Chapter 1

General Introduction

1.1 The Taxonomy of *Streptomyces*

The genus *Streptomyces* was proposed by (Waksman & Henrici, 1943) and is one of the largest genera from the phylum Actinobacteria. This genus also belongs to the family *Streptomycetaceae* on the basis of morphological and physiological characteristics. Streptomycetes are Gram-positive, soil-dwelling bacteria and have genomes with a high GC-content (Gust *et al.*, 2003). Streptomycetes produce an extensive branching substrate mycelium and most strains also produce an aerial mycelium and spores. Many strains produce a volatile metabolite, geosmin, which gives a distinctive earthy odour (Gust *et al.*, 2003). Streptomycetes display a complex secondary metabolism and produce around two-thirds of clinically useful antibiotics such as tetracycline, neomycin, chloramphenicol and streptomycin. The development of numerical taxonomic systems, which make use of phenotypic features helped to resolve the classification within the family *Streptomycetaceae* and allowed the reclassification of six genera; *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasatoa* and *Microellobosporia* to the *Streptomyces* genus (Anderson & Wellington, 2001).

1.2 Characteristics of the genus *Streptomyces*

Streptomyces have a superficial resemblance to filamentous fungi as both grow as branching hyphae, form a vegetative mycelium and eventually form specialised reproductive structures called aerial hyphae, which emerge from the colony surface into the air (Flardh & Buttner, 2009) and finally develop spores that aid in dispersal.

The streptomycetes are highly abundant in nature with spore formation thought to occur during lack of nutrient supply in the soil environment. Consequently, the dormant spores remain quiescent for long periods until conditions support their growth; this is a successful survival strategy in allowing this group of microorganisms to survive and reproduce in a heterogeneous, particulate environment such as the soil (Flardh & Buttner, 2009). A number of species have been studied in terms of the regulation of antibiotic biosynthesis such as *Streptomyces rimosus*, *S. fradiae* and *S. hygroscopicus* amongst others (Anderson & Wellington, 2001). Despite this, the developmental biology of relatively few species has been studied; most extensively *S. coelicolor* A3(2), *S. griseus* and in recent years *S. venezuelae* (Chaudhary *et al.*, 2013).

1.2.1 *Streptomyces coelicolor*

In this thesis, the focus is mainly on *S. coelicolor,* the best characterized species among *Streptomyces*. The genome of this organism was sequenced in 2002 (Bentley *et al.*, 2002) and its large (~8,7mb) linear genome described. The lifecycle of *S. coelicolor* is illustrated in Fig. 1.1. When a spore comes across conditions suitable for growth, it germinates and a germ tube emerges from the spore and elongates to form long branching filaments that generate a network of substrate or vegetative mycelium. Manufacture of cell walls is carried out through insertion of new cell wall material at the hyphal tip and cross walls separate the hyphae into cellular compartments. Each compartment contains multiple copies of the chromosome. In coordination with septation, DNA and other constituents are moved towards the growing tip into new branches (Flardh, 2003b). Eventually, hyphae emerge into the air and form an aerial mycelium on the surface of the

colony (Chater, 1998, Elliot *et al.*, 2008). The aerial hyphae develop reproductive structures called spores and undergo a number of maturation steps that turns the aerial mycelium from white to grey through the production of spore coat pigment encoded by the *whiE* locus (Elliot *et al.*, 2008, Kelemen *et al.*, 1998). At this, stage *Streptomyces* spores are available for dispersal in the environment allowing both survival of environmental hardship and colonization of new locations within the soil matrix (Flardh & Buttner, 2009) (Fig. 1.1).

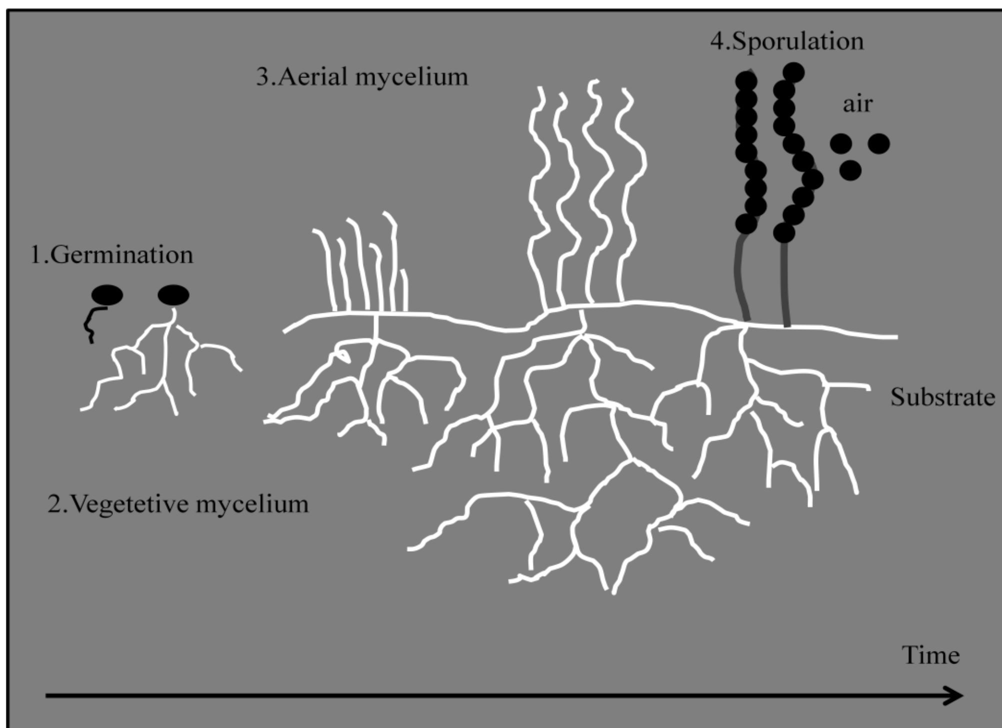

Fig. 1.1 The life cycle of *S. coelicolor.* (1) Spore germination; (2) a germ tube emerges from the spore and elongates to form a long branching filament that generates the network of substrate or vegetative mycelium; (3) the differentiation of an aerial mycelium that further (4) develop into chains of spores suited for dispersal to the environment (modified from (Chater, 1998, Flardh & Buttner, 2009))

1.2.2 *Streptomyces venezuelae*

Although most studies on the genetics of *Streptomyces* have been carried out using the model species *S. coelicolor*, the chloramphenicol-producing organism, *S. venezuelae* presents many advantages over *S. coelicolor* for molecular studies. One advantage is that *S. venezuelae* grows and differentiates faster than *S. coelicolor* (approximately four days rather than seven days for *S. coelicolor*). *S. venezuelae* is also able to sporulate in both submerged and on solid culture, while *S. coelicolor* is limited to sporulation on agar (Glazebrook *et al.*, 1990). This limits the developmental studies than can be carried out in *S. coelicolor* and has led to the emergence of *S. venezuelae* as a model organism.

In this thesis we began using *S. coelicolor* as our model, but subsequently moved to *S. venezuelae* due to the ability of the latter organism to sporulate in liquid culture where > 90% total biomass is converted into spores (Glazebrook *et al.*, 1990). In theory, this should remove many of the drawbacks associated with the inability of *S. coelicolor* to sporulate in liquid culture.

