Regulation of morphological and physiological differentiation in actinomycetes

Regulation of Morphological and Physiological Differentiation in Actinomycetes

Habilitationsschrift

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Summary

Actinomycetes are among the most fascinating microorganisms. Their developmental lifecycle, including morphological and physiological differentiation, and the rich repertoire of secondary metabolites, about 70 - 80 % of bioactive secondary metabolites are being produced by actinomycetes, have resulted in a large research community studying these microbes. In general, the research topics pursued include: i) regulation and the genetics of the morphological development; ii) regulation of secondary metabolism; iii) primary metabolic pathways and their control; iiii) the interplay between primary and secondary metabolism and morphogenesis and iiiii) search for new bioactive secondary metabolites and the improvement of their productivity.

The last point may be the driving force to study actinomycetes, even though their handling requires special skills. Research projects driven by the quest to find new molecules or to improve production of a certain metabolite have resulted in very detailed results on specific systems (strains or biosynthetic routes). However, results are rarely transferable to other systems (strains or pathways) due to the great diversity among actinomycetes in both, their physiology and in the secondary metabolites made. Hence, knowledge on regulatory pathways remains very limited. Some model organisms, such as *Streptomyces coelicolor* A3(2) and *S. griseus*, have emerged and they demonstrate that some general pathways are shared but that common features in regulation of differentiation are rarely identified. Advances in molecular biology have resulted in a growing number of reports that establish links on the genetic level between primary and secondary metabolism and morphogenesis. With the completion of the *Streptomyces* genome project, gene chips may soon be available and open up new possibilities for studying regulation and the response to stress factors leading to differentiation and secondary metabolism.

Nevertheless, more detailed physiological studies are needed to address and to understand the complex regulatory network that leeds to cellular differentiation. Especially the design of cultivations systems that allow to distinguish between the different effects of primary metabolism

on cell differentiation, such as repression by glucose (carbon), nitrogen and phosphorous are needed. Instead, a multitude of strains that produce a great variety of secondary metabolites is currently being studied under different cultivation regimes making it difficult to identify common signals in regulation.

The aim of this review is to compile and to discuss the recent advances in, and our current understanding of the regulatory events leading to and controlling the differentiation processes. The lifecycle and the known signaling cascades that partially control differentiation are discussed first. Following a discussion of the influence of the primary metabolism and the nutritional state of the cell, several additional factors that have been implicated in regulation are analyzed.

Taken together, examples indicate that regulation is exerted at transcriptional and translational level. Moreover, recent findings suggests several partly independent signaling pathways that regulate differentiation, such as the coexistence of two independently acting signaling pathways leading to morphogenesis in *S. griseus*. Triggers for differentiation include environmental stress conditions, but also the accumulation of primary metabolites including those of the stringent response. The growing number of sensor kinase/response regulator two component systems identified, many of which are membrane associated and involved in regulating differentiation, may be each a starting point of a signal cascade. So far ligands and targets of these systems remain to be identified. At some point the signals from the various pathways have to converge in order to set off differentiation. A putative level just above the pathway-specific regulators may be such a node.

The completion of the *Streptomyces* genome project will provide the tools urgently needed to understand the molecular nature of the known signal pathways and to identify new players in the processes leading to cellular differentiation.

1. Introduction

The use of microorganisms to produce natural products and processes that benefit and improve our socioeconomic lifestyles has been part of human history since the days of early civilizations. Actinomycetes, and members of the genus *Streptomyces* in particular, are ubiquitous in nature and have been an excellent source for biologically active secondary metabolites, such as antibiotics, including anti-tumor, anti-fungal and anti-viral metabolites, immunosuppressants and others. Table 1.1 was recently compiled, listing some 62 useful secondary metabolites out of the over 9000 biologically active molecules isolated so far from Actinomycetes, which account for 2/3 of the known antibiotics made by microorganisms and about 60% of all secondary metabolites with biological activities other than antibiotic. In both cases nearly 80% of the molecules are made by members of the genus *Streptomyces* (Kieser et al., 2000). Their function in nature can, in most instances, not clearly be defined. Some molecules are designed as competitive weapons, others are implicated in metal transport, or molecules might act as stimulants for symbiosis, sexual hormones or effectors for differentiation (Demain, 1989). The driving force for the vast structural diversity of secondary metabolites and the evolution of their complex pathways is not well understood, as is the regulation of the processes leading to the production of secondary metabolites.

In this work, the latest results on regulation of secondary metabolites will be reviewed and discussed.

There are other commercially valuable products of the primary metabolism of actinomycetes and other gram-positives that are not mentioned in this table. Amino acid production by *Corynebacteria* may be the most relevant process to mention (Aida et al., 1986).

Table 1.1. List of useful actinomycete antibiotics and their classification. (source: Kieser et al., 2000).

Antibiotic	Producer	Chemical class[1]	Target[2]	Application
Actinomycin D	*S. spp.*	Peptide	Transcription	Antitumor
Antimycin A	*S. spp.*	Macrolide	Cytochrome system	Telocidal
Avermectin	*S. avermitilis*	Macrolide (PK)	Chloride ion channels	Antiparasitic
Bambermycin	*S. bambergiensis*	Substituted amino-glycosides (complex of at least 4 moenomycins	Peptidoglycan	Growth promotant
Bialaphos	*S. hygroscopicus*	Peptide	Glutamine synthetase	Herbicidal
Bleomycin	*S. verticillus*	Glycopeptide	DNA strand breakage	Antitumor
Candicidin	*S. griseus*	Polyene macrolide (PK)	Membrane (pore former)	Antifungal
Cephamycin C	*Nocardia lactamdurans* (and others)	β-lactam	Peptidoglycan	Antibacterial
Chloramphenicol	*S. venezuelae*	N-dichloracyl phenylpropanoid	R	Antibacterial
Chlorotetracyline	*S. aureofaciens*	Tetracycline (PK)	R	Antibacterial
Clavulanic acid	*S. clavuligerus*	β-lactam	β-lactamase inhibitor	Combined with a β-lactam as antibacterial
Cycloserine	*S. orchidaceus*	Substituted cyclic peptide	Peptidoglycan	Antibacterial
Daptomycin	*S roseosporus*	Lipopeptide	Lipoteichoic acid?	Antibacterial
Daunorubicin (daunomycin)	*S. peucetius*	Anthracycline (PK)	DNA intercalation	Antitumor
Desferrioxamine	*S. pilosus*	Peptide	Iron chelation	iron purging in iron overload
Doxorubicin (adiamycin)	*S. peucetius* var. *caesius*	Anthracycline (PK)	DNA intercalation	Antitumor
Erythromycin	*Sac. erythraea*	Macrolide (PK)	R	Antibacterial
FK506 (tacrolimus)	*S. hygroscopicus*	Macrolide (PK)	Binds to FK protein	Immunosup-pressant
Fortimicin	*Micromonosproa olivoasterospora*	Aminoglycoside	R	Antibacterial
Fosfomcin	*S. spp.*	Phosphoric acid	Peptidoglycan	Antibacterial
Gentamycin	*Micromonospora spp.*	Aminoglycoside	R	Antibacterial
Hygromycin B	*S. hygroscopicus*	Substituted aminoglycoside	R	Antihelminitic
Kanamycin	*S, kanamyceticus*	Aminoglycoside	R	Antibacterial

Antibiotic	Producer	Chemical class[1]	Target[2]	Application
Lasalocid	*S. lasaliensis*	Polyether (PK)	Membrane (ionophore)	Anticoccidial; growth promotant
Lincomycin	*S. lincolnensis*	Sugar-amide	R	Antibacterial
Milbemycin	*S hygroscopicus*	Macrolide (PK)	Chloride ion channels	Antiparasitic
Mithramycin	*S. argillaceus*	Aureolic acid	DNA alkylation	Antitumor
Mitomycin C	*S caespitosus* *S. verticillatus*	Benzoquinone	DNA cross-linking	Antitumor
Monensin	*S. cinnamonensis*	Polyether (PK	Membrane (ionophore)	Anticoccidial; growth promotant
Natamycin	*S. nataensis*	Tetraene polyene (PK)	Membrane (pore former)	Antifungal
Neomycin	*S. fradiae*	Aminoglycoside	R	Antibacterial
Nikkomycin	*S. tendae*	Nucleoside	Chitin biosynthesis	Antifungal; insecticidal
Nocardicin	*Nocardia uniformis*	β-lactam	Peptidoglycan	Antibacterial
Nosiheptide	*S. actuosus*	Thiopeptide	R	Growth promotant
Novobiocin	*S. niveus*	Coumerin glycoside	DNA gyrase (β–subunit)	Antibacterial
Nystatin	*S. noursei*	Polyene macrolide (PK)	Membrane (pore former)	Antifungal
Oleandromycin	*S. antibioticus*	Macrolide (PK)	R	Antibacterial
Oxytetracycline	*S. rimosus*	Tetracycline (PK)	R	Antibacterial
Paromomycin	*S. rimosus* forma *paromomycinus*	Aminoglycoside	R	Antiamoebal
Phleomycin	*S. verticillus*	Glycopeptide	DNA strand breakage	Antitumor
Polyoxins	*S. cacaoi var. asoensis*	Nucleoside-peptide	Chitin biosynthesis	Antifungal (plant protection)
Pristinamycin	*S pristinaespiralis*	Peptidic macrolactone + polyunsatturated macrolactone (PK)	R	Antibacterial
Puromycin	*S. alboniger*	Purine nucleoside	R	Research
Rapamycin	*S. hygroscopicus*	Macrolide (PK)	Binds FK protein	Immunosup-pressant
Rifamycin	*Amycolatopsis mediterranei*	Ansamycin (PK)	RNA polymerase	Antibacterial
Ristocetin	*Nocardia lruida*	Glycopeptide	Peptidoglycan	Antibacterial
Salinomycin	*S. albus*	Polyether (PK)	Membrane (ionophore)	Anticoccidial; growth promotant
Spectinomycin	*S. spectabilis*	Aminocyclitol	R	Antibacterial
Spinosyns	*Sac. spinosa*	Tetracyclic mocrolide (PK)	Unknown	Insecticidal
Spiramycin	*S. ambofaciens*	Macrolide (PK)	R	Antibacterial
Streptogramins	*S. graminofaciens*	Macrocyclic lactons	R	Antibacterial

Antibiotic	Producer	Chemical class[1]	Target[2]	Application
Streptomycin	*S. griseus*	Aminoglycoside	R	Antibacterial
Sterptothricin	*S. lavendulae*	N-glycoside	R	Growth promotant; plant protection
Teichoplanin	*Actinoplanes teichomyceticus*	Glycoprotein	Peptidoglycan	Antibacterial
Tetracycline	*S aureofaciens*	Tetracycline (PK)	R	Antibacterial
Thienamycin	*S. cattleya*	β-lactam	Peptidoglycan	Antibacterial
Thiostrepton	*S. azureus*	Thiopeptide	R	Growth promotant
Tobramycin	*S. tenebrarius*	Aminoglycoside	R	Antibacterial
Tylosin	*S. fradiae*	Macrolide (PK)	R	Growth promotant
Validamycin	*S. hygroscopicus*	Aminoglycoside	R	Plant protection
Vancomycin	*Amycolatopsis orientalis*	Glycopeptide	Peptidoglycan	Antibacterial
Virginamycin	*S. virginiae*	Macrocyclic lactone (PK) + macrocyclic peptidolactone	R	Growth promotant

[1] PK = polyketide, [2]R = binds to ribosomes and thus inhibits protein synthesis

Not only the production of valuable metabolites but also the complex lifecycle of actinomycetes has culminated in numerous research projects in the course of which genetic tools where developed, genomes sequenced, and studies on the morphological and physiological differentiation were conducted. In part driven by the availability of an ordered cosmid library (Redenbach et al., 1996) maintained at the John Innes Centre (Norwich, UK) *Streptomyces coelicolor* A3(2) and *S. lividans*, a strain closely related to *S. coelicolor* serve as model systems for many studies. *S. griseus*, producer of streptomycin, is another well studied strain in which A-factor, the quorum sensing autoregulator, has been discovered (Horinouchi, et al., 1983/86/90; Horinouchi and Beppu, 1992/94; Ueda et al., 1998/2000). Other strains were studied, especially when an isolate produced an interesting metabolite that would warrant the effort. These studies impressively demonstrate the physiological diversity within the actinomycetes or the streptomycetes which probably reflects the adaptation to a very competitive and hostile environment, the soil, in which cells grow as vegetative mycelium provided nutrients are available and environmental conditions are favorable. Environmental stress, however, will induce a highly regulated cascade of events that

result in a morphological and a physiological differentiation with the aim to produce spores that are resistant to low nutrient and water availability (Figure 2.1). The costs for this developmental program are high: Since the program is initiated after, e.g., nutritional stress had developed, parts of the substrate mycelium are sacrificed and lysed to liberate resources until the sporulation has been completed (Wildermuth, 1970; Miguélez et al., 1999). Once started, differentiation will neither be reversed nor stopped. Nucleases were discovered that specifically degrade the DNA in the substrate mycelium (Nicieza et al., 1999), a process with significant analogies to the late steps of apoptosis, the programmed cell death, of eucaryotic cells. Moreover, to defend the partly lysed mycelium from being invaded by other soil organisms, a physiological differentiation starts which leads to the production of highly bio-active (antibiotic) molecules. These compounds are highly oxidized and from a metabolic point of view, very expensive molecules, and therefore are naturally produced in small amounts (Prosser and Tough, 1991). Nevertheless, the process appears to be money well spent as examples showed that viable spores could be recovered from 70 year old soil samples (Morita, 1985). Other adaptations of Actinomycetes to the native environment include the largely missing regulation of amino acids biosynthesis (Kroening and Kendrick, 1987). Biosynthesis is constitutive as in the nutrient limited environment repression of biosynthesis is not required.

Differences to Gram-negative microorganisms and low G+C Gram-positives can be found in the central carbon metabolism: There is no evidence that *Streptomyces* has a glucose-phospho-transferase system (PTS) and the recently described fructose PTS system of *Streptomyces* appears to lack the Ser-phosphorylation of Hpr (Histidine Protein) that plays a regulatory role in low G+C Gram positives (Titgemeyer et al., 1995). Similar to other systems, pathways for uptake and utilization of carbon sources other than glucose are generally inducable and repressed by glucose. However, cAMP is not implemented in carbon catabolite repression (CCR), and whether the fructose-PTS participates in CCR in unclear. In contrast, the ATP-dependent glucose kinase (GlkA) plays a crucial role in CCR (Angell et al., 1994; Hostalek et al., 1976).

Most streptomycetes use the Embden-Meyerhof-Parnas (EMP) pathway for glucose utilization

(Cochrane, 1961). Known exceptions are *S. antibioticus* that apparently only uses the hexose monophosphate shunt (Salas et al., 1984) and *S. clavuligerus* that can not utilize glucose as carbon and energy source but can use starch instead (Garcia-Dominguez et al., 1989). Also interesting to note is that several streptomycetes switch pathways from glycolysis (EMP) during primary metabolism to hexose monophosphate shunt during secondary metabolism (Dijkhuizen, Hunter, Oliver personal comm.) and that there is no evidence of any streptomycete using the Entner-Doudoroff pathway.

In addition to the physiological there are also genetic differences to other microorganisms. The G+C content of the DNA within the coding regions is typically greater 70 %, and, most noteworthy, the chromosome found in all streptomycetes studied so far is linear (Lin et al., 1993; Lezhava et al., 1995). Giant linear plasmids could also be identified (Keen et al., 1988). The high G+C content of the DNA has resulted in a different codon usage in streptomycetes which often hinders efficient transcription of heterologous DNA, and vice-versa interferes with efficient expression of *Streptomyces* DNA in hosts such as *Escherichia coli*. In addition, the ends of the chromosome are rather unstable which leads to large deletions and to recombinations resulting in an often unstable phenotype (Roth et al., 1982).

After this brief introduction into the fascinating biology of actinomycetes the following sections will discuss the latest results on the regulation of differentiation and the primary metabolism of streptomycetes in more detail.

2. The Lifecycle of Actinomycetes

A bacterium that looks like a mold and that grows into a differentiated colony - more representing a primitive organism than a unicellular bacterium ?**!**

On solid medium, colonies of *Streptomyces* are readily distinguished from other bacteria and yeast by their typical sculptured surface that, upon sporulation, becomes a velvet-like surface. In addition, pigmented secondary metabolites are often excreted into the surrounding medium.

The major stages of the *Streptomyces* lifecycle are shown in Figure 2.1. Starting with a dormant spore (1), the cycle begins when favorable environmental conditions are sensed. Water availability and temperature (mild heat treatment improves germination) are the most obvious stimuli that could be sensed by a dormant spore and induce germination. Divalent cations (often Ca^{2+}) and CO_2 are also required. Germ tube emergence from the unigenomic spore coincides roughly with completion of the first round of DNA replication. Synchronous DNA replication continues until the germ tube contains 32 genomes, and the rate of hyphal extension has increased to approximately 40 µm per hour on rich medium (the length of an *E. coli* cell every 2-3 minutes) (reviewed in Champness and Chater, 1994). Growth of the mycelium occurs mainly at the tip, and the growth rate doubles with each replication of the chromosome in the initially aseptate germ tube (Chater and Losick, 1996). At this accelerating rate of tip growth cell wall biosynthesis would soon become limiting. The emergence of a new tip relieves this problem every time the maximum rate of extension has been reached, thus initiating the mycelial growth pattern (2). Therefore, the rate of tip extension does not increase, nevertheless a more or less exponential increase in biomass is achieved by an exponential increase in the number of branches formed. Kretschmer (1991) suggests that the trigger signal for branching is an accumulation of cell wall precursors beyond levels that can be consumed by the tip. The highest concentration of these precursors would be expected at the farthest distance from the growing tip, the region where branching is observed. Alternatively, branching could be induced by an imbalance in the ratio of genomes to cytoplasm as DNA replication continues, especially in the subapical cells. Branching with subsequent septation would correct this imbalance (Champness and Chater, 1994). Occasional septa are formed which often are close to branch points. It remains to be determined what controls the positioning and the formation of the septa.

