes* [36], we identified lipolytic enzymes in the secretory fraction, including the previously characterized triacylglycerol lipase, designated glycerol-ester hydrolase A (GehA; PPA2105). GehA is recognized as one of the virulence factors involved in the pathogenesis of acne [37-39], and is thought to be the main enzyme responsible for the hydrolysis of sebum triacylglycerides, resulting in the release of glycerol and free fatty acids. The released fatty acids are thought to be inflammatory; they favor ductal hypercornification and increase adhesion between *P. acnes* and cells of the hair follicle, promoting colonization of *P. acnes* and biofilm formation [37,40-42]. Furthermore, GehA itself is a strong chemotactic factor [43]. Other secreted esterases identified include a putative lysophospholipase (PPA2142) and a putative phosphoesterase (PPA1498) with unknown specificities. Proteases, another class of secreted hydrolases, were also detected, e.g. a peptidase S8/S53 family protein (PPA0598) among others; their substrate specificities remain to be elucidated.

Table 1 Twenty proteins constitute the common secretome of *P. acnes*

Gene ID	Updated annotation	Protein family/domains/origin of closest ortholog	Secretion signal[a]	Phylo-type	Semi-quantification[b]/ comments
PPA0532	conserved hypothetical protein	*Corynebacterium*	SP, TAT	IA, IB, III	++
PPA0533	conserved hypothetical protein	*Corynebacterium*	SP	IA, IB, II, III	+
PPA0534	conserved hypothetical protein	*Corynebacterium*	SP	IA, IB	+
PPA0598	putative protease	peptidase S8/S53 superfamily; *Arthrobacter*	SP, (TAT)	IA, IB	+
PPA0644	putative endo-glycoceramidase	cellulase, glycoside hydrolase family 5; *Corynebacterium*	(SP), TAT	IA, IB, III	++/42% identity to PPA2106
PPA0687	CAMP2	CAMP factor superfamily; *Mobiluncus curtisii, Streptococcus*	SP	IA, IB, II, III	+++
PPA0721	putative invasion-associated protein; NlpC/P60 family	NlpC/P60 family; *Kribbella flavida, Streptomyces*	SP, TAT	IA, IB, II, III	+++/NlpC/P60 is found in cell wall hydrolases
PPA0816	glyceraldehyde 3-phosphate dehydrogenase	GAPDH	no	IB, II, III	+
PPA1310	putative protease	PDZ superfamily; *Kribbella flavida, Streptomyces*	Internal SP (wrong N-terminus)	IA, IB, II, III	+/PDZ is a signaling domain
PPA1498	putative phosphoesterase	metallo-dependent phosphatase superfamily; *Rhodococcus*	TAT	IA, IB, II	+
PPA1662	putative lysozyme	CH-type (chalaropsis-type) lysozyme, glycoside hydrolase family 25; *Streptomyces*	SP, TAT	IB, II, III	+++
PPA1715	hypothetical protein, specific to *P. acnes*		SP	IA, IB	+/17 PT repeats
PPA1939	hypothetical protein, specific to *P. acnes*		SP	IA, IB, II, III	+++
PPA2097	putative 5'-nucleotidase, metallo-phosphoesterase	UshA (5'-nucleotidase/2',3'-cyclic phosphodiesterase and related esterases); *Jonesia denitrificans*	SP, TAT	IB, II	+
PPA2105	triacylglycerol lipase	lipase class 2, esterase/lipase superfamily; *Rhodococcus*	SP	IA, IB, II, III	++
PPA2106	putative endoglycoceramidase	cellulase, glycoside hydrolase family 5; *Nocardioides*	SP, (TAT)	IA, IB, II, III	+/42% identity to PPA0644
PPA2142	putative lysophospholipase	alpha/beta-hydrolase superfamily, PldB; *Corynebacterium*	SP, TAT	IB, II	+
PPA2164	putative beta-N-acetyl-glucosaminidase	glycoside hydrolase family 3; *Arthrobacter*	SP	IA, IB, II	++
PPA2175	hypothetical protein, with SH3 and RlpA domains, specific to *P. acnes*	SH3 (type 3) domain; peptidoglycan-binding domain; C-terminus: DPBB_1 (RlpA-like double-psi beta-barrel)	SP, TAT	IB, II	+++/SH3: Src homology 3 domain
PPA2239	putative peptidase/glycosyl hydrolase	DUF348 superfamily; G5 domain; C-terminus: DPBB_1 (RlpA-like double-psi beta-barrel); *Janibacter*	SP	IA, IB, II, III	++/G5: a potential N-acetylglucosamine recognition domain