1.3 Developmental biology of *Streptomyces*

The life cycle of *Streptomyces* begins with germination from uninucleoid spores under appropriate growth conditions. The bacteria grow as mesh of branching substrate mycelia. During conditions of environmental stress and nutrient depletion, extracellular signals are produced that lead to developmental changes (Nodwell, 1998, Nodwell *et al.*,

1996, Pope *et al.*, 1996, Willey *et al.*, 1993). The substrate mycelia begin to lyse and provide nutrients for the growth of aerial hyphae.

1.3.1 Physiological differentiation of streptomycetes

Streptomycetes have a complex morphological and physiological differentiation process during their life cycle. The completed genome sequence 8.7-Mb of *S. coelicolor* has revealed that about 400 kb (more than 20 gene clusters) are concerned with secondary metabolites synthesis (Gust *et al*., 2003). Streptomycetes and related actinomycetes are potentially the source of novel secondary metabolites which can be used for anti-infective agents, anti-cancer agents or other pharmaceutically useful compounds (Bibb, 2005). They have the ability to metabolise many different compounds including sugars, alcohols, amino acids, and aromatic compounds by producing extracellular hydrolytic enzymes. Their metabolic diversity is thought to be due to their extremely large genomes which encodes many transcription factors that control gene expression, allowing them to respond to specific environmental needs (Bibb, 2005).

Streptomyces produce many industrially significant antibiotics (Hopwood, 1999) and other pharmacologically active compounds (Rodriguez-Garcia *et al.*, 2005). Other than the production of antibiotics, *Streptomyces* have received increased attention for the synthesis of other bioactive agents such as immunomodulatory compounds (e.g. Rapamycin from *S. hygroscopicus*) (Lomovskaya *et al.*, 1997, Cot *et al.*, 2011). Antibiotic production begins at the same time that the developmental shift begins and small molecule signals regulate antibiotic and secondary metabolite production. The correlation of aerial hyphal growth and secondary metabolite production is likely to act as

a mechanism to protect the remaining nutrients from being used by other microorganism during morphological development. As the aerial hyphae continue to grow, they coil and septate to produce uninucleoid spores for dispersal (Chater, 2001).

S. coelicolor produces at least five secondary metabolites and has been exploited to study its physiological developmental processes genetically. The two pigmented antibiotics, the red undecylprodigiosin and the blue polyketide actinorhodin, as well as the spore associated grey polyketide pigment allowed the isolation of genetic determinants controlling physiological and/or morphological differentiation of *S. coelicolor* (Hopwood *et al.*, 1995, Chater, 1998). In addition, *S. rimosus* is also a well studied system for studying antibiotic synthesis in streptomycetes. There are more than 100 publications related to analysis of the production of the tetracycline derivative, oxytetracycline (OTC). This antibiotic is used principally in aquaculture and is approved within the European Union and elsewhere for fish health care, despite some evidence that tetracycline resistant infections are becoming more widespread. There are reviews about *S. rimosus* genetics, paying specific attention to the application of modern molecular approaches that will be invaluable for the development of new tetracyclines by combinatorial biosynthesis and novel recombination approaches (Petkovic *et al.*, 2006).

According to (Nikaido, 2009) the production of antibiotics annually is about 100,000 tons, which are used in agriculture, food, and health. Their exploitation has impacted populations of bacteria, inducing antibiotics resistance. This resistance may be due to genetic changes such as mutation or acquisition of resistance genes through

horizontal transfer, which most often occurs in organisms of different taxonomy (Procopio *et al.*, 2012).

1.3.2 Morphological differentiation of streptomycetes

Gram-positive bacteria of the genus *Streptomyces* grow by tip extension and form branched hyphae and mycelia (Hempel *et al.*, 2008). When nutrients are plentiful they grow on solid medium as branched filaments with thin septa in the multinucleoidal hyphae. During starvation, differentiation is induced and the streptomycetes begin to form an aerial mycelium. It is from these aerial hyphae that uninucleoidal spores are generated that are capable of starting a new differentiation cycle. As streptomycetes grow as hyphal filaments, the single-cell state, which is typical for bacteria which divide by binary fission, is only attained when the streptomycetes differentiate and form spores (Elliot *et al.*, 2008).

Energy to fuel this erection of aerial hyphae is generated through cell cannibalism of vegetative hyphae (Gonzalez-Pastor *et al.*, 2003). As such, this process is coincident with the biosynthesis of secondary metabolites including antibiotics (Chater, 1998). It was argued that such physiological differentiation of vegetative mycelium and aerial hyphae may serve as a way to protect the colony from competitor microorganisms and so protect emerging aerial structures. The next phase corresponds to the sporulation process, which takes place in the apical part of mature aerial hyphae. Sporulation involves the alteration of the aerial hyphae into spore chains. The streptomycete reproductive process occurs by the formation of long chains of spores in aerial hyphae. This process is commenced by the organization of chromosomes at regular intervals within the hypha.

The large size and linearity of the *Streptomyces* chromosome, the formation of multigenomic hyphae during growth and the multiple cell division events that convert aerial hyphae into chains of prespore compartments may impose special requirements on the partitioning system. *S. coelicolor* contains *parA* and *parB* partitioning genes, arranged in a two gene operon (Jakimowicz *et al.*, 2002, Jakimowicz & Wezel, 2012). These cytoplasmic regions are separated into unigenomic compartments through a synchronous septation occurring upon the ladder-like assembly of the cell division protein FtsZ at regular intervals (Schwedock *et al.*, 1997, Willemse *et al.*, 2012). During sporulation septation, deposits of glycogen are transiently stored in the apical compartments of aerial hyphae. These may serve to supply energy during spore maturation as well as precursors for threhalose stores that will confer osmoprotection to the mature spores and provide them with energy and carbon source during germination (Yeo & Chater, 2005). In a later phase of spore maturation, the cell wall of the prespore compartments mature by thickening, synthesizing a polyketide gray pigment and rounding off into exospores. However, unlike the endospores formed by *Bacillus subtilis*, streptomycete spores are not resistant to treatment with chemicals, UV and heat. They are, indeed, resistant to desiccation and physical forces, and are covered by several hydrophobic layers, which are believed to facilitate their dispersion in the environment (Wildermuth *et al.*, 1971).

1.3.2.1 Growth of streptomycete substrate hyphae

Studies of the cell division process during the morphological development of streptomycetes have been performed for many years although surprisingly little is known about germination or tip extension of substrate hyphae. The coiled-coil protein DivIVA is

involved with *mreB*-independent (Mazza *et al.*, 2006) and polarized growth in Actinobacteria. In *S. coelicolor*, DivIVA is essential for growth and accumulates at growing hyphal tips, whilst partial depletion and over expression have dramatic effects on tip extension, cell shape determination. This protein is recruited to future branch points before branch emergence from the primary hypha (Flardh, 2003a). DivIVA is a molecular marker of new branch sites in *S. coelicolor* as this protein accumulates at hyphal tips and branch points; this was demonstrated in *S. coelicolor* strain K112, which produces a DivIVA-EGFP (enhanced green fluorescent protein) fusion protein (Flardh, 2003a, Flardh *et al.*, 2012, Hempel *et al.*, 2012, Hempel *et al*., 2008). FilP, is also involved in growth and morphogenesis of *S. coelicolor*. FilP is an intermediate filament-like protein that has been predicted to give a coiled-coil structure. Purified FilP forms filamentous structures *in vitro* and discontinuous filaments are observed in growing vegetative hyphae of the merodiploid strain *filP:filP-egfp.* In addition, FilP is needed for normal growth and morphology (Ausmees *et al.*, 2007, Bagchi *et al.*, 2008). However, the detailed mechanism of FilP cytoskeletal functions remain unknown. Another protein, Scy (*Streptomyces* cytoskeletal element), is a unique bacterial coiled-coil protein, which together with DivIVA creates and retains polarized growth via the organization of Scy-DivIVA assemblies. Scy, DivIVA and FilP, the filament forming protein, establish a multi-protein, polar assembly in *Streptomyces* that is known as the Tip Organizing Centre (Ditkowski *et al.*, 2013, Holmes *et al.*, 2012). Scy also recruits ParA to the hyphal tips and regulates ParA polymerization, while CslA (a cellulose synthase-like protein), which interacts with DivIVA is located at the hyphal tip (Xu *et al.*, 2008).

