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CHARACTERISATION OF ANTIBIOTIC-RESISTANT *PROPIONIBACTERIUM ACNES* FROM ACNE VULGARIS AND OTHER DISEASES

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"Discovery consists of looking at the same thing as everyone else does and thinking something different"

Albert Szent-Gyorgyi

To my family

ABSTRACT

Propionibacterium acnes (P. acnes) is an anaerobic, Gram-positive bacterium that belongs to the normal microflora. The skin is the major habitat but it can also be found in other body regions. *P. acnes* plays an important role in the pathogenesis of acne vulgaris. The general perception is that *P. acnes* is a microorganism with low virulence, but during the last years, the prevalence of severe, life-threatening infections caused by *P. acnes* has increased, especially in immuno-compromised patients and in those with prosthetic devices. Long courses of antibiotics have been a mainstay of acne treatment. The consequence has been the development of antibiotic-resistant *P. acnes*.

The aim of the present investigation was to perform a characterisation of *P. acnes* antibiotic-resistant clinical isolates collected from acne patients and various other diseases. The resistance pattern, genetic diversity and molecular resistance mechanisms have been studied.

We have found that acne patients in Stockholm treated with antibiotics had a significantly higher risk of carrying resistant *P. acnes* strains, than acne patients who did not receive such a treatment. Furthermore, we have demonstrated that antimicrobial resistance has emerged among *P. acnes* strains isolated from different severe, life-threatening infections in Europe. The prevalence of tetracycline resistant isolates was lower as compared to erythromycin and clindamycin resistant isolates. The bacterial resistance in *P. acnes* isolates obtained from various diseases mirrors the situation with antimicrobials presently in use in different countries.

We have developed a new pulsed-field gel electrophoresis protocol as typing method for *P. acnes* strains. Pulsed-field gel electrophoresis is a powerful tool in epidemiology for the determination of clonal identity of bacteria. We have demonstrated that antibiotic-resistant *P. acnes* population is polyclonal and that skin isolates do not represent a separate pulsed-field type when compared with the bacterial population from other sites than the skin. We have shown that an acne patient may be colonized with different *P. acnes* strains with various resistance phenotypes, suggesting that certain bacterial clones are more prone to acquire resistance against a specific antibiotic.

The resistant strains from acne and other diseases showed well-known mutations in the 23S rRNA and 16S rRNA, but also new mechanisms of resistance have evolved. It is conceivable that mobile genetic elements carrying resistance genes have developed and can be transferred to other pathogenic bacteria.

There is a complex relationship between antibiotic resistance and outcome in acne vulgaris. It is still an open question how much of the antibiotic efficacy in acne is due to the anti-propionibacterial or anti-inflammatory effect. Treatment with oral tetracycline combined with a topical retinoid proved to be a good clinical alternative to oral isotretinoin, regardless of the presence of antibiotic-resistant *P. acnes* on the skin.

The resistance seems to move from the acne patients to the community. We have shown that carriage of resistant *P. acnes* isolates occurs, not only in acne patients and their close contacts, but also in the general population. Close contacts within families were found to carry the same clonal type of antibiotic-resistant *P. acnes* as acne patients. The cost of resistance may be ameliorated by compensatory mutations causing the stabilization of the antibiotic-resistant bacterial population. Efforts should be made in preventing the development of resistance and the accumulation of antibiotic resistant *P. acnes* strains.

Key words: *P. acnes*, acne vulgaris, clinical isolates, antimicrobial resistance, PFGE.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Oprica C,** Emtestam L, Lapins J, Borglund E, Nyberg F, Stenlund K, Lundeberg L, Sillerström E, Nord CE. Antibiotic-resistant *Propionibacterium acnes* on the skin of patients with moderate to severe acne in Stockholm. Anaerobe 2004; 10: 155-164.

II. **Oprica C**, Nord CE. European surveillance study on the antibiotic susceptibility of *Propionibacterium acnes*. Clinical Microbiology and Infection 2005; 11: 204-213.

III. **Oprica C**, Löfmark S, Lund B, Edlund C, Emtestam L, Nord CE. Genetic basis of resistance in *Propionibacterium acnes* strains isolated from diverse types of infection in different European countries. Anaerobe 2005; 11: 137-143.

IV. **Oprica C,** Emtestam L, Hagströmer L, Nord CE. Clinical and microbiological comparisons of isotretinoin versus tetracycline in acne vulgaris. Submitted to Acta Dermato-Venereologica.

V. **Oprica C**, Fang H, Emtestam L, Nord CE. Carriage of antibiotic-resistant *Propionibacterium acnes* by close contacts of acne patients and healthy volunteers. Manuscript.

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LIST OF ABBREVIATIONS

ADA	adapalene
ATCC	American Type Culture Collection
bp	base pair
BPO	benzoyl peroxide
CD	cluster of differentiation
CFU	colony-forming units
CL	clindamycin
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DLQI	dermatology life quality index
DNA	deoxyribonucleic acid
EM	erythromycin
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
GLC	gas-liquid cromatography
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leucocyte antigen
IGF	insulin-growth factor
IL	interleukin
ISO	isotretinoin
LIN	linezolid
LTB4	leukotriene B4
MIC	minimum inhibitory concentration
MLS	macrolide-lincosamide-streptogramin B
MMP	matrix metalloproteinases
MQ	MilliQ-water
NCCLS	The National Commmittee for Clinical Laboratory Standards
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PPAR	peroxisome proliferator-activated nuclear receptor
PYG	peptone-yeast-glucose
RAPD	random amplified polymorphic DNA
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TET	tetracycline
TLR	toll-like receptor
TNF	tumour necrosis factor
UPGMA	unweighted pair group method using arithmetic averages
UV	ultra-violet

1 *PROPIONIBACTERIUM ACNES*

1.1 Important members of the cutaneous microbiota

Normal human skin is colonized by a large variety of organisms that exist as commensals on its surface. There are quantitative differences in different regions of the skin, related to temperature difference, moisture content, pH, oxygen concentration, ultraviolet radiation, interactions with other microbes and the presence of various concentrations in skin lipids. All these factors may influence microbial survival and growth on the skin. The skin is not a favourable place for the bacteria to live due to its dryness and because the fluids on its surface have a relatively high osmotic pressure that will favour the survival of Gram-positive bacteria and will tend to exclude the Gram-negative species (Wilson, 2005). The skin microflora resides on the skin surface, in the ducts of hair follicles, sebaceous glands and sweat glands (Noble, 1990). The major groups of microorganisms from the skin are various genera of bacteria and yeasts (**Table 1)** (Granato, 2003; Hay *et al.*, 2004). There is an unexpected stability of the normal microflora in response to changes in environment suggesting close co-evolution of the skin microflora and the skin environment (Bojar *et al.*, 2004).

Table 1. The indigenous microbiota of the skin.

Genus	Characteristics	Most prevalent species
Staphylococcus	Facultative anaerobic, Gram-positive cocci	*S. hominis, S. haemolyticus* *S. epidermidis, S. capitis*
Micrococcus	Aerobic, Gram-positive cocci	*M. luteus, M. varians*
Corynebacterium	Aerobic/facultative anaerobic, Gram-positive pleomorphic rods	*C. jeikeium, C. urealyticum* *C. bovis*, *C. minutissimum*
Propionibacterium	Anaerobic Gram-positive rods	*P. acnes, P. granulosum, P. avidum*
Acinetobacter	Aerobic, Gram-negative coccobacilli	*A. calcoaceticus* var. *lwoffi* and var. *anitratus*
Brevibacterium	Aerobic, Gram-positive rods	*B. epidermidis, B. otitidis, B. mcbrellneri, B. casei*
Dermabacter	Gram-positive rods	*D. hominis*
Malassezia	Lipophilic yeasts	*M. restricta, M. globosa, M. sympodialis*

1.2 Characteristics and classification of propionibacteria

Propionic acid-producing bacteria were first described by von Freudenreich and Orla-Jensen in 1906 and were originally isolated from cheese (von Freudenreich *et al.*, 1906). Propionibacteria have been classified over the years as *Bacillus* spp., *Corynebacterium* spp., anaerobic diphteroids and *Propionibacterium* spp. The cutaneous propionibacteria are *P. acnes* and *P. granulosum*, which are isolated mainly from sebum rich areas (head, chest and back), *P. avidum* (mainly in moist areas as axillae, inguina and perianal area), *P. propionicum* (on the eyelids and mouth) and *P. lymphophilum* (it is not known if it should be regarded as part of the normal flora). A sixth commensal strain previously known as *P. innocuum* has been reclassified as *Propioniferax innocua* (Bojar *et al.*, 2004). Commensally propionibacteria are distinct from the "classical" propionibacteria isolated from diary products: *P. freudenreichii, P. jensenii, P. thoenii* and *P. acidipropionici* (Eady *et al.*, 1994). The sequencing of 16S rRNA genes has shown that classical propionibacteria, commensally ones and *P. propionicum* form separate clusters

(Eady *et al.*, 1994). The habitat of different *Propionibacterium* spp. as well as the tests that may be used in order to speciate different strains are shown in **Table 2**.

Table 2. Habitats and biochemical characteristics of human propionibacteria – (adapted from Bojar *et al.*, 2004; Funke *et al.*, 1997).

		P. acnes	*P. granulosum*	*P. avidum*	*P. propionicum*
Habitat	Skin	+++	+++	+++	-
	Eye	+	-	-	+++
	Oral cavity	++	+	-	+++
	Large intestine	+++	-	-	-
	Vagina	+	-	-	+
Differentiation	Catalase	+	+	+	-
	Growth conditions	Anaerobic	Anaerobic	Anaerobic & aerobic	Anaerobic
	Indole test	+	-	-	-
	Nitrate to nitrit	+	-	-	+
	Esculin hydrolysis	-	-	+	-
	Fermentation of sucrose, maltose	-	+	+	+
	Casein hydrolysis	+	-	+	-
	β-hemolysis	+/-	+	+	-

Propionibacterium genera consist of Gram-positive anaerobic non-motile non-spore forming rods. These rods can grow in oxygen at reduced rates since they possess oxygen de-toxifying enzymes. Propionibacteria have a high G+C content of DNA (59-67 moles%) (Eady *et al.*, 1994). The G+C contents and DNA sequences are significantly different among bacteria belonging to *Propionibacterium* spp. (Johnson *et al.*, 1972).

The propionibacteria are usually found as harmless commensals on the human skin (Bojar *et al.*, 2004). They have preference for hair follicles and they grow at varying depths beneath the skin surface where oxygen levels are optimal. They also survive on skin surface where oxygen utilization by aerobes (micrococci, brevibacteria and some *Corynebacterium* spp.) and facultative anaerobes (staphylococci and some *Corynebacterium* spp.) will help to offer an oxygen-depleted environment (Wilson, 2005). The Gram-positive cell wall of propionibacteria give them stability and resistance against drying, osmotic shock and mechanical stress. Their optimal pH for growth is 5.5-6.0 and they utilize free fatty acids and glycerol as carbon and energy sources. The main end-products of glucose metabolism are propionic and acetic acid, which help in their differentiation from other Gram-positive rods (Eady *et al.*, 1994). Propionibacteria produce lipases, that liberate free fatty acids from the lipids in sebaceous glands, and proteases, that can liberate arginine used as an energy source from skin proteins (Wilson, 2005). Vitamins are necessary for their growth (biotin, nicotinamide, panthotenate and thiamine) and the growth of some organisms can be inhibited due to their production of propionic acid, bacteriolytic enzymes and bacteriocins. In most adults, the sebum-rich areas of the skin will support the growth of propionibacteria which will help in maintaining the ecosystem of healthy skin. In the gut and mouth they will stabilize different microbial populations and fill a niche that could be colonized by pathogenic bacteria (Bojar *et al.*, 2004).

1.3 *P. acnes* general characteristics and natural habitats

P. acnes is the least fastidious propionibacteria and can grow on a medium containing only eight amino acids (Wilson, 2005). On the other hand, it may be unrecognized in cultures because of their slow growth (Hall *et al.*, 1994). The colony appearance varies with different culture media, age and culture conditions, but is generally 0.5-4 mm in size, dome-shaped and beige to pink in colour. The cells are 0.5-0.8 μm in width and 3-4 μm in length and the morphology may differ from diphteroidal or club-shaped with one round end and the other tapered or coccoid, bifid or branched (Moncla *et al.*, 2003).

P. acnes is the predominant species with regard to prevalence and population among propionibacteria, while *P. granulosum* density is significantly less abundant (McGinley *et al.*, 1980). *P. acnes* earned its name because it was first isolated from the skin of acne patients (Gilchrist, 1901). The name is improper since *P. acnes* is present in nearly 100% of healthy persons (Jappe *et al.*, 2002). There is a transient colonization after birth and then the skin levels of propionibacteria are very low until the adrenarche when the sebum production starts to increase. The levels of *P. acnes* continue to increase throughout teenage years into the twenties (Leyden *et al.*, 1998). *P. acnes* accounts for half of the skin microbiota (McDowell *et al.*, 2005). The organism is isolated from the skin surface, but the multiplication is in the duct of pilosebaceous follicles. Only 17% of the follicles in normal individuals are colonized by propionibacteria (Eady *et al.*, 1994). The density at one site varies widely among individuals and between different sites in the same person. High levels have been found on the face and scalp (10^5-10^6 organisms/cm^2) and very low levels on the arms and legs (10^2 organisms/cm^2) (McGinley *et al.*, 1980).

The skin is the major habitat for *P. acnes* but it can be also isolated from oral cavities (superficial sites and periodontal pockets) (Sutter, 1984), upper respiratory tract, external ear canal, conjunctiva, large intestine, urethra and vagina (Brook, 2002). The true incidence of colonization is underestimated since it is difficult to isolate *P. acnes* from other mixed anaerobic flora.

A minority harbours *P. acnes* in the colon and the population density in faeces is 10^8-10^{10} colony forming units (CFU)/gram. The colonization depends upon the ability to compete and interact with the rest of the normal microflora (Eady *et al.*, 1994). *P. acnes* plays an important role in the ecosystem of the colon. It produces intracellular nitrate and nitrite reductase that are involved in ammonia production and extracellular proteinase might contribute to the break-down of dietary proteins (Eady *et al.*, 1994).

The Meibomian glands from the external eye secrete meibum, a sebaceous-like substance which can promote *P. acnes* growth. The ducts of these glands are probably the bacterial multiplication site (McNatt *et al.*, 1978).

Recently it was found that *P. acnes* normally resides in peripheral lung tissue and mediastinal lymph nodes (Ishige *et al.*, 2005).

1.4 *P. acnes* serotypes and biotypes

P. acnes isolates are classified into five biotypes on the basis of fermentation tests of ribose, erythritol and sorbitol, and two types by the agglutination tests (Kishishita *et al.*, 1979). The type I stains have galactose as a cell wall sugar and are distributed among all five biotypes and the type II strains have only glucose and mannose and consist only of biotype 2 isolates. In healthy volunteers sero-biotypes IB1, IB3, IB4 and IIB2 were the most common ones. Recent phylogenetic analysis of the non-ribosomal housekeeping gene *rec*A revealed that *P. acnes* type I and II represent distinct lineages (McDowell *et*

al., 2005). Random amplification of polymorphic DNA (RAPD) has also been used to distinguish between type I and II strains (Perry *et al.*, 2003). The biochemical differences between the two types therefore represent deeper differences in their phylogeny. Bacteriophage or bacteriocin typing has not been used despite the description of phages (Jong *et al.*, 1975) and bacteriocins (Fujimura *et al.*, 1978). Polyacrylamide gel electrophoresis was applied to the study of whole-cell proteins of cutaneous propionibacteria in an attempt to characterise possible protein patterns that may be typical for strains isolated from acne skin. No particular protein pattern was characteristic for skin isolates; in fact the *P. acnes* strains from all sources appeared to be identical (Nordstrom, 1985). Only five of 50 ribosomal proteins were shown to be different between *P. acnes* serotype I and II using two-dimensional polyacrylamide gel electrophoresis (Dekio *et al.*, 1989).

1.5 Pathogenicity factors of *P. acnes*

P. acnes is generally considered as a microorganism with low virulence, but may become pathogenic under certain circumstances in compromised patients. *P. acnes* in the skin can adhere to oleic acid from the sebum and this fatty acid promotes co-aggregation of the bacteria. The organism can also express a surface-located fibronectin binding protein, which is important for attachment during medical devices associated infections (Yu *et al.*, 1997). *In vitro P. acnes* is capable of surviving for 8 months in anaerobiosis without subculture, suggesting that it could survive *in vivo* at low oxidation-reduction potentials for long periods of time. *P. acnes* may persist in human tissues and can damage the tissue by metabolites (acetate, propionate, indole, porphyrin), enzymes as well as degradation products of glucose and fructose. Histamine, tryptamine and short-chain fatty acids were found in culture supernatants of *P. acnes*. These substances may cause inflammation without the mediation of immune system (Csukas *et al.*, 2004). The exocellular enzymes and other bioactive products that may act as true virulence determinants are presented in **Table 3**.

Table 3. Exocellular products of *P. acnes* (modified from Eady *et al.*, 1994).

Product	Substrate	Possible role
Lipase	Triglycerides	Nutrition; production of irritant free fatty acids; promote cell adherence
Phospholipase C	Phospholipids	Perturbation of membrane function
Proteinase	Collagen, keratine	Nutrition; complement activation; release chemotaxins; proteolysis in colon; tissue invasion
Hyaluronidase, neuraminidase	Mucopolysaccharides	Tissue invasion
Acid phosphatase	Sugar phosphatase	Nutrition
Bacteriocins		Antagonism of other bacteria
Histamine, tryptamine	Arterial muscles	Mediation of acute inflammation

P. acnes is very resistant to phagocytosis and can persist intracellularly within macrophages for a long time, provoking an ongoing immune reaction and a long-standing inflammation. The reason may be related to the cell wall structure. The *P.*

acnes persistence in tissue may explain the longevity of inflammatory acne lesions (Webster *et al.*, 1985). From this point of view *P. acnes* resembles mycobacteria in their slow-growing, phagocytose-resistant phenotype and by being capable in promoting granuloma formation. *P. acnes* is a powerful immunological adjuvant. Pre-treatment with heat-killed cells of *P. acnes* has been shown to provide protection against infection and anti-tumour activity in a variety of animal models (Eady *et al.*, 1994).