At least three of the five *P. acnes* strains secrete the listed proteins; see additional file 2 for a complete list of identified proteins. Re-annotation was performed for each gene. [a] SP, signal peptide; TAT, twin-arginine motif; [b] Semi-quantification based on Coomassie stained gels

CAMP factors and other secreted proteins

A set of five highly similar *P. acnes* genes (PPA687, PPA1198, PPA1231, PPA1340, PPA2108) in the genome of *P. acnes* KPA encodes homologs to Christie-Atkins-Munch-Petersen (CAMP) factors, which are co-haemolytic proteins, found mainly in streptococcal species [25,44,45]. CAMP factors have been characterized as pathogenic determinants that exert lethal effects when administered to rabbits and mice [46]. In addition, streptococcal CAMP factors have been reported to act as pore-forming toxins [47]. In agreement with previous work [45], all *P. acnes* strains examined here were positive for the co-haemolytic CAMP reaction (data not shown). Our secretome data showed that all tested *P. acnes* strains secreted CAMP2 (PPA0687). In addition, the skin isolate KPA secreted CAMP4 (PPA1231).

Secretion of the other three CAMPs was not observed in any strain using our approach. A previous study reported variable production of CAMP factors in different *P. acnes* isolates, as detected by western blotting experiments using different anti-CAMP sera [45]; the authors reported an abundance of CAMP1 in type IB and II strains. We did not find CAMP1 among the secreted proteins; a discrepancy that could be due to the detection limits of the different techniques used, i.e. our MS analysis detects the most prominently secreted factors, whereas immunoblotting is a more sensitive technique.

A key enzyme of glycolysis, GAPDH, was also secreted by three out of the five *P. acnes* strains tested. At first glance it is peculiar why a glycolysis enzyme should be secreted; however, a number of studies have identified GAPDH as an anchorless, multifunctional protein, displayed on the surface of several fungi and Gram-positive pathogens, which contributes to adhesion and virulence [48,49]. In *Streptococcus pyogenes*, this cell-associated and soluble protein is also known as streptococcal surface dehydrogenase (SDH) and as a plasmin receptor (Plr); its complement C5a-binding activity was shown to play a role in evasion of neutrophil recruitment to sites of infection [50]. Moreover, in *S. agalactiae*, GAPDH is an immunomodulatory factor, exhibiting B lymphocyte-stimulatory activity [51].

In addition to the above-mentioned proteins, several (conserved) hypothetical secreted proteins were detected. Three of these hypothetical proteins are encoded by a gene cluster (PPA0532-0534), with homologs only in *Corynebacterium* spp. Three additional secreted proteins (PPA1715, PPA1939, PPA2175) are unique to *P. acnes*; PPA1715 contains characteristic repeats of the dipeptide proline-threonine (PT), similar to other putative adhesins (discussed below), and PPA1939 was secreted most strongly by all tested strains. Future work will determine the function of this abundantly secreted protein.

Strain-specific secretion of putative adhesions

As expected, the secretomes of the type IB strains, KPA and P6, share a higher degree of similarity with each other than with the other three strains tested. Nevertheless, we identified a few prominent differences between KPA and P6: (i) KPA secreted both CAMP4 and CAMP2. By contrast, P6 exclusively secreted CAMP2; (ii) KPA was the only strain which secreted PPA2141, a protein unique to *P. acnes* and with no homology to proteins stored in any database. A likely explanation for the KPA-specific expression of the gene encoding PPA2141 is a duplication of a 12 bp repeat within the 5′-end of the gene in strains 266 and P6 (Fig. 3A). This duplication results in the insertion of four amino acids just after the predicted cleavage site of the signal peptide, which potentially alter secretion; (iii) likewise, PPA1880, which also has no existing homology to other proteins but contains characteristic PT repeats (Fig. 3B), was secreted exclusively by P6. Interestingly, PPA1880 possesses a phase variation-like signature - a stretch of guanine residues, located within the putative promoter region. Sequencing of the upstream region of PPA1880 revealed a variable number of guanine residues in the three strains (11 nt in P6, 13 nt in KPA and 15 nt in 266) (Fig. 3C). Changes in the number of guanine residues alter the length of the spacer region of the putative promoter. Thus, observed differences in spacer lengths - 18 nt in P6 (close to the consensus length), 20 nt in KPA and 23 nt in 266 - may explain why PPA1880 expression is P6-specific. Alternatively, if the guanine tract is assumed to be part of the N-terminus of PPA1880, frameshifts leading to truncated proteins would be introduced in KPA and 266, but not in P6 (additional file 3).