1.3.2.2 Erection of aerial hyphae

Two major classes of regulatory genes involved in morphological differentiation in *S. coelicolor* have been identified through genetic studies of mutants blocked in development. One group are the *whi* (white) genes, mutants of which can form aerial hyphae, but cannot give rise to mature spores. As such, *whi* mutants produce aerial hyphae but are not able to form the grey spore-associated pigment and therefore have a white colony phenotype (Chater, 1972, Hopwood *et al.*, 1970).White mutants are described in Section 1.3.2.3. Mutations in the other group of genes, called *bld* (bald), which are blocked at the earliest stages of differentiation, allow growth as a substrate mycelium but prevent the erection of aerial mycelia and the colonies of these mutants have a shiny and bald appearance. Not only are *bld* mutants unable to erect the spore forming aerial mycelium, they are also defective in antibiotic production, regulation of carbon utilization and production or response to extra-cellular signals (Champness, 1988, Nodwell *et al.*, 1996). Most *bld* mutants were isolated during genetic studies of *S coelicolor* and even though plenty of *bld* mutants have already been isolated, only few *bld* genes have been studied at the molecular level. Most of the known *bld* genes encode regulatory factors and have pleiotropic effects on both morphological and physiological differentiation. Consistent with their morphological block, many *bld* mutants (*bldA*, *bldB*, *bldC*, *bldG*, *bldH*, and others) (Fig. 1.2) of *S. coelicolor* are also unable to make pigmented antibiotics such as actinorhodin and undecylprodigiosin (Pope *et al.*, 1996, Willey *et al.*, 1991).

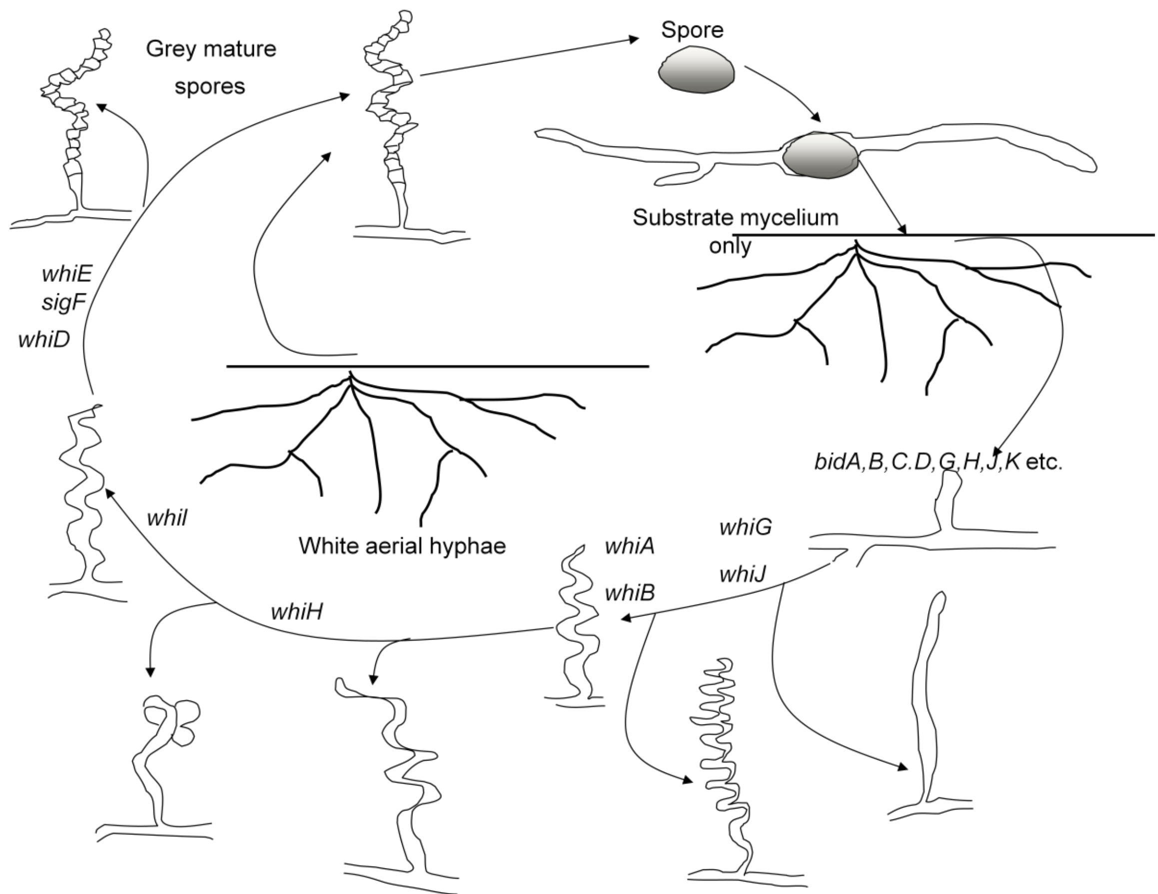

Fig. 1.2 Roles of developmental genes in the morphological differentiation of *S. coelicolor* (Chater, 1998).

bldA encodes a tRNA that distinguishes the rare leucine codon UUA, which is uncommon in *Streptomyces* DNA (Lawlor *et al.*, 1987) and allows TTA codon-containing genes to be dependent on the expression of *bldA* for translation of their mRNA and consequently initiation of aerial growth (Nguyen *et al.*, 2002, Takano *et al.*, 2003). Other *bld* loci have been characterized at the molecular level, for example, *bldB* is the most pleiotropic of the *bld* mutants as mutations in this gene cannot be rescued by any of the mechanisms that can restore aerial development in mutants of other *bld* genes. BldB is needed for catabolite repression, morphogenesis and antibiotic production and contains

a putative DNA binding site (Pope *et al.*, 1998). Another protein is BldD, a DNA binding protein that acts as a transcription factor, regulating its own transcription as well as *whiG* and *bldN* (Elliot *et al.*, 1998). *bldC* encodes a small DNA-binding protein (Hunt *et al.*, 2005) while *bldG* codes for an anti-anti-sigma factor (Bignell *et al.*, 2000) homologous to a *B. subtilis* anti-sigma factor. Although several *bld* loci encode products that are related to gene expression, one product from the *bldJ* gene (Willey *et al.*, 1993) is a small secreted peptide that is imported inside the hyphal cells by the BldK oligopeptide permease complex (Nodwell *et al.*, 1996). The *bldK* locus consists of five genes that specify homologs of ABC transporters. It was inferred that BldK is an oligopeptide importer. *bldM* encodes another transcription factor (Molle & Buttner, 2000) and *bldN* encodes an RNA polymerase sigma factor (Bibb *et al.*, 2000).