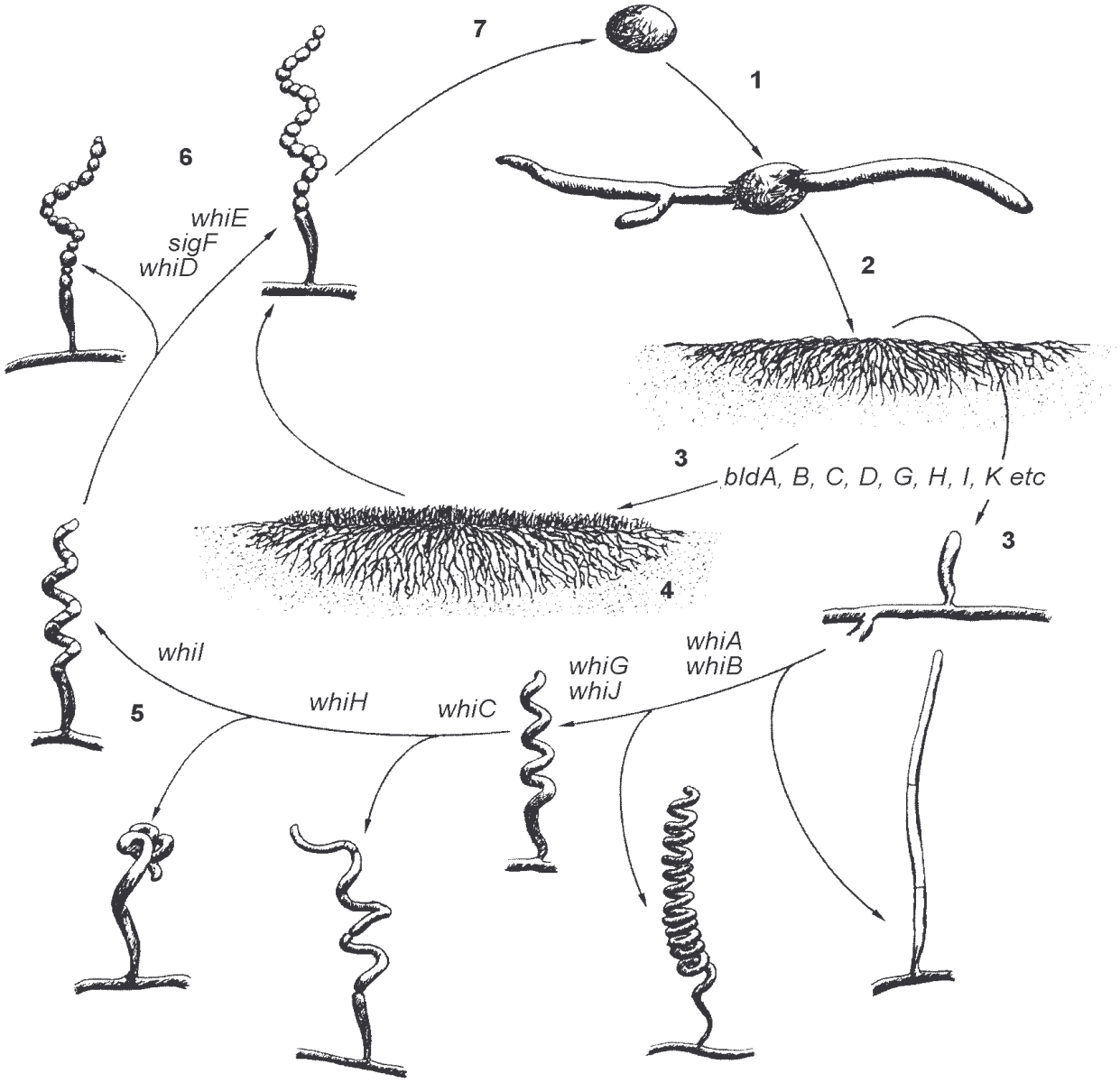


Figure 2.1. Lifecycle of actinomycetes. The inner circle describes the macroscopic appearance of the colony, the outer circle provides a more detailed depiction of the single steps in aerial hyphae development. Names of the genes involved are indicated. The numbers refer to the main stages in development: 1: germination; 2: formation of a substrate mycelium; 3: development of aerial hyphae and curling of the hyphae; 4: production of secondary metabolites; 5: formation of chains of uninucleate spores; 6: unigenomic prespores; 7: spore maturation. See text for details. (modified, from Kieser et al., 2000).

Most branches are close to septa which are more frequently found in the older parts of the mycelium. After septation, the mycelium can be divided into the apical part corresponding to the growing tip where cell wall biosynthesis takes place, and the sub-apical part which lacks cell wall

biosynthesis but where, nevertheless, DNA replications takes place. When a branch is formed in a sub-apical compartment, it regains some apical properties, such as cell wall biosynthesis. This vegetative mycelium develops into the colony by spreading over the and penetrating into the substrate until environmental stress factors are sensed and a complex regulatory network is being triggered leading to the morphological and physiological differentiation of the colony (3). Changes that are observed during this phase include:

- Initiation of lysis of some compartments of the substrate mycelium to provide nutrients for the newly developing aerial hyphae (Wildermuth, 1970; Miguélez et al., 1999).
- Initiation of aerial hyphal growth.
- Increased production of some extracellular proteins
- Onset of secondary metabolism (4)

The aerial hyphal development into spores involves growth (mainly tip growth; Miguélez, et al., 1994) into multigenomic, often curled hyphae (5) which undergo multiple synchronous cell divisions to yield unigenomic prespore compartments in which glycogen is transiently accumulated (6). Subsequently the prespore compartments round up and a thickened spore wall, pigmented with gray, polyketide-related aromatic compounds is formed (7). Trehalose, a major constitute of the spore, is probably associated with their resistance to desiccation (Braña et al., 1986).

Again, the development from an aerial hypha to multiple spores is a highly regulated and controlled process (Chater 1993, Bruton et al., 1995, Potúèková et al., 1995). During sporulation, the aerial hyphae require nutrients to be supplied by diffusion through the substrate mycelium Miguélez et al., 1994). These nutrient may in part derive from the lysing compartments of the substrate mycelium. Furthermore, the differentiation process requires that the continuous elongation and DNA replication in the aerial hyphae stops, that genes required for septation are activated and that the metabolism is directed toward the synthesis of spore components before it is eventually shut

down.

In *Streptomyces coelicolor* A3(2) glycogen deposits and oil droplets have been identified at two distinct locations in the mycelium. So called phase I glycogen deposits are observed roughly at the interface between substrate mycelium and aerial hyphae. It is argued that they supply carbon and energy for the aerial hyphae (Plaskitt and Chater, 1995). Alternatively, solubilization of the phase I deposits is believed to increase the osmotic potential resulting in water uptake, contributing to the development of sufficient turgor for drive extension of the aerial hyphae into the air (Chater, 1989a). Phase II glycogen deposits, found in the developing spore chains, are probably used as storage product for the biosynthesis of the spore pigment. Two glycogen branching isoenzymes have been identified, *glgB*I and *glgB*II, that are involved in synthesis of phase I and phase II glycogen deposits, respectively (Bruton et al., 1995). A recent analysis (Schneider et al., 2000) revealed that each of the two isoenzymes is part of a distinct, largely duplicated operon of at least 4 genes in the order *pep1-treS-pep2-glgB*, of which Pep1 shows relatedness to the α-amylase superfamily and TreS to a maltose-trehalose interconverting enzyme. Together with Pep2, these enzymes are thought to boost the localized supply for trehalose to the aerial mycelium for an increased growth and improved spore yields (Schneider et al., 2000). Each operon is located near to, and diverges from another gene likely to be involved in glycogen degradation: *glgP*, which encodes a putative glycogen phosphorylase, in the case of the *glgB*I cluster, and *glgX*, which specifies a putative debranching enzyme, in the case of the *glgB*II cluster (Schneider et al., 2000). Hence, the two spatially separated glycogen deposits are synthesized by pathways employing genetically distinct enzymes. Evidence for a developmentally distinct regulation of the two pathways came from *bld* mutants (bald: impaired in aerial hyphae formation) that where devoid of any phase I glycogen deposits (Plaskitt and Chater, 1995).

Other regulatory elements controlling sporulation have been identified. These include the *whi* genes, mutants of which are impaired in spore maturation (Chater et al., 1989), and at least 3

developmentally regulated sigma-factors that regulate morphogenesis. Details are discussed in chapter 3.1.1 and 3.1.2, respectively.

This brief description of the colonial development makes clear that a *Streptomyces* colony can well be regarded as a multicellular differentiated organism, very much like that of the fruiting body forming myxobacterial colony (Kaiser, 1984).

The above described livecycle is representative for actinomycetes growing on solid support. Under laboratory conditions medium requirements for efficient sporulation may vary from strain to strain. When growing in liquid media this life cycle will normally not be observed. Most *Streptomyces* hardly if ever will sporulate in submerged culture and morphological changes are mostly limited to a progressing fragmentation of mycelia in aging cultures. Exceptions are strains of *S. griseus* (e.g., Birkó et al., 1999). With all mycel-forming organisms, liquid medium cultivations will result in the formation of mycelial aggregates, pellets or balls, the size and shape of which will depend on the strain, the medium and the agitation scheme used (Prosser and Tough, 1991; Whitaker, 1992). Some, mostly chemically defined minimal media, will support a more disperse growth. The addition of not metabolised compounds such as sucrose (Hopwood et al., 1985), polyethylene $glycol_{8000}$ (Hodgson, 1982), Junlon (Hobbs et al., 1989), starch (Doull and Vining, 1989) or agar at low concentration (Hobbs et al., 1989; Magnolo et al., 1991) might further enhance dispersed growth which is desired for physiological studies, thus eliminating any mass transfer problems for oxygen and nutrients that might be encountered in a mycelial ball. Unfortunately, however, disperse growth is often accompanied by a complete inhibition of secondary metabolism (Prosser and Tough, 1991; Whitaker, 1992). This indicates the linkage between morphological and physiological differentiation and points to some common early signals involved in both differentiation processes.

3. Regulation

The following section reserves separate chapters for the morphological and the physiological differentiation even though common signals and signal cascades, especially regulating the early events, are linking both processes. One reason for this division is that the respective studies where done under very different conditions. Most of the work on morphological development is done on solid support grown cultures, while regulation of secondary metabolism has been studied in both, surface and submerged grown cultures. In addition, the individual treatment of both differentiation processes should provide a better understanding of the common, as well as of the different regulatory elements characterized so far among the genetically diverse streptomycetes.

3.1 Regulation of the Morphological Differentiation

The complex *Streptomyces* lifecycle and its developmental program, which involves the entire colony, requires a tight control. Spore germination is induced by some triggers, signaling favorable environmental conditions, the nature of which still remains to be determined. A prerequisite for the development of mature spores is the septation of the aerial hyphae. Two proteins, FtsQ and FtsZ are required for septation in bacteria. The *ftsZ* gene product is a highly abundant protein that somewhat resembles eucaryotic tubulin (Lutkenhaus, 1993) that is essential for *E. coli* and *Bacillus subtilis* cells to generate colonies (Beall and Lutkenhaus, 1991; Dai and Lutkenhaus, 1991). FtsZ is also present in streptomycetes (Dharmatilake and Kendrick, 1994; McCormick et al., 1994), but dispensable. Deletion of *ftsZ* in *S. coelicolor* resulted in more or less normal colonies (McCormick et al., 1994). A *S. coelicolor* deletion mutant for a second cell division gene, *ftsQ,* was also viable and able to form a branched mycelium. Its mycelium, however, was devoid of any cross walls (McCormick and Losick, 1996). Both deletion mutants, however, failed to develop the uninucleoid

compartments in the aerial hyphae and subsequently failed to produce spores. This demonstrates that septation is not required for branching, vegetative growth, or aerial hyphae formation. Only at the late steps in the development of the aerial mycelium into spores require the intact cell division and septation functions, which virtually eliminates septation as possible trigger for initiation of differentiation.

But how is morphological development regulated? A comprehensive answer to this question remains subject for intense studies. The paradigm for the onset of differentiation is that the growing colony experiences environmental stress upon which the developmental program is initiated. The nature of the environmental signals is still under investigation. They are likely to correlate with the nutritional status or the cell density of the colony, but other stress signals, such as heat-, cold-, or osmotic shock (Vohradsky et al., 2000), or occurrence of direct contact between hyphae, an internal counting of cell cycles or branching order (Champness and Chater 1994) should be considered.

Two main classes of genes or loci are associated with morphological differentiation in *Streptomyces coelicolor*: the *bld* genes which are required for the erection of aerial hyphae, and the *whi* genes, which are needed for the formation of mature spores (Table 3.1). These genes/loci could be grouped into a signaling cascade. Others, also included in Table 3.1, are genes or loci that do not fit into the *bld*-*whi* cascade but are essential for differentiation as discussed in the following sections.

3.1.1 Development of Aerial Hyphae - the *bld*- and *whi*- Genes

Some of the *bld* and *whi* genes have been isolated and partially characterized at a molecular level (*bldA, B, D, G, J, K, M, N; whiA, B, E, G, H, I, J*), others describe classes of mutants that were grouped, based on complementation studies, for their ability to restore morphological development and / or secondary metabolism in other *bld* or *whi* mutants (Table 3.1).

Table 3.1. Genes involved in morphogenesis. The genes were grouped into the *bld* gene-related functions, the *whi*-related genes and other genes that have been isolated based on phenotypes of altered morphogenesis.

Gene	*Sourceorganism*	*Product*	*Function*	*References*
***bld*-related genes involved in aerial mycelium formation**				
bldA	*S. coelicolor*	rare UUA-lucyl tRNA	transcription of TTA codons - mostly secondary metabolite pathway regulators (see Table 3)	Merrick, 1976; Lawlor et al., 1987; Leskiw et al., 1991
bldB	*S. coelicolor*	DNA binding protein	Putative transcription activator	Merrick, 1976; Pope et al., 1998; Harasym et al., 1990
bldC	*S. coelicolor*		ADP-ribosylation	Merrick, 1976
bldD		DNA binding protein	Putative transcription activator; ADP-ribosylation	Merrick, 1976; Elliot et al., 1998; Elliot and Leskiw, 1999
bldE	*S. coelicolor*	?	bald and Red$^-$	Hodgson, 1980
bldF	*S. coelicolor*	?	no aerial hyphae, no spores and no secondary metabolites	Puglia and Cappelletti, 1984; Passantino et al., 1991
bldG	*S. coelicolor*	anti-anti sigma-factor		Champness, 1988
bldH	*S. coelicolor*	?	ADP-ribosylation	Champness, 1988
bldI	*S. coelicolor*	?	?	Harasym et al., 1990
bldJ (formerly *bld261*)	*S. coelicolor*	'signal 1' non-ribosomal oligopetide	first signal in proposed *bld* cascade	Nodwell and Losick, 1998
bldK	*S. coelicolor*	ABC oligopeptide transporter	Import of 'signal 1' (BldJ)	Nodwell et al., 1996
bldL	*S. coelicolor*	?	putative response to 'signal 1'	Nodwell et al., 1999
bldM (formerly *whiK*)	*S. coelicolor*	response regulator, FixJ subfamily	Different allels arrest aerial hyphal development at distinct stages	Molle and Buttner, 2000
bldN	*S. coelicolor*	ECF subfamily sigma-factor	Required for transcription of *bldMp1*	Bibb et al., 2000
nrsA (formerly *orf1590*)	*S. griseus*	56 kDa protein, putative repressor of differentiation to avoid early sporulation	putative DNA binding protein; supressor of *bld* class IIIA mutants	McCue et al., 1996
SapB	*S. coelicolor*	peptide	Surfactant coating aerial hyphae	Guijarro et al., 1988; Willey et al., 1991
***whi*-related genes involved in sporulation and spore maturation**				
whiA	*S. coelicolor*	*whiG*-independant, autoregulated protein	coiled aerial hyphae, few or no sporulation septa; *whiA* and *whiB* needed for orderly cessation of growth	Chater, 1972; McVittie, 1974; Aínsa et al., 2000; Flärdh et al., 1999

Gene	*Sourceorganism*	*Product*	*Function*	*References*
whiB	*S. coelicolor*	putative transcription factor, expression developmentally regulated	*whiA* and *whiB* needed for orderly cessation of growth; coiled aerial hyphae, few or no sporulation septa	Chater, 1972; McVittie, 1974; Davis and Chater 1992; Soliveri et al., 2000
whiC	*S. coelicolor*		mostly uncoiled aerial hyphae, very low level of sporulation	Chater, 1972
whiD	*S. coelicolor*	spore wall biosynthesis	chains of spores formed but spore wall thin	Chater, 1972; McVittie, 1974
whiE locus	*S. coelicolor*	polyketide spore pigment gene cluster; gray polyketide	morphologically intact but unpigmented spores; very low level of SapB	Chater, 1972; McVittie, 1974; Kelemen et al., 1998
whiF	*S. coelicolor*		rod-shaped, un-pigmented spores, reduced in abundance	Chater, 1972; McVittie, 1974;
whiG	*S. coelicolor*	sigma-factor for early sporulation genes	straight aerial hyphae, no sporulation septa	Chater, 1972; McVittie, 1974; Chater, 1989b
whiH	*S. coelicolor*	autoregulatory DNA binding protein, repressor	Loosely coiled, partially fragmented aerial hyphae; no sporulation septa	McVittie, 1974; Chater and Losick, 1996
whiI	*S. coelicolor*	response regulator, *whiG*-dependent transcription	Tightly coiled, partially fragmented aerial hyphae	McVittie, 1974; Aínsa et al., 1999
whiJ	*S. coelicolor*			
whiK **renamed *bldM***	*S. coelicolor*	response regulator, FixJ subfamily		Molle and Buttner, 2000
sigF	*S. coelicolor*	sigma-factor	late sporulation genes, *whiE* genes	Potúèková et al., 1995; Kelemen et al., 1998
adsA	*S. griseus*	ECF subfamily sigma-factor	aerial hyphae development	Yamazaki et al., 2000
sapA	*S. coelicolor*	spore coat protein	?	Guijarro et al., 1988
facC	*S. griseus*	extracellular peptide	required for submerged sporulation	Birkó et al., 1999
ftsQ	*S. coelicolor*	cell division protein	Septation	McCormick and Losick, 1996
ftsZ	*S. coelicolor*	cell division protein ; FtsZ, tubulin-like protein	Septation	Dharmatilake and Kendrick, 1994; McCormick et al., 1994
other genes involvel in morphogenesis				
amfA *amfB* *amfR*	*S. griseus*	Hly family of ATP-dependent secretory protein response regulator by phosphorylations by sensor kinases	Induces rapid mycelium formation	Horinouchi, 1996 Ueda et al., 1993/98

Gene	*Sourceorganism*	*Product*	*Function*	*References*
ramA *ramB* *ramR*	*S. coelicolor*	Hly family of ATP-dependent secretory protein response regulator by phosphorylations by sensor kinases	Induces rapid mycelium formation	Ma and Kendall, 1994
craA	*S. griseus*	DNA-binding protein, carbon source-dependent transcription	negative regulator for differentiation	Ueda et al., 1999
crpA *crpB* *scbR*	*S. coelicolor*	positive regulator negative regualtor	Influences mycelium formation, spore formation and secondary metabolism	Onaka et al., 1998
amfC	*S. coelicolor*	?	Spore maturation	Yonekawa et al., 1999
brgA	*S. coelicolor*	resistance to 3-aminobenzamide (ADP-ribosyltransferase inhibitor)	ADP-ribosylation	Shima et al., 1996a
ssgA	*S. griseus* *S. coelicolor*	cytoplasmic protein	needed for sporulation, either with or without *ssfR*	Kawamoto et al., 1997; Jiang and Kendrick, 2000; van Wezel et al., 2000
ssfR	*S. griseus*	transcriptional regulator, similar to catabolic regulators of the primary metabolism	may regulate *ftsZ* and *whiG*, proposed signal path of sporulation upon nutrient limitation	Jiang and Kendrick, 2000
amfC	*S. griseus* *S. coelicolor*	A-factor dependent, AdpA independent route for morphogenesis	*S. griseus*: aerial mycelium and spore maturation *S. coelicolor*: also act	Kudo et a;., 1995 Yonekawa et al., 1999
relA	*S. coelicolor*	ppGpp synthetase	ppGpp synthesis and stringent response	Chakraburtty and Bibb, 1997
obg	*S. griseus* *S. coelicolor*	GTP-binding	Sensing GTP-pool / starvation	Okamoto et al., 1997; Okamoto and Ochi, 1998
cya	*S. coelicolor*	adenylate-cyclase	cAMP synthesis	Süsstrunk et al., 1998
ornA	*S. griseus* *S. coelicolor*	oligoribonuclease A, AdpA-dependent expression in *S. griseus*	needed for vegetative growth and initiation of differentiation	Ohnishi et al., 2000
arpA	*S. griseus*	A-factor Receptor	DNA-binding protein	Onaka and Horinouchi, 1997

While the mutations of these complementation groups could be mapped to the chromosome, their genes remain to be identified. It can be expected that the completion of the *Streptomyces* genome project will provide further insight into these loci, their products and functions. Nevertheless, a signaling cascade has been proposed for the morphological development in *S.*

coelicolor according to which the early steps of aerial hyphae development are governed by the action of the *bld* genes. It is assumed that some 5 signals are produced that are sensed by the following gene/locus of the cascade. The first signal, the non-ribosomal oligopeptide (BldJ), is subsequently imported by BldK (oligopeptide transporter) thus inducing the next, still hypothetical, signals involving the action of *bldL, A, H, I, G, C, D, M* (Figure 3.1). BldD-dependent production of SapB (Nodwell et al., 1999), a surfactant that coats the outer surface of aerial hyphae allowing the aerial hyphae to break the surface tension of the aqueous environment of the substrate mycelium and grow into the air (Tillotson et al., 1998), is currently the last known step of this signaling pathway of aerial hyphae development. A second signaling pathway, comprised of the *whi* genes, is implicated in the further differentiation of the aerial hyphae leading to the mature spores. The first steps regulate the orderly cessation of growth (*whiA/B*, Aínsa et al, 2000; Flärdh et al., 1999), followed by the steps inducing curling of and septa formation in the aerial hyphae (*whiJ, C, H, I*). Finally, spore maturation is completed when the spore wall is formed with the spore pigments incorporated (*whiD, and whiE*; Figure 3.1).