A number of major differences were detected between strains belonging to phylotypes IA and IB: In comparison to the two type IB strains (KPA and P6), strain 266, a type IA strain, exhibited (i) reduced lysozyme (PPA1662) secretion, and (ii) increased secretion of the lipase GehA; (iii) in addition, strain 266 exclusively secreted PPA2127. PPA2127 (also designated PA-25957) is a host cell-surface attachment protein with dermatan-sulphate-binding activity and has immunoreactive properties [26]. The corresponding gene is associated with a putative phase variation signature; variable expression in different *P. acnes* strains has been observed and attributed to mutated start codons or alterations in the length of the homopolymeric cytosine tract in the 5′-end of the gene [26]. Comparison of PPA2127 gene sequences from KPA, P6 and 266 revealed that the start codon was mutated in KPA. In strain P6 the length of the cytosine tract was altered, leading to a frameshift and the introduction of a premature stop codon (Fig. 3D). In addition, the number of PT repeats within the C-terminus of PPA2127 varied. These repeats were more numerous in strain 266 as compared to the two type IB strains (Fig. 3E).

Strain 329, a type II strain, secreted a few proteins (Fig. 1D, spots 39-41) which could not be assigned to any known protein. MALDI-MS identification and subsequent homology searches against the genomes of *P. acnes* and the whole NCBI database retrieved no significant matches, indicating that these proteins are unique to strain 329.

Strain 487, a type III strain, secreted fewer factors than any of the other strains. One protein, PPA1758, an outer membrane lipoprotein of the periplasmic binding proteins (PBPs) superfamily, was secreted solely by