The microbiological principle of biofilms has been proposed to be applicable to acne (Burkhart *et al.*, 2003). Thus, *P. acnes* would be protected by antibiotics and the host immune system, which may explain the observation of more resistant bacteria on the skin surface than in the follicles (Coates *et al.*, 2002).

2 VARIOUS DISEASES WITH WHICH *P. ACNES* HAS BEEN ASSOCIATED

2.1 Acne vulgaris

2.1.1 Epidemiological data and natural history

Acne vulgaris is a chronic inflammatory disorder of the pilosebaceous units and in USA represents the single most common reason for persons aged between 15 and 45 years to visit a dermatologist (Stern, 1996). At some time during adolescence or as adults the majority of men and women have acne (Stern, 2000) and the mean prevalence of the disease is between 70% and 87% (Dreno *et al.*, 2003b). It is a problem for the patient, by affecting the quality of life, but also a huge problem from the socioeconomic point of view. Between 1996 and 1998, 6.5 million new prescriptions at a value of more than 1 billion $ per year were provided in USA for systemic acne drugs (Zouboulis *et al.*, 2005).

The prevalence of the disease is higher among men than women, but women are more likely to seek medical advice for the acne treatment (Stern, 1992; Stern, 1996). The evaluation of the true prevalence of adolescent acne is difficult due to the definition of the disease used in various studies (Dreno *et al.*, 2003b). Nevertheless, the same pattern of evolution regarding the age of onset was noticed, which is 11 years in girls and 12 years in boys, reflecting the earlier onset of puberty, with a predominance of comedonal acne (Dreno *et al.*, 2003b). Significant prepubertal acne is seldom found to be a sign of an endocrine abnormality (Simpson *et al.*, 2004). A decrease in the disease severity is also observed with time: from 57% of boys and 19% of girls with severe acne in 1931 (Bloch, 1931) to 35% and 13% respectively in 1981 (Fellowes *et al.*, 1981). The consequences of this condition are cosmetically and by 18 years one fourth of adolescents have scars attributed to acne inflammation (Kilkenny *et al.*, 1998). In most cases there is spontaneous resolution in the late adolescence or early twenties (Dreno *et al.*, 2003b). It may persist beyond 25 years of age in 7-17% of individuals (Goulden *et al.*, 1997). On the other hand, true late-onset acne has been described and, at the age of 40 years important lesions are present in 1% males and 5% females (Cunliffe *et al.*, 1979). It was shown that patients with disease persistent into their thirties have a family history of acne (Goulden *et al.*, 1997). Other factors of poor prognostic are represented by acne lesions beginning before puberty (Dreno *et al.*, 2003b) and smoking, although the data regarding the last point are contradictory (Jemec *et al.*, 2002; Schafer *et al.*, 2001). The factors responsible for acne resolution or relative persistence in women are poorly understood (Simpson *et al.*, 2004).

2.1.2 Aetiology of acne

Acne affects the sebaceous follicles in the face, chest, shoulders and back. The aetiology is multifactorial and the sequence of events in the initiation of acne has to be elucidated. There are four important pathophysiological factors contributing to acne development:

- seborrhoea
- comedo formation
- ductal colonization with *P. acnes*
- inflammation and immunological host reactions

The genetic factor plays an important role. There is a familial clustering and severe acne forms appear frequently within the same family (Zouboulis *et al.*, 2005). Family history is also associated with earlier onset of acne, increased number of retentional lesions and therapeutical difficulties (Ballanger *et al.*, 2006). Genetic modification of the androgen receptors will affect the peripheral response to androgens (Sawaya *et al.*, 1998). The biological efficacy of natural retinoids could be greatly impaired due to a mutation of the gene encoding for cytochrome P 450 and may lead to abnormal sebocyte differentiation and hyperkeratinization of the follicular canal (Paraskevaidis *et al.*, 1998).

2.1.2.1 Seborrhoea

Sebum production and its roles. Sebum is the first glandular product of the human body and its composition is species-specific. Two main biosynthetic pathways are involved in the synthesis of sebum lipids: the first leads to the synthesis of triglycerides, free fatty acids, wax esters and sterol esters and the second to the synthesis of squalene and cholesterol (Downie *et al.,* 2004). Sebum is degraded as it passes through the duct and the surface lipids contain sebum together with lipids from the keratinocytes and from eccrine glands (Zouboulis, 2004). Squalene is unique to humans and represents an intermediate in cholesterol biosynthesis. Its high abundance in sebum may suggest inefficient conversion of squalene to cholesterol, due to the fact that enzymes catalysing the steps beyond squalene are not expressed (Downie *et al.,* 2004). Bacterial hydrolases convert some of the triglycerides to free fatty acids and glycerol as sebum passes the sebaceous ducts, but the sebaceous glands also produce free fatty acids without the presence of bacteria (Zouboulis, 2001).

Sebum composition may be altered by starvation (which decreases the amount of sebum production by 40%), hormonal or drug administration (retinoids or antibiotics) or some diseases such as AIDS (Pierard *et al.*, 2000). The composition of native sebum and epidermal lipids is presented in **Table 4**.

Table 4. Average lipid composition (%) of the skin (adapted after Simpson *et al.*, 2004).

Constituent	Native sebum (%)	Epidermal lipids (%)
Glycerides (plus free fatty acids)	57.5	65
Wax esters	26	0
Squalene	12	0
Cholesterol esters	3	15
Cholesterol	1.5	20

Lipid film controls moisture loss and contributes to body odour and protects the skin from bacterial or fungal infections. It contains immunoglobulin A, which can inactivate bacterial, viral and different antigenic materials. Certain surface free fatty acids were shown to inhibit the growth of *Staphylococcus aureus* (*S. aureus*) *in vitro* (Shuster, 1976), and products of its hydrolysis are fungistatics (Simpson *et al.*, 2004). Sebum transports vitamin E to the upper layers of the skin protecting stratum corneum from damaging oxidation (Downie *et al.*, 2004). Cunliffe proposed that the sebaceous gland excretes small amounts of androgenic steroids that act as pheromones (Cunliffe, 1989). Sebum represents an anaerobic milieu for *P. acnes* and the colonization with this bacterium may have an immunomodulatory function, for example protection against malignant hematological diseases (Simpson *et al.*, 2004).

The role of different constituents of lipids in acne is uncertain. They may be involved in ductal hypercornification or in stimulation/inhibition of bacteria. Acne patients have higher levels of squalene and wax esters (Simpson *et al.*, 2004).

Control of the sebaceous gland. Male and female acne patients have a hyper production of sebum and the level of sebum correlates with the severity of acne (Burton *et al.,* 1971). The signals coming from the pituitary influence the adrenal and gonadal hormonal production that finally will control the pilosebaceous units (Ebling, 1974; Pochi *et al.,* 1974). Acne starts at the time of adrenarche when the adrenal glands begin to produce dehydroepiandrosterone sulphate (DHEAS) which is a precursor of testosterone (Zouboulis *et al.,* 2005). The targets are sebaceous glands of the face, back and chest armoured with highly sensitive androgen receptors. Androgen receptors are localized to the basal layer of the sebaceous gland and the outer root sheath keratinocytes of the hair follicle (Thiboutot, 2004). Testosterone is converted to dihydrotestosterone (DHT) by the iso-enzyme 5α-reductase type I, which is 5-10 times more potent than testosterone and has a major role in the development of sebaceous glands and acne (Thiboutot *et al.,* 1995). The activity of this iso-enzyme in skin prone to acne is greater than in non-acne-prone skin. The skin is also a steroidogenic tissue but the clinical significance is not yet known (Thiboutot, 2004). Sebocytes and keratinocytes have enzymes that can synthesize androgens de novo from cholesterol, or transform weak androgens into more powerful derivates. These enzymes are highly active in the skin of acne patients and may represent a novel target of therapy (Pawin *et al.,* 2004). High levels of sebum could result from high androgen production, increased availability of free androgen due to a relative reduction of sex hormone binding globulin or an increased target response mediated through 5α-reduction of testosterone or an increased ability of the intracellular receptors to bind androgens (Simpson *et al.,* 2004). Acne patients are generally not endocrine misfits and an end-organ hyper response of the glands to androgens is the main cause of the seborrhoea (Cunliffe, 1998). Plasma levels of testosterone are not abnormally high in male acne patients (Pochi *et al.,* 1965; Simpson *et al.,* 2004), while the androgenic balance is modified to some extent in 50-70% of female patients. An endocrine evaluation is indicated in adult female patients with one of the following features: sudden onset of severe disease, acne resistant to therapy, irregular menstrual periods, signs of hyperandrogenism (hirsutism, cushingoid features, increased libido, clitoromegaly, deepening of the voice, acanthosis nigricans, androgenic alopecia and possible insulin resistance), or relapse shortly after starting isotretinoin (ISO) treatment (Simpson *et al.,* 2004; Thiboutot, 2004).

Nevertheless, no correlation was found between sebum secretion in women and lesion counts in facial regions (Youn *et al.*, 2005) and oral administration of type I 5α-reductase inhibitor did not improve acne lesions (Leyden *et al.*, 2004). These findings support the idea that sebum secretion simply increases the likelihood of developing acne and is not a direct and unique cause of the disease (Youn *et al.*, 2005). Sebum excretion varies from follicle to follicle (Pierard, 1986) and this may explain why acne does not appear simultaneously on all susceptible sites. In acne there is a heterogeneity regarding sebum production by diverse follicles and certain follicles may be prone to acne lesion formation (Simpson *et al.*, 2004).

Other hormones may also influence the sebaceous secretion; for example the sebum production is decreased in individuals with growth hormone deficiency and in acromegaly the rate of secretion is high (Simpson *et al.*, 2004).

Peroxisome proliferators-activated nuclear receptor (PPAR). PPARs play a key role in lipid metabolism and inflammation. Androgen acts to increase PPAR expression and it was found that sebaceous lipid synthesis is stimulated by the presence of both

androgens and PPAR ligands, i.e., leukotriene B4 (LTB4) (Rosenfield *et al.,* 1998). Free fatty acids, linoleic acid and androgen activate these receptors which bind to retinoid X receptors inducing alterations of sebocyte proliferation and synthesis of free fatty acids (Pawin *et al.,* 2004).

Neuromediators and sebaceous secretion. Substance P is a neuropeptide which is associated with multiple cellular responses and it is produced by peri-sebaceous nerve endings that are more numerous in acne patients than in controls. Sebaceous glands have receptors for substance P, mediating stimulation of sebaceous gland proliferation and differentiation. This finding may explain the possible exacerbation of acne from the neurological point of view (Pawin *et al.,* 2004; Toyoda *et al.,* 2002).

Diet and sebum composition. It is still an open question if there is a link between westernized diet and acne. Sebaceous glands can make lipids from a variety of substances and starvation studies indicate that the type of food may not be important in general sebum production. Nevertheless, the percentage of squalene increases in the follicle with a meal rich in unsaturated fat (Pawin *et al.,* 2004). Adolescents in westernized societies may be hyperinsulinemic due to a highly glycemic diet and this may initiate a cascade that affects the sebaceous gland and involve insulin-growth factor (IGF), androgens and retinoid signalling pathways (Thiboutot *et al.,* 2002).

2.1.2.2 Comedo formation

Comedones are due to abnormalities in the proliferation and differentiation of ductal kertinocytes/corneocytes (Simpson *et al.*, 2004). They are temporary structures which undergo cyclic growth that extends over days or weeks (Cunliffe *et al.*, 2004). Obstruction of the pilosebaceous follicle occurs in the infrainfundibulum (Pawin *et al.*, 2004). Ductal hypercornification represents an important characteristic of the disease which gives rise to the "horny" plug within the follicular duct (Dreno *et al.*, 2003a; Jeremy *et al.*, 2003). Microcomedones are the first acne lesions which can be seen histologically and can be found in 28% of biopsies of apparently normal-looking skin (Pawin *et al.*, 2004) while comedones are the clinically visible lesions.

In the induction of this process several factors have been proposed as being involved:

- sebaceous lipid composition – hyperseborrhoea decreases the linoleic acid level by dilution and this diminishes the epidermal barrier function and might render the comedonal wall permeable to inflammatory substances (Cunliffe *et al.*, 2004). Free fatty acids are found to modulate sebocyte proliferation and differentiation *in vitro* (Zouboulis, 2001);
- androgens – abnormalities of intracellular metabolism of androgens could play a role in this process (Dreno *et al.*, 2003a). A correlation is found between comedone numbers and DHEAS levels in prepubertal individuals (Cunliffe *et al.*, 2004);
- local cytokine production – initiation of the process may be due to a change in sebum composition/secretion, that will cause the release of interleukin 1α (IL-1α) by follicular cells and consequently the start of comedogenesis (Dreno *et al.*, 2003a). IL-1α is also secreted by keratinocytes in response to local irritation which could explain the frequency of comedones of the chin or around the scalp (Pawin *et al.*, 2004);
- hyperproliferation of keratinocytes in the affected area and apparently normal skin, which may justify the treatment of the entire surface of the acneiform zone (Pawin *et al.*, 2004);
- changes in the expression of keratinocyte integrins of the infrainfundibulum (Pawin *et al.*, 2004);

- it was recently shown that components of the cell wall of *P. acnes* may stimulate keratinocyte proliferation. As a result the bacterium is involved in the formation of the microcomedo (Jugeau *et al.*, 2005).

2.1.2.3 P. acnes role in acne

P. acnes is the major bacterium that has been implicated in the pathogenesis of acne for more than 100 years. The association was reinforced after the antibiotics started to be used in acne treatment. However, acne is not an infection and Koch's postulates are not applicable to the disease. It is difficult to allocate to a bacterium a pathogenic role when it is present on both normal skin and in a disease condition (Bojar *et al.*, 2004).

The bacterial population correlates with the amount of lipids produced in different body regions (McGinley *et al.*, 1980), but there is no correlation between bacterial density on the skin surface and acne severity (Simpson *et al.*, 2004). There is a much closer relation between follicular propionibacteria obtained by punch biopsy and the disease severity. There is no difference between male and female patients and the age of the patient was not found to influence microbial population density. Follicles in female facial skin and male back skin showed higher numbers of propionibacteria in patients than in controls (Till *et al.*, 2000).

Skin colonization of propionibacteria is not uniform, but there are lower numbers in normal follicles than in inflamed follicles. This bacterium may be involved in the inflammatory process or, conversely, an inflamed lesion may provide the growth conditions for the bacteria (Simpson *et al.*, 2004). It was proposed that the follicular ducts undergo cyclic changes and that inflammation may occur at a specific stage of the cycle, i.e., when the follicular conditions are suitable for microbial colonization (Aldana *et al.*, 1998). The duration of the disease may be explained by the total number of follicles capable of developing appropriate conditions for colonization (Till *et al.*, 2000). It has been proposed that the colonization of the follicles involves free fatty acids and bacterial lipases (Simpson *et al.*, 2004). It is possible that the microenvironment produced by *P. acnes* is more important than the absolute numbers of bacteria. There is evidence that expansion of *P. acnes* occurs more slowly in individuals without acne, so the population densities are not reached until beyond puberty (Eady *et al.*, 2003a).

2.1.2.4 Inflammation and immunological host reactions

The first evidence of *P. acnes* significance in acne came from an *in vivo* study in which the injection of concentrated viable, but not dead *P. acnes* or other bacteria into a sterile cyst of Steatocystoma multiplex produced inflammation (Kirschbaum *et al.*, 1963). Moreover, the clinical improvement of acne with antibiotics reinforces this hypothesis. Some authors have questioned the bacterial role and suggested that the pilosebaceous gland itself may produce immune factors and *P. acnes* will amplify this initial immune response (Zouboulis, 2001). The initial infiltrate consist of CD4+ T cells and later, in severe established lesions, there is a classical cutaneous type IV reaction initiated by leakage of IL-1α into the dermis and perpetuated by a T cell response to antigens (Eady *et al.*, 1994). It was demonstrated that *P. acnes* has mitogenic activity, i.e., *P. acnes* stimulates lymphocyte proliferation by specific antigens and the production of non-specific mitogens (Jappe *et al.*, 2002).

It is possible that changes in the local microenvironment give rise to an increase in the *P. acnes* population and that bacterial antigens activate Langerhans cells migrating to the local lymph nodes and presenting the antigens to CD4+ T cells. The latter migrate to the skin, release cytokines and activate the mononuclear cells leading to inflammation. Clearance of the antigenic stimulus will down-regulate the inflammatory

response and the lesion will be healed. Another scenario would be that the initial events may be non-specific and followed by an antigen specific response (Holland *et al.*, 1998). The increase in sebum production at puberty will lead to a deficiency in linoleic acid and consequently an increase in water within the follicle due to perturbations in barrier function. The colonization of *P. acnes* is then promoted. Deep in the follicles the microorganism and/or its products interact with keratinocytes and sebocytes which then produce IL-1α that diffuse into the dermis causing non-specific activation of mononuclear and endothelial cells. Also, disruption of keratinocytes by extracellular products of *P. acnes* will lead to release of IL-1α. Release of bacterial antigens in the dermis will further exacerbate inflammation through activation of CD4+ T cells as described earlier (Farrar *et al.*, 2004). Farrar *et al.* have focused on the role of heat-shock proteins produced by *P. acnes* in response to nutritional stress at puberty. The heat-shock proteins will activate CD4+ cells via presentation on Langerhans cells. With time this response will diminish due to homology between bacterial and human heat-shock proteins, explaining the resolution of acne (Farrar *et al.*, 2004). In **Table 5** the most important factors responsible for the inflammatory process are summarized.

Table 5. Immunological factors responsible for acne inflammation (adapted from Pawin *et al.*, 2004).