3.1.2 σ-Factors and other Regulators

At least six σ-factors have been identified that are directly or indirectly involved in regulating differentiation. One of those is the recently characterized σ^{BldN} (*bldN*, formerly *whiN*), a sigma factor that belongs to the extracytoplasmic function (ECF) subfamily RNA polymerase sigma factors (Ryding et al., 1999). σ^{BldN} shares some 27% identity on protein level to the *B. subtilis* σ^{X} (Bibb et al., 2000). A σ^{bldN} homolog, σ^{AdsA},was recently characterized in *S. griesus* (Yamazaki et al., 2000). Both, σ^{bldN} and σ^{AdsA} deletion were impaired in aerial mycelium formation and lost the ability to produce spores. Both sigma-factors regulate development: While σ^{AdsA} in *S. griseus* regulates its own expression in an A-factor-dependent manner (Yamazaki et al., 2000), σ^{bldN} expression depends on BldG and BldH implying the involvement of an anti-anti-sigma factor and an additional sigma-

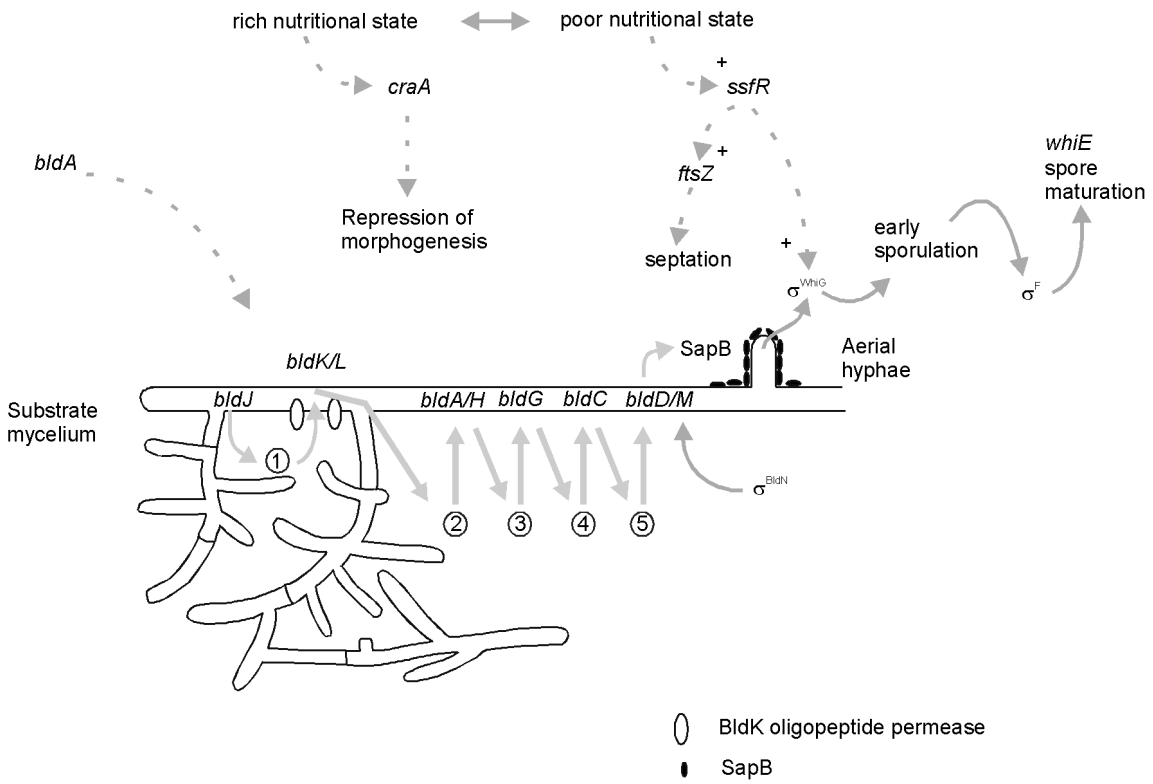


Figure 3.1. The proposed *bld* and *whi* signaling pathways for the development of aerial hyphae and spores. (adapted from Kelemen and Buttner 1998) Dashed arrows indicate proposed interaction. The numbers indicate putative signals of the *bld* cascade. See Table 3.1 for description and references of the genes.

factor (Bibb et al., 2000). σ^{BldN} of *S. coelicolor* regulates expression from promoter p1 of *bldM* which itself is a response regulator (Molle and Buttner, 2000; Bibb et al., 2000).

Another sigma-factor, σ^{WhiG} (*whiG*) which regulats the early steps in sporulation, has a high degree of similarity to the motility and chemotaxis specific σ^{FilA}-factors found in *Salmonella thyphimurium.* Interestingly, σ^{whiG} is present throughout the colony (young vegetative to aerial mycelium) without any strong evidence for developmental regulation of *whiG* transcription. According to our current understanding, regulation of σ^{whiG} occurs at the post-translational level, similar to that of the homolog σ^{FilA} of *Salmonella typhimurium*, which is regulated by a specific anti-σ protein (Ohnishi et al., 1992) which remains to be identified in case of σ^{whiG}. σ^{whiG} regulates genes involved in the early steps of morphogenesis. Three known exceptions are *whiA* and *whiB*, an autoregulated DNA-binding and a putative transcription factor, respectively (Aínas et al., 2000,

Flärdh et al., 1999), and *whiH* a putative autoregulated repressor (Chater and Losick, 1996).

σ^F (*sigF*) is involved in regulating spore maturation. σ^F is related to *Bacillus* σ^B (stress response), σ^F and σ^G (forespore-specific σ-factors) (Potúèková et al., 1995). SigF mutants make thin-walled, unpigmented spores. Yet another sigma factor, σ^A, is required for the normal cell wall structure (Paget at al., 1999). Deletion mutants of *sigA* were more sensitive to muraminidases, depended on high concentrations of Mg^{2+} for normal growth and sproulation and overproduced actinorhodin in the absense of Mg^{2+} (Paget et al., 1999), which may be a response to stress induded by the impaired cell wall biosynthesis or lysis of the substrate mycelium, a condition usually encountered during morphogenesis.

At least two additional sigma factors of the ECF subfamily, σ^E and σ^R, have recently been discovered in *S. coelicolor*. Both may also participate in the regulatory network implicated in differentiation. σ^E (*sigE*) regulates expression of the extracellular agarase (*dagA*) (Buttner et al., 1988) and transcription of the *hrdD* gene encoding a sigma factor that is structurally very similar to the essential sigma factor of *S. coelicolor* (σ^{HrdB}) with unknown function. σ^R (*sigR*) modulates expression of the thioredoxin system (oxidative stress) (Paget et al., 1998). While σ^E and σ^R are not directly involved in the regulation of morphogenesis, they are involved in sensing stress and regulation the stress response which in turn may induce the onset of differentiation.

In additon to sigma factors, transcription activators (*bldA*,*D* and *whiA,B*) and response regulators (*bldM*, *whiH* and *whiI*) have been identified and partly characterized (see Table 3.1 for references). BldD has recently been shown to recognize its own promoter. It binds to the DNA just upstream and overlapping with the -10 region of the promoter (Elliot and Leskiw, 1999). Other promoters that are recognized by BldD remain to be identified. WhiI appears to be involved in repressing its own expression and that of another σ^{WhiG}-dependent sporulation-specific regulatory gene, *whiH* (Aínsa et al., 1999).

The best characterized of the *bld* genes is *bldA*, which encodes a leucyl tRNA for the UUA

codon. This codon is very rare in the G+C rich DNA of *Streptomyces* (Lawlor et al., 1987). Champness and Chater (1994) analyzed the occurrence of this rare TTA codon in 100 well-established sequences (summarized in Figure 3.2). Interestingly, only the TTA codon shows a specialized distribution. TTA codons are rarely found in genes from the primary metabolism, but found frequently in genes regulating secondary metabolism. But within all the genes involved in morphogenesis characterized so far, only the *nrsA* gene (McCue et al., 1996), a supressor gene for certain bald phenotypes in *S. griseus* (Kelemen and Buttner, 1998) contains a TTA codon. Since, however, *bldA* mutants in *S. coelicolor* have a bald phenotype, it remains to be determined how BldA is involved in regulation morphological differentiation. The absence of TTA codons in all known *bld* and *whi* genes suggests an additional global regulatory step in front of the *bld* signaling pathway.

The best studied global regulator is 2-isocaprylol-3*R*-hydroxymethyl-γ-bytrolactone, termed A-factor, from *S. griseus* (Yamazaki et al., 2000). A-Factor, an autoregulator sensing cell density, is

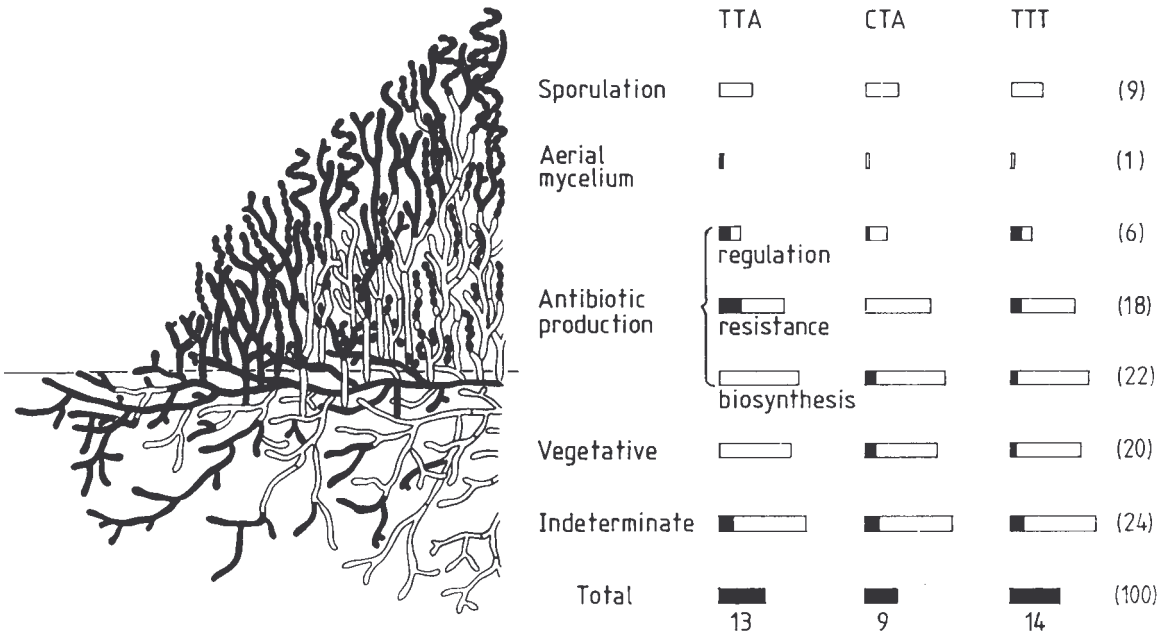


Figure 3.2. The three rarest codons found in *Streptomyces* are TTA, CTA, and TTT. Analysis of 100 well-established DNA sequences from diverse genes in different *Streptomyces* spp. have been classified according to their developmental role. Only the TTA codon shows a distribution that can be correlated with development. The numbers in brackets indicate the number of genes analyzed in each category and the black areas of the boxes indicate the percentage of genes with the respective codon. (source: Champness and Chater, 1994)

an example for quorum sensing in *Streptomyces*. A-factor biosynthesis and function was first identified in *S. grieseus* (Horinouchi et al., 1983), but A-factor homologes seem to be common regulators for differentiation in *Streptomyces*. A-factor and A-factor receptor homologes found in numerous *Streptomyces* (See Table 3.2.) are highly specific for the producing strain. A detailed discussion on these molecules is presented in chapter 3.2.2.1.

Several additional genes involved in morphological development have been described. Factor C is an example for an extracellular signal peptide. Encoded by *facC*, which was recently sequenced (Birkó et al., 1999), the mature 286 aa extracellular form is implicated in submerged sporulation of *S. griseus*. Homologes were found in several streptomycetes that can sporulate in submerged culture (Birkó et al., 1999). The molecular level of interaction between FacC and other sporulation genes remains to be determined.

The *amfR/A/B* operon, which codes for a putative protein or peptide translocator/response-regulator pair (see chapter 3.2.2.2.), and the *nrsA* and *amfC* genes of *S. griseus* were isolated for their ability to restore aerial mycelium formation in an A-factor deficient bald mutant of *S. griseus* (Ueda et al., 1993; Babcock and Kendrick, 1990; Kudo et al., 1995). AmfC (218 aa) does neither restore A-factor biosynthesis nor induce streptomycin biosynthesis, and disruption of *amfC* in wild-type *S. griseus* severely reduced the abundance of spores due to infrequent sporulation. *amfC* homologous nucleotide sequences were found in all of 12 tested *Streptomyces* strains suggesting a common role in morphogenesis (Kudo et al., 1995). Unlike in *S. griseus*, mutants of *S. coelicolor*, deficient in *amfC* are severely affected in secondary metabolism (Yonekawa et al., 1999). Almost no transcription of the actinorhodin pathway-specific transcriptional activator *actII-orf4* was detected resulting in a greatly reduced production of actinorhodin. In addition, synthesis of undecylprodigiosin was delayed. Additional copies of *amfC* abolished pigmentation of the otherwise morphologically intact spores, suggesting an effect on *whiE* transcription only (Yonekawa et al., 1999). The molecular link between AmfC and the regulation of secondary metabolite production remains to be elucidated. A putative protein or peptide translocator/response-regulator

pair, the *ramA/B/R* gene cluster, has also been isolated from *S. coelicolor* (Ma and Kendall, 1994). Both *ramA/B/R* and *amfA/B/R* appears to be related in their functions (see chapter 3.2.2.2.).

3.1.3 Links to Primary Metabolism

Most interesting is the observation that the defects in morphogenesis and in secondary metabolite formation of all *bld* mutants, with the exception of *bldB*, are partially restored by growth on poor carbon sources such as mannitol (Champness, 1988; Champness and Chater, 1994; Wiley et al. 1991). Formation of aerial hyphae is restored in the *bldA* mutant, yet it remains deficient for actinorhodin biosynthsis (Champness, 1988). Evidence that *bld* mutants in general are defective in sensing the nutritional state of the cell came from studies on the regulation of the glucose-sensitive, galactose-dependent *galP1* promoter, the glucose-repressed *dagA* (agarase) and the glucose-repressed, glycerol-dependent *gly* (glycerol utilization operon) in these mutants (Pope et al., 1996). This carbon source dependence of the bald phenotypes led to the suggestion that the defect of regulation of carbon utilization is epistatic to the defects in morphogenesis and secondary metabolism and that the inability of these mutants to differentiate is a consequence of their defect in sensing or in signaling starvation (Pope et al., 1996). Recently Ueda and coworker (1999) have identified the *craA* gene that when expressed high level, acts as a repressor for the initiation of morphological development (Figure 3.1 and chapter 3.3.2). Expression of *craA* is carbon source-dependent and highest on glucose. Thus, CraA expression during vegetative growth blocks the onset of differentiation as long as glucose is available (Ueda et al., 1999).

A new gene, *ssfR*, has recently been described (Jiang and Kendrick, 2000) to be involved in regulating sporulation in *S. griseus*. This gene has a close homology to Ic1R, a transcriptional regulator that belongs to the family of catabolite regulators of the primary metabolism. Ic1R is a repressor for the *aceBAK* operon which is involved in the synthesis of the enzymes of the glyoxalate bypass in *E. coli* that are required for growth on acetate (Gui et al., 1996; Pan et al., 1996).

Disruption of *ssfR* in *S. griseus* did not affected production of streptomycin, however, prevented submerged sporulation on glucose-containing medium (Jiang and Kendrick, 2000). It was proposed by the authors that in response to nutritional stress, regulation of expression of *ftsZ* (septation) and of *whiG* (sporulation sigma factor) depends on SsfR. This would add a new signal level to the *bld-whi* cascade (Figure 3.1). Downstream of *ssfR, ssgA* was localized, a gene that is essential for submerged sporulation of *S. griseus* (Kawamoto et al., 1997). Despite being required for submerged sporulation, SsgA does not depend on SsfR (Jiang and Kendrick, 2000). More recently, the *ssfR/ssgA* homologs in *S. coelicolor*, the *ssgA/R* genes, have been characterized (van Wezel et al., 2000). The authors determined that, in contrast to the findings in *S. griseus*, a *ssgA* knock out mutant in *S. coelicolor* failed to sporulate and had a reduced actinorhodin biosynthesis (undecylprodigiosin production was not affected), while overexpression of *ssgA* resulted in enhanced septum formation and production of spore-like bodies in submerged culture. It was further suggested by the lack of consensus sequences indicative for DNA-binding motifes that SsgA might interact directly with the cell division machinery.