Little is known about how the products of *bld* genes participate in morphological differentiation, but a possible clue was provided by the identification of a small hydrophobic protein of unusual structure called SapB (the spore associated-protein) (Guijarro *et al*., 1988), which appears to be directly involved in erecting aerial hyphae (Willey *et al*., 1991). Production of SapB is impaired by mutations in various *bld* genes, and the capacity of one such *bld* mutant to differentiate is restored by the application of the protein in experiments where SapB is supplied exogenously (Willey *et al*., 1991). Aerial mycelium formation and spore production are driven by at least two different pathways. One pathway is dependent on SapB, which operates during growth on rich medium, such as a glucose carbon source and appears to be important for morphogenesis under these conditions (Champness, 1988, Merrick, 1976, Willey *et al.*, 1993). This

pathway is dependent on the *bld* genes. Aerial mycelia are able to produce aerial hyphae because SapB coats the mycelia surface and allows emergence of aerial hyphae despite the energy barrier generated by the surface tension of the air-water interface (Willey *et al.*, 1991). SapB-rescued *bld* mutants from the inability to generate aerial filaments that did not go on to sporulate to some degree, whilst rescued strains displayed a morphology that resembled short branching vegetative hyphae that were simply released from the colony surface to stand erect. This was consistent with a primary role for SapB as a biosurfactant. Similar to hydrophobins (Wosten, 2001), SapB self-assembles at the air-colony interface through hydrophobic interactions where it reduces the surface tension and enables the upward growth of aerial hyphae (Fig. 1.3). Recent studies show that all *bld* mutants are defective in production of SapB, which may explain why they fail to produce aerial hyphae. On the other hand, the production of aerial hyphae seems independent of most *bld* genes products and no SapB is produced when grown on minimal medium with an alternate carbon source, such as mannitol. Most *bld* mutants can form aerial hyphae on poor carbon sources, although *bldB* and *bldN* are exceptions to this (Champness, 1988, Champness & Chater, 1994, Merrick, 1976).

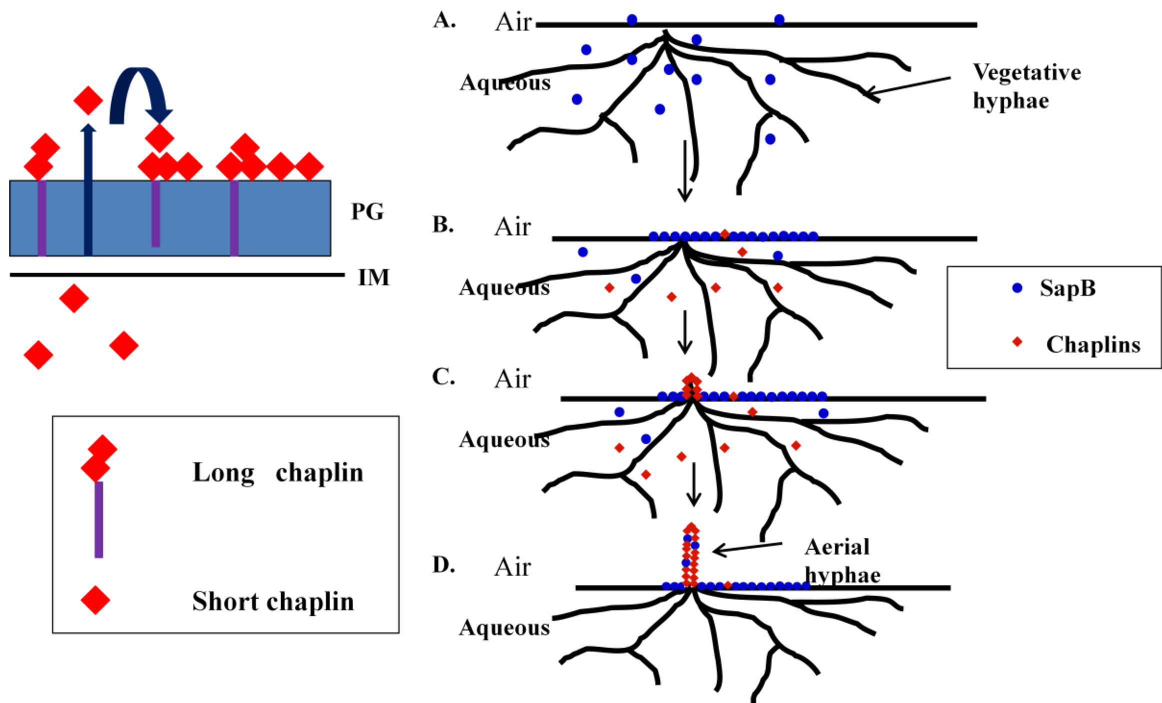

Fig. 1.3 Model for the role of the chaplins and SapB in streptomycete morphogenesis. The long chaplins are covalently attached to the cell wall of the aerial hyphae at their C-terminal end. The central, peptidoglycan-spanning domain of the long chaplins (thick blue line) presents the tandem hydrophobic chaplin domains (purple) on the cell wall surface. The short chaplins consist of a single chaplin domain (red), and are proposed to heteropolymerise with the long chaplins to form extended hydrophobic chaplin filaments on the cell surface; as a consequence the filaments are anchored into the cell wall. (A and B) SapB (black circles) is secreted and self-assembles into an amphiphilic sheet at the air-water interface, reducing surface tension and facilitating the emergence of aerial hyphae. (C) The chaplins (hatched diamond) and rodlins are secreted and polymerise to form the hydrophobic sheath around the aerial hyphae, further facilitating their escape into the air. SapB may also form part of the hydrophobic sheath. (D) The chaplin filaments are organized into higher order structures by the rodlins (RdlAB) to give the characteristic 'basketwork of paired rodlets' ultrastructure seen on the surface of *S. coelicolor* spores and aerial hyphae. (modified from (Elliot *et al*., 2008).

In addition to having a wall of peptidoglycan, aerial hyphae and spores of *S. coelicolor* have another outer layer consisting of amphipathic proteins called chaplins and rodlins which make the aerial hyphal surface hydrophobic (Claessen *et al*., 2003, Claessen *et al*., 2002, Elliot *et al*., 2003). A network of protein filaments are formed by the rodlins, RdlA and RdlB, which, in conjunction with the chaplains, give spores a characteristic "rodlet" surface structure. Despite this, rodlins have no consequence to the emergence of aerial hyphae which is dependent on the generation of a hydrophobic sheath of chaplins (Claessen *et al*., 2002).