Type of immunity	Involved factors
Acquired immunity	CD 4 lymphocytes
	circulating antibodies
	P. acnes acting as a superantigen
Innate immunity	polynuclear neutrophils
	complement deposition
	toll-like receptors
	cytokines
	β defensins
	matrix metalloproteinases
	reactive oxygen species
	PPAR receptors
	melanocortin receptors

2.1.2.5 Acquired immunity

- Cellular immunity – an infiltrate composed of CD4+ T cells is present around the non-involved follicles of acne patients and seems to be the earliest event in acne lesion. The topical use of anti-inflammatory based therapies is thereby validated and the initiation of inflammation is a response to a specific antigen (Jeremy *et al.*, 2003). CD8+ cells occasionally occur perivascularly and periductally and CD1+ cells are present in low numbers in these locations (Jappe *et al.*, 2002). This may indicate that keratinocytes and sebocytes function as antigen-presenting cells and activate natural killer T cells by presenting lipid antigens with the help of CD1+ molecules (Koreck *et al.*, 2003).
- Humoral immunity – antibodies to *P. acnes* are present in all humans and the correlation to the disease severity in acne patients is unclear. It has been shown that the levels of IgG1 and IgG3 were significantly higher in patients with severe acne compared to patients with moderate acne, while IgG2 was significantly higher in moderate and severe acne patients as compared to healthy controls (Ashbee *et al.*, 1997). The dominant antigen was found to have a carbohydrate component (Burkhart *et al.*, 1999b).

- *P. acnes* has superantigenic activity by some membrane fractions that augment the inflammation. This aspect may be responsible for acne fulminans and acute reactions produced sometimes after the first week of ISO treatment (Pawin *et al.*, 2004).

2.1.2.6 Innate immunity

- Polynuclear neutrophil chemotaxis – the early hypothesis was that enzymes produced by *P. acnes* split triglycerides into glycerol and free fatty acids. Free fatty acids diffuse through the follicular wall and attract polynuclear neutrophils which are capable of disrupting the follicle by lysosomal hydrolytic enzymes. The consequence is represented by the diffusion of inflammation into the dermis. Lipase inhibitors were shown to reduce free fatty acids but failed to treat the disease (Weeks *et al.*, 1977). *P. acnes* has also been shown to have potent immunostimulatory effects. It produces low molecular weight material that easily passes through the pilosebaceous wall and may attract polynuclear neutrophils (Pawin *et al.*, 2004).
- Complement deposition is stimulated by extracellular products of *P. acnes* and has been shown in early and late acne. The C3 fraction was found in the dermal vessel walls, sometimes together with immunoglobulins, and this constitutes evidence of the formation of immune complexes around the acne lesions. *P. acnes* is a strong promoter of both classical and alternative complement pathways: the alternative pathway is triggered by cell wall carbohydrates and the classical pathway requires the presence of anti-bacterial antibodies. The activation of complement leads to release of inflammatory mediators (Burkhart *et al.*, 1999a).
- Toll-like receptors (TLRs) may identify pathogen-associated molecular patterns that occur on bacteria. The TLRs act via the nuclear complexes AP-1 and NF-kB (Pawin *et al.*, 2004). *P. acnes* triggers inflammatory cytokine responses in acne by activation of TLR-2 expressed on the cell surface of macrophages surrounding pilosebaceous follicles (Kim *et al.*, 2002). TLR-2 and TLR-4 expression is also increased in the epidermis at the sites of acne (Jugeau *et al.*, 2005). TLRs regulate the production of pro-inflammatory cytokines, which contribute to inflammation and tissue destruction (Simpson *et al.*, 2004).
- Pro-inflammatory cytokines, i.e., IL-1α, tumour necrosis factor-α (TNF-α), IL-6 and IL-8 are produced by activated keratinocytes and macrophages (Webster, 1995). An *in vitro* study showed that *P. acnes* may induce human keratinocytes to produce IL-1α, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) and that cells in the stationary phase induce a higher level of cytokines than those in the exponential phase. This may explain why only a proportion of the follicles are affected at one time (Farrar *et al.*, 2004). Cytokines stimulate the expression of adhesion molecules on endothelial cells, have chemotactic properties, activate leukocytes and stimulate the formation of leukotrienes and prostaglandins (Koreck *et al.*, 2003). Porphyrins are produced by *P. acnes* and their interaction with molecular oxygen generates toxic oxygen species which may damage the keratinocytes and lead to cytokine release (Brüggemann *et al.*, 2004). It has been shown that cytokines may, in a second phase, inhibit the secretion of lipids from the sebaceous gland (Downie *et al.*, 2002). The remission of the individual lesions is then influenced. These findings help to explain the natural history of the disease.
- β defensins-1 and -2 are antimicrobial peptides that are up-regulated in acne lesions, i.e., a protective mechanism for controlling pathogens in acne patients (Jones, 2005).

- Matrix metalloproteinases (MMPs), mainly MMP-9, are produced by keratinocytes and polynuclear neutrophils. These endopeptidases have a role in the destruction of the pilosebaceous wall and possibly in scars (Pawin *et al.*, 2004). *P. acnes* increases the expression of MMP-9 either directly or indirectly through TLRs (Jugeau *et al.*, 2005). Gene array expression profiling recently revealed upregulation of genes involved in inflammation and matrix remodelling (i.e., MMPs, inflammatory cytokines and antimicrobial peptides) (Trivedi *et al.*, 2006).
- Reactive oxygen species (superoxide radical anion, hydrogen peroxide and hydroxyl radical) are generated by neutrophils for the purpose of lysis of invading microorganisms and have a role in the irritation and destruction of the follicular wall. Antibiotics used for the treatment of acne significantly inhibit generation of reactive oxygen species by neutrophils, as compared to other antibiotics.
- PPAR receptors induce production of IL-1 and TNF-α and are therefore involved in acne inflammation (Pawin *et al.*, 2004).
- Melanocortin-1 receptor expression was demonstrated in sebocytes of normal human skin by immunohistochemistry. Receptor activation by α-MSH may decrease the production of IL-8 and thus it may act as a modulator of inflammatory responses in the pilosebaceous unit (Bohm *et al.*, 2002; Pawin *et al.*, 2004).

The involvement of *P. acnes* is not always necessary for the development of acne inflammation. Although bacteria stimulate cytokine expression, the sebaceous glands express cytokines at steady state without bacterial influence. There is no correlation between the levels of any cytokine and the number of bacteria. IL-1 has been shown to occur in normal sebaceous glands and mRNA for IL-1α, IL-β and TNF-α are expressed in normal skin (Zouboulis, 2001). Furthermore, the *in vitro* addition of IL-1α in the human follicular infundibulum resulted in hypercornification similar to that seen in comedones (Guy *et al.*, 1996). In addition, free fatty acids are produced in the absence of bacteria (Zouboulis, 2001).

LTB4 is a pro-inflammatory mediator synthesized from arachidonic acid and was found to induce recruitment of monocytes and neutrophils. The production of pro-inflammatory cytokines is also stimulated. These events are independent of bacteria. Leukotriene precursors are synthesized in the sebaceous gland. A study using an agent that specifically blocks the formation of LTB4 showed an important reduction in the number of inflammatory lesions in acne patients (Zouboulis *et al.*, 2003a). Future therapies will have to reduce pro-inflammatory lipids in the sebum and inhibit accumulation of inflammatory cells.

2.2 Different infections due to *P. acnes*

P. acnes is more often associated with various infections than any other bacteria belonging to the same group (Brook *et al.*, 1991). The general perception was that *P. acnes* is a microorganism with low virulence and it was considered to be a contaminant in cultures obtained by percutaneous punctures or biopsies. The number of reports describing different diseases in which *P. acnes* was repeatedly isolated has abruptly augmented during the last years and this bacterium is increasingly recognized as an opportunistic pathogen (Jakab *et al.*, 1996).

In case of clinical signs of infection (i.e., local tenderness and swelling on examination or purulence noted at surgery), pure culture of *P. acnes* pleads against contamination since this bacterium is rarely the only isolated species. Many other skin bacteria are easier to grow in the laboratory than propionibacteria (Skinner *et al.*, 1978).

It is accepted that at least two separately positive *P. acnes* cultures from a normally sterile body site may define a case of significant infection (Brook *et al.*, 1991). Recent studies showed that *P. acnes* was recovered as frequently as coagulase-negative staphylococci from the prosthetic hips of patients undergoing arthoplasty (McDowell *et al.*, 2005).

In case of severe infections, the identification of *P. acnes* by a combination of two PCR analysis, one for the detection of 16S rRNA and the other for the lipase genes, was proposed as an easier, faster and more accurate method (Nakamura *et al.*, 2003).

2.2.1 Primary infections in previously healthy persons

A few cases of primary infections caused by *P. acnes* have been documented: primary purulent folliculitis dissimilar from acne vulgaris (Maibach, 1967), acute meningitis (Schlesinger *et al.*, 1977), osteomyelitis (Suter *et al.*, 1992), sepsis (Praderio *et al.*, 1998) and different eye infections (endophtalmitis, conjunctivitis, blepharitis, keratitis, canaliculitis, dacryocystitis, peri-orbital cellulites and abscesses) (Eady *et al.*, 1994). Bacterial DNA was also found in gallstones (Wu *et al.*, 1997). An outbreak of *P. acnes* postoperative shoulder arthritis has been found in non-debilitated patients resulting from a poor efficiency of the ventilation (Lutz *et al.*, 2005).

2.2.2 Secondary/opportunistic infections

Predisposing factors for secondary/opportunistic infections include: previous surgery (cerebro-spinal fluid shunts or implantation of prosthetic devices in cardiac, orthopedic or eye surgery), diabetes, invasive diagnostic procedures, immunodeficiency or immunosuppression, malignancy (Bolognia *et al.*, 1997; Eady *et al.*, 1994) and intravenous drug use (Brook *et al.*, 1991). It is possible that infections may appear a short time after surgery (as a result of a high inoculum) or longer time after. The last possibility is explained by the fact that *P. acnes* resides intracellulary and remains in a dormant state for months or years (Jakab *et al.*, 1996). The location of the infection may help the diagnosis. For example *P. acnes* causes infections more often after shoulder arthoplasty than after replacement of other joints (Zimmerli *et al.*, 2004). It has been shown that *P. acnes* has the ability to form biofilms on the surface of different implanted biomaterials, allowing the bacteria to survive in a protected environment (Ramage *et al.*, 2003). The clinical diagnosis of *P. acnes* infection may be difficult in high-risk patients and in those with foreign bodies since the signs of sepsis are often absent.

Possible infections include: abscesses at various sites, meningitis associated to cerebral spinal fluid shunts (Skinner *et al.*, 1978), osteomyelitis or arthritis (Yocum *et al.*, 1982), endocarditis associated with implanted valves and other intravascular diseases (Horner *et al.*, 1992; Nord, 1982), pleuro-pulmonary infections (Claeys *et al.*, 1994; Finegold *et al.*, 1975; Mohsenifar *et al.*, 1979), peritonitis (Dunkle *et al.*, 1976), biliary infections (Funke *et al.*, 1997), endophtalmitis after extracapsular cataract surgery and posterior chamber intraocular lens implantation (Augsten *et al.*, 1999, Friedman *et al.*, 1978), blood stream infections (Nord, 1982; Spanik *et al.*, 1996), lymph gland infections (Chanet *et al.*, 2005), obstretic and gynecological infections (Brook *et al.*, 1991), head and neck infections (Lipkin *et al.*, 1987), botryomycosis (Esteban *et al.*, 1994; Schlossberg *et al.*, 1980), infections of the skin (Moncla *et al.*, 2003) and inflammation around the nerve root in patients with sciatica (Brook *et al.*, 1991; Eady *et al.*, 1994; Stirling *et al.*, 2001).

2.3 Other diseases and syndromes

Oral diseases such as dental caries, periodontal diseases and gingivitis are documented. *P. acnes* has been shown to participate in building-up the dental plaque together with *Streptococcus mitis* and *Streptococcus sanguis* and may have a contribution to inflammation and tissue destruction (Ciardi *et al.*, 1987; Eady *et al.*, 1994).

Kawasaki disease – P. acnes has been isolated from the blood or lymph nodes of a minority of affected patients and a cytopathogenic protein was isolated from these strains (Tomita *et al.*, 1987).

Sarcoidosis – P. acnes DNA has been isolated from lymph nodes of patients with sarcoidosis (Eishi *et al.*, 2002). Recent findings have shown that *P. acnes* normally resides in those areas and, consequently, the association may not be specific to sarcoidosis (Ishige *et al.*, 2005). RAPD showed that strains from patients with/without sarcoidosis may differ genetically.

SAPHO syndrome (synovite-acné-pustulose-hyperostose-ostéite) – viable *P. acnes* has been isolated from the osteolytic lesions of affected patients (Nault *et al.*, 1985). The syndrome is different from arthralgia associated with the most severe forms of acne which is produced by immune complexes in the joints (Eady *et al.*, 1994).

3 TREATMENT CONSIDERATIONS

3.1 *P. acnes* general susceptibility to antibiotics

P. acnes, as well as the other *Propionibacterium* spp., are generally but not universally susceptible to benzylpenicillin, amoxicillin, ticarcillin, cefazolin, cefoxitin, cefotetan, third-generation cephalosporins (ceftriaxone, cefotaxime), chloramphenicol, clindamycin (CL), erythromycin (EM), imipenem, meropenem, tetracycline (TET), vancomycin, rifampicin, fluoroquinolones, linezolid (LIN), and the combinations of penicillins and β-lactamase inhibitors (Bansal *et al.*, 1984; Chow *et al.*, 1978; Denys *et al.*, 1983). *Propionibacterium* spp. are generally resistant to 5-nitroimidazole agents (metronidazole, tinidazole and ornidazole), aminoglycosides and mupirocin (Brook, 2002; Eady *et al.*, 2003b).

3.2 Antibacterial acne treatment

Many drugs have been developed for acne treatment during the last years: antibiotics, topical benzoyl peroxide, topical retinoids, topical azelaic acid, topical salicylic acid, oral isotretinoin, hormonal therapy and zinc salts (Dreno *et al.*, 2004; Gollnick *et al.*, 2003).

P. acnes is the major target of using antibacterial treatment, which can be administrated either topical or systemic, even though the reduction in bacterial numbers does not correlate with the clinical efficacy (Leyden *et al.*, 1975). Successful acne treatment with antimicrobials reduces the number of propionibacteria but does not completely eliminate the bacteria from the skin, and the reduction is temporary (Eady *et al.*, 2003a).

3.2.1 Oral antibacterial treatment

3.2.1.1 Systemic antibiotics

Systemic administrated antibiotics represent the most widely used therapy. They are indicated in: moderate-to-severe acne associated with papules, pustules, nodules; in patients in whom topical therapy has failed; in patients with moderate acne with tendency for scarring and in acne affecting shoulders, back or chest (Zouboulis *et al.*, 2003b). Antibiotics with therapeutic value in acne are lipid-soluble substances which may concentrate in the pilosebaceous unit (Bojar *et al.*, 2004). The antibiotics used in acne treatment are concentrated by leukocytes and are delivered at the site of inflammation where the local concentrations can become higher than the serum concentrations (Murdoch *et al.*, 1991). The following antibiotics are used:

Cyclines of the first generation: TET hydrochloride, oxytetracycline (natural products) and second generation: lymecycline, doxycycline, minocycline (semi-synthetic derivates). These substances are antibiotics of choice on the basis of efficacy, antibiotic resistance and safety profile. The second generation is preferred due to pharmacokinetic advantages and lymecycline and doxycycline have less side effects as compared to minocycline (Dreno *et al.*, 2004).

Macrolides are composed of 14- (EM and oleandomycin) or 16-membered lactones ring (josamycin, spiramycin, tylosin) to which amino and/or sugars are attached. During the last years, semi-synthetic derivates such as azithromycin (a 15-membered azalides) and telithromycin (a 14-membered ring ketolides) have been

introduced (Roberts, 2002). EM or azithromycin (Kapadia *et al.*, 2004) are rarely used (mainly when TET is contra-indicated) due to resistance and cross-resistance to CL (Dreno *et al.*, 2004). Roxithromycin, a semi-synthetic derivate of EM has been shown to be effective in inflammatory acne (Akamatsu *et al.*, 2002).

Clindamycin is a semi-synthetic derivate of lincosamide that is not so commonly used due to a possible side-effect which is pseudomembranous colitis. That occurs when antibiotics such as clindamycin, ampicillin and 3rd generation cephalosporins suppress the normal flora, allowing *Clostridium difficile* to grow and produce toxins (Oldfield, 2004).

Trimethoprim (a diamino-pyrimidine*) and co-trimoxazole* (a 1:5 combination of trimethoprim and sulfamethoxazole*)* are of limited use and are considered as a third choice of treatment due to severe allergic reactions (Cunliffe *et al.*, 1999).

Levofloxacin belongs to the group of fluoroquinolones and has been shown to be efficient as an acne treatment (Kawada *et al.*, 2002). However, levofloxacin is not currently used in acne due to side effects and development of antibiotic resistance of the normal microflora (Dreno *et al.*, 2004). It is also preferable to save these broad spectrum antibiotics for the treatment of systemic life-threatening infections (Katsambas *et al.*, 2004).

3.2.1.1.1 Mechanisms of action of orally administrated antibiotics

- Reduction of *P. acnes* levels by:
  - ⇒ inhibition of protein synthesis. Cyclines bind reversibly to the 30S ribosomal subunit and prevent the association of aminoacyl-tRNA with the codon on mRNA, thus preventing translation and protein synthesis; macrolides and CL bind reversibly to the 50S ribosomal subunit at or near the same site, in domains II and V of the 23S rRNA and prevent elongation of the peptide chain;
  - ⇒ inhibition of enzymes in the bacterial pathway for the production of tetrahydrofolic acid (trimethoprim and sulfamethoxazole);
  - ⇒ inhibition of bacterial gyrase (quinolones) (Dreno *et al.*, 2004).
- Inhibition of production of pro-inflammatory mediators by *P. acnes* (cyclines, macrolides and CL) (Eady *et al.*, 2003a).
- Inhibition of lymphocyte mitosis, inhibition of phagocytosis, decrease of cytokine production (TNF-α, IL-1 and IL-6) and increase in the secretion of anti-inflammatory cytokine (IL-10), inhibition of leucotaxis, inhibition of reactive oxygen species production (macrolides and cyclines) (Dreno *et al.*, 2004; Jain *et al.*, 2002).
- Decrease activation of C3 and inhibition of collagenases and MMPs (cyclines) (Dreno *et al.*, 2004; Eady *et al.*, 2003a; Jain *et al.*, 2002).
- Modulation of cutaneous production of α-MSH (minocycline) (Sainte-Marie *et al.*, 1999).
- Roxithromycin may serve as anti-androgen but only in the case of hypersensitivity to androgens, by modulating end-organ hypersensitive condition (Inui *et al.*, 2001).

