

**A NOVEL TWO-COMPONENT SIGNAL TRANSDUCTION  
SYSTEM IN *PROPIONIBACTERIUM ACNES* AND ITS ASSOCIATION  
WITH A PUTATIVE EXTRACELLULAR SIGNALLING PEPTIDE**

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## Abstract

*Propionibacterium acnes*, a resident micro-organism of human skin, is thought to be involved in the development of inflammatory acne, which is an exclusive human disease affecting more than 80% of the whole population. Quorum sensing is the regulation of gene expression in response to cell density. As it is often involved in pathogenicity of bacteria, its signal transduction pathway has been suggested as potential target for new drug development. This project identified a putative unique quorum sensing system of *P. acnes*, consisting of a putative signalling peptide, and divergently transcribed histidine kinase and response regulator. The aim of this project is to investigate the relationship among these three components as being elements of a putative quorum sensing system. Using purified proteins and *in vitro* phosphorylation assays, the histidine kinase was demonstrated to phosphorylate the response regulator indicating they may constitute a legitimate pair of a two-component system, despite being encoded by divergently transcribed genes. By mapping transcriptional start site, it was found that the signalling peptide and histidine kinase were co-transcribed from the same start site, suggesting that the signalling peptide was associated with the two-component system. Gene expression analysis also revealed these three genes were co-regulated during the growth of *P. acnes*, which is consistent with these three genes functioning together as a part of quorum sensing system. The results of this project suggested that the signalling peptide, histidine kinase and response regulator are associated with each other and may constitute a quorum sensing system.

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# **Chapter I**

## **Introduction**

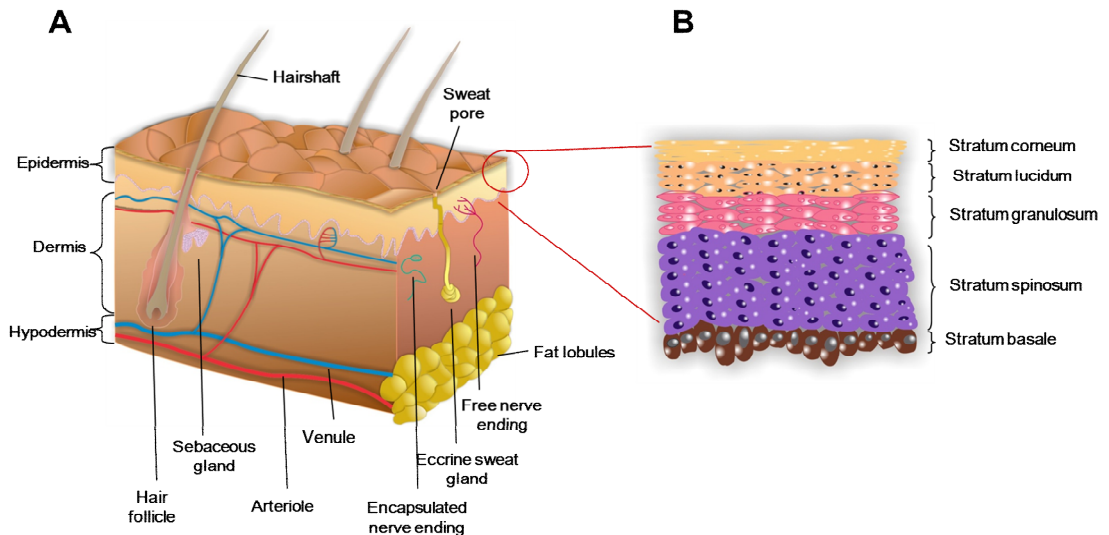
This chapter provides the background of this PhD project and introduces the reader to the skin and acne, with special emphasis on the association between *Propionibacterium acnes* and acne. Then the biology of *P. acnes* is briefly described with examples of its involvement in other diseases as an opportunistic pathogen. Finally two-component signal transduction systems involved in quorum sensing (QS) are introduced, including their roles in the pathogenesis and virulence and their potential as targets for the development of new antimicrobials in the future.

### **1.1 The skin**

#### **1.1.1 Structure and function of human skin**

The skin is one of the largest organs of the body in terms of its surface area and weight. It provides a protective covering for the internal tissues and organs and has several specialized structures known as appendages, including hair follicles, eccrine sweat glands, sebaceous glands and apocrine glands (Figure 1.1) (Wilson, 2008). There is also regional variation in the skin, which is manifested primarily in terms of skin thickness, composition, density of appendages and biochemical differentiation. There are three layers in the skin: epidermis, dermis and hypodermis (Figure 1.1). Interaction

among these layers is important during skin development and for maintenance of skin homeostasis in the adult (Haake *et al.*, 2001).



**Figure 1. 1 The major structures of human skin.**

The skin contains three major layers which are epidermis, dermis and hypodermis. The epidermis can be further divided into five layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. Several specialized structures are present in the skin: the hair follicle, sebaceous gland and eccrine sweat gland. Hair follicles are situated in the dermis with the erector pili muscle attached to each follicle. Sebaceous glands are also associated with hair follicle. Eccrine sweat glands are in the dermis, but they are not associated with hair follicles. Blood vessels and nerves course through the dermis, which transmit sensations of pain, itch and temperature. The hypodermis has a layer of fat, connective tissue and large blood vessel and nerves. This layer is important in the regulation of temperature of the skin and the body.

The epidermis is a stratified, continually renewing epithelium. Approximately 90-95% of epidermal cells are keratinocytes. They are organized into those five major layers corresponding to progressive stages of differentiation, which is a genetically programmed and tightly regulated series



of morphological and metabolic events (Figure 1.1). Also found in the epidermis are melanocytes, Langerhans cells and Merkel cells. Melanocytes are melanin-producing cells that populate the retinal pigment epithelium. Langerhans cells are dendritic cells in the suprabasal layer of the epidermis and they present foreign antigens in the epidermis (Farrar, 2000). Therefore, they have a very important role in immune surveillance. Merkel cells are specialized intraepithelial cells of the skin. Epidermal Merkel cells produce nerve growth factor and dermal Merkel cells produce nerve growth factor receptors, which enable them to function as mechanoreceptors and may contribute to the development of the nerve plexus in the upper dermis (Haake et al., 2001).

As a connective-tissue component, the dermis protects the body from mechanical injury. It binds water, aids in thermal regulation and contains receptors of sensory stimuli (Haake et al., 2001). Collagen and elastic connective tissue are the main types of fibrous connective tissue of the dermis. Non-fibrous, connective tissue molecules such as fine filamentous glycoproteins, proteoglycans and glycosaminoglycans also exist in the dermis (Marples, 1965). Monocytes, macrophages and dermal dendrocytes are a heterogeneous collection of cells that constitute the mononuclear phagocytic system of cells in the skin. Mast cells in the dermis are specialized secretory cells located in sheaths of epidermal appendages, around the blood vessels and nerves of the subpapillary plexus. They synthesize an array of mediators, which are the primary effector in the onset of an allergic reaction (Haake et al., 2001).

The tissue of the hypodermis serves as a reserve energy supply, protects the skin and allows for the mobility over underlying structures. It is an adipose tissue-rich subcutaneous region. Mesenchymally derived adipocytes are the primary cells in the hypodermis. They are organized into lobules defined by septa of fibrous connective tissue, within which nerves, vessels and lymphatics are located. Actively growing hair follicles extend into the subcutaneous fat and the apocrine and eccrine sweat glands are normally confined to this depth of the skin (Haake et al., 2001).

### **1.1.2 The sebaceous gland and pilosebaceous unit**

Sebaceous glands are found in all areas of the body except the palms, soles and dorsum of the feet. They vary in size and distribution depending upon body site with the largest glands on the face and smaller glands on the extremities (Marples, 1965). The sebaceous gland, when it is associated with a hair follicle, is known as a pilosebaceous unit and it is these sebaceous follicles that are affected in the development of acne. One of the functions of the sebaceous gland is to secrete sebum, which is a mixture of relatively neutral lipids synthesized by the glands (Nikkari, 1974). Increased sebum secretion is a major aetiological factor in the development of acne. The recent development of experimental models for the *in vitro* study of human sebaceous glands have identified several, previously unknown functions of this organ. These include the production of foetal vernix caseosa, an influence on the three-dimensional organization of the skin surface lipids, the integrity of the skin barrier and an influence on follicular differentiation (Zouboulis *et al.*, 2008b). Sebaceous glands also contribute to the transport

of fat-soluble antioxidants from and to the skin surface, natural photoprotection, pro- and anti-inflammatory skin properties and to the innate antimicrobial activity of the skin (Zouboulis *et al.*, 2008b). These multiple homeostatic functions make the sebaceous gland the most important cutaneous endocrine gland on the body.

## **1.2 Acne**

Acne is a complex, yet commonly occurring skin disease. It is mainly a disorder of human sebaceous follicles of the face, the upper chest and upper back (Jappe, 2003). Acne has many different symptoms including comedones, papules, pustules, nodules, cysts and pilosebaceous inflammation, amongst which inflammatory lesions of acne are of the greatest concern to patients because they can lead to life-long post-acne scars (Nakatsuji *et al.*, 2009). Acne is also often considered as a chronic disease as it often has a prolonged course, a pattern of recurrence or relapse, manifestation as acute outbreaks or slow onset, and with psychological and social impacts that can affect quality of life (Thiboutot *et al.*, 2009).

### **1.2.1 Prevalence of acne**

Prevalence of acne strongly depends on its definition, the age categories of the studied populations and communities in which the studies were based, but estimates of prevalence could still be determined. In white Caucasian populations nearly 85% of individuals aged 12-25 present a variant clinical picture of acne (Zouboulis *et al.*, 2005). Due to the severity of

their clinical condition, 15-30% of acne patients require medical treatment and it is the most common diagnosis for patients 15-45 years of age (Inglese *et al.*, 2007). Spontaneous regression is common, but in about 5% of cases acne persists beyond the age of 25 and 2-7% of them experience life-long post-acne scars (Zouboulis *et al.*, 2005; Jappe, 2003). It has been estimated that between 30% and 100% of the adolescent population is affected by acne in industrialized countries, while it is considerably lower in developing countries (Tan *et al.*, 2007; Cordain *et al.*, 2002). Overall incidence is similar for both men and women, and peaks at 17 years of age (Williams *et al.*, 2003).

### **1.2.2 Social and economic impact of acne**

Although acne is not a life-threatening disease and has not been the focus of much clinical and laboratory research, it significantly influences quality of life of affected individuals and can lead to significant socioeconomic problems. Significant psychological problems may result from acne, such as stigmatization from peers, lower self-esteem, interpersonal difficulties, anxiety, depression and higher unemployment rates (Law *et al.*, 2009). Acne is associated with severe depressive symptoms, which may lead to psychosocial impairment, suicidal ideation and a recurring course in extreme cases (Akiskal, 2005). According to surveys conducted by the National Ambulatory Medical Care Survey and National Disease and Therapeutic Index during 1995-1998, acne is also the leading dermatological diagnosis with more than 6.1 million prescriptions for women and 3.8 million prescriptions for men per year in America (Stern, 2000). For systemic

anti-acne medication only, 6.5 million new prescriptions at a value of over \$ 1 billion per year were provided to American patients during 1996-1998 (Stern, 2000). The worldwide cost for systemic and topical acne treatment was calculated to represent 12.6% of the overall costs for the treatment of all skin disease (Stern, 2000).

### **1.2.3 Pathogenesis of acne**

Although the aetiology of acne is not fully understood yet, it is widely accepted that acne is a multi-factorial skin disease with four primary and significant pathogenic factors interacting in a complex manner to produce acne lesions: (1) enhanced sebaceous gland activity and increased sebum production; (2) alteration in the keratinisation process; (3) proliferation of *P. acnes* in the follicle and (4) release of inflammatory mediators into the skin (Thiboutot et al., 2009, Toyoda & Morohashi, 2001).

#### **1.2.3.1 Sebum, sebaceous gland and hormones**

The enhanced sebaceous gland activity and overproduction of sebum induced by androgens has been listed as the first factor in the pathogenesis of acne (Toyoda & Morohashi, 2001). The sebaceous gland acts as an independent endocrine organ in response to changes in androgens and hormones (Zouboulis *et al.*, 2008a). The hormone control of sebaceous gland function is very complex. Different hormones produced by the adrenal gland and gonads interact with each other under the influence of the pituitary, which is the main driver (Pochi & Strauss, 1974; Ebling, 1974). Androgens are

essential for increasing the size of sebaceous glands, stimulating sebum production and keratinocyte proliferation (Pochi & Strauss, 1969). Results using human models also suggest that the androgen is involved in the proliferation/differentiation of sebocytes and infrainfundibular keratinocytes. However, a more recent study shows that acne patients have normal circulating androgen levels suggesting that acne severity does not correlate with serum androgen levels (Kurokawa *et al.*, 2009). It may only play a role in initiating acne development. Or the local overproduction of androgens and high expression and responsiveness of androgen receptors may determine the formation of acne lesions (Kurokawa *et al.*, 2009).

#### **1.2.3.2 Hyperkeratinisation**

The second crucial factor in the pathogenesis of acne is hyperkeratinisation and the subsequent obstruction of sebaceous follicles, which results in microcomedones and comedones. During the development of microcomedones and comedones, the normal pattern of keratinisation is changed, with hyperproliferation of keratinocytes lining the follicle wall occurs and reduced desquamation of the epithelium (Knaggs *et al.*, 1994). Although the mechanism underlying the follicular hyperkeratinisation remains uncertain, several factors may be involved in it. Interleukin (IL) -1 $\alpha$  has been reported to induce hyperkeratinisation in follicular infundibulum and the formation of comedones both *in vitro* and *in vivo* (Ingham *et al.*, 1992). The local follicular deficiency of linoleic acid is another important factor involved in follicular hyperkeratinisation (Downing *et al.*, 1986). Androgens are also potential factors involved in follicular hyperkeratinisation. The

androgen-controlling effect is also evidenced by a reduction in the number of comedones when a patient is prescribed antiandrogen therapy (Stewart *et al.*, 1986).

### **1.2.3.3 Inflammation**

The inflammatory reaction is fuelled by a variety of pathological processes including neutrophil infiltration, cytokine production, a type IV hypersensitivity, lymphocytes releasing reactive oxygen radicals and enzymes and rupture of the follicle wall. Two mechanisms have been proposed for the initial cellular infiltrate: neutrophils and T lymphocytes. Neutrophils were firstly suggested by Kligman in 1974 and it was concluded that this event was also followed by microscopic rupture of the sebaceous follicle wall and subsequent formation of a clinically visible inflamed lesion (Kligman, 1974). Much literature also described that these neutrophils were attracted by neutrophil chemoattractants produced by *P. acnes* that diffuse through the thinned follicular epithelium (Leyden *et al.*, 1998, Webster, 1998). Meanwhile, histological studies have provided compelling evidence that T lymphocytes are involved in the initiation of inflammation. A 1988 study demonstrated that the CD4+ T cells were the initial infiltrate of all developing inflammatory lesions predominantly and neutrophils were seen later in the course of inflammation (Norris & Cunliffe, 1988). A more recent study using biopsy specimens observed large numbers of CD4+ T cells and macrophages around uninvolved follicles from acne patients, but none were found in non-acne controls (Jeremy *et al.*, 2003). Moreover, there is no neutrophil observed and a reduction in the number of Langerhans cells in the

perifollicular epidermis suggesting that it is a specific immune response (Jeremy et al., 2003). It was hypothesized that subsequent production of cytokines activates local endothelial cells, up-regulating inflammatory vascular markers including E-selectin, vascular cell adhesion molecule (VCAM) -1, intercellular adhesion molecule (ICAM) -1 and human leukocyte-associated antigen (HLA) -Dr (Jeremy et al., 2003). On the other hand, the initiation of inflammation could occur non-specifically through IL-1 $\alpha$  induction. Dermal IL-1 $\alpha$  would lead to the non-specific accumulation of mononuclear cells and the initiation of antigen-independent inflammation (Kozłowska *et al.*, 1998). Such initially non-specific events could lead to a specific amplification phase of inflammation via a T cell response to comedonal antigens (Jeremy et al., 2003). It is also suggested that the inflammation can result from comedonal components after the release of reactive oxygen radicals and enzymes by neutrophils and rupture of the follicle wall (Guy & Kealey, 1998; Leyden et al., 1998; Webster, 1998; Akamatsu & Horio, 1998). Inflammation may also result from a type IV hypersensitivity reaction to *P. acnes*.

#### **1.2.3.4 *P. acnes* proliferation**

As the predominant resident microorganism, *P. acnes* can be found on sebaceous gland-rich areas of skin (Holland, 1989). The follicular infundibulum of comedones is highly colonised by *P. acnes* where the anaerobic and lipid rich environment is ideal for *P. acnes* overgrowth. (Toyoda & Morohashi, 2001). The association between *P. acnes* and acne has been shown through the use of antibiotic treatment of acne. Initial studies



demonstrated that antibiotics such as tetracyclines, macrolides, sulfonamides and clindamycin were therapeutic and resulted in a decrease in the population density of *P. acnes*. Although antibiotics are known to be able to modulate the immune system and may have a more direct anti-inflammatory activity on acne, other studies also strengthen the hypothesis that *P. acnes* plays an important role in acne. For example, the failure of erythromycin treatment of acne in a number of individuals was related to the development of erythromycin resistance of *P. acnes* (Eady *et al.*, 1989). Furthermore, viable *P. acnes* cells are known to be able to induce prominent inflammation *in vivo* and trigger human keratinocytes to produce IL-1 $\alpha$ , tumor necrosis factor (TNF) - $\alpha$  and granulocyte-macrophage colony stimulating factor (GM-SCF) resulting in prominent inflammation *in vitro* (Jappe, 2003; Graham *et al.*, 2004). Meanwhile, dead *P. acnes* cells were unable to reproduce these effects (Jappe, 2003; Walters *et al.*, 1995; Ingham *et al.*, 1998).

It has also been suggested that toll-like receptors (TLRs) may contribute to inflammation in acne through the activation of TLR2 on macrophages by *P. acnes*, resulting in the release of proinflammatory cytokines IL-12 and IL-8 (Kim *et al.*, 2002). It indicated that *P. acnes* may contribute to inflammation in acne through activation of TLRs expressed on keratinocytes and sebocytes and induction of proinflammatory cytokines (Koreck *et al.*, 2003). Interestingly, variations in cytokine stimulation by *P. acnes* in different phases of growth have been demonstrated. *P. acnes* cells in stationary phases induced higher levels of cytokines than those in the exponential phase of growth (Graham *et al.*, 2004). It is also hypothesized that different *P. acnes* growth phases in different sebaceous follicles may result from differences in the microenvironment (Farrar & Ingham, 2004).

A recent description of phylogenetically distinct *P. acnes* clusters raises the possibility that certain *P. acnes* strains may cause an opportunistic infection, so worsening acne lesions (McDowell *et al.*, 2005). *P. acnes* has also been shown to have potent T-cell mitogenic activity, which may contribute to the inflammation in acne through the activation of T cells (Jappe *et al.*, 2002). In patients with severe acne, an increased cellular and humoral immunity to *P. acnes* has been detected and the severity of the disease was correlated with the production of antibodies (Holland *et al.*, 1993; Ashbee *et al.*, 1997; Jappe *et al.*, 2002). However, as *P. acnes* is unlikely to be in contact with T cells or macrophages, it is likely to be significant only if rupture of the follicle occurs. Therefore, the direct effects of *P. acnes* on T cells and macrophages are likely to augment the established immune response rather than initiate it (Farrar & Ingham, 2004).

Free fatty acids produced by lipase secreted from *P. acnes* may also act as comedogenic and acnegenic factors in sebaceous follicles and may irritate the follicular walls as well as the surrounding dermis following follicular rupture (Toyoda & Morohashi, 2001). It is now believed that *P. acnes* is not the cause of acne, but is a significant contributing factor to the inflammatory stages of the disease (Farrar & Ingham, 2004).

#### **1.2.3.5 Other factors**

It is well known that emotional stress can result in exacerbation of acne. Clinical evidence also suggests that the nervous system and psychological factors can influence the course of acne (Koo & Smith, 1991). It has been found that neuropeptide substance P can accelerate lipid synthesis and

induce significant increase in the area of sebaceous glands as well as in the size of individual sebaceous cells (Toyoda & Morohashi, 2001). This study also suggested that neuropeptide substance P may stimulate the proliferation and the differentiation of sebaceous glands and up-regulate lipid synthesis in sebaceous cells (Toyoda & Morohashi, 2001). These findings have been confirmed *in vitro* by a more recent study, in which neuropeptide substance P increases immunoreactivity and the expression of proinflammatory factors (Hong *et al.*, 2008).

As acne is driven by hormones and growth factors acting on the sebaceous glands and the keratinocytes lining the pilary canal, nutritional components that potentially have an influence on hormonal production may also contribute to the development of acne (Kurokawa *et al.*, 2009). For example dairy products contain  $5\alpha$ -reduced steroid hormones and other steroid precursors of dihydrotestosterone (DHT) that drive sebaceous gland function (Darling *et al.*, 1974). Drinking milk can cause a direct rise in serum insulin levels and in insulin-like growth factor (IGF) -1, whose levels closely parallel acne activity during teenage years (Hoyt *et al.*, 2005). High glycemic load foods also cause IGF-1 mediated elevations in DHT (Charakida *et al.*, 2007). Vitamin A is needed for normal follicular function, but it is often deficient in teenagers (Kurokawa *et al.*, 2009). Dietary fatty acids can be either proinflammatory or anti-inflammatory in inflammation (Treloar *et al.*, 2008). Iodine may also enhance inflammation (Danby, 2007).

Evidence of hereditary factors in acne has been described and an association of frequency and severity of acne in families in the descendants was also observed (Bataille *et al.*, 2002; Goulden *et al.*, 1999). Moreover,

there is direct genetic association of acne with androgen and lipid abnormalities, such as familial hyperandrogenism and inadequate activity of steroid 21-hydroxylase (Bekaert *et al.*, 1998; Ostlere *et al.*, 1998).

Recently, smoking has been reported to be a clinically important contributor to acne prevalence and severity (Schäfer *et al.*, 2001). It has been found that cigarette smoke contains high amounts of arachidonic acid and polycyclic aromatic hydrocarbons which induce a phospholipase A2-dependent inflammatory pathway (Tithof *et al.*, 2002).

#### **1.2.4 Treatment of acne**

Treatments are used to reduce the disfigurement, the psychological distress and to prevent scarring. These treatments include the use of anticomedogenic, anti-inflammatory and antimicrobial substances.

##### **1.2.4.1 Retinoids**

Retinoids have been suggested to have a direct pharmacological action on neutrophils and anti-inflammatory properties (Jappe, 2003). Topical tretinoin inhibits the release of lysosomal enzymes from polymorphonuclear leucocytes, which play a very important role in follicular wall damage and subsequently release inflammatory mediators into the dermis (Camisa *et al.*, 1982). Although it is not clearly understood, oral isotretinoin reduces sebaceous gland size to up to 90% by decreasing proliferation of basal sebocytes, suppressing sebum production and inhibiting sebocyte differentiation *in vivo* (Jappe, 2003). It also normalizes the pattern of

keratinisation within the sebaceous gland follicle, inhibits inflammation and reduces growth of *P. acnes* (Ganceviciene *et al.*, 2007). However, its discontinuation may be followed by recurrence and serious side effects like teratogenicity, myalgias, arthralgias (Alestas *et al.*, 2006; Kurokawa *et al.*, 2009). Therefore, identifying the appropriate acne patient for isotretinoin treatment is very important nowadays.

#### **1.2.4.2 Hormonal treatment**

Hormonal treatments consist of the ovarian suppression of androgen production by oral contraceptives, androgen receptor blockers (such as cyproterone acetate (CPA) and spironolactone), adrenal suppression of androgen production by corticosteroids and inhibitors of 5 $\alpha$  reductase (Jappe, 2003). New non-androgenic-progestin-containing cyclical oral contraceptives with strong anti-androgenic activity and reduced concentrations of ethinyl estradiol may replace the classic CPA/ethinyl estradiol and chlormadinone acetate/ethinyl estradiol oral contraceptives, because of comparatively less side-effects (Koltun *et al.*, 2008). Topical therapy with CPA in solid lipid nanoparticles can facilitate the penetration of the compound into the follicle canal suggesting additional therapeutic opportunity (Iraji *et al.*, 2006).

#### **1.2.4.3 Antimicrobial substances**

Oral antibiotics have been suggested to improve inflammatory acne by inhibiting the growth of *P. acnes* in the deeper parts of the follicle. However, only lipophilic drugs that penetrate the microcomedones are bacteriologically

and clinically efficient, which are tetracycline, doxycycline and minocycline. Several antibiotics also have additional anti-inflammatory effects, improving acne by decreasing leucocyte chemotaxis and alteration of cytokine production (Jappe, 2003; Kurokawa et al., 2009). Topical erythromycin is also well accepted, because it reduces *P. acnes* colonization in the follicle, suppresses the chemotaxis of inflammatory cells and directly decreases pro-inflammatory free fatty acids in sebum (Fulton, 1974; Webster et al., 1981). Tetracycline, erythromycin and nadifloxacin reduce reactive oxygen species formation by neutrophils and acne inflammation. New antibiotics include limecycline, a second-generation tetracycline and roxithromycin, which possess anti-inflammatory and anti-androgenic properties (Fenner et al., 2008).

#### **1.2.4.4 New strategies for acne treatment**

Monotherapy is mostly not efficient. Most retinoids do not have a direct effect on *P. acnes*. Although antibiotics have anti-inflammatory properties, use of antibiotics risks the development of antibiotic resistance. A review of combination therapy studies, using topical retinoids and antimicrobial agents together, showed remarkably consistent results: combination therapy achieves significantly greater and faster acne clearing versus antimicrobial therapy alone (Thiboutot et al., 2009). A combination of a topical retinoid plus an antimicrobial agent is an effective first-line therapy for targeting both inflammatory and non-inflammatory acne and their multiple pathogenic features in most patients (Thiboutot et al., 2009). However, the usage of

antibiotics should be minimized. Oral antibiotic treatment should be stopped once control of acne is achieved (Jappe, 2003).

*P. acnes*-based vaccination is another tempting therapy in the future. A study performed in the 1970s showed that an oral antigen treatment with heat inactivated lyophilized acne bacteria improved acne in 80% of cases (Niedre, 1975; Jappe, 2003). By targeting a cell wall-anchored sialidase of *P. acnes*, a recent study demonstrated that the serum containing anti-sialidase effectively neutralized the cytotoxicity of *P. acnes in vitro* and *P. acnes*-induced IL-8 production in human sebocytes (Nakatsuji *et al.*, 2008). Moreover, it also provided protective immunity against *P. acnes in vivo* (Nakatsuji *et al.*, 2008).

Plant extracts from *Azadirachta indica*, *Sphaeranthus indicus*, *Hemidesmus indicus*, *Rubia cordifolia* and *Curcuma longa* have shown anti-inflammatory activity *in vitro*. Such extracts appear to suppress the activity of *P. acnes*-induced reactive oxygen species and pro-inflammatory cytokines (Jain & Basal, 2003). Agents that target specific components of the *P. acnes* biofilm and inhibit the biofilm formation may have a role as future anti-acne drugs as well (Burkhart & Burkhart, 2003).

Photodynamic therapy provides an alternative method to those who are unable to take standard therapy or failed these treatments. Furthermore, it also reduces the use of antibiotics (Taylor & Gonzalez, 2009).

As discussed in section 1.2.3.5, several studies have shown that dairy intake, vitamin A and high glycemic foods are associated with acne (Kurokawa *et al.*, 2009). So, full acne control also requires dietary control.

### **1.3 Cell-cell communication by quorum sensing**

#### **two-component system in Gram-positive bacteria**

The concept of cell-cell signalling and coordinated microbial group behaviour was first revealed by two independent studies. One was reported in 1965 by Tomasz, the results of which suggested that an extracellular peptide helped regulate competence in *Streptococcus pneumoniae* (Tomasz, 1965). The other discovery was reported in 1970 by Nealson and colleagues. It revealed that *Vibrio fischeri* only produced bioluminescence at high cell density, and that this luminescence could be induced at low cell density by growing *V. fischeri* in conditioned medium from a high-density culture (Nealson *et al.*, 1970). This phenomenon was referred to as “autoinduction”, with the associated signal factor being referred to as the “autoinducer” (Visick & Fuqua, 2005). Since then, due to the identification of the *N*-acyl homoserine lactones (AHLs), an autoinducer used widely in Gram-negative bacteria, the bacterial multicellularity was accepted by the scientific community and brought to the forefront of microbiology research (Shapiro, 1998). The sophisticated signal transduction networks used by bacteria can benefit them by allowing access to resources that can not effectively be utilized by single cells, collectively defending against antagonists, optimizing population survival by differentiating into distinct cell types, using cellular division of labour and moreover, integrating intercellular signals with other information to regulate gene expression and cellular differentiation (Shapiro, 1998). Nowadays, there are intense interests in microbial signalling systems. Scientists are trying to interpret these signalling systems in all aspects of microbial social behaviour, interspecies communication and the cost/benefits



derived from an apparent multicellular existence. In this section, the QS, two-component system (TCS) of Gram-positive bacteria is introduced and discussed with illustrations by a few examples of QS TCS in Gram-positive bacteria.

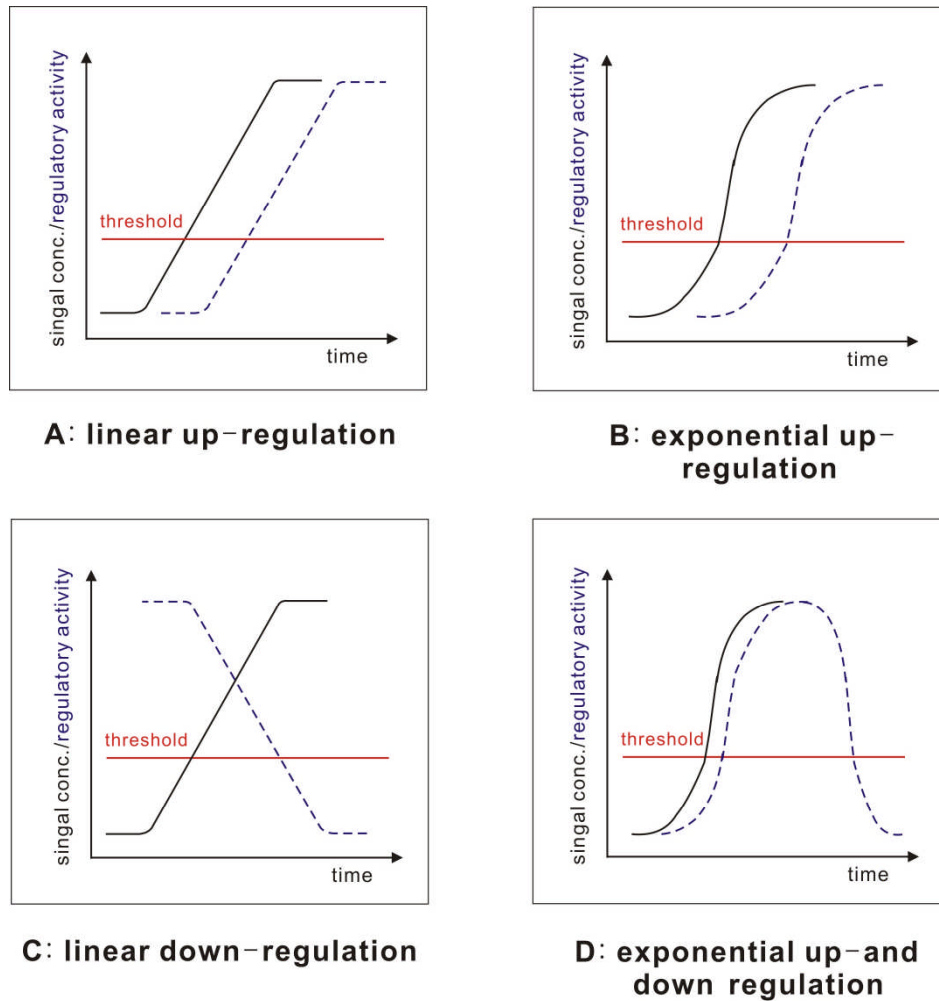
### **1.3.1 Quorum sensing in cell-cell communication**

QS is a cell-cell communication mechanism. The term QS was specifically introduced to refer to the coordinated gene expression in bacterial population in response to cell density, which recognises threshold signal concentrations to induce a synchronized population response (Fuqua *et al.*, 1994). QS allows bacteria to switch between two distinct gene expression programs: one that is favoured at low cell density for individual, asocial behaviours, and the other that is favoured at high cell density for social behaviours (Ng & Bassler, 2009). Many QS systems have been discovered in both Gram-negative and Gram-positive bacteria and involved in many bacterial processes. However, Gram-positive bacteria and Gram-negative bacteria employ very different QS systems to coordinate their gene regulation. Firstly, most Gram-positive bacteria utilize amino acids or short peptides as signalling molecules, while Gram-negative bacteria utilize fatty acid derivatives such as AHLs (Whitehead *et al.*, 2001). Secondly, signalling molecules like peptides in Gram-positive bacteria are often modified from the initially synthesized precursor, and are often exported across the cytoplasmic membrane by specialized transporters. In contrast, QS signalling molecules in Gram-negative bacteria are permeable to membranes (Magnuson *et al.*, 1994; Mayville *et al.*, 1999). Thirdly, the location of the cognate receptors of

signalling molecules in Gram-negative bacteria are cytoplasmic, while the sensor for oligopeptide autoinducers in Gram-positive bacteria are integral membrane proteins (Ng & Bassler, 2009). The remainder of this section will focus on QS systems in Gram-positive bacteria.

### **1.3.2 Kinetics of QS systems**

In QS systems, the signalling molecules are produced continually by each cell of the population. Once they accumulate to a certain density threshold concentration, they activate the regulatory system that controls the expression of target genes. The simplest mode of the signal production is that the amount of signalling molecules increases with an increasing number of cells per volume in a linear fashion (Figure 1.2, panel A and C) (Podbielski & Kreikemeyer, 2004). However, the production of the signalling molecules may also exponentially increase, if the QS system contains processing enzymes, sensors or regulators (Figure 1.2, panel B and D) (Podbielski & Kreikemeyer, 2004). When the density threshold is passed, the regulatory response could be a linear up-regulation (Figure 1.2, panel A), exponential up-regulation (Figure 1.2, panel B) or, less often, a linear down-regulation of the dependent genes (Figure 1.2, panel C) (Minogue *et al.*, 2002). In some QS-regulation circuits, the signalling molecules could be actively degraded once the threshold level has been passed allowing up- and subsequent fast down-regulation of the dependent genes (Figure 1.2, panel D) (Waters *et al.*, 2003).



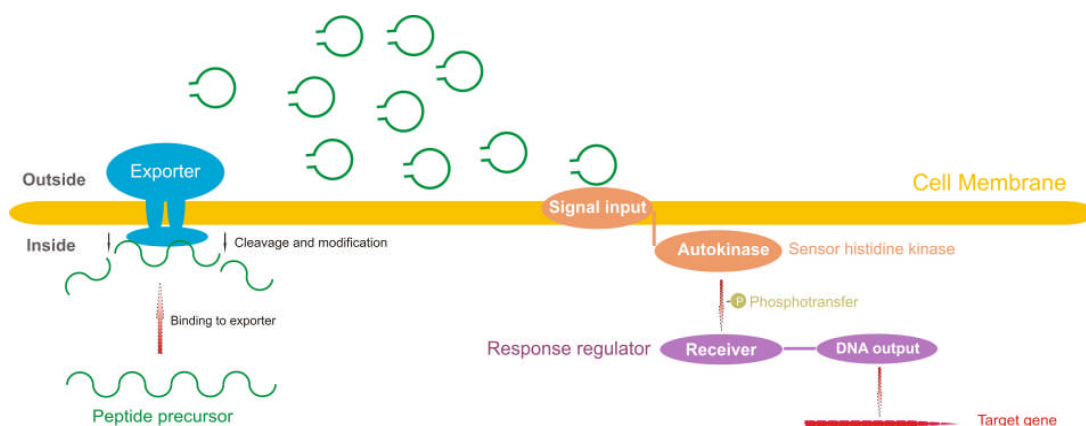
**Figure 1. 2 Kinetic patterns of QS regulation.**

In QS bacteria, signalling molecules (bold black line) are produced either in a linear (A and C) or exponential (B and D) fashion. Once the signalling molecule reaches the concentration threshold (red line), the regulatory response (dashed blue line) will start. It could be a up-regulation in a linear or exponential fashion (A and B), or a down-regulation (C). It also could be an initial up-regulation and subsequent down-regulation (D) (Podbielski & Kreikemeyer, 2004).