Numerous genes have been described in recent years that are not directly involved in morphogenesis but are implicated in triggering the various signal cascades. Due to the multitude of potential stress factors that need to be sensed independently, these genes can not readily be grouped into a single signaling pathway. They may interact on a common level above that of the *bld* and *whi* genes, but additional, *bld*-independent pathways should not be ruled out, especially in view of new information that becomes available on the regulation of the primary metabolism and catabolite repression. Some of theses genes will interact with morphological development (*ssfR, craA*, see also chapter 3.3.2.) or secondary metabolism only (*acoA*, *citA*, chapter 3.3.1, *cya*, chapter 3.4.1), others will affect both, morphological and physiological differentiation (*relA*, chapter 3.3.5.2; *obg*, chapter 3.3.5.3.).

3.2 Regulation of the Physiological Differentiation

The solubilization of parts of the vegetative mycelium to provide nutrients for the sporulating aerial mycelium could be rather indiscriminate: other soil bacteria might benefit from this availability of nutrients. However, *Streptomyces* colonies defend their resources by poisoning the local environment with antibiotics to which the producer is resistant (Chater and Merrick, 1979; Chater, 1992). The high expense of the synthesis of these molecules under nutrient limited conditions has led to the development of a complex regulatory network. Despite intensive research this network is still far from being understood. Primary metabolism, secondary metabolism and morphological differentiation are linked for a coordinated development. Most *bld* mutants discussed earlier (chapter 3.1.) are not only deficient in morphological development, but also deficient for secondary metabolite production and for sensing the nutritional state of the cell. Similarly, A-factor has pleitrophic effect on morphogenesis and secondary metabolism in *S. griseus*.

The pathway-specific regulators of secondary metabolism are rather well characterized but our understanding of the global signaling pathways activating these pathway-specific regulators is only rudimentary. Best understood is the A-factor-mediated differentiation in *S. griseus* (Yamazaki et al., 2000), but many new players have been identified that could not be integrated into the established signaling cascades.

3.2.1 Pathway Specific Regulation

The genes for the biosynthesis of secondary metabolites are usually clustered. Well studied are gene clusters for numerous antibiotics. Again, *S. coelicolor* has served as a model, and the biosynthesis of its two colored secondary metabolites, actinorhodin (Act) and undecylprodigiosin (Red), provided information that could be applied to other systems. Both gene clusters, the actinorhodin (*act*) and the undecylprodigiosin (*red*) cluster, contain within the biosynthetic genes

pathway-specific activators *actII-orf4* and *redE*. Transcription of *actII-orf4* is developmentally regulated and translation is *bldA*-dependent (BldA = leucyl-tRNA recognizing the rare UUA codon) (Gramajo et al., 1993). The *actII-orf4* product, a transcriptional activator, then induces transcription of the *act* biosynthetic genes. Level of transcription sharply increased when cultures entered stationary phase. Following the increase of *actII-orf4* transcripts, other *act* structural biosynthetic genes were upregulated (Gramajo et al., 1993; Arias et al., 1999). Actinorhodin production can be enhanced and uncoupled from cell development by constitutively overexpressing *actII-orf4*. Overexpression of *actII-orf4* also restored Act production in *bld* mutants (*bldA, D, F, G and H;* Passantino et al., 1991). Interestingly, expression of *actII-orf4* was regulated on the level of transcription and not, as is suggested by the presence of the very rare UUA codon, at the level of translation. Recently a gene, *orf10*, located within 4.8 kb adjacent to the right end of the *act* biosynthetic cluster (downstream of *actVB-orf6*) in *S. coelicolor* was identified that resembles a LysR-type transcriptional regulator. Disruption of *orf10* in *S. coelicolor* had no effect on Act production but extra copies restored Act production in an *actIII* mutant. Disruption of *orf10* in the closely related *S. livdans* induced Act production. The molecular interactions of the *orf10* gene product remain to be determined (Martínez-Costa et al., 1999).

A similar growth-phase-dependent expression was observed for *redE*, the pathway-specific regulator for undecylprodigiosin. Again, regulation of *redE* expression occurs at the transcriptional level, and upregulating *redE* expression resulted in transcription of the structural genes for undecylprodigiosin biosynthesis (Takano et al., 1992). Both genes, *actII-orf4* and *redE* encode DNA-binding proteins belonging to the growing family of **S**treptomyces **A**ntibiotic synthesis **R**egulatory **P**rotein (SARP; Wietzorrek and Bibb, 1997).

The isolation of *bldA* mutants, normally Act^- and Red^-, that produced undecylprodigiosin (*pwb*) led to the identification of *redZ*. RedZ is a response regulator that activates transcription of *redE* (White and Bibb, 1997). Interestingly, RedZ lacks the phosphorylation pocket at its amino-terminal domain usually found in response regulators (Guthrie et al., 1998). The mechanism by

which RedZ activated *redE* remains to be determined.

The same pathway-specific regulation has been identified for the dunorubicin gene cluster of *Streptomyces peucetius*, in which *dnrN* and *dnrI* are the homologues to *redZ* and *redE*, respectively. Again, there was no evidence for phosphorylation of DnrN, the response regulator activating expression of *dnrI*. DnrI is a member of the SARP-family DNA-binding proteins that activates transcription of the structural genes for daunorubicin biosynthesis (Furuya and Hutchinson, 1996; Madduri and Hutchinson, 1995; Tang et al., 1996; Wietzorrek and Bibb, 1997). Also the transcriptional activator, CcaR, of the cephamycin C gene cluster of *S. clavuligerus* displays homology to other pathway-specific activators of the SARP family. CcaR is required for activation of both, the clavulanic acid and cephamycin C biosynthesis (Alexander and Jensen, 1998; Pérez-Llarena et al., 1997). A phosphate controlled expression involving a SARP-like protein has recently been proposed for the oxytetracyclin biosynthesis in *S. rimosus* (McDowall et al., 1999).

In contrast, expression of *strR*, the activator of the well studied streptomycin (*str*) cluster in *S. griseus*, is transcriptionally regulated by the A-factor-dependent expression of AdpA, a different transcription activator (see Figure 3.3., chapter 3.2.2.1.; Yamazaki et al., 2000).

Activation of the bialaphos (*brp*) genes in *S. hygroscopicus* is also dependent on a putative DNA-binding protein BrpA (Anzai et al., 1987; Raibaud et al., 1991). Methylenomycin biosynthesis in *S. coelicolor* provides a rare example for a negative control of a pathway of a secondary metabolite. The repressor, MmyR, has been classified as a terR-like repressor (Chater and Bruton, 1985).

Interestingly, very little is known about the regulation of the complex polyketides biosynthesis. The only pathway specific regulator for a macrolide antibiotic described so far is SrmR of the spriamycin cluster in *S. ambofaciens*. The activator gene *srmR*. encodes a protein that did not reveal significant homology to known proteins (Geistlich et al., 1992; Richardson et al., 1990). However, short stretches of homologies to putative transcriptional regulators and to putative regulatory proteins in *S. coelicolor* were found in a recent database search (9/2000) for the N-

terminal and C- terminal regions, respectively.

Pathway specific regulators for the larger macrolide (type I - polyketide synthases) gene clusters such as the erythromycin of *Saccharopolyspora erythraea* (Donadio et al., 1993) or the rapamycin gene cluster *S. hygroscopicus* remain to be identified. A sensor kinase response-regulator gene pair was found next to the rapamycin cluster (~ 100kb in size), its involvement in regulation of rapamycin biosynthesis is not known (Schwecke et al., 1995).

3.2.2 Global Regulations

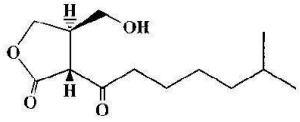
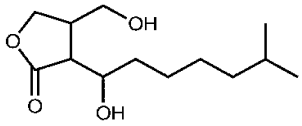
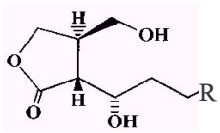
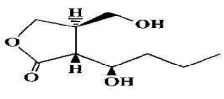
The more complex regulation of differentiation and secondary metabolism begins at the level above the pathway specific regulation. The connection between inter- and intra-cellular messaging, the response to a variety of environmental stimuli and the evaluations of the signals involved, requires multiple steps and the interconnection of different signaling paths. Moreover, the status of the primary metabolism and the overall nutritional state of the cells has to be evaluated for an integrated response leading to cellular differentiation. A part of this complexity has already been discussed in connection with morphogenesis. A more in-depth discussion of the various known regulatory systems will follow below.

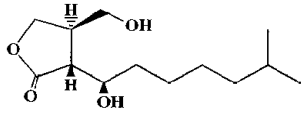
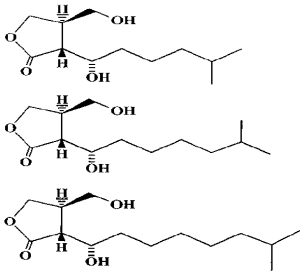
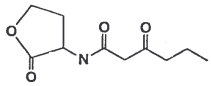
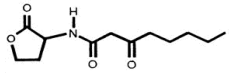
3.2.2.1 Population Density and Intercellular Signaling (A-Factor)

The best studied and most completely understood signal pathway in streptomycetes is the A-factor driven morphological differentiation and induction of streptomycin biosynthesis in *S. griseus* (Horinouchi and Beppu, 1992/1994; Horinouchi, 1996; Yamazaki et al., 2000). A-Factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) belongs to a large family of structurally similar compounds used for cell-cell signaling (quorum sensing) (Table 3.2). Occurrence of autoinducers is by far not limited to streptomycetes. Actually, the bacterial luminescence system of *Vibrio fischeri*

was the first example that introduced the autoinducer concept of cell-density-dependent regulation (Nealson et al., 1970; Eberhard et al., 1981). These autoinducers are active in nanomolar concentrations and hence considered to be microbial hormones. The list presented in Table 3.2. demonstrates that autoinducers are common signal molecules in probacteria and even lower fungi. Similar to their counterparts in streptomycetes, the homoserine lactones (HSL) found throughout Gram-positives and Gram-negatives are signals for cell density, which upon reaching a threshold value induce cell-density related functions. Typical cell-density related functions include luminescence, production of secondary metabolites, and bacterium-host interactions such as virulence factors or induction of nodulation. Morphological and physiological differentiation are the processes controlled by autoinducers in streptomycetes and filamentous fungi (for references see Table 3.2.).

Table 3.2. Quorum sensing in microorganisms: Examples for γ-butyrolactone and homoserine lactone derived autoinducers.

Organism	***Gene***	***Compound***	***Activity***	***References***
Actinomycetes - γ-butyrolactones				
S. griseus	*afsA*	A-Factor 	Induction of aerial mycelium formation, streptomycin and yellow pigment biosynthesis	Horinouchi and Beppu, 1992; Ohnishi et al., 1999; Yamazaki et al., 2000
S. coelicolor		Acl-2a 	Controlling morphogenesis	Sugiyama et al., 1998
S. virginiae	*barX*	virginiae butanolides, VB-A, B, C, D 	Induction of virginiamycin production	Sakuda and Yamada, 1991; Kinoshita et al., 1997/1999
Streptomyces lavendulae FRI-5	*farX*	IM-2 	Controlling production of blue pigment and nucleoside antibiotics showdomycin and minimycin	Sato et al., 1991; Mizuno et al., 1996; Kitani et al., 1999

Organism	*Gene*	*Compound*	*Activity*	*References*
S. viridochromogenes		Factor-I 	Induction of anthracycline production	Gräfe et al., 1982
S. bikiniensis *S. cyaneofuscatus*			Interspecific induction of morphogenesis and secondary metabolism	Gräfe et al., 1983
Other bacteria - homoserine lactones				
Vibrio fischeri	*luxI*	*N*-(β-caproyl) homoserine lactone 	Controlling expression of luminescence	Eberhard et al., 1981
Vibrio harveyi	*luxL/M*		Controlling expression of luminescence	Bassler et al., 1994
Agrobacterium tumfaciens	*traI*	AAI (*N*-(3-oxooctanoyl)-L-homoserine lactone) 	Controlling virulence (*vir*) genes on Ti plasmid and Ti plasmid conjugal transfer	Fuqua and Winans, 1996; Zhu et al., 1998
Erwinia carotovora	*expI / carI*	*N*-(3-oxohexanoyl)-L-homoserine lactone	Regulating carbapenem antibiotic synthesis and virulence factors	Bainton et al., 1992; Pierson et al., 1998
Pseudomonas aureofaciens	*phzI*	*N*-(3-hexanoyl)-L-homoserine lactone	Regulating phenanzine biosynthesis	Pierson et al., 1998
P. stewartii	*esaI*	*N*-(3-oxohexanoyl)-L-homoserine lactone	Regulating exopolysaccharide capsule formation	Pierson et al., 1998
Rhizobium leguminosarum	*rhiI*	*N*-(3-hydroxy-7-*cis*-tetradecanoyl)-L-homoserine lactone	Controlling rhizome interactions and root nodulation	Pierson et al., 1998
Serratia liquefaciens MG1	*swrI*	*N*-butanoyl-L-homoserine lactone	Controlling production of serrawettin Ws, a surfactant required for swarming	Eberl et al., 1996; Lindum et al., 1998
Enterobacteriaceae		several homoserine lactones isolated	Implicated infood spoilage	Gram et al., 1999
Pseudomonas aeruginosa	*lasI*	*N*-(3-oxododecanoyl)-L-homoserine lactone	Virulence factors	Seed et al., 1995; Pearson et al., 1999
Filamentous fungi - butyro lactone				
Aspergillus terreus		Butyrolacton I	Controlling differentiation and secondary metabolite formation	Schimmel et al., 1998

Why exactly bacteria use N-acyl-L-homoserine lactones as messengers is not clear. Dunlap

(1996) argues that intracellular levels of HSL and acyl groups may vary with the flow of carbon, nitrogen, and energy through the amino acid and fatty acid biosynthetic pathways. Hence, increased precursor concentrations, as a consequence of perturbations in these flows, is associated with the onset of stationary phase. When combined to form *N*-acyl-L-HSL, these metabolites would become membrane-permeant intercellular signals that activate functions adaptive to high cell density (Huisman and Kolter, 1994). It remains to be proven if this hypothesis is applicable for the butyrolactone synthesis in *Streptomyces*. So far neither the pathway nor the growth-related regulation of γ-butyrolactone biosynthesis are known. The current model for autoregulator mediated differentiation in *Streptomyces* has derived from detailed studies on A-factor of *S. griseus*.

Is is suggested that A-factor is the product of the condensation of a glycerol with a β-keto fatty acid mediated by the function of AsfA (Ando et al., 1997a). The presumptive β-keto fatty acid may contain either acetyl-CoA or acyl carrier protein at the end (Ohnishi et al., 1999). Expression of AfsA appears to be regulated by *afsB*, a DNA-binding protein of which homologous sequences are readily detected in streptomycetes (Horinouchi et al., 1996). A-factor is produced in a growth-dependent manner, probably by a low level expression of *afsA*. Such low basal expression is suggested for the *lux*-system in *Vibrio fischeri* (Dunplap, 1996). A reduced mRNA stability or missing substrate due to the high growth rate in exponentially growing cultures are discussed as causes for the low autoinducer biosynthesis in *Vibrio fischeri* when grown in culture. At higher cell density, when the culture enters stationary phase and metabolic activities change, precursors for autoinducer biosynthesis may be more readily available (Dunlap, 1996). Whether this model is applicable in *Streptomyces* remains to be proven. Nevertheless, A-factor biosynthesis and/or concentration reaches a critical level when a culture enters stationary phase and A-factor then binds to the A-factor receptor protein (ArpA) setting off the regulatory cascade shown in Figure 3.3 (Yamazaki et al., 2000). ArpA is a DNA-binding protein that binds as a dimer to the promoter of *adpA* (A-factor dependent protein A) preventing transcription of this gene. Upon binding of A-

factor to ArpA, the complex dissociates from the DNA and transcription of *adpA* commences. AdpA then activates transcription of *strR*, encoding for the pathway-specific activator StrR for

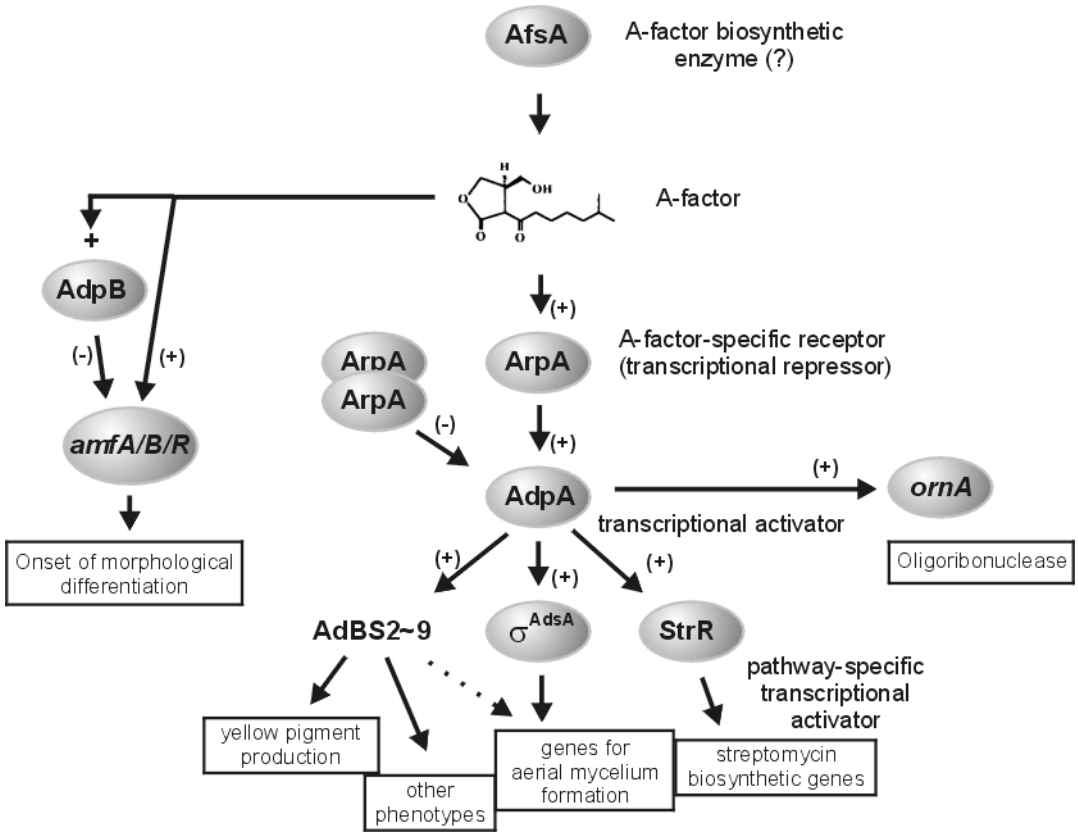


Figure 3.3. A-Factor-dependent regulation of differentiation in *S. griseus*. See text for details. (modified after Yamazaki et al., 2000)

streptomycin biosynthesis, and transcription of *aphD*, the gene conferring streptomycin resistance (Ohnishi et al., 1999). Transcription of *adpA* also results in the enhanced transcription of a gene encoding a new oligoribonuclease, *ornA*, which is located downstream of *adpA*. OrnA is believed to be required for the recycling of mRNAs to provide precursors for newly initiated transcriptional activities leading to a rapid shift in gene expression (Ohnishi et al., 2000). In addition, the *adsA* gene encoding σ^{AdsA}, a homolog to *bldN* in *S. coelicolor*, required for induction of aerial hyphae formation is activated by AdpA (Figure 3.3.; Yamazaki et al., 2000). Moreover, additional AdpA-dependent DNA-binding proteins were identified that are involved in inducing the production of a yellow pigment and other phenotypes. The multiple action of AdpA significantly amplifies the A-factor signal and thus ensures a rapid differentiation.