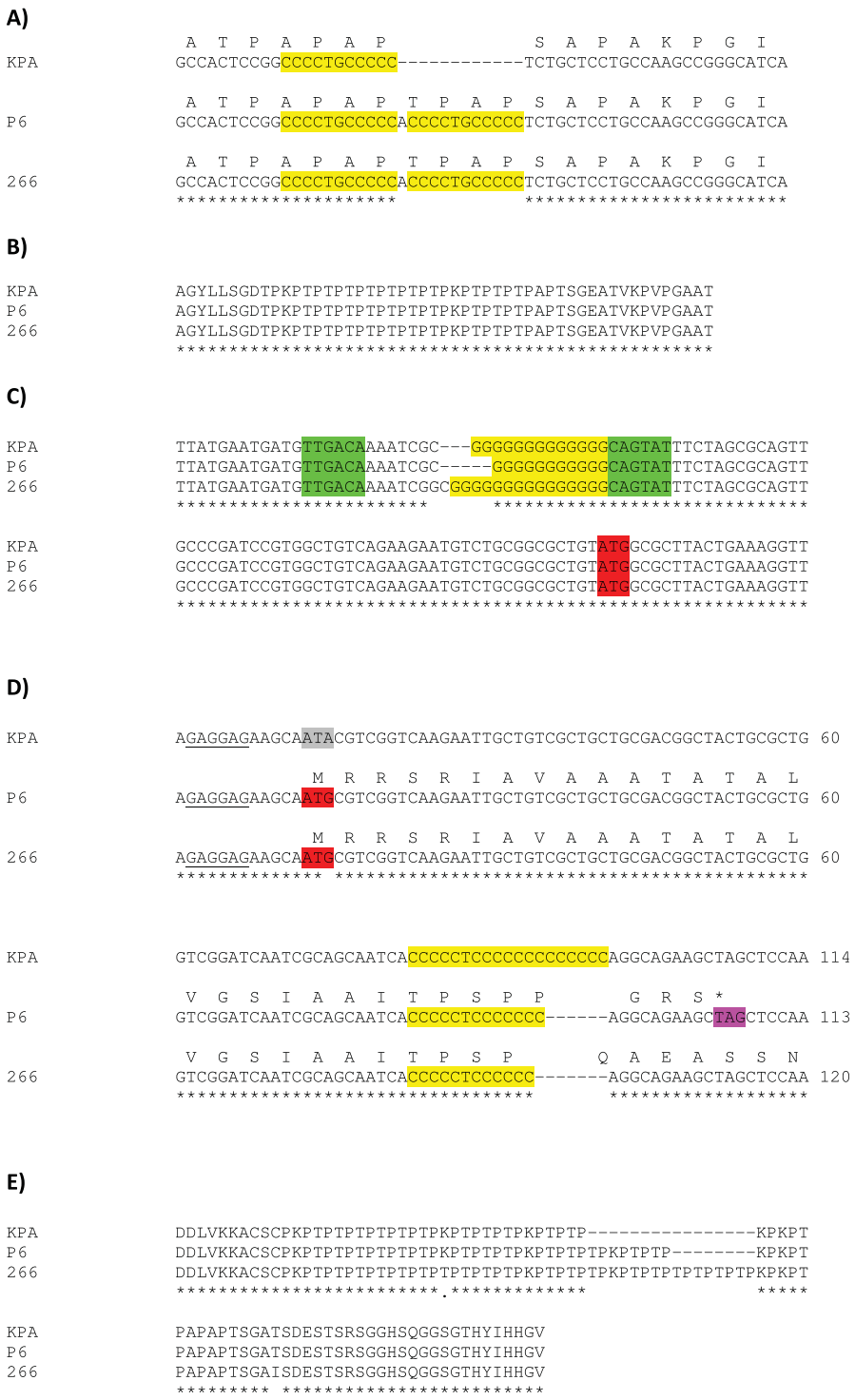

Figure 3 Changes in repetitive sequences involved in strain-specific expression and secretion of putative adhesins of *P. acnes*. (a) Insertion of a 12 bp repeat in the 5'-end of PPA2141 in *P. acnes* strains P6 and 266 results in an altered N-terminus. PPA2141 is secreted only by strain KPA. (b) Proline-threonine (PT) repeats at the C-terminus of PPA1880; these repeats are conserved in the indicated *P. acnes* strains. (c) Changes in the number of guanine residues in the upstream region of PPA1880, resulting in altered sizes of the spacer region of the possible promoter (in green: putative -35 and -10 region of the promoter; in red: predicted start codon). An alternative consequence of these alterations is shown in Fig. S2. PPA1880 is only secreted by strain P6. (d) Sequence differences of PPA2127: mutated start codon in strain KPA and variation of the C tract in the 5' end of the gene. PPA2127 is only secreted by strain 266. (e) Different numbers of PT repeats at the C-terminus of PPA2127.

strain 487. PPA1758 exhibits a 38% protein identity to the membrane-associated glycylmethionine binding protein (GmpC) of *Staphylococcus aureus* [52], indicating a potential role as a dipeptide transporter for PPA1758.

Secretome of *P. acnes* 266 in stationary growth phase

To investigate growth phase-dependent secretion, *P. acnes* was grown to stationary phase. We selected strain 266 for this analysis as it was found to aggregate strongly upon reaching the stationary phase (additional file 4). 2-DE/MALDI-MS analysis of strain 266 culture supernatants revealed approximately half of the identified spots (33 out of 65) corresponded to proteins already identified as being secreted during the mid-exponential phase (Fig. 4 and additional file 5). The other spots corresponded to proteins mainly involved in key metabolic pathways and that are known to be primarily located in the bacterial cytoplasm. Thus, it is most likely that lysis of *P. acnes* occurred in the stationary phase, releasing the most abundant cytosolic proteins. Enzymes of key pathways such as glycolysis, pyruvate metabolism and the tricarboxylic acid cycle were identified, including phosphoglyceromutase, phosphoglycerate kinase, oxaloacetate decarboxylase, fumarate hydratase, and succinyl-CoA synthetase. In addition, we detected amino acid-converting proteins, i. e. serine hydroxymethyltransferase, tryptophanase and ornithine carbamoyltransferase. Other identified proteins included elongation factors, catalase, 10 kDa chaperonin as well as the fatty acid biosynthesis enzyme acyl-carrier-protein S-malonyltransferase. Only two proteins with a typical signal peptide, which were not detected in the exponential phase-secretome, were identified: PPA2152, an extracellular solute-binding protein, and PPA2210, another protein containing a long stretch of PT repeats. PPA2210, designated as dermatan-binding protein PA-5541, was previously identified as being immunoreactive [26] and shares many properties with the above-mentioned protein PPA2127 (PA-25957). To unambiguously identify the stationary phase secretome of *P. acnes* future work is required to reduce the number of 'contaminating' (i.e. cytoplasmic) proteins; for instance, the choice of the culture medium might influence cell lysis. In addition, it is necessary for comparative reasons to determine the complete proteome of the cytoplasmic fraction.