3.2.1.2 Oral isotretinoin (13-cis-retinoic acid)

This drug has been used in acne vulgaris for more than 20 years and it is the only treatment that affects all etiological factors involved in acne pathogenesis. It indirectly inhibits *P. acnes* growth by changing the follicular milieu (King *et al.*, 1982). Bacterial suppression under ISO administration correlates with decreased sebum secretion

(Flemetakis *et al.*, 1989) and persists after the treatment has stopped (Leyden *et al.*, 1986). It is prescribed for severe nodular/conglobated acne but also moderate acne, i.e., scars, inflammatory acne resistant to conventional treatment, relapsing or extensive acne, dysmorphophobic patients or those with excessive seborrhoea. It is also administrated in acne fulminans, Gram-negative folliculitis and pyoderma faciale (Gollnick *et al.*, 2003; Orfanos *et al.*, 1998). After ISO treatment, the numbers of Gram-negative bacteria decrease sharply in nares and skin (Leyden *et al.*, 1986) and there is an increase in *S. aureus* colonization and cutaneous infections (Leyden *et al.*, 1987) probably due to an enhancement of staphylococcal adherence to epithelia caused by retinoids (Lianou *et al.*, 1989).

3.2.2 Topical antibacterial treatment

3.2.2.1 Topical antibiotics

Topical antibiotics are useful in mild to moderate inflammatory acne and the most commonly used are EM (the most effective against inflammatory lesions) and CL. Topical administrated TET is less effective (Eady *et al.,* 1990; Johnson *et al.,* 2000). They are administrated in concentrations of 1-4%, with or without the addition of zinc (Holland *et al.,* 1992). A liposome-encapsulated 1% CL was found to give better clinical results in comparison to the conventional topical CL (Honzak *et al.,* 2000). The combination of EM with zinc is more effective on non-inflammatory lesions and decreases the percentage of resistant strains as compared to EM alone (Dreno, 2004). Topical EM and CL are more effective combined with a topical retinoid than used alone (Dreno, 2004).

Meclocycline (an oxytetracycline derivate) (Knutson *et al.,* 1981) and nadifloxacin (a synthetic fluoroquinolone derivate) have shown promising results with a low incidence of resistant propionibacteria (Kurokawa *et al.,* 1991; Plewig *et al.,* 2006).

3.2.2.1.1 Mechanisms of action of the topical antibiotis

- anti-inflammatory – suppression of leucocyte chemotaxis, anti-lipase activity and reduction in pro-inflammatory free fatty acids (Dreno, 2004);
- antibacterial – both CL and EM have a moderate effect in reducing the number of bacteria (Toyoda *et al.*, 1998);
- reduction in non-inflamed lesions by reducing perifollicular lymphocytes involved in comedogenesis (Simpson *et al.*, 2004).

3.2.2.2 Benzoyl peroxide (BPO)

BPO represents a highly lipophilic bacteriostatic and possibly bactericidal agent that reduces the *P. acnes* population and has not been associated with bacterial resistance (Thiboutot, 2000). It has also been shown to have anti-inflammatory properties (inhibits the production of reactive oxygen species from neutrophils). Combinations with EM or CL are more effective than each product alone (Dreno, 2004). The antibiotic kills all susceptible isolates and BPO eliminates the resistant isolates at the site of application. In addition a synergistic activity of BPO and EM has been demonstrated. Skin application of EM enhances the radical formation by BPO which could explain the high efficacy of the combined therapy (Burkhart *et al.*, 2000).

3.2.2.3 Azelaic acid

Azelaic acid is a C9 dicarboxylic acid that has a mild comedolytic and mild anti-inflammatory effect (Gollnick *et al.,* 2003). It has been shown to reduce the number and

function of *P. acnes*, although recent data have brought some uncertainty (Nazzaro-Porro, 1987; Simpson *et al.,* 2004). There is no report regarding *P. acnes* resistance to azelaic acid (Dreno, 2004).

3.2.2.4 Retinaldehyde

Retinaldehyde differs from natural retinoids in demonstrating antibacterial activity upon topical use. This is due to the aldehyde group in the isoprenoic lateral chain, which confers both retinoid and antibacterial activities (Pechère *et al.,* 2002). The combination between retinaldehyde (0.1%) with glycolic acid (6%) may be used together and can amplify the efficacy of other anti-acne treatments (Dreno *et al.,* 2005).

3.3 Antibacterial treatment of different infections caused by *P. acnes*

The management of *P. acnes* infections includes a combination of intravenous, intramuscular or oral antibiotics and surgical procedures (drainage of the infected site, debridements or removal of the infected device) (Brook, 2002; Jakab *et al.*, 1996). *P. acnes* susceptibility of isolates collected from severe infections needs to be verified to assure proper use of antibiotics (Rasmussen *et al.*, 1997). Tissue specimens and not swabs are recommended to be cultivated due to a better sensitivity (Zimmerli *et al.*, 2004). The prognosis is generally favourable if an appropriate antibiotic is administrated (Funke *et al.*, 1997).

Benzylpenicillin is the treatment of choice for severe infections. Other drugs should be considered in the case of penicillin allergy or concern about resistance: third generation cephalosporins, vancomycin, imipenem, meropenem, gatifloxacin and moxifloxacin (Brook, 2002; Zimmerli *et al.*, 2004). It has been shown that *in vitro* combinations of vancomycin and cefotaxime, or quinupristin/dalfopristin and cefotaxime may have synergistic effects (Mory *et al.*, 2005). Other authors (Komagata *et al.*, 1998) consider that no synergistic combination is known to be useful in infections caused by *P. acnes*. In polymicrobial infections the treatment should cover all pathogens (Brook, 2002).

The data from the literature support the idea of using perioperative prophylactic antibiotics for surgery involving skin or mucosa in the presence of any previously implanted biomaterial (Halpern *et al.*, 1988). In patients with prosthetic heart valves, antibiotics are currently used, although it is still controversial whether they should be administrated to patients with prosthetic joints (Ferrar *et al.*, 2005).

4 ANTIBIOTIC RESISTANCE IN *P. ACNES*

4.1 The widespread problem of *P. acnes* resistance

Antibiotics have been prescribed for acne treatment for more than 40 years but EM resistance emerged before EM or CL were introduced as topical acne treatments and was described in a clinical *P. acnes* isolate collected from the Mayo Clinic (Martin *et al.*, 1972). The first report of resistant *P. acnes* strains collected from acne patients was published in USA in 1979, but was not considered to be significant (Crawford *et al.*, 1979; Guin *et al.*, 1979). Soon after the introduction of topical EM and CL in acne treatment resistant strains were reported in the USA (Leyden *et al.*, 1983). Since then reports about *P. acnes* resistance collected from acne patient have been published in many parts of the world (Kurokawa *et al.*, 1988; Oakley *et al.*, 1995; Ross *et al.*, 2003; Tan *et al.*, 2001). It has been demonstrated that between 1991 and 1997 the proportion of patients carrying resistant bacteria in UK doubled, consequently 60% of patients in Leeds region were found to carry resistant strains (Eady, 1998). The decrease of colonization rates during late 1989 and 1999 may be explained by a change in prescribing practices due to publicity about development of resistance. However during 2000 the resistant rates started to increase again (Coates *et al.*, 2002). A survey conducted in Europe showed that 50% of acne patients are colonized by CL and EM resistant *P. acnes* and 20% by TET resistant *P. acnes* (Ross *et al.*, 2003).

Despite the fact that USA is the largest market for antibiotics, the last study concerning this problem was published in 1983 and since then there are no prevalence data from the USA (Eady *et al.*, 2003a).

4.2 Factors that may promote resistance development

Several factors may be involved in the development of resistance to antibiotics, such as:

- Low dose of antibiotic which may promote overgrowth of resistant *P. acnes* strains or *de novo* acquisition of resistance within the normal microflora (Dreno *et al.*, 2004).
- There are data that prescribing practices for acne influence the resistance rates (Ross *et al.*, 2003). Many acne patients are colonized by resistant strains and therefore it is important to prove the selectivity of a certain drug by using quantitative microbiology ($\log_{10}$ CFU/cm^2 skin) (Eady *et al.*, 2003a).
- The likelihood of becoming colonized with resistant *P. acnes* strains increases with the treatment duration and, in previously untreated patients, resistant strains appear after 12-24 weeks of treatment (Eady, 1998). Courses of 4-6 months are likely to result in resistance development (Dreno *et al.*, 2004).
- Sequential or simultaneous use of chemically different antibiotics which may determine the selection of multiple resistant propionibacteria (Eady, 1998).
- It is possible that poor treatment compliance may promote the selection of resistant isolates by altering the antibiotic pressure (Eady, 1998).

4.3 *P. acnes* resistance mechanisms

Ross *et al.* (1997) have shown the genetic base of resistance against EM and CL in cutaneous *P. acnes* and classified different resistance phenotypes according to the patterns of cross-resistance to MLS antibiotics: **m**acrolides (14- and 16- member rings),

lincosamides and type B **s**treptogramins. The most prevalent mechanism was due to three different point mutations in genes encoding domain V, the peptidyltransferase loop of the 23S rRNA, and it has been demonstrated that each of these mutations is associated with a specific cross-resistance phenotype to MLS antibiotics. The corynebacterial transposon Tn 5432 carrying *erm*(X) resistance gene has been detected in *P. acnes* strains highly resistant to MLS antibiotics (Ross *et al.*, 2002). *Erm* (*e*rythromycin *r*ibosome *m*ethylase) genes encode for methyl transferases that methylate the N^6 position of *Escherichia coli* (*E. coli*) A2058 in the 23S rRNA. This position represents the overlapping binding site for MLS antibiotics and is responsible for cross-resistance to these antibiotics (**Table 6**) (Leclercq, 2002). The *Erm*(X) gene confers resistance to higher concentrations of MLS antibiotics than the point mutations do. It is therefore possible that the incidence of the transposon will increase if topical antibiotics are widely used and mutational resistance may not be sufficiently protective *in vivo* against the antibacterial substances (Ross *et al.*, 2002).

Table 6. Genetic mechanisms of MLS antibiotic-resistance in *P. acnes* isolates. The individual nucleotides are enumerated in the order of their occurrence based on the equivalent 23S rRNA sequence of *E. coli*.

	23S rRNA base mutation/resistance gene	**Resistance phenotype**
Group I	A→G transition at *E. coli* equivalent base 2058	MLS resistance
Group II	*erm*(X)	High level MLS resistance, especially to telithromycin and clindamycin
Group III	G→A transition at *E. coli* equivalent base 2057	Low level resistance to 14- membered-ring and susceptibility to 16-membered-ring macrolides
Group IV	A→G transition at *E. coli* equivalent base 2059	Resistance to 14-and 16- membered-ring macrolides; elevated but variable resistance to lincosamides

In general, resistance to TET has been shown to be associated with resistance to EM and CL. A single G-C transition in the 16S rRNA of the small ribosomal subunit at *E. coli* equivalent base 1058 was found to be responsible for clinical TET resistance in *P. acnes* (Ross *et al.*, 1998). TET resistant isolates may display various grades of cross-resistance to minocycline and doxycycline (Ross *et al.*, 2001). If a *P. acnes* strain exhibits resistance to a single member of an antibacterial class, it will exhibit various resistance degrees against all members of that class (Eady *et al.*, 2003a).

The administration of increasing antibiotic doses will drive several resistance factors that are required to produce clinically applicable increases in minimum inhibitory concentrations (MICs). Some experts recommend starting therapy with a full therapeutic dose that will permit achievement of a sufficiently high concentration. Isolates with reduced susceptibility will then be affected and may not survive by acquiring new mutations (Eady *et al.*, 2003a), although the physicians should be aware of possible side effects (Dreno *et al.*, 2004).

Trimethoprim/sulfamethoxazole resistant *P. acnes* strains have seldom been isolated (Eady *et al.*, 1993; Ross *et al.*, 2001) and propionibacteria with reduced susceptibility to chloramphenicol and fusidic acid have evolved (Ross *et al.*, 2001).

4.4 Effects of resistance on acne patients outcome

4.4.1 Oral antibiotic treatment

There are studies which have proved a correlation between the presence of antibiotic-resistant *P. acnes* and the clinical response to orally administered EM and TET (Eady *et al.*, 1989; Leyden *et al.*, 1983; Ozolins *et al.*, 2004). The clarification of the correlation found between EM resistant *P. acnes* and the clinical response to EM resides in the fact that the MIC of EM for resistant strains was much higher (512-2048 mg/L) than the concentration possible to be reached in follicles after oral administration of the drug (Coates *et al.*, 2003). The link between resistance and treatment outcome of oral TET is more difficult to prove since the effect is more dependent upon follicular concentration than in the case of EM and strains considered resistant in the laboratory may be clinically susceptible (Eady *et al.*, 2003). MICs of resistant strains were 4-64 mg/L for TET and 2-16 mg/L for minocycline thereby overlapping with plasma levels. As a result, the concentration in follicles may be sufficient to inhibit the resistant strains (Coates *et al.*, 2003). Minocycline has the highest lipophilicity of all cyclines but the partition in the skin surface sebum seems not to be sufficient to inhibit some resistant isolates (Eady *et al.*, 2003a).

4.4.2 Topical antibiotic treatment

An association has been found between the occurrence of antibiotic-resistant *P. acnes* and the clinical response to topical antibiotics (Eady *et al.*, 1989; Leyden *et al.*, 1983; Mills *et al.*, 2002). Topical administration may deliver enough active substance to inhibit strains with a high MIC value but the effect is generally concentration and formulation dependent. It has been shown that topical CL and EM can be selective or inhibitory for resistant *P. acnes* depending on the concentration or formulation of the agent (Eady *et al.*, 2003a).

4.4.3 Other factors influencing the clinical outcome

Besides antibiotic-resistant *P. acnes* there are many other reasons for a poor clinical response in acne patients: inadequate dose or treatment duration of antibiotic used, poor compliance, high sebum excretion rate (more than 2.5 $\mu g/cm^2/min$ will result in a poor treatment response with oral antibiotics), folliculitis due to staphylococci, enterobacteria or yeasts (Eady, 1998).

5 AIMS OF THE STUDY

General aim

The general aim of the present thesis was to perform a characterisation of antibiotic-resistant *P. acnes* clinical isolates from acne patients and various other diseases. The resistance pattern, epidemiological relatedness and molecular resistance mechanisms were analysed.

Specific aims

The specific aims of the studies were to:

- evaluate the association between previous antibiotic treatment and the occurrence of antibiotic-resistant *P. acnes* strains collected from the skin of acne patients in the Stockholm area;
- analyse the prevalence of antibiotic-resistant *P. acnes* strains in patients with different diseases in 13 European countries;
- identify the genetic diversity among antibiotic-resistant *P. acnes* strains by using PFGE as a typing method and to determine the mechanisms of resistance in CL-EM and TET resistant strains by PCR and gene sequencing;
- compare the efficacy of two different drugs used for treatment of acne: TET (the most commonly prescribed antibiotic in acne patients) and ISO, considered as the "golden standard" of acne therapy, from the clinical and microbiological perspective;
- investigate the spread and persistence of resistant *P. acnes* strains in the general population and to examine the genetic relatedness of *P. acnes* resistant isolates from patients and their close contacts using PFGE.

6 MATERIALS AND METHODS

6.1 Patients and controls (Papers I, IV and V)

All patients and controls were informed and they or their parents gave their oral and written informed consent to take part in the study.

The participants in Paper **I** were 100 male and female acne patients between 12 and 45 years of age. The patients had moderate to severe inflammatory acne (papulo-pustular acne and nodular lesions) and had received oral antibiotic therapy during the last 2-6 months. A control group of 30 acne patients with the same characteristics as above but without any antibiotic treatment for the last 2 months was also enrolled. The study was conducted exclusively in the Stockholm area.

In Paper **IV,** male and female patients with moderate or severe inflammatory acne vulgaris (at least grade 3 on the face, back or chest according to Leeds acne scoring system), between 15-35 years of age, were enrolled in the study. The patients were randomly assigned to one of two treatments. The first group (TET/ADA group) received a 24-week treatment with TET hydrochloride (Tetracyklin®; NM Pharma) (500 mg twice daily, 1 hour before meals) and topical adapalene (ADA) once a day in a thin film on the affected area (Differin® gel 0.1%; Galderma Nordic AB). The second group (ISO group) received oral ISO (Roaccutane®; Roche, Basel, Switzerland), 1 mg/kg/day, in two doses. ISO is a potent teratogen and for women patients treated with this drug a pregnancy test was performed and oral contraception with Diane 35 (Schering Nordiska AB) was started before, has continued during the treatment period and 6 weeks post-therapy. After the cessation of oral treatment, in the 2-month follow-up period, the TET/ADA group of patients received topical ADA once a day as a maintenance therapy, while the patients from the ISO group did not use any maintenance therapy. The study was conducted at the Division of Dermatology and Venereology, Karolinska University Hospital Huddinge.

In Paper **V,** five male and female patients aged 15-35 years old with moderate to severe inflammatory acne were included. These patients had received oral lymecycline (Tetralysal®; Galderma Nordic AB), 300 mg twice daily and daily topical ADA (Differin® gel 0.1%; Galderma Nordic AB) for 4 months. At least two of their close contacts living in the same household as the treated acne patients and with close and/or regular contact with the acne patients were included in the study. Twelve male and female volunteers, within the same range of age as the contact group, were included as a control group in the study. They were healthy persons, with no acne, no hospitalization and no history of antibiotic treatment, with no known contact with acne patients or persons treated with antibiotics.

6.2 Clinical evaluation (Paper IV)

The assessment of the clinical efficacy was based on the counting of the lesions and on the acne grading system (Burke *et al.*, 1984). At each visit (baseline, follow-up visits at 2, 4, 6 months of treatment and 2 months after the treatment had stopped) lesions were counted on the face, back and chest and categorised into non-inflammatory, superficial inflammatory and deep inflammatory.