A second A-factor-dependent pathway, essential for morphological differentiation in *S. griseus* has recently been uncovered. This path is σ^{AdsA}-independent, but dependent on a second A-factor dependent protein, AdpB. AdpB controls transcription of *amfR* (Ueda et al., 1993/1998): in the absence of A-factor AdpB acts as repressor by binding to the promoter region of *amfR*. Addition of A-factor relieves the repression resulting in AmfR synthesis and the onset of morphogenesis (Ueda et al., 1993/1998). The molecular details of these interactions are still unknown.

Searching for similar signal pathways in other *Streptomyces* revealed some common features, but also major differences. γ-butyrolactone autoinducers and their respective, highly ligand-specific receptor proteins have been identified in many strains of *Streptomyces*. Structural differences in their C-2 side chains resulted in a classification of butyrolactone autoregulators identified so far into three groups: (i) A-factor type possessing a 6-keto group, (ii) virginiae butanolide type (VB), e.g. VB-A to VB-E controlling virginiamycin biosynthesis in *S. virginiae*, possessing a 6-α-hydroxy group, and (iii) the IM-2 type possessing a 6-β-hydroxy group. Although the structural differences among these factors are small, the producer strains show a high degree of ligand specificity toward the corresponding autoregulator (Miyake et al., 1989; Nihira et al., 1988, Waki et al., 1997), which is mediated through the autoinducer receptor proteins.

The working model for A-factor-mediated regulation in *S. griseus* describes some common regulatory features that have also been identified in other streptomycetes, but major differences are observed as well. Virginiae butanolide (VB) in *S. virginiae,* for example, is only produced immediately before virginiamycin production is started and controls production of this metabolite only (Kinoshita et al., 1997). Also the chromosomal organization of the genes for the autoinducer and the receptor proteins are distributed differently: While the VB genes (*barX*/*R*) in *S. virginiae* are located adjacent to each other are the *afsA* and *arpA* in *S. griseus* distributed (Kinoshita et al., 1997; Ohnishi et al., 1999).

A further variation of the γ-butyrolactone-mediated signaling has been found in *S. lavendulae*

FRI-5. During early growth, cultures of *S. lavendulae* produce the antibiotic D-cycloserine but later switch to the production of the nucleoside antibiotics showdomycin and minimycin. IM-2, the autoinducer of this strain binds to its receptor protein FarA (Waki et al., 1997), which, in the absence of IM-2 represses its own expression. Further analysis has led to the hypothesis that FarA might be involved in regulating the switch in the type of antibiotic produced in early and late stages of the culture (Kitani et al., 1999).

A-factor-mediated signaling in *S. coelicolor* A3(2) is much less understood. Six different γ–butyrolactones and three arpA-like genes have been identified so far that are implicated in controlling morphological and physiological differentiation (Anisova et al., 1984; Onaka et al., 1998). Results from databank searches suggest the presence of more than eight AdpA-like homologues in *S. coelicolor* (Yamazaki et al., 2000). Two of the *arpA*-like genes of *S. coelicolor*, *cprA* and *cprB*, have been characterized in more detail. The deduced amino acid sequence revealed a significant similarity in the helix-turn-helix DNA-binding motive suggesting that both proteins share the same DNA binding sites, one acting as positive and the other as negative regulator of differentiation (Onaka et al., 1998; Sugiyama et al., 1998). Interestingly, disruption of the ArpA-like repressor protein CprB resulted in earlier and precocious sporulation and in overproduction of actinorhodin. No changes were detected in undecylprodigiosin biosynthesis (Onaka et al., 1998), adding to the differences in pathway-specific regulation mentioned earlier (*redE/redZ* pair and *actII-orf4*, chapter 3.2.1).

Further examples of γ-butyrolactone-mediated signaling in *Streptomyces* are given in Table 3.3. Little is known on the molecular interactions of these autoinducers with their receptor proteins and about the targets of the receptor proteins.

In summary, γ-butyrolactone-mediated signaling is widespread in *Streptomyces*, but the strains have diverged significantly during evolution. While in some strains both, morphological and physiological differentiation are regulated by one autoinducer, only one branch of the differentiation

is regulated by one or more autoinducers in others which implies that a signal, common to both events may have been lost.

In addition to butyrolactones and HSLs, peptides or small proteins are often implicated in quorum sensing and regulation of cell-density dependent functions, such as ComC for competence for transformation in *Streptococcus pneumoniae* (Lee and Morrison, 1999), AgrD for virulence in *Staphylococcus aureus* (Ji et al., 1995) or ComX and CSF for competence and sporulation in *B. subtilis* (Lazazza and Grossman, 1998; Lazazzera et al., 1999). Whether BldJ or even SapB (chapter 3.1.1.) qualify as a quorum sensing molecule remains to be seen.

3.2.2.2 Phospopo Relay Systems and other Regulatory Pairs

In procaryotes, two-component signal transduction systems are employed in situations in which the cell needs to respond to environmental signals such as osmotic response and phosphate control in *E. coli* or sporulation in *B. subtilis* (Parkinson, 1995; Stock et al., 1995). The general paradigm is that a sensor protein mostly associated with the membrane senses the signal and autophosphorylates. The phosphoryl group is then transferred to a companion protein which often acts as a transcriptional regulator.

Three such two-component regulatory systems have been identified in *S. coelicolor* and *S. lividans*, *absA1/A2* (Brian et al., 1996), *cutR/S* (Chang et al., 1996), and *afsQ1/Q2* (Ishizuka et al., 1992), all of which are implicated in secondary metabolite production (Table 3.3.).

1.) The *absA* locus has been studied in detail. Recently, the molecular nature of the Abs$^-$ phenotyp, loss of production of all antibiotics in *S. coelicolor*, has been resolved (Anderson et al., 1999). The Abs$^-$ phenotype is the result of point mutations in the conserved regions of AbsA1, a his-kinase sensor transmitter family protein. One mutant allel carries two point mutations, I^{360} to L and R^{365} to Q, altering the 'G-box' motif, which is involved in nucleotide binding (Stock et al., 1995). The second mutant allel carries a single point mutation changing L^{253} to R, that is located in the 'X-

box' which has recently been proposed to be involved in the aspartylphosphatase activity, common to many sensor-transmitters (Hsing et al., 1998). Supressor mutants for these Abs$^-$ alleles were identified that carry either partial deletions of *absA1/A2*, or second site point mutations in other conserved regions of either *absA1* or *absA2*, that are involved in signal transfer to the response regulator or in DNA binding of the response regulator (Anderson et al., 1999). Hence, it was concluded that the mutant alleles causing the Abs$^-$ phenotye of the *absA1/A2* system, which earlier has been shown to negatively regulate secondary metabolite biosynthesis (Brian et al., 1996), lock this system in the negative control state. The ligand and the target for the *abs*-system remain to be determined.

2.) The second regulator pair for secondary metabolism, *cutR/S*, has been isolated form *S. lividans* (Chang et al., 1996). Inactivation of *cutR/S* increased Act and Red production while multiple copies of *cutR* reduced Act production, again implying a negative regulation of the secondary metabolism by CutR/S. Southern blot analysis as well as DNA and protein sequence comparison with the genes from the *abs*-locus revealed that the *cut*-locus is distinct from *abs* and that *cut* may also be present in *S. coelicolor* A3(2). Chang and coworker (1996) suggest that both, *abs* and *cut* may interact in a hierarchical manner, which remains to be resolved. As for the first system, nothing is known about the ligands and the target of this regulator.

3.) The third and probably universal sensor kinase / response regulator system is the *afsQ1/Q2* regulatory pair which has been identified in many actinomycetes (Ishizuka et al., 1992). The deduced amino acid sequence of AfsQ2 implies that this senor kinase is a transmembrane protein. The N-terminal sensor domain is located outside the cytoplasmic membrane, while the C-terminal domain including the histidine residue at position 294 that is subject to phosphorylation remains cytoplasmic. *asfQ1* appears to be a transcriptional activator since it induces actinorhodin and A-factor biosynthesis in *S. lividans*. Disruption of the *asfQ1* or *afsQ2* in *S. coelicolor* A3(2) did not have a detectable effect on secondary metabolism or mophogenesis (Ishizuka et al., 1992). Thus, in contrast to the two loci described above, the *afsQ1/Q2* system can be regarded as a positive, but

dispensable regulator of secondary metabolism and A-factor biosynthesis. Again, neither the ligands nor the targets for AfsQ2 and AfsQ1, respectively, have been identified.

Table 3.3. Two-component regulatory pairs identified in *S. coelicolor* and *S. lividans* affecting secondary metabolism.

Locus	*Genes*	*Lignads/Function*	*Effects*	*References*
Procaryotic - type two-comonent regualtory systems				
absA1/A2	*absA1* *absA2*	Sensor kinase Response regulator Negative regulator for antibiotic synthesis	mutant in *absA1*: Act$^-$, Red$^-$, CDA$^-$, Mmy$^-$; deletions: Act↑, Red↑; multicopy: Act↑, Red↑	Brian et al., 1996; Aceti and Champness, 1998; Anderson et al., 1999; Champness, 1999
cutR/S	*cutR* *cutS*	Response regulator Sensor kinase Negative regulator for antibiotic synthesis	disruption Act↑, Red↑, multicopy: *cutR*: Act↓	Chang et al., 1996
afsQ1/Q2	*afsQ1* *AfsQ2*	Response regulator Sensor kinase	His-phosphotransfer system, on plasmid: suppresser of *absA*; multicopy: Act↑, Red↑ and A-factor↑ in *S. linidans* 66	Ishizuka et al., 1992
Eucaryotic - type Ser-Thr-Tyr Phosphorelay				
afsR/K/R2	*afsR* **(formerly *afsB*)** *afsK* *afsR2* (*afsS*)	Ser-Thr phosphoprotein Ser-Thr protein kinase unknown	AfsK:membrane associated Posphokinase deletions: Act↓, Red↓; multicopy of any: Act↑, Red↑ small ORF downstream of *afsR* that results in the same effects than *afsR/S*	Floriano and Bibb, 1996; Matsumoto et al., 1994; Umeyama et al., 1999; Horinouchi and Beppu, 1984; Horinouchi et al., 1986; Stein and Cohen, 1989 Vögtli et al., 1994
pkg2 *pgk3* *pgk4*	*pkg2*	Transmembrane Ser-Thr kinase cytoplasmic Ser-Thr kinases	in *S granticolor*: morphology of aerial hyphae	Vomastek et al., 1998; Nádvorník et al., 1999
Systems w/o phosphorylation or unknown partner proteins				
amfABR	*amfR*	response regulator	Required for morphogenesis in *S. griseus*	Horinouchi, 1996 Ueda et al., 1993/98
ramABR	*ramR*	response regulator	Induces rapid mycelium formation in *S. coelicolor*	Ma and Kendall, 1994
ptpA	*ptpA*	Phosphotyrosine phosphatase	target unknown, multicopy: Act↑, Red↑	Li and Strohl, 1996
Other global regulators				
Aba	*abaA*	unknown	locus of 5 ORFs (ORFA to E), ORFB + 137 bp downstream induces *act* in *S. lividans*	Fernández-Moreno et al., 1992
abaB	*orf1* *abaB*	sulfur metabolism ? LysR-type transcriptional regulator	? in *S. lividans*: Act↑, Red↑	Scheu et al., 1997

Locus	*Genes*	*Lignads/Function*	*Effects*	*References*
absB	*absB*	Rnase III homolog	mutant in *rnc* homolog: Act$^-$, Red$^-$	Aceti and Champness, 1998; Price et al ., 1999
orf10, downstream of *act*	*orf10*		negative control of Act in *S. lividans*	Martínez-Costa et al., 1999
mia	ND	90 nt sequence; putative inhibitor or sequestering an activator	multicopy: Act↓, Red↓, CDA↓	Champness et al., 1992
micX	ND	unknown possibly antisense	multicopy: Act↑, Red↑	Romero and Mellado, 1995; Rudd and Hopwood, 1979

Act: actinorhodin; Red: undecylprodigiosin; CDA: calcium dependent antibiotic, Mmy: methylenomycin; ND: not determined; ↑: upregulated; ↓: downregulated

In addition to the procaryotic-typ two-component systems, a growing number of eucaryotic-type two component sensor-kinase/response-regulator pairs have been identified (Table 3.3.). The well-studied *afsR/K* system in *Streptomyces* is comprised of the regulatory protein AfsR that is phosphoylated on Ser and Thr residues by the adjacently encoded AfsK kinase (Matsumoto et al., 1994). Mutants of *afsK* had a reduced Act production but were not affected in morphological development (Horinouchi et al., 1990). AsfR has a high degree of homology to RedD and ActII-Orf4, the pathway specific regulators of the *act* and *red* clusters yet AsfR can not substitute for RedD or ActII-Orf4 (Floriano and Bibb, 1996). Moreover, removing *asfR* resulted in a loss of Act and Red production on minimal medium (SMM; Kieser et al., 2000) without affecting expression of the pathway-specific activators. Interestingly, Red production could be restored by growth on media with low phosphate concentrations, both, Act and Red production, were restored on rich medium (Floriano and Bibb, 1996) or by introducing multiple copies of the *redD* and *actII-orf4*. Furthermore, *asfR* mutants also had a reduced production of the Calcium-dependent antibiotic (CDA), while methylenomycin (Mmy) synthesis was not affected by AsfR. Introduction of multiple copies of *asfR* stimulated expression of *redD and actII-orf4* and production of the respective antibiotics but had no effect on CDA or Mmy. These results suggest that AsfR/K is required for Act and Red at high phosphate concentrations and that the *asfR/K* system operates independently from

the pathway-specific regulators. The molecular nature of this interaction remains to be determined.

Homologues of *afsR/K* were recently described in *S. griseus* (Umeyama et al., 1999). In contrast to *S. coelicolor*, *afsR/K* mutants in *S. griseus* were conditionally defective for morphological differentiation. Disruption of either *asfR* or *asfK* resulted in the disability to form aerial hyphae or spores on media containing glucose at concentration higher that 1%. This phenotype was supressed in media in which glucose was replaced by mannitol or glycerol (Umeyama et al., 1999). A-factor and streptomycin synthesis were not affected in these mutants. As for the *asf*-system in *S. coelicolor* the ligands and targets for AsfK and AsfR remain to be determined.

The discovery of a third gene of the *asfR/K* locus in *S. griseus*, *afsR2* (*afsS* in *S. coelicolor*), located some 213 nt downstream of *afsR* (Vögtli et al., 1994) has raised some question on the regulation by AfsR. Mutation analysis demonstrated that AfsR2 alone was required for induction of Act production in *S. lividans*. Introduced in high copy number, AfsR2 stimulates Act and Red biosynthesis through activation of the pathway-specific activator *actII-orf4* and *redD*. A segment of the 63 aa AfsR2 protein shows similarity to a domain found in various procarytic sigma factors. However, the most conserved regions that are associated with core binding, promoter recognition and DNA-melting are missing in AfsR2. In addition, the small size does not support the idea that AfsR2 acts as a sigma factor itself (Vögtli et al., 1994). Moreover, the pronounced copy number effect may suggest that AsfR2 may titer out a repressor for the pathway-specific regulators. Another *asfR2*-like sequence that is implicated in the regulation of nystatin biosynthesis has recently been found in *S. noursei* (Sekurova et al., 1999). A more widespread distribution of AsfR2-like proteins may point to a common regulation of the pathway-specific regulators.

A set of Ser-Thr kinases, Pkg2, Pgk3 and Pkg4, have been isolated from *S. granaticolor* (Vomastek et al., 1998; Nádvorník et al., 1999). While Pgk2 is a membrane-spanning Ser/Thr kinase, Pgk3 and Pgk4 are cytoplasmic kinases. The Pgk2$^-$ phenotye shows a lack in morphological differentiation which, however, was conditional and depended on the growth medium. The malt

extract, yeast extract and glucose medium restored morphogenesis, while a medium containing cornsteep, starch and yeast extract clearly showed the Pgk$^-$ phenotype (Nádvorníc et al., 1999). It is conceivable, that similar to AsfR/K, Pgk2 may be required for regulation of morphogenesis at higher phosphate concentration.

Additional phosphotyrosine phosphatases have been recently characterized, which in multicopy increase Act production in *S. lividans* (*ptpA*; Li and Strohl, 1996). Again, that mode of action, ligands and targets are unknown.

Two gene clusters, the *ramA/B/R* and *amfA/B/R* of *S. coelicolor* and *S. griseus*, respectively, are required for initiation of aerial hyphae development (Ueda et al., 1993; Ma and Kendall, 1994) as has been described in chapter 3.1. Both are two component systems in which genes A and B are considered to form a protein or peptide translocator, while gene R represents the response regulator protein. AmfR depends on the phosphorylation of Asp54 (Ueda et al., 1993). Transcription of *amfR* is normally repressed by AdpB, but repression is relieved in an A-factor dependent manner (Ueda et al., 1998). Interestingly, neither a kinase nor a phosphatase was found in the vicinity of *amfR*. Nevertheless, AmfR is essential for morphogenesis, and Ueda and coworker (1998) suggested that AmfR, in analogy to SpoA0 in *B. sultilis* (Burbulys et al., 1991; Hoch, 1993), may receive its signal from a variety of different receptors, and that AmfR therefore might be the final target of the phospho-relay, committing the cell to differentiate.

Analysis of another global regulator in *S. coelicolor*, the *abaA* locus, revealed five putative ORFs. Subcloning experiments revealed that OrfB together with 137 bp downstream of it is responsible for Act overexpression in *S. lividans* (Fernández-Moreno et al., 1992). Disruption of *orfB* did not affect the morphological development or the production of methylenomycin in *S. coelicolor*. Act, Red and CDA productions, however, were abolished or severely reduced. No details are known on the molecular mode of action.

A similar overproduction of Act was observed when a DNA fragment, isolated adjacent to the

right end of the *act* cluster, was transformed into *S. lividans.* The fragment contained 6 ORFs, one of which, *orf10* has homology to LysR-type transcriptional regulators (Martínes-Costa et al., 1999). In contrast to *abaA*, disruption of *orf10* had no significant effect in *S. coelicolor*, but resulted in actinorhodin overproduction in *S. lividans*. This implies, even though no target was found, that Orf10 protein negatively regulates actinorhodin production in *S. lividans*. Orf10 apparently represses its own and the transcription of *orf11*, located downstream. A model, according to which Orf10 protein may regulate an unknown transcription factor that, in turn, negatively controls *actII-orf4* gene expression has been proposed (Martínes-Costa et al., 1999).