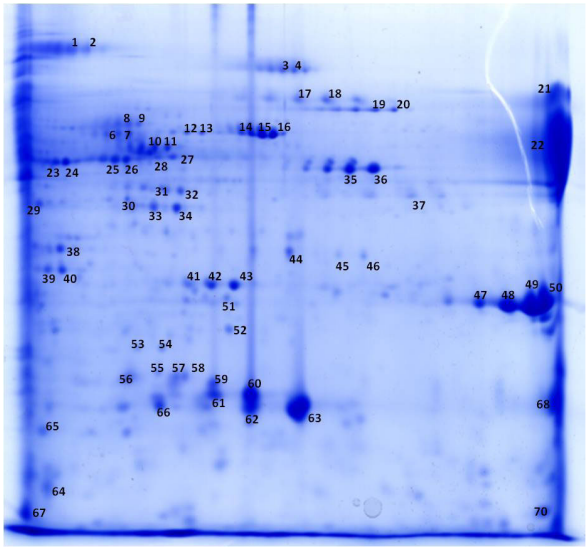

Figure 4 Stationary phase secretome of *P. acnes* strain 266. Strain 266 was grown in BHI medium for 72 h, culture supernatants were harvested and precipitated. Proteins were separated on a 2-DE gel and visualized by staining with Coomassie brilliant blue G-250. Information about the identified protein spots is provided in additional file 5.

Conclusions

Despite the ubiquitous presence of *P. acnes*, our knowledge of this bacterium remains limited, in particular regarding the factors allowing its growth on human tissues. Many studies have shown that *P. acnes* has the ability to act as an opportunistic pathogen, with suggested etiological roles in a variety of inflammatory diseases. Due to its immune-stimulatory activity, it seems plausible that *P. acnes* causes inflammation within blocked sebaceous follicles or when it grows in tissue sites unaccustomed and/or hostile to this anaerobic bacterium. Hence, the ability of *P. acnes* to acquire and process growth substrates from its host, especially in the harsh environment of human skin, is dependent on the factors this bacterium secretes. The detection and identification of such factors are therefore important steps in further understanding *P. acnes* pathogenesis. Our study has highlighted the prevalence of secreted hydrolases likely to be involved in degrading human tissue components. Other identified proteins such as immunoreactive adhesins have a putative role in virulence. Secreted factors may also fulfill other functions such as defending against competing organisms and the evasion of the immune response. Functional characterization of these secreted factors is a necessary and logical next step, which requires the development of appropriate tools, e.g. a mutagenesis approach to create *P. acnes* knock-out mutants. Another challenge for the future lies in the elucidation of the molecular basis for observed differences in virulence between *P. acnes* isolates. The relationship between phylotypes (based on recA/tly sequences) and strain properties remains obscure; some properties, for instance the ability to trigger production of proinflammatory cytokines/chemokines in keratinocytes, seem to be phylotype-specific [21,22], whereas other properties, e.g. biofilm formation,

are not [53]. Recent work has shown that an extended typing method based on serotyping in tandem with sequence comparison of three genes (trigger factor, p60, and mce) could distinguish invasive from non-invasive *P. acnes* isolates [54]; thus, this approach may be more appropriate for typing *P. acnes* isolates. In addition, our secretome analyses has revealed differences not only between but within phylotypes. A more extensive comparative analysis of *P. acnes* isolates incorporating robust phylotype identification will help to further our understanding of strain specificities.