To evaluate the patients' perception and assessment regarding the two treatments, the dermatology life quality index (DLQI) (Finlay *et al.*, 1994) was completed by all patients before the treatment started and after the treatment had stopped.

6.3 Sampling and microbiological procedures (Papers I, IV and V)

The *P. acnes* isolates were examined by a quantitative sampling method. A 5-mL plastic syringe filled with 12% sterile gelatine (Difco Laboratories, USA) was cut at the needle end of the syringe and gelatine was pressed against the skin surface for 10 seconds without rotation. A 2-mm slice of gelatine with the bacterial bearing surface (approximately area 1 cm^2) was cut-off, put in a sterile glass tube with 2 mL pre-reduced peptone-yeast-glucose (PYG) medium (Difco Laboratories, Detroit, MI, USA) and immediately transported to the laboratory, as previously described for the collection of the skin microflora (Borglund *et al.*, 1984; Lidefelt *et al.*, 1991; Sullivan *et al.*, 2001b). It was shown previously that the follicular casts from patients colonized with high numbers of resistant bacteria on the skin surface not always contain antibiotic resistant *P. acnes.* Consequently the follicular colonization with resistant bacteria probably starts from the skin surface (Coates *et al.*, 2002). In order to increase the diversity of the bacterial population more than one sample was collected in each subject. In Paper **I**, three samples per patient and per control were taken from the face, neck or trunk areas. In Paper **IV**, skin samples were taken at baseline, after 2, 4, 6 months of treatment and at 2 months after the treatment had stopped. The samples were taken from five areas (forehead, right cheek, left cheek, back and chest). In Paper **V**, three samples were taken from the patients and their close contacts at baseline, after 2, 4 months of treatment and at 2 months after the treatment had stopped. The samples were taken from three areas (face, back and chest). Skin samples obtained from control subjects were collected twice from the face at 3-days intervals.

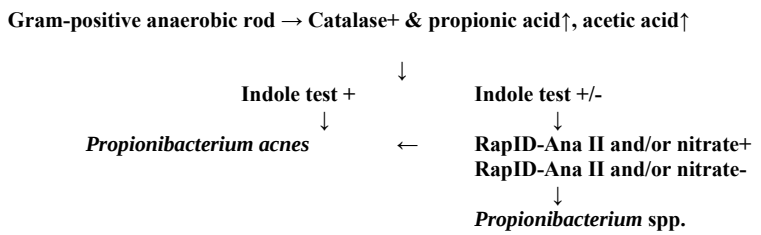
Each of the collected samples was diluted to 10^{-2} in pre-reduced PYG medium and then inoculated on non-selective blood agar medium: Columbia agar base (Acumedia, Baltimore, MD, USA), 0.01% tryptophan and 5% horse blood citrate. The plates were incubated for 7 days at 37ºC in anaerobic jars with a disposable hydrogen and carbon dioxide generator envelope (BBL®, GasPak®; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). After incubation, different colony types were morphologically identified and counted. Marples and McGinley (Marples *et al.*, 1974) have previously shown that cutaneous propionibacteria can be presumptively selected on the basis of colony morphology upon primary subcultivation. The lower limit of detection was 20 CFU/cm^2 skin. From each sample, one colony of each different morphological type was isolated in pure culture and incubated anaerobically and aerobically (for testing air tolerance) 48 h at 37ºC (Lidefelt *et al.*, 1991).

6.4 *P. acnes* identification (Papers I-V)

P. acnes identification was performed by Gram-staining, biochemical tests (McGinley *et al.*, 1978) and determination of the fatty acid metabolites from glucose, after 48 h anaerobic culturing in pre-reduced PYG medium, using gas-liquid chromatography (Varian 3400; Chromacol Ltd., London, UK) analysis (Cummins *et al.*, 1986) – see **Fig. 1**. The production of free fatty acids was interpreted according to the Anaerobe Laboratory Manual (Holdeman *et al.*, 1977). An amount of 750 μL of the PYG-culture was centrifuged for 5 min at 10 000 rpm, and the supernatant was used for the analysis of volatile fatty acids. For non-volatile fatty acid detection, methanol, 50% sulphuric acid, distilled water and chloroform were added to 250 μL of the PYG culture and mixed. The chloroform phase containing dissolved non-volatile fatty acids was analysed with gas-liquid chromatography (GLC).

The *P. acnes* strains identified by these tests were frozen at -70ºC and the determination of resistant strains was performed at the same time.

Figure 1. Flow-chart used for *P. acnes* identification. In case of unclear indole test, the RapID-Ana II System (Remel Inc., Lenexa, USA) for identification of anaerobic isolates (Marler *et al.*, 1991) and/or nitrate test were used for detection (McGinley *et al.*, 1978).



6.5 Bacterial isolates from 13 European countries (Paper II)

Laboratories in each country included in the study (Croatia, Czech Republic, Denmark, Finland, Germany, Great Britain, Greece, Hungary, Italy, the Netherlands, Norway, Slovenia and Sweden) collected consecutive, non-duplicate isolates of *P. acnes*, together with information regarding sampling day and type of infection. The isolates were sent to the Division of Clinical Bacteriology, Karolinska University Hospital Huddinge, Karolinska Institute, Stockholm, Sweden. The *P. acnes* strains were identified as previously described, frozen (-70ºC) and susceptibility testing was performed simultaneously on all isolates.

6.6 Antibiotic susceptibility testing (Papers I-V)

The tested antibiotics were clindamycin (CL), erythromycin (EM), tetracycline (TET) (Papers **I-V**), trimethoprim/sulfamethoxazole in Paper **I**, linezolid (LIN) (Papers **II**, **IV** and **V**), benzylpenicillin and vancomycin in Paper **II**. The MIC of each antibiotic was determined by the agar dilution method according to the The National Committee for Clinical Laboratory Standards (NCCLS, 2004). This is the reference method for anaerobic bacteria and it is performed using brucella base agar supplemented with hemin, vitamin K_1 and 5% sterile defibrinated horse blood (Papers **II-V**) or PDM Antibiotic Sensitivity Medium Agar (Biodisk, Solna, Sweden) supplemented with 5% sterile defibrinated horse blood (Paper **I**). Different antimicrobial concentrations were obtained by incorporating each substance when preparing the agar plates. The inocula consisted of approximately 1 x 10^5 CFU per spot applied to the agar plates with a modified Steers replicator. An agar plate without antimicrobial agent was used as a growth control. The agar plates were incubated in anaerobic jars for 48 h at 37ºC. At least three control strains were used for each experiment. The MIC was defined as the lowest concentration of the antimicrobial agent that resulted in a marked change in the appearance of growth in comparison with the control plate, as described in the NCCLS protocol. Resistance to TET was defined at MIC ≥ 2 mg/L, to CL at MIC ≥ 0.25 mg/L, to EM at MIC ≥ 0.5 mg/L, to trimethoprim/sulfamethoxazole at MIC ≥ 1 mg/L, to LIN at MIC ≥ 8 mg/L, to benzylpenicillin at MIC ≥ 0.25 mg/L, and to vancomycin at MIC ≥ 4 mg/L (EUCAST, 2003). The MIC_{50} was determined as the antibiotic concentration at which 50% of the isolates were inhibited and consequently at the MIC_{90} value 90% of the isolates were inhibited.

6.7 Genotyping with PFGE (Papers I, II, III and V)

Cells of pure cultures were grown for 3 days on sheep-blood agar plates with glucose 5.8 g/L and the bacteria were embedded in low-melting agarose (Sea Plaque GTC agarose; Bio Whittaker Molecular Application, Rockland, ME, USA) for plug preparation. The lysis buffer consisted of 6 mM Tris pH 7.5, 0.5% lauroyl sarcosyl, 1% polyethylenglycol, 0.5% brij, 0.2% deoxycholate containing 625 U/mL of mutanolysin (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 12.5 mg/mL of lysozyme (Sigma-Aldrich, Inc.). Mutanolysin in mixture with lysozyme was necessary for inducing lysis (Fliss *et al.*, 1991). In the lysis buffer, polyethylene glycol was used serving as an osmotic stabilizer and as an enhancer of the lysis process (Chassy *et al.*, 1980). Lysis buffer was then replaced by the proteolysis buffer: 1% SDS (sodium dodecyl sulphate), 1 mg/mL protease (Sigma-Aldrich, Inc.). The proteolysis step was carried out at 37ºC for 12 h. The agarose plugs were washed four times with 10 mL $T_{10}E_1$ buffer (10 mMTris HCl, 1 mM Na_2EDTA [Ph 8]) at room temperature with gentle shaking, digested 3 h with 20 U of *Spe* I restriction enzyme (Promega, Madison, WI, USA) at 37ºC. The plugs were loaded in a 1% agarose gel (SeaKem$_R$ GTG agarose; FMC BioProducts, Rockland, ME, USA). Electrophoresis was run by using a contour-clamped homogenous electric field apparatus (Gene Path^{TM} System, Bio-Rad Laboratories, Hercules, CA, USA). The running conditions were as follows: voltage gradient 6V/cm, ramped with an initial switching time of 0.5 s and a final switching time 5 s for 16 h at 14ºC and 120º angle. The gels were stained with ethidium bromide (1 μg/mL) for 30 min, rinsed in tap water (15 min) and photographed under UV irradiation.

6.8 Gel image analysis and calculation of dendrograms (Papers I, II, III and V)

Calculation of similarity matrices and creation of dendrograms was performed using the Molecular Analyst Software program (Bio-Rad Laboratories) in Papers **I**, **II** and **III** and the Gel Compar II (Applied Maths, Kortrijk, Belgium) in Paper **V**, by the unweighted pair group method with arithmetic averages (UPGMA). The similarity coefficients were calculated according to the method of Dice, expressed as percentages (Dice, 1945). *P. acnes* ATCC 6919 was used as control for each gel experiment to allow comparisons.
Capital letters were used to designate the main genetic lineages of *P. acnes.* The isolates were also visually inspected. Criteria established by Tenover *et al.* (1995) are to be regarded as relative recommendations rather than strict guidelines and are applicable for small sets of epidemiologically related isolates ($\leq$ 30) and for investigating outbreaks. The criteria for strain identity are considered not appropriate for studies of larger populations of organisms collected over extended periods of time. The accepted level of band difference within a specific strain depends on the genomic heterogeneity within a certain species of bacteria (Lund, 2003). Visual analysis revealed that isolates from the same cluster had fewer than 3 bands difference and were considered to represent the same strain, while the number of band differences between isolates belonging to different clusters was greater than four (Tenover *et al.*, 1995). All isolates could be typed by PFGE. The preparation and digestion of randomly chosen DNA strains was repeated under the same conditions to assess the reproducibility of the method, revealing identical results. To validate the results, PFGE analysis was carried out with multiple enzymes (*Spe* I and *Not* I). Both enzymes have AT-rich recognition sites. No

differences regarding the discriminatory power were found. A minimum of 70% cophenetic correlation coefficient is generally considered to be necessary to ensure that the dendrogram accurately represents the similarities between the PFGE patterns (McEllistrem *et al.*, 2004). The cophenetic correlation coefficient was calculated for each dendrogram resulting in 75% (Paper **I**), 82% (Paper **II**), 78% respective 94% (Paper **III**) and 95%/70%/90% in Paper **V**.

6.9 PCR and sequencing of resistant isolates (Paper III)

A total number of 36 CL-EM and 27 TET resistant *P. acnes* strains collected in Papers I and II from acne patients and various diseases were analysed. Ten susceptible *P. acnes* strains were also investigated. Control strains *P. acnes* ATCC 6919 and *P. acnes* ATCC 11828 were used.

6.9.1 DNA preparation

The bacterial strains were cultured under anaerobic conditions on blood agar plates (BBL GasPak Anaerobic System, Cockeysville, MD, USA) at 37ºC for 48 h. Approximately 1 µL of bacteria was suspended in 100 µL MQ water before heated at 95ºC for 15 min and centrifuged (10 000 rpm, 7 min, 4ºC). The DNA containing supernatant, free from cellular debris, was stored at -20ºC until used.

6.9.2 Clindamycin and erythromycin resistant isolates

PCR and sequencing of genes encoding the conserved domain V of 23S rRNA were performed in order to detect the basis for resistance among CL and EM resistant strains. To locate mutations in this region, amplification of a section of the DNA, corresponding to *E. coli* equivalents as previously described (Ross *et al.*, 1997), was accomplished with a pair of 20-bp primers (**Table 7**). The reactions were performed in a 50 µL volume containing 1 µL DNA, 10 x PCR reaction buffer, 1.5 mM $MgCl_2$, PCR nucleotide mix including 200 µM each of dNTP (Promega Corp., Madison, WI, USA), 0.5 µM of each primer (ThermoHybaid GmbH, Ulm, Germany) and 0.5 U of Taq DNA polymerase (Promega Corp.). All PCR analysis were subjected to an initial denaturation at 94ºC (10 min), followed by 30 cycles of 94ºC (30 s), 52ºC (30 s), 72ºC (1 min) and a final elongation at 72ºC (10 min).

Electrophoresis in 1.5% agarose gels was used for detection of amplified products. The PCR products were purified using a QIAquik PCR purification kit (QIAGEN GmbH, Hilden, Germany) to remove free primers and nucleotides. The sequence analysis using ABI 310 Genetic Analyser was performed according to the manufacturer (Perkin Elmer, Foster City, CA, USA). Alignment analysis was made using the ClustalW interactive multiple sequence alignment from European Bioinformatics Institute (http://www.ebi.ac.uk).

6.9.2.1 Extended PCR for erm(X) gene detection

To detect whether the *erm*(X) gene was carried by CL and EM resistant strains, a pair of primers was designed (Primer Premier version 5.0) based on the sequence of the *P. acnes erm*(X) gene, GenBank accession no. AF411029 (**Table 7**). The PCR reactions were performed as described above, with exception of using 2 mM $MgCl_2$. The amplification products were run on electrophoresis in 3% agarose gels and stained by

ethidium bromide for visualization. Strain 98-4277-2 of *Arcanobacterium pyogenes*, kindly provided from Dr. B.H. Jost, was used as a positive control (Jost *et al.*, 2003).

6.9.3 Tetracycline-resistant isolates

Amplification of a 444 bp section of DNA encoding the 16S rRNA was accomplished with a pair of primers designed with the program Primer Premier version 5.0 (**Table 7**). The reactions were performed as above, with 1.5 mM $MgCl_2$.The PCR products were purified and the sequence analysed.

Table 7. Description of oligonucleotide primers used in the PCR assays.

Target gene	Name	Sequence 5′-3′	Amplicon
23S rRNA	23SF	CGA AAT TCC TTG TCG GGT AA	241 bp
	23SR	GTA TTT CAA GGT CGG CTC CA	
***erm*(X)**	ermXF	ATA ACG GCA GTT GAA GTG GA	167 bp
	ermXR	CGA AGA ATG GCA GTG GTG	
16S rRNA	16SF	GAC ATG GAT CGG GAG TGC	444 bp
	16SR	TCG GGT GTT ACC AAC TTT CA	

6.10 Reserpine test of tetracycline-resistant *P. acnes* (Paper III)

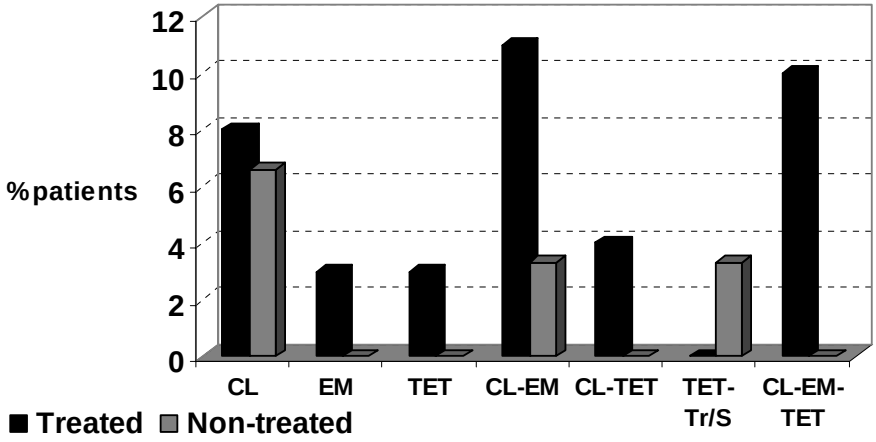
In order to verify the contribution of active efflux to the TET resistance phenotype, the *in vitro* activity of TET was compared for all 27 strains, with and without the efflux pump inhibitor reserpine (Neyfakh *et al.*, 1991). The hypothesis was that the active efflux and the target gene mutation contribute independently to TET resistance. TET was obtained from Lederle Laboratories (Pearl River, NY, USA) and reserpine from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Reserpine solution was freshly prepared by using dimethyl sulfoxide as a solvent. MIC determination of TET was performed with the agar dilution method (NCCLS, 2004) using brucella base sheep blood agar with and without addition of reserpine (20 μg/mL) (Oh *et al.*, 2003).

7 RESULTS

7.1 Antibiotic-resistant *P. acnes* in acne patients (Paper I)

Acne patients treated with antibiotics were found to have a higher risk of carrying resistant *P. acnes* strains as compared to the non-treated group of patients: OR=3.8; 95% CI=[2.1-6.7]; p=0.01. The prevalence of TET resistant strains in the treated group was lower as compared to CL and EM resistant bacteria, although oral TET was the most commonly prescribed drug for acne treatment (98% patients). Seventeen percent of the patients carried TET resistant strains and 34% carried CL and/or EM resistant *P. acnes* strains in this group. Ten percent of the patients from the treated group were colonized with strains resistant to CL-EM-TET (**Fig. 2**). Logistic regression analysis showed that there was no difference between males and females with regard to carrying resistant *P. acnes* strains.

Fig. 2. Percentage of patients colonized with antibiotic-resistant *P. acnes* strains with different resistance profiles in acne patients treated (n=100) and non-treated (n=30) with antibiotics.