The *abaB* locus isolated from *S. antibioticus* also contains a putative LysR-type transcriptional regulator that upon expression in *S. lividans* elicits Act production. Southern analysis with chromosomal DNA from five streptomycetes revealed the presence of homologous DNA sequence to *abaB* in all *Streptomyces* tested (Scheu et al., 1997). A second gene of this locus has homology to genes involved in sulfur metabolism (Scheu et al., 1997).

The recent characterization of the *absB* gene as a homolog to RNase III, a dsRNA-specific endoribonuclease indicates the involvement of posttranslational modification as a means to control expression of genes for secondary metabolites (Price et al., 1999). Most substrates for RNase III contain a ~ 20 bp double-helical region without the need for a specific DNA sequence (Zhang and Nicholson, 1997). It has previously been shown that RNase III in *E. coli* can up- or downregulate expression of genes posttranscriptionally by cleavage of stem-loop structures within the noncoding regions of certain mRNAs (Court, 1993). It remains to be determined if the level of activity of the *absB* gene or gene product is developmentally regulated, and whether and how RNase III might interact with secondary metabolism.

Other DNA sequences have been described to exert pleiotropic effects on secondary metabolism. *micX*, encoding a putative antisense mRNA, and *mia* (Table 3.3.) are two examples of less well characterized loci.

3.3 Metabolism and Nutritional State

There is ample evidence that changes in the nutritional state of a culture can prevent or induce the onset of differentiation. It is therefore not possible to discuss cellular differentiation in *Streptomyces* without taking into consideration their primary metabolism and its regulation. This, however, should not be done without regarding the natural environment, the soil, where streptomycetes as saprophytes mostly degrade plant material. Plant material is generally rich on carbon (complex polysaccharides) but poor on nitrogen. As a consequence, a multitude of carbohydrate catabolic pathways have been discovered in actinomycetes. These pathways are subject to catabolite repression which, however, is distinct from carbon catabolite repression found in *E. coli* or *B. subtilis* (see below). Interestingly, carbohydrate transport into the cell is mostly accomplished by constitutive low-affinity-high-capacity permeases. Inducible high-affinity permeases have only low transport capacity. In respect to the generally low nitrogen concentration in soil, streptomycetes have to grab what they can. Not surprising, transport of amino acids is accomplished mostly by high-affinity systems. In addition, about half of the amino acid catabolic routes are constitutive and amino acid biosynthesis is rarely feed-back inhibited. Our current knowledge of the primary metabolism of *Streptomyces* and understanding of its control have recently been reviewed (Hodgson, 2000).

Unfortunately, physiological studies in streptomycetes are difficult to conduct and therefore rarely found. Already small colonies or mycelial pellets may contain cells at different states of differentiation which will interfere with analysis of metabolic fluxes and the analysis of enzyme activities. Furthermore, strains that overproduce secondary metabolites may already be deregulated in their primary metabolism, e.g. precursor supply. Additional complications derive from the observation that several isoenzymes are present that have different functions in catabolism, anabolism, or secondary metabolism. For example, the *p*-aminobenzoic acid synthetase of *S. griseus*

required for folic acid and candicidin synthesis (Gil et al., 1985) has two isoenzymes. One isoenzyme is involved in primary metabolism and the second, regulated by phosphate and aromatic amino acids, is required for antibiotic synthesis. In *S. parvulus*, producer of actinomycin D, two isoenzymes of kynurenin formamidase were found, one of which was constitutive and postulated to have a role in NAD synthesis and tryptophan breakdown, and the second inducable isoenzyme is involved in actinomycin D synthesis (Brown et al., 1986). Finally, the constitutive expression of many pathways is accompanied by a low level expression of the respective enzymes. Detailed kinetic analysis of these pathways may require a 1000 fold or better purification and enrichment of the respective enzymes (Stuart and Hunter, 1993; Alves et al., 1997).

The recent review by Hodgson (2000) has compiled and discussed in a very complete manner the current state of our understanding of the primary metabolism and its control. Hodgson discussed the various results considering the organisms phylogeneic relatedness (distribution within 19 major and 40 minor clusters). As expected from their natural environment and as indicated by the diversity in secondary metabolites produced, streptomycetes also excel through an enormous metabolic diversity in primary metabolism. The limited number of primary metabolic pathways studied indicate that only a few, namely Embden-Myerhof-Parnas (EMP, glycolysis), tricarboxylic acid (TCA)-cycle, and the shikimate pathway, were commonly found in streptomycetes. Other pathways often are species specific in both, the enzymes involved and in their regulation. This, together with diverse experimental settings used in the varies studies makes it difficult to determine the regulatory interface between primary and secondary metabolism. As a result of Hodgson's review on the primary metabolism, the author points out that the missing tight regulation of the primary metabolism and the rather tightly controlled secondary metabolism suggests, that the old idea of secondary metabolism being a form of overflow metabolism (Bu'Lock, 1961) that is initiated during conditions of unbalanced growth (missing of a major nutrient), may be due for revival. The biological role of secondary metabolites would thus shift from that of self defense or a signal for cell-cell interactions to dealing with unbalanced growth conditions by removing overflow primary

metabolites under 'feast' conditions, such as ample supply of amino acids. Removal of overflow products would be advantageous to the cell, regardless the structure of the secondary metabolite. This hypothesis leads to an alternative view on how the vast diversity of secondary metabolite pathways may have developed: driven by the need to remove a variety of primary metabolites, the composition of which would be expected to be different for any given environmental niche, rather than by evolution as adaptation to a hostile environment. This thought is attractive as it is hard to conceive how, for example, production of a molecule that we value as an immunosupressant such as rapamycin or FK506 may provide a selective advantage for a soil inhabiting microorganism.

It is furthermore interesting to note that reactions in pathways of the primary metabolism are often catalyzed by enzymes that are more closely related to eucaryotic homologues then to those found in other bacteria. The exposure to decaying material of higher organism may well be responsible for horizontal gene transfer. Acquisition of new genetic material might have contributed to the large size (~ 8 megabases) of the linear *Streptomyces* chromosome. The almost completed *Streptomyces* sequencing project (www.sanger.ac.uk/Projects/S_coelicolor/) indicates that this genome contains more genes than the yeast genome. The genetic instability near the ends of the *Streptomyces* chromosome or the existence of giant linear plasmids (Cullum et al., 1988; Birch et al., 1990; Chang and Cohen, 1994) may be elemental for such exchanges of foreign DNA, which, if beneficial, could recombine into a more stable location on the chromosome or otherwise be lost again.

As mentioned, the metabolic diversity makes it difficult to pinpoint key regulatory events that will induce differentiation in *Streptomyces*. Nevertheless, several studies in recent years demonstrate the link between primary metabolism, a change in nutritional state of a culture and secondary metabolism. Two such systems have already been mentioned: The inhibition of morphological development under nutrient-rich conditions by the *craA* gene product, and the induction of sporogenesis under starvation by SsfA (Figure 3.1). Yet another gene, *spaA*,appears to be involved in sensing starvation (Schneider et al., 1996). To date, very little is known about the

underlying molecular events of these processes. However, the hypothesis that onset of differentiation and secondary metabolism is preceded by a downshift in the rates of protein biosynthesis and growth, often caused by a change in or a depletion of the source of carbon, nitrogen or phosphate (Martin and Demain, 1980; Hobbs et al., 1990; Lounes et al., 1995; Wilson and Bushell, 1995), has generally been accepted, but also challenged (Granozzi et al., 1990). Translation of these observations into molecular interactions established catabolite repression, stringent response and the GTP-pool as key players in the control of differentiation. The following chapters will focus on events in the primary metabolism that trigger differentiation, mostly the initiation of the secondary metabolite production. The studies were mostly performed in liquid culture, which generally does not permit the observation of morphological differentiation. Some of our own findings are included.

3.3.1 Central Carbon Metabolism

It is somewhat surprising that the biosynthetic pathways for secondary metabolites are being studied in great detail for quite some time now, but that only very limited information is available on the key metabolic routes of the primary metabolism. Scattered information is available on the presence or absence, or the regulation of Embden-Myerhoff-Parnas (EMP, glycolysis), pentose phosphate (PP) pathway, Tri-carbocylic acid (TCA) -cycle and glyoxylate shunt. However, rarely were these studies done in the same host. The discorvery of new metabolic routes, for example an alternative anaplerotic pathway to the glyoxylate cycle described by Han and Reynolds (1997) always leaves the question open, how common pathways and their regulation are among streptomycetes. Furthermore, fundamental events, such as glucose uptake, remain to be characterized for *Streptomyces*. So far, the only phosphoenolpyruvate-dependent phosphotransferase system (PEP:PTS) identified in *S. lividans, S. coelicolor* and *S. griseofuscus* is that for fructose (Titgemeyer et al., 1995).

Salas and coworkers (1984) studied in *S. antibioticus* the fluxes through the glucose-catabolic pathways in respect to the developmental state of the culture. ^{14}C-labeling studies showed that the PP pathway and the TCA-cycle were active in dormant spores under non-germinating conditions. Under germinating conditions and early stages of germination, EMP and TCA-cycle were predominantly active, but the PP pathway continuously increased in its participation in glucose catabolism until in mid-exponential growth the PP pathway was the main catabolic route for glucose consumption. The TCA-cycle was active and drained for biosynthetic purposes. A similar study in *S. coelicolor* (Obanye et al., 1996) revealed a shift from EMP to PP pathway coinciding with the onset of production of methylenomycin during the transition from exponential to stationary phase. Interestingly, the data presented show that the culture resumes growth after approximately 5 hours while methylenomycin is being produced, but no data were provided on the residual concentrations of glucose or nitrogen (alanine) during this second growth phase. The demand for reducing power in form of NADPH for the biosynthesis of methylenomycin is proposed as the driving force for the shift to the PP pathway in which glucose-6-phosphat dehydrogenase and 6-phosphogluconate dehydrogenase provide reduced NADPH, which may also be needed for the synthesis of polyketide antibiotics (Ikeda et al., 1988).

A key enzyme for regulation of the glycolytic pathway is the irreversible ATP-dependent phosphofructokinase (ATP-PFK). In most bacteria ATP-PFK, a tetramer of 25 kDa subunits, is allosterically inhibited by PEP and activated by ADP and GDP. The *S. coelicolor* ATP-PFK has recently been purified and characterized kinetically (Alves et al., 1997). The enzyme was strongly inhibited by PEP *in vitro*, but showed highest (70%) similarity to the inorganic pyrophosphate-dependent (PP_i)-PFK from *Amycolatopsis methanolica* (actinomycete), although the two enzymes differ strongly in their regulatory properties. The PP_i-PFKs isolated from bacteria are usually dimeric and not subject to regulation on activity level (Alves et al., 1997). Interestingly, the *A. methanolica* PP_i-PFK is a tetrameric enzyme and shares similarities with both, PP_i- and ATP-PFKs (Alves et al., 1996). How PEP or ADP and GDP regulate ATP-PFK of *S. coelicolor in vivo* remains

to be studied.

Evidence for the involvement of the TCA-cycle in the morphological and physiological differentiation has recently been published. Disruption of the aconitase from *S. viridochromgenes* Tü494 yielded mutants that were deficient in aerial mycel (bald phenotype), sporulation and in the production of the phosphinotricine tripeptide (PTT). Not buffering the medium, but addition of L-glutamate could rescue the bald phenotype (Schwartz, et al., 1999), but the mutants remained deficient for sporulation. About 10% of the wild-type levels of PTT was detected in the aconitase mutant supplemented with L-glutamate. Similar defects in morphological development and the production of secondary metabolites were described for an aconitase (*acoA*) mutant in *S. coelicolor*, that is unable to grown on L-glutamate (Viollier et al., 2001b). Growth on glucose medium resulted in excretion of large quantities of citrate, acetate and pyruvate. Buffering of the medium could partly restore growth defects of the *acoA* mutant, but growth was still retarded, most likely due to the citrate accumulation. Citrate chelates divalent cations which could in part be responsible for the developmental phenotype (Viollier et al., 2001b). A citrate synthase mutant (*citA*) in *S. coelicolor* (Viollier et al., 2001a) also excreted large quantities of organic acids. The defects of the *citA*$^-$ mutant could be rescued on buffered medium or by growth in close proximity of the wild-type and most *bld* mutants (*bldA*, *bldB*, *bldC*, *bldG*, *bldH*), except for *bldC* and *bldJ* (see Figure 3.1). The *citA*$^-$ mutant in turn could complement *bldK* which suggests that citrate synthase is involved in the signal cascade for morphological differentiation in *S. coelicolor* (Viollier et al., 2001a).

While these studies demonstrate that the enzymes of the TCA-cycle play a central role in coordinating the initiation of the differentiation, the molecular level of this interaction is largely unknown.

3.3.2 Catabolite Repression

Catabolite repression, exerted mainly by glucose, of expression of a variety of genes,

including many operons coding for exoenzymes for degradation of complex carbon sources, such as starch, cellulose, xylan, chitin, agarose, and others has long been known (Hodgson, 2000 and references therein). The glycerol catabolic genes, glycerol kinase and NAD-independent L-glycerol-3-phosphate dehydrogenase too, are repressed coordinately by various carbon sources, of which glucose has the strongest effect (Seno and Chater, 1983). Moreover, glucose repression of the morphological and physiological development has been demonstrated by the conditional defects of most *bld* mutants (chapter 3.1.1) but the molecular level of interaction between glucose repression and morphogenesis or secondary metabolism remains to be elucidated.

The general mechanism of glucose repression in streptomycetes is distinct from that of other bacteria and not well understood. While in entric bacteria the glucose PEP:PTS together with cAMP regulate catabolite repression (Postma et al., 1993), no PTS system for glucose could be found in *Streptomyces* (Novotná and Hostálek, 1985). Furthermore, cAMP is not implicated in glucose repression as indicated by the absence of fluctuations in cAMP level with changes in carbon source (Chatterjee and Vining, 1982; Hodgson, 1982). Nevertheless, cAMP may play a key role in differentiation (chapter 3.5.1.).

The ATP-dependent glucose kinase (*glkA*) has been identified as a key player in glucose-mediated gene repression in streptomycetes. In *S. coelicolor*, the most genetically characterized streptomycete, inactivation of an ATP-dependent glucose kinase (*glkA*) resulted in the inability to utilize glucose and in a pleiotrophic loss of glucose repression (Hodgson, 1982; Seno and Chater, 1983), but it had no effect on glucose transport (Hodgson, 1982). Replacing GlkA with an unrelated glucose kinase from *Zymomonas mobilis*, or with a normally cryptic glucose kinase of *S. coelicolor*, conferred back the ability to utilize glucose but not that of glucose repression (Angell et al., 1994). Moreover, a study with a glucose kinase (*glkA*) mutant of *S. coelicolor* by Kwakman and Postama (1994) showed that overexpression of GlkA relieves carbon catabolite repression for the glycerol and agarose catabolism. Furthermore, carbon catabolite repression of the glycerol kinase in the *glkA* mutant was relieved in the presence of glucose, arabinose, galactose, citrate, and glutamat, which in

the wild-type represses expression of this enzyme. Since these carbon sources, except for glucose, are not metabolized via GlkA, the authors suggest that GlkA may titer out a negative regulator, and that GlkA itself rather than any flux through glycolysis may be involved in carbon catabolite repression. How GlkA mediates glucose repression remains to be elucidated. Inducer exclusion, however, does play an important role, especially considering the many constitutive sugar permeases found in streptomycetes (Hodgson, 2000).

While several genes and operons, such as agarase (*dag*), amylase (*aml*), or the glycerol (*gly*) operon are glucose-repressed in a glucose-kinase-dependent manner, repression of others (chitinase, *chi*) does not require glucose kinase activity (Hodgson, 2000 and references therein). Whether a common mechanism for regulation of catabolite repression in *Streptomyces* exists, how induction by the macromolecules, such as chitin or starch, works, is still not well understood. The following, more detailed examples should point out the variety of mechanisms that have so far been identified.

In Gram-negative bacteria glucose repression is mediated mostly by the absence of positive regulators. In contrast, repression in *Streptomyces* seems to operate through negative regulation, in some cases mediated through the, in procaryotes rather unusual, binding of proteins to direct repeats that overlap with RNA polymerase binding sites. A 12-base-pair direct repeat upstream of the *chi63* chitinase gene of *S. plicatus* has been studied in some detail (Delic et al., 1992; Ni and Westpheling, 1997). Single base changes in a pair of 12-bp perfect direct repeats that overlap with the putative RNA polymerase binding site of the *chi-63* promoter resulted in glucose-resistant and chitin-independent expression, suggesting that this sequence is an operator that binds a negative regulator. Further mutations revealed that the -10 (TATTCT) and -35 (TTGACC) regions, that are similar to those typical for eubacterial RNA polymerase recognition sequences, are required for RNA polymerase interaction. The authors suggest that a common protein which binds at the direct repeat sequences may, as part of a signaling cascade, mediate glucose repression as well as chitin induction. Similar 5' regulatory regions have been identified in a number of chitinase genes from different *Streptomyces* (Ni and Westpheling, 1997), all of which contain eubacterial-type RNA

polymerase binding sites and a pair of direct repeat sequences. Interestingly, these direct repeats are all located at the same face of the DNA helix and either overlapping with or in close proximity to the putative RNA polymerase recognition sequence. Hence, these genes may have a similar mechanism of transcription regulation.

Subsequently, additional promoter regions of catabolic enzymes have been identified that have of direct or inverted repeat motifes. The direct repeats identified upstream of the *galP1* promoter had a different sequence compared to those found in the *chi63* promoter, but their location in respect to the RNA polymerase binding site was very similar (Mattern et al., 1993; Brawner et al., 1997). Evidence was presented that these repeats are involved in negative regulation of *galP1* and that a new RNA polymerase holoenzyme is involved in transcription. The promoter of the *aml* (amylase) of *S. limosus* also has a pair of direct repeats and a pair of inverted repeats that are implicated in growth-phase-dependent, glucose-sensitive and maltotriose-dependent expression of the gene (Virolle and Gagnat, 1994). *amy* and *malE*, both containing direct repeats,

Several LacI/GalR-like transcriptional regulators, comprised of a N-terminal DNA binding domain and a C-terminal ligand binding and dimerization domain are involved in mediating carbon catabolite repression. These regulators recognize these operator motifs of the other pormoters as binding sites. Examples include Reg1 or MalR that negatively control the amylase and the maltose operons in *S. lividans* and *S. coelicolor* (Nguyen et al., 1997; van Wezel et al., 1997a/b), and GlyR that controls expression of the glycerol catabolic operon in *S. coelicolor* (Hindle and Smith, 1994). Similar to mutations in the operator sequences upstream of the *amy* and *malE* genes, mutations in *reg1* enhance expression and abolish glucose repression. Reg1 mutants were also affected in chitinase expression which became glucose-insensitive and was no longer induced by chitin. Hence, Reg1 appears to be a pleiotrophic regulator for exoenzymes. In *S. lividans* it could either positively regulate chitinase, maybe at the level of the sugar uptake system, or negatively regulate chitinase, α-amylase, as well as endoglucanase (*celB*) and xylanase (*xlnB*) in the presence of glucose (Nguyen et

al., 1997). Consensus motives for Reg1 binding sequences were proposed recently (Nguyen, 1999): the direct repeats display a consensus sequence CTTGCAG for both half-sites, and the consensus for the inverted repeats were CTTGCAG and CTGCAAG for the left-hand and the right-hand sequence, respectively. The length of the repeat sequences varies, as does the spacing between the repeats. It is interesting to note that the *glyR* promoter is very similar to *galP1* (Brawner et al., 1997) and hence may be itself subject to catabolite repression.