### 1.3.3 Quorum sensing in Gram-positive bacteria

The QS system in Gram-positive bacteria generally consists of a signalling molecule and a TCS containing a histidine kinase (HK) and a response regulator (RR) (Waters & Bassler, 2005). Peptides are the most

common and well-studied QS signalling molecules in Gram-positive bacteria and are often referred as autoinducing peptides (AIPs). Mostly, these peptides are generated from longer precursors. The peptide precursor is directed by its leading sequence to a dedicated exporter, which is commonly an ABC transporter system. During export, the peptide precursors are often cleaved and modified to their final stable and functional form. Once the secreted peptide accumulates to a certain concentration threshold, it binds to the signal input domain of the HK resulting in the activation of the TCS and the transduction of information via a series of phosphorylation reactions. The binding of the peptide to the signal input domain of HK stimulates its intrinsic autophosphorylation activity and leads to subsequent transfer of a phosphoryl group to the receiver domain of the cognate RR. The phosphotransfer between HK and RR results in the activation of the DNA output domain of the RR. Phosphorylated RR is then activated and acts as a DNA-binding transcription factor to modulate expression of target genes (Figure 1.3) (Hoch & Silhavy, 1995; Reading & Sperandio, 2006; Ng & Bassler, 2009). In most cases, the genes encoding AIP, HK and RR form an operon and the gene expression is autoinduced by producing positive feedback to accelerate the transition from the low cell density to the high cell density QS mode (Ji *et al.*, 1995; Peterson *et al.*, 2000).



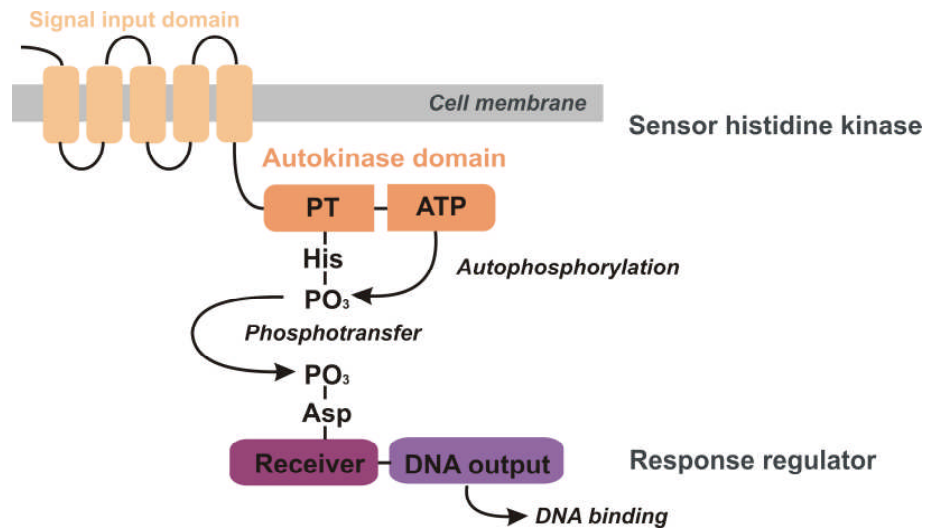
**Figure 1. 3 A canonical QS TCS in Gram-positive bacteria.**

In Gram-positive bacteria, the peptide precursor is produced continually in the cell. After the production, the peptide precursor, directed by its leading sequence, binds to the dedicated exporter. During the exportation, the exporter normally cleaves the peptide precursor and modifies it to its functional form. Once the extracellular signalling molecule accumulates to a concentration threshold, it binds to the signal input domain of the cognate HK resulting in the autophosphorylation of its autokinase domain. The phosphoryl group is then transferred to the receiver domain of the cognate RR and subsequently activates the DNA output domain resulting in the regulation of gene expression of the target gene.

### 1.3.3.1 The basic structure of a TCS

The TCS is one of the major regulatory systems that can process and transduce environmental signals (Hoch & Silhavy, 1995). In both Gram-positive and Gram-negative bacteria, the TCS is very important for bacterial adaptability and pathogenesis. As in the regulatory system in QS system, the TCS plays an essential role in the QS regulation and subsequently changes gene expression of a bacterium. The simplest TCS consists of a HK as a signal sensor and a RR as a transcription factor (Figure 1.4). Most HKs in TCSs are integral membrane proteins with their signal input domains embedded into the cell membrane containing several

transmembrane helices. Whereas, their autokinase domains are cytoplasmic. The autokinase domain also consists of two sub-domains: a phospho transfer (PT) sub-domain and an ATP-binding sub-domain. Upon the recognition of the specific signal the HK catalyzes an ATP-dependent autophosphorylation to a conserved histidine residue in its PT sub-domain. This phosphoryl group is subsequently transferred to a conserved aspartic acid residue in the receiver domain of the cognate RR resulting in the activation of DNA-binding domain of the RR (Hoch & Silhavy, 1995; Stephenson & Hoch, 2002).



**Figure 1. 4 The structure of the basic TCS.**

The basic structure of a TCS contains a HK and a RR. The HK includes a signal input domain and an autokinase domain. The signal input domain is normally an integrated protein embedded in the cell membrane and has several transmembrane helices. The autokinase domain contains a PT sub-domain and an ATP sub-domain. The RR includes a receiver domain and a DNA output domain. Upon the recognition of the environmental signal by the signal input domain, the HK catalyzes an ATP-dependent autophosphorylation on the histidine residue of the PT sub-domain. This phosphoryl group is subsequently transferred to the aspartic residue on the receiver domain of the RR resulting in the activation of the DNA output domain and binding to the target gene (Stephenson & Hoch, 2002).

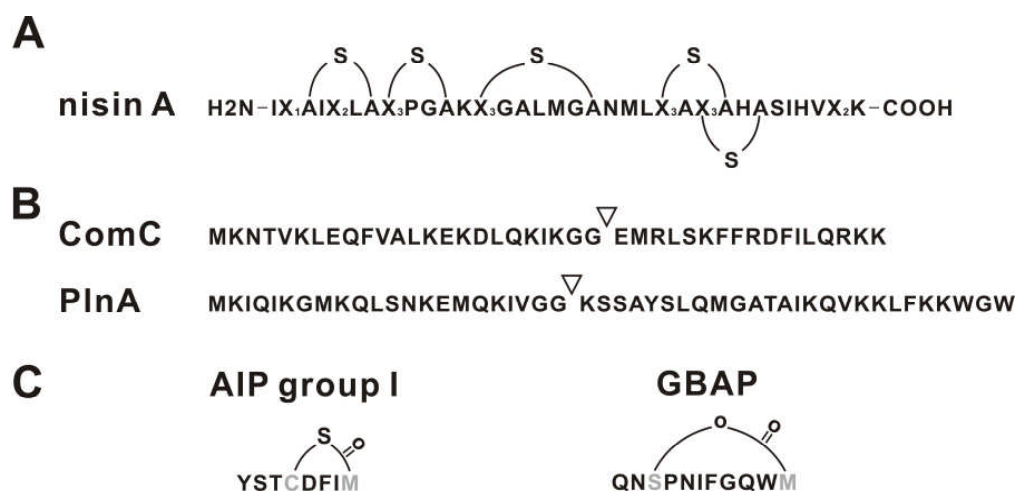
The regulation in bacteria by the TCS is not only controlled by the specific recognition between the environmental signal and the corresponding HK or the specific phosphorylation between HK and its cognate RR, but also achieved by specific phosphatase activities of the HK (Ohlsen *et al.*, 1994; Perego *et al.*, 1994). They limit the activation of RR by removing the phosphoryl group (Stephenson & Hoch, 2002). As the TCS provides different levels of control, where information can be fed into the system to influence

the activation of the RR, it also has been considered as a potential drug target (Perego et al., 1994; Stephenson & Hoch, 2002).

### **1.3.3.2 Processing and modification of autoinducing peptides**

Each Gram-positive species uses a signal different from that used by other species and the cognate receptor is exquisitely sensitive to the signal's structure. As described above (Section 1.3.3), AIPs are not diffusible across the membrane. Signal release is mediated by exporters, which mostly are either ABC exporters or dedicated exporters. AIPs are also frequently processed during exportation and are in several cases subject to post-translational modification before or after export. So far, three different kinds of processing or modification of AIPs have been identified in Gram-positive bacteria. Lantibiotics, like nisin from *Lactococcus lactis* or subtilin from *Bacillus subtilis*, are class I antimicrobial peptides, but they also function as QS communication molecules (Sturme *et al.*, 2002). They contain modifications such as dehydrated amino acids and typical ( $\beta$ -methyl)-lanthionines, which are modified from the precursor peptides by several enzymes encoded by genes subjected to autoregulation (Figure 1.5, panel A) (Kleerebezem *et al.*, 1997). Dedicated ABC exporters are responsible for these modification reactions and they occur intracellularly. The AIP is activated by proteolytic removal of the pre-sequence, also known as the leader sequence. This proteolytic removal is done by either the dedicated ABC exporter or by a specific leader peptidase encoded by a gene also subjected to autoregulation (Van der Meer *et al.*, 1993; McAuliffe *et al.*, 2001).





**Figure 1. 5 Structural diversity of AIPs found in Gram-positive bacteria.**

(A) Lantibiotic nisin A of *Lactococcus lactis* (X<sub>1</sub>: dehydrobutyrine, X<sub>2</sub>: dehydroalanine, X<sub>3</sub>: 2-aminobutyric acid, A-S-A: lanthionine, X<sub>3</sub>-S-A:  $\beta$ -methyl-lanthionine). (B) Double-glycine-type peptide precursors (PlnA of *Lactobacillus plantarum* C11 and ComC of *Streptococcus pneumoniae*); ▽ indicates the cleavage position between the leader peptide sequence and the mature AIP sequence. (C) Cyclic thiolactone- and lactone-peptides (AIP group I: group I cyclic thiolactone peptide of *Staphylococcus aureus*; GBAP: cyclic lactone peptide GBAP(gelatinase biosynthesis-activating pheromone) of *Enterococcus faecalis*) (Waters & Bassler, 2005, Ng & Bassler, 2009, Sturme et al., 2002).

Another type of AIPs are linear peptides that are often involved in the regulation of genetic competence and the production of class II antimicrobial proteins called bacteriocins (Figure 1.5, panel B). These AIPs contain a typical double-glycine-type leader peptide, which is then cleaved from the precursor peptide during export by the dedicated ABC exporter (Sturme et al., 2002). In this case, the ABC exporter has an N-terminal extension characterizing a peptidase domain (Havarstein *et al.*, 1995b). This typical processing of AIPs is widely spread amongst Gram-positive bacteria and has been reported in many studies, such as peptide ComC in *Streptococcus pneumoniae*, the bacteriocin-inducing peptide IP-673 in *Lactobacillus sake*

LTH673, PlnA in *Lactobacillus plantarum* C11, CbnB2 and CbnS in *Carnobacterium piscicola* LV17B and CTC492 in *Enterococcus faecium* CTC492 (Sturme et al., 2002).

A specific type of modification to AIPs is found to contain a cyclic thiolactone or cyclic lactone-structure, which are involved in the *agr*- and *fsr*-systems in *Staphylococcus aureus* and *Enterococcus faecalis*, respectively (Figure 1.5, panel C) (Sturme et al., 2002). It is proposed that the AIP is cleaved off as an internal part of the precursor peptide and then processed and modified via an unknown mechanism by the dedicated exporter during the exportation (Ji et al., 1997; Jiro et al., 2001).

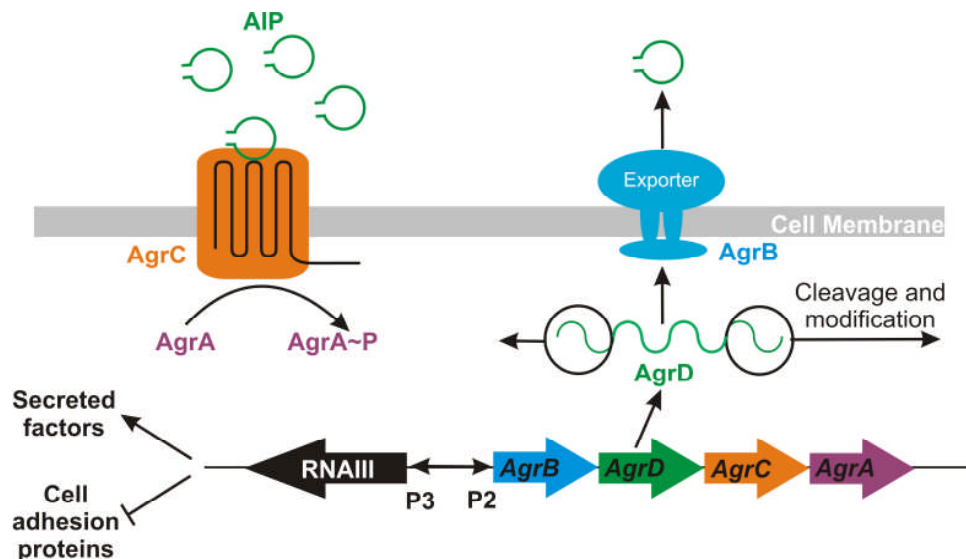
A new type of modification has been predicted recently for the ComX pheromone involved in competence development in *B. subtilis* (Sturme et al., 2002). It is thought that this 10 amino acid pheromone is processed from a 55 amino acid precursor peptide and modified by a protein, ComQ, which contains a putative isoprenoid-binding domain required for its *in vivo* function. It has been suggested that the modification on ComX is likely to be an isoprenoid (Ansaldi et al., 2002; Bacon-Schneider et al., 2002).

### 1.3.3.3 Examples of quorum sensing in Gram-positive bacteria

#### -The Agr QS system of *Staphylococcus aureus*

The *agr* QS system in Gram-positive bacteria is a fascinating example that uses a peptide signal. It is also probably the best studied system. *S. aureus* is normally a human commensal, but can become an invasive pathogen. It can promote disease in almost any tissue in the human body including skin infections, toxic shock syndrome, endocarditis and food

poisoning (Miller & Bassler, 2001). Interestingly, a variety of virulence factors required for successful invasion of *S. aureus* are regulated by QS peptide. *S. aureus* expresses protein factors that promote attachment and colonization at low cell density, whereas at high cell density it represses these traits and initiates secretion of toxins and proteases that are presumably required for dissemination (Lyon & Novick, 2004). This switch in gene expression programs is regulated by the *agr* QS system. The *agr* QS system consists of an AIP encoded by *agrD* and a TCS with a HK and RR, encoded by the *agrC* and *agrA* genes, respectively (Figure 1.6) (Novick *et al.*, 1995). This system also contains a dedicated transpoter, AgrB, that exports and adds the thiolactone ring modification to the AIP (Saenz *et al.*, 2000). Upon binding of the AIP to AgrC, the activated AgrA induces the expression of RNAIII, a regulatory RNA, which represses expression of cell-adhesion factors while inducing expression of secreted virulence factors (Novick *et al.*, 1993). The activated system also provides positive feedback and increases the expression of the *agrBDCA* operon ensuring the entire population switch from low-cell-density to the high-cell-density model (Novick *et al.*, 1995). *S. aureus* can be categorized into four different groups depending on the sequence of their thiolactone-containing AIPs (Dufour *et al.*, 2002). The specificity of the interactions between an AIP and its cognate HK and its interference on other strains is discussed in the following section 1.3.4. It has been shown by clinical analysis that each *S. aureus* group is the primary causative agent of a specific type of *S. aureus* disease suggesting that cell-cell communication has been instrumental in establishing a specific niche of each strain (Novick, 2003).



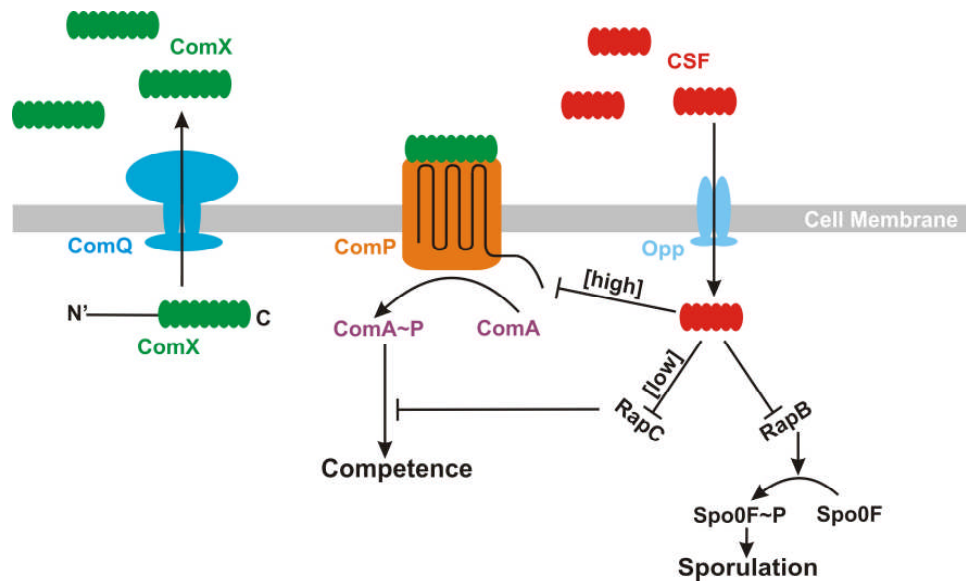
**Figure 1. 6 The agr QS system of *S. aureus*.**

The core *agr* operon encodes a dedicated transporter (AgrB), the signalling molecule (AgrD), a HK (AgrC) and a RR (AgrA). After production, AgrD is processed into its functional form and exported by AgrB. Upon the sensing of AgrD by AgrC, activated AgrC phosphorylates AgrA resulting in the interaction between AgrA~P and Agr-dependent promoters P<sub>2</sub> and P<sub>3</sub>. This leads to an increased transcription rate from these promoters and the transcription of the RNAIII molecules, which controls the gene expression of virulence factors and cell adhesion factors (Waters & Bassler, 2005).

*-The ComP/ComA QS competence/sporulation system of Bacillus subtilis*

*B. subtilis* is a soil bacterium that uses QS to alternate between competence for DNA uptake and sporulation. This occurs when environmental conditions are deteriorated and nutrients become limiting which is normally at the transition between logarithmic and stationary phase growth (Grossman, 1995; Schauder & Bassler, 2001). In *B. subtilis*, two AIPs mediate QS regulation of competence and sporulation; ComX and competence and sporulation factor (CSF). In the competence state, ComX is

processed and modified by ComQ and detected by the ComP/A TCS containing HK and RR, respectively (Figure 1.7) (Magnuson et al., 1994). The activation of this TCS results increased expression of ComK, a transcription factor that controls the expression of structural genes required to develop competence (Van Sinderen *et al.*, 1995). ComK is also involved in a transcriptional loop providing positive feedback and likely promotes the commitment of the cells to the competence pathway (Hahn *et al.*, 1996). The second AIP, CSF, is a pentapeptide, which is cleaved from the C-terminus of the precursor peptide PhrC and involved in both competence and sporulation processes (Solomon *et al.*, 1996). CSF accumulates extracellularly as a function of increasing cell density. Then it is imported into *B. subtilis* by an ABC-type oligopeptide transporter named Opp performing its signalling role intracellularly (Lazazzera *et al.*, 1997). At low internal concentration of CSF, it binds to a protein called RapC. RapC binds to ComA and prevents competence development (Core & Perego, 2003). In this case, the CSF indirectly increases the activity of ComA and promotes the competence development. However, when the internal concentration of CSF is high, it reduces the expression of ComS resulting increased proteolysis of ComK. ComK is involved in the processing of ComX (Turgay *et al.*, 1998). Therefore, CSF indirectly inhibits competence and drives *B. subtilis* into the sporulation process. It also inhibits the RapB phosphatase activity, which mediates the dephosphorylation of Spo0F (Perego, 1997). This activity increases the level of phosphor-Spo0A favouring a switch from competence to the sporulation. A study also showed that CSF affects ComP-ComA signalling cascade through an unknown mechanism (Solomon et al., 1996).



**Figure 1. 7 The ComP/ComA competence/sporulation QS system of *B. subtilis*.**

In *B. subtilis*, two AIPs (ComX and CSF) are produced to regulate two different development pathways: competence and sporulation. In the competence state, ComX is processed and exported by ComQ. Active ComX is recognized by ComP leading to the phosphorylation of ComA, which regulates expression of structural genes required to develop competence. Meanwhile, extracellular CSFs accumulate as an indication of cell density and then are internalized by Opp. When the internal CSF concentration is low, it binds to RapC and prevents RapC from binding to ComA. It indirectly promotes the development of competence. When the internal CSF concentration is high, it binds to ComP to inhibit the interaction between ComP and ComA and down-regulates the development of competence. It also dephosphorylates RapB resulting in the development of sporulation (Waters & Bassler, 2005).

*-The ComD/ComE QS competence system of Streptococcus pneumoniae and S. mutans*

*S. pneumoniae* was one of the original Gram-positive QS systems identified. The genetic transformation of *S. pneumoniae* requires the recipient bacterium to become competent to acquire exogenous DNA molecules (Miller & Bassler, 2001). The transition of competence is partially controlled

by the ComD/ComE QS system (Miller & Bassler, 2001). Competence stimulating peptide (CSP), a 17-amino acid peptide, is the peptide signal required for the competence development and is encoded by the *comC* gene (Havarstein *et al.*, 1995a). Within the same operon, there lies a HK, encoded by *comD* and a RR, encoded by *comE* (Pestova *et al.*, 1996). Following cleavage from a 41-amino acid precursor peptide, CSP is secreted by the ComAB ABC transporter apparatus (Havarstein *et al.*, 1995b). At high cell density, CSP is detected by ComD resulting in the autophosphorylation in HK ComD and phosphotransfer to RR ComE. Phosphor-ComE subsequently activates transcription of the *comX* gene, which is an alternative sigma factor required for transcription of structural genes involved in competence development (Lee & Morrison, 1999). When phosphorylated, ComE also autoregulates the expression of the *comCDE* operon, *comAB* and *comX* and induces genes involved in stress response and protein synthesis. The competence state of *S. pneumoniae* only occurs during exponential growth (Lee & Morrison, 1999; Peterson *et al.*, 2004). It has been shown that the bacterium loses the ability to take up exogenous DNA in later stages of growth suggesting that a regulatory system(s) exists to drive it out of the competence state (Miller & Bassler, 2001). Moreover, a small fraction of the 124 genes induced by the CSP system are identified for transformation in a recent transcriptome analysis, which suggests that the CSP system might be also involved in other types of regulation (Peterson *et al.*, 2004).

*S. mutans* specifically and firmly colonizes the surface of the tooth by protein adhesins and produces biofilms containing a water-insoluble, sticky extracellular matrix of complex polysaccharides (Podbielski & Kreikemeyer, 2004; Li *et al.*, 2001b). Unlike other streptococci, it continues the fermentation

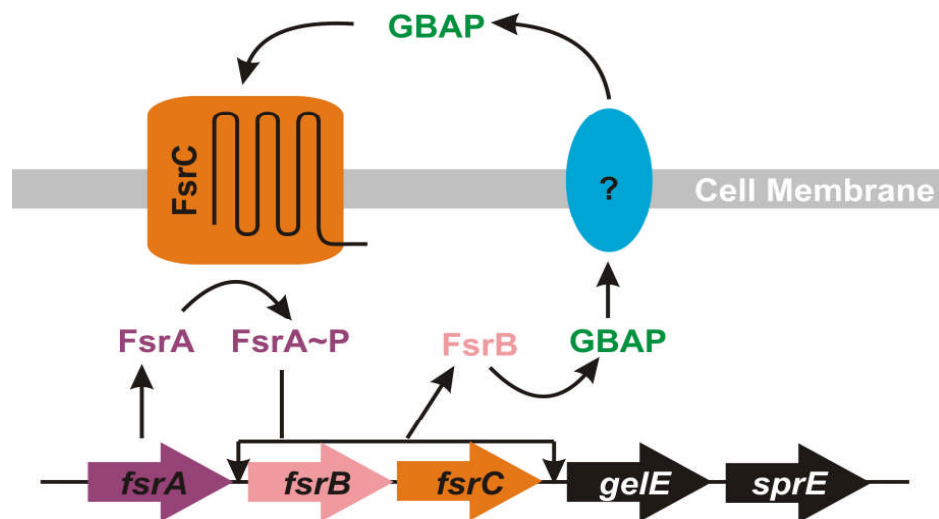
of sugar until high amounts of lactic acid are produced, resulting in the formation of caries (Li *et al.*, 2001a). It has been shown that both biofilm formation and acid tolerance of *S. mutans* are associated with a QS system that closely resembles the *com* regulon of *S. pneumoniae*. Using defined *S. mutans com* mutants and synthetic ComC signalling molecules, it was demonstrated that when cells live at a high cell density in a biofilm, competence is more pronounced and simultaneously acid tolerance is better (Li *et al.*, 2002; Li *et al.*, 2001a). Furthermore, a normal biofilm can only be formed with a functional *com* regulon, indicating that the *com* regulon is a very important player in the ability of *S. mutans* to cause caries lesions (Li *et al.*, 2001a).

#### *-Evolutionary conserved agr systems in other Gram-positive bacteria*

*E. faecalis* colonizes human intestines, respiratory tract and skin without causing diseases. However, this bacterium is also of prominent medical importance because of its ability to harbour multiple antibiotic resistance genes and to exchange these genes with other bacteria (Podbielski & Kreikemeyer, 2004). It has been shown that the *fsr* QS system exhibits significant sequence homology to the *S. aureus agr* QS system (Podbielski & Kreikemeyer, 2004). In the *fsr* system, the *fsrA*, *B* and *C* genes encode a RR, a processing enzyme and a HK respectively (Figure 1.8). Instead of having an independent gene encoding for the AIP as in *S. aureus agr* system, *fsrB* contains the signalling peptide as part of its C-terminus, called gelatinase biosynthesis activating pheromone (GBAP) peptide (Nakayama *et al.*, 2001). At the transition from exponential growth to stationary phase, GBAPs are



accumulated and activate the *fsrA/fsrC* TCS phosphorylation cascade resulting in the induction of the *gelE* and *sprE* genes (Qin *et al.*, 2001). The *gelE* gene and *sprE* gene, encoding a gelatinase and serine protease respectively, also up-regulate the transcription of the *fsrBC* operon (Qin *et al.*, 2001). However, different from *S. aureus agr* system that is involved in a regulatory RNAIII, the induction is directly promoted by the binding of FsrA to the respective promoters (Nakayama *et al.*, 2001).



**Figure 1. 8** The evolutionary conserved *fsr* QS system of *E. faecalis*.

After FsrB is produced, it undergoes an auto-processing step, which liberates the signalling molecule GBAP from the C-terminal portion of the protein. GBAP is then exported by a still underdefined pathway and accumulates to a crucial density threshold. The interaction between GBAP and FsrC activates the FsrC/FsrA phosphorylation cascade and subsequently up-regulates the transcription of the *fsrBC* operon and the adjacent gelatine-cysteine protease operon (Podbielski & Kreikemeyer, 2004).

Other evolutionary conserved *agr* systems have also been found in *Lactobacillus plantarum*, *Clostridium perfringens*, *Clostridium botulinum*, *Clostridium difficile* and *Listeria monocytogenes*. However, the chemical identity of AIPs of these bacteria is not yet known (Wuster & Babu, 2008).

#### **1.3.4 Diversity and specificity of quorum sensing systems**

The signal recognition and transduction pathway in QS are highly specific between species and even strains of the same species. The highest sequence diversity is found in the N-terminal and linker part of the HK, the AIP and peptide-processing genes (Sturme et al., 2002). For example, *S. aureus* strains can be classified on the basis of the sequence of their thiolactone-containing AIP. Four different AIPs have been identified so far, therefore, four different groups of *S. aureus* have been classified (Figure 1.9). The mature AIPs are seven to nine residues in length with a five-member ring formed between the sulphur atom from a central cysteine and the C-terminus via a thiolactone bond. The two bulky hydrophobic residues of each AIP are involved in cognate receptor binding and the ring structure is critical for activity (Ng & Bassler, 2009). It has been shown that the AIP produced by one *S. aureus* strain inhibits *agr*-expression in some of the other strains. The specificity of the Agr system not only enables the specific recognition between cognate AIPs and receptors, but also the repression of other AIPs by competitive binding to noncognate receptors (Jarraud et al., 2000; Ji et al., 1997). The specific activation or inhibition is thought to be dependent on both amino acid sequence and stereochemical structure of AIPs. It is hypothesized that either activation or inhibition is due to AIPs interacting with

the cognate receptor at overlapping binding sites (Lyon *et al.*, 2002a). This interference phenomenon with specific AIPs may allow *S. aureus* strains belonging to one of the AIP groups to predominantly colonize in special locations and to be associated with a defined subset of diseases. For example, AIP group I *S. aureus* mainly colonized in the vagina. Also, AIP group I and II are associated with enterotoxin-mediated disease and endocarditis, while AIP group III and IV are associated with toxic shock toxin-mediated disease and exfoliative toxin-mediated disease, respectively (Jarraud *et al.*, 2000; Jarraud *et al.*, 2002; Podbielski & Kreikemeyer, 2004).

### *S. aureus* AgrD

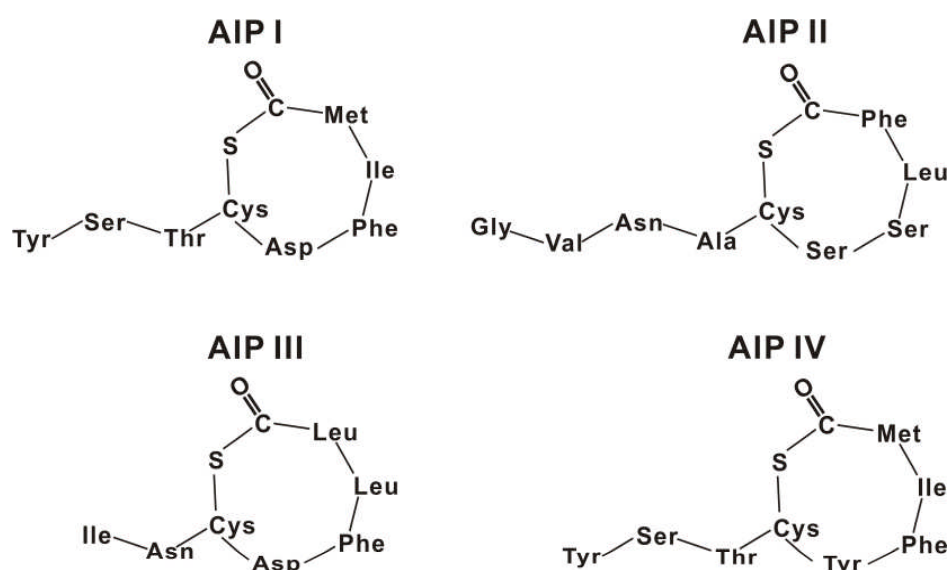


Figure 1. 9 Structures of four different AIPs in *agr* systems in *S. aureus*.

Moreover, the signalling molecule can be recognized by related species as well. Cross-inhibition has been detected between *S. aureus* strains and *S. epidermidis* or *S. lugdunensis* and this intra- and interspecies cross-talk can

be either inhibitory or inducing (Ji *et al.*, 1997; Otto *et al.*, 2001). Cross-induction has been observed for the ComX-peptides from *B. subtilis* and *B. mojavensis* strains, the ComC-peptide in *S. pneumoniae* and the SalaA lantibiotic peptides in *S. salivarius* and *S. pyogenes* (Tortosa *et al.*, 2001; Whatmore *et al.*, 1999; Upton *et al.*, 2001).

### **1.3.5 Biological significance of activation and inhibition of QS systems in bacteria**

As QS systems coordinate gene expression in the population and sense signals from the environment, they are very important to most bacteria to adapt to a given environment or develop virulence factors. The coordinated gene expression and its consequent physiological responses may also lead to competitive advantages and constant interactions between bacteria within a specific population or among bacteria in the whole ecosystem (Sturme *et al.*, 2002). As illustrated above (Section 1.3.3.3), in Gram-positive bacteria QS regulation is associated with the production of virulence factors in *S. aureus*, the development of natural competence and sporulation in *B. subtilis* and *S. pneumoniae*, biofilm formation of *S. mutans* and the production of antibacterial peptides by lactic-acid bacteria. These QS regulations are very important not only to bacterial adaptability, but also determine the pathogenicity of the bacteria. For example, the QS-regulated competence systems in *Bacillus* and *Streptococcus* spp. enable them to take up free DNA from the environment and integrate parts of this foreign DNA into their genomes. By adding new genetic features in these bacteria, it may increase adaptation to the environment or competitive advantages or have a function

in DNA repair (Sturme *et al.*, 2002; Lorenz & Wackernagel, 1994). Moreover, despite being a specific language of a specific group, QS regulation and QS signalling molecules are also used widely between strains, or species, or even kingdoms. Cross-talk by AIPs between strains and species within microbial systems is one of the most important ways that bacteria communicate with each other and may play an essential role in microbial ecosystem development and function (Sturme *et al.*, 2002). Cross-talk can either be inhibitory or cooperative to other bacteria or interfere with host responses. For example, the production of antimicrobial peptide can be induced by AIP in lactic-acid bacteria (McAuliffe *et al.*, 2001). In this way, it may enable these bacteria to have advantages in competing with other bacteria and subsequently in colonization of ecological niches or accessing nutrients (Ennahar *et al.*, 2000). Moreover, cross-inhibition has also been shown in the colonization of plant material, other raw materials such as milk and surfaces such as the skin or mucosal epithelium in the gastrointestinal tract (Sturme *et al.*, 2002).

### **1.3.6 The QS system as a potential target for antimicrobial therapy**

A number of features of QS systems make them attractive targets for antimicrobial therapy. Firstly, QS systems, especially TCSs, are ubiquitous in bacteria. They contain elements for bacteria to sense the environment and coordinate gene expression either for adaptability or pathogenicity (Stephenson & Hoch, 2002). The signal transduction pathway of QS-TCS system is highly specific, therefore, a designed inhibitor could selectively inhibit the pathogen without affecting members of the natural flora (Podbielski

& Kreikemeyer, 2004). Moreover, as the specific signalling pathway can be completely blocked, it also exerts less selective pressure for the development of resistance mutations compared to antibiotics (Podbielski & Kreikemeyer, 2004). Secondly, the sequence and structure of HK and RR are highly conserved, especially around the active sites, therefore, a finely designed inhibitor targeting several common elements may be able to inhibit a wide range of virulence and antibiotic resistance systems (Hoch & Silhavy, 1995). Finally, the TCS system has not been identified in the animal kingdom, but QS system has been described in the interactions between pathogen and host. QS systems also control certain biological functions like the internalization into eukaryotic cells suggesting that an inhibitor could interfere with this process and act inside of eukaryotic cells instead of outside like antibiotics (Podbielski & Kreikemeyer, 2004). Furthermore, the signal transduction pathway of TCS is distinct from the Ser/Thr signaling pathways commonly used in eukaryotic cells indicating that a designed inhibitor could also minimize toxicity effects in animals (Stephenson & Hoch, 2002).

Nowadays, the research of developing interfering molecules is mainly based on the well-documented *agr* QS system for *S.aureus*. Interestingly, the suppression between AgrD molecules of one group and AgrD-mediated signalling in other groups can already be of therapeutic relevance by nature. Administration of heterologous AgrD molecules that interfere with RNA III activation results in decreased lesion size (Balaban *et al.*, 1998, Mayville *et al.*, 1999). It was found that any amino acid exchange affecting the internal thiolactone structure or the sequence of the N-terminal tail of AgrD had negative effects on the signalling capabilities of this molecule (Lyon *et al.*, 2000; Philip *et al.*, 2001). It has been shown that the binding of AgrD to the

cognate AgrC has two steps involving the thiolactone ring as the first step and the N-terminal tail as the second (Lyon *et al.*, 2002b). Providing different AgrD molecules or only the thiolactone structure blocks the first binding process, but does not activate the AgrD and the following phosphorylation cascade, as it lacks the second binding event (Lyon *et al.*, 2002b). It was also observed that *S. epidermidis* AgrD interferes with *S. aureus agrD* signalling (Otto *et al.*, 2001). Moreover, therapeutic approaches using QS inhibitors, such as RNAlII inhibiting peptide and its non-peptide analog, have been shown to prevent or treat infections caused by staphylococcal strains, even including antibiotic-resistant strains like MRSA (Balaban *et al.*, 2007; Kiran *et al.*, 2008b). Moreover, it has been observed that the interference AgrD with non-cognate AgrC kinases prevents these kinases from binding to their cognate AgrD and inhibits their signalling cascades (Wright III *et al.*, 2004). Although further understanding of the interaction between AgrD and AgrC is required, this inhibitory effect has therapeutic potential by creating chimeric AgrC protein (Wright III *et al.*, 2004; Sperandio, 2007). There are still many obstacles to overcome before interfering molecules can be used as alternative therapeutics in any infections.

### **1.3.7 The TCSs of *P. acnes* identified in the genome sequence**

Since the complete genome sequence of *P. acnes* strain KPA171202 (DSM 16379) has been revealed in 2004, some of *P. acnes*' mysterious and contradictory traits have become or will become easier to track down. This completely sequenced skin isolate belongs to *P. acnes* type I and falls into a subgroup of strains isolated from acne lesions, dental infections and failed

prosthetic hip implants (Bruggemann, 2005). The sequenced strain exhibits 100% identity on the 16S rRNA level to several clinical *P. acnes* isolates as well as to the well-studied laboratory strain P37 (Bruggemann *et al.*, 2004). In the genome of *P. acnes* KPA101202, the environmental signal transduction seems to be restricted to 10 TCSs as well as a few orphan HKs and RRs (Table 1.1) (Bruggemann *et al.*, 2004). As illustrated in Table 1.1 A, the functions of many identified TCSs are unknown. A few systems, such as PPA0118/119, PPA0359/360, PPA1337/1338 and PPA2112/2113, have been implicated in controlling lifestyle related to oxygen availability (Bruggemann *et al.*, 2004). These systems show some similarity to characterized respiration regulatory systems ArcAB or ResED, although no striking similarity could be detected (Bruggemann *et al.*, 2004). There are also several orphan putative HKs and RRs identified in the genome of *P. acnes* (Table 1.1 B and C). So far, functions of these proteins are unknown and how they are involved in the sensing and regulation pathway is unclear.