Methods

Bacteria and growth conditions

The following *P. acnes* strains were used: 266 (type IA), P6 and KPA171202 (both type IB), 329 (type II), and 487 (type III). Strains 266, 329 and 487 were kindly provided by Oliver Knapp and Michel Popoff (Institut Pasteur). Strain KPA171202 was obtained from DSMZ (German German Collection of Microorganisms and Cell Cultures) and strain P6 was isolated from a cancerous prostate [55]. All *P. acnes* strains were cultured at 37°C on Brucella agar plates under anaerobic conditions for three days. Plate-grown bacteria were resuspended and washed in brain heart infusion (BHI) broth. Twenty ml BHI broth was inoculated with *P. acnes* (OD_{600} 0.01) and grown for 12-72 h at 37°C and 160 rpm in an anaerobic jar. After 14-18 h, the cultures typically reached the mid-exponential growth phase with an OD_{600} of 0.5-0.6. Stationary phase was obtained after 72 h of growth.

Precipitation of extracellular proteins

The exponential cultures were centrifuged for 15 min at 20,000 × *g* and 4°C, and the supernatant was filtered through a 0.22-μm-pore-size membrane filter to remove residual bacteria. Extracellular proteins were precipitated using a modified trichloroacetic acid (TCA) method as described previously [56]. In brief, the filtrate (100 ml) was mixed with 100% TCA to a final concentration of 10% and incubated overnight at 4°C. The mixture was centrifuged for 30 min (20,000 × *g* and 4°C) and the resulting pellet resuspended in 100 ml of acetone and dissolved using an ultrasonic water bath. The mixture was centrifuged as before, washed twice with acetone and the resulting pellet air dried.

Two-dimensional gel electrophoresis

Protein samples were solubilized for 30 min at ambient temperature in 9 M urea-1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-70 mM dithiothreitol (DTT)-2% Servalyte 2-4 (Serva). Protein species were separated by a small-gel 2-DE system [57]. The samples containing 200 μg of protein were applied to the anodic side of the isoelectric focusing gel containing ampholytes in the pH range 2-11. The SDS-PAGE of the second dimension was performed using 15% acrylamide gels (7 cm × 8 cm). Protein spots were visualized by staining with Coomassie Brilliant Blue G-250 [58].

MALDI-MS

Protein spots were identified by MALDI-MS after in-gel tryptic digestion of excised spots [59]. The peptide mixture was solubilized in 1 μl 33% acetonitrile/0.3% trifluoroacetic acid. For MALDI-MS measurement, 0.25 μl of the solubilized peptides were mixed with 0.75 ml a-cyano-4-hydroxycinnamic acid (CHCA) and spotted onto a MALDI plate. A 4700 Proteomics Analyzer (Applied Biosystems) with a mass range of 800-4000 Da was used for MS and at least 3 MS/MS spectra were measured per spot. Peptide mass fingerprinting (PMF) and MS/MS data were searched against the complete NCBI Database (Version 20090513). Proteins were identified using MASCOT 2.1 http://www.matrixscience.com allowing a peptide mass tolerance of 30 ppm and ± 0.3 Da for the fragment mass tolerance. A maximum of one missed cleavage, oxidation of methionine, N-terminal acetylation of the peptide, propionamide at cysteine residues and N-terminal pyroglutamic acid formation were considered in these searches. The identification criteria were: minimum 30% sequence coverage; or minimum 15% sequence coverage and one MS/MS confirmation; or sequence coverage below 15% and at least two MS/MS confirmations.

DNA isolation, PCR and sequencing

DNA from *P. acnes* was isolated using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre). Typing of *P. acnes* strains by *recA/tly* sequencing was performed as described previously [23]. For the analysis of the repetitive elements of PPA1880, PPA2127, and PPA2141 the PCR primers listed below were used to amplify 400-500 bps of the corresponding genomic region in strains P6, KPA and 266. PCR reactions were carried out using the Platinum Pfx DNA polymerase (Invitrogen), which has a proofreading 3′-5′ exonuclease activity. PCR products were subsequently sequenced using the same primers. Primers: PPA1880_N_for CACTGTACGGAC AGGTCTGG, PPA1880_N_rev CCATCCATATCG-CACTTGTC; PPA1880_C_for GGCCAGCGAGACC TCTGATT, PPA1880_C_rev GGATGGGCAACAATTC GATG; PPA2127_N_for ATTCTCTACACGGCAT-GAGC, PPA2127_N_rev ATCCAGCCTTAACCAAC GCA; PPA2127_C_for CAAGACTGCTGAGCAGCTCG, PPA2127_C_rev GCCGATGGTGATCAGAATCC; PPA 2141_N_for CAACCTCGCTACGAAGTGGA, PPA2141 _N_rev GGTCCTTGAGAACGGTATCG.