The isolation of the *sblA* gene (Gagnat et al., 1999) points to a new mechanism of glucose repression. The new gene allows for the glucose-resistant expression of a heterologous α-amylase (*aml*) in *S. lividans*. *sblA* encodes a 274 aa protein that shares significant homology to proteins of the inositol monophosphatase (IMP) family that are thought to be involved in the degradation of small phosphorylated substrates. Transcription of *sblA* is enhanced during transition-phase, indicating a growth-phase-dependent regulation. In addition, eight direct repeats are located upstream of the 5' region, one of which overlaps with the putative -35 region (Gagnat et al., 1999). The substrate for SblA or its mode of action remains to be determined.

Glucose repression is not limited to primary metabolism. Two enzymes of the actinomycin biosynthetic pathway, phenoxazione synthase (*phsA*) and actinomycin synthase I, in *S. antibioticus* are glucose repressed (Hsieh and Jones, 1995 and references therein).

More recently, Ueda and coworker (1999) describe a gene in *S. griseus* that is expressed at high level in media where glucose or galactose is present, while no expression was observed on media containing maltose or mannitol. This putative glucose-induced gene, termed *craA* (**c**arbon-source-dependent **r**egulation of **a**erial mycelium formation), repressed initiation of morphological differentiation. It was suggested that the availability of the carbon source regulates *craA* expression (Figure 3.1.). However, it remains to be determined how glucose actually accomplishes this regulation and why maltose or glucose produced by the anaplerotic routes does not.

A *trans*-acting locus has been identified in *S. coelicolor* (Ingram et al., 1995), that maps near

argA and the glucose kinase gene which is controlling transcription from *galP1*, expression of the *gly* operon and of the chitinase gene *chi63*. The locus was named *ccrA* for **c**ontrol of **c**atabolite **r**epression. In *ccrA1* mutants, expression of these genes and operons became glucose-insensitive and independent of the addition of the respective inducer. Yet, addition of the respective inducer resulted in expression level several fold higher than observed in the wild-type strain. In contrast, expression from *galP2*, a promoter that is not subject to glucose repression, was not affected by *ccrA*. The authors suggest that CcrA might act as a cAMP receptor protein (CRP)-like protein in *Streptomyces* that interacts with RNA polymerases to allow transcription from *ccrA*- regulated promoters (Ingram et al., 1995).

Other examples for carbon catabolite repression include repression of some catabolic enzymes by amino acids which is mediated by their carbon skeleton that remains after deamination. Kominek (1972) reported citrate repression of glucose catabolism in *S. niveus*, producer of novobiocin. Interestingly, this organism used TCA-cyle intermediates as preferred carbon source. Chatterjee and Vining (1981) observed that acetate and citrate were good repressors of the *S. venezuelae* maltase.

3.3.3 Production and Excretion of Organic Acids

Directly related to the central carbon metabolism of *Streptomyces* is the phenomenon of excretion of substantial amounts of organic acids. Mostly pyruvate, acetate, and TCA-cycle intermediates but also lactate has been detected indicating a fermentative metabolism have been detected (Perlman and Wagman, 1952; Doskoèil et al., 1959; Minas, 2003). Excretion of α-keto acids has been described for several *Streptomyces*. *S. venezuelae* (Ahmed et al., 1984), *S. rimosus* (Bormann and Hermann, 1968), and *S. hygroscopicus* (Gräfe et al., 1975) have been shown to excrete pyruvic and α-keto glutaric acids, as does *S. lividans* TK24 (Madden et al., 1996). *S. aureofaciens* (Doskoèil et al., 1959) has been shown to only excrete pyruvic acid, while *S. griseus*

(Hockenhull et al., 1954), in addition to pyruvic and α-keto glutaric acids, also excreted lactic acid, indicative for a fermentative pathway. Gräfe and coworker (1975) suggest that the NADH/NAD^+ ratio regulates pyruvate dehydrogenase, citrate synthase, and malate dehydrogenase activities and that the internal pool of Coenzyme A, for which pyruvate- and α-ketoglutarate dehydrogenase are competing, could be implicated in regulation of these enzymatic steps. Changes in oxygen availability would readily change these pool sizes resulting in accumulation of acids.

Our own experiments on organic acid production revealed that organic acids are also excreted by cultures of *S. coelicolor* A3(2). Figure 3.4 shows a cultivation of this strain in a glutamate-maltose-containing medium (MG medium described by Doull and Vining, 1989). A slightly diauxic growth pattern is observed. Glutamate is the preferred carbon source and is consumed during the first phase of rapid growth. Upon depletion of glutamate, acetic acid is the first acid that transiently

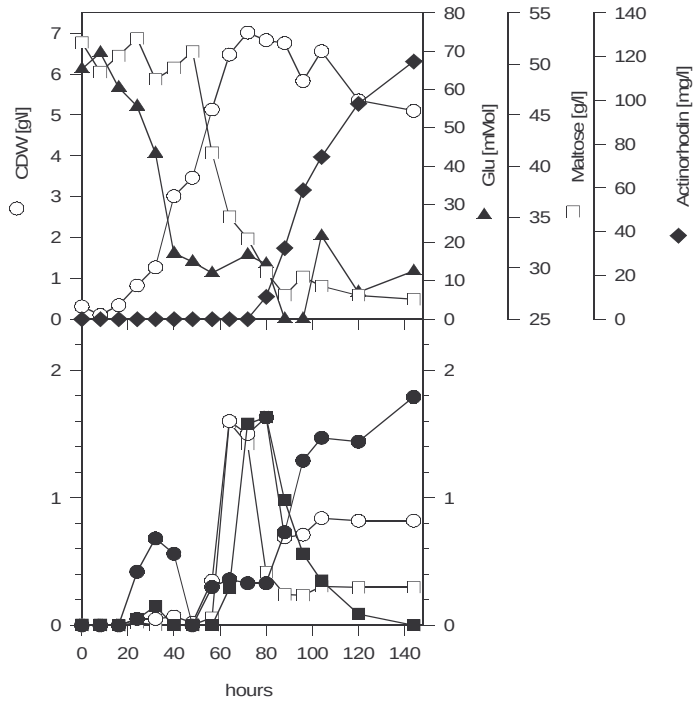


Figure 3.4. Shake flask cultivation of *S. coelicolor* A3(2) in MG minimal medium (Doull and Vining, 1989). The upper panel shows biomass accumulation (open circles), glutamate and maltose (filled triangles and open squares, respectively) and actinorhodin production (filled diamonds). The lower panel depicts the transient excretion of organic acids: pyruvic (open squares), acetic (filled circles), succinic (open circles), and lactic (filled squares) acid. (source: Minas, 2003)

accumulates. Acetate is readily reassimilated at the beginning of the second phase of rapid growth on maltose as carbon source. Acetate reappears in the medium later during growth on maltose indicating an overflow. Coinciding with the depletion of the maltose, pyruvate, succinate and lactate transiently appear and actinorhodin production starts. Accumulation of acetic acid, the typical overflow metabolite from glycolysis, during stationary phase can be attributed to maltose utilization in a nitrogen limited culture (Minas, 2003). In contrast, carbon limited cultures do not excrete acetic acid during stationary phase.

Acid excretion can drastically lower the pH which may have secondary effects on growth and development of the cells (Redshaw et al., 1976; Surowitz and Pfister, 1985a; Süsstrunk et al., 1998; Viollier et al., 2001 a/b). In some strains, acid secretion is followed by reassimilation of the acids which also raises the pH of the medium. In other strains or mutants, acids are not reassimilated and block cell differentiation (Gräfe et al., 1975; Madden et al., 1996; Surowitz and Pfister, 1985a; Süsstrunk et al., 1998; Viollier, et al., 2001a/b). Thus, the effect of glucose repression on differentiation might be related to acid toxicity in strains where excreted acids are not reassimilated.

Several studies, including our own have demonstrated that key enzymes of the TCA-cycle, citrate synthase and aconitase, are implicated in both acid production and triggering differentiation processes (Schwartz et al., 1999; Viollier et al., 2001a/b). Surowitz and Pfister (1985a) have measured activities of the key enzymes of the glycolytic pathway and the TCA-cycle in *S. alboniger* grown in the presence and absence of glucose. Interestingly, only phosphofructokinase and pyruvate kinase are both induced over 5 fold in glucose containing media, while citrate synthase and pryrvate kinase activities remained unchanged. Pyruvate accumulation is thus the result of an imbalance between glycolysis and TCA-cycle caused by an relatively increased flux through the glycolysis. The authors also showed that cultures growing on mannose or sucrose were repressed in the uptake of these sugars shortly after addition of glucose to the culture. Utilization of glucose commenced immediately suggesting a constitutive pathway. The addition of adenine, and to a lesser extent adenosine and guanosine prevents acid production on glucose in *S. alboniger* by an unknown

mechanism. No direct interaction of adenine with the enzymes could be found (Redshaw et al., 1976, Surowitz and Pfister, 1985a). Interestingly, adenine also relieved the block in aerial mycel formation. In contrast, puromycin biosynthesis in this strain remained under glucose repression irrespective of the extracellular pH.

While pyruvate decarboxylase and citrate synthase were not affected by growth on glucose in *S. alboniger*, accumulation of α-keto acids in *S. venezuelae* (Ahmed et al., 1984), *S. rimosus* (Bormann and Hermann, 1968), and *S. hygroscopicus* (Gräfe et al., 1975) appears to be the result of inhibition of pyruvate decarboxylase and/or α-ketoglutarate dehydrogenase activities. Further details on the regulation of these enzymes are not available.

Madden and coworker (1996) demonstrated that in addition to the carbon sources, the nitrogen source too has a great impact on acid excretion in *S. lividans*. Glucose grown cultures would excrete between 6 and 18% of the total consumed carbon as organic acids, mostly pyruvate and α-ketoglutarate when nitrate or amino acids were used as nitrogen source. In contrast, addition of ammonium to the medium repressed acid production. This observation clearly links the carbon metabolism with the nitrogen metabolism, which has been shown to be very much implicated in the repression/induction of secondary metabolism (discussed in chapter 3.3.5.).

3.3.4 Storage Products

The accumulation of several storage products has been observed in *Streptomyces*. The deposit of **glycogen** at different times and locations in the mycelium, mediated by two distinct glycogen operons, and their implication in morphogenesis has been discussed in chapter 2 (Braña et al., 1986; Schneider et al., 2000), as has the interconversion of the immobile, inert glycogen deposits into metabolically active metabolites (e.g. glucose-1-phosphate) and into metabolically inert but physiologically relevant trehalose (Schneider et al., 2000).

Accumulation of the glucose disaccharide **trehalose** was observed throughout the colony in

all phases of colony development (Braña et al., 1986). A preferential accumulation was found in aerial hyphae and spores, where trehalose can make up to 12% of the spore dry weight. Trehalose is synthesized by the condensation of GDP-D-Glucose and glucose-6-phosphate resulting in trehalose-6-phosphate (Elbein, 1967a/b; Hey and Elbein, 1968). A trehalose-6-phosphate phosphatase and a trehalase were subsequently identified in *S. hygroscopicus* (Hey-Ferguson et al., 1973). The general role for trehalose accumulation, especially in spores, is that of a storage compound. However, Braña and coworker (1986) concluded from their studies in *S. antibioticus* on the location of trehalose deposits (mainly in the tips of the aerial hyphae and the spores) and the kinetics of trehalose synthesis that trehalose has a second function as osmoprotectant, protecting against deydration. While the amount of trehalose found in the cells varied depending on culture condition, no direct role in cellular differentiation was attributed to trehalose.

In contrast to trehalose, **triacylglycerols** (TAG) may be involved in supplying precursors for secondary metabolites. Olukoshi and Packter (1994) found neutral lipids accumulations in *S. lividans, S. albus, S coelicolor* and *S. griseus*. TAG accumulation was highest in *S. lividans* and stimulated by a high C:N ratio. TAG synthesis was highest in the stationary phase which may be the result of switching from membrane phospholipid biosynthesis to TAG synthesis under conditions when cultures where limited by nitrogen, but not carbon. Interestingly, actinorhodin is formed in *S. coelicolor* only after glucose has been exhausted (on YEME medium). TAG has been suggested as the supplier of the C_2 units required for actinorhodin biosynthesis (Olukoshi and Packter, 1994).

The accumulation of **polyhydroxybutyrate** (PHB) in streptomycetes has occasionally been reported (Kannan and Rehacek, 1970; Packter and Flatman, 1983; Ranade and Vining, 1993). PHB accumulation in *S. venezuelae* is observed during exponential growth phase and PHB disappears coinciding the production of the secondary metabolite chloramphenicol (Ranade and Vining, 1993). However, even though PHB could provide the precursors for chloramphenicol biosynthesis, the authors conclude that the small amount of accumulated PHB would be insufficient to ensure the precursor requirement for the production of the antibiotic.

3.3.5 Nitrogen Metabolism

The review on the *Streptomyces* primary metabolism by Hodgson (2000) provides a most comprehensive overview on our current understanding of the nitrogen metabolism in these organisms. As it is not within the scope of this review to repeat all these details, I will focus in my discussion only on aspects that are related and/or relevant for morphological differentiation and secondary metabolism.

Many antibiotic molecules have structures that contain nitrogen atoms derived from nitrogenous primary metabolites or the carbon skeleton of amino acid derived precursors. In these cases, the relationship between primary and secondary metabolism is very obvious. Examples are the peptide antibiotics or β-lactams, where amino acids are condensed with minor modifications to form the antibiotic. L-glutamine and L-arginine are used as nitrogen donors in the biosynthesis of aminocyclitol antibiotics such as streptomycin (Walker and Walker, 1982) and the carbon skeleton of amino acids provides the precursors for the macrolides avermectin or tylosin which do not contain nitrogen in their aglycon (Hafner et al., 1991). In cases where a specific nitrogen source, such as an amino acid, is required for the biosynthsis of a secondary metabolite, exogenous addition of the compound or genetic manipulation of the pathway leading to the precursor can improve production of the secondary metabolite, which has been demonstrated by an increased cephamycin C production in *S. clavuligerus* upon lysine addition (Braña and Demain, 1988 and references therein). Often however, global inhibition of secondary metabolism can be observed on certain sources of nitrogen. In addition, several studies show that the source of nitrogen and the carbon to nitrogen (C:N) ratio have pronounced effects on the production of secondary metabolites. Similar to the situation in carbon metabolism, again a great variability exists for the various catabolic and anabolic routes for nitrogen utilization in different strains of *Streptomyces*. This makes it difficult to define general models for regulation by nitrogen. Especially the amino acid metabolic routes are

controlled differently, depending on whether a particular amino acid provides precursors for secondary metabolite biosynthesis or not (for review see Hodgson, 2000). For example, Inbar and Lapidot (1988) showed that extracellular L-glutamate represses production of actinomycin D in *S. parvulus* even though a relatively high intracellular pool of L-glutamate is needed for actinomycin D biosynthesis. Interestingly, the cells have the capacity to maintain high intracellular L-glutamate concentration without affecting actinomycin D production. The authors showed that the carbon skeleton of the intracellular glutamate was not derived from the extracellular glutamate, but rather from fructose catabolism. In addition, a new intracellular pyrimidine derivative was detected whose carbon skeleton and nitrogen was derived from exogenous L-glutamate. The authors suggest that this and a second pyrimidine derivative that was derived from fructose, serve as nitrogen storage for actinomycin D synthesis (Inbar and Lapidot, 1988).

3.3.5.1 Nitrogen Repression

'Nitrogen repression' of secondary metabolism refers to an inhibition of, reduction of, or delay in onset of secondary metabolism by the presence of a readily catabolized nitrogen source (Braña and Demain, 1988). This repression takes place also in the presence of a second, non-repressing nitrogen source. However, it should be noted that the concentrations needed to elicit nitrogen repression, usually by ammonium and some amino acids, is very high (10 - 120 mM).

The regulation of nitrogen metabolism is best understood in entric bacteria (Magasanik, 1996). When entric bacteria are grown on media containing a high concentration of (~1 mM) ammonia, NH_4^+ can be directly incorporated into glutamate by glutamate dehydrogenase (GDH). The high K_M of GDH restricts this route to high ammonium concentrations. Under nitrogen-limited conditions, ammonium is assimilated into glutamine by glutamine synthetase (GSI), and glutamate is synthesized from glutamine by glutamate synthase (GOGAT = glutamine: α-oxoglutarate aminotransferase). Activity of GSI is feed-back inhibited by nine different products of the glutamine

metabolism and expression of GSI (*glnA*) is activated under N-limited conditions mediated by the activated form of NtrC of the Ntr system which senses the glutamine:α–ketoglutarate ratio. NtrC will be phosphorylated by NtrB at a low ratio and dephosphorylated at a high ratio (Magasanik, 1996).

Not all bacteria have this system. Bacilli have no GDH and use only the GS-GOGAT system for ammonium assimilation (for review Fisher and Sonenshein, 1991; Schreier, 1993). The bacillus GS is only feed-back regulated by GlnR and TnrA (Wray et al., 1996) rather then adenylation.

In contrast to other Gram-positive bacteria, streptomycetes contain at least two GS enzymes, GSI (*gnlA*) which is similar to other bacterial GSs and GSII (*glnII*), a heat-labile octameric protein that resembles the eucaryotic GSs. Expression of *glnA* and *glnII* are regulated by GlnR, a response-regulator which positively regulates glutamine synthetase in *S. coelicolor* (Fischer, 1992) and GlnRII, respectively (Weißschuh et al., 2000). GSI appears activated by adenylation (Fink et al., 1999).

As has been discussed in chapter 3.3.3, excretion of organic acids in *S. lividans* can be repressed by ammonium in the medium, while nitrate resulted in the excretion of up to 18% of the total consumed carbon as organic acids which in turn affect differentiation. In contrast, Ahmed and coworker (1984) report copious excretion of organic acids when *S. venezuelae* was grown on readily utilizable nitrogen sources such as ammonium sulfate, urea and certain amino acids. Not surprising, ammonium ions have also been reported to affect the production of numerous secondary metabolites in different strains (Table 3.4.). For example, cephamycin C production by *S. clavuligerus* was reduced by ammonium in a concentration-dependent manner whether or not other sources of nitrogen, such as asparagine, were present in the medium (Aharonowitz and Demain, 1979).

Table 3.4. Antibiotics produced by actinomycetes whose synthesis is hindered by ammonium salts (adapted from Omura and Tanaka, 1984; Braña and Demain, 1988).