**Table 1. 1 (A) Putative TCS pairs identified in the *P. acnes* genome.**

Locus	Function	Homology
PPA0056/5	Unknown	PPA0055 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> HL067PA1 with more than 99% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator of <i>Streptomyces avermitilis</i> with 98% identities. PPA0056 was annotated as a hypothetical protein; the closest homologue is a conserved hypothetical protein of <i>P. acnes</i> HL013PA1 with 89% identities; apart from <i>Propionibacteria</i> , the closest homologue is a putative lipopolysaccharide biosynthesis protein of <i>Vibrio coralliilyticus</i> with 25% identities.
PPA0118/9	Potassium transport	KdpD/KdpE;
PPA0290/89	Unknown	PPA0289 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> HL056PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator of <i>Sanguibacter keddieii</i> with 96% identities. PPA0290 was annotated as a sensor kinase; the closest homologue is a histidine kinase of <i>P. acnes</i> HL046PA2 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase of <i>Intrasporangium calvum</i> with 92% identities.
PPA0360/59	Unknown	PPA0359 was annotated as a response regulator containing a REC receiver domain and a trans_reg_C DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> SK187 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a phosphate regulon transcriptional regulatory protein, PhoB, of <i>Streptomyces sviveus</i> with 99% identities. PPA0360 was annotated as a sensor kinase containing a HisKA domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> HL110PA4 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is histidine kinase of <i>Streptomyces flavogriseus</i> with 84% identities.
PPA0415/6	Unknown	PPA0415 was annotated as a sensor histidine kinase; the closest homologue is a histidine kinase of <i>P. acnes</i> HL013PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a sensor histidine kinase of <i>Aeromicrobium marinum</i> with 90% identities. PPA0416 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> SK187 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is transcriptional regulatory protein UhpA of <i>Aeromicrobium marinum</i> with 97% identities.

Table 1. 1 (A) Putative TCS pairs identified in the *P. acnes* genome (continue).

Locus	Function	Homology
PPA0925/6	Unknown	PPA0925 was annotated as a sensor kinase containing a HisKA_3 domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> HL110PA3 with 96% identities; apart from <i>Propionibacteria</i> , the closest homologue is a signal transduction histidine kinase of <i>Saccharomonospora viridis</i> with 92% identities. PPA0926 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> J139 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a transcriptional regulator, LuxR family protein, of <i>Dietzia cinnamea</i> P4 with 98% identities.
PPA0945/7	Unknown	PPA0945 was annotated as a histidine kinase containing a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> HL082PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase of <i>Micrococcus luteus</i> with 82% identities. PPA0947 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like DNA binding domain; the closest homologue is a response regulator of <i>P. acnes</i> HL103PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator of <i>Micrococcus luteus</i> with 96% identities.
PPA1337/8	Unknown	PPA1337 was annotated as a histidine kinase containing a HisKA domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> SK187 with 84% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase of <i>Kribbella flavida</i> with 88% identities. PPA1338 was annotated as a response regulator containing a REC receiver domain and a trans_reg_C DNA binding domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> HL030PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator receiver of <i>Thermobifida fusca</i> with 95% identities.
PPA2027/8	Unknown	PPA2027 was annotated as a sensor kinase containing a HisKA_3 domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> HL025PA1 with 93% identities; apart from <i>Propionibacteria</i> , the closest homologue is a sensor kinase of <i>Streptomyces bingchenggensis</i> with 96% identities. PPA2028 was annotated as a response regulator containing a REC receiver domain and a LuxR_C_like domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> HL030PA2 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator of <i>Streptomyces sp.</i> with 97% identities.

**Table 1. 1 (A) Putative TCS pairs identified in the *P. acnes* genome (continue).**

Locus	Function	Homology
PPA2113/2	Unknown	PPA2112 was annotated as a response regulator containing a REC receiver domain and a trans_reg_C domain; the closest homologue is response regulator receiver domain protein of <i>P. acnes</i> HL025PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a transcriptional regulator of <i>Mycobacterium sp.</i> with 100% identities. PPA2113 was annotated as a sensor kinase containing a HisKA domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> J165 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is histidine kinase of <i>Catenulispora acidiphila</i> with 96% identities.

**Table 1.1 (B) Orphan putative TCS HK identified in the *P. acnes* genome.**

Locus	Function	Homology
PPA0068	Unknown	PPA0068 was annotated as a sensor kinase containing a HisKA_3 domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> SK187 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase of <i>Nocardioideae bacterium</i> with 84% identities.
PPA1073	Unknown	PPA1073 was annotated as a sensor kinase containing a HisKA_3 domain and a HATPase_c domain; the closest homologue is a histidine kinase of <i>P. acnes</i> SK187 with 91% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase of <i>Nakamurella multipartite</i> with 84% identities.
PPA1265	Unknown	PPA1265 was annotated as a histidine kinase containing a H_kinase_N domain, a HisKA_2 domain and a HATPase_c domain; the closest homologue is a sensor histidine kinase of <i>P. acnes</i> HL097PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a histidine kinase, dimerisation/phosphoacceptor of <i>Nocardioides sp.</i> with 99% identities.

**Table 1.1 (C) Orphan putative TCS RR identified in the *P. acnes* genome.**

<b>Locus</b>	<b>Function</b>	<b>Homology</b>
PPA0714	Unknown	PPA0714 was annotated as a response regulator containing a REC receiver domain; the closest homologue is a conserved hypothetical protein of <i>P. acnes</i> HL013PA1 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is <i>Streptomyces hygrosopicus</i> with 77% identities.
PPA0772	Unknown	PPA0772 was annotated as a response regulator containing a REC receiver domain and a ANTAR domain; the closest homologue is a response regulator receiver domain protein of <i>P. acnes</i> SK187 with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator of <i>Streptomyces ghanaensis</i> with 90% identities.
PPA2043	Unknown	PPA2043 was annotated as a response regulator containing a REC receiver domain and a trans_reg_C domain; the closest homologue is a response regulator receiver domain protein with 100% identities; apart from <i>Propionibacteria</i> , the closest homologue is a response regulator, MprA, of <i>Actinomyces sp.</i> with 98% identities.

Among these putative TCS pairs, PPA0945/47 is a novel putative pair that has a unique genetic organization compared with a classic TCS. In a classic TCS, the HK and RR are transcribed in the same direction in the genome and are normally from the same promoter. However, in the PPA0945/47 pair, the HK (PPA0945) and RR (PPA0947) are transcribed divergently in the genome. Moreover, there is another hypothetical protein (PPA0946) encoded between the PPA0945 and PPA0947. PPA0946 is also transcribed in the same direction as PPA0945 in the genome of *P. acnes*. Therefore, we propose that PPA0945 and PPA0947 are from a cognate pair of TCS and PPA0946 is associated with this system.



**Figure 1. 10 The genetic organization of proposed QS TCS identified in the *P. acnes* genome.**

This putative QS TCS contains an HK (PPA0945, orange arrow), hypothetical protein (PPA0946, green arrow) and a RR (PPA0947, purple arrow). PPA0945 and PPA0946 are transcribed in the same direction, whereas PPA0947 is transcribed divergently from them in the genome.

In the following texts, PPA0945, PPA0946 and PPA0947 is assigned as possible quorum sensing (Pqs) A, PqsB and PqsC, respectively.

## 1.4 Aims and objectives of the study

Acne is an exclusive human disease, which affects more than 80% of the whole population and influences the quality of life. Although *P. acnes* has been implicated in the pathogenesis of acne for more than 100 years and significant progress has been made to understand the relationship between acne and *P. acnes*, the exact causes of initiation of acne have not yet been elucidated, especially at the molecular level of *P. acnes*. Current drugs available for the successful treatments of acne are mostly in the risk of the development of antibiotic resistance. Therefore, finding new drug targets of acne treatment now becomes an emerging field in both research and clinical studies. As QS TCS signalling pathway has many attractive features as a potential drug target, it is highly possible that new drug targets could be identified by characterization of the signalling pathway of a QS TCS. With the newly sequenced *P. acnes* genome, it is now possible to characterize the putative QS TCS signalling pathway in *P. acnes*. To understand the signalling pathway of this putative QS TCS, the first step would be to confirm that this unique QS TCS forms a legitimate system despite being transcribed divergently. So, the main aim of this project is to investigate the relationship among these three components of the putative QS TCS.

This main aim could be further divided into specific objectives that are presented in this thesis as follows:

1. Identification of putative QS TCS by preliminary sequence analysis of PqsA, PqsB and PqsC;
2. Investigation of the relationship of PqsA and PqsC using purified proteins and phosphorylation assays to demonstrate they are a cognate pair of a TCS;
3. Investigation of the relationship of PqsB and the putative TCS (PqsA and PqsC) by identifying the transcriptional start site (s) of these three genes;
4. Investigation of the relationship of PqsB and the putative TCS (PqsA and PqsC) using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) during *P. acnes* growth.

## **Chapter II General materials and methods**

### **2.1 Equipment**

#### **2.1.1 Balances**

Chemicals and reagents were weighted using either a METTLER TOLEDO XS2002S (Mettler-Toledo) or an A&D INSTRUMENTS HR-200-EC balance (Mettler-Toledo).

#### **2.1.2 pH meter**

The pH of solutions was determined using a JENWAY 3520 pH meter (Jenway). Before usage, the instrument was calibrated between pH 6.9 and either pH 4.0 or pH 9.2 as required using standard buffer solutions supplied by Radiometer Analytical.

#### **2.1.3 Centrifuges**

Samples in universal bottles or Falcon tubes were centrifuged in a MSE HARRIER 18/80R centrifuge (MSE), while samples in microcentrifuge tubes were centrifuged in a Sigma 1-14 microcentrifuge (Sigma Centrifuges). Centrifugation speed and times are stated in specific sections.



#### **2.1.4 Incubators**

Anaerobic incubations were performed in a MACS-MG-1000 anaerobic workstation (Don Whitley Scientific), while aerobic incubations were performed in a SANYO MIR-553 incubator (Sanyo). Incubations that required shaking were performed in a SANYO ORBI-SAFE incubator (Sanyo). Incubation times, temperatures and rotating speeds are stated in specific sections.

#### **2.2 Sterilization**

Small quantity of liquids and small equipments were sterilised using a pressure cooker at 15 psi for 15 min. Large quantity of liquids and large equipments were sterilized by the autoclaving service provided within the Faculty of Biological Sciences, University of Leeds. Heat-sensitive solutions were sterilized by mechanical filtration using Millex-OR filter unit with pore size 0.22  $\mu\text{m}$  (Millipore).

#### **2.3 Media**

All the media components were purchased from Oxoid and all the chemical reagents were purchased from Sigma. Addresses of suppliers are listed in Appendix III.

### **2.3.1 Luria-Bertani (LB)**

Tryptone (1 g), yeast extract (0.5 g) and NaCl (0.5 g) were dissolved in distilled water (100 ml) and sterilised by autoclaving. The medium was stored at room temperature in a dark place.

### **2.3.2 Tryptone yeast extract glucose (TYG)**

Tryptone (1 g), yeast extract (0.5 g) and glucose (0.25 g) were mixed in distilled water (100 ml) and sterilised by autoclaving. The medium was stored at room temperature in a dark place.

### **2.3.3 Super optimal broth (SOB)**

Tryptone (20 g), yeast extract (5 g), NaCl (1 M, 5 ml) and KCl (1 M, 1.25 ml) were dissolved in 494 ml distilled water and sterilised by autoclaving. After cooled down, sterile magnesium salts solution (5 ml,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 M;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1 M) was added. The medium was stored at room temperature in a dark place.

### **2.3.4 SOB with catabolite repression (SOC)**

Sterilized glucose solution (2 M, 5 ml) was added into sterilised SOB medium (500 ml). The medium was stored at room temperature in a dark place.

### **2.3.5 Synthetic media**

#### **2.3.5.1 Vitamin solution**

Pyridoxine HCl (1.25 g) and nicotinic acid (0.25 g) were dissolved in 70 ml distilled water. The pH was adjusted to pH 5.6 before adding pantothenic acid (125 mg) and biotin (15 mg). The solution was made up to 100 ml using distilled water and dispensed into suitable aliquots. The solution was stored at – 20 °C.

#### **2.3.5.2 Salts solution**

FeSO<sub>4</sub> · 7H<sub>2</sub>O (1 g), MnSO<sub>4</sub> · 4H<sub>2</sub>O (1 g), CaCl<sub>2</sub> · 2H<sub>2</sub>O (1 g), ZnCl<sub>2</sub> (0.25 g), CoCl<sub>2</sub> · 6H<sub>2</sub>O and CuSO<sub>4</sub> · 5H<sub>2</sub>O were mixed in 100 ml HCl (1 M) and dispensed into 10 ml aliquots. The solution was stored at – 20 °C.

### **2.3.5.3 Amino acids**

L-alanine (100 mg), L-arginine (200 mg), L-asparagine (100 mg), L-aspartic acid (200 mg), L-glutamic acid (500 mg), glycine (100 mg), L-histidine (100 mg), L-hydroxyproline (100 mg), L-isoleucine (100 mg), L-cysteine (100 mg), L-leucine (100 mg), L-lysine (100 mg), L-methionine (100 mg), L-phenylalanine (100 mg), L-proline (100 mg), L-serine (200 mg), L-threonine (200 mg), L-tryptophan (200 mg), L-tyrosine (100 mg) and L-valine (100 mg) were dissolved in 400 ml distilled water.

### **2.3.5.4 Other components**

$K_2HPO_4$  (3.5 g),  $KH_2PO_4$  (3.5 g),  $MgSO_4 \cdot 7H_2O$  (0.2 g), NaCl (2 g),  $(NH_4)_2SO_4$  (1.5 g), sodium citrate (0.5 g), PIPES (10 g) and glucose (5 g) were dissolved in 500 ml distilled water.

For 1 L medium, amino acid solution (400 ml), other components (500 ml), vitamin solution (2 ml), salts solution (2 ml) and 100% Tween 80 (1 ml) were dissolved and adjusted pH to 5.6. The medium was made up to 1 L using distilled water and then filter sterilised. The medium was stored at room temperature in a dark place.

### **2.3.6 Reinforced clostridium agar (RCA)**

Reinforced clostridium agar (26.25 g) was dissolved in 500 ml distilled water and sterilised. After cooling to 45-50 °C, the media was dispensed into Petri dishes and allowed to set.

### **2.3.7 LB agar**

Tryptone (1%, w/v), yeast extract (0.5%, w/v), NaCl (0.5%, w/v) and agar bacteriological (1%, w/v) were dissolved in distilled water and sterilised. After cooling to 45-50 °C., the media was dispensed into Petri dishes and allowed to set.

### **2.3.8 SOB agar**

Agar bacteriological (5 g) was added SOB medium (500 ml) and sterilised by autoclaving. The medium was stored at 4 °C.

### **2.3.9 Heated blood agar**

Blood agar base (20 g) was added to distilled water (500 ml) and sterilised by autoclaving. After cooled to 50 °C, defibrinated horse blood (12.5 ml) was added and the medium was heated to 70 °C for 20 min. Following

cooling to 50 °C, the medium was dispensed into Petri dishes. The medium was stored at 4 °C.

## **2.4 Bacterial strains and plasmids**

*E. coli* strains and plasmids used in this study are listed in the following tables.

**Table 2. 1 *E. coli* strains used in this study**

<b><i>E. coli</i> strain</b>	<b>Genotype</b>	<b>Source/Reference</b>	<b>Details</b>	<b>Used in this study</b>
DH5 $\alpha$	<i>supE44</i> , $\Delta$ <i>lacU169</i> ( $\Phi$ <i>80lacZ</i> $\Delta$ M15), <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i>	Lab collection (Hanahan, 1983)	Transforms with high efficiency	Cloning
BL21(DE3)	$F^-$ , <i>ompT</i> , <i>hsdS<sub>B</sub></i> ( <i>r<sub>B</sub>m<sub>B</sub></i> ), <i>gal</i> , <i>dcm</i> , $\lambda$ (DE3)	Lab collection (Studier & Moffatt, 1986)	Contains a chromosomal copy of an isopropyl-beta-D-thiogalact opyranoside (IPTG) inducible T7 RNA polymerase	Protein overproduction

**Table 2. 2 Plasmids used in this study.**

<b>Plasmid name</b>	<b>Relevant characteristics</b>	<b>Antibiotic selection</b>	<b>Source/Reference</b>
pCR <sup>®</sup> 4-TOPO <sup>®</sup>	Cloning vector	Kanamycin 50 µg/ml	Invitrogen
pGEX-6P-1	Expression vector, N-terminal tag: 26 kDa glutathione S-transferase (GST)	Ampicillin 100 µg/ml	Amersham
pET30b	Expression vector, C-terminal tag: six histidine residue	Kanamycin 50 µg/ml	Novagen
pHRE001	665 bp autokinase domain of <i>pqsA</i> cloned into pCR <sup>®</sup> 4-TOPO <sup>®</sup>	Kanamycin 50 µg/ml	This study
pRRC001	Full-length <i>pqsC</i> cloned into pCR <sup>®</sup> 4-TOPO <sup>®</sup>	Kanamycin 50 µg/ml	This study
pHRE002	Autokinase domain of <i>pqsA</i> cloned into pGEX-6P-1 between the <i>Bam</i> HI and <i>Xho</i> I sites	Ampicillin 100 µg/ml	This study
pRRC002	Full-length of <i>pqsC</i> cloned into pET30b between the <i>Nde</i> I and <i>Xho</i> I sites	Kanamycin 50 µg/ml	This study



## **2.5 Standard culture conditions**

### **2.5.1 Aerobic incubation**

Cultures of *E. coli* were incubated at 37 °C without shaking in a Sanyo MIR-553 incubator or with shaking (180 rpm) in a Sanyo ORBI-SAFE TS net wise incubator. The culture media and incubation times are stated in specific sections.

### **2.5.2 Anaerobic incubation**

Cultures of *P. acnes* were incubated at 34 °C in MACS-MG-1000-anaerobic workstation. The culture media and incubation times are stated in specific sections.

## **2.6 Preparation of RNase-free equipment and solution**

The equipment was soaked in distilled water containing diethyl pyrocarbonate (DEPC, 1%, v/v) in an appropriate container and incubated overnight at 37 °C. Following the incubation, the distilled water was discarded and the equipment was autoclaved. The RNase-free solution was prepared by adding DEPC (1%, v/v) into the specific solution and incubated overnight at 37 °C. Following the incubation, the solution was autoclaved and stored at room temperature.

## **2.7 Measurement of bacterial concentration**

The concentration of bacterial cultures was estimated by measuring the optical density at a wavelength of 600 nm ( $OD_{600}$ ) using a JENWAY 6305 UV/Vis spectrophotometer.

## **2.8 Measurement of nucleic acid concentration**

The concentration of nucleic acid was measured and calculated using a Thermo Scientific NanoDrop™ 1000 spectrophotometer at the wavelength of 260 nm.

## **2.9 Primer design**

Primers used in the study were designed to amplify either a specific DNA fragment or a certain length of a target gene. For primers used for normal PCR reactions, the primer properties were calculated using Oligonucleotide Properties Calculator ([http://www.basic.northwestern.edu/biotools/oligo\\_calc.html](http://www.basic.northwestern.edu/biotools/oligo_calc.html)). Both basic and salt adjusted melting temperatures ( $T_m$ ) were calculated and the specificity of the primer was checked by BLAST provided by the software.

Primers used in real-time PCR reactions were picked up by the software Primer 3 (<http://frodo.wi.mit.edu/>) according to the target gene sequence. These primers were designed to amplify a DNA fragment within the range of 80-90 bp and the specificity of each primer was checked using a

BLAST search (nucleotide; [www.ncbi.nlm.nih.gov/blast/Blast](http://www.ncbi.nlm.nih.gov/blast/Blast)). These primers and their amplified DNA fragments were also further checked for the secondary structure using software DNA and RNA Mfold (<http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi> and <http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>, respectively). The melting temperature was calculated using the software mentioned above.

Primers for normal PCR reactions were purchased from Invitrogen and primers for real-time PCR reactions were purchased from Thermal Scientific. Routinely the primers were high pressure liquid chromatography (HPLC) purified by the vendor.

## **2.10 Sonication**

The cell pellet of a late exponential/stationary phase culture was resuspended in 5 ml of the appropriate buffer and then sonicated on ice for 3 cycles of 30 s at 13-15 amplitude microns in Soniprep 150 (Sanyo). The sonicated cell suspension was centrifuged at  $12,000 \times g$  for 20 min at 4 °C.

## **2.11 Preparation of *E. coli* competent cells**

### **2.11.1 RF1 solution**

RbCl (1.21 g), MnCl<sub>2</sub> · 4H<sub>2</sub>O (0.99 g), potassium acetate (0.29 g), CaCl<sub>2</sub> · 2H<sub>2</sub>O (0.15 g) and glycerol (15 g) were dissolved in 100 ml distilled water and

the pH was adjusted to 5.8 using 0.2 M acetic acid. The solution was filter sterilised, dispensed into 33 ml aliquots and stored at room temperature.

### **2.11.2 RF2 solution**

MOPS (0.21 g), RbCl (0.12 g), CaCl<sub>2</sub> · 2H<sub>2</sub>O (1.1 g) and glycerol (15 g) were dissolved in 100 ml distilled water and the pH adjusted to 6.8 using 0.1 M NaOH. The solution was filter sterilized, dispensed into 10 ml aliquots and stored at – 20 °C.

### **2.11.3 Procedures**

The appropriate strain of *E. coli* was streaked out for single colonies on an SOB agar plate from a frozen stock. A single colony of 2-3 mm in diameter were selected to inoculate 10 ml of SOB broth and incubated overnight at 37 °C with shaking. The overnight culture was then used to inoculate 100 ml of SOB to an OD<sub>600</sub> of 0.05 in a 250 ml flask. This culture was incubated at 37 °C with shaking until an OD<sub>600</sub> of 0.3 to 0.5 was reached. Then the culture was chilled on ice for 15 min and the cells were harvested by centrifugation at 6, 000 rpm for 10 min at 4 °C. After resuspending the cell pellet in 33 ml of ice-cold RF1, the suspension was incubated on ice for 15 min. The cells were harvested again by centrifugation as above and resuspended in 8 ml of ice-cold RF2. Following the incubation of the suspension on ice for another

15 min, 100- $\mu$ l aliquots were dispensed into sterile, pre-chilled 1.5 ml microcentrifuge tubes and stored at  $-70^{\circ}\text{C}$ .

## **2.12 Genomic DNA extraction of *P. acnes***

Chromosomal DNA of *P. acnes* was isolated using a modified protocol of DNeasy Tissue Kit (Qiagen). *P. acnes* was grown anaerobically for 48 h in 10 ml TYG broth. Cells were harvested by centrifugation for 10 min at 5,000 rpm (Sanyo, MSE Harrier 18/80) and the pellet was resuspended in 180  $\mu$ l of lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% (v/v) Triton X-100, 20 mg/ml lysozyme and 625 U/ml mutanolysin, pH 8.0). After incubation at  $37^{\circ}\text{C}$  for 2 h, 25  $\mu$ l Proteinase K (supplied with the kit) and 200  $\mu$ l Buffer AL (supplied with the kit) were added separately and incubated at  $70^{\circ}\text{C}$  for 30 min. DNA was precipitated by adding 200  $\mu$ l ethanol (96-100%, v/v), washed with 500  $\mu$ l Buffer AW1 (supplied with the kit) and Buffer AW2 (supplied with the kit) and then eluted with 100  $\mu$ l Buffer AE (supplied with the kit). Isolated chromosomal DNA was stored at  $-20^{\circ}\text{C}$ .

## **2.13 Growth of *P. acnes***

*P. acnes* was always inoculated freshly from a liquid nitrogen stock onto a RCA plate and incubated at  $34^{\circ}\text{C}$  for 4 to 7 days anaerobically. Then

colonies were transferred into a 10 ml TYG broth and incubated at 34 °C for 40-48 hours anaerobically. The broth culture was used to inoculate a 100 ml TYG broth to an OD<sub>600</sub> of 0.2. The OD<sub>600</sub> readings of the 100 ml culture were followed to create the growth curve. The growth curve was plotted using incubation time against natural log of OD<sub>600</sub> readings. For growing *P. acnes* in a synthetic medium, above procedures were followed. Then cells were taken from mid-exponential phase of a TYG culture and used to inoculate 100 ml synthetic medium to an OD<sub>600</sub> of 0.2. The OD<sub>600</sub> readings were followed and the growth curve was plotted the same as above.

## **2.14 DNA manipulation**

Basic techniques were performed as described in Molecular Cloning: A Laboratory Manual (Sambrook *et al.*, 1989).

### **2.14.1 DNA Agarose gel electrophoresis**

Tris/Borate/EDTA buffer (TBE buffer, 5 ×) were made by dissolving Tris-HCl (27 g), boric acid (13.75 g) and EDTA (10 ml, 0.5 M, pH 8.0) in 500 ml distilled water. Unless otherwise stated, agarose (1%, w/v) was dissolved in 1 × TBE buffer in gentle heat using a microwave and cooled approximately 50 °C. Then SYBR® Safe DNA gel stain (10,000 × concentrate; Invitrogen) was diluted and added to the gel. The mixture was poured into the prepared

base and allowed to set with the comb in place. Following the loading of the sample, the gel was run at a constant voltage of 5-10 V/cm for 40-60 min.

#### **2.14.2 DNA extraction from a agarose gel**

DNA from agarose gels was electrophoresed and stained as in 2.12.1. The required fragment was cut from the agarose gel using a sterile scalpel. The DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

#### **2.14.3 DNA purification from PCR product**

DNA to be purified from PCR products was extracted using the High Pure PCR Product Purification Kit (Roche Applied Science) as described by the manufacturer.

#### **2.14.4 General polymerase chain reaction (PCR)**

Unless stated, DNA fragments were amplified using specific primers by GoTaq® DNA polymerase (Promega) in GoTaq® Green Master Mix (1 ×; Promega) according to the manufacturer's instructions. Deoxynucleosidetriphosphates (dNTPs) were obtained commercially as 10 mM stock solutions (Promega). All primers and their melting temperature are listed in the Appendix I.

### **2.14.5 Plasmid DNA purification**

Plasmid DNA was isolated using PureYield™ Plasmid Miniprep System as described by the manufacturer (Promega).

### **2.14.6 DNA sequencing**

Amplified DNA sequences to be confirmed were sequenced using the Automated DNA Sequencing Facility, Faculty of Biological Sciences, University of Leeds.

## **2.15 Molecular cloning of DNA**

### **2.15.1 Cloning of DNA into non-expression vector**

Cloning of DNA for sequencing or non-expression purposes was performed using purified DNA fragments and a TOPO TA cloning® kit (Invitrogen) or a pGEM-T® Easy vector system (Promega) as described by manufacturer's instructions.

### **2.15.2 Cloning of DNA into expression vector**

Cloning of DNA for the purpose of expression was performed using purified DNA fragments and a purified expression vector such as pGEX-6P-1 or pET30b. The DNA fragment and the expression vector was ligated by T4 ligase as described by manufacturer (Promega).



### **2.15.3 Transformation of *E. coli* DH5 $\alpha$ /BL21(DE3)**

Following ligation, aliquots of the reaction (5  $\mu$ l for the transformation of *E. coli* DH5 $\alpha$  and 0.5  $\mu$ l for the transformation of *E. coli* BL21(DE3)) were mixed with 100  $\mu$ l aliquots of competent cells. After incubation on ice for 20 min, the mixture was heat-shocked at 42 °C for 40 s and then placed back in ice for 1 min. SOC (900  $\mu$ l) was added to the mixture and incubated at 37 °C for 1.5 h with shaking. Following this incubation, two different volumes of the culture (50  $\mu$ l and 200  $\mu$ l for *E. coli* DH5 $\alpha$  and 20  $\mu$ l and 100  $\mu$ l for *E. coli* BL21 (DE3)) were spread onto LB agar plates containing appropriate antibiotics (Table 2.2). Additionally, for the transformation of *E. coli* DH5 $\alpha$ , the remaining cells were collected by centrifugation, resuspended in 100  $\mu$ l SOC and then spread onto a selective plate. The plates were incubated overnight at 37 °C.

### **2.15.4 Restriction digest**

Restriction digests were typically set in a total volume of 10  $\mu$ l with 20 units of restriction enzyme (New England Biolabs). The appropriate 10  $\times$  buffer and 20 units of restriction enzyme (supplied with restriction enzyme, New England Biolab) were both added in volumes of 10 $\mu$ l containing DNA (50 to 100 ng). The reaction mixtures were typically incubated at 37 °C for 2 hours followed by a termination step of 65 °C for 20 min, unless otherwise stated. If necessary, the DNA was cleaned as described in section 2.14.2 and 1.14.3.

## **2.16 RNA manipulation**

Basic techniques were performed as described in Molecular Cloning: A Laboratory Manual (Sambrook et al., 1989).

### **2.16.1 Purification of total RNA sample from bacteria**

The total RNA was isolated using RiboPure™ - Bacteria Kit (Ambion) with a modified protocol of the manufacturer's. In brief, cellular metabolism was stopped by adding a mixture of 5% (v/v) phenol in ethanol: 1 ml was added for every 9 ml of bacterial culture. Cells were collected by centrifugation at 6,000 rpm for 10 min at 4 °C as in section 2.1.3. Cell pellets were resuspended using 350 µl of RNeasy and the suspension was transferred into a 0.5 ml screw cap tube containing ~ 250 µl of ice-cold Zirconia Beads. The mixture was homogenized in a Beads Beater with 3 cycles of 1 min and cooled on ice for 1 min between each cycle. After this treatment, the mixture was centrifuge at 13,000 × *g* for 5 min at 4 °C. The bacterial lysate was transferred to a fresh 1.5 ml tube and 0.2 volumes 100% (v/v) chloroform was added. After shaking vigorously for 30 s and incubation for 10 min at room temperature, the aqueous and organic phases were separated by centrifugation at 13,000 × *g* for 5 min at 4 °C. The aqueous phase was transferred to a fresh 1.5 ml tube and a 0.5 volumes 100% (v/v) ethanol was added. The mixture was transferred to the Filter Cartridge and centrifuged at 13,000 × *g* for 1 min. The filter was washed once with 700 µl Wash Solution 1 and then twice with 500 µl aliquots of Wash Solution 2/3.

The excess wash was removed by centrifugation as above and finally the total RNA was eluted by adding 25 to 50  $\mu$ l of preheated Elution Solution (95-100 °C). The RNA yield was maximized by repeating the elution step.

## **2.16.2 RNA FA gel electrophoresis**

### **2.16.2.1 Formaldehyde agarose (FA) gel buffer (10 ×)**

EDTA (1.46 g), 3-[N-morpholino]propanesulfonic acid (MOPS; 20.93 g) and sodium acetate (2.05 g) was dissolved in 400 ml of distilled water and pH was adjusted to 7.0 with NaOH. The solution was made up to 500 ml with distilled water.

### **2.16.2.2 FA gel running buffer (1 ×)**

FA gel buffer (10 ×, 25 ml), formaldehyde (37%, 5 ml) and RNase-free water (220 ml) were mixed and used directly in the gel electrophoresis. The buffer was made just before use.

### **2.16.2.3 FA gel preparation (1%, w/v)**

Agarose (0.3 g) and FA gel buffer (10 ×, 3 ml) were dissolved in 27 ml distilled water in a microwave and cooled to 65 °C. Formaldehyde (37%, 540  $\mu$ l) and SYBR® Safe DNA Stain (10,000 ×, 3  $\mu$ l) were added into the solution. The mixture was poured into the prepared base and allowed to set with the

comb in place. Prior to running, the gel was equilibrated in 1 × FA gel running buffer for at least 30 min at room temperature.

#### **2.16.2.4 RNA sample preparation and gel electrophoresis**

The RNA running sample was prepared by mixing 1 volume of 5 × RNA loading buffer with 4 volumes of RNA sample. The mixture was incubated at 65 °C for 5 min and then transferred on ice for at least 2 min.

The gel was run at a constant voltage of 5-10 V/cm for 40-60 min in 1 × FA gel running buffer.

#### **2.16.3 DNase treatment**

All the RNA samples were treated by TURBO™ DNase (Ambion) to degrade contaminating DNA using a slightly modified protocol of the manufacturer and then extracted from the reaction. In brief, the RNA sample was diluted to  $\leq 200$  µg/ml using RNase-free water and 50 µl aliquots were dispensed to RNase-free tubes. TURBO™ DNase Buffer (5.6 µl, 10 ×) and TURBO DNase (1 µl) were added to the RNA sample and mixed gently. After incubation at 37 °C for 30 min, another 1 µl TURBO DNase was added into the reaction and incubated for another 30 min. The reaction was stopped by adding RNase-free EDTA (0.6 µl, 0.5 M). The RNA was extracted using 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1). After centrifugation at 13,000 × *g* for 2 min, the aqueous layer was collected into a new

RNase-free tube. For the maximum recovery, RNase-free water (1 volume) was added into the previous tube and centrifuged as above. The RNA was further purified by extracting with 1 volume of chloroform: isoamyl alcohol (49:1). The aqueous phase was transferred into a new RNase-free tube and the RNA was precipitated by 0.1 volume of RNase-free sodium acetate (3 M, pH5.2) and 2 volumes of 100% (v/v) ice-cold ethanol. After incubation on dry ice for 30 min, the RNA was collected by centrifugation as above for 20 min at 4 °C. The pellet was washed using 500 µl of 70% (v/v) ethanol and collected again by centrifugation as above for 30 min at 4 °C. Following air drying at room temperature for 10 min, the pellet was resuspended with RNase-free water.

#### **2.16.4 Reverse transcriptase polymerase chain reaction (RT-PCR)**

RT-PCR was performed using QIAGEN OneStep RT-PCR Kit according to the manufacturer's instructions. Primers and annealing temperature used are listed in the Appendix I.

#### **2.16.5 cDNA synthesis**

cDNA was synthesised using SuperScript™ Reverse Transcriptase (Invitrogen) in the presence of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen) according to the manufacturer's instructions. Primers used are listed in the Appendix I.

## **2.17 Protein manipulation**

### **2.17.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

#### **2.17.1.1 Sample buffer (2 ×, 5 ×)**

For 2 × sample buffer, Tris (0.24 g), SDS (0.8 g), glycerol (4 ml), DTT (0.62 g) and bromophenol blue (40 mg) were dissolved in 20 ml distilled water and adjusted pH to 6.8. Equal volume of the sample buffer was added into the protein sample before loading onto the gel.

For 5 × sample buffer, Tris (0.6), SDS (2 g), glycerol (10 ml), DTT (1.55) and bromophenol blue (100 mg) were dissolved in 20 ml distilled water and adjusted pH to 6.8. The sample buffer was diluted to 1 × before loading onto the gel.

#### **2.17.1.2 Electrophoresis running buffer (10 ×)**

Two different types of electrophoresis running buffers were used in this study for the purpose of separating large proteins or peptides and small proteins.

To prepare 10 × Tris-Glycine running buffer, Tris (15.15 g), glycine (72.05 g) and SDS (5 g) were dissolved in 500 ml distilled water and the solution was diluted to 1 × before use. It was used to separate large proteins.

To prepare 10 × Tris-Tricine running buffer, Tris (60.55 g), tricine (89.6 g) and SDS (5g) were dissolved in 500 ml distilled water and the solution was diluted to 1 × before use. It was used to separate peptides and small proteins.

### 2.17.1.3 Staining buffer

Coomassie brilliant blue (R) (0.15 g) was dissolved in a mixture of glacial acetic acid (10 ml), methanol (45 ml) and distilled water (45 ml). The solution was filtered using filter paper and stored at room temperature.

### 2.17.1.4 Destaining buffer

Glacial acetic acid (100%, v/v, 50 ml), methanol (25 ml) and distilled water (425 ml) were mixed together.

### 2.17.1.5 Resolving gel

The gel solution was prepared as follows:

**Table 2. 3 Components of resolving gel.**

Components	
Acrylamide/bis-acrylamide (40%, w/v)	1.25 ml
Tris (1 M, pH 8.8)	1.875 ml
Sodium dodecyl sulfate (SDS, 10%, w/v)	50 $\mu$ l
Distilled water	1.8 ml
Ammonium persulphate(10%, w/v)	20 $\mu$ l
TEMED	5 $\mu$ l

The ammonium persulphate solution was prepared freshly for each SDS-PAGE. The ammonium persulphate solution and TEMED were added just before the gel was poured.