In contrast to the inessential nature of the rodlins in the formation of aerial hyphae, this process depends on both the chaplins (Capstick *et al*., 2007, Claessen *et al*., 2004) and SapB which are accumulated on the surface of substrate and attach to the aerial hyphae (Guijarro *et al.*, 1988, Kodani *et al.*, 2004, Willey *et al*., 1991, Willey *et al*., 1993). The capability to lower surface tension is thought to be required for the emergence of aerial hyphae from the water into the air (Willey *et al*., 1991, Willey *et al*., 1993). The genome of *S. coelicolor* encodes eight homologous chaplin proteins, ChpA-H, which all have a N-terminal signalling sequence for secretion, which implies how these proteins might be attached to the outside of the cell. ChpD-H have a conserved hydrophobic domain found in the five smaller chaplins, whilst ChpA-C are twice the size of the three larger proteins. ChpA-C are also covalently anchored to the peptidoglycan cell wall mediated by their C-terminal domain, while the small chaplins are considered to interact with the large ones (Claessen *et al*., 2003, Di Berardo *et al*., 2008, Elliot *et al*., 2003). When the peptide SapB, encoded by the *ramS* gene, undergoes posttranslational

modifications, that is likely to be mediated by the putative lantibiotic synthetase RamC (Kodani *et al*., 2004, Willey *et al.*, 2006), SapB is thought to be transported to the outside of the cells by the RamA and RamB, components of an ATP-binding cassette transporter (Willey *et al*., 2006). The genes required for SapB synthesis encode a response regulator *ramR* whose product activates the operon including *ramS*, *ramA*, *ramB* and *ramC* (Keijser *et al.*, 2002, Nguyen *et al*., 2002, O'Connor & Nodwell, 2005).

Under suitable growth conditions, both the chaplins and SapB are produced in a *bld*-gene dependent manner and are required for aerial hyphae to be formed at normal levels. On minimal media, SapB is not produced and the process is only dependent on the chaplins, which on nutrient-poor media are produced independently of several of the *bld* genes (Capstick *et al*., 2007). According to these studies, there are two regulatory pathways controlling aerial hyphal formation, the first is active on nutrient-rich media and involves the *bld* genes and the production of both SapB and the chaplins. The second is a *bld*-gene independent pathway corresponding to aerial hyphae on nutrient-poor media through the chaplins and is independent of SapB (Capstick *et al*., 2007, Willey *et al.*, 1991). The regulatory networks that are involved in morphological development using *S. coelicolor* as a model have been well characterised and are described above. However, as *S. coelicolor* sporulates only on solid medium and erects the aerial mycelium that comprises about 5% of the total biomass, this represents a challenge to understanding the molecular mechanisms that underpin development in this species. *S. venezuelae* sporulates in liquid culture (Glazebrook *et al*., 1990), which has been exploited to characterise these process in more detail.

1.3.2.3 Differentiation of aerial hyphae to spores

Mutants of *S. coelicolor* that are defective in sporulation have been classified through their white colour. These mutants, first described in 1970 (Hopwood *et al.*, 1970), fail to produce the polyketide grey pigment related with sporulation process. The "early" *whi* loci which proceed in the following hierarchical order are *whiG, whiJ>whiA,whiB>whiH>whiI* (Figs. 1.2 and 1.4) (Chater, 1993). These genes are needed for further development of aerial filaments for segregation into unigenomic prespore compartments. Furthermore early *whi* genes activate expression of the "late" *whi* genes involved in cell wall spore formation and spore maturation (Chater, 1998, Kaiser & Stoddard, 2011).

whiG is a bacterial sigma factor (σ^{WhiG}) that is a key early *whi* gene (Chater, 2001), which directs the transition to a sporulation-specific mode of gene expression in aerial hyphae (Kaiser & Stoddard, 2011). A *whiG* mutant presents an appearance with characteristic aerial hyphae, which have vegetative-like septa and rare branches. When *whiG* is over expressed, this leads to premature and ectopic sporulation in the vegetative mycelium, which suggests that WhiG applies a key regulatory role in the primary sporulation program in *S. coelicolor* (Flardh *et al.*, 1999). Several researchers have considered that an anti-sigma factor might be involved in inhibiting WhiG activity during sporulation (Kaiser & Stoddard, 2011). WhiG belongs to the FliA-family of sigma factors that reconcile the chemotactic response in *Escherichia coli* and *B. subtilis* (Chater, 1998). Whilst, *whiG* transcription is described as having a more or less constant level all the way through development, it was disputed that the activity of WhiG is equivalent to FliA

(Kelemen *et al.*, 1996). Once active, WhiG:RNA polymerase holoenzyme is responsible for the transcription of two early sporulation regulatory genes, *whiH* (Ryding *et al.*, 1998) and *whiI* (Ainsa *et al.*, 2000). These two genes are controlled directly and indirectly respectively. Nevertheless, an increase in expression of both genes during sporulation septation might be explained by the release of specific signals by relief of repression. WhiH and WhiI belong to two different families of transcription factors (Ainsa *et al*., 2000, Ryding *et al.*, 1998). WhiH is a member of the GntR family of transcriptional regulators. These typically respond to acid carbon metabolites, which modulate their transcriptional auto-repression, as well as repression of genes involved in carbon metabolism (Ryding *et al.*, 1998). WhiI is an atypical response regulator that is unlikely to be phosphorylated by a cognate sensor histidine kinase as its predicted phosphorylation pocket lacks two key conserved residues needed for phosphorylation. Another two early regulatory *whi* genes, expressed during sporulation include *whiA* and *whiB* (Ainsa *et al*., 2000, Chater, 2001, Flardh *et al*., 1999) and more details of these genes as well as *whiD* are provided in Section 6.1.

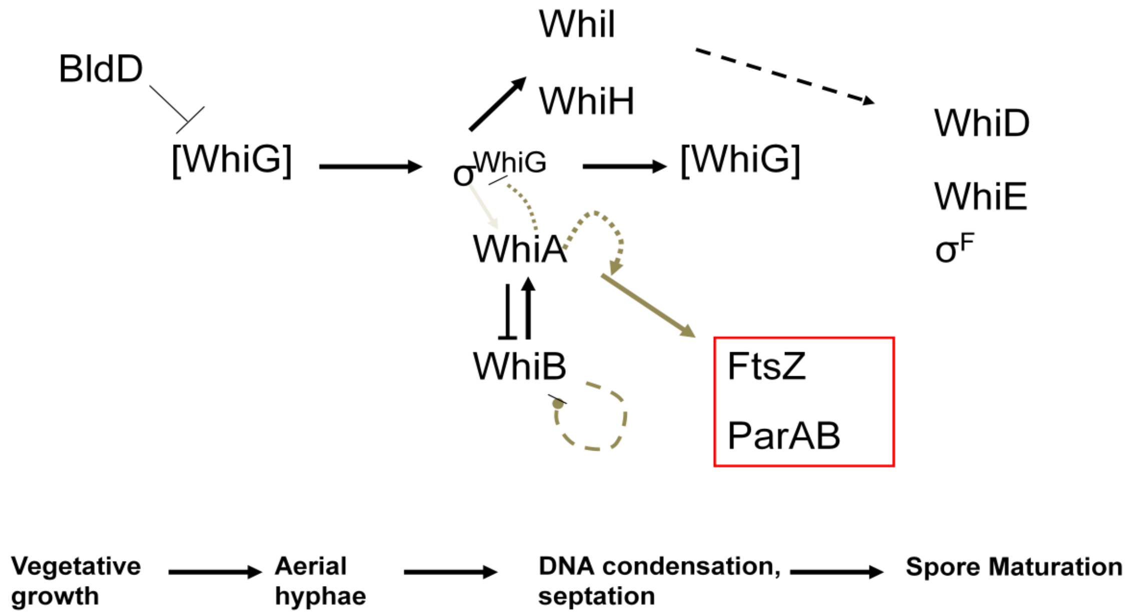

Fig. 1.4 The sporulation regulatory network in *S. coelicolor*. The known *whi* clusters for *S. coelicolor* beginning with initiation of aerial hyphae and proceeding to spore maturation. The only direct interaction known is between σ^{whiG} and *whiH* and *whiI*. WhiH is required for FtsZ expression and proper septation. WhiI is required for DNA condensation. WhiA and WhiB are required for cessation of elongation. All five early whi genes are required for SigF and WhiD production and proper spore maturation. (modified from (Chater, 2001, Kaiser & Stoddard, 2011)).