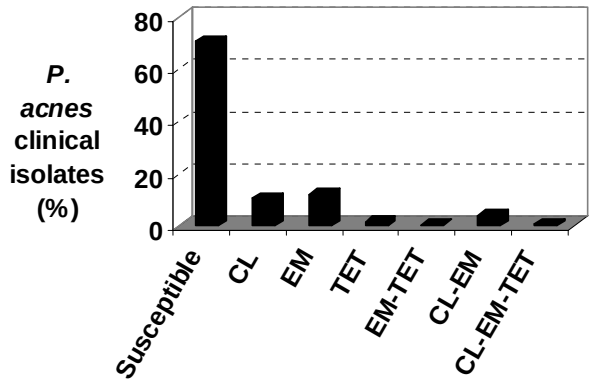
The group treated with antibiotics had strains with higher MICs for CL (64 mg/L comparative with 16 mg/L) and TET (32 mg/L comparative with 4 mg/L); for EM the range of sensitivity was the same (≤0.008->128 mg/L) and for trimethoprim-sulfamethoxazole the range was higher for the non-treated group (4 mg/L compared to 0.5 mg/L).

7.2 Antibiotic-resistant *P. acnes* in various diseases (Paper II)

Overall 29% of *P. acnes* isolates were resistant to at least one of the tested antimicrobial agents and most of them were resistant to CL and/or EM. Less than 3% of the collected *P. acnes* strains were TET resistant (**Fig. 3**).

All clinical isolates were found susceptible to low concentrations of benzylpenicillin (MICs 0.008-0.125 mg/L), vancomycin (MICs 0.25-2 mg/L) and linezolid (MICs 0.25-2 mg/L).

Fig. 3. Resistance profile of *P. acnes* clinical isolates from various diseases (n=304).



The rates of resistance from the most common sources are presented in **Table 8**.

Table 8. Resistance to antibiotics expressed as percentage of *P. acnes* isolates from different sources.

Source of isolation (number tested)	% of resistant isolates		
	CL ≥ 0.25 mg/L	EM ≥ 0.5 mg/L	TET ≥ 2 mg/L
Blood (105)	20	31.4	1.0
Skin/soft tissue infections (77)	16.9	7.8	3.9
Abdominal infections (22)	18.2	18.2	-
Head/neck (19)	5.3	10.5	5.3
Bone (15)	-	13.3	6.7
Meningitis (14)	21.4	21.4	-
Eye infections (13)	15.4	-	-
Prosthesis (10)	20	10	-
Lung/pleural fluid (6)	-	16.7	-
Biliary tract infections (4)	-	-	25
Endocarditis (13), lymph nodes (2), females genital (2), arthritis (1)	-	-	-
Faeces (1)	-	-	100

The prevalence of resistance ranged from 83% in Croatia and 60% in Italy to 7% in Great Britain and no antibiotic-resistant isolate in the Netherlands. Different selection pressures operated for each country or region, but generally the highest prevalence of resistance was detected in isolates from the southern Europe (Greece and Italy) where 54.5% of *P. acnes* strains were resistant to antibiotics and the lowest prevalence (6.2%) was found in the western European countries (Germany, Great Britain and the

Netherlands). In comparison to other countries from the region, lower resistance rates were found for isolates from Hungary and Czech Republic, results which are in agreement with those of a study investigating skin samples (Ross *et al.*, 2003).

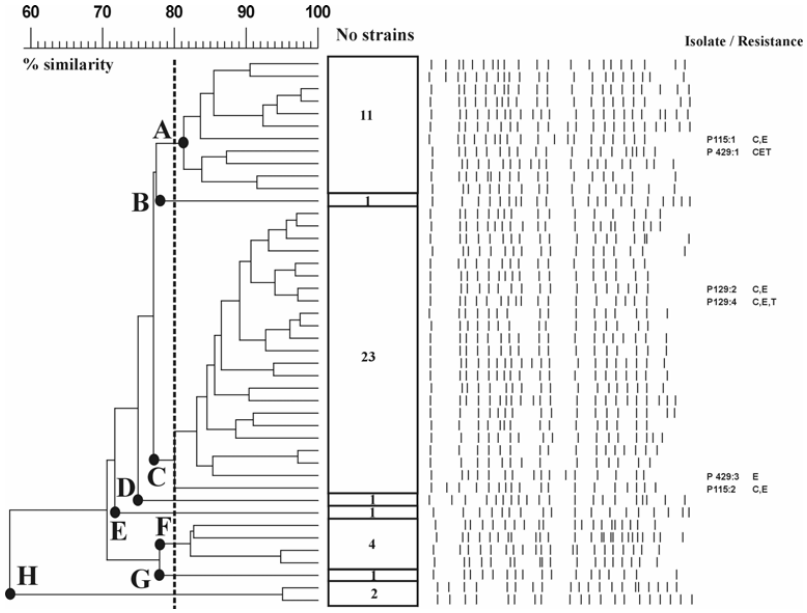
Interestingly, some correlations were found between outpatient antibiotic sales in countries of the European Union expressed as daily dose/1000 inhabitants/day (Cars *et al.*, 2001) and the resistant rates found in the present study. In Finland, the country with the highest use of TET (5.5), a higher rate (11.8%) of TET resistance was found. In Italy, currently with the lowest TET consumption (0.56), no isolates were TET resistant. Similar correlations were observed between the highest sales of macrolides and lincosamides in Italy (5.07) and high rates of resistance to EM (42.1%) and CL (21.1%). Sweden has reported a large reduction in antibiotic use, but the rates of resistance to CL and EM were 11.5% for both antimicrobial agents and 3.8% for TET. The very low use of antibiotics in the Netherlands was reflected in the present results by the absence of resistant isolates.

7.3 Genetic diversity of antibiotic-resistant *P. acnes* (Papers I and II)

7.3.1 PFGE typing of resistant isolates from acne patients (Paper I)

All of the resistant strains (n=69) were typed using PFGE. One representative of the isolates per individual, presenting the same antibiogram and the same PFGE pattern, was included in dendrogram anlysis. Eight main genetic lineages composed between 1 to 23 isolates in each were noticed. The similarity between major types ranged from 58% to 98% and a relatively tight clustering was noticed. Two isolates that had a more distant pulsed-field pattern formed a single clone and clustered with the other *P. acnes* at 58% similarity **(Fig. 4).**

Fig. 4. Dendrogram of antibiotic-resistant *P. acnes* from acne patients. The dotted line represents the clonal group level set at 80%. A-H represent the main genetic lineages.



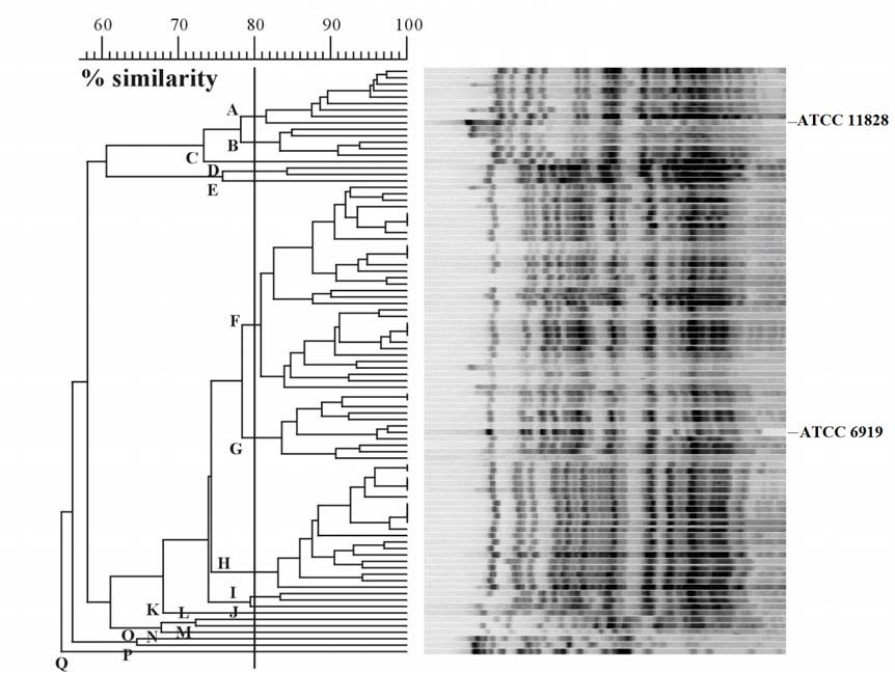
One patient (P 129) was colonized with two antibiotic-resistant *P. acnes* strains with different resistant patterns: CL (MIC 4 mg/L), EM (MIC > 128 mg/L) respective CL (MIC 32 mg/L), EM (MIC >128 mg/L) and TET (MIC 4 mg/L), both strains belonging to the same clone C. Patient 429 was colonized with strains with different resistance patterns belonging to different clones (A and C). Patient P 115 carried two different strains (belonging to clone A and C) with different degrees of CL resistance (MIC 0.25 mg/L and 0.5 mg/L).

Eighty-three percent of the CL resistant *P. acnes* strains belonged to a single clone.

7.3.2 PFGE typing of resistant isolates from other diseases (Paper II)

All of the resistant isolates (n=89) together with two *P. acnes* reference strains belonging to different serotypes: ATCC 6919 (serotype I) and ATCC 11828 (serotype II) were typed by using PFGE. Seventeen clones composed between 1 to 32 isolates were identified **(Fig. 5).**

Fig. 5. Dendrogram analysis of 89 resistant strains and two *P. acnes* reference strains divided into the 17 major clonal groups. The vertical line corresponds to the cut-off level of 80% used to delineate PFGE types.



7.3.2.1 Correlations of pulsed-field types and resistance pattern or infection type

Sixty-nine percent of clindamycin resistant strains were grouped in type F. CL resistant *P. acnes* strains with the PFGE class F pattern showed MICs ranging from 0.25 to 1 mg/L. *P. acnes* showing other PFGE patterns had CL MICs from < 0.064 to 2 mg/L. Clone H contained 46% of EM resistant *P. acnes* isolates, with MICs of 0.5-1 mg/L**,**

while other EM resistant isolates had MICs of 0.5-128 mg/L. Sixty percent of isolates from soft tissue infections belonged to clone F. The two reference strains were found to belong to different clones, with less than 60% similarity.

7.4 Genetic basis of *P. acnes* resistance (Paper III)

7.4.1 Clindamycin-erythromycin resistant isolates

The previously described mutations were identified in the group of 36 CL-EM analysed isolates. The genotype I was the most prevalent (46% of strains collected from acne and 28.5% of clinical isolates), followed by genotype IV (9%, respective 14.2%) and genotype III (9% of acne isolates). In general, more strains from acne compared to those from other diseases carried one of the mutations: OR=2.33, [CI=1.16-4.69]. In 37% of the isolates from acne and 57.1% of the isolates from other diseases there was no mutation detected. Pairwise alignment and comparison using ClustalW/EMBL did not reveal any other unknown mutation in the resistant strains within the DNA sequence of the investigated fragment. No CL-EM resistant isolates carried the *erm*(X) gene.

The correlation between CL and EM MIC values and different mutation types indicated a certain pattern (see Table 6), with two exceptions: one strain from the phenotypic group I showed a lower level of resistance to EM and CL (0.5 mg/L) and one strain from group III showed a higher level of EM resistance (128 mg/L), as compared to isolates from the same group.

7.4.1.1 Relationship between CL-EM resistance genotype and PFGE analysis data

CL-EM resistant isolates were grouped in five clusters by using a cut-off value of 75% among strains. Isolates carrying the mutation types I or IV were clustered within three different clones, and the two isolates carrying mutation type III belonged to a single clone.

7.4.2 Tetracycline-resistant isolates

PCR and DNA sequencing of the 27 TET resistant strains revealed that 44% carried a G–C transition at *E. coli* equivalent base 1058 in the 16S rRNA of the small ribosomal subunit. These strains were all isolated from Swedish acne patients. The MIC value of TET in strains carrying the mutation (n=12) was 32 mg/L, whereas the MIC range in strains with no detectable mutation was 2-8 mg/L. The MIC values in all the TET resistant strains were unaffected by the presence of the efflux pump inhibitor reserpine.

7.4.2.1 Relationship between TET resistance genotype and PFGE analysis data

The clonal group level was set at 75% similarity. Among the clinical isolates, those harbouring the mutation at 16S rRNA were clustered in one clone. In strains where no mutation was found, a greater variability was noticed; these strains were distributed among all seven clones with a 35-75% range of similarity between them.

7.5 Clinical and microbiological comparisons of oral isotretinoin versus tetracycline in acne patients (Paper IV)

A total of 52 patients were randomized, 26 patients to receive TET/ADA and 26 patients to receive oral ISO. Three patients abandoned the study after randomization and were not included in further analysis. Analysis of clinical efficacy parameters was

performed on the intention-to-treat population, which included all patients who had at least one post-baseline evaluation. For patients who prematurely discontinued the treatment, the last observations were carried forward.

7.5.1 Clinical results

Both the ISO and TET/ADA medication reduced the number of superficial inflammatory, deep inflammatory and non-inflammatory lesions ($p<0.001$). ISO demonstrated an advantage over TET/ADA. A significant difference between treatments was seen after 2 months (non-inflammatory lesions) and 4 months (superficial inflammatory lesions). There was no difference between the treatment groups with regard to deep inflammatory lesions after 6 months of therapy. After the treatment had stopped, patients in the ISO group had fewer lesions as compared to the TET/ADA group. There was no correlation between age or acne duration and the clinical response in any of the two groups. Total lesion counts were summarized and analysed by sub-group for gender difference. Both male and female patients in the ISO group had more pronounced reductions after 6 months of treatment (87.3% and 90.6% respectively) as compared to the male and female patients treated with TET/ADA (43.3% and 62.5%), $p<0.05$. The advantage of ISO persisted in the drug-free period. In female patients treated with ISO the total number of lesions continued to decrease noticeably with 43.1 % after the treatment stopped, while in the other sub-groups the lesion numbers increased.
The evolution over time regarding reduction of face acne grading was better for ISO treated patients (an interaction between time and treatment $p=0.008$). Comparisons between groups at different time points demonstrated no difference between ISO and TET/ADA during the 6-month treatment period, but a significant difference after the follow-up period ($p=0.009$).

7.5.1.1 DLQI scores

There was a significant improvement in both treatment groups after 24 weeks of therapy as described by DLQI ($p<0.001$). Analysis of the gender difference showed improvement in life quality for male patients in both groups and in female patients treated with ISO, but not in female patients treated with TET/ADA.

7.5.2 Microbiological results

The microbiological results are reported in terms of the average number of bacteria per cm^2 and the prevalence of patients in each group having a particular finding.

Both treatments produced a significant reduction from baseline evaluation in total *P. acnes* counts, but treatment with ISO was more effective than TET/ADA, starting 2 months of treatment, and the difference persisted for the whole study period. There was no significant variation in the density of TET or CL-EM resistant bacteria in any of the two groups, although it was a tendency for patients treated with TET/ADA to gain resistant strains of *P. acnes* and for patients treated with ISO to preserve or to loose them. After adjusting for baseline differences and by using logistic regression, there was no difference between the two groups with regard to the carriage of TET or EM resistant strains after 6 months of treatment. During the same period, the odds for a patient in TET/ADA group to carry CL resistant strains as compared to the ISO group was 0.18 (95% CI=[0.05-0.71], $p=0.014$). Two months after the treatment had stopped, there was a significant difference between treatments, and the probability to carry

resistant strains for CL and TET was higher in the TET/ADA group as compared to the ISO group (OR=0.06, 95% CI=[0.013-0.37], p<0.01, and OR=0.05, 95% CI=[0.006-0.49], p<0.001, respectively). After 6 months of treatment, patients of both groups carrying TET resistant *P. acnes* strains were found to carry CL-EM resistant strains more often than patients with no TET resistant strains (p<0.05).

No correlation was found between the density or presence of resistant *P. acnes* strains for each of the investigated regions (face, back and chest) and the clinical response, expressed as total lesion number or acne grading.

7.6 The spread of antibiotic-resistant *P. acnes* (Paper V)

Four male and one female acne patients with a mean age of 16 years and a range of 14-18 years were treated with oral lymecycline. Acne contacts consisted of seven parents and six siblings (10 females and 3 males) with a mean age of 31 years and a range of 13-54 years. Controls consisted of eight females and four males and their mean age was 31 years (range 23-47 years).

7.6.1 Microbiological results

Two patients were colonized with EM and CL resistant strains, respectively, at baseline. During the 4-month study period, four of the five patients carried resistant bacteria for at least one of the tested antibiotics and three of them carried strains resistant to CL-EM-TET. The resistant *P. acnes* strains were also recovered 2 months after the treatment had stopped in all four patients.

Three contacts (23%) were colonized with CL resistant strains at baseline and two of them had lost their resistant strains after 2 months. During the following 4 months, four of the 13 contacts carried resistant *P. acnes* strains: three for TET and one for CL-EM. After the treatment had stopped, no resistant *P. acnes* were isolated from the contacts. One contact carried resistant bacteria at two different determinations. Taken together the results showed that 46% of contacts carried resistant *P. acnes* strains at least once during the study.

In the control group, four subjects (33%) carried CL (2 subjects), TET (1 subject) and TET-EM (1 subject) resistant *P. acnes* isolates. No difference was noticed between the contacts and controls regarding carriage of resistant bacteria at baseline (p>0.05).

No LIN resistant *P. acnes* strains were detected on the skin of acne patients, close contacts or healthy subjects.

7.6.2 PFGE patterns

PFGE analysis was performed for all of the *P. acnes* resistant isolates collected from patients and contacts during the study (families 2, 3 and 4). One representative of the isolates from each sampling source per individual, presenting the same antibiograms and the same PFGE pattern, was included in dendrogram analysis. These isolates were considered to represent the same strain repeatedly detected, therefore only one isolate was analysed.

Family 2: At baseline, both contacts and the patient carried a CL resistant isolate belonging to the same clone B and the patient was also found to harbour a CL resistant *P. acnes* belonging to a different clone A. After 4 months of treatment and at the follow-up visit the strain from clone A became predominant on the patient skin, but

with a different resistance pattern (CL-EM-TET), whereas no resistant *P. acnes* was detected in any of the contacts.

Family 3: At baseline, neither patient nor contacts harboured resistant isolates. Two months after the treatment had started, CL, CL-EM and CL-EM-TET resistant *P. acnes* strains were recovered from the patient, all belonging to the same clone A. Certain heterogeneity was noticed within this clone, and two of the strains collected at the follow-up visit, were found to have 82% similarity with the other strains from the same group. Two months after the patient had started the treatment, the contact carried a TET resistant *P. acnes* strain that did not belong to clone A found in the patient, but still related to clone A, with a Dice coefficient of 72.13% and a cophenetic correlation of 70%.