### 2.17.1.6 Stacking gel (4%, w/v)

**Table 2. 4 Components of stacking gel.**

Components	
40% (w/v) Acrylamide/bis-acrylamide	0.4 ml
1 M Tris (pH 6.8)	2 ml
10% (w/v) SDS	40 $\mu$ l
Distilled water	1.535 ml
10% (w/v) Ammonium persulphate	20 $\mu$ l
TEMED	5 $\mu$ l

The ammonium persulphate solution was prepared freshly for each SDS-PAGE. The ammonium persulphate solution and TEMED were added just before the gel was poured.

### 2.17.1.7 SDS-PAGE gel preparation and electrophoresis

The SDS-PAGE gel was prepared and run using the Mini protein III electrophoresis system (Bio-Rad). It was cast in two sections: a lower resolving gel and an upper stacking gel. After the resolving gel solution was prepared as in Section 2.17.1.5, it was pipetted between the electrophoresis plates and overlaid with 200  $\mu$ l water-saturated butanol and allowed to polymerise for at least 30 min at room temperature. Then the water-saturated butanol was removed and the prepared stacking gel (Section 2.17.1.6) was poured. The comb was carefully inserted between electrophoresis plates and



gels were allowed to polymerise for another 30 min. After the gel was polymerised, the comb was removed and the gel was washed thoroughly with distilled water. Then it was assembled into the electrophoresis cell. Electrophoresis was carried out in 1 × running buffer (2.17.1.2) at 200 V until the dye reached the bottom of the gel.

Proteins were visualised after staining overnight by submersion in staining buffer (2.17.1.3) and destaining by submersion in destaining buffer (2.17.1.4).

#### **2.17.1.8 Protein sample preparation and loading**

*E. coli* cell pellets were resuspended in 100 µl sample buffer (2.17.1.1) and heated in a boiling water bath for 3 to 5 min. Samples were cooled to room temperature before being loaded onto the gel. Purified proteins or cell lysate was mixed with 2 × sample buffer at the ratio of 1:1 (v/v) and then loaded onto the gel.

#### **2.17.2 Measurement of protein concentration**

The concentration of protein was measured using BCA<sup>TM</sup> protein assay kit (PIERCE). The method is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. It combines the well-known reduction of Cu<sup>+2</sup> to Cu<sup>+1</sup> by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the Cu<sup>+1</sup>. Standards and working reagent were prepared as described by manufacturer.

All the reactions were performed using microplates according to the vendor's instructions. Results were obtained by measuring absorbance at 562 nm.

### **2.17.3 Dialysis**

The tubing (Sigma-Aldrich) was prepared as described in Molecular cloning: a laboratory manual (Sambrook et al., 1989). Dialysis was performed with 10 to 15 ml of the protein sample in 2 L of the desired buffer and incubated overnight at 4 °C with stirring.

### **2.17.4 N-terminal sequencing**

N-terminal sequencing was performed by the proteomics facility at the University of Leeds.

### **2.17.5 Circular dichroism (CD) analysis**

Purified GST fused PqsA and full-length PqsC were diluted to a concentration of 0.2 mg/ml using distilled water. For each sample, 350 µl was loaded into the cuvette and scanned at wavelengths from 260 nm to 190 nm using a speed of 50 nm/min. CD signals were measured in a 1 mm path-length cell at 37 °C. Measurements were repeated 5 times for each protein sample. CD spectrums were automatically generated by the CD analysis software.

### **2.17.6 Overexpression of proteins**

An overnight culture was used to inoculate a 500 ml LB broth containing the appropriate antibiotic in a 2 L flask to an OD<sub>600</sub> of 0.05. The culture was incubated at 37 °C with shaking as above to an OD<sub>600</sub> between 0.6 to 1.0. IPTG was added to a final concentration of 1 mM to induce production of recombinant protein and culturing continued for another 3 h. Cells were aliquoted into 50 ml in Falcon tubes and harvested by centrifugation at 6000 rpm for 10 min at 4 °C as in 2.1.3. Cell pellets were stored at - 20 °C if necessary.

### **2.17.7 Purification of proteins**

#### **2.17.7.1 Purification of GST-fused autokinase domain of PqsA**

Cell pellets harvested from 500 ml of induced culture were resuspended in 25 ml of ice-cold binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and sonicated as described in 2.10. After centrifugation, the cell lysate was kept on ice until use. Autokinase domain of PqsA was produced as glutathione S-transferase (GST) fusion proteins and purified according to the manufacturer's instructions (GE Healthcare Life Sciences). Briefly, for every 1 L of induced culture, a 50% slurry of glutathione sepharose 4B (1.33 ml) was prepared using ice-cold binding buffer and original 75% slurry. The slurry was loaded onto a 2 ml gravity column (TALON, BD Biosciences). After the slurry settled, the cleared cell lysate was loaded onto the column and allowed to flow through. The column

was washed with  $3 \times 5$  ml of ice-cold binding buffer. The bound protein was eluted with  $5 \times 1$  ml of elution buffer (50 mM Tris, 10 mM reduced glutathione, pH 8.0). All the drained samples were collected and stored at  $-20$  °C for further analysis by SDS-PAGE.

#### **2.17.7.2 Purification of full length PqsC**

Cell pellets harvested from 1 L of induced culture were resuspended in 100 ml of  $1 \times$  IB wash buffer (20 mM Tris pH7.5, 10 mM EDTA, 1% (v/v) Triton X-100). Aliquots (20 ml) were dispensed and sonicated on ice as described in 2.10. The PqsC was first purified as inclusion bodies, solubilised and then refolded as described in Manufacturer's instructions (Protein refolding kit, Novagen). Generally, the inclusion bodies were collected by centrifugation at  $10,000 \times g$  for 10 min at  $4$  °C and then washed using 0.1 culture volume of  $1 \times$  IB wash buffer. After the inclusion bodies were collected by centrifugation as above, they were resuspended in 0.1 volume of  $1 \times$  IB wash buffer, transferred to a clean centrifuge tube with known tare weight and collected by centrifugation as described above. The wet weight of the inclusion bodies was obtained by weighing the tube and subtracting the tare weight. The inclusion bodies were then resuspended in  $1 \times$  IB solubilisation buffer (50 mM CAPS, pH 11.0) containing 0.3% (w/v) N-lauroylsarcosine and 1 mM DTT. The suspension was incubated at room temperature with rotation until most of the inclusion bodies were solubilised. The suspension was clarified by centrifugation at  $10,000 \times g$  for 10 min at room temperature and the supernatant was transferred into a pre-prepared dialysis tube (Section

2.17.3). The protein was refolded by dialysis at 4 °C in 2 × 50 volumes of the sample of dialysis buffer (20 mM Tris pH 8.5) containing 0.1 mM DTT and another 2 × 50 volumes of the sample of dialysis buffer without DTT. After it was refolded, the protein was dialysed into storage buffer (20 mM Tris pH 8.5, 40% (v/v) glycerol) and stored at - 20 °C for analysis by SDS-PAGE.

## **2.18 *In vitro* Phosphorylation assays**

### **2.18.1 *In vitro* Autophosphorylation assay**

Following the purification of GST-fused autokinase domain of PqsA, the recombinant protein was dialysed into kinase test buffer (Tris-HCl, 50 mM; KCl, 20 mM; DTT, 1 mM; Glycerol, 40%, v/v; pH 8.0). GST-fused PqsA (2.5 µM at final concentration) was added into a 20 µl reaction containing 50 mM EPPS (pH 8.0), 20 mM MgCl<sub>2</sub>, 100 µM EDTA and 5% (v/v) glycerol. The autophosphorylation reaction was initiated by adding ATP containing 50 µCi of [ $\gamma$ -<sup>32</sup>P] ATP to a final concentration of 100 µM. Each reaction was incubated at room temperature for 0, 1, 2, 3, 5, 10, 15 and 20 min. Reactions were stopped immediately by mixing with 5 µl of 5 × SDS-PAGE sample buffer and transferred onto dry ice. The samples were analysed by SDS-PAGE. The gels were run until the dye front had travelled through 75% of the length of resolving gel. The lower portion of the gel containing unincorporated [ $\gamma$ -<sup>32</sup>P] ATP was removed and the gel was washed in distilled water. The gel was covered with cling film and exposed to a bioimaging plate

(BAS IIIs, Fujifilm) for 2 h at room temperature. The gel was visualised using a Fuji BAS1000 phosphoimager.

For the *in vitro* autophosphorylation assay of HK-CD1579, purified HK-CD1579 (1  $\mu$ M at final concentration) was added into a 20  $\mu$ l reaction containing the same solutions as above. The reaction was initiated the same as above and incubated at room temperature for 0, 2, 4, 6, 8, 10, 15 and 20 min. Following procedures were the same as above.

### **2.18.2 *In vitro* Phosphotransfer assay**

A GST-fused version of the autokinase domain of PqsA was incubated with purified PqsC at a ratio of 1:25 at the final concentration. The reactions were performed the same as described in section 2.18.1, with the exception that the incubation times were 20, 40 and 60 min.

HK-CD1579 was incubated with Spo0A at a ratio of 1:10 at the final concentration. The reactions were incubated for 0, 2, 4, 6, 8, 10, 15 and 20 min at room temperature. For the specificity test, the GST-fused autokinase domain of PqsA was incubated with Spo0A at a ratio of 1:20. Two sets of experiment were prepared for this test. Reactions from the experiment with HK-CD1579 were incubated at room temperature for 0, 2, 4, 6, 8, 10, 15 and 20 min, while reactions from the experiment with GST-fused autokinase domain of PqsA were incubated at room temperature for 20 and 60 min.

## **2.19 Rapid amplification of cDNA ends (RACE)**

RACE was performed using a slightly modified protocol of 5'/3' RACE kit (Roche Applied Science) that included a pre-treatment with terminator<sup>TM</sup> 5'-phosphate-dependent exonuclease (Epicentre<sup>®</sup> Biotechnologies) before the RACE experiment. Primers used in this experiment and their annealing temperatures are listed in Appendix I.

### **2.19.1 Terminator treatment**

RNA sample (10 µg) was mixed with 10 × terminator exonuclease 10 × reaction buffer (2 µl), terminator exonuclease (1 µl) and an appropriate amount of RNase-free water in a 20 µl reaction. After incubating at 30 °C for 1 h, the reaction was terminated by phenol extraction and ethanol precipitation. Generally, RNase-free water (180 µl) was added to the reaction to give the total volume of 200 µl. RNA was extracted with 200 µl acid phenol (pH 5.5) and centrifuged for 3 min at 13,000 × rpm. Aqueous phase was transferred into a new RNase-free tube and 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol was added to the reaction. Following thorough mixing, the reaction was incubated on ice for 30 min and then the RNA was pelleted by centrifugation at 18, 000 × *g* for 30 min at 4 °C. The RNA pellet was washed using 500 µl of 70% (v/v) ethanol and pelleted again by centrifugation as above for 5 min. The supernatant was carefully removed and the RNA pellet was resuspended in an appropriate amount of RNase-free water after it was air-dried for 10 min at room temperature.

### **2.19.2 First-strand cDNA synthesis**

After the RNA sample was treated by the terminator, it was used to synthesise the first-strand cDNA using the vendor's protocol. The RNA sample (12  $\mu$ l) was mixed with cDNA synthesis buffer (4  $\mu$ l), deoxynucleotide mixture (2  $\mu$ l), cDNA synthesis primer SP1 (1  $\mu$ l, 12.5  $\mu$ M) and Transcriptor reverse transcriptase (25 U) and incubated at 55 °C for 1 h. The reaction was terminated by incubation at 85 °C for 5 min. The resulting cDNA was purified using a High Pure PCR production purification kit (Roche Applied Science) as per the vendor's instructions in the 5'/3' RACE kit.

### **2.19.3 Poly(A) tailing of first-strand cDNA**

Purified cDNA (19  $\mu$ l) was mixed with 10  $\times$  reaction buffer (2.5  $\mu$ l) and 2 mM dATP (2.5  $\mu$ l) and incubated for 3 min at 94 °C. After chilling on ice, terminal transferase (80 U) was added to the mixture and incubated for 30 min at 37 °C. The reaction was terminated by incubation at 70 °C for 10 min.

### **2.19.4 PCR amplification of dA-tailed cDNA**

Following tailing reaction, dA-tailed cDNA (5  $\mu$ l) was mixed with oligo-dT-anchor primer (1  $\mu$ l, 12.5  $\mu$ M), specific primer SP2 (1  $\mu$ l, 12.5  $\mu$ M), deoxynucleotide mixture (1  $\mu$ l), GoTaq<sup>®</sup> DNA polymerase (1.5 U, Promega), 5  $\times$  reaction buffer (10  $\mu$ l, supplied with GoTaq<sup>®</sup> DNA polymerase) and 31.7



µl distilled water in a 50 µl reaction. PCR was performed using 30 cycles of 95 °C for 30 s, specific annealing temperature of each SP2 primer for 30 s and 72 °C for 1 min. After the first PCR reaction was finished, it was used in a second PCR amplification as the template. In the second reaction, a sample of the first PCR reaction (1 µl) was mixed with PCR anchor primer (1 µl, 12.5 µM), specific primer SP3 (1 µl, 12.5 µM), deoxynucleotide mixture (1 µl), GoTaq<sup>®</sup> DNA polymerase (1.5 U, Promega), 5 × reaction buffer (10 µl, supplied with GoTaq<sup>®</sup> DNA polymerase) and distilled water (35.7 µl) in a 50 µl reaction. The reaction was done as described above, but using the annealing temperature appropriate for each SP3 primer. The sequences of primers and their specific annealing temperatures are listed in the Appendix I.

### **2.19.5 Cloning of amplified DNA fragment into non-expression vector and sequencing**

The products of the second PCR reaction were run on a 1% (w/v) agarose gel and the amplified DNA fragment was extracted as described in 2.14.2. The purified PCR product (7 µl) was cloned into pGEM<sup>®</sup>-T Easy vector according to the manufacturer's instructions and introduced by transformation into *E. coli* DH5α cells as described in 2.15.3. Plasmid DNA was isolated from transformants using a Wizard<sup>®</sup> Plus SV Minipreps DNA Purification System (Promega). After confirming the presence of an insert by cutting an aliquot of the plasmid with *EcoRI*, plasmid DNA was sent for DNA sequencing as described in 2.14.6.

## **2.20 Quantitative RT-PCR**

The mRNA of PqsA, PqsB, PqsC and a reference gene encoding DNA gyrase subunit A (r-PPA0010) was quantified using a standard curve method (Nolan *et al.*, 2006). Generally, it involves four steps : (1) optimisation of primer concentrations; (2) production of standard curve ; (3) quantitative RT-PCR and (4) data analysis. After the mRNA was quantified, the data of PqsA, PqsB and PqsC was compared to the data of r-PPA0010, which was used to give ratio of gene expression for each gene at each timepoint.

### **2.20.1 Optimisation of primer concentrations**

#### **2.20.1.1 Amplification of DNA fragment used for optimisation**

Two DNA fragments, F1 and F2, were amplified using isolated genomic DNA of *P. acnes* DSM 16379 as template and specific forward and reverse primers. Amplification was achieved using *Taq* DNA polymerase with 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min according to the manufacturer's instructions (Promega). To amplify fragment F1d, DNA sequence GCTCAGGTCTCACGAACTGGC and GCTCAGCTACTCAGCC GGG were used as forward and reverse primer, respectively. To amplify fragment F2, DNA sequence CGGTGACGTCATGGGTAAGT and GCTCA CTCACCGTTGTCCTCC were used as forward and reverse primer, respectively. F1 was used for the optimisation of the primer pairs of PqsA, PqsB and PqsC, while F2 was used for the optimisation of the primer pair of r-PPA0010.

After F1 and F2 were amplified, they were diluted to  $1 \times 10^8$  copies/ $\mu\text{l}$  in distilled water containing 100 ng/ $\mu\text{l}$  yeast rRNA (Invitrogen). Then fragments were further diluted to  $1 \times 10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  copies/ $\mu\text{l}$  in 100 ng/ $\mu\text{l}$  yeast rRNA. All samples were stored at  $-20^\circ\text{C}$ .

### 2.20.1.2 Optimization of primer concentration for each gene

The primer sequences used for quantitative PCR are listed in the Appendix I. For each primer pair of PqsA, PqsB, PqsC and r-PPA0010, different concentrations of each forward primer were mixed with different concentrations of each reverse primer. These primer matrices were prepared at their final concentrations as follows :

**Table 2. 5 Primer matrices used for optimization**

Primer stock		Forward primer (nM)			
		100	200	300	400
Reverse primer (nM)	100	100/100	200/100	300/100	400/100
	200	1000/200	200/200	300/200	400/200
	300	100/300	200/300	300/300	400/300
	400	100/400	200/400	300/400	400/400

The primer matrix of PqsA, PqsB and PqsC was mixed with  $10^4$  copies of F1 and iQ SYBR Green Supermix as described by the vendor (Bio-Rad), while the primer matrix of r-PPA0010 was mixed with  $10^4$  copies of F2 and iQ SYBR Green Supermix. All PCR reactions were prepared in duplicate and performed as described by the manufacturer. Data was analysed using MxPro QPCR Software provided by the Mx3000P QPCR System (Stratagene). The primer combination that fulfilled the following criteria was selected and used in the qPCR reaction of gene expression analysis : (1) absence of, or minimal primer dimers in the dissociation curve ; (2) lowest  $C_t$  value of the primer combination which resulted in the most sensitive and efficient reaction ; (3) highest end point fluorescence, which is likely to signify the highest number of amplicon products being formed and (4) absence of signal in negative control (Nolan et al., 2006).

### **2.20.2 Production of standard curve**

The standard curve of PqsA, PqsB, PqsC and r-PPA0010 was prepared using series dilutions of F1 and F2 from section 2.20.1.1 and optimised primer concentration from section 2.20.1.2 by PCR reactions. Briefly, the optimised primer combinations of PqsA was mixed with  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$  and  $10^3$  copies of F1 individually. Then the mixture was added with iQ SYBR Green Supermix as indicated in the vendor's instructions. Same reactions were prepared for PqsB and PqsC. For r-PPA0010, the optimized primer combination was mixed with  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  copies of F2 and then added with iQ SYBR Green Supermix. Each reaction was prepared in

triplicate. PCR reactions were performed the same as in section 2.20.1.1. Standard curves were produced using the software provided.

### **2.20.3 Two-tube RT-qPCR**

The quantitative RT-PCR assay was carried out as a two-tube reaction for PqsA, PqsB, PqsC and r-PPA0010. In the RT reaction, cDNA was synthesized by SuperScript™ III Reverse Transcriptase (Invitrogen). Total RNA (200 ng) was mixed with 2 pmole of PPA0945RealTR, PPA0946RealTR, PPA0947RealTR and PPA0010RealTR individually in a total volume of 13 µl. After heating at 65 °C for 5 min, the mixture was incubated on ice for 2 min. Then 5 × First-Strand buffer (4 µl, provided), DTT (0.1 M, 1 µl, provided), RNaseOUT™ Recombinant Rnase Inhibitor (Invitrogen) and SuperScript™ III RT (1 µl, provided) were added into the reaction. Following incubation at 55 °C for 60 min, the reaction was inactivated by another incubation at 70 °C for 15 min. The negative controls that omitted SuperScript™ III RT were also prepared as described above for each reaction.

In quantitative RT-PCR reactions, optimized primer combination for PqsA, PqsB, PqsC and r-PPA0010 was mixed with iQ SYBR Green Supermix individually. Then cDNA template (5 µl) obtained from above (Section 2.20.3) was added into each mixture. The product (5 µl) of negative control reaction from above (Section 2.20.3) was also mixed with the mixture for the downstream negative control experiment. Triplicates were prepared for each reaction. PCR reactions were performed the same as in section

2.20.1.1. Data was analysed using MxPro QPCR Software provided by the Mx3000P QPCR System.

#### **2.20.4 Data analysis**

Data analysis was performed using MxPro QPCR Software provided by the Mx3000P QPCR system. Data was analysed using the following criteria : (1) abnormal plots were identified and omitted ; (2) the background fluorescence level was checked and also the background fluorescence level of the negative control was checked to ensure there was not a significant upwards drift ; (3) the threshold was set at the same level for all the data ; (4) the slope of the standard curve was between -3.2 and -3.5 ; (5) melting curve was checked to ensure that there was a single peak suggesting the product was specific ; (6) all triplicates were examined to ensure the  $C_t$  value was within 0.5  $C_t$  of each other ; if not, abnormal data were omitted for analysis ; (7)  $C_t$  value of the sample and the negative control was examined to ensure a minimum difference of 5  $C_t$  between them.

The same baseline was set for each data analysis to ensure the correct  $C_t$  could be calculated for the comparison. According to the generated standard curve of each gene, initial copy number of PqsA, PqsB, PqsC and r-PPA0010 in the RNA sample was calculated using the obtained  $C_t$ . Then for each RNA sample that was harvested at different incubation time, gene expression ratio was calculated by comparing the copy number of PqsA, PqsB and PqsC with the corresponding r-PPA0010. Relative quantification was expressed by the changing ratio of each target gene compared with the r-PPA0010.

## Chapter III Overproduction of PqsA and Pqs C and *in vitro* phosphorylation assays between them

### 3.1 Chapter introduction

As annotated in the genome of *P. acnes* KPA171202, *pqsA* and *pqs C* are a putative histidine kinase and a response regulator, respectively. These two genes are in juxtaposition in the genome, but transcribed divergently. However, the genes of most of the TCSs identified so far are parts of the same transcriptional unit (Stephenson & Hoch, 2002). The aim of this chapter was to confirm biochemically that *pqsA* and *pqsC* are functionally related, not co-localised by chance.

As introduced previously (Section 1.3.3.1), in the prototypical two-component signalling pathway, a histidine kinase is activated by a stimulus leading to autophosphorylation on a conserved histidine residue. This is followed by transfer of the phosphoryl group to a conserved aspartate residue within the receiver domain of the cognate response regulator. Phosphorylation of the response regulator leads to activation of an output domain, which is frequently a DNA-binding domain that controls transcription initiation. Most histidine kinases are bifunctional and can catalyse both the phosphorylation and dephosphorylation of their cognate response regulator.

Both histidine kinases and response regulators comprise paralogous gene families and the members of each family share significant homology at both the primary sequence level and the structural level. So far, nearly 350 members of the histidine kinase superfamily have been identified and they consist of distinct subgroups (Grebe & Stock, 1999). The signal input

domains are variable in sequence reflecting the many different environmental signals to which histidine kinases are responsive, whereas the autokinase domains are more highly conserved and can be identified by a set of conserved primary sequence motifs (West & Stock, 2001). These sequences are generally most conserved in the largest of the histidine kinase subfamilies, but in other subfamilies these so called boxes tend to be less conserved, and in some cases may be so distorted that they can only be identified through detailed sequence alignments (Grebe & Stock, 1999). Many identified HKs of Gram-positive bacteria fall into HK subfamily 7 and 10. The subfamily 7 contains DegS from *B. brevis*, ComP and several other HKs from *B. subtilis* (Grebe & Stock, 1999; Louw *et al.*, 1994; Msadek *et al.*, 1995). The subfamily 10 includes the kinases that regulate competence for genetic transformation in *Streptococcus* spp (Cheng *et al.*, 1997; Havarstein *et al.*, 1996; Pestova *et al.*, 1996).

Response regulators have been grouped together as a response regulator superfamily according to their homology in their DNA output domains. Most response regulators are multidomain proteins. They are very diverse in their domain organization and full-length sequences. The response regulator superfamily includes CheY, NtrC, FixJ and OmpR, which are named after their founding member. The CheY family contains the short single-domain response regulators, whereas all others are multidomain proteins (Volz, 1995). Most of them are positive transcriptional regulators containing DNA recognition sequences in their DNA output domain. The NtrC family contains RRs with full-length homologies to NtrC possessing two domains after the RR. The last domain of NtrC is homologous to the FIS protein (Johnson *et al.*, 1988; Koch *et al.*, 1988). The FixJ family includes



sequences with one extra FixJ-type domain on their carboxy termini (Volz, 1995). The OmpR family are all two domain proteins including PhoP, PhoB, VirG and other full-length OmpR homologs (Volz, 1995).

The similarity of histidine kinases and response regulators raises the possibility of cross-talk between different two-component pathways, but it is unclear to what extent it occurs *in vivo* (Laub & Goulian, 2007). In most cases, the signal transduction between a histidine kinase and its cognate response regulator is highly specific and cross-talk has been seen only after introducing various genetic perturbations and is thus unlikely to be present in or physiologically relevant to the wild-type organism (Laub & Goulian, 2007). The mechanism of preventing cross-talk has been suggested as the bifunctional activity of a histidine kinase, where a bifunctional histidine kinase acts as a phosphatase for its cognate regulator. This phenomenon has been found in a number of systems. For example, the PhoR-PhoB TCS senses and responds to changes in phosphate availability in *E.coli*. The VanS-VanR TCS regulates the expression of genes conferring vancomycin resistance in enterococci and other Gram-positive bacteria. Cross-talk from the kinase VanS to the response regulator PhoB can occur only in the absence of PhoR, while the kinase PhoR can cross-talk to the response regulator VanR only in the absence of VanS (Fisher *et al.*, 1995; Haldimann *et al.*, 1997; Silva *et al.*, 1998). In each case, the cross-talk is likely a consequence of eliminating the phosphatase activity normally provided by the other histidine kinase, which is bifunctional. In other words, any inadvertent cross-phosphorylation of PhoB by VanS is eliminated by PhoR-dependent dephosphorylation of PhoB, and vice versa for cross-phosphorylation of VanR by PhoR. The role of phosphatases in preventing cross-talk was also explored in both systematic

search and mathematical analysis (Alves & Savageau, 2003; Verhamme *et al.*, 2002). It has been suggested that monofunctional histidine kinases are more likely to appear in two-component systems where the response regulator integrates inputs from multiple kinases (Alves & Savageau, 2003). In addition, even for systems with monofunctional histidine kinases, there may be additional phosphatases that protect against unwanted cross-talk, such as CheZ of *E. coli* for chemotaxis (Stock *et al.*, 2000).

Response regulators also help to limit cross-talk between some signalling pathways by competition of binding sites. Studies of the CpxA-CpxR and EnvZ-OmpR systems in *E. coli* have shown that cross-talk from the kinase CpxA to the regulator OmpR requires the absence of both EnvZ and CpxR, whereas, conversely, cross-talk from EnvZ to CpxR requires the absence of CpxA and OmpR (Siryapron & Goulian, 2008).

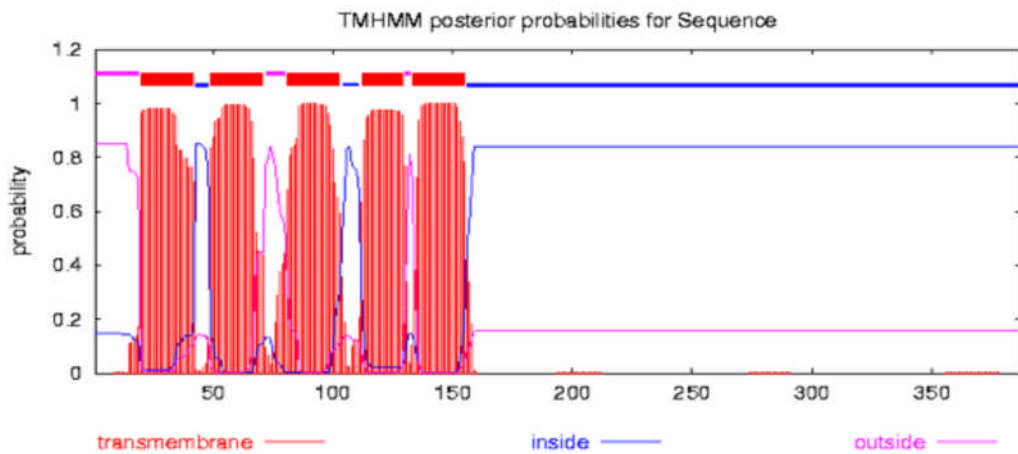
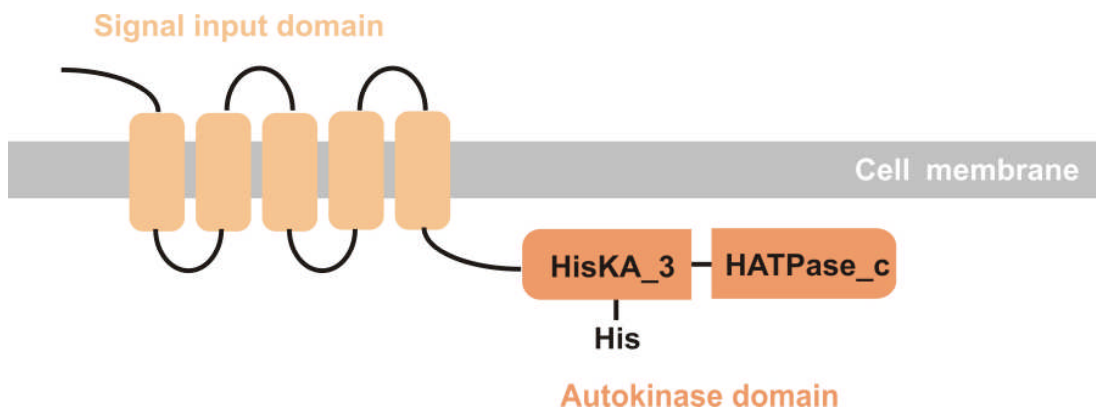
The low abundance of histidine kinases relative to response regulators also minimizes cross-talk to noncognate substrates (Siryapron & Goulian, 2008). In *E. coli*, EnvZ and OmpR are present at roughly 100 and 3500 molecules per cell, respectively (Cai & Inouye, 2002). Consistently, cross-talk has been reported by many groups when a histidine kinase is overproduced. For instance, overproducing the histidine kinase NtrB resulted in cross-phosphorylation of the response regulator CheY (Ninfa *et al.*, 1988).

In this chapter, *pqsA* and *pqsC* were expressed using an *E. coli*-based system to determine whether PqsA could phosphorylate PqsC, *i.e.* to obtain biochemical evidence that the two are functionally related.

### 3.2 Bioinformatic analysis of *pqsA*, *pqsB* and *pqsC*

PqsA was annotated as a putative histidine kinase in the genome of *P. acnes* KPA171202. This prediction was confirmed by searching the amino acid sequence of PqsA using the TMHMM (TransMembrane prediction using Hidden Markov Models; <http://www.cbs.dtu.dk/services/TMHMM/>) server and Pfam database (<http://pfam.sanger.ac.uk/>) (Krogh *et al.*, 2001; Sonnhammer *et al.*, 1998; Finn *et al.*, 2010). The complete sequence of PqsA contains two domains: the signal input domain and autokinase domain as a cytoplasmic domain. Searching sequences in the TMHMM database further revealed 5 transmembrane helices within the signal input domain between amino acid residues 1 to 155 (Figure 3.1 panel A). Comparison against entries in the Pfam database revealed that in the autokinase domain of PqsA it has a dimerisation and phosphor-acceptor domain (from amino acid residues 183 to 248) and an ATPase domain (from amino acid residues 290 to 381) of histidine kinases (Figure 3.1 panel B and C). The phosphor-acceptor domain belongs to HisKA\_3, a sub-family of histidine kinase family, while the ATPase domain is from the family of HATPase\_c representing the structurally related ATPase domains of histidine kinases. Similarity searches by BLAST amino acid sequence of PqsA against the bacterial protein database found that PqsA was the most homologous to other histidine kinases from other *P. acnes* strains, such as *P. acnes* SK187, with between 55% to 80% identity. As these identical residues were found both in signal input domain and autokinase domain, it suggested that PqsA was a common histidine kinase and conserved for many stains of *P. acnes*. Apart from histidine kinases found in *P. acnes*, the most homologous histidine kinase was identified in

*Micrococcus luteus* with around 37% identity. However, these identical residues were all from the autokinase domain of each histidine kinase. These homologous histidine kinases have not been assigned for any biological function, therefore, the precise role of PqsA could not be predicted from the bioinformatics.

**A****B****C**

HMM ERARIARELHDSVQSLSAIAKIQELARLLLEKPEKAREELLETLELIREALAEVRRLLIDLRFA  
 PqsA ERQGLAEELHDVVAKDLAVITMLAGSLA--LNPNEDVITASKSENTSRAALDYLQRLLELVLRNR

**Figure 3. 1 Secondary structures and domains predicted within PqsA.**

(A) Five transmembrane helices predicted by TMHMM; (B) Predicted domains and sub-domains by Pfam. PqsA was predicted to contain 5 transmembrane helices in its signal input domain and a phosphor-acceptor sub-domain (HisKA\_3) with a conserved histidine residue and a structurally related ATPase domain (HATPase\_c) in its autokinase domain; (C) Alignment of the autokinase domain of PqsA and HMM. 'HMM' shows the consensus of HMM; 'PqsA' shows the sequence of the autokinase domain of PqsA; the identical matches are highlighted in black and the conservative substitutions are highlighted in grey.

PqsC was annotated as a putative response regulator in the sequenced genome of *P. acnes* KPA171202. Two significant matches were found within the Pfam database: an N-terminal response regulator receiver domain, and a C-terminal bacterial regulatory protein domain, that belongs to the LuxR family (Figure 3.2). LuxR/FixJ is a protein family of response regulators, which includes a DNA-binding, helix-turn-helix domain of about 65 amino acids. It was named after LuxR of *Vibrio fischeri* as a transcriptional activator for quorum-sensing control of luminescence. By BLAST analysis of the amino acid sequence of PqsC against the bacterial protein database, PqsC was found most homologous to response regulators of different strains of *P. acnes*. Then the next to the most homologous proteins were also response regulators found in *Micrococcus luteus* and *Streptomyces pristinaespiralis* with 44% and 41% identity, respectively, but the homologous proteins have no known function so far. Moreover, the similarities extended along the full-length of PqsC, suggesting that the biological functions of them may be closely related.

A



B

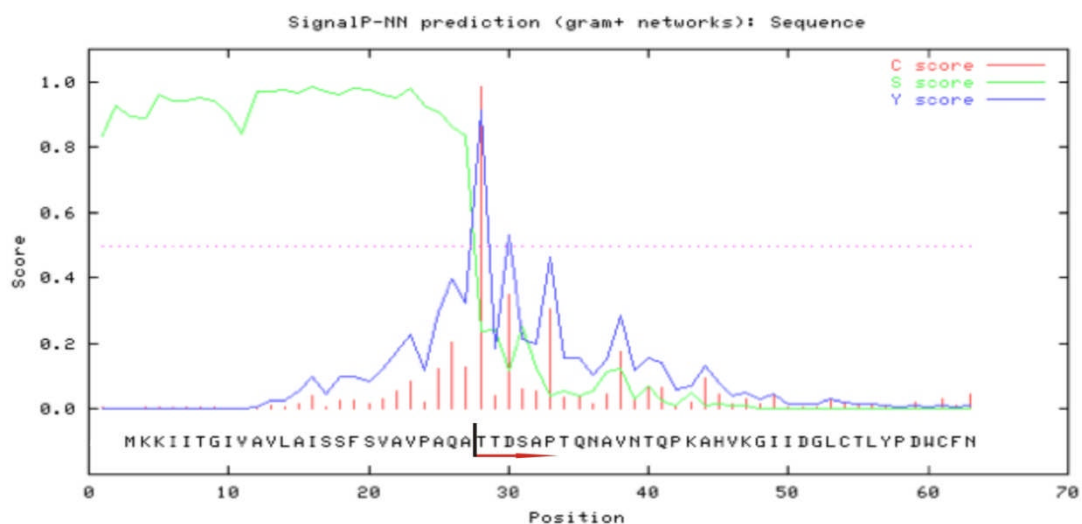


**Figure 3. 2 Domains predicted within PqsC.**

(A) Domains predicted within PqsC. PqsC was predicted to contain a response regulator receiver domain and a transcriptional regulator that belongs to the LuxR family; it includes a DNA binding and helix-turn-helix domain. (B) Alignment of PqsC and Hidden Markov Model (HMM). 'HMM' shows the consensus of HMM; 'PqsC' shows the sequence of PqsC; the identical matches are highlighted in black and the conservative substitutions are highlighted in grey.

PqsB was annotated as a hypothetical protein in the genome of *P. acnes*, and analysed by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). It predicts the presence and location of signal peptidase I cleavage sites in amino acid sequences from Gram-positive and Gram-negative prokaryotes (Bendtsen *et al.*, 2004). A significant cleavage score (C score) was obtained between amino acid residue 25 and 26, between alanine and threonine (Figure 3.3). The signal peptide score (S score) showed that the sequence from residue 1 to 15 was part of a signal peptide or peptide leader that may be involved in directing the peptide precursor to transporters. The findings of the C score and the S score was then interpreted by the Y score, a derivative

of the C score combined with the S score resulting in a better cleavage site prediction than the raw C score alone (Nielsen *et al.*, 1997).



**Figure 3. 3 Peptidase cleavage site of PqsB predicted by SignalP**

The red arrow indicates the predicted cleavage site; C score is the cleavage score indicating the predicted cleavage site; S score is reported for every single amino acid position, with high scores indicating that the corresponding amino acid is part of a signal peptide and low scores indicating that the amino acid is part of a mature protein; Y score is a derivative of the C score combined with the S score resulting in a better cleavage site prediction than the raw C score alone (Nielsen *et al.*, 1997).