The *B. subtilis sigB* homologue, *sigF* is another gene responsible for the later stages of sporulation (Potuckova *et al.*, 1995). A disrupted *sigF* mutant produces spores that have thin cell walls and are smaller and more sensitive to detergents compared to the wild type. *sigF* (σ^F) encodes a second sporulation sigma factor that is active at the later stages of sporulation and is most similar to σ^B of *B. subtilis* that encodes a protein responsible for the general stress response and controls gene expression during stationary phase (Potuckova *et al.*, 1995). Spores of *sigF* mutants are formed with a light green colony phenotype, have thinner spore walls, less condensed DNA, and are more sensitive to detergent then the wild type spores (Potuckova *et al.*, 1995)

Mutations in some loci in *S. coelicolor* allow the formation of sporulation septa, but still affect the later stages of sporulation, including pigmentation due to their inability to produce the pigment and were therefore white or less grey than the wild type strain. When spores mature they develop a grey colour due to the production of this spore-associated polyketide pigment. This is encoded by the *whiE* locus, which contains a group of genes encoding a polyketide synthase complex that produces the grey polyketide pigment at the spore cell wall. This locus encodes enzymes involved the biosynthesis of the pigment and regulator of seven genes (ORFI-VII) and another gene (ORFVIII) that is transcribed in the opposite direction (Davis & Chater, 1990, Kelemen *et al*., 1998).

Sporulation septation and spore maturation call for transcription of the following later genes: *ftsZ*, which assemble the FtsZ rings and are key to the location of septation (Flardh *et al*., 1999) and *ssgA-G* that encode a group of homologous proteins, SsgA-like proteins (SALPs). These proteins are found in sporulating actinomycetes and are involved in several aspects of sporulation (spore specific cell division, chromosome partitioning/condensation, spore wall synthesis, septum location and autolytic spore separation (Noens *et al.*, 2005)). Additional genes that also have an impact on late sporulation processes have been reported. One example is *mreB*, which affects the deposition of peptidoglycan surrounding the spores (Carballido-Lopez, 2006, Mazza *et al*., 2006). In addition, SALPs proteins in *S. coelicolor* which include (SsgA-SsgF) have been reported to control different steps in the conversion of aerial hyphae into spores (Noens *et al.*, 2007). SALPs have only been found in actinomycetes, in both more morphologically complex genera as well as in non sporulating species, and are small

acidic proteins with no similarity to any known proteins (Traag & van Wezel, 2008). An *ssgA* null mutant in *S. coelicolor* has a significant impact on sporulation processes such as the formation of sporulation septa. An *ssgD* mutant lacked a thick peptidoglycan layer surrounding normal spores, *ssgE* and *ssgF* mutants affect the efficiency of separation of adjacent spores in the chains (Noens *et al*., 2005). SsgA was reported as a protein responsible for sporulation and suppressor of fragmented growth of *S. griseus* B2682 and was shown to be essential for submerged sporulation (Kawamoto & Ensign, 1995, Kawamoto *et al.*, 1997). Similar to *whiG*, over-expression of *S. griseus ssgA* in liquid-grown mycelium of *S. coelicolor* induced mycelial fragmentation and spore formation (van Wezel *et al.*, 2000a). On solid media *ssgA* mutants have a conditional "white" phenotype, capable of producing spores on mannitol-containing medium, but not in the presence of glucose (Jiang & Kendrick, 2000, Van Wezel *et al.*, 2000b).

Several genes have also been classified that are involved in septation: *ftsZ* from *S. coelicolor* and *S. griseus* and *ftsQ* from *S. coelicolor* (McCormick & Losick, 1996). In addition, many cell division genes from *E. coli* and *B. subtilis* are also involved in streptomycete sporulation; the *mre* genes *mreB, mreC* and *mreD*, were identified in *E. coli* by complementation of a deletion mutant which grew as spherical rather than rod-shaped cells and are important in the synthesis of the septum-specific peptidoglycan (Burger *et al.*, 2000). Cell wall growth polarization is different in streptomycetes from that of *E. coli* and *B. subtilis* (Hempel *et al*., 2008), which like most rod-shaped bacteria extend the cell and obtain their rod shape by insertion of new peptidoglycan units along the lateral wall (DePedro *et al.*, 1997). This is dependent on the actin-like MreB proteins

(Cabeen & Jacobs-Wagner, 2005). In contrast to rod-shaped bacteria, an *mreB*-independent mechanism facilitates streptomycete tip extension that is also independent of FtsZ and cell division (Mazza *et al.*, 2006). The *S. coelicolor* genome contains two *mreB* genes, but they are involved principally in sporulation and have no effect on tip extension in the vegetative mycelium (Mazza *et al*., 2006). Actually, most rod-shaped relatives of *Streptomyces* within the phylum Actinobacteria, like mycobacteria and corynebacteria, lack *mreB* genes and also assemble their cell wall at the cell poles (Chauhan *et al.*, 2006). There are many genes that are essential for cell division in rod-shaped bacteria, but when deleted in streptomycetes simply result in a white phenotype. This indicates that sporulation in streptomycetes is analogous to cell division in rod-shaped bacteria (Bennett *et al.*, 2007, Errington *et al.*, 2003). Deletions of *S. coelicolor* *ftsZ* and *ftsQ* are absolutely or, to a great extent, blocked for septation, respectively (McCormick & Losick, 1996). *ftsL* and *DivIC* (*ftsB/ygbQ* genes in *E. coli)* homologues *S. coelicolor* have also been characterized (Bennett *et al*., 2007, Bramkamp *et al.*, 2006, Noirclerc-Savoye *et al.*, 2005). The surfactant peptide SapB also happens to be localized at hyphal tips whilst DNA translocases of the *ftsK* family (also known as the SpoIIIE family) (Flardh & Buttner, 2009) are involved in sporulation (Ausmees *et al*., 2007, Barre, 2007, Begg *et al.*, 1995, Wang *et al.*, 2007).

Recent research has shown that bacterial cells are highly systematized and homologues to eukaryotic actin, tubulin, and intermediate filament proteins have been identified (Graumann, 2007). FtsZ, a prokaryotic homologue of β-tubulin plays a key role in morphogenesis of coccal cells as well as in division of both coccal and rod cells by

forming a ring-shaped structure during cell division (Cabeen & Jacobs-Wagner, 2005), while MreB, an actin homolog in prokaryotic cells that often polymerizes into helical structures, is responsible for guiding the formation of rod-shaped bacteria (Cabeen & Jacobs-Wagner, 2005). In addition to cocci and rods, there are many other bacteria showing more complicated shapes. In these bacteria, intermediate filament-like proteins have been shown to play crucial roles (Ausmees *et al.*, 2003, Cabeen & Jacobs-Wagner, 2005). These prokaryotic cytoskeletal elements may influence the cell shape by guiding cell wall peptidoglycan synthesis (Cabeen & Jacobs-Wagner, 2005).