Family 4: The strains collected from the patient and both contacts belonged to the same cluster, however clearly divided into two subgroups. One subgroup consisted of multi-resistant strains from the patient (2 strains from the patient were 100% identical) and the other subgroup consisted of a CL-EM resistant *P. acnes* from one contact or a TET resistant *P. acnes* from another contact.

8 DISCUSSION

The drawback with regard to the usefulness of long-term treatment with antibiotics is the possible effect on microbial ecology. The normal microflora represents a barrier against colonization by pathogenic bacteria and colonization by already present microorganisms, i.e., colonization resistance. Acne patients who are in general heavily treated with antibiotics may suffer such disturbances (Sullivan *et al.*, 2001a). EM, CL and TET have been shown to strongly suppress the oropharyngeal and gastrointestinal microflora (Sullivan *et al.*, 2005) and this may promote an overgrowth of pathogenic bacteria or yeast. Topical antibiotics exert selective pressure at the site of application but probably also at other sites of the body by transfer of the product and can easily be transmitted to the patient's close contacts (Eady, 1998). Oral antibiotics exert selective pressure for resistance development at all body sites where there is a normal microflora. Not only *Propionibacterium* spp. may become resistant, but also coagulase-negative staphylococci on the skin, *S. aureus* in the nares, streptococci in the oral cavity, enterobacteria in the gut, etc. (Eady, 1998).

In a group of patients receiving oral TET, pronounced changes in the colon microflora and new colonization with TET resistant strains occurred (Borglund *et al.*, 1984).

TET, minocycline (Eady, 1998), CL (Borglund *et al.*, 1984) and EM resistant coagulase-negative staphylococci (Dreno *et al.*, 2001) were identified in acne patients. Antibiotic-resistant coagulase negative staphylococci were found to colonize skin and nares after topical administration of EM and may transfer their resistant genes to *S. aureus* (Vowels *et al.,* 1996). *S. aureus* has developed worldwide resistance to penicillin due to β-lactamase production in > 90% of the cases, and methicillin resistance is now a major problem with resistance levels of > 50% in certain areas of the world. *S. aureus* strains are often multiresistant, and include resistance to EM and TET, with resistance to quinolone developing rapidly.

Many years ago it was claimed that even though acne patients are colonized by antibiotic-resistant bacteria, they are not at a higher risk of infections (Layton *et al.*, 1989). A recent study showed that acne patients treated with antibiotics are more often colonised by TET resistant *Streptococcus pyogenes* in the oropharynx than acne patients not treated with antibiotics (Levy *et al.*, 2003). In addition, the odds of an upper respiratory tract infection to develop in individuals who use an antibiotic to treat acne was shown to be about two times larger than in those not using antibiotics (Margolis *et al.*, 2005).

The bacterial populations are facing a wide range of antibiotic concentrations after each administration of the drug. On the other hand, the spontaneous variability of microbial populations may provide a wide possibility of potentially selected resistant variants. The antibiotic concentration able to select one of these resistant variants is the concentration that can inhibit the susceptible population, but not the isolates harbouring a mechanism of resistance. In other words, if a selective antibiotic concentration is above the MIC of the susceptible population, a selection effect is expected. If the MICs of both the susceptible and the variant populations are surpassed, no selection takes place. The same is true if the antibiotic concentration is below the MICs of both populations. Therefore, the selection of a particular variant may occur in a very narrow range of concentrations, between the MICs of the susceptible and the resistant populations (Baquero *et al.*, 1993).

Epidemiological studies are necessary in order to track patterns of antibiotic resistance and these data may help in designing guidelines for acne treatment. The breakpoint divides organisms into susceptible (sensitive) and resistant groups based on their *in vitro* susceptibility to antibiotics and their clinical efficacy. The interpretative criteria for susceptibility and resistance breakpoints differ in different European countries, USA and Japan. Breakpoints tend to be lower in Europe than in USA which results in higher resistance rates in Europe than in USA (Goldstein *et al.*, 2006). Susceptibility breakpoints used in this thesis were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2003).

Except for a small previous study (Borglund *et al.*, 1984), the situation regarding the prevalence of antibiotic-resistant *P. acnes* in acne patients in the Stockholm region was largely unknown. The data in Paper I demonstrated that the resistance among *P. acnes* strains from acne patients has emerged. Acne patients treated for at least 2 months with oral antibiotics were more likely to develop resistant *P. acnes* strains than acne patients who had not received antibiotic treatment. Even though TET is highly prescribed as acne treatment, the prevalence of TET resistant strains was lower compared to EM or CL resistant strains. This finding is in agreement with previous studies (Ross *et al.*, 2003; Tan *et al.*, 2001). Oral TET is probably less selective as compared to topical CL or EM. Another possibility would be that TET resistant strains are *in vitro* less fit and have growth rates below those fully sensitive (Coates *et al.*, 2002). Resistant isolates were also found in non-treated acne patients as a result of previous treatments (patients' histories were difficult to be ascertained for longer periods and topical CL is commonly prescribed in Sweden) or due to the transfer of resistant isolates from close contacts. One single strain was found to be resistant to trimethoprim/sulfamethoxazole. This type of resistance has been described previously (Eady *et al.*, 1993; Ross *et al.*, 2001) and may indicate that new mechanisms of resistance have evolved.

The results in Paper II demonstrated that there is a potential risk for serious and life-threatening infections caused by CL, EM and TET resistant *P. acnes*. A previous study showed that 5% of patients with acute infections caused by propionibacteria died despite the appropriate antibiotic treatment (Brook *et al.*, 1991). Under certain circumstances many organisms with low pathogenicity, such as propionibacteria, may be involved in different infections: *Bacillus* spp. (Sliman *et al.*, 1987), alpha-streptococci (Swensen *et al.*, 1982) and anaerobic diphteroids (Johnson *et al.*, 1970). Similar to *P. acnes* collected from the skin of acne patients (Ross *et al.*, 2003), TET resistance was less frequently encountered among isolates from various infections. Fortunately, all the clinical isolates were found to be uniformly susceptible to bactericidal antibiotics used for the treatment of serious infections. These data are in agreement with a recent study performed on clinical isolates of *P. acnes* collected from central nervous system infections (Mory *et al.*, 2005). Antibiotics used for acne treatment are not normally used in the treatment of severe infections.

The normal microbial flora may act as a reservoir for antibiotic resistance genes and under some circumstances there may be a transfer of these genes to pathogenic bacteria during their temporary colonization of the same site (Cohen, 1992). It has been demonstrated that Gram-positive genes are now found in Gram-negative species (Roberts, 2002). It is not impossible that other mobile genes encoding for resistance against various antibiotics used in the treatment of severe diseases may be transferable to *P. acnes*.

LIN is the first marked oxazolidinone with a structure unrelated to any antibiotic agent and with a bacteriostatic activity against most susceptible Gram-positive aerobic and anaerobic bacteria (Edlund *et al.*, 1999). LIN may show some bactericidal activity against some streptococci. It was successfully used in the treatment of complicated skin and soft tissue infections, surgical site infections, vancomycin-resistant *Enterococcus faecium* infections, pneumonia and infections caused by methicillin-resistant *S. aureus* (Wilcox, 2005). LIN inhibits bacterial ribosomal protein synthesis by stopping the first step in which bacteria assemble ribosomes from their dissociated subunits. This unique mechanism makes cross-resistance with other antimicrobial agents unlikely (Wilcox, 2005). Nevertheless, LIN resistant enterococci have been isolated from patients treated with this antibiotic (Auckland *et al.* 2002). Recently an *E. faecium* strain in a patient never exposed to LIN was found to be resistant and it was suggested that spontaneous mutations in the 23S rRNA gene conferring LIN resistance are maintained in the absence of the selective pressure (Bonora *et al.*, 2006). LIN resistance has also emerged in *S. aureus* strains (Gales *et al.*, 2006). We have tested *P. acnes* susceptibility to LIN, which may be a promising future treatment of acne vulgaris and serious infections caused by *P. acnes*. None of the tested isolates was found to be resistant against LIN, but clinicians should be aware that propionibacteria may become resistant in the future against this antimicrobial agent.

Another example is retapamulin, a semisynthetic pleuromutilin developed as a topical treatment of uncomplicated skin infections, which showed good *in vitro* activity against clinical isolates of propionibacteria (Goldstein *et al.*, 2006). Daptomycin is a cyclic lipopeptide comprised of a hydrophilic core and lipophilic tail that will be introduced in Europe in 2006. It has a novel mode of action: the tail binds irreversibly to the cell membrane of Gram-positive bacteria via a calcium-dependent process, leading to depolarisation of the cell membrane due to efflux of potassium and other ions. This unique mode of action and the rapid bactericidal activity should serve to minimise the potential for development of resistance (Rybak, 2006). This antimicrobial agent was found to be highly active against propionibacteria (Goldstein *et al.*, 2003).

The international variations in resistance rates emphasize the importance of selective pressure exerted by improperly used antimicrobial agents in general medicine. The emergence of bacterial resistance in *P. acnes* clinical isolates collected from various infections mirrors the situation with the antimicrobials presently in use. Surprisingly, despite a restrictive policy of antibiotic prescription and the fact that Sweden has reported the lowest sales rates for macrolides and lincosamides, the overall rate for *P. acnes* resistant strains in Sweden was higher than for other northern and western European countries. However, sales data cannot be equated with antibiotic exposure without consideration of the patient compliance, low compliance to treatment or self medication, which all are very important contributors to the resistance problem (Albricht *et al.*, 2004).

Early phenotypic methods used to distinguish between different strains within the same species were serotyping, biotyping, phage-typing, bacteriocin typing and antibiotic resistance typing. These methods are not considered sufficient discriminatory today (see 1.4). Examples of novel methods suggested for bacterial typing are: ribotyping, RAPD, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) (Lund, 2003). In ribotyping, extracted DNA is digested with a restriction enzyme, followed by electrophoretic fragment separation on an agarose gel, transferred to a membrane and probed with radiolabelled rRNA genes (Tannock, 1999). RAPD is a low stringency PCR reaction using short primers that anneal at low temperatures, followed by electrophoretic separation of the acquired DNA fragments of

a specific pattern (Tannock, 1999). MLST is based on the sequence analysis of approximately six 400-500 bp long stretches within the housekeeping genes for each bacterium. One of the advantages of MLST over other molecular typing methods is that sequence data are portable between laboratories and have led to the creation of global databases. Exchange of molecular typing data is performed via the Internet (Feil *et al.*, 2001) but drawbacks with this method are higher costs as compared to PFGE and the requirements for a skilled operator.

PFGE was developed in 1984 by Schwartz and Cantor (Schwartz *et al.*, 1984) and is often considered to be the golden standard among epidemiological typing methods. It has full typability, good reproducibility and very good discriminatory power. It is a type of electrophoresis allowing separation of large DNA fragments (up to 10 Mb) generated by rare-cutting restriction enzymes, which enables restriction fragment length polymorphism analysis of the entire bacterial genome. Challenges in using this method include the fact that it is a labour-intensive and time-consuming technique. Some factors may influence the electrophoretic patterns of DNA, i.e., repeated subculturing of the isolates used to prepare the genomic DNA. This may determine spontaneous loss of plasmids or other recombinational events that alter the final profile.

Molecular typing methods have not been applied in large epidemiological studies on *P. acnes*. Dairy propionibacteria have been analysed previously using RAPD (Rossi *et al.*, 1998) and two studies have used PFGE to analyse a small number of *P. acnes* isolated from endophtalmitis and postsurgical infections (Jallo *et al.*, 1999; Ting *et al.*, 1999). A trustworthy typing method can respond to many questions regarding apparition and dissemination of resistant strains in the general population. PFGE profiles can establish strain relatedness among bacteria with the same pattern of multiple antibiotic resistances and detect if a certain clone is more prone of becoming resistant, or if the patients colonized with this clone respond less well to therapy. Furthermore investigation of resistance spread from one person to another may be facilitated (Sahm, 1996).

Application of PFGE to the study of *P. acnes* has been limited since isolation of high-quality genomic DNA from propionibacteria has proved difficult. The cell-wall skeleton of *P. acnes* contains glucosamine with free amino groups, which are responsible for the resistance to lysozyme (Kamisango *et al.*, 1982). We have developed a method for the lysis of the thick cell wall of a Gram-positive bacterium. This technique uses mutanolysin and lysozyme suspended in a buffer containing polyethylene glycol that serves as an osmotic stabilizer and enhancer of the lysis (Chassy *et al.*, 1980). Mutanolysin, an endo-N-acetyl muramidase, splitts the β-1-4-N-acetylmuramyl-N-acetylglucosamine linkage of peptidoglycan-polysaccharide cell-wall polymer, a highly conserved linkage among bacterial species. This muralytic enzyme has a wide range of activity against bacterial strains resistant to lysozyme (Lichtman *et al.*, 1992).

The *P. acnes* population was found to be polyclonal on the skin of acne patients, although a tight clustering was noticed. This could be explained by a triple selection: 1) isolates were collected from acne patients, a group heavily exposed to antibiotic treatment; 2) patients came from the same area and were sampled at the same time; 3) all isolates were resistant. Most of the isolates may derive from a limited number of ancestral clones similar to methicillin-resistant *S. aureus* (Tenover *et al.*, 1995). We found that an acne patient may be colonized with different *P. acnes* strains with various resistance phenotypes, suggesting that certain strains are more able to acquire a specific resistance. Another possibility is carriage of the same pulse-type of *P. acnes* harbouring

various types of antibiotic resistance. A large number of CL resistant isolates from the skin of acne patients were clustered within the same clone.

Pulsed-field analysis of clinical isolates from various diseases revealed a higher level of genomic diversity, reflected by a large number of different patterns and by the presence of strains having a unique position in the dendrogram and a profile that was distinct from the other strains. A correlation was observed between strains with low-level resistance to EM or CL and certain pulsed-field types. It is therefore concluded that the majority of CL or EM resistant *P. acnes* isolates are members of a single clone that has spread in different areas of Europe, similar to previous findings regarding clonal distribution of CL resistant strains from acne patients. A large number of strains from soft tissue infections were clustered within one clone. Future work with more isolates from clinical sources will make it possible to determine whether certain genotypes are linked to certain clinical conditions.

The results from the presented studies showed that the *P. acnes* skin population may be the same as the population from other sites than the skin. It is important to note the geographical spread of some clones in related areas, but also in more distant countries. Strains from neighbouring countries belonging to the same clone may have similar or different patterns of antibiotic resistance. In the first case, it may represent the same clone in geographically closed regions, and in the second case the clone may be formed by strains more prone of becoming resistant to different antimicrobial agents. Recently, RAPD typing of clinical isolates of *P. acnes* showed that two strains of different serotypes belonged to two different clones (Perry *et al.*, 2003). In the present study *P. acnes* ATCC 6919 (serotype I) and *P. acnes* ATCC 11828 (serotype II) were also found to belong to different clones with less than 60% similarity. The *P. acnes* sero-biotype IB3 had the highest lipase activity and may have the greatest influence on skin rash in acne patients (Higaki *et al.*, 2000). However, the roles that *P. acnes* serotype I and II play in various clinical infections and the possible differences in production of virulence factors have not been studied. In the future, PFGE could be a fast tool to distinguish between different *P. acnes* serotypes.

Bacteria may become resistant to macrolides and lincosamides through target site modification by methylation or mutation that prevents antibiotic binding to its ribosomal target, through efflux of the antibiotic and by drug inactivation (Leclercq, 2002). Modifications of the ribosomal target will confer resistance to both types of antibiotics, while the last two mechanisms will affect only one of the drugs.

The most prevalent mechanisms of CL and EM resistance were point mutations giving rise to genotypes I and IV, in agreement with previous studies (Ross *et al.*, 2001). The phenotypic effect of a mutation in this conserved phylogenetic region situated on the large ribosomal subunit (the peptidyl transferase center) in domain V of the 23S rRNA may vary according to the environmental conditions. The advantage conferred in the presence of antibiotics must be weighted against the disadvantage of having these substitutions in the absence of antibiotics. A second-site mutation in rRNA may allow the first mutation to be maintained with no cost for *P. acnes* (Vester *et al.*, 2001). Position A2058 is a key nucleotide involved in macrolide interaction on the ribosome. In the *P. acnes* strains, the A2058G substitution gives high level resistance to all antibiotics from the MLS group, but the highest level of resistance to 14-member-ring macrolides (Vester *et al.*, 2001). One strain from the genotype I group showed a lower level of resistance to EM and CL (0.5 mg/L), possibly due to a compensatory mutation. Position A2057 is limited only to propionibacteria and to clarithromycin-resistant *Helicobacter pylori* and confers low level resistance to 14-member-ring macrolides and no resistance to 16-member-ring macrolides. Type III mutation was

only identified in two strains from Swedish acne patients. This resistance genotype has previously been identified among skin isolates from UK (Ross *et al.*, 2001). Possibly, the relatively sparse prevalence of the group III resistance genotype might be due to the fact that this mutation renders a cost for the bacteria which is not balanced by the relatively low level of EM resistance. *P. acnes* strains carrying this mutation showed a high level of EM resistance (128 mg/L) which may be explained by a secondary mutation in another region, or by the presence of another resistance mechanism. Position A2059 confers resistance to both 14- and 16-member-ring macrolides, but gives significantly higher resistance to josamycin (a 16-member-ring macrolide) as compared to the A2058G mutation (Ross *et al.*, 1997). Both classes interact with 2058 and 2059 positions, but probably the last one may be shifted toward position 2059 (Vester *et al.*, 2001). This position confers elevated but variable resistance to lincosamides and no resistance to type B streptogramins. By definition, these types of mutations are considered to be non-transferable, but the possibility exists of spreading them through a transformation mechanism followed by homologous recombination, as described for pneumococci (Leclercq, 2002).

Nearly 40 *erm* genes have been reported so far, but only the *erm*(X) has been found to be present in *P. acnes* strains. All CL-EM resistant strains were tested for the presence of the *erm*(X) resistance gene, but the results were negative. This result was not unexpected, since in a previous report it accounted for less than 10% of resistance among the tested strains (Ross *et al.*, 2002).