### 3.3 Amplification of DNA fragments of *pqsA* and *pqsC*

The *pqsA* and *pqsC* genes were amplified and cloned to facilitate purification of their products. Segments encoding the autokinase domain of PqsA and full-length PqsC were amplified and inserted into plasmids that facilitate heterologous expression in *E. coli* (pET system manual, Novagen; GST gene fusion system handbook, Amersham Biosciences). The details are outlined below. The cloning of a segment encoding full-length PqsA was not

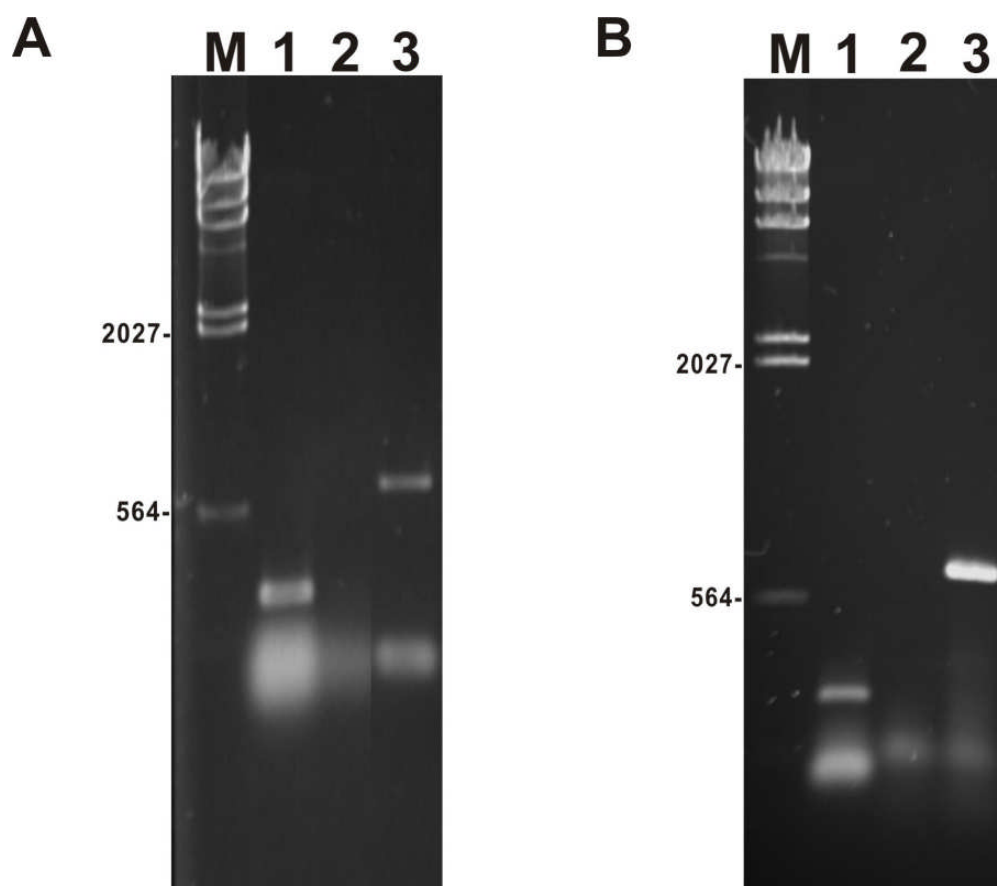


attempted, as the inclusion of the transmembrane domain would have meant using protocols for the purification of membrane proteins (Ma *et al.*, 2008; Saidijam *et al.*, 2003). Furthermore, it was well documented that many transmembrane proteins of Gram-positive organisms, such as *B. subtilis* are toxic to an *E. coli* overexpressing host, and are difficult to produce in large enough quantities to allow biochemical studies (Szurmant *et al.*, 2008). Previous studies have shown that the autokinase domain of other histidine kinases is sufficient to direct phosphorylation of their cognate response regulators *in vitro* (Rowland *et al.*, 2004).

Primers were designed to amplify a segment encoding the autokinase domain of PqsA. The forward and reverse primers for the amplification of the autokinase domain incorporated at their 5' ends restriction sites for *Bam*HI and *Xho*I (Appendix I). These sites were included to facilitate insertion into the corresponding sites of plasmid pGEX-6P-1 and the production of the autokinase domain of PqsA with a N-terminal glutathione S-transferase (GST) tag that facilitated purification by affinity chromatography using immobilized glutathione (GST Gene Fusion System, Amersham Biosciences). For *pqsC*, primers were designed to amplify a segment encoding full-length PqsC. The forward and reverse primers for the amplification of PqsC incorporated at their 5' ends restriction sites for *Nde*I and *Xho*I (Appendix I). These sites were included to facilitate insertion into the corresponding sites of plasmid pET30b and the production of PqsC with a C-terminal oligohistidine tag (pET system, Novagen).

Genomic DNA of *P. acnes* KPA171202 was extracted using a modified protocol (Section 2.12) and used as the template in PCR. As shown in Figure

3.4, amplicons corresponding to segments encoding the autokinase domain of PqsA (Panel A) and full-length PqsC were obtained (Panel B). The gel mobility of these amplicons was consistent with their predicted sizes of 665 and 672-bp, respectively (Lanes 3). No amplicons were produced in the absence of DNA template (Lanes 2). A positive control produced an amplicon of 266 bp product (Lanes 1), which corresponded to a segment containing the promoter of heat shock protein 60 (Dr Mark Farrar, University of Leeds). The primers and conditions for PCR are described elsewhere (Section 2.14.4).

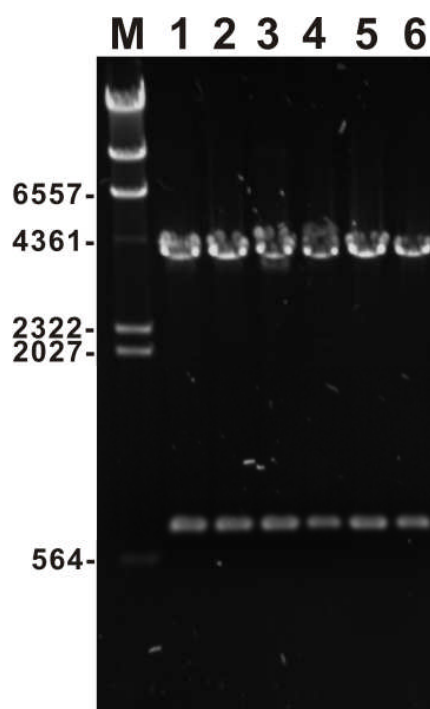


**Figure 3. 4 Agarose (1%, w/v) gel electrophoresis of the products of amplification of the autokinase domain of PqsA and full length PqsC.**

(A) Amplification of the autokinase domain of PqsA. Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1: positive control using genomic DNA of *P. acnes* and primers to amplify the promoter sequence of the heat shock protein 60; the primers should amplify a 266 bp product; Lane 2: negative control using all the primers without DNA template; Lane 3: amplified autokinase domain of PqsA PCR product with *Bam*HI and *Xho*I restriction sites. (B) Amplification of full length PqsC. Lane M, 1 and 2 are the same as in panel A, respectively; Lane 3: amplified PqsC product with *Nde*I and *Xho*I restriction sites.

### **3.4 Cloning of autokinase domain of PqsA and full-length PqsC**

The amplicons corresponding to the autokinase domain of PqsA and full-length PqsC were purified from agarose gels (Section 2.14.2) and ligated to plasmid - pCR<sup>®</sup>4-TOPO<sup>®</sup>, a cloning vector that is designed for cloning PCR products for DNA sequencing (Section 2.15.1). Afterwards, the amplified DNA segments were verified by DNA sequencing. The ligation mixes were then transformed into *E. coli* DH5 $\alpha$  (Section 2.15.3). Plasmid DNA was isolated from individual transformants, and the presence of the required inserts confirmed first by restriction enzyme analysis (Section 2.15.4) and then by DNA sequencing. Representative restriction enzyme digests of three independent isolates of each of the two constructs are shown in Figure 3.5. Plasmids containing amplicons encoding the autokinase domain of PqsA and full-length PqsC were called pHRE001 and pRRC001, respectively.

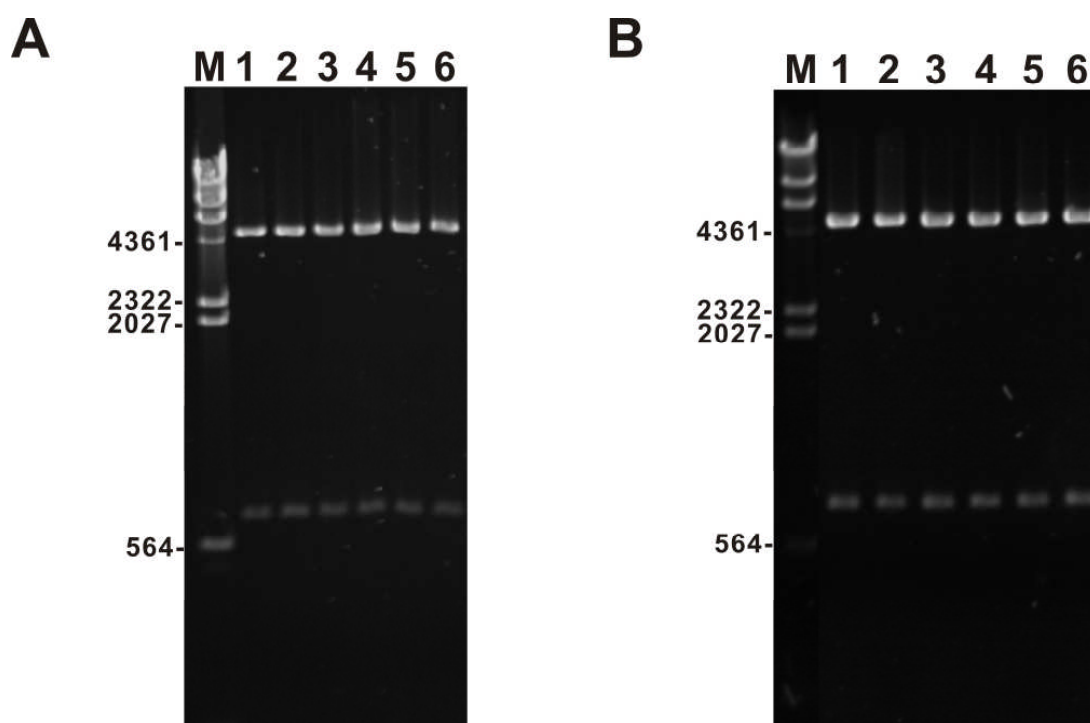


**Figure 3. 5** Restriction digests of pHRE001 and pRRC001 on 1% (w/v) agarose gel confirming the presence of the correct insert in each plasmid.

Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1 to 3: pRRC001 digested with *Nde*I and *Xho*I; Lane 4 to 6: pHRE001 digested with *Bam*HI and *Xho*I.

Constructs with the correct insert were digested again with the corresponding restriction enzymes and separated by gel electrophoresis. The segment of the autokinase domain of PqsA and full-length PqsC was then isolated from the gel (Section 2.14.2) for insertion into the expression vectors pGEX-6P-1 and pET30b, respectively. Plasmid pGEX-6P-1 was digested with *Bam*HI and *Xho*I, whereas plasmid pET30b was digested with *Nde*I and *Xho*I. The plasmid backbones of pGEX-6P-1 and pET30b were then purified as described in section 2.14.3 before being ligated with the segment of the autokinase domain of PqsA and full-length PqsC, respectively. The ligation mixtures were transformed to *E. coli* DH5 $\alpha$  and transformants selected using

the antibiotic appropriate for each construct (Section 2.15.3). After the presence of the required insert had been confirmed by restriction enzyme analysis, recombinant plasmids were transformed into the *E. coli* expression host BL21(DE3). Representative restriction enzyme digests of six independent isolates of each of the two constructs are shown in Figure 3.6. Plasmids containing amplicons that encode the autokinase domain of PqsA and full-length PqsC were called pHRE002 and pRRC002, respectively.



**Figure 3. 6 Restriction digests of pHRE002 and pRRC002 on 1% (w/v) agarose gel confirming the presence of the correct insert in each plasmid.**

(A) Restriction digest of pHRE002. Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1 to 6: pHRE002 digested with *Bam*HI and *Xho*I; (B) Restriction digest of pRRC002. Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1 to 6: pRRC002 digested with *Nde*I and *Xho*I.

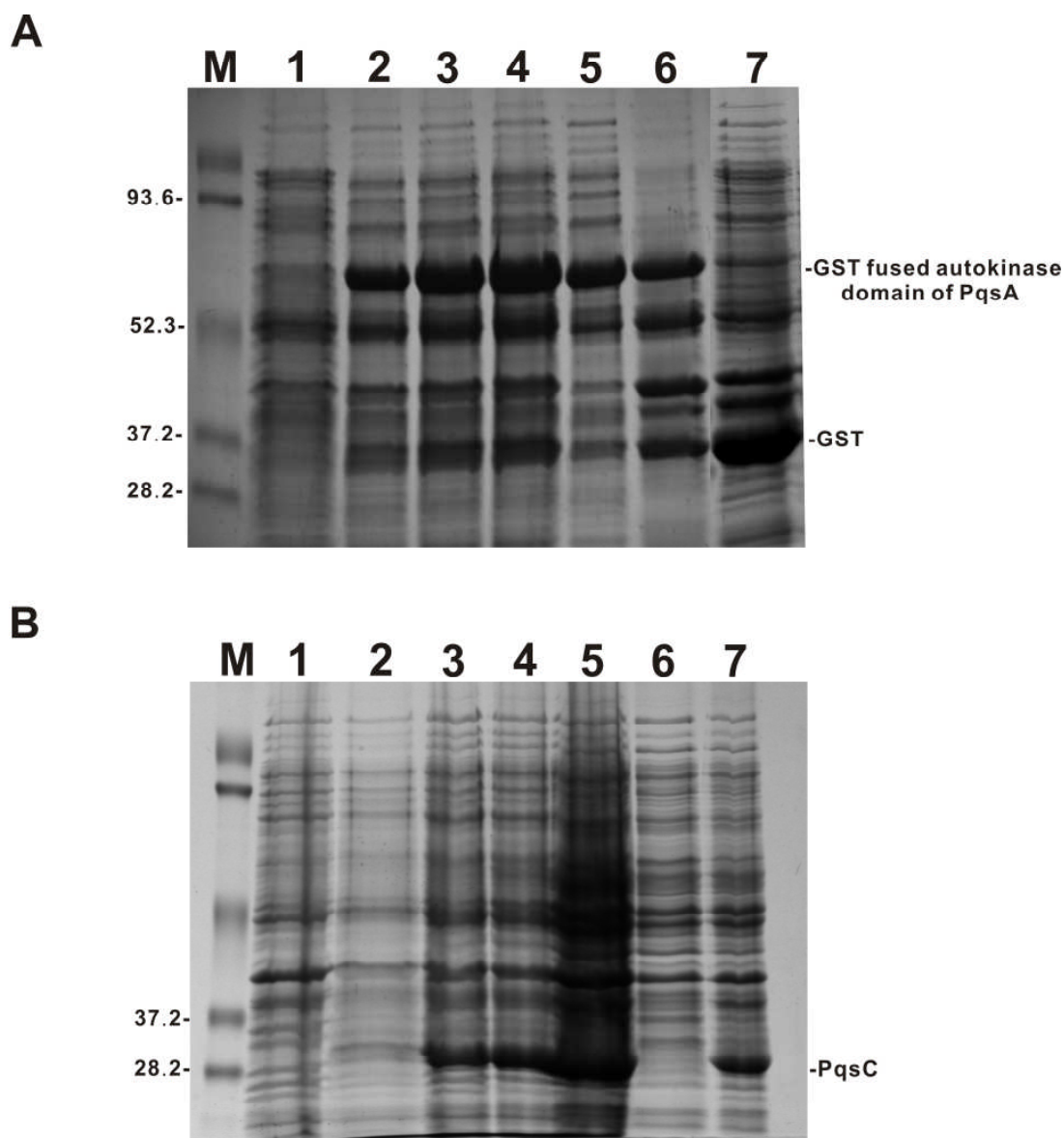
### 3.5 Overexpression of *pqsA* and *pqsC*

Overproduction of the autokinase domain of PqsA and full-length PqsC was induced by adding IPTG to exponentially grown cultures at an OD<sub>600</sub> of 0.6 (Section 2.17.6). Samples of each of the cultures were taken before induction, and after 1, 2, and 3h. The cells in each sample were collected by centrifugation, resuspended and lysed by sonication (Section 2.10). The cell lysate was cleared by centrifugation and samples of the supernatant (soluble fraction) and pellet (insoluble fraction) were collected and analysed by SDS-PAGE (Section 2.17.1).

The autokinase domain of PqsA was produced as a recombinant with GST fused at its N-terminus. The expected mass of this recombinant protein is 50 kDa. Engineering GST as a fusion can significantly increase the activity and amount of target protein present in the soluble fraction, because GST itself is highly soluble (GST gene fusion system handbook, Amersham biosciences). PqsC was produced as a fusion with an oligohistidine-tag at its C-terminus. This recombinant has an expected mass of 24 kDa. Both proteins were successfully overproduced (Figure 3.7). Major bands corresponding to protein of GST-PqsA and PqsC were detected after, but not before induction of cells containing plasmids pHRE002 and pRRC002, respectively. The level of both of the induced polypeptides was highest in the samples taken 3 h after induction (Figure 3.6, panel A, lane 4 and panel B, lane 5, respectively). For the overproduction of PqsA experiment, no expression was detected in the negative control, which was cells containing pHRE002 prior to induction (Panel A, lane 1). In another negative control, 3 h induced pGEX-6P-1 without *pqsA* inserted, only GST was overproduced

following induction (Panel A, lane 7). The overproduced autokinase domain of PqsA fused with GST was detected in both the supernatant (Lane 5) and cell pellet (Lane 6) indicating this polypeptide was partly soluble. Only the soluble fraction was used in subsequent experiments. PqsC was also overproduced after induction for 3 h (Panel B, lane 5). There was no expression in negative controls: uninduced cells containing pRRC002 (Lane 1) and 3 h induced cells containing pET30b without insert (Lane 2). However, the overproduced PqsC could only be detected in the insoluble fraction (Lane 7). In this case, this polypeptide had to be purified from inclusion bodies and refolded.



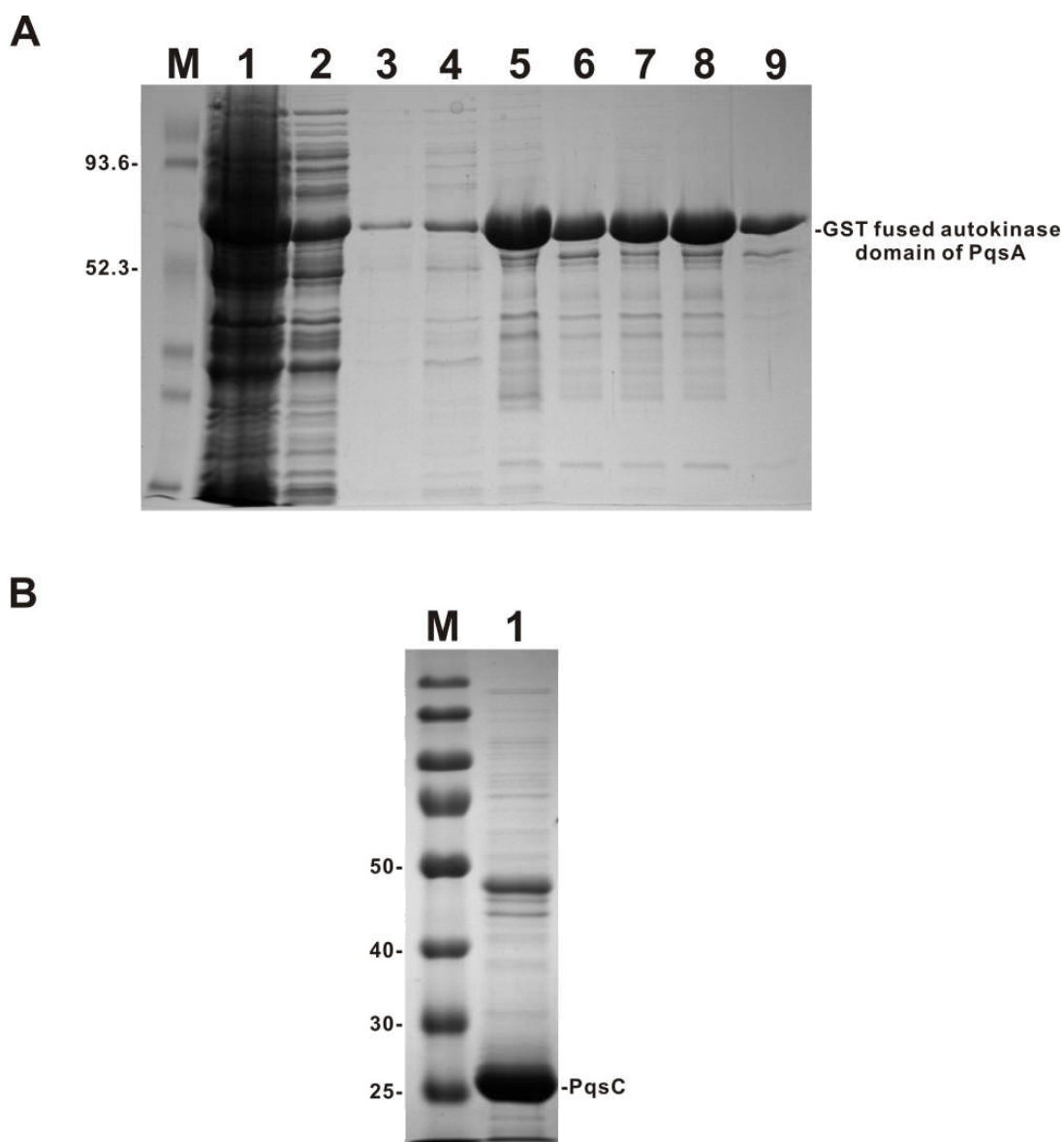


**Figure 3. 7 Overproduction of PqsA and PqsC in *E. coli* on SDS-PAGE (10%, w/v).**

(A) Overproduction of GST-PqsA. Lane M: protein standards; the numbers at the left of each panel indicates the size (kDa) of the markers; (prestained SDS-PAGE standards, low range, Bio-Rad); Lane 1: uninduced GST-PqsA; Lane 2 to 4: GST-PqsA induced for 1, 2 and 3 h, respectively; Lane 5: soluble fraction of overproduced GST-PqsA; Lane 6: insoluble fraction of overproduced GST-PqsA; Lane 7: pGEX-6P1 induced for 3 h. (B) Overproduction of PqsC. Lane M: same as in panel A; Lane 1: uninduced PqsC; Lane 2: pET30b induced for 3 h; Lane 3 to 5: PqsC induced for 1, 2 and 3 h, respectively; Lane 6: soluble fraction of overproduced PqsC; Lane 7: insoluble fraction of overproduced PqsC.

### **3.6 Purification of PqsA and PqsC**

Following protein overproduction, the GST fused autokinase domain of PqsA was purified as soluble protein under native conditions by affinity chromatography using immobilized glutathione (Section 2.17.7.1) and PqsC was purified from inclusion bodies under denaturing conditions and then refolded by dialysis (Section 2.17.7.2). Samples were analysed by SDS-PAGE (Figure 3.8). Both overproduced polypeptides were successfully purified and accounted for more than 90% of the protein in the preparation.



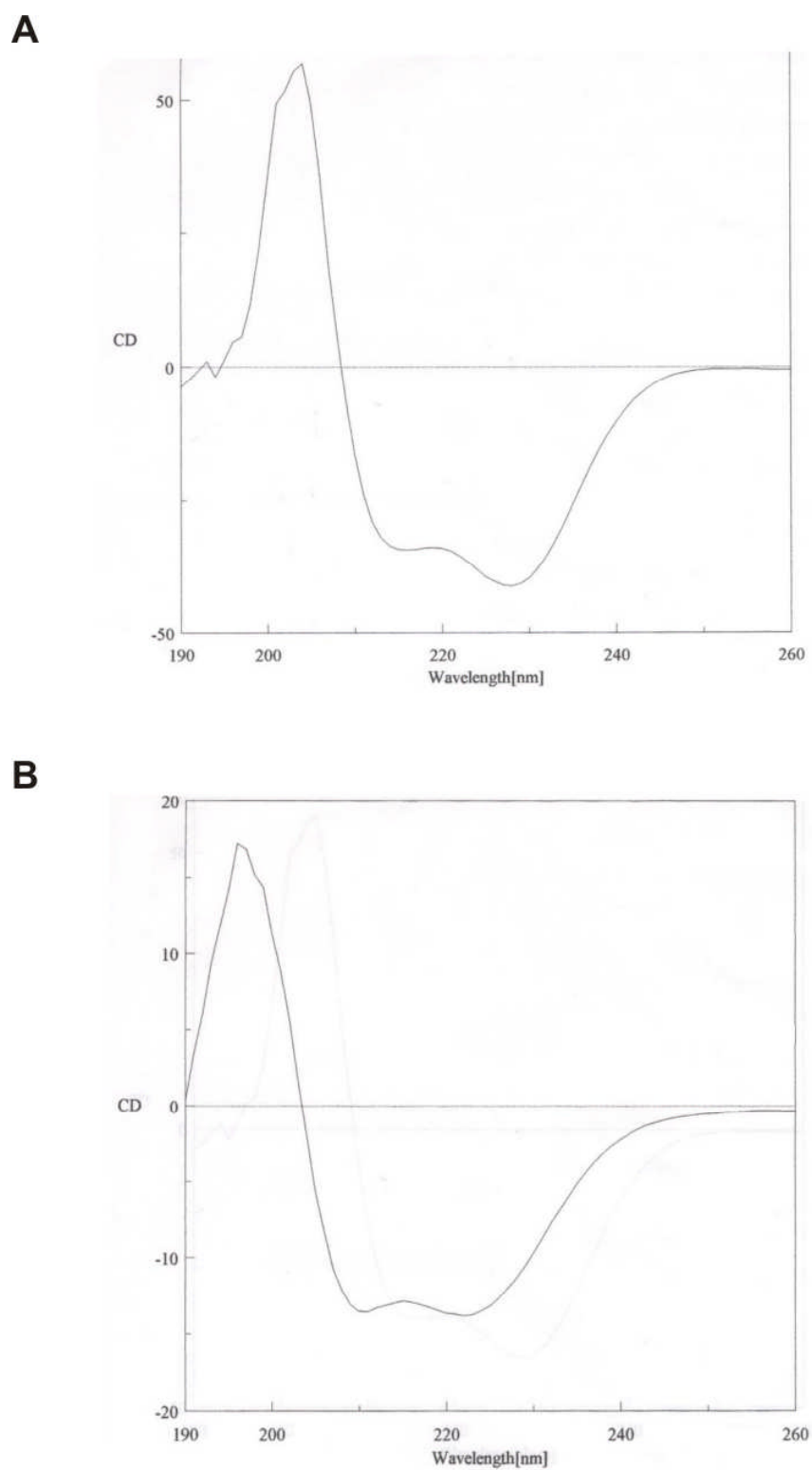
**Figure 3. 8 Purification of GST fused autokinase domain of PqsA and PqsC on SDS-PAGE.**

(A) Purification of the GST fused autokinase domain of PqsA. Lane M: protein standards; the numbers at the left of each panel indicates the size (kDa) of the markers (prestained SDS-PAGE standards, low range, Bio-Rad); Lane 1: unbound protein from cell lysate; Lane 2 to 4: wash 1, 2 and 3, respectively, with wash buffer containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3; Lane 5 to 9: elution 1, 2, 3, 4 and 5, respectively, with elution buffer containing 50 mM Tris, 10 mM reduced glutathione, pH 8.0.

(B) Purification of PqsC. Lane M: protein standards; the numbers at the left of each panel indicates the size (kDa) of the markers (Protein ladder (10 to 250 kDa), New England Biolabs); Lane 1: purified PqsC.

The amino acid sequence of each purified protein was verified by N-terminal sequencing. The first 10 residues obtained for GST fused autokinase domain of PqsA were Gly-Pro-Leu-Gly-Ser-Ala-Glu-Glu-Gln-Ala. The first 10 residues obtained for PqsC were Ser-Val-Arg-Leu-Val-Val-Thr-Asp-Asp-His. These sequences were identical to those predicted from the genome sequence. These results confirmed that the GST fused autokinase domain of PqsA and PqsC were successfully overproduced and purified.

The structure of the protein was also confirmed by CD analysis. The CD spectrum showed that both GST fused autokinase domain of PqsA and PqsC represented an alpha-helix secondary structure as expected (Figure 3.9). Importantly, it demonstrated that purified PqsC was refolded from inclusion bodies under the experimental conditions *in vitro* and suitable for the downstream phosphorylation assays.



**Figure 3. 9 CD analysis of the GST fused autokinase domain of PqsA and refolded PqsC.**

(A) CD spectrum of the GST fused autokinase domain of PqsA. (B) CD spectrum of refolded PqsC. The experiment was performed with the wavelength scan start from 260 nm to 190 nm at the speed of 50 nm/min.

### 3.7 Autophosphorylation of the GST fused autokinase domain of PqsA

Prior to the phosphotransfer studies of the GST fused autokinase domain of PqsA and PqsC, the autophosphorylation activity of the GST fused autokinase domain of PqsA was assayed. The purified GST fused autokinase domain of PqsA was incubated with radiolabelled ATP ( $\gamma[^{32}\text{P}]\text{-ATP}$ ). Samples were withdrawn at different time points and analysed by SDS-PAGE in combination with phosphorimaging (Section 2.18.1). The GST fused autokinase domain of PqsA was clearly phosphorylated and the amount increased with time (Figure 3.10).



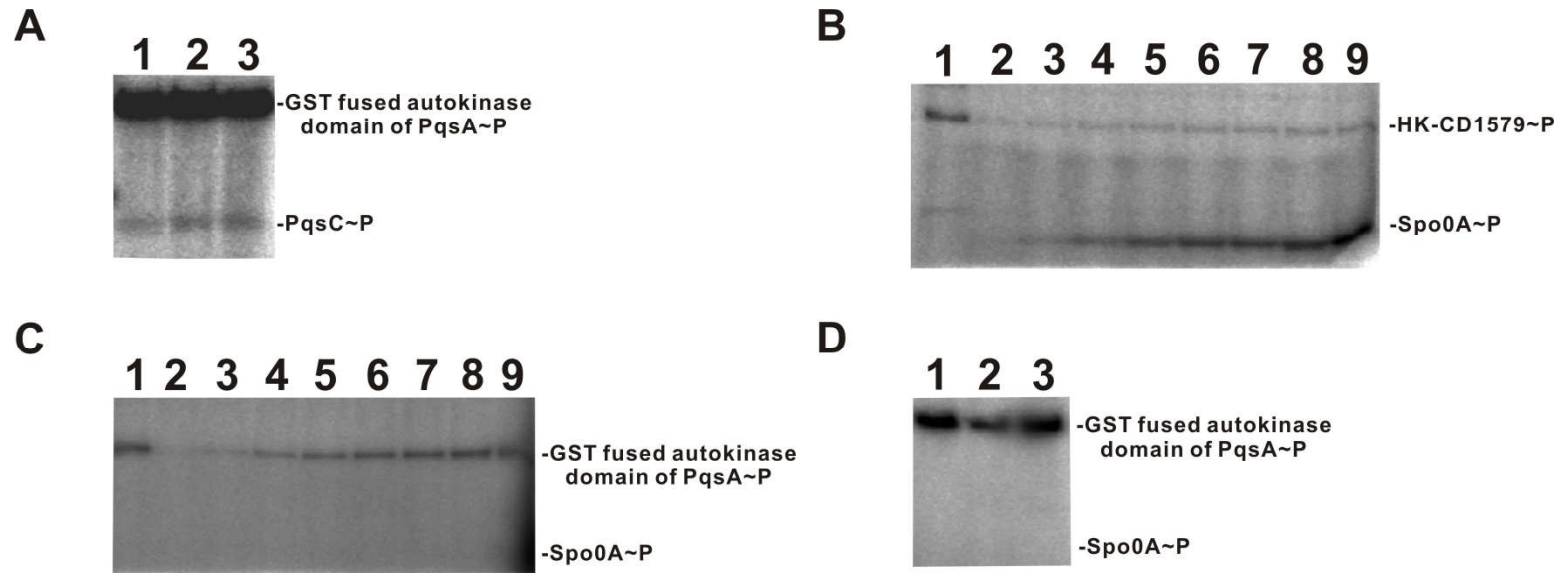
**Figure 3. 10 Autophosphorylation of GST fused autokinase domain of PqsA on SDS-PAGE.**

Lane 1 to 8: purified GST fused autokinase domain of PqsA incubated with  $\gamma[^{32}\text{P}]\text{-ATP}$  for 0, 1, 2, 3, 5, 10, 15 and 20 min, respectively.

### 3.8 Phosphotransfer between the GST fused autokinase domain of PqsA and PqsC

When a histidine kinase is activated by autophosphorylation, it catalyses the transfer of its phosphoryl group to the active site aspartate residue in the N-terminal regulatory domain of the cognate response regulator (Stock *et al.*, 1989). Phosphorylation of the response regulator is thought to alter its

structure, allowing the C-terminal output domain to bind to DNA and regulate gene expression (Lewis *et al.*, 2002). The ability of PqsA to phosphorylate PqsC was determined by phosphorylation assays. The purified GST fused autokinase domain of PqsA was incubated with PqsC in the presence of  $\gamma$ [<sup>32</sup>P]-ATP (Figure 3.11, panel A). As shown in Figure 3.11 panel A, the radiolabelled phosphoryl group was transferred from the GST fused autokinase domain of PqsA to PqsC. It suggested that PqsA and PqsC was a legitimate pair of a TCS as previously hypothesised, despite being transcribed divergently in the genome. Furthermore, to measure whether the phosphotransfer between them was specific, the purified GST fused autokinase domain of PqsA was incubated with an unrelated response regulator, Spo0A (kindly donated by Dr Sarah Underwood, University of Leeds) of *Clostridium difficile*, in the presence of  $\gamma$ [<sup>32</sup>P]-ATP. When Spo0A was incubated with its cognate histidine kinase, HK-CD1579 (kindly donated by Dr Sarah Underwood, University of Leeds), it was phosphorylated by HK-CD1579 (Figure 3.11, panel B). However, when it was incubated with the GST fused autokinase domain of PqsA, the histidine kinase of *P. acnes* could not transfer the phosphoryl group to Spo0A, unlike HK-CD1579 (Figure 3.11, panel C and D). It indicates that the phosphorylation of PqsC by PqsA is the result of a specific interaction between the histidine kinase and response regulator.



**Figure 3. 11 Phosphotransfer activity of the GST fused autokinase domain of PqsA to PqsC on SDS-PAGE.**

(A) Phosphotransfer between GST fused autokinase domain of PqsA and PqsC. Lane 1-3: GST fused autokinase domain of PqsA incubated with PqsC for 20, 40 and 60 min, respectively. (B) Phosphotransfer between HK-CD1579 of *Clostridium difficile* and Spo0A. Lane 1: control reaction, in which HK-CD1579 alone incubated for 20 min; Lane 2-9: HK-CD1579 incubated with Spo0A for 0, 2, 4, 6, 8, 10, 15 and 20 min, respectively. (C) Phosphotransfer between the GST fused autokinase domain of PqsA and Spo0A. Lane 1: control reaction, in which GST fused autokinase domain of PqsA alone incubated for 20 min; Lane 2-9: GST fused autokinase domain of PqsA incubated with Spo0A for 0, 2, 4, 6, 8, 10, 15 and 20 min, respectively; (D) Phosphotransfer between the GST fused autokinase domain of PqsA and Spo0A. Lane 1: control reaction, in which GST fused autokinase domain of PqsA alone incubated for 60 min; Lane 2-3: GST fused autokinase domain of PqsA incubated with Spo0A for 20 and 60 min, respectively.



### 3.9 Discussion

This chapter shows that PqsA can be autophosphorylated and then transfer a phosphate specifically to PqsC. Therefore, these polypeptides would appear to be a legitimate pair of a TCS. They appear to be the first example of a TCS where a response regulator is not co-transcribed with the histidine kinase. Response regulators have previously been identified that are divergently transcribed from the histidine kinase, but only in atypical systems where there are two response regulators, one of which is co-transcribed with the histidine kinase. Examples of these atypical systems include the CbbRRS system involved in the metabolic route for carbon dioxide assimilation in *Rhodopseudomonas palustris* (Romagnoli & Tabita, 2006), the Roc system regulating the expression of fimbrial *cup* gene in *Pseudomonas aeruginosa* (Kulasekara *et al.*, 2005) and the Cor system activating the expression of phytotoxin coronatine in *Pseudomonas syringae* (Ullrich *et al.*, 1995). Previously identified response regulators in these three component TCSs at least has one response regulator containing a helix-turn-helix effector domain, whereas the other response regulator has been proposed to antagonize the DNA-binding activity of the system (Kulasekara *et al.*, 2005). The recently identified CbbRRS system has two response regulators and neither of them contains an DNA-binding domain (Romagnoli & Tabita, 2006). However, none of these cases is the same as this novel TCS in of *P. acnes*. Although there are three components in this system, PqsB between PqsA and PqsC is not homologous to any response regulator. Moreover, it has been predicted as a signalling peptide and may be modified after removing the leader sequence. Therefore, the TCS consisting

PqsA and PqsC of *P. acnes* is the first identified TCS containing only a histidine kinase and a single response regulator that are encoded by adjacent genes, but transcribed divergently.