Re-Annotation

All identified proteins were re-annotated, i.e. homology searches against sequence databases such as GenBank, and protein-domain/family databases, i.e. Pfam and InterPro, were performed. Homologous proteins in other bacteria were only discussed if sequence similarity to *P. acnes* proteins exceeded 25% on the protein level, with an overlap of the query and subject sequence of at least 90%.

Accession numbers

The sequences reported in this study were deposited in GenBank. Sequences of *recA/tly* for the typing of the five strains have accession numbers HM461111 to HM461117. Sequence data from PPA1880, PPA2141, and PPA2127 have accession numbers HM461118 to HM461123.

List of abbreviations

BHI: brain heart infusion; CAMP: Christie-Atkins-Munch-Petersen; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GehA: glycerol-ester hydrolase A; MALDI-MS: matrix-assisted-laser-desorption/ionization mass spectrometry; SP: signal peptide; TAT: twin-arginine translocation; 2-DE: two-dimensional gel electrophoresis.

Additional material

Additional file 1: Secreted proteins of different *P. acnes* strains. Bacteria were grown in BHI medium to an OD (600 nm) of 0.6. Proteins in the culture supernatants were precipitated using 10% TCA and separated on 2D-PAGE gels. (A) Second and (B) third replicate of the experiment shown in Figure 1

Additional file 2: MS-based identification of all protein spots originating from exponential phase culture supernatants of five *P. acnes* strains. This table lists all MS-identified proteins that were separated by 2-DE (see Figure 1).

Additional file 3: Alternative consequence of guanine stretch alterations upstream of PPA1880. The homopolymeric guanine stretch could be part of the N-terminus of PPA1880. The different lengths of the G tract would lead to the formation of truncated proteins in strains KPA and 266 due to the appearance of a premature stop codon in the respective reading frame. Only in strain P6 a full protein would be synthesized.

Additional file 4: Adherence/agglutination of *P. acnes* strains grown to stationary phase. 2 ml BHI medium per well was inoculated with the indicated five *P. acnes* strains ($OD_{600\ nm}$ 0.01) and grown to stationary phase (72 h) under anaerobic conditions (37°C, 110 rpm). Strain 266 agglutinated stronger than the other strains. Shown are two independent experiments.

Additional file 5: MS-based identification of all protein spots originating from the stationary phase culture supernatant of *P. acnes* strain 266. This table lists all MS-identified proteins that were separated by 2-DE (see Figure 4).

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Author details

[1]Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany. [2]Proteomics Core Facility, Max Planck Institute for Infection Biology, Berlin, Germany.

Authors' contributions

CH: protein sample preparations and data analyses; TNM: PCR analyses; UZA, MS, PRJ: 2-DE/MALDI-MS experiments and data analyses; CH, PRJ, TFM: assisted in the design of the study, and critically read the manuscript; HB: conceived and designed the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Paper II

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Paper III

Mak TN, Fischer N, Laube B, Brinkmann V, Metruccio ME, Sfanos KS, Mollenkopf H, Meyer TF, Brüggemann H. 2012 *Propionibacterium acnes* host cell tropism contributes to vimentin-mediated invasion and induction of inflammation. *Cellular Microbiology.* 14(11):1720-33

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Paper IV

Fassi Fehri L, **Mak TN**, Laube B, Brinkmann V, Ogilvie LA, Mollenkopf H, Lein M, Schmidt T, Meyer TF, Brüggemann H. 2011 Prevalence of *Propionibacterium acnes* in diseased prostates and its inflammatory and transforming activity on prostate epithelial cells. *International Journal of Medical Microbiology*. 301(1):69-78

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