For the remaining CL-EM resistant *P. acnes* strains that had no identified mutation but showed resistance phenotypes, other mechanisms may be involved, such as mutations in ribosomal proteins (L4 or L22) (Gregory *et al.*, 1999), transition at position 2611 in domain V of 23S rRNA (Vannuffel *et al.*, 1992) or deletions within domain II of 23S rRNA (Dam *et al.*, 1996). These possible mechanisms are only able to confer macrolide resistance and should be accompanied by other mechanisms for determination of the double resistance to EM and CL.

The two strains belonging to type III mutation were clustered together in PFGE analysis, but more strains have to be analysed in order to validate the possible clonality of strains harbouring this resistance mechanism.

Bacteria commonly use three strategies to become resistant to TET. The most common is energy-dependent drug efflux; the second mechanism involves protection or alteration of the ribosome and the third confers enzymatic inactivation by the product of the *tet*(X) locus. The single 16S rRNA base mutation causing alteration of the binding site to the ribosome was found in 12 of the 27 *P. acnes* strains. Unlike a previous study (Ross *et al.*, 2001), all strains in the present study carrying this mutation were found to have MIC values of ≥32 mg/L. The strains carrying this mutation were all collected from acne patients. To interprete the epidemiological importance of this observation, PFGE was used as a typing method and it was noticed that all these isolates were clustered in one clone. This clone was found only in acne patients heavily treated with different antibiotics. Seven strains with a low level of TET resistance and no detected mutation within the investigated 16S rRNA region were shown to be closely genetically related to the strains carrying the specific mutation, indicating that the mutation associated with TET resistance is a relatively recent event. TET efflux offers another way of limiting the access of the antibiotic to ribosomes, and represents the most common mechanism of TET resistance (Speer *et al.*, 1992). It has been shown that reserpine, a multi-drug-resistance efflux pump inhibitor, could increase the intra-cellular concentration of antibiotic in bacteria exhibiting efflux pump mediated resistance

(Neyfakh *et al.*, 1991). Our results do not support the idea of efflux pumps in TET resistant clinical isolates of *P. acnes.*

Other resistance mechanisms, except the described ones, seem to operate in case of *P. acnes.* However, the absence of the *erm*(X) gene in CL-EM resistant strains and of active efflux pump in TET resistant *P. acnes* was not an unexpected finding. *P. acnes* has been shown to carry three copies of the rRNA (Ross *et al.*, 1998) and the fewer *rrn* operons a bacterium has, the greater the likelihood that resistance will be conferred by mutations, rather than by other mechanisms (Vester *et al.*, 2001). The reason for this is not clear, although it may be that the degree of resistance is linked to the proportion of modified ribosomes. The mutation must be present in multiple copies in organisms containing more than three copies of *rrn.* All erythromycin-resistant isolates of *Propionibacterium* spp. have been homozygous for the mutation (Ross *et al.*, 1997). Gene conversion may explain how triple mutations may occur in the same base by replacement of the wild-type allele with the mutant allele using the mutant allele as template (Kobayashi, 1992).

The genetic linkage on a conjugative transposon, between CL-EM and TET resistance determinants is possible. The combination between *erm*(B) and *tet*(M) genes has been found in streptococci, staphylococci and enterococci (Chopra *et al.*, 2001). This may also explain the high frequency of isolation of multiple resistant *P. acnes* strains.

Guidelines of acne treatment have been published during the last years (Dreno *et al.*, 2004; Gollnick *et al.*, 2003) and generally the acne severity and extent, as well as the patient's choice and the cost will direct the first option of therapy. ISO is considered a very efficient acne treatment for severe forms, but it is connected with a wide variety of side effects (Goldsmith *et al.*, 2004). It is of interest to compare clinical and bacteriological responses after ISO therapy with other efficient alternative treatments not associated with such risks. An alternative treatment in case of moderate/severe acne is the mixture between an oral antibiotic and a topical retinoid, followed by topical retinoid as maintenance therapy.

A classification in mild, moderate and severe acne is important and easy to make in the clinic for correctly guiding the treatment. For research purposes, acne grading can also be performed with a 0-10 scale, or by a lesion counting method (Burke *et al.*, 1984). By using these quantification methods both treatments improved the clinical conditions, although ISO was more effective in the treatment of the majority of lesions. Furthermore, after the discontinuation of therapy, ISO was found to have a better long-term efficacy as compared to TET/ADA. Female patients in the ISO group received anticonceptional treatment (the standard procedure in Sweden for oral ISO treatment) which has antiseborrheic activity, for the study duration and one month after treatment with ISO had stopped. In the drug-free period, these patients were the only sub-group that continued to reduce the lesion numbers.

Treatment with ISO had a better indirect effect in reducing the propionibacteria numbers, starting with 2 months of treatment, by altering the follicular microclimate (Orfanos *et al.*, 1998) and this effect was more persistent. No correlation was found between this reduction and clinical efficacy (acne grading or number of lesions), which is in agreement with a previous report showing that effectiveness of oral antibiotics in treating the disease cannot be explained by a reduction in the number of viable bacteria (Cove *et al.*, 1980). The decrease in numbers of *P. acnes* is a parameter of therapeutical effectiveness, and other environmental factors may modulate the inflammatory aspects of acne (Burkhardt *et al.,* 1999a).

Previous data (Eady *et al.*, 2003a) and the results presented in Papers I and II indicate that EM and CL are more selective for *P. acnes* resistance, as compared to TET The patients entering in the study had moderate and severe acne and many of them were colonized at baseline with resistant propionibacteria. The quantitative microbiology is therefore the method of choice for proving selectivity of an antibiotic treatment. The population of resistant bacteria was not significantly affected by the treatment with any one of the two drugs. It is possible that certain clonal types have increased capability to survive depending of their degree/mechanism of resistance or that antibiotic-resistant and antibiotic-susceptible *P. acnes* may show different responses to oral ISO (Coates *et al.*, 2005). In addition, as demonstrated in Paper II, there are non-skin reservoirs of resistant *P. acnes* strains which form a continuum with the skin population. They may not be affected by ISO treatment and they represent the source of re-colonization with resistant strains that may multiply and spread on the skin. At the present moment, except topical BP at the site of application, there is no method to eradicate completely antibiotic-resistant *P. acnes* strains (Coates *et al.*, 2005).

The concentration of TET inside the pilosebaceous follicle may be high enough to inhibit the population with low levels of MIC but not sufficiently high to suppress the bacteria with elevated levels of MIC. Finally, equilibrium may be settled between these two sub-populations of resistant propionibacteria, which may explain the unmodified levels of total resistant *P. acnes*. The addition of an antibiotic to an ecologically stable environment can result in a destabilization of that environment. Other organisms may be selected and may occupy the cutaneous niche, e.g., staphylococci. These results may also indicate that once a patient becomes colonized with resistant bacteria, this is stabilized within the population and may persist a long time, regardless of the treatment. The cost of resistance may be ameliorated by compensatory mutations in previously low-fitness resistant clones ensuring their unimpaired fitness for survival and causing the stabilization of the resistant strains (Nagaev *et al.*, 2001). Further studies are necessary to compare the *in vivo* and *in vitro* fitness of wild type and resistant *P. acnes*. It is possible that mutants may indefinitely remain as part of the resident microflora (Ross *et al.*, 1997).

There are multiple factors that may explain the lack of correlation between the clinical response or the life quality score and the presence of resistant *P. acnes*. The clinical significance of a resistant strain may be determined by comparing the MIC of the antibiotic with the concentration that can be achieved at the site where the bacteria proliferate. In acne the problem resides in the fact that there is no method to detect the level of antibiotics within the pilosebaceous follicle, and a patient whose response to treatment is good will have a majority of follicles in which the concentration is high (Eady *et al.*, 2003b). Each lesion behaves as a separate infection, independent of the other lesions, and each follicle may contain a mixture of susceptible, moderately resistant and highly resistant *P. acnes* strains. Consequently, the clinical response will be highly dependent of this factor. It is unlikely that all follicles will have resistant bacteria, and therefore carrying of resistant *P. acnes* strains will not lead to total failure, but to a limited response.

In addition, biological actions of tetracyclines affecting inflammation, proteolysis, angiogenesis, apoptosis, metal chelation and bone metabolism have been demonstrated. Their therapeutic effects include the following diseases: acne, rosacea, bullous dermatoses, neutrophilic diseases, pyoderma gangrenosum, sarcoidosis, aortic aneurysms, cancer metastasis, periodontitis and autoimmune disorders (Sapadin *et al.*, 2006). It is still an open question how much of the antibiotic efficacy in acne is due to the anti-propionibacterial or to the anti-inflammatory effect. A low dose of doxycycline

was shown to be clinically effective even though the number of bacteria on the skin did not change (Skidmore *et al.*, 2003). In addition, ADA topical treatment probably enhanced the clinical response by targeting both non-inflammatory and inflammatory lesions (Dreno *et al.*, 2004).

TET combined with a topical treatment is a good clinical option and efforts should be made in preventing the development of resistance and the accumulation of resistant bacteria.

Acne patients are considered to be reservoirs of antibiotic-resistant *P. acnes* strains. EM resistant *P. acnes* strains were found in the same family in acne patients treated with long-term antibiotic therapy and in their close contacts. The dermatologists treating the acne patients were also colonized with EM or CL resistant propionibacteria. No resistant isolates were found in dermatologists not involved in acne treatment and no TET resistant bacteria were isolated (Ross *et al.*, 2003). Unexpectedly, we found CL, EM and TET resistant *P. acnes* strains not only on the skin of close contacts of acne patients, but also on the skin of one third of the healthy, no-acne contacts and non-antibiotic treated subjects. The resistance pattern of *P. acnes* from the skin of patients and close contacts was not the same.

The acquisition of the resistant strains could be due to transmission of a resistant isolate from another person (Eady *et al.*, 2003a), from the environment (Qureshi *et al.*, 2004), by acquirement of a mobile resistance element from another bacteria or by *de novo* mutation within the chromosomal DNA of a susceptible strain. It has been shown that *P. acnes* can survive on different surfaces for long periods of time at room temperature in air and this finding may explain the high rate of resistant bacteria in the control group through direct transfer of resistant strains from the environment (Qureshi *et al.*, 2004). The horizontal transfer of the *erm*(B) gene has been recently shown to occur between two obligate anaerobes, *Clostridium difficile* and *Butyvibrio fibrisolvens* (Spigaglia *et al.*, 2005).

PFGE provided some clarifications regarding the spread of resistant *P. acnes*. The resistant *P. acnes* isolates detected from the patients and their close contacts were clustered in the same clone in two cases and closely related, but not in the same clone, in another case. Three different epidemiological situations are described in the present study. In the first situation, the patient and his both contacts were colonized with the same strain, identical with regard to resistance pattern and bands, most probably a direct transfer between patient and contacts. The patient also carried two different types of CL resistant *P. acnes* isolates at baseline and this result is in agreement with our previous study (Oprica *et al.*, 2004). After 4 months of lymecycline treatment, one of the strains had disappeared from the skin. The other strain continued to persist and had acquired resistance against EM and TET. Persistence of TET resistant bacteria in the oral cavity of children who had never been exposed to TET was connected with the presence of *tet*(M) gene (Lancaster *et al.*, 2005). It is possible that certain resistance genes may be responsible for persistence of resistant *P. acnes* isolates on the skin.

In the second case, the following possibilities are likely to have occurred: i) the patient and his contact carried different clones of propionibacteria and a *tet* resistance gene or a mutation was acquired by susceptible *P. acnes* after the treatment started; ii) the patient and the contact initially harboured the same susceptible strain but after the insertion of a resistant gene and/or mutation within susceptible *P. acnes* the pulsed-field pattern had changed; iii) a different source and not the patient was the origin of TET resistant *P. acnes* strains isolated from the contact. In the last case, both contacts and patient carried the same pulsed-field type of *P. acnes* which on the contacts' skin and in the absence of the pressure from the antibiotic treatment, have probably lost the

resistance gene or have gained compensatory mutations. The discrepancies between *P. acnes* resistance patterns of patient and contacts may explain these findings.

The fact that a resistant strain may colonize the contact person in the absence of the antibiotic selective pressure may be explained either by the transfer of active drug as topical application (Miller *et al.*, 1996) or by excretion of the orally administrated antibiotic into the sweat. The excretion of ciprofloxacin and β-lactams in the sweat has been demonstrated and may explain why staphylococci so rapidly become resistant to these drugs (Høiby *et al.*, 1997; Høiby *et al.*, 2000). The carriage of resistant bacteria was transitory on the skin of acne contacts and controls. Interestingly, in the contact group no resistant strains were isolated after the antibiotic treatment had stopped, although the inoculum of resistant *P. acnes* on the skin of acne patients was still high. This finding could support the idea of the persistence of resistant strains as long as there is an "indirect" contact with the antibiotic excreted into the sweat or sebum. It may explain why resistant strains disappeared from the skin of contacts once the selective pressure of the antibiotic had disappeared. Another possible cause for reduced transfer between patients and contacts at high inoculum levels could be an improved attachment to the donor surface when bacterial concentrations are high (Montville *et al.*, 2003). These persons could nevertheless have pools of resistant strains at one moment and can act as transmitters of resistant bacteria in the general population either directly or via inanimate objects.

9 CONCLUDING REMARKS

Acne is one of the very few diseases in which long antibiotic treatments are recommended. Dermatologists treating acne patients should evaluate which is the best treatment for the patient or for the community and consider the antibiotic resistance problem not only in propionibacteria, but also in the normal microflora.

Non-antibiotic treatment should therefore be considered as the first alternative in mild to moderate acne and ISO should be appropriately prescribed in acne to prevent physical and psychological problems. In USA, ISO is prescribed in severe recalcitrant acne but leading dermatologists have recommended extending indications for its use. Nowadays, experts focus on the combination therapy that targets more pathogenical factors and may enhance the efficacy (Dreno, 2004; Dreno *et al.*, 2004).

P. acnes resistance to antibiotics does not necessarily imply that acne will be resistant to treatment, but it is important to prevent development of resistance and accumulation of antibiotic-resistant *P. acnes*. EM, CL and TET resistant *P. acnes* is mainly due to mutations and is likely to persist after the selective pressure of the antibiotic has been removed (Simpson *et al.*, 2004). It has been suggested that resistant organisms might develop and retain mutations ensuring their unimpaired fitness for survival (Morell, 1997). Other mechanisms of *P. acnes* resistance except the described ones, e.g., mobile genetic elements carrying resistance genes, have developed. The risk is the transfer of resistant genes between *P. acnes* and other pathogenic bacteria.

It is important to extend the life span of existing antibiotics because there are no examples of antibacterial agents against which bacteria have not developed resistance. Since resistance to new drugs can quickly appear, other strategies should be used, such as development of new classes of drugs with unique antibacterial mechanisms.

The antibiotic resistance seems to move from the acne patients to the community. The carriage of single and multiple resistant *P. acnes* strains can occur as a transitory event in acne close contacts, but also in the general population. Until now it has not been shown how the resistance spreads in the general population. Environmental selection pressures, other than antibiotics, may contribute to the spread and maintenance of resistant bacteria in areas where antibiotics have not been used (Salyers *et al.*, 1997). Apparently close contacts within families carry the same type of resistant *P. acnes* and mutations or transfer of different resistance genes are probably responsible for differences in resistance patterns.

Measures proposed for reversal of antibiotic resistance have emphasized regulation or limitation of antibiotic use and control of the spread of resistant bacteria (Phillips, 1998). However, it should be noted that resistance does not always follows the use of antibiotics (e.g., *Streptococcus pyogenes* are still sensitive to penicillin despite the selective pressure to which other respiratory pathogens have responded). Furthermore the resistance can diminish in some organisms for reasons that are unrelated to antibiotic use: disappearance of “hospital staphylococcus” of the 1960s as the diminution in the incidence of β-lactamase-producing gonococci in UK (Warren *et al.*, 1993).

The following conclusions can be drawn from the presented work:

- Acne patients in Stockholm treated with antibiotics have a significantly higher risk of carrying resistant *P. acnes* strains, than those with acne who have not received such treatment.

- In acne patients, isolates resistant to EM and CL are more common than isolates resistant to TET. Resistance to TET is often associated with resistance to EM and CL.
- Antimicrobial resistance has emerged among *P. acnes* isolates from different severe, life-threatening infections. The prevalence of TET resistant isolates is lower as compared to EM or CL resistant isolates.
- The antibiotic-resistant rates in *P. acnes* clinical isolates from various infections mirror the situation with the antimicrobials presently in use in different countries.
- Using PFGE as typing method of resistant bacteria demonstrated that the *P. acnes* resistant population is polyclonal. Antibiotic-resistant *P. acnes* skin isolates do not represent a separate pulsed-field type when compared with the bacterial population from other sites than the skin.
- Acne patients may be colonized with different *P. acnes* strains with various resistance phenotypes. This suggests that certain clones are more able to acquire resistance against a specific antibiotic.
- Different resistance genotypes were distributed throughout Europe in strains from acne and other diseases but new mechanisms of resistance have evolved.
- The PFGE analysis showed a clonal distribution for the CL resistant isolates from acne patients and other diseases, for the EM resistant isolates from various diseases and for the TET resistant isolates from acne patients carrying the 16S rRNA mutation.
- Oral treatment of acne patients with TET combined with a topical retinoid proved to be a good alternative to ISO, although ISO had a prolonged remission after treatment. The anti-inflammatory properties of the TET could be important in the treatment of inflammatory acne. The clinical response may be good regardless of the presence of resistant *P. acnes* on the skin.
- The cost of resistance may be ameliorated by compensatory mutations causing the stabilization of the antibiotic-resistant bacterial population. It is possible that certain clonal types have increased capabilities to survive.
- Carriage of resistant *P. acnes* isolates occurs not only in acne patients and close contacts of acne patients but also in the general population.
- Apparently close contacts within families carry the same type of antibiotic-resistant *P. acnes*, which may spread furthermore. Mutations or transfer of different resistance genes probably result in differences in resistance patterns.
- Efforts should be made in preventing development of resistance and accumulation of resistant bacteria.

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