The direct phosphoryl group transfer (Figure 3.11) also suggested that no intermediate was required for the relay of phosphate between PqsA and PqsC. Intermediates are required for the relaying of phosphate between the histidine kinase KinA and response regulator Spo0A in *B. subtilis*. In the initiation of sporulation in *B. subtilis*, both Spo0A and Spo0F are related to response regulator components of the TCS used to control environmental responses in bacteria (Burbulys *et al.*, 1991). The Spo0F response regulator was found to be the primary substrate for phosphorylation by the sporulation-specific histidine kinase, KinA (Burbulys *et al.*, 1991). Phosphorylated Spo0F was the phosphodonor for a phosphotransferase, SpoB, which transferred the phosphate group to the second response regulator, the transcription regulatory protein Spo0A (Burbulys *et al.*, 1991). Most of the intermediate response regulators, like Spo0F, are response regulators that do not contain DNA-binding domains. Although there are several orphan response regulators in the genome of *P. acnes*, they are all response regulators containing both receiver domains and DNA-binding domains. Therefore, it is highly unlikely that an additional protein is involved in the TCS signal transduction pathway of PqsA and PqsC in *P. acnes*.

Initially, an attempt was made to overproduce full-length *pqsA*. The corresponding open-reading frame was cloned into both pGEX-6P-1 and pET30b. However, following the induction, no polypeptide of the expected size was overproduced to a detectable level. Rather than attempting to

improve the production of full-length PqsA, the established strategy of producing only the cytoplasmic domain of a membrane-bound protein, in this case the autokinase domain, was adopted (Georgellis *et al.*, 1997). However, using a truncated version of a histidine kinase for the downstream phosphorylation assays was a risk, because in some cases the conformational changes in the autokinase domain resulting from the activation of the signal input domain is essential for the phosphotransfer to its cognate response regulator (Romagnoli & Tabita, 2006). However, in the case of PqsA, the autokinase domain alone could transfer the phosphoryl group to PqsC.

Many methods were tried to optimize the overexpression and purification of PqsC including producing it as a recombinant with the *peIB*, periplasmic localization signal at the N-terminus, lowering the induction temperature to between 20 °C and 25 °C and reducing the concentration of IPTG to 0.4 mM. However, these efforts failed to produce a significant amount of soluble PqsC. Inclusion bodies were formed during the overexpression of full-length *pqsC* resulting in the insoluble protein.

The attempt of purifying PqsC under denaturing conditions using immobilised metal-affinity chromatography was also tried. The oligohistidine-tag had been added to the C-terminus of PqsC. The strong denaturant 8 M urea was used to completely solubilise inclusion bodies and the protein refolding was tried to refold using less concentrated urea by multi-step dialysis. However, the solubilised protein always became aggregated or precipitated when the urea was completely removed at the final step of dialysis. After several attempts, PqsC was finally solubilised and

successfully refolded using the protocol of Protein refolding kit (Novagen). The structure of refolded PqsC was confirmed by CD analysis showing  $\alpha$ -helix in the refolded protein.

## **Chapter IV Determination of transcriptional start site(s) of putative signalling peptide, sensor histidine kinase and response regulator**

### **4.1 Chapter introduction**

Identification of the transcription start sites (TSSs) of *pqsA*, *pqsB* and *pqsC* are very important for this study, because the transcription of DNA into RNA ultimately leads to changes in cell function. In bacteria, these changes or gene transcription regulation often reflect changes in their environments. Moreover, transcriptional regulation is one of the major mechanisms controlling gene expression in prokaryotes. Identification of the exact position of a 5' TSS of an RNA molecule is crucial for the identification of the regulatory regions that immediately flank it. Determining TSSs of these putative genes will provide more information on gene transcription and regulation, help to identify the promoter sequences and investigate the relationships among PqsA, PqsB and PqsC.

Transcription is the biosynthesis of RNA, in which DNA acts as a template and the process follows the same base pairing principle as the replication of DNA (Turner *et al.*, 2000). The synthesis is initiated at the promoter sequence by the RNA polymerase. In bacteria, a single RNA polymerase is responsible for the synthesis of messenger, transfer and ribosomal RNAs (Turner *et al.*, 2000). Bacterial RNA polymerase consists of a core polymerase containing  $\beta$ ,  $\beta'$ ,  $\omega$  and two  $\alpha$  subunits along with one of several sigma factors (Turner *et al.*, 2000, Record Jr *et al.*, 1996). The core

polymerase is competent for transcription elongation and termination, while transcript initiation requires an associated sigma subunit. The initiation is the most frequent target for regulation by different transcription factors. The sigma factor is involved in promoter recognition. They are related but distinct in different forms of RNA polymerase. Apart from their role in recognizing, binding to specific promoter sequences and directing to the target promoter, they also play a very important role during the transcription. For example, they are targets for transcription activators, are involved in promoter melting near the transcription start site, inhibit nonspecific initiation and regulate early transcription by clearing and releasing the promoter from RNA polymerase (Buck *et al.*, 2000). Most bacteria contain multiple sigma factors including a primary sigma factor that recognizes the majority of promoters and alternative sigma factors that respond to specific environmental stresses (Ghosh *et al.*, 2010). For example, *Streptomyces coelicolor* has a staggering 63 sigma factors, while *Bacillus subtilis* has 18 sigma factors (Gruber & Gross, 2003).

The promoter sequence is an important piece of genetic information, which is recognized by the RNA polymerase and allows it to start the transcription. The process of transcription begins with binding of RNA polymerase to the promoter. Once bound at a promoter site, RNA polymerase typically contacts ~80 bp of DNA extending from as much as ~60 bp upstream of the start point of transcription to ~20 bp downstream (Ross & Gourse, 2009). Within this extended promoter recognition region reside the key contact points that allow RNA polymerase to distinguish promoter from non-promoter DNA (Helmann, 2009). The precise nature of these sequences varies depending on the promoter and the holoenzyme (Helmann, 2009). For

sigma 70 class promoters, the key recognition elements are conserved hexamers located near -35 and -10 with consensus sequences of TTGaca and TAtaaT, respectively (where upper case letter denote more highly conserved positions). In the case of alternative sigma factors related to sigma 70, there are often distinct sequence motifs generally centred at these same two positions. Moreover, as the promoter sequence is asymmetrical, the RNA polymerase can also get directional information in addition to information about the starting point for transcription (Turner *et al.*, 2000).

TSS is the first nucleotide of a transcribed DNA sequence where RNA polymerase begins synthesizing the RNA transcript. Knowledge of the exact position of a 5' TSS of an RNA molecule is crucial for the identification of the regulatory regions that immediately flank it. The critical issue in mapping a true site of transcription initiation is to be able to distinguish it from a 5' end generated by RNA endonucleolytic cleavage or the possible exonucleolytic degradation (Belasco, 2010). Recently studies also found an alternative mRNA decay pathway involved in RppH, an RNA pyrophosphohydrolase, which preferentially acts on single strand 5' termini and converts the 5'-terminal triphosphate to a monophosphate (Deana *et al.*, 2008). Although no homologue to RppH is found in the genome of *P. acnes* KPA171202 so far, it contains several putative ribonucleases with no known function. It is preferable to distinguish 5' capped mRNA from decapped mRNA when investigating the TSS for the gene of interest.

Several methods have been employed to identify the TSS including S1 nuclease mapping, primer extension and 5' rapid amplification of cDNA ends (5' RACE) (Sambrook *et al.*, 1989). However, many factors influence the

choice of method, such as the strength of the promoter and the abundance of the transcript to be analyzed. S1 nuclease or primer extension is suitable for detection of abundant transcripts, which do not require amplification of the signal, while low abundance transcripts may require signal amplification, either by cloning the promoter region on a multicopy plasmid, or by amplification of cDNA copies produced from the transcript (Ross & Gourse, 2009). This study utilized 5' RACE to investigate the TSS of PqsA, PqsB and PqsC.

The 5' RACE method involves primer extension for first strand cDNA synthesis from extracted total RNA, followed by PCR amplification of the cDNA products using procedural modifications that create a primer binding site flanking the 3' end of the cDNA (Ross & Gourse, 2009). Products of this amplification can be sequenced directly, or cloned and sequenced to identify the 5' mRNA end (5'/3' RACE kit, 2<sup>nd</sup> generation, Roche applied science).

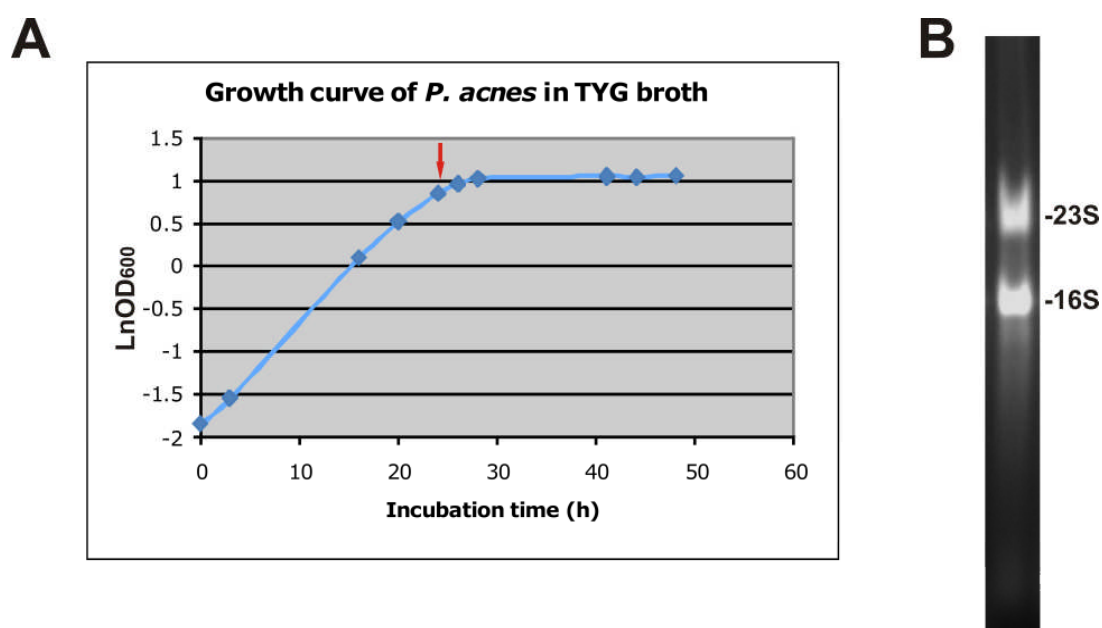
This chapter describes the identification of TSS of PqsA, PqsB and PqsC. RNA was extracted from cells harvested at the late exponential phase using a slightly modified protocol of RiboPure™ - Bacteria Kit (Ambion) (Section 2.14.1). After DNase treatment and verification, TSSs were identified by 5' RACE combined with Terminator™ 5'-phosphate-dependent exonuclease treatment.

## **4.2 RNA extraction from *P. acnes***

Liquid culture of *P. acnes* was obtained as described in section 2. 13. The OD<sub>600</sub> readings of the culture were followed to create the growth curve.



The growth curve was plotted using incubation time against natural log of OD<sub>600</sub> readings (Figure 4.1, panel A). When *P. acnes* grew to late exponential phase, cells were taken and immediately stabilized using phenol/ethanol solution and then harvested by centrifugation. The total RNA of *P. acnes* was extracted using a slightly modified protocol of RiboPure™ - Bacteria Kit as described in section 2.16.1. The extracted RNA sample was run on a denaturing gel (Figure 4.1, panel B). As shown in the figure, the bands corresponding to 23S and 16S rRNA were indicating RNA was successfully isolated from *P. acnes* cells.

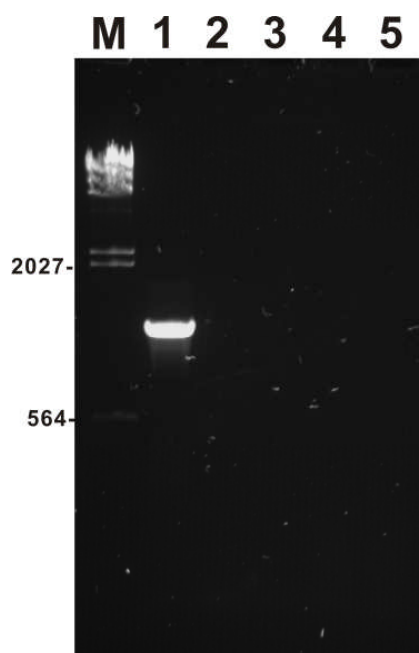


**Figure 4. 1 RNA extraction of *P. acnes*.**

(A) Growth curve of *P. acnes* KPA171202. X axis indicates the incubation time in hours; Y axis indicates the natural log of OD<sub>600</sub> readings; the blue diamond indicates the reading which was taken during the growth. (B) Extracted total RNA sample from *P. acnes* KPA171202 on 1 % (w/v) formaldehyde agarose gel.

### **4.3 Preparation of RNA sample for transcription analysis**

After isolated, the RNA was treated with TURBO™ DNase to remove contaminating genomic DNA as described in section 2.16.3. To verify that there was no DNA contamination in the RNA sample, the treated RNA sample was used as template in the PCR reactions with specific primers for each target gene (Figure 4.2). As the amplifications were done without prior incubation with reverse transcriptase, no amplified fragment should be detected if the RNA sample is free of contaminating genomic DNA. The amplified fragment of PqsA, PqsB and PqsC should result in a 170 bp, 153 bp and 186 bp product, respectively. However, no amplified fragment could be detected for all three genes indicating that the RNA sample was free of DNA contamination (Figure 4.2, lane 3 to 5). Positive and negative controls were also included in the reaction. The band detected in the positive control demonstrated that PCR reactions worked well (Lane 1) and no band in the negative control showed no detectable primer dimer was formed during the reaction (Lane 2). The gel demonstrated that the DNase treatment was successful and the extracted RNA sample was free of DNA.

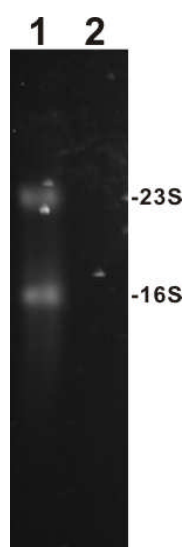


**Figure 4. 2 Confirmation of DNA-free RNA sample by PCR reactions.**

Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1: positive control using genomic DNA of *P. acnes* and primer RTF and RTR; the primers should amplify a 1164 bp product; Lane 2: negative control included all the primers without DNA template in one reaction; Lane 3: PCR reaction used RNA as template and primer PPA0945RTPCRF and PPA0945RTPCRR to amplify the DNA fragment of PqsA; Lane 4: PCR reaction used RNA as template and primer PPA0946RTPCRF and PPA0946RTPCRR to amplify the DNA fragment of PqsB; Lane 5: PCR reaction used RNA as template and primer PPA0947RTPCRF and PPA0947RTPCRR to amplify the DNA fragment of PqsC. PCR reactions were performed for 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min.

Following DNase treatment and confirmation of absence of genomic DNA contamination, the RNA sample was treated by Terminator<sup>TM</sup> 5'-phosphate-dependent exonuclease. This was to ensure the downstream experiments could identify the true TSS of each target gene, rather than random 5'end of a degraded mRNA. After the treatment of exonuclease, the

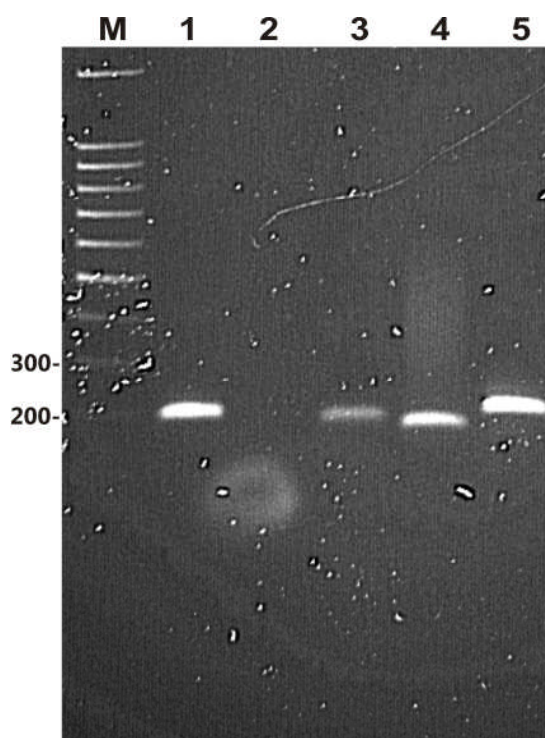
RNA samples were run on a denaturing gel again to confirm the successful enzyme treatments (Figure 4.3). As 23S and 16S RNA have 5'-monophosphate groups, they were removed by the exonuclease. Therefore, the absence of 23S and 16S in the gel indicated that the treatment of Terminator™ 5'-phosphate-dependent exonuclease was successful.



**Figure 4. 3 Terminator™ 5'-phosphate-dependent exonuclease treated RNA sample on 1% (w/v) FA gel.**

Lane 1: the RNA sample before treatment; Lane 2: the RNA sample after treatment.

RT-PCR reactions were also performed to confirm the presence of target mRNA using gene specific primer the same as above for PqsA, PqsB and PqsC individually. Positive and negative controls were also included in the reaction. Figure 4.4 shows the corresponding DNA fragment was amplified for each target gene, which demonstrated the presence of mRNA in the RNA sample.



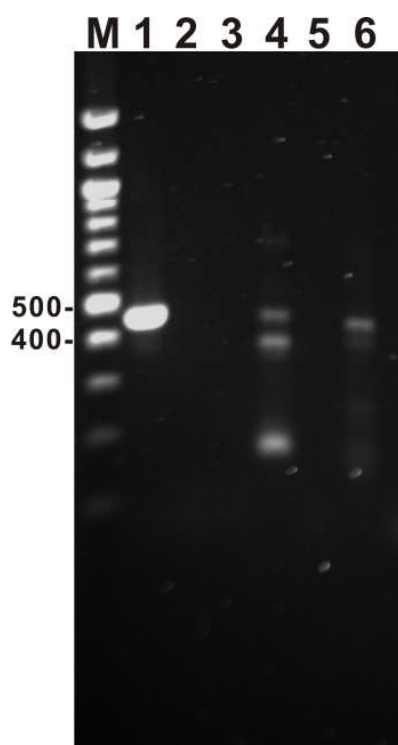
**Figure 4. 4 RT-PCR for *pqsA*, *pqsB* and *pqsC* on 1% (w/v) agarose gel.**

Lane M: DNA standards (100 bp DNA ladder, BioLabs); the numbers indicate the size (bp) of the markers; Lane 1: positive control used primer RTF2 and RTR2; the primers should amplify a 218 bp DNA fragment; Lane 2: negative control included all the primers without template in one reaction; Lane 3: amplified fragment of PqsA using RNA sample and primer PPA0945RTPCRF and PPA0945RTPCRR; Lane 4: amplified fragment of PqsB using RNA sample and primer PPA0946RTPCRF and PPA0946RTPCRR; Lane 5: amplified fragment of PqsC using RNA sample and primer PPA0947RTPCRF and PPA0947RTPCRR. PCR reactions were performed for 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min.

#### **4.4 5' RACE of *pqsA*, *pqsB* and *pqsC***

5' RACE was performed using 5'/3' RACE kit as described by manufacturer (Section 2.19). For PqsA, first strand cDNA was synthesized with primer PPA0945SP1 and then purified using High Pure PCR product purification kit as indicated in the handbook of 5'/3' RACE kit. Following the

purification, the maximum amount of cDNA was used to be tailed with dATP by terminal transferase provided with the kit. Then this tailed cDNA was used as the template in the first PCR reaction. The DNA fragment was amplified by oligo dT-anchor primer and PPA0945SP2. The second PCR was performed using PCR anchor primer and primer PPA0945SP3 and neat sample of the first PCR reaction as DNA template. As shown in Figure 4.5, there was no fragment visible in the first PCR reaction, but after the second PCR reaction, one DNA product which was ~ 450 bp was amplified (Lane 6). The positive control in the first PCR reaction produced a specific product (Lane 1) and there was no DNA amplified in the negative control (Lane 2), which indicated that the first PCR reaction worked well. There were multiple bands detected in the positive control of the second PCR reaction (Lane 4). It also demonstrated that the PCR reaction worked under the conditions used. These multiple bands might have been caused by the suboptimal annealing temperature of the forward primer used in the positive control. However, this annealing temperature was optimal for both primers used in the second PCR reaction of 5' RACE, provided by the evidence that there was one single product of the second PCR reaction (Lane 6). Then this RACE product was gel extracted and cloned into pGEM-T<sup>®</sup> Easy vector. Following the cloning, they were transformed into *E. coli* DH5 $\alpha$ . After being purified from *E. coli*, plasmids were digested with *EcoRI* to confirm the target insert (data not shown). The confirmed plasmids were sent for DNA sequencing to reveal the TSS of PqsA. The details of the revealed DNA sequence are shown in Figure 4.7.



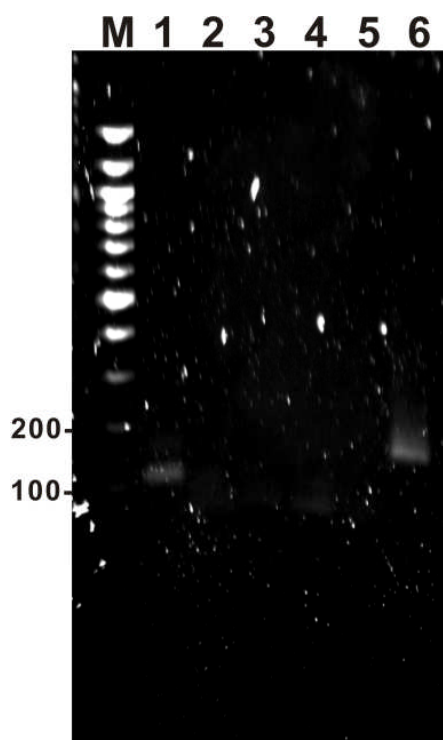
**Figure 4. 5' RACE of *pqsA* on 1% (w/v) agarose gel.**

Lane M: DNA standards (100 bp DNA ladder, BioLabs); the numbers indicate the size (bp) of the markers; Lane 1: positive control for the first PCR reaction using purified cDNA and primer RTF1 and PPA0945SP2; Lane 2: negative control for the first PCR reaction using all the primers without template; Lane 3: the first PCR amplification of 5'RACE of *pqsA* using purified cDNA and oligo dT- anchor primer and primer PPA0945SP2; reactions were performed for 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 1 min; Lane 4: positive control for the second PCR amplification using first PCR amplification as template and primer RTF1 and PPA0945SP3; Lane 5: negative control for the second PCR amplification using all the primers without template; Lane 6: the second PCR amplification of 5' RACE of *pqsA* using the first PCR reaction as template and PCR anchor primer and primer PPA0945SP3; reactions were performed for 30 cycles of 95 °C for 30 sec, 53 °C for 30 sec and 72 °C for 1 min.

The 5' RACE for PqsB was performed using the same strategy as for PqsA, but only the gene specific primers used for amplification were different.

The first strand cDNA was synthesized by primer PPA0946SP1 and purified. Then the same primer was used together with oligo dT-anchor primer to amplify the first DNA fragment of PqsB in the first PCR reaction. The second PCR reaction was done using primer PPA0946SP2 and PCR anchor primer. After two staggered PCR amplifications, a fragment of ~ 150 bp DNA was detected by the agarose gel electrophoresis (Figure 4.6, lane 6) and no amplification could be detected in the first PCR reaction (Lane 3). Both positive controls in the first and second PCR showed a specific DNA product after the amplification (Lane 1 and 4, respectively) and no DNA fragment detectable in both negative controls (Lane 2 and 5, respectively) indicating that both PCR reactions worked well. Following cloning into pGEM-T<sup>®</sup> Easy vectors and the transformation, plasmids were sent for DNA sequencing. The details of the DNA sequence revealed are shown in Figure 4.7.





**Figure 4. 6 5' RACE of *pqsB* on 1% (w/v) agarose gel.**

Lane M: DNA standards (100 bp DNA ladder, BioLabs); the numbers indicate the size (bp) of the markers; Lane 1: positive control for the first PCR reaction using purified cDNA and primer PPA0946F and PPA0946SP1; Lane 2: negative control for the first PCR reaction using all the primers without template; Lane 3: the first PCR amplification of 5'RACE of PqsB using purified cDNA and oligo dT- anchor primer and primer PPA0946SP1; reactions were performed for 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min; Lane 4: positive control for the second PCR amplification using first PCR amplification as template and primer PPA0946F and PPA0946SP2; Lane 5: negative control for the second PCR amplification using all the primers without template; Lane 6: the second PCR amplification of 5' RACE of PqsB using the first PCR reaction as template and PCR anchor primer and primer PPA0946SP2; reactions were performed for 30 cycles of 95 °C for 30 sec, 52 °C for 30 sec and 72 °C for 1 min.

The DNA sequencing results showed the same TSS for both *pqsA* and *pqsB* (Figure 4.7). For the 5' RACE for *pqsA*, the result revealed that the TSS

was 238 bp upstream of the putative start codon of *pqsA*, which was 'A' in the DNA sequence. According to the genomic database, this 'A' is part of an 'ATG', which is the third amino acid residue in the predicted protein sequence of *pqsB*. Although different primers were used, the gene specific primer of *pqsB*, the 5' RACE result for *pqsB* revealed the same 'A' for the TSS. These results of *pqsA* and *pqsB* were confirmed by several different DNA sequencing results of different plasmids (data not shown). This demonstrated that PqsA and PqsB are transcribed together from the same TSS. These two genes are also related to each other. This result also suggested that the 'ATG', that encoding the third predicted amino acid residue, was the true start codon of *pqsB*, not as predicted in the genomic database. Furthermore, this result showed that the TSS of *pqsB* was overlapped with the start codon. This kind of mRNA is referred as leaderless mRNA (Moll *et al.*, 2002). As revealed, there was no ribosomal binding site for *pqsB*.

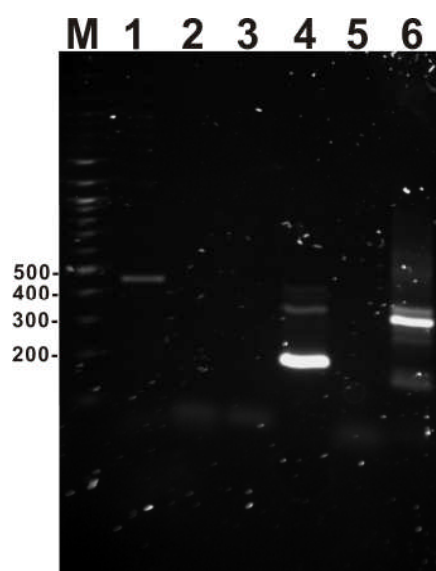


**Figure 4. 7 DNA sequencing result of 5' RACE of *pqsA* and *pqsB*.**

The DNA sequence in green and orange colour represents the sequence of *pqsB* and *pqsA*, respectively. The predicted start and stop codon for each gene is underlined and highlighted with the arrow indicating the transcriptional direction. The blue rectangle circles the primer PPA0945SP3 or PPA0946SP2 binding sites in the cDNA synthesis. The identified TSS for *pqsA* and *pqsB* is circled in red colour and the identified true start codon of *pqsB* is also underlined in red colour.

For *pqsC*, the first strand cDNA was synthesized using primer PPA0947SP1. Primers PPA0947SP2 and PPA0947SP3 were used to amplify the DNA fragment in the first and second PCR, respectively. After the second PCR amplification, a ~ 300 bp product was amplified for the 5' RACE reaction (Figure 4.8, lane 6). And there was no detectable amplification in the

first PCR reaction (Lane 3). The positive control in the first PCR showed a single product around 450 bp (Lane 1). The one in the second PCR amplification had a significant product ~200 bp and a fainter band of ~350 bp (Lane 4). The non-specific product may be because the annealing temperature was not optimal for the primer PPA0947F. No amplification detected in both negative controls (Lane 2 and 5) suggested that both PCR reactions should be specific for the used primers.



**Figure 4. 8 The first and second PCR amplifications of 5' RACE of *pqsC*.**

Lane M: DNA standards (100 bp DNA ladder, BioLabs); the numbers indicate the size (bp) of the markers; Lane 1: positive control using purified cDNA as template and primer PPA0947F and PPA0947SP2; Lane 2: negative control using all the primers without template; Lane 3: the first PCR amplification of 5' RACE of *pqsC*; reactions were performed for 30 cycles of 95 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min. Lane 4: positive control using first PCR amplification as template and primer PPA0947F and PPA0947SP3; Lane 5: negative control using all the primers without template; Lane 6: the second PCR amplification of 5' RACE of *pqsC*; reactions were performed with 30 cycles of 95 °C for 30 sec, 51 °C for 30 sec and 72 °C for 1 min.

The same procedures of gel extraction, cloning, transformation and plasmid purification were followed to prepare the plasmids for DNA sequencing. After confirmation of the correct insert by restriction digest, they were sent to the sequencing service. The DNA sequencing result of *pqsC* revealed the 'G' as the TSS, which was 116 bp upstream of the start codon of *pqsC* (Figure 4.9). This result has been confirmed by several different sequencing reactions of different plasmids from the 5' RACE reactions of *pqsC* (data not shown).

GCATGCGAGGATGGGAAGTAATGTGAGGTTGACAAGATACTTCGCGTCGC  
 AGGCGTTATATGTCTGATCGTTGGCGCCGCGCGATCACCGTGGGTGAGGA

GGTTCACCTGGGCTCAGCAGCGCCCAACGTGAGTGTAAGGAGGAGTGATG  
 TCAGTTCGACTCGTGGTTACTGACGATCATCCGATCATGAGGCAGGCTCTT  
 GCCGGCTATTTCTCTCGTGTTGAGGAGATGGAGGTTGTCGGCCAGGCCACT  
 AATGGTCGTGAAGCCGTTGAGCTTGTTGATCAGCTATTGCCCGACGTTGTC

CTTATGGACCTCAAGATGCTGAAATGGACGGTCTCACAGCTACCCGAATG  
 ATCGTC.....

putative start codon

primer PPA0947SP3 binding

**Figure 4. 9 DNA sequencing result of 5' RACE of *pqsC*.**

The DNA sequence of *pqsC* is shown in purple with the start codon underlined and highlighted (The DNA sequence is shown in reverse order to Figure 4.7). The arrow above the start codon indicates the transcription direction. The identified TSS of PqsC is underlined and circled in red colour. The primer PPA0947SP3 binding sites are shown in blue rectangles.

#### **4.5 Identification of the promoter sequence of *pqsA*, *pqsB* and *pqsC***

After the TSS of each gene was determined by modified 5' RACE protocol, possible promoter sequence of *pqsA*, *pqsB* and *pqsC* was identified by comparing the DNA sequences around the -10 and -35 region with the corresponding DNA sequence in *E. coli*. As shown in Figure 4.10, the possible promoter sequence of the co-transcribed PqsA and PqsB was 'TTGtgg' and 'TAcAt' around -35 and -10 region, respectively (conserved positions are shown in upper case letters). 'TTGtgg' was located between positions -12 to -7, while 'TAcAt' was located between positions -36 to -31. All the highly conserved positions were identical to the ones in *E. coli* providing evidence that the identified sequences were very likely the true promoter sequence of *pqsA* and *pqsB*. Using the same method, the promoter sequence of *pqsC* was identified. Around the -35 region, the sequence was Tgctgc with only one conserved residue compared with *E. coli* (conserved position is shown in upper case letters). This sequence was located between positions -37 and -32. The sequence 'TAatgT' starting from position -12 to -7 was identified as the promoter sequence around -10 region (conserved positions are shown in upper case letters). This sequence contained all the conserved positions compared to *E. coli*. However, no promoter could be found by searching the upstream sequence of the TSS for each gene in the bacterial promoter database BROM (<http://linux1.softberry.com/berry.Phtml?topic=bprom&group=programs&subgroup=gfindb>).

**A**

CTGCACGTGCTAGCCTCCTCGCATCACTTTTGCCTGACCGTTGTGGAGGGC  
predicted -35 region

CCCTCAAAGGAGATACATTGTGCCATGAAGAAGATCATCACCGGCATCGT  
predicted -10 region putative start codon

GGCTGTCTTAGCAATCAGCTCCTTCTCGGTGCGCGTCCCCGCTCAGGCCAC  
 CACTGACTCCGCCCCACTCAGAACGCTGTGAATACGCAACCCAAGGCCA  
 TGTTAAGGGAATCATAGACGGCCTTTGCACCCCTTACC CCGACTGGTGTTT  
putative stop codon

TAACTGATCCCGCGCCTATGCCCAATGCATGGGCTGTCTAACACGCGGAG  
putative start codon

AACGCATCTAATGAATAGTGGTTCGCCTGGCCGTAGCTTCATCTTCAA.....  
putative start codon

**B**

putative -35 region putative -10 region

ACTGCTGCGGCATGCGAGGATGGGAAGTAATGTGAGGTTGACAAGATAC

TTCGCGTCGCAGGCGTTATATGTCTGATCGTTGGCGCCGCGGATCACCG

TGGGTGAGGAGGTTCACTTGGGCTCAGCAGCGCCCAACGTGAGTGTAAG

putative start codon

GAGGAGTGATGTCAGTTCGACTCGTGGTACTGACGATCATCCGATCA.....  
putative start codon

**Figure 4. 10 Putative promoter sequences of *pqsA*, *pqsB* and *pqsC*.**

(A) Identified putative promoter sequences of *pqsA* and *pqsB*. The DNA sequences in green and orange colour represent the sequence of *pqsB* and *pqsA*, respectively. The start and stop codon for each gene is underlined and highlighted with the arrow indicating the transcriptional direction. The identified putative promoter sequences are shown in blue. (B) Identified putative promoter sequence of *pqsC*. The DNA sequence of *pqsC* is shown in purple with the start codon underlined and highlighted. The arrow above the start codon indicates the transcription direction. The identified putative promoter sequences are shown in blue.

## 4.6 Discussion

The 5' RACE experiment revealed the same TSS for PqsA and PqsB, and this TSS overlapped with the start codon of PqsB. It firstly suggests that PqsB was translated from a leaderless mRNA, which does not contain conventional ribosomal binding site. The leaderless mRNAs found so far are either preceded by only a few nucleotides or start directly with a 5'-terminal AUG (Moll *et al.*, 2002). In the last decade, many bacterial genomes have been sequenced, but only about 35-40 leaderless mRNAs have been identified with certainty. They appear to be rather frequent in Gram-positive bacteria, as they have been found in many Gram-positive genera such as *Streptococci*, *Lactococci*, *Streptomyces* and *Corynebacterium* (Moll *et al.*, 2002). They are also relatively abundant in some eubacterial species, which seems to correlate with the absence of a functional homologue of ribosomal protein S1. The leaderless mRNA is quite common in archaea judging from the complete genome sequence of some archaea. For instance, an analysis of 144 genes of the crenarchaeal *Sulfolobus solfataricus* revealed that genes encoded by monocistronic transcripts, as well as genes located at the 5'-proximal end of operons, lack a Shine-Dalgarno (SD) sequence and generally have little or no sequence upstream of the translational starts (Tolstrup *et al.*, 2000). It is commonly believed that the translational efficiency of prokaryotic mRNAs is intrinsically determined by both primary and secondary structures of their translational initiation regions (Moll *et al.*, 2002). However, for leaderless mRNAs starting with the AUG initiating codon occurring in bacteria, archaea and eukaryotes, there is no evidence for ribosomal recruitment signals downstream of the 5'-terminal AUG that seems to be the only necessary and constant element (Moll *et al.*, 2002). Binding of



leaderless mRNAs to the ribosome is dependent on the presence of the initiator tRNA. Studies with *E. coli* revealed that the ratio of initiation factor (IF)2 to IF3 plays an important role in translation initiation of leaderless mRNA, indicating that the translational efficiency of this mRNA class can be modulated depending on the availability of components of the translational machinery (Laursen *et al.*, 2005). It was suggested that leaderless mRNA is recognized by a 30S-IF2-fMet-tRNA<sub>f</sub><sup>Met</sup> complex equivalent to that formed during translation initiation in eukaryotes (Grill *et al.*, 2001). However, it remains to be established whether translational control of leaderless mRNAs in Gram-positive bacteria is governed by the same mechanisms as in *E. coli*. Moreover, the faithful translation of leaderless mRNAs in heterologous systems shows that the ability to translate leaderless mRNA is an evolutionary conserved function of the translational apparatus (Jenssen, 1993). Although only about 35-40 leaderless mRNAs have been identified with certainty in bacteria, it is predicted that the number of leaderless mRNAs will increase considerably in the future (Moll *et al.*, 2002).