1.4 Involvement of phospholipids in bacterial growth

Lipids are molecules that display a wide diversity in structure and biological function. The major role of lipids is to form the lipid bilayer that acts as a permeability barrier between cells and organelles and the surrounding environment. Neutral diacylglycerol glycans are major membrane-forming components in many Gram-positive bacteria and in the membranes of plants, while Gram-negative bacteria utilize a saccharolipid (Lipid A) as a major structural component of the outer membrane. *E. coli* has three major phospholipids (PLs) and several different fatty acids along with many minor precursors and modified products, the number of individual PL species numbers many hundreds. In more complex eukaryotic organisms with greater diversity in both the PLs and fatty acids, the number of individual species is in the thousands (Vance & Vance, 2008). Bacteria compete for limited nutrients by the production of antibiotics that serve to inhibit the growth of their competitors in the soil environment. The majority of antibacterial compounds in clinical use are natural products of soil-dwelling organisms

are produced by *Streptomyces* spp. and related members of the Actinomycetales. Many of these compounds target the synthesis of the peptidoglycan cell wall layer or disrupt membrane function (Hachmann *et al.*, 2009). Triacylglycerols (TAGs) are the most common lipid-based energy reserves in animals, plants and eukaryotic microorganisms. In bacteria, polyhydroxyalkanoic acids are the most abundant type of neutral lipids are, but some examples of large amounts of TAG accumulation have been found, mainly in the actinomycetes *Mycobacterium, Nocardia, Rhodococcus,* and *Streptomyces* (Arabolaza *et al.*, 2008, Olukoshi & Packter, 1994).

The mycobacterial cell envelope is the site of action of many antimycobacterial agents for the chemotherapy of tuberculosis. In order to develop new drugs that interfere with envelope biosynthesis in mycobacteria it is necessary to first develop an understanding of how component of the cell is synthesised (Jackson *et al.*, 2000). The enzymes that are involved in the synthesis of mycobacterial PLs are potential targets for drugs that interfere with envelope biosynthesis in this bacterial group as well as in other actinobacterial genera of medical or industrial relevance, such as *Streptomyces* and *Corynebacterium*.

1.4.1 Cardiolipin

Cardiolipin (CL) is an unusual PL with a dimeric structure, carrying four acyl groups and two negative charges (Fig. 1.5). It is found in bacterial and mitochondrial membranes, which are designed to generate an electrochemical potential for substrate transport and ATP synthesis. The name cardiolipin refers to the tissue from where it was first isolated in 1942 from beef heart (Sandoval-Calderon *et al.*, 2009, Schlame *et al.*, 2000). CL, also called diphosphatidylglycerol, with its unique dimeric molecular structure in which two phosphatidyl moieties are linked by a glycerol (Mileykovskaya & Dowhan, 2009). A number of structural analogues of CL are also found in bacteria such as from the genera *Streptococcus* (phosphatidylglycero-phosphoglycerol, D-glucopyranosylcardiolipin, D-alanylcardiolipin) and *Clostridium* (plasmenylcardiolipin) (Schlame *et al*., 2000).

CL is formed by a condensation reaction of two phosphatidylglycerol (PG) molecules in bacteria (Zhang & Rock, 2008). A common variation on the basic mechanism of PL biosynthesis is the formation of glycolipids by the reaction of a uridine diphosphate sugar with diacylglycerol (DAG). Glucosyl- and diglycosyl-diacylglycerol lipids are abundant in many Gram-positive bacteria, and these lipids act as membrane anchors for the lipoteichoic acid constituents of the cell wall. In addition, ornithine derived lipids are produced in phosphate-limiting environments so that available phosphate can be diverted to macromolecular synthesis. Ornithine lipids are also crucial structural components of the membranes of photosynthetic bacteria.

Quantitative detection of PLs is challenging and unequivocal visualization of CL domains in *E. coli* and *B. subtilis* membranes has been accomplished by means of the CL-specific fluorescent dye, 10-N-nonyl acridine orange (NAO) (Matsumoto *et al.*, 2006). NAO is a dye that can be used as a specific probe for the detection of CL in living cells (Kaewsuya *et al.*, 2007) and has been applied in liposomes and mitochondria through its interaction with CL. For example, NAO incorporation in the inner membranes of mitochondria can be detected as red fluorescence emitted by binding of NAO dimers. Increasing amounts of CL in membrane vesicles added to NAO changed the green fluorescence to red fluorescence and provides an elegant way to discriminate CL from other anionic PLs. CL-rich domains (Mileykovskaya & Dowhan, 2000) were visualized with the CL-specific fluorescent dye NAO and used to demonstrate CL localization to septa and the polar membrane regions of *E. coli* cells. The same group proposed a model for the mechanism of CL-specific staining in which the nonyl group of NAO inserts at the hydrophobic surface created by the two outer acyl chains of CL between the phosphate head groups (Mileykovskaya *et al.*, 2001). Fluorescent domains of NAO-stained CL was observed at septal and polar regions of exponential *B. subtilis* cells, however this fluorescence was not observed in cells blocked in CL synthase activity that lacked detectable quantities of CL through a *clsA* null mutation (Kawai *et al.*, 2004). In sporulating cells, fluorescent domains were clearly seen in the membranes during prespore engulfment and subsequently in prespore membranes during the various stages of *B. subtilis* sporulation. Interestingly, spore membranes have a high CL content, although its localization in the membranes has not been observed. The fluorescence

images of NAO in *B. subtilis* cells are more distinct than those seen in *E. coli*, although this is perhaps due to the simpler structure of the Gram positive envelope. The preferential localization of CL at the poles of *E. coli* and *B. subtilis* cells is consistent with its enrichment in minicells, which are formed by aberrant cell division close to the pole (Koppelman *et al*., 2001). The phosphatidylethanolamine (PE) and CL-rich domains localization at the septum raises interesting questions as to the subcellular localization of the enzymes involved in PE and CL synthesis. The first committed step in PE synthesis in *B. subtilis* is carried out by phosphatidylserine synthase (PssA) (Fig. 1.5). Its product, phosphatidylserine (PS), is then converted to PE by PS decarboxylase (PsD). PG synthase (PgsA) catalyses the initial step for the synthesis of phosphatidylglycerol (PG). This is then used by CL synthase to produce CL. When GFP was fused to these enzymes in *B. subtilis*, they were found to septally localized, even when expression levels, and consequently fluorescence intensity, were low (Nishibori *et al.*, 2005). As a result it seems likely that these enzymes concentrate at the septum under normal conditions.