Secondly, this result also suggests that PqsA and PqsB must share transcriptional regulation as they are transcribed by the same promoter. As it has been demonstrated that PqsA and PqsC constitute a legitimate pair of a TCS (Chapter 3), this result further provides the evidence that PqsB is also related to this unique TCS. As identified by bioinformatics analysis, PqsB is a signalling peptide which could be cleaved, modified and exported out of the membrane, this result also suggested that PqsB could be a QS signal for this unique TCS. Although no promoter could be found using the bacterial promoter database, putative promoter sequences were hypothesised by comparing the sequences around -35 and -10 region with those in *E. coli*

(Figure 4.10). This indicates that the promoter of PqsA, PqsB and PqsC may belong to a family that yet has not been identified. The recognition between the promoter and the cognate sigma factor is dependent on the accuracy of the promoter sequence. Sigma 70 class promoters have the key recognition around the -35 and -10 region, while promoters recognized by the sigma N holoenzyme contain conserved elements near -24 and -12 (Wigneshweraraj *et al.*, 2008). In addition to these core promoter elements, other sequences throughout the extended promoter recognition region can have a large influence on promoter strength (Ross & Gourse, 2009). For instance, the region upstream of the -35 element can contain phase A- and T-rich sequences which provide a site of interaction for the carboxyl-terminal domain of the two alpha-subunits (Estrem *et al.*, 1998; Ross *et al.*, 1993). This upstream promoter element can increase promoter strength by 100-fold or more (Helmann, 2009). In *E. coli*, upstream promoter elements are also found as part of many different promoters and these elements are common in other bacteria (Ross *et al.*, 1998; Helmann, 1995). In other cases, the region upstream of the -35 recognition element may instead serve as a binding site for positively acting transcription factors (Dove *et al.*, 2003). The region between the core promoter elements may also be involved in the promoter strength. One class of sigma 70 promoters contains an 'extended' -10 element that includes important recognition sequences for sigma, upstream of the conventional -10 element (Helmann, 2009). It can compensate or even substitute for a poor -35 region (Mitchell *et al.*, 2003). In addition, the region between the -10 element and +1 is known as the discriminator sequence and can influence promoter properties and strength (Haugen *et al.*, 2006).

Finally, as revealed in this study, the TSS of PqsB was the 'A' in an 'ATG', which was predicted as the third amino acid residue from the start codon in the database (Bruggemann *et al.*, 2004). And this 'ATG' also encodes a start codon. This indicated that the previously predicted start codon of PqsB was not correct. The third amino acid, which was ATG, was the true start codon of PqsB and the genome database should be amended.

For PqsC, the modified 5' RACE revealed that its TSS was 116 bp upstream of the start codon. Compared to PqsA and PqsB, this result suggested that PqsC was a canonical mRNA, which contains the ribosomal binding site. The control of translation initiation of a canonical mRNA has been studied in more detail than that in leaderless mRNA. Bacterial ribosomes already initiate translation during transcription, therefore, translation initiation is the rate-limiting and most highly regulated phase of the four phases in protein biosynthesis (Laursen *et al.*, 2005). The ribosome, fMet-tRNA<sub>f</sub><sup>Met</sup>, mRNA and three protein initiation factors IF1, IF2 and IF3 are involved in the translation initiation phase. In the translation initiation phase, the bacterial 70S ribosome composed of a large 50S and a small 30S subunit is promoted to subunit dissociation by the binding of IF3 to the 30S (Petrelli *et al.*, 2001). IF1 stimulates the activities of IF3 and hence also the dissociation of the ribosomal subunits (Gualerzi & Pon, 1990). The SD sequence of canonical mRNAs interacts with the anti-SD sequence of the 16S rRNA and the initiation codon is adjusted in the peptidyl tRNA binding site by the initiation factors (Yusupova *et al.*, 2001; La Teana *et al.*, 1995). The 30S preinitiation complex, consisting of the 30S ribosomal subunit, three initiation factors and mRNA in a standby position where fMet-tRNA<sub>f</sub><sup>Met</sup> is bound in a codon-independent manner, is relatively unstable and undergoes a

rate-limiting conformational change (Pon & Gualerzi, 1984). This promotes the codon-anticodon interaction and forms the more stable 30S initiation complex. IF2 stimulates association of the 50S ribosomal subunit to the complex, while IF1 and IF3 are ejected (Laursen *et al.*, 2005). Then IF2 is released from the complex and initiator fMet-tRNA<sub>f</sub><sup>Met</sup> is adjusted to the correct position in the peptidyl tRNA binding site (Laursen *et al.*, 2005). The newly formed 70S initiation complex, which holds fMet-tRNA<sub>f</sub><sup>Met</sup> as a substrate for the peptidyltransferase centre of the 50S ribosomal subunit, is ready to enter the elongation phase of translation.

## **Chapter V Gene expression analysis of putative signalling peptide, sensor histidine kinase and response regulator**

### **5.1 Chapter introduction**

This chapter describes the use of quantitative RT-PCR to determine the expression of *pqsA*, *pqsB* and *pqsC* during the growth of *P. acnes*. Gene expression is the process by which a gene's DNA sequence is converted into the functional proteins of the cell. Several steps in the gene expression process may be regulated including transcription, mRNA degradation, translation and post-translational modification. Gene expression profiling is the simultaneous measurement of the cellular levels of the mRNA of many different genes. The regulation of gene expression is vital to bacterial growth and adaptability. It allows bacteria to receive environmental signals and react accordingly. By analysing differential gene expression, the mechanisms involved in the response to the environment can be understood. The gene expression pattern of several genes under certain conditions could also reveal the relationship among these genes (Fiedler *et al.*, 2010). Therefore, in this chapter gene expression analysis was used to investigate the relationship among PqsA, PqsB and PqsC during growth and to find out whether these genes are regulated. Moreover, it also revealed how gene expression changed during the growth of *P. acnes*.

Reverse transcription followed by PCR represents a powerful tool for the detection and quantification of mRNA. Quantitative RT-PCR distinguishes itself from other methods available for gene expression analysis because of its high sensitivity, accuracy, good reproducibility and fast results (Derveaux *et al.*, 2010). It relies on measuring the increase in fluorescent signal, which is

proportional to the amount of DNA produced during each PCR cycle. Individual reactions are characterized by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence (Nolan *et al.*, 2006). It is also referred to as a parameter known as the threshold cycle ( $C_t$ ) (Nolan *et al.*, 2006). The more target there is in the starting material, the lower the  $C_t$ . This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays (Nolan *et al.*, 2006).

Generally there are two methods for the quantitative detection of the amplicon established: the gene-specific probes or specific double strand DNA binding agents based on fluorescence resonance energy transfer such as TaqMan (Applied Biosystems) and the non-specific fluorescent intercalating double strand DNA binding dyes such as SYBR green (Pfaffl, 2004). For researchers who are new to using quantitative RT-PCR techniques, SYBR green is a common choice as it is cost effective and easy to use (Morrison *et al.*, 1998). It displays relatively low fluorescence when it is free in solution, but over 1000-fold higher fluorescence when it is bound to double-stranded DNA (Morrison *et al.*, 1998). This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescent signal. The only limitation of the SYBR green assay is the inherent non-specificity of this method. It is possible that the measured fluorescence may include signal contamination of non-specific product resulting in artificially early  $C_t$  values and giving an inaccurate representation

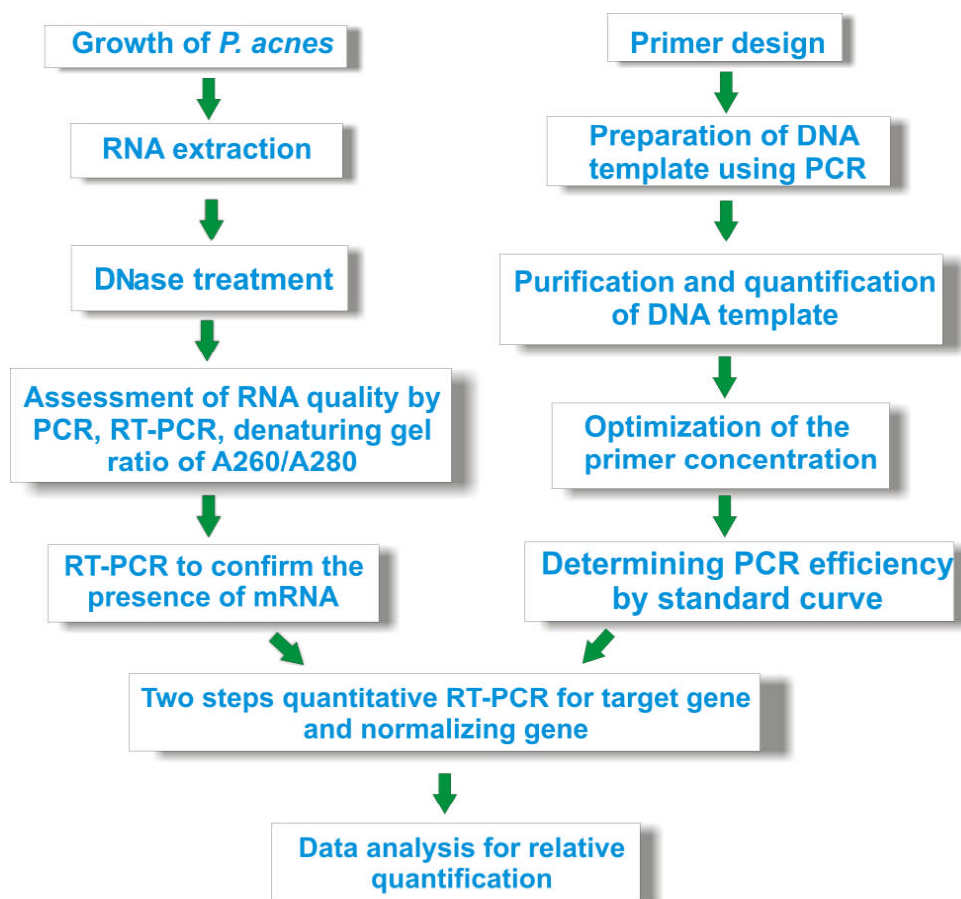
of the true target concentration (Introduction to quantitative PCR, Stratagene). Both non-specific and specific detection produce comparable dynamic range and sensitivity, while SYBR green detection is more precise and produces a more linear decay plot than the TaqMan probe detection (Schmittgen *et al.*, 2000). It has been demonstrated that for single PCR product reactions with well-designed primers, SYBR green can work extremely well, with spurious non-specific background only showing up in very late cycles (Pfaffl, 2001). Also, the non-specific product can be detected easily and reliably by performing melting curve analysis on the PCR products from every run. Moreover, compared to TaqMan detection, SYBR green is easier to use, especially for new users (Introduction to quantitative PCR, Stratagene). Therefore, SYBR green was used in this study.

There are also two basic quantification methods and each is suitable for different applications: absolute quantification and relative quantification. This study used the relative quantification method. Relative quantification measures the relative change in mRNA expression levels and the majority of scientific questions regarding gene expression can be answered by relative quantification (Pfaffl, 2004). Relative quantification is measured using the relative concentration of the gene of interest in unknown samples compared to a calibrator or control sample. The calibrator is a baseline for the expression of a given target gene. In this case, differences in  $C_t$  value between an unknown sample and the calibrator are expressed as fold-changes relative to the calibrator. In addition to comparing the expression of the target gene alone in a control versus experimental sample, the result can be normalised with a normalising target, typically a gene whose expression is constant in both the calibrator and experimental samples

(Introduction to quantitative PCR, Stratagene). The commonly used normalizing gene is a housekeeping gene whose expression should be constant under the experimental conditions of the assay (Pfaffl, 2004). To investigate the changes in the level of gene expression of *pqsA*, *pqsB* and *pqsC* during growth, DNA gyrase A (r-PPA0010) was used as the normalizing gene for this study.

In this chapter, SYBR Green was used to measure the relative mRNA levels of *pqsA*, *pqsB* and *pqsC* compared with the reference gene – r-PPA0010 during the growth of *P. acnes*. It describes the preparation of RNA sample, determination of PCR reaction efficiency and relative quantification of transcript of the target gene. As for strategies shown in Figure 5.1, generally, total RNA samples were harvested during the growth of *P. acnes*. Following the extraction, samples were prepared by digesting the possible DNA contamination using TURBO<sup>TM</sup> DNase. Meanwhile, primers were designed and DNA template that was used for the primer optimization was generated. After determining the PCR efficiency by standard curve, real-time RT PCR was performed for all genes including *pqsA*, *pqsB*, *pqsC* and r-PPA0010. Relative quantification of *pqsA*, *pqsB* and *pqsC* during *P. acnes* growth was obtained by comparing the gene expression of these genes to the gene expression of r-PPA0010.





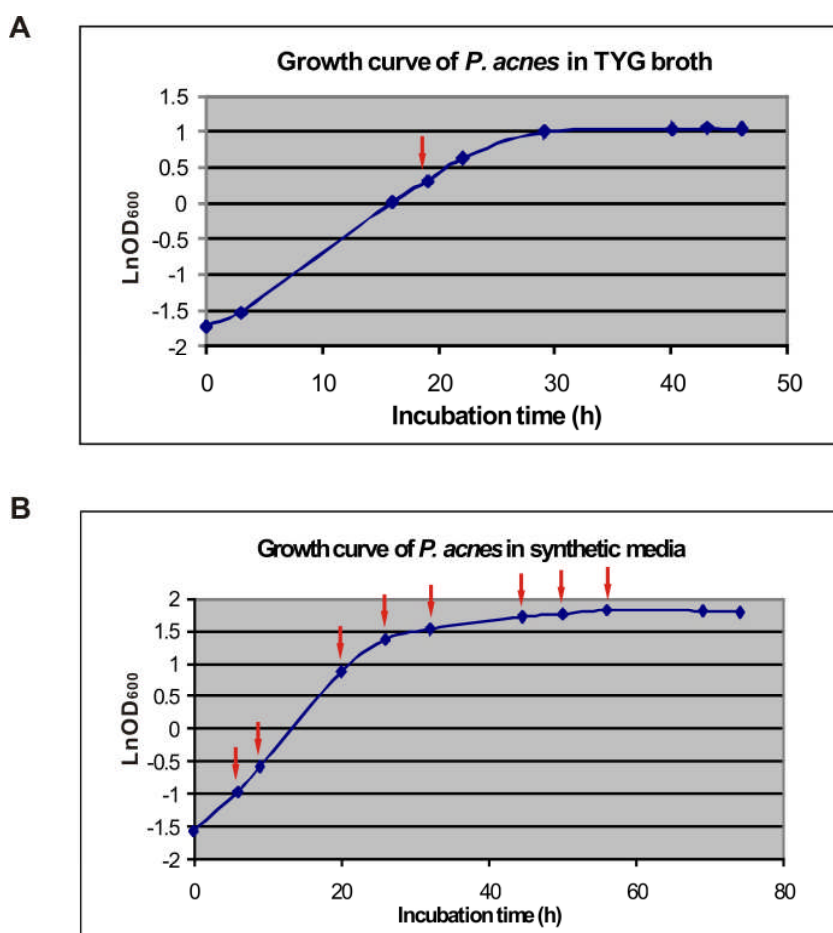
**Figure 5. 1** The strategy used for gene expression analysis of *pqsA*, *pqsB* and *pqsC*.

*P. acnes* was grown in synthetic medium for RNA isolation. Following the RNA extraction, samples were treated with TURBO™ DNase and the absence of DNA was verified by PCR, RT-PCR and denaturing gel. Meanwhile, primers were designed and DNA template was generated by PCR. Following the purification and quantification, primers were optimized using the DNA template. Then the efficiency of PCR reactions was determined by the optimized primer and purified DNA template. Finally, two steps of quantitative RT-PCR were performed for each target gene and the reference gene followed by the data analysis for relative quantification.

## **5.2 Preparation of RNA samples of *P. acnes***

### **5.2.1 Growth of *P. acnes* in synthetic media**

To be able to more easily associate changes in gene expression with changes in a particular growth parameter(s), synthetic medium (18 AA) was used (Holland *et al.*, 1979). As a control, cells were also grown in standard TYG broth. Figure 5.2 shows the growth curves of *P. acnes* in both media. During the growth of *P. acnes* in the synthetic medium, cells were taken, treated with phenol to stop cellular metabolism and harvested for RNA isolation. The time when cells were harvested covered from early exponential, mid-exponential, late-exponential/early stationary and stationary phase. Specifically, cells were harvested at 6, 9, 20, 26, 32, 44.5, and 50 hours after inoculation. Harvested cells were immediately used for RNA extraction.



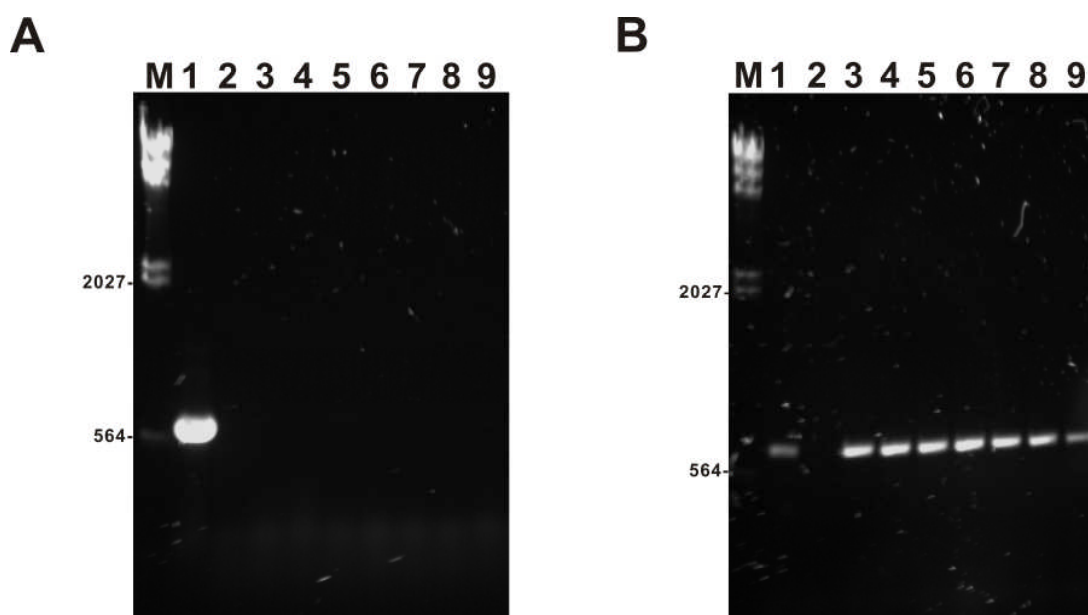
**Figure 5. 2 Growth curves of *P. acnes* in TYG and synthetic media.**

(A) Growth curve of *P. acnes* in 100ml TYG broth. The red arrow indicates the time when cells were harvested and used for inoculating the following synthetic medium. (B) Growth curve of *P. acnes* in 400 ml synthetic medium. Red arrows indicate times when cells were harvested for RNA extraction.

### 5.2.2 Preparation of RNA sample for real-time RT-PCR

RNA in each of the harvested samples was extracted, treated with TURBO™ DNase and purified as described previously (Section 2.16.1 and 2.16.3). PCRs were performed to confirm that DNA was not a significant contaminant (Figure 5.3, panel A). The primers used amplified a segment of PqsC. The template for the positive control was *P. acnes* genomic DNA (Lane 1). Template was omitted from one reaction to provide a negative

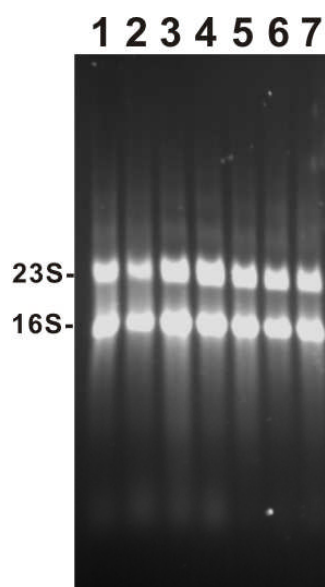
control (Lane 2). No DNA fragment was amplified in any RNA sample indicating that the RNA samples were free of DNA contamination (Lane 3 to 9). Then RT-PCRs were performed using the same primer pair for all RNA samples to confirm the presence of mRNA. The positive and negative controls were the same as in the PCRs. As shown in Figure 5.3, panel B, amplicons of the expected size were detected for all the RNA samples suggesting that mRNA was present in every RNA sample.



**Figure 5. 3 Verification of RNA samples by PCRs and RT-PCRs.**

(A) Confirmation of DNA-free RNA samples by PCRs. Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1: positive control used primer PPA0947F and PPA0947R and genomic DNA of *P. acnes* as DNA template; Lane 2: negative control used all the primers without DNA template; Lane 3-9: PCRs used RNA sample harvested at 6, 9, 20, 26, 32, 44.5 and 50 hours incubation, respectively, as DNA template; reactions were performed for 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. (B) Confirmation of the presence of mRNAs in RNA samples by RT-PCRs. Lane M: DNA standards; Lane 1: positive control positive control used primer PPA0947F and PPA0947R and genomic DNA of *P. acnes* as DNA template; Lane 2: negative control used all the primers without DNA template; Lane 3-9: RNA sample harvested at 6, 9, 20, 26, 32, 44.5 and 50 hours incubation, reactions were performed under the same conditions as in panel A.

The RNA samples were then run on a denaturing gel to check their integrity. The bands corresponding to 23S and 16S rRNA indicated that the RNA had not been degraded by contaminating nucleases and was thus suitable for gene expression analysis by quantitative RT-PCR (Figure 5.4).



**Figure 5. 4 Verification of RNA samples for integrity by denaturing gel.**

Lane 1 to 7: RNA sample harvested at 6, 9, 20, 26, 32, 44.5 and 50 hours incubation, respectively.

Verified RNA samples were quantified as described in section 2.11. All RNA samples were successfully extracted from *P. acnes* with high concentration (Table 5.1). The A260/280 ratio of each sample was around 2 indicating that each sample was in good quality.

**Table 5. 1 Measurement of the concentration for each RNA sample.**

<b>Sample ID</b>	<b>ng/ul</b>	<b>A260</b>	<b>A280</b>	<b>260/280</b>
<b>6h</b>	221.15	5.529	2.728	2.03
<b>9h</b>	251.39	6.285	3.156	1.99
<b>20h</b>	174.46	4.361	2.253	1.94
<b>26h</b>	266.31	6.658	3.353	1.99
<b>32h</b>	260.72	6.518	3.324	1.96
<b>44.5h</b>	266.09	6.652	3.331	2
<b>50h</b>	261.86	6.546	3.34	1.96
<b>56h</b>	245.34	6.133	3.065	2
<b>69h</b>	264.75	6.619	3.32	1.99
<b>74h</b>	172.16	4.304	2.192	1.96

## **5.3 Primer optimization**

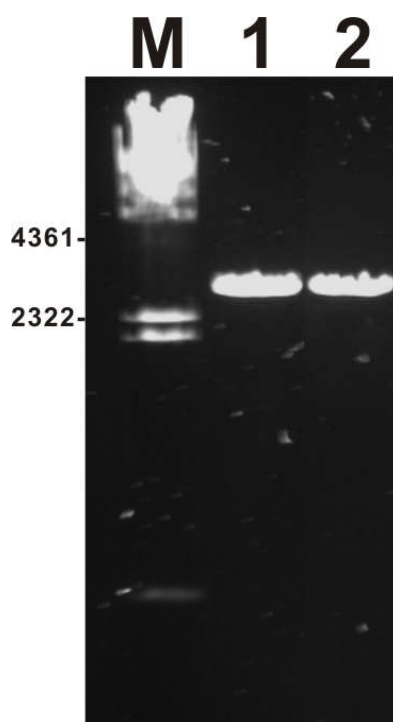
### **5.3.1 Primer design**

Primers used for quantitative RT-PCR were designed as described in section 2.9. Each primer was engineered to contain between 19-21 nucleotides with an annealing temperature of around 60°C and to amplify a DNA fragment between 80-90 base pairs. Primers were further checked by online servers for possible secondary structure. For each primer, the specificity was examined using BLAST to identify its binding site in the *P. acnes* genome. All the primers were HPLC purified.

### **5.3.2 Preparation of DNA template F1 and F2**

DNA fragments used for primer optimization were amplified by PCRs as described in section 2.20.1. Primers were designed to amplify a fragment, designated F1, that contains *pqsA*, *pqsB* and *pqsC* and another fragment, designated F2, that contains r-PPA0010. F1 was used for optimization of

primers for *pqsA*, *pqsB* and *pqsC*, while F2 was used to optimize primers for r-PPA0010. The expected sizes of the corresponding amplicons were 2,419 bp and a 2,395 bp product, respectively (Figure 5.5). Amplified fragments were then purified as described in section 2.14.1. Following quantification, F1 and F2 were diluted with RNase-free water to a concentration of  $10^8$  copies/ $\mu$ l. These stocks were then diluted serially using RNase-free water containing yeast tRNA at a concentration of 100 ng/ $\mu$ l. Concentrations of F1 and F2 were produced at  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2$  copies/ $\mu$ l.



**Figure 5. 5 Amplification of DNA template F1 and F2.**

Lane M: DNA standards ( $\lambda$  DNA/*Hind* III fragments, Invitrogen); the numbers indicate the size (bp) of the markers; Lane 1: amplified DNA template F1 containing HK-PPA0945, SP-PPA0946 and RR-PPA0947. Lane 2: amplified DNA template F2 containing full length r-PPA0010.



### 5.3.3 Optimization of primer concentration

The PCR conditions for each of the target gene were optimized as described in section 2.20.1. F1 and F2 were used as the template at the concentration of  $10^4$  copies/ reaction. The final concentration of primers was varied between 100 nM to 400 nM and melting curves were analysed for evidence of non-specific product. The primer concentrations that produced the lowest  $C_t$  (without evidence of non-specific product) were selected as the optimal combination of primers (Table 5.2).

**Table 5. 2 Optimized primer concentration used in quantitative RT-PCR.**

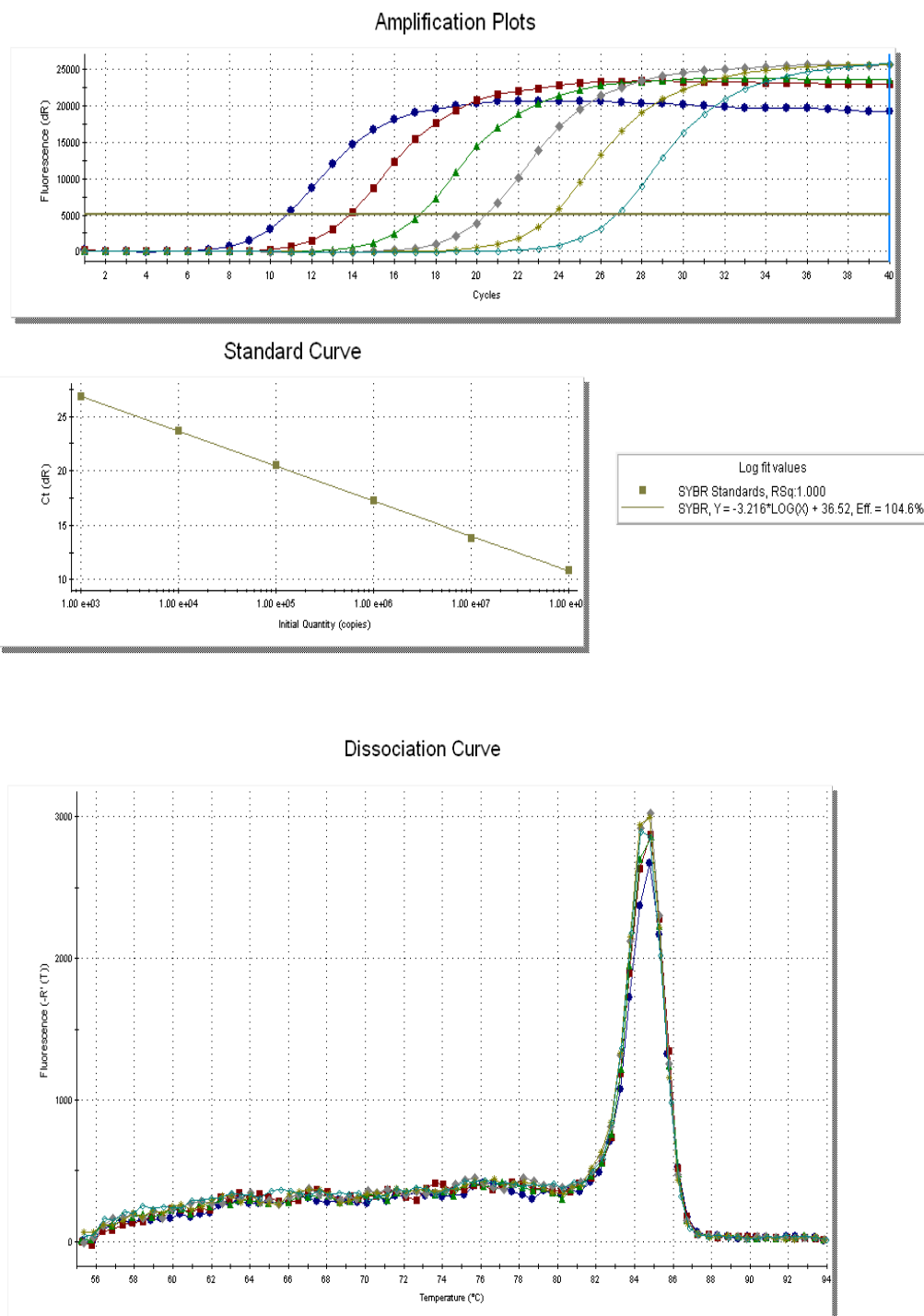
	Forward and reverse primer (nM)
<b>PqsA</b>	400 and 300
<b>PqsB</b>	200 and 400
<b>PqsC</b>	400 and 400
<b>r-PPA0010</b>	300 and 300

### 5.4 Determination of PCR efficiency of *pqsA*, *pqsB*, *pqsC* and r-PPA0010

The efficiency of the PCR reaction for each target gene was determined by performing standard curved analysis using quantitative RT-PCR. For PqsA, PqsB and PqsC, six serial dilutions of F1 ranging from  $10^8$  to  $10^3$  copies/ reaction were used as DNA template with each of the primers at the optimal concentration (Table 5.2). For r-PPA0010, six serial dilutions of F2 ranging from  $10^7$  to  $10^2$  copies/ reaction were used as DNA template. The standard curve was generated by plotting the initial quantity of each DNA

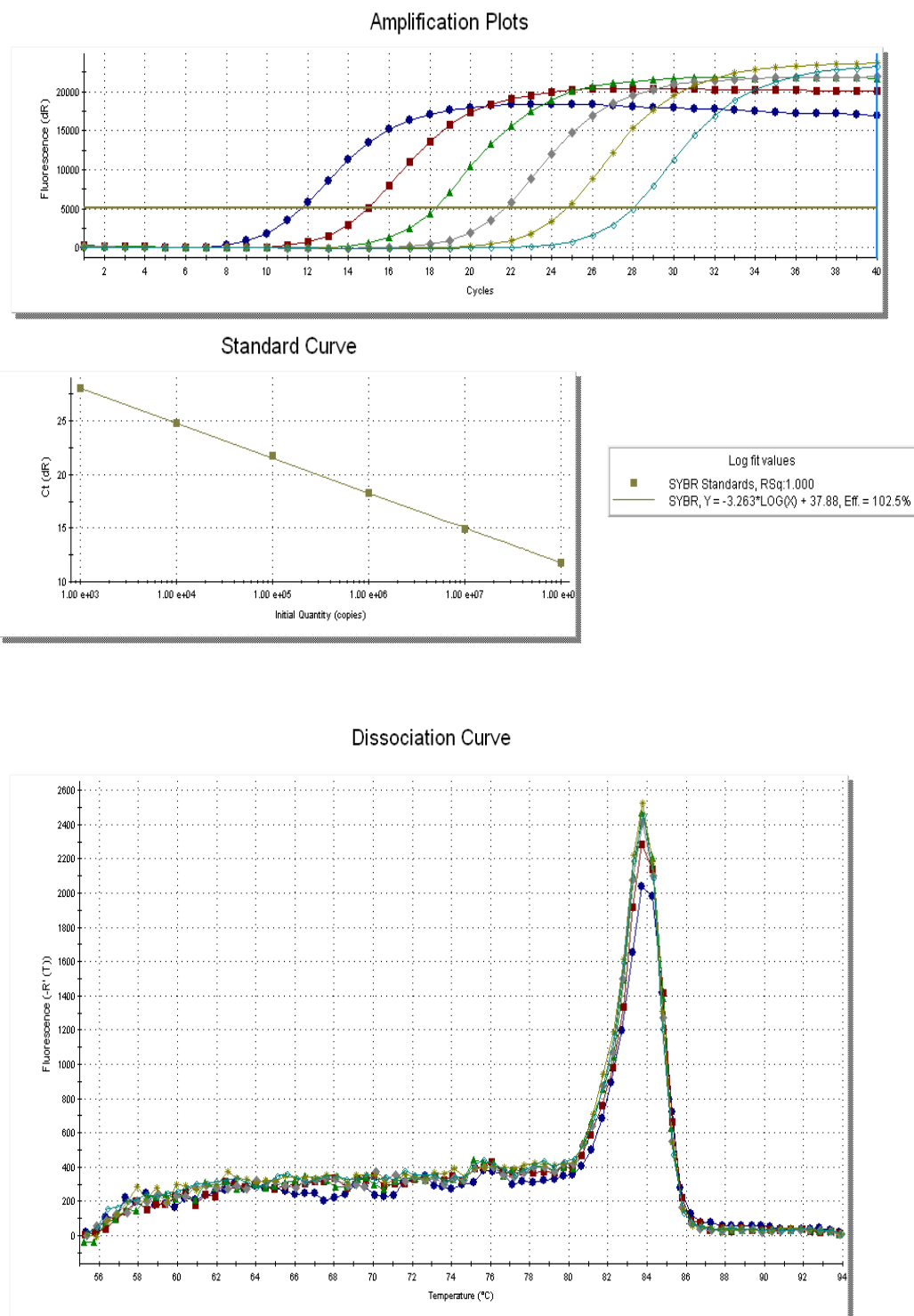
template against  $C_t$ . The PCR efficiency and coefficient of determination (Rsq) of each PCR reaction were automatically calculated by the MxPro QPCR Software provided by the Mx3000P QPCR system (Stratagene).

For *pqsA*, the PCR efficiency was 104.6% with a slope of -3.216 (Figure 5.6).  $C_t$  values obtained from each serial dilutions of F1 were listed in Table 5.3. All the Rsq for each serial dilutions were 1. Theoretically the PCR reaction is based on exponential amplification, the standard curve should generate a straight line. Practically, a linear fit with a slope between approximately -3.1 and -3.5 or a reaction efficiency between 90% to 110% is acceptable for most applications requiring accurate quantification (Introduction to quantitative PCR, Stratagene). Moreover, the melting curve showed a single peak in the plot indicating one specific product for the PCR amplification of *pqsA*. Therefore, the generated standard curve of *pqsA* was acceptable for the quantification of *pqsA* transcripts. The same was found for *pqsB*, *pqsC* and r-PPA0010. The PCR efficiency of *pqsB*, *pqsC* and r-PPA0010 was calculated for 102.5%, 99.7% and 99.2%, respectively (Figure 5.7, 5.8 and 5.9), while the corresponding slope was -3.263, -3.328 and -3.342, respectively (Table 5.4, 5.5 and 5.6). The melting curves of *pqsB*, *pqsC* and r-PPA0010 indicated that the level of any non-specific product was insignificant.



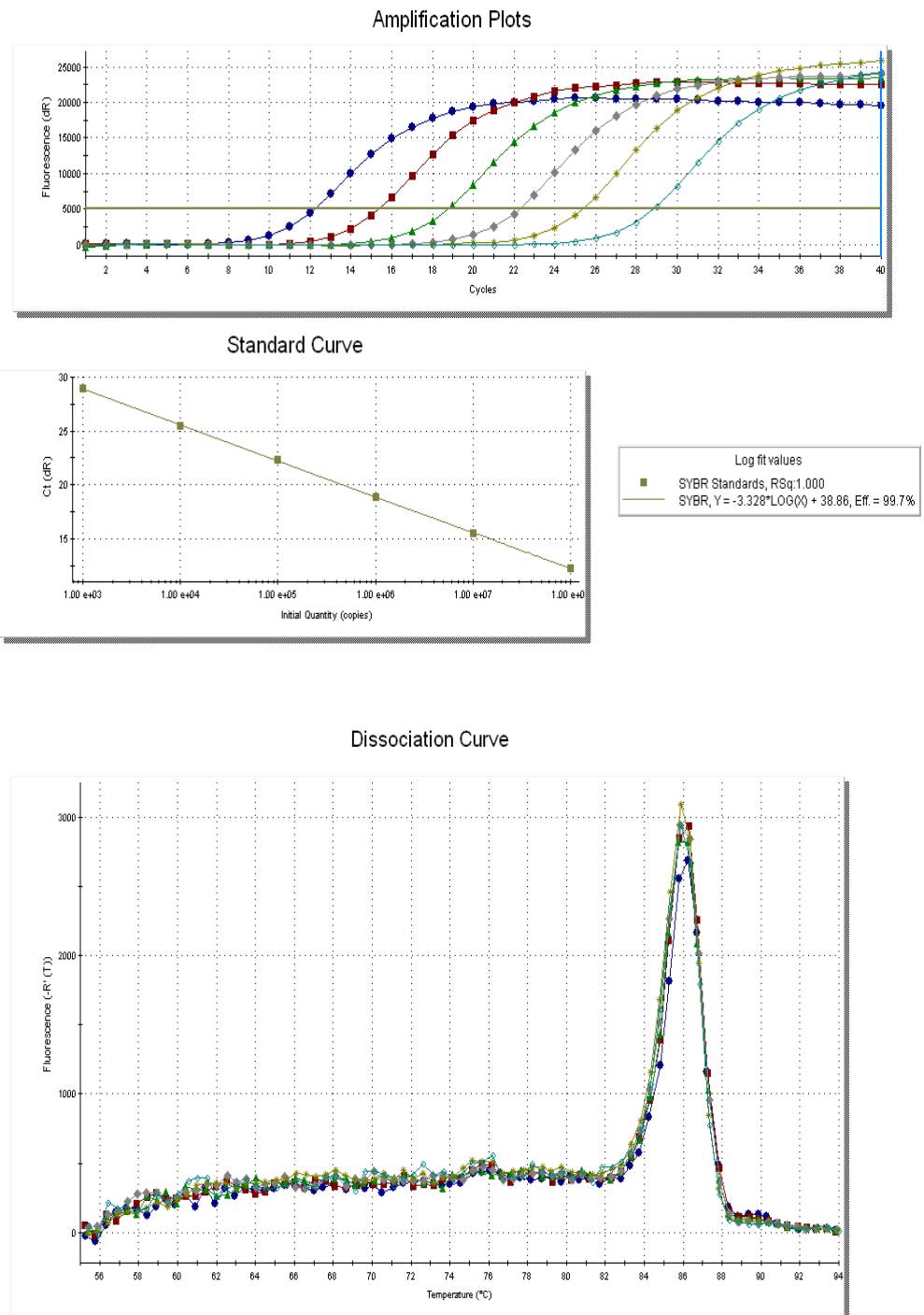
**Figure 5. 6 Amplification plots, standard curve and dissociation curves of *pqsA*.**

The PCR efficiency of PqsA was 104.6% with the slope of -3.216. The dissociation curve showed a single peak indicating the specific product generated for PqsA.



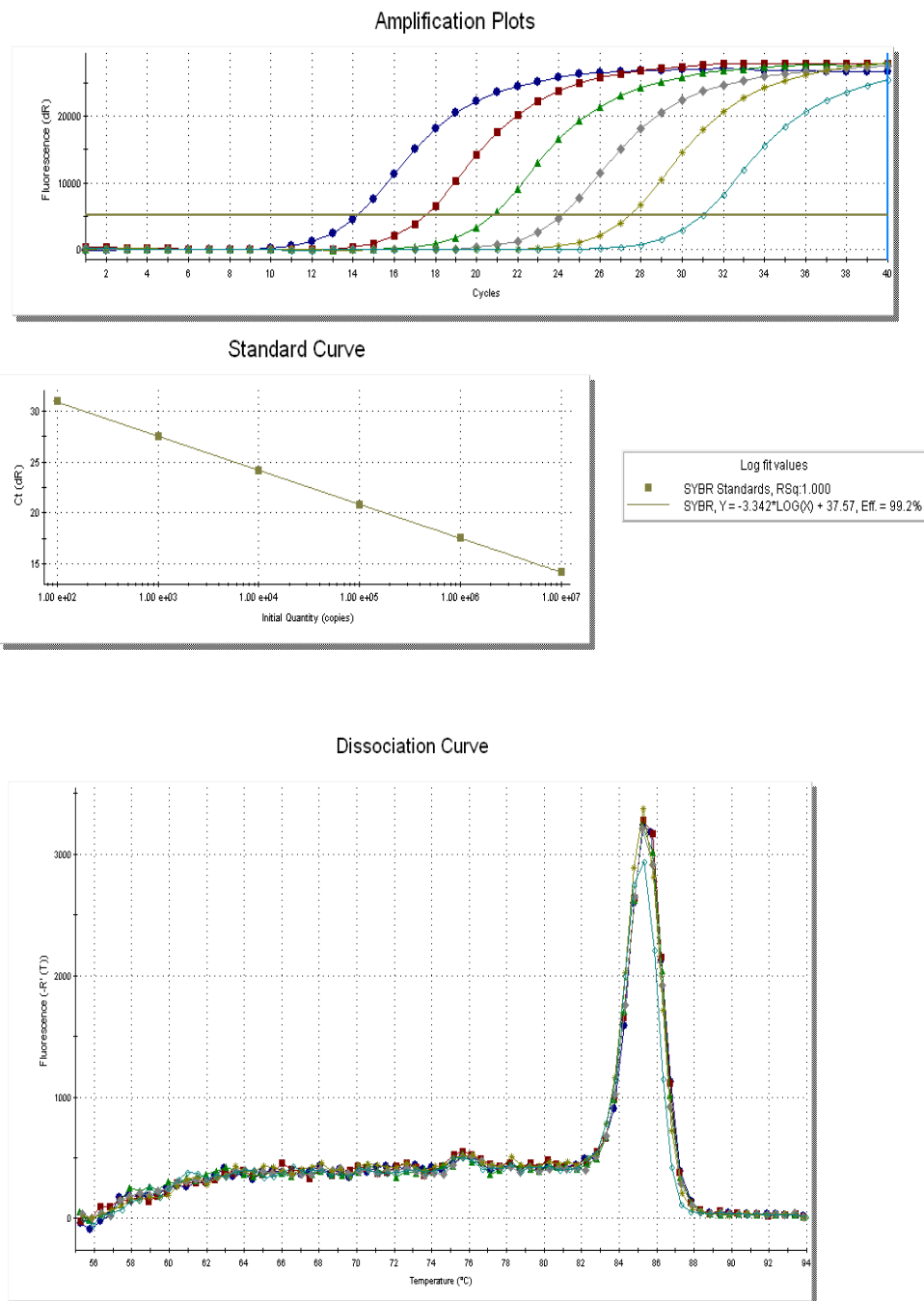
**Figure 5. 7 Amplification plots, standard curve and dissociation curves of *pqsB*.**

The PCR efficiency of PqsB was 102.5% with the slope of -3.263. The dissociation curve showed a single peak indicating the specific product generated for PqsB.



**Figure 5. 8 Amplification plots, standard curve and dissociation curves of *pqsC*.**

The PCR efficiency of PqsC was 99.7% with the slope of -3.328. The dissociation curve showed a single peak indicating the specific product generated for PqsC.



**Figure 5. 9 Amplification plots, standard curve and dissociation curves of r-PPA0010.**

The PCR efficiency of r-PPA0010 was 99.2% with the slope of -3.342. The dissociation curve showed a single peak indicating the specific product generated for r-PPA0010.

**Table 5. 3  $C_t$  obtained for *pqsA*, *pqsB*, *pqsC* and r-PPA0010 for standard curves.**

	<i>pqsA</i>	<i>pqsB</i>	<i>pqsC</i>	r-PPA0010
<b>10<sup>8</sup> copies</b>	10.85	11.76	12.29	Not used
<b>10<sup>7</sup> copies</b>	13.89	14.98	15.47	14.22
<b>10<sup>6</sup> copies</b>	17.28	18.29	18.84	17.54
<b>10<sup>5</sup> copies</b>	20.5	21.74	22.36	20.78
<b>10<sup>4</sup> copies</b>	23.74	24.79	25.44	24.2
<b>10<sup>3</sup> copies</b>	26.81	28.03	28.91	27.49
<b>10<sup>2</sup> copies</b>	Not used	Not used	Not used	31

### **5.5 *pqsA*, *pqsB* and *pqsC* are co-ordinately expressed during the growth of *P. acnes***

Quantitative RT PCR was performed for *pqsA*, *pqsB*, *pqsC* and r-PPA0010 for each RNA sample harvested at 6, 9, 20, 26, 32, 44.5 and 50 hours incubation of *P. acnes*. Experiments were performed as described in section 2.20.3 and data was analysed as described in section 2.20.4. A template control was included for each of the genes of interest.  $C_t$  values obtained for each mRNA (and negative control) at each time point are listed in Table 5.4. Then copy number of each transcript was calculated according to the individual standard curve for each target gene and reference gene. After comparing the copy number of *pqsA*, *pqsB* and *pqsC* with the reference gene – r-PPA0010, the relative gene expression was obtained for each incubation time. Then the pattern of gene expression for each target gene

during *P. acnes* growth was generated by plotting the incubation time against the ratio of the copy number of target genes and reference gene (Figures 5.10 and 5.11). The graph was plotted with the incubation time as the X axis, the growth of *P. acnes* as the first Y axis and the ratio of gene expression as the second Y axis. The entire experiments were repeated to confirm the results were reproducible.

It was found that during the *P. acnes* growth, the gene expression of *pqsA*, *pqsB* and *pqsC* were firstly down-regulated, and then up-regulated until the late exponential phase/early stationary phase. The changes in the gene expression of each gene are shown in Figure 5.10 and 5.11. Specifically, for *pqsA* the gene expression changed from the lowest ratio of 0.137 at 20 hours to the highest ratio of 0.293 at 32 hours, a 2.1 fold increase in the gene expression. *pqsB* changed from the lowest ratio of 19.229 at 9 hours to the highest ratio of 79.058 at 32 hours, a 4.1 fold increase in the gene expression, while for *pqsC*, it increased from the lowest ratio of 0.175 at 9 hours to the highest ratio of 0.918 at 32 hours, a 5.2 fold increase in the gene expression. The gene expression of all these three genes followed a similar pattern during growth suggesting that these three genes might be co-regulated.

It was also noticed that the gene expression of *pqsB* was always much higher than that of *pqsA* or *pqsC* during the growth of *P. acnes*. At 32 hour, when the expression of all three genes reached the highest level, *pqsB* was expressed nearly 100 fold higher than *pqsA* or *pqsC*. It suggested that although these three genes were co-regulated, their products might not be produced to similar levels. Moreover, it had been confirmed that *pqsA* was

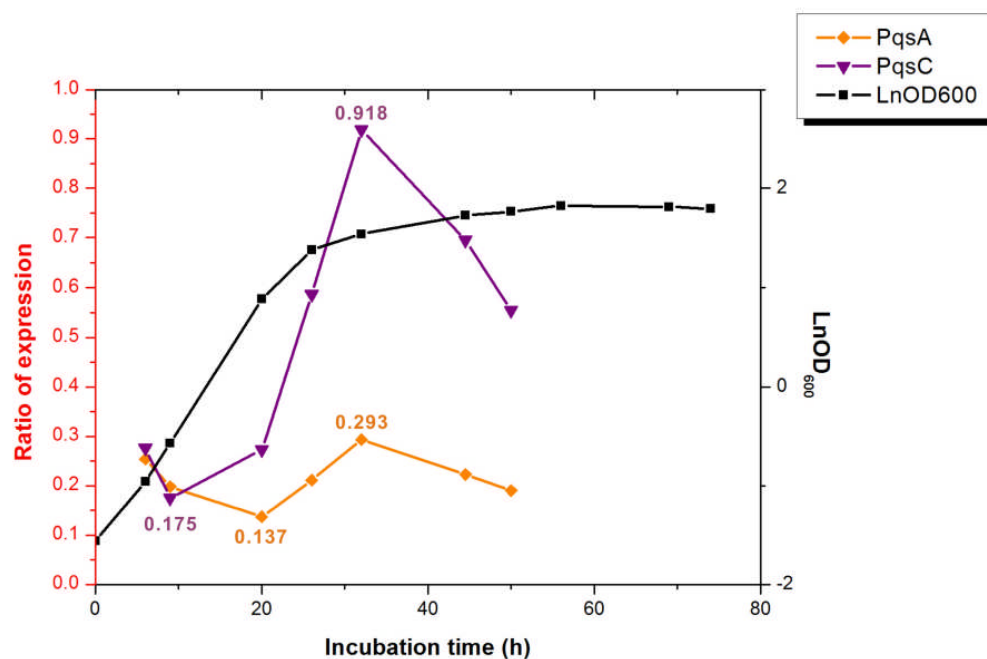


co-transcribed with *pqsB* from the same transcript as shown in the previous chapter. The large difference in the level of RNA corresponding to *pqsA* and *pqsB* suggested that the segment of the polycistronic mRNA encoding *pqsA* may be more unstable than that of *pqsB* or a significant amount of transcription terminates before *pqsA*, but after *pqsB*.

There was slight down-regulation of all target genes at the beginning of exponential phase as shown in Figures 5.10 and 5.11. This may be because cells used for inoculating the new medium were taken from the mid-exponential phase of the previous culture. The expression of the target gene changed when cells need to adapt to a new environment. It was also highly possible that this phenomenon was related to the cell density, as cells were diluted when inoculated to a new medium. In addition, the expression of all three genes was down regulated when cells were grown to stationary phase. This provided some evidence that the gene expression of *pqsA*, *pqsB* and *pqsC* might be cell-density related.

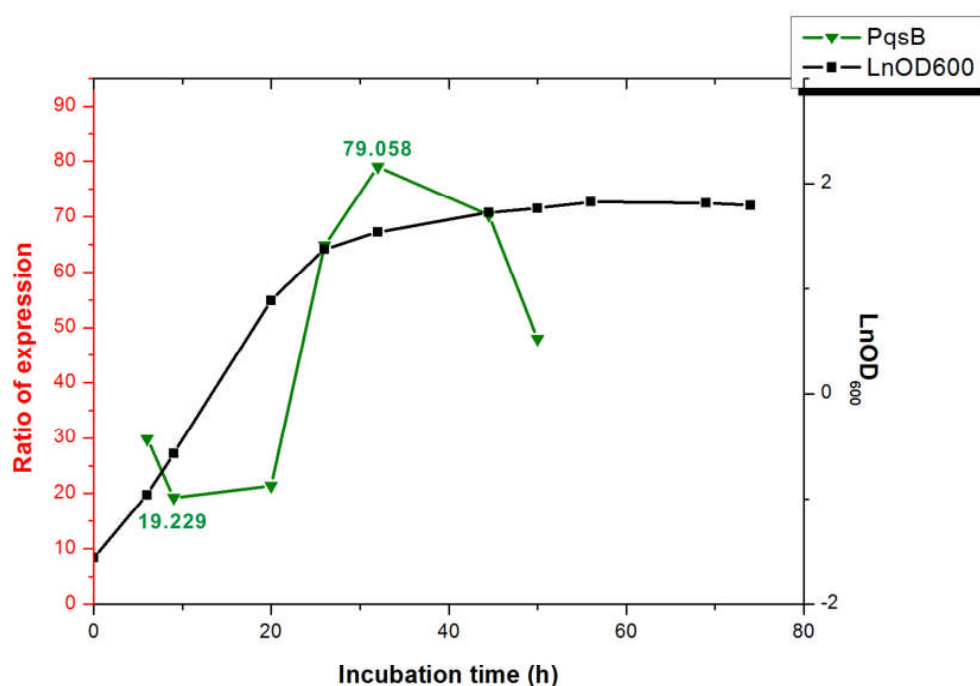
**Table 5. 4  $C_t$  obtained for *pqsA*, *pqsB*, *pqsC* and r-PPA0010 for each RNA sample and no template control.**

	<i>pqsA</i>		<i>pqsB</i>		<i>pqsC</i>		r-PPA0010	
	Sample	-ve control	Sample	-ve control	Sample	-ve control	Sample	-ve control
<b>6h</b>	21.97±0.16	35.5±0.03	16.36±0.05	35.9±0.29	23.68±0.14	36.48±0.11	20.46±0.23	35.74±0.09
<b>9h</b>	21.7±0.18	31.21±0.25	16.35±0.19	35.5±0.47	23.69±0.21	36.99±0.41	19.81±0.1	31.41±0.15
<b>20h</b>	22.21±0.2	33.45±0.21	16.2±0.14	33.92±0.43	23.06±0.27	34.51±0.1	19.81±0.16	33.45±0.31
<b>26h</b>	22.74±0.08	34.09±0.15	15.78±0.03	35.35±0.52	23.12±0.13	35.74±0.1	20.99±0.04	34.67±0.02
<b>32h</b>	23±0.06	33.92±0.23	16.23±0.06	34.21±0.42	23.22±0.12	35.78±0.51	21.74±0.16	33.54±0.23
<b>44.5h</b>	23.17±0.22	33.96±0.06	16.18±0.08	35.47±0.45	23.4±0.06	35.6±0.35	21.52±0.22	35.26±0.45
<b>50h</b>	23.24±0.11	34.42±0.25	16.9±0.04	34.42±0.55	23.46±0.12	30.97±0.15	21.7±0.29	34.04±0.4



**Figure 5. 10** Relative gene expression of *pqsA* and *pqsC* during the growth of *P. acnes*.

The X axis shows the incubation time of *P. acnes*; the left Y axis shows the ratio of gene expression of *pqsA* and *pqsC* against r-PPA0010; the right Y axis shows the natural log of OD<sub>600</sub> readings of the growth; the growth curve is indicated by the black line; the changes in the relative gene expression of *pqsA* and *pqsC* are indicated by the orange and purple line, respectively.



**Figure 5. 11** Relative gene expression of *pqsB* during the growth of *P. acnes*.

The X axis shows the incubation time of *P. acnes*; the left Y axis shows the ratio of gene expression of *pqsB* against r-PPA0010; the right Y axis shows the natural log of OD<sub>600</sub> readings of the growth; the growth curve is indicated by the black line; the changes in the relative gene expression of *pqsB* is indicated by the green line.

## 5.6 Discussion

By using real-time RT-PCR, this chapter investigated the gene expression of *pqsA*, *pqsB* and *pqsC* during the growth of *P. acnes*. To obtain more accurate regulation of gene expression, each target gene was interpreted by normalizing the data against the gene expression of a housekeeping gene – r-PPA0010. Relative gene expression of *pqsA*, *pqsB* and *pqsC* was calculated and plotted with the growth curve. The repeated experiments also showed similar results. The result firstly revealed the same gene expression pattern for all three genes, which was firstly down-regulated

and then up-regulated until the late-exponential phase of the growth. All genes were expressed to the highest level at the late-exponential phase/early stationary phase, and then down-regulated during the stationary phase. Because all three genes were expressed in the same way during growth, it suggested that these three genes were co-regulated. This is consistent with these three genes functioning together as part of a QS TCS.

Although the expression of *pqsA* and *pqsC* followed the same expression pattern with *pqsB*, it has been noticed that the expression of them did not change as much as that in PqsB. Firstly, this maybe because as a histidine kinase, PqsA will be in the state of dephosphorylation, once it transfers the phosphoryl group to its cognate response regulator. It acts like a recycled protein, which turns over to its original conformation and could be used again to receive environmental signals (Mizuno, 1998). Moreover, the response regulator may also undergo dephosphorylation, which is stimulated by the cognate histidine kinase and ATP (Aiba *et al.*, 1989). It has been confirmed that the phosphorylated response regulator is relatively unstable, and is readily converted to its unphosphorylated form. For example, in *E. coli* the *in vitro* half-lives of phospho-CheY and phospho-OmpR are a few seconds and several hours, respectively (Bourret *et al.*, 1991). Such rapid and controlled conversion of the phosphorylation/dephosphorylation state of a certain receiver is obviously crucial for the underlying signal transduction mechanism, because the state of a receiver may directly control its own biological activity by converting the ON/OFF state of the covalently attached signal output domain (Mizuno, 1998). Therefore, for the signal transduction pathway, both histidine kinase and response regulator are not required as much as signals for the signal transduction pathway.

It has been revealed that *pqsA* and *pqsB* were from the same transcript as described in the previous chapter. However, this chapter discovered that *pqsA* and *pqsB* were expressed at different RNA levels despite sharing a promoter. This suggested that the segment of the polycistronic mRNA encoding *pqsA* may be more unstable than that of *pqsB* or a significant amount of transcription terminates before *pqsA*. This might be a pattern for the gene regulation in bacteria that genes are expressed to different levels when from a part of the same transcriptional unit. Regulating mRNA stability is one of the essential mechanisms in gene expression (Aiso & Ohki, 2003). A study from screening the whole genome of *E. coli* revealed that mRNAs of the histidine kinase genes are synthesized efficiently, but rapidly degraded, resulting in the cellular level of the histidine kinase mRNAs being very low (Aiso & Ohki, 2003).

Moreover, differential mRNA stability of an operon encoding several genes has been discovered in other bacteria. In the pathogen *Rhodococcus equi*, although *vapA* has been found cotranscribed with the downstream *vapICD* genes, this initial transcript was subsequently processed to give rise to a 700 nt *vapA* with a half-life of 7.5 min, while *vapI*, *vapC* and *vapD* transcripts have an average half-life of 1.8 min (Byrne *et al.*, 2008). It has been suggested that the intergenic RNA structure was responsible for the increasing stability of the transcript, and this has also been shown for a large number of bacterial systems (Grunberg-Manago, 1999). It is hypothesized that differential mRNA stability within a polycistronic transcript is to attain differential gene expression, which is very important for the regulation of gene expression in bacteria. For example, differential mRNA stability of the six cistronic *puf* operon of *Rhodobacter capsulatus* allows for different

stoichiometries of reaction centre complexes and light-harvesting pigments (Heck *et al.*, 2000). In addition, in most cases of differential stability of a polycistronic transcript, they are functionally related but are different, nonhomologous proteins and expressed at different levels, just like what was found in this putative QS TCS in *P. acnes* (Byrne *et al.*, 2008). The differential stability may also depend on the different growth phases of the bacteria. For instance, two classes of mRNA have been described in group A streptococci and they can be distinguished by 1) stability in the stationary phase of growth; 2) kinetics of decay in exponential phase and 3) effect of depletion of RNases J1 and J2 and polynucleotide phosphorylase on decay in exponential phase (Bugrysheva & Scott, 2010). Also, the structure of a mRNA seems to be important for determining the class to which it belongs and presents a model to explain differential mRNA decay (Bugrysheva & Scott, 2010). Although the mechanism that produces different levels of expression of PqsA, PqsB and PqsC is unclear, it is likely that the levels of expression are a critical component of their function.

## Chapter VI Conclusion and future work

*P. acnes* is a human skin commensal residing within sebaceous follicles. It is speculated that *P. acnes* contributes to healthy skin by producing propionic acid, which is thought to deter colonization of the skin by severe pathogens (Cogen *et al.*, 2008). Meanwhile, it is also well established that *P. acnes* is one of the pathophysiological factors responsible for the formation of skin disorders such as acne (Bojar & Holland, 2004; Kurokawa *et al.*, 2009). Moreover, it is also considered as an opportunistic pathogen, since the bacterium is frequently isolated from a variety of inflammatory disease sites (Sorensen *et al.*, 2010). In addition, *P. acnes* is known for its ability to elicit extensive immunostimulatory activity including innate and adaptive immune responses as well as complement activation (Kim *et al.*, 2002; Jappe *et al.*, 2002). Currently, most drugs available for the treatment of acne are designed to limit the growth of *P. acnes* in the affected follicles (Jappe, 2003). However, they all risk the development of antibiotic resistance and more recent studies have revealed that there is an emerging need for the identification of new drug target for the treatment of acne (Jappe, 2003). A study has observed a prevalence of bacterial resistance to erythromycin of 52% for *P. acnes* strains that were colonizing patients with predominantly inflammatory lesions and 42% of *P. acnes* strains from patients without any previous application of erythromycin (Dreno *et al.*, 2001). As acne lesions are dominantly colonized by *P. acnes* and *S. epidermidis*, both inhabitants have to be considered in the treatment using antimicrobial agents. However, more than 30% of *S. epidermidis* strains have been detected resistant to erythromycin, roxithromycin and clindamycin (Nishijima *et al.*, 2000). Moreover, not only *P. acnes* and *S. epidermidis* develop antibiotic resistance due to acne treatment,



but also the resistance is spread among bacteria and even patients and their close contacts (Mills *et al.*, 2002).

Qs which contain TCSs in the signal transduction pathway have been identified as potential targets for the development of new drugs (Stephenson & Hoch, 2002; Sintim *et al.*, 2010; Costerton *et al.*, 2007). As introduced in section 1.3.6, the high specificity in the signal transduction pathway makes it a very attractive drug target and also provides less selective pressure for the development of resistance mutations compared with antibiotics (Podbielski & Kreikemeyer, 2004). Especially, so far the TCS system has not been identified in the animal kingdom and the signal transduction pathway of a TCS is distinct from the Ser/Thr signalling pathways commonly used in eukaryotic cells (Stephenson & Hoch, 2002). Moreover, TCSs not only contain elements for bacteria to sense the environment, but also to coordinate gene expression either for adaptability or pathogenicity (Stephenson & Hoch, 2002). Therefore, characterizing the signal transduction pathway in the QS system of *P. acnes* may provide a platform for identifying new drugs or treatment for acne. As acne is affecting more than 80% of people all over the world, an effective treatment would have both financial and social benefits. The availability of a genome sequence increases the number of tools that can be used to study *P. acnes* KPA171202 (Bruggemann *et al.*, 2004).

Despite *P. acnes* being identified as the causative agent of acne, relatively little research has focused on the microorganism itself at the molecular level. The broad objective of this thesis was to obtain experimental evidence for a histidine kinase (PqsA) and response regulator (PqsC)

interacting functionally as part of a two-component system that might be associated with the precursor of a signal peptide (PqsB) used for quorum sensing. The genes for the histidine kinase and response regulator of interest were unusual in being divergently transcribed.

It was found that PqsA phosphorylates PqsC *in vitro* (Chapter 3), suggesting that they interact functionally in *P. acnes*. Moreover, evidence was obtained that PqsA is transcribed from a promoter located upstream of PqsB (Chapter 4), consistent with the latter being functionally associated with the PqsA/PqsC two-component system. Furthermore, the gene expression analysis revealed a similar pattern of gene expression for PqsA, PqsB and PqsC during the growth of *P. acnes* (Chapter 6). This is consistent with these three genes functioning together as part of a QS TCS.

As PqsA and PqsC were purified, future studies could involve investigating the structure of these proteins by crystallization. Structures of many histidine kinases and response regulators of TCSs have been revealed in both Gram-positive and Gram-negative bacteria. By analysing the structure of these proteins, it helps to understand the mechanism of signal recognition and transduction in the TCS, classify the protein into an homologous family and identify the genes regulated by the system (Kim *et al.*, 2010). Moreover, development of structural genomics as well as resolution of three dimensional structures of proteins from the TCS is the first step for developing molecules blocking their activity (Jagusztyn-krynicka & Wyszynska, 2008). Structure-based drug design has emerged as a valuable pharmaceutical lead discovery tool, showing potential for accelerating the

discovery process, while reducing developmental costs and boosting potencies of the drug that is ultimately selected (Grey & Thompson, 2010).

As discussed before, a few TCSs have been identified containing three components (Romagnoli & Tabita, 2006; Kulasekara *et al.*, 2005; Ullrich *et al.*, 1995). These systems have a histidine kinase and two response regulators with one of them transcribed divergently (Kulasekara *et al.*, 2005). Although very unusual, it is still possible that PqsA may phosphorylate another response regulator in the genome. Therefore, investigating the phosphotransfer between PqsA and other orphan response regulators of *P. acnes* would also be an interesting area to study further. This study provides some methods for purifying orphan response regulators and investigating the phosphotransfer between a histidine kinase and a response regulator. The same strategies could also be tried for orphan response regulators.

As it has been confirmed that PqsA and PqsB were differentially transcribed, studies on analysing the RNA half life of each transcript would be interesting. And this could be performed by quantitative RT-PCR, which has already been optimized in this study. It will provide more information on gene regulation, transcriptional and post-transcriptional regulatory mechanisms and RNA degradation pathways. They are very important determinants of the extent of gene expression and play key roles in maintaining its accuracy (Ghosh & Jacobson, 2010).

PqsB has been predicted as a signalling peptide, which potentially is modified and exported outside of the cell membrane (Chapter 3). Therefore, detecting the PqsB in the supernatant of *P. acnes* culture will provide strong evidence of it being a QS signal of this putative QS TCS. High pressure liquid

chromatography followed by mass spectrometry could be used for the detection. It may provide more information on the structure, post-modification and transportation of PqsB. If PqsB could be detected using these techniques, PqsB could be purified and added back into the *P. acnes* culture. Changes in gene expression of PqsA, PqsB and PqsC could be measured by quantitative RT-PCR after the induction. This would provide strong evidence that these three genes constitute a legitimate QS TCS of *P. acnes*. Confirming the relationship among these three genes is very important for developing new drugs to block its signal transduction pathway. Moreover, by knowing the amino acid sequence or the structure of PqsB, an inhibitor could be developed to interfere with the signalling pathway of the QS TCS (Kiran *et al.*, 2008a). Once the structure of PqsB is determined, the specificity of the interactions between the signalling molecule and the histidine kinase could be revealed. This would provide very useful information for developing interference molecule to block or weaken the signal transduction pathway. Studies of structure-function analysis for autoinducing peptides have revealed that *agr* cross-inhibition was determined to require the peptide ring structure. However, it was relatively tolerant of amino acid replacements (Lyon *et al.*, 2002b; George *et al.*, 2008), as well as replacement of the thiolactone bond with a lactam (MDowell *et al.*, 2001; Lyon *et al.*, 2002a). Therefore, autologous and heterologous autoinducing peptides interact with AgrC via unique yet overlapping binding sites resulting in a process of competitive inhibition (Geisinger *et al.*, 2009).

It is also obvious that identifying the genes regulated by this putative QS TCS is crucial for investigating this system and studying *P. acnes*. To identify genes regulated by PqsC, chromatin immunoprecipitation (ChIP) is a

powerful method to measure protein-DNA interactions *in vivo*, and it can be applied on a genomic scale with microarray technology (ChIP-chip) (Wade *et al.*, 2007). Or the most recent method for mutagenesis of *P. acnes* could be tried for knocking out PqsC to find the genes regulated by it (Sorensen *et al.*, 2010).

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## Appendix I: List of primer sequences used in this study

### Primer sequences used in this study

Primer	Sequence	Annealing temperature
PPA0945DF	GCGCGGATCCGCCGAGGAACAAGCTGCCG	66 °C
PPA0945R	GCGCCTCGAGTCAGGTCTCACGAACTGGCTTT G	64 °C
PPA0947F	GGCCATATGTCAGTTCGACTCGTGGTTAC	58 °C
PPA0947R	GCGCCTCGAGGCGACGCAGCCGGGGGA	68 °C
PPA0945SP1	TTGAGGCGTAAGGAACCTGC	54 °C
PPA0945SP2	CCGAATCCGATGCCGATTGCGC	60 °C
PPA0945SP3	GACAACCATCCAGACTGTCCG	56 °C
PPA0946SP1	GTCGGGGTAGAGGGTGC	60 °C
PPA0946SP2	CCTTGGGTTGCGTATTCACAG	54 °C
PPA0947SP1	GACGACTACGCACTCCCAGTT	56 °C
PPA0947SP2	CAATCTGATGAGACCCCCACCAC	61 °C
PPA0947SP3	GTGAGACCGTCCATTTCCAGGC	56 °C
RTF1	GGCCATATGAATAGTGGTTCCGCCTGGCCGTA	57 °C
RTR1	GCGCCTCGAGGTCTCACGAACTGGCTTTGC	56 °C
RTF2	GCA CCC TCT ACC CCG AC	60 °C
RTR2	CGG ACA GCA CCA ATA CGG	59 °C
HSP60F	TCTAGAATTCGTCATCGTTGGTAGTGC	58 °C
HSP60R	TCTAGATTCCTCCGCTCCGGTG	59 °C
PPA0945RealTF	CGACTGGAATGGGTATTGCTC	60 °C
PPA0945RealTR	GCTTAACCAACCAACCAACCAATTG	60 °C

<b>Primer</b>	<b>Sequence</b>	<b>Annealing temperature</b>
PPA0946RealTF	CTCAGAACGCTGTGAATACGC	60 °C
PPA0946RealTR	GCAAAACACCAGTCGGGGTAGAG	60 °C
PPA0947RealTF	GCTGGACGGTCTCACAGCTAC	60 °C
PPA0947RealTR	GACGAGCAAAAGTCGTCAGG	60 °C
PPA0010RealTF	CCCGTACTGGTCAGCGTTTA	60 °C
PPA0010RealTR	GCCGTGTGCTTGTACAGGTT	60 °C



## Appendix II: List of Abbreviations

AHLs	<i>N</i> -acyl homoserine lactones
AIPs	Autoinducing peptides
CD	Circular dichroism
ChIP	Chromatin immunoprecipitation
CPA	Cyproterone acetate
CSF	Competence and sporulation factor
CSP	Competence stimulating peptide
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
dNTPs	Deoxynucleosidetriphosphates
EDTA	Ethylenediaminetetraacetic acid
EPSP	3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid
FA	Formaldehyde agarose
GBAP	Gelatinase biosynthesis activating pheromone
GM-SCF	Granulocyte-macrophage colony stimulating factor
GST	Glutathione S-transferase
His	Histidine
HK	Histidine kinase

HLA	Human leukocyte-associated antigen
HPLC	High-performance liquid chromatography
ICAM	Intercellular adhesion molecule
IF	Initiation factor
IGF	Insulin-like growth factor
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertani
MOPS	3-[N-morpholino]propanesulfonic acid
PCR	Polymerase chain reaction
Pqs	Possible quorum sensing
PT	Phosphor transfer
QS	Quorum sensing
RACE	Rapid amplification of cDNA ends
RCA	Reinforced clostridium agar
RR	Response regulator
Rsq	Coefficient of determination
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SOB	Super optimal broth
SOC	SOB with catabolite repression
SD	Shine-Dalgarno

SDS	Sodium dodecyl sulfate
SDS -PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris/Borate/EDTA
TCS	Two-component system
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLRs	Toll-like receptors
T <sub>m</sub>	Melting temperatures
TMHMM	TransMembrane prediction using Hidden Markov Models
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
TSS	Transcription start site
TYG	Tryptone yeast extract glucose
VCAM	Vascular cell adhesion molecule

### **Appendix III: List of suppliers**

#### **Ambion**

Address:

Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington,  
WA3 7OH, UK

#### **Amersham**

Address:

GE Healthcare Life Sciences, Amersham Place, Little Chalfont,  
Buckinghamshire, HP7 9NA, UK

#### **BD Biosciences**

Address:

Edmund Halley Road, Oxford Science Park, Oxford, OX4 4DQ, UK

#### **Bio-Rad**

Address:

Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead,  
Hertfordshire, HP2 7DX, UK

**Don Whitley Scientific**

Address:

Don Whitley Scientific Limited, 14 Otley Road, Shipley, West Yorkshire,  
BD17 7SE, UK

**Epicentre Biotechnologies**

Address:

Cambio Ltd, The Irwin Centre, Scotland Road, Dry Drayton, Cambridge,  
CB23 8AR, UK

**Fujifilm**

Address:

Fuji Photo Film (UK) Ltd, Unit 5-10 and 12 and 15-16, St. Martins Way,  
Bedford, Bedfordshire, MK42 0LF

**Invitrogen**

Address:

Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley,  
PA4 9RF, UK

**Jenway**

Address:

Bibby Scientific Limited, Beacon Road, Stone, Staffordshire, ST15 0SA, UK

**Mettler-Toledo**

Address:

Laboratory Analytical Instruments

Mettler-Toledo Ltd, 64 Boston Road, Beaumont Leys, Leicester, LE4 1AW,  
UK

**Millipore**

Address:

Suite 3 and 5, Building 6, Croxley Green Business Park, Watford, WD18  
8YH, UK

**MSE**

Address:

MSE (UK) Ltd, Worsley Bridge Road, Lower Sydenham, London, SE26 4AZ,  
UK

**New England Biolabs**

Address:

New England Biolabs (UK) Ltd, 75/77 Knowl Piece, Wilbury Way, Hitchin,  
Herts, SG4 0TY, UK

**Novagen**

Address:

Merch Chemicals Ltd, Boulevard Industrial Park, Padge Road, Beeston,  
Nottingham, NG9 2JR, UK

**Oxoid**

Address:

Oxoid Limited, Wade Road, Basingstoke, Hampshire, RG24 8PW, UK

**Promega**

Address:

Branch Office, Delta House, Southampton Science Park, Southampton,  
SO16 7NS, UK

**Qiagen**

Address:

Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK

**Radiometer Analytical**

Address:

Hach Lange Ltd, Pacific Way, Salford, Manchester, M50 1DL, UK

**Roche Applied Science**

Address:

Roche Diagnostics Ltd, Charles Avenue, Burgess Hill, RH15, 9RY, UK

**Sanyo**

Address:

Sanyo E and E Europe BV, Biomedical Division (UK Office), 9 The Office Village, North Road, Loughborough, Leicestershire, LE11 1QJ, UK

**Sigma-Aldrich**

Address:

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK

**Sigma Centrifuges**

Address:

SciQuip Ltd, 2 and 3 Merrington Hall Farm, Merrington, Shrewsbury, Shropshire, SY4 3QJ, UK



**Stratagene**

Agilent Technologies UK Ltd, Lakeside, Cheadle Royal Business Park,  
Stockport, Cheshire, SK8 3GR, UK

**Thermal Scientific**

Address:

Stafford House, Boundary Way, Hemel Hempstead, Hertfordshire, HP2 7GE,  
UK