According to (Koppelman *et al*., 2001), *E. coli* cell division is initiated by formation of the FtsZ ring in the middle of the cell. Eventually, other cell division proteins are recruited to this ring and collectively they form the divisome, which carries out the process of daughter cell separation. Localization of the cell division proteins has been studied extensively in this organism, although it is still not known how they are directed to the correct cellular address (Margolin, 2000). It seems possible, that proteins of the divisome recognize an appropriate combination of proteins and lipids. As a result, the process of membrane invagination might also require a particular composition of PLs

at mid-cell in order to facilitate membrane curvature and invagination. Membrane curvature might form an important component of this process and, as such, the shape of PLs might also affect membrane invagination. For example whether the individual PLs form cylinders, cones or even inverted cones. For example, CL is cone shaped, but forms a cylindrical shape in the absence of these ions. (Koppelman *et al*., 2001).

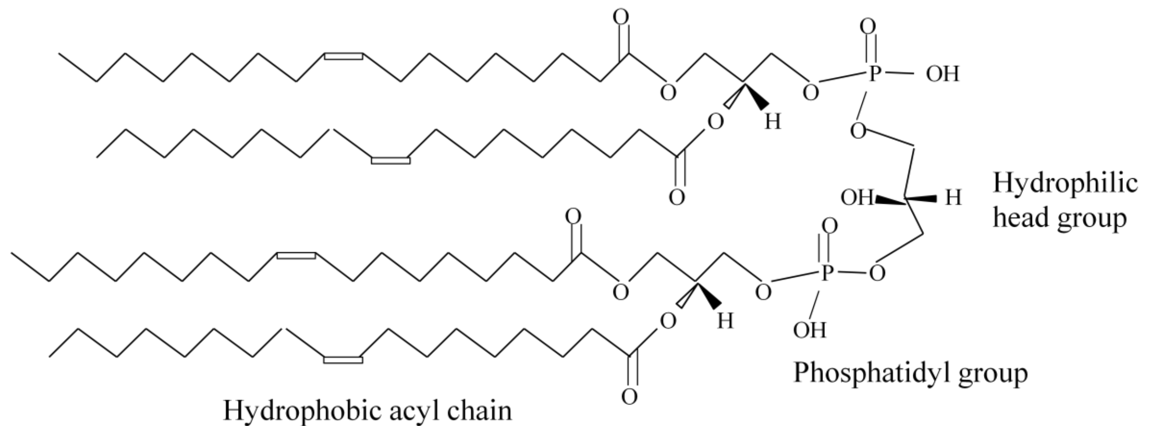

Fig. 1.5 Cardiolipin structure. CL consists of two phosphatidyl groups connected by a glycerol and has four hydrophobic acyl chains and a small hydrophilic head group.

1.5 Biosynthesis of phospholipids by *Streptomyces*

In silico reconstruction of PL biosynthesis in *S. coelicolor* was carried out and allowed the identification of genes responsible for the biosynthesis of PLs (Borodina *et al.*, 2005, Sandoval-Calderon *et al.*, 2009). These PLs and the enzymes that catalyse their biosynthesis are described in Fig. 1.6. It is expected that a deficiency in some of these PL would affect the structural and functional organization of the bacterial membrane, presumably resulting in the death of the bacterium. Contrary to PE, PG, PS, and CL, which are frequently encountered in all living organisms, phosphatidylinositol (PI) is an essential PL of eukaryotic cells, but has seldom been found in prokaryotic cells. Actually, the distribution of PI in prokaryotes seems limited to some Actinobacteria (*Mycobacterium, Corynebacterium, Nocardia, Micromonospora, Streptomyces, and Propionibacterium*), as well as *Myxobacterium* and *Treponema*. In terms of other lipids found in bacteria, neutral lipid storage compounds are synthesized during the post-exponential phase during growth in liquid culture in *S. coelicolor* (Arabolaza *et al.*, 2008).

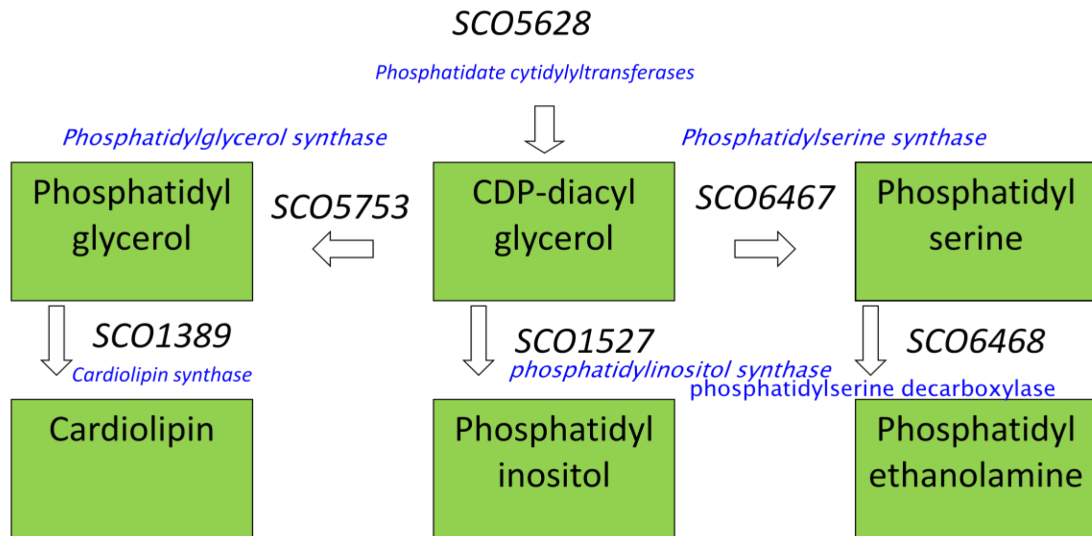

Fig. 1.6 Major PLs and their biosynthetic enzymes in *S. coelicolor* (reproduced from Sandoval *et al*., 2009).

1.5.1 Phospholipid extraction form bacteria

Several techniques dealing with the PLs extraction have previously been published. Although some authors used whole cells, the first step generally consists of a mechanical (Schauner *et al.*, 1999) or enzymatic cell disruption (Hoischen *et al.*, 1997). The second step consists of the extraction of lipids from cellular fragments. Most of the methods are based on chloroform/methanol/water extractions as described by (Folch *et al.*, 1957) or (Bligh & Dyer, 1959, Limonet *et al.*, 2007). The last steps consist of the separation, detection and the determination of PL. In microbiological studies, although high performance liquid chromatography coupled with laser light-scattering evaporative (Hoischen *et al.*, 1997), electrospray ionisation coupled with collision-induced dissociation mass spectrometry, fast atom bombardment mass spectrometry, or ^{31}P NMR

were occasionally used. However, the most often employed technique is two dimensional thin layer chromatography (2D-TLC) with separation using a combination of chloroform, methanol and ammonia followed by a specific staining (Schauner *et al*., 1999, Hoischen *et al*., 1997). Nevertheless, the 2D-TLC technique is time-consuming and usually necessitates a large amount of sample. After separation, CL, PI, PG, and phosphatidic acid (PA) were identified by comparison with reference substances (Nampoothiri *et al.*, 2002).

1.6 Aims of this thesis

Although much is now know about the proteins that govern growth and development of *Streptomyces*, little is known about the role that PLs play in directing these proteins to the correct cellular address so that peptidoglycan is incorporated into the cell wall at hyphal tips and at cross walls during the laying down of sporulation septa. As such, it was the aim of this thesis to determine the changes in PL content that take place during growth and development of *Streptomyces* as well as the changes in expression of PL biosynthetic genes during the process of morphological differentiation.