Antibiotic	**Microorganism**	**Reference**
Actinorhodin	*S. coelicolor*	Hobbs et al., 1990; Liao et al., 1995
Anthracylines	*S. peuceticus*	Dekleva et al., 1985
Cephamycin C	*S. cattleya,*	Lilley et al., 1981
	S. clavuligerus,	Aharonowitz and Demain, 1979
	S. lactamdurans	Castro et al., 1985
Chloramphincol	*S. venzuelae*	Shapiro and Vining, 1984
Clavulanic acid	*S. clavuligerus*	Romero et al., 1984
Erythromycin	*Saccharopolysporaerythraea*	Flores and Sanchez, 1985
Gilvocarcin V	*S. arenae*	Byrne and Greenstein, 1986
Leucomycin	*S. kitasatoensis*	Omura et al., 1980a
Lincomycin	*S. lincolnensis*	Young et al., 1985
Milbemycin	*S. hygroscopicus*	Warr et al., 1994
Nanomycin	*S. rosa*	Tanaka et al., 1984
Mourseothricin	*S. noursei*	Gräfe et al., 1977
Novobiocin	*S. niveus*	Kominek, 1972
Rifamycin	*Nocardia mediterranei*	Jiao et al., 1979
Spiramycin	*S. ambofaciens*	Omura et al., 1980b
Tylosin	*S. fradiae*	Masuma et al., 1983

Despite this and the many other examples listed in table 3.4, the effect of ammonium on the production of secondary metabolites varies largely. Depending on the strain, the culture conditions and the experimental procedures used, the effect of ammonium on secondary metabolite formation can range from being inhibitory to stimulating and may very much depend on other growth-limiting factors such as the carbon source, phosphate levels or the availability of trace elements or ammonium trapping reagents (Omura and Tanaka, 1984). Evidence from several studies (reviewed in Braña and Demain, 1988; Hodgson, 2000) indicate that the nitrogen source can influence the formation of the enzymatic machinery for the production of secondary metabolites, and it can reduce the activity of biosynthetic pathways. How this regulation is achieved remains to be determined.

We have used the maltose-glutamate (MG) medium (Doull and Vining, 1989) throughout our

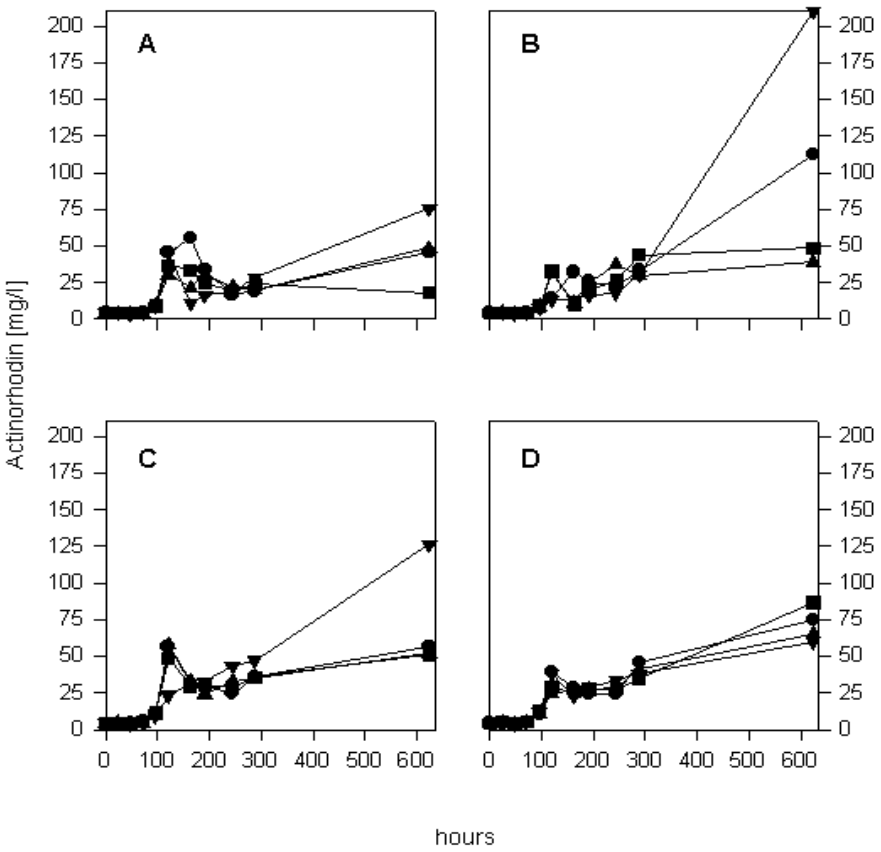


Figure 3.5. *S. coelicolor* WM2303 grown in MG medium (Doull and Vining, 1989). The medium was supplemented with 0, 2.5, 5, 7.6 mM ammonium (circle, square, triangle, inverted triangle) at time zero (panel A), 18.5 (panel B), 36 (panel C) and 48 (panel D) hours into the cultivation. Cultivation conditions are described elsewhere (Minas et al., 2001).

fermentation studies with the *S. coelicolor* A3(2) mutant *S. coelicolor* CH999 (*argA*, *proA*, *redE60*, Δ*act*, SCP1$^-$, SCP2$^-$; Khosla et al., 1992) that does not produce any secondary metabolites. Transforming *S. coelicolor* CH999 with plasmid pIJ2303 encoding the entire *act* cluster of *S. coelicolor* (Malpartida and Hopwood, 1984) resulted in strain WM2303. This strain regained the ability to produce actinorhodin under its natural developmental control. MG medium supports disperse growth and yet ample production of actinorhodin. Interestingly, strain CH999 only grows on the glutamate. The maltose provided with the medium is not utilized. Hence, effects of carbon catabolite repression can be excluded from regulation in these studies. Using glutamate as the sole carbon source results in the release of ammonia into the medium reaching concentrations of 500 to 600 mg/l without affecting Act production. This observations prompted us to evaluate the possibility that increased ammonium concentrations can trigger actinorhodin biosynthesis. Results

summarized in Figure 3.5 show that addition of ammonium can indeed improve Act production. Interestingly, however, timing of the addition is critical as early or late supplementation had no effect. The time at which the culture reacts to the ammonium stimulation correlates with the growth phase that is characterized by the excretion of organic acids (Minas, 2003).

Results of a recent study on yeast (Palková et al., 1997) ascribe a new function to ammonium. It may serve as a small volatile messenger for long-distance cell-cell communication. The authors showed that yeast colonies, grown on amino acid containing medium, use directed pulses of ammonia excretion to signaling colonies nearby. These ammonia pulses mediate the asymetrical growth of two neighboring colonies by causing inhibition of growth of the facing parts of both colonies. If a similar role for ammonium can be attributed to the differentiating colonies of streptomycetes is currently being evaluated.

3.3.5.2 Stringent Response

Closely connected with nitrogen metabolism, especially to the amino acid availability, is the stringent response, the adaptation of the cellular activities to nutrient depletion. Stringent response is characterized by the immediate reduction of the overall rates of RNA and DNA synthesis, a reduction in the rates for synthesis of proteins, carbohydrates, phospholipids, cell wall constituents, and the uptake of low-molecular-weight precursors for these macromolecules. In contrast, stationary phase genes or amino acid biosynthesis genes are induced (Lengeler et al., 1999). Figure 3.6. depicts the molecular events underlying the stringent response. Two genes, *relA* and *spoT* are the key players in the stringent response and the synthesis of alarmoron ppGpp. During amino acid starvation, synthesis of ppGpp is triggered by the absence of charged tRNA at the A site of a ribosome. Binding of ppGpp stalls the ribosome, and the large (50S) subunit of the stalled ribosome binds via the L11 peptide (*rplK*) to the RelA protein (ppGpp synthethase) and triggers the synthesis

of pppGpp.

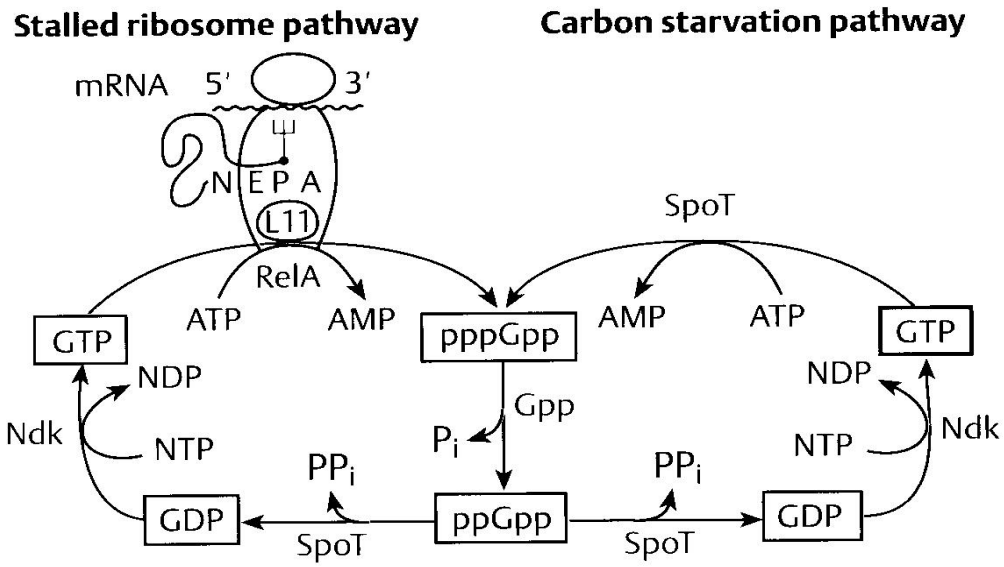


Figure 3.6. Generalized model for (p)ppGpp synthesis and hydrolysis which regulates stringent Response. See text for details (source: Lengeler et al., 1999).

Alternatively, severe starvation for carbon induces a RelA-independent route of ppGpp formation. pppGpp synthesis can be induced through an unknown pathway involving SpoT (ppGpp synthetase II activity). pppGpp is converted by 5'-phosphohydrolase (Gpp) to the active ppGpp alarmoron. The turn over of (p)ppGpp is accomplished by a second activity of SpoT ((p)ppGpp 3'-phosphohydrolase) hydrolyzing ppGpp to GDP. Finally, nukleoside 5'-diphosphate kinase (Ndk) closes the cycle.

In *S. lividans* and *S. coelicolor* a RelA/SpoT homologue (Rel) has been identified (Martínez-Costa et al., 1996/1998). In contrast to the *E. coli* RelA protein, *Streptomyces* Rel is also capable of degrading (p)ppGpp, a function associated with SpoT in *E. coli*, but *Streptomyces* Rel may not be able to catalyze SpoT-like (p)ppGpp synthesis under carbon starvation suggesting that also a SpoT homologue might be present in *Streptomyces* (Martínez-Costa et al., 1998).

Stringent response has been implicated with triggering the initiation of secondary metabolism. An inverse correlation was determined between intracellular accumulation of ppGpp and growth rate (Strauch et al., 1991). A marked ppGpp accumulation occurs in *S. coelicolor* cultures upon nutritional shift-down and entry into stationary phase, which coincides with the detection of

transcripts for biosynthesis genes of the actinorhodin pathway. However, if stringent response is induced by the addition of serine hydroxamate, a competitive inhibitor for seryl-tRNA synthetase, ppGpp levels raise, but cells continue to grow for about two generations and secondary metabolism is initiated only after cells have entered stationary phase (Strauch et al., 1991). A similar correlation between ppGpp accumulation and secondary metabolism was reported for bioalaphos synthesis (*bar*) in *S. hygroscopicus* (Holt et al., 1992). Initiation of secondary metabolism was observed in a short hesitation phase (2 hours) during exponential growth at which the culture underwent dramatic changes as indicated by an altered protein biosynthesis pattern, reduced levels of ATP and GTP, increased ppGpp levels, and activation of *brpA* (regulator of *bar* genes) -controlled genes.

Deletion of *relA* in *S. coelicolor* abolished ribosomal ppGpp synthesis and almost completely abolished Act production while Red biosynthesis was not affected. In contrast, extra copies of *relA* enhanced production of both antibiotics. Overproduction of *actII-orf4*, the actinorhodin pathway-specific regulator (chapter 3.2.1.) in the Rel^- background also restored Act production, a situation that is reminiscent to the AbsA and AbsB phenotype (chapter 3.2.2.2) (Martínez-Costa et al., 1996). In a more recent study using a different genetic background, the *relA* deletion in *S. coelicolor* severely affected growth and morphological development. In addition, neither actinorhodin nor undecylprodigiosin were produced, while CDA synthesis was not affected by lack of stringent response (Chakraburtty and Bibb, 1997). The biosynthesis of Act and RED were both blocked at the level of their respective pathway specific regulators, *actII-orf4* and *redD*. However, these effects were only observed in nitrogen-limited cultures. Both, wild-type and reA^- strain grew normal and produced secondary metabolites under phosphate-limited growth conditions (Chakraburtty and Bibb, 1997; Kang et al., 1998). The authors conclude that an alternative, RelA-independent route for ppGpp synthesis might exist that can activate stringent response under limitations other than nitrogen.

3.3.5.3 GTP-Pool

Closely linked with stringent response is the GTP pool, which in part serves as precursor supply for ppGpp synthesis. The isolation and characterization of relaxed mutants of several *Streptomyces* has led Ochi (1986; 1987a/b; 1988) to propose that morphological differentiation results from a decrease in the GTP pool. Indeed, GTP-binding (G)-proteins in eucaryotes (e.g. the Ras and Ras-like proteins) participate in numerous signal transduction processes such as hormonal regulation of adenylate cyclase (Bourne et al., 1991). Much less is known about G protein-mediated signaling in procaryotes. The best known procaryotic G protein may be Era of *E. coli* (Ahnn et al., 1986; Johnstone et al., 1999). Genes of G proteins similar to Era, were identified in *Streptococcus mutans* (*sgp*, Wu et al., 1995), *Pseudomonas aeroginosa* (*pra*, Chopade et al., 1997), *P. putida* (*flhF*, Pandza et al., 2000) or in *B. subtilis* (*obg*, Welsh et al., 1994). Common to these G proteins is that they are essential for growth and viability, their expression is regulated in response to stress (starvation, heat/cold shock, entry into stationary phase, sporulation), and that they appear to be membrane associated. G proteins can complement each other: SPG can complement a temperature-sensitive *E. coli era* mutant (Era^{ts}) suggesting a common mode of action (Pillutla et al., 1995; Johnstone et al., 1999 and references therein). Era of *E. coli* is the best studied bacterial G protein. It is involved in diverse biological processes: In the temperature-sensitive *E. coli era* mutant, temperature upshift to 42°C, resulted in Era^{ts} depletion, the depression of certain heat shock proteins, an increased utilization of TCA cycle intermediates, increased pyruvate kinase I levels and decreased (p)ppGpp levels (Lerner and Inouye, 1991). Evidence suggests that Era, probably together with RNase III, is involved in RNA metabolism (Johnstone et al., 1999).

G proteins have also been identified in *Streptomyces*. Several, specifically GTP-binding proteins and at least one guanylylated protein have been detected in *S. griseus* (Itoh et al., 1996). One of the GTP-binding proteins has been suggested to be an *obg* homologue which is essential for growth (Okamoto et al., 1997; Okamoto and Ochi, 1998). Decreasing the GTP pool size by addition

of decoyinie, an inhibitor of GTP synthesis, or increasing the Obg content of the cell 2-3 fold by expression of *obg* from a high copy number vector supressed the morphological development only. Secondary metabolism was not affected. The authors suggest that the balance between GTP and Obg could be the signal to trigger cell development. As in *Bacillus* (Vidwans et al., 1995) Obg in its GTP-bound form may stimulate growth and prevent morphological development. In contrast, overexpression of *obg* in *S. coelicolor* resulted also in a reduction of Act production. Red formation seems unaffected (Okamoto and Ochi, 1998). Reduction of the GTP level by decoyinine relieved suppression of morphological development on nutrient rich medium and and supression of Act production in Obg-overexpressing strains.

The current model for Obg action is presented in Figure 3.7. Activated Obg-GTP promotes growth and inhibits development. Starvation toward the end of exponential growth will lower the GTP levels until, after reaching a threshold value, Obg is deacativate to the Obg-GDP form, which

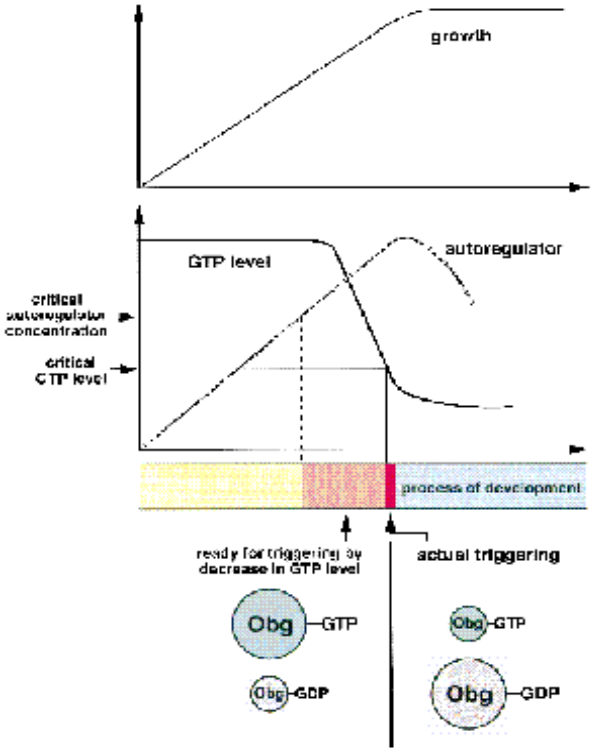


Figure 3.7. Obg-GTP/GDP mediated induction of differentiation according to Okamoto and Ochi (1998): A-factor accumulated in the medium to reach a threshold concentration that renders cells sensitive to sensing the GTP pool size. During starvation GTP levels decrease and Obg-GTP (preventing development) converts into the GDP-bound form triggering development. (source: Okamoto and Ochi, 1998).

will trigger the onset of differentiation processes. A-factor is incorporated into this model as a molecule that with increasing concentration renders the cell more sensitive to receive and to respond to GTP levels (Okamato and Ochi, 1998).

A study by Gaal and coworker (1997) showed that high ATP or GTP concentrations are needed for *E. coli* ribosomal promoters (*rrn* P1) to stabilize the very short-lived complexes with RNA polymerase and to initiate transcription. Six of the *rrn* P1 promoters needed about 10 times more ATP for initiation than a control promoter, while one, *rrnD* P1, was sensitive to GTP concentrations. The authors proposed a model according to which the nutritional state of the *E. coli* cell, reflected by purine NTP pools, controls the rate of rRNA transcription. Transient imbalances between NTP generation (respiration) and consumption (mostly protein synthesis: ATP for charging tRNAs, GTP for tRNA binding to the ribosome) will result in a readjustment of the rRNA synthesis in respect to translation and growth rate (Gaal et al., 1997). This model, however, was contradicted by Petersen and Moller (2000) who found the NTP pool sizes largely invariant with growth rate in the range from 0.5 to 2.3 generations/hour.

Whether the differentiation processes in *Streptomyces* are more directly controlled by NTP levels, GTP in particular, or by ppGpp-mediated responses remains to be determined. Again, the divergence among mutants of different *Streptomyces* strains make a direct comparison difficult. Moreover, effects related to the nitrogen source and its metabolism such as repression by high amonium ion concentration, may not be clearly separated from those of stringent response and GTP pool. The situation becomes even more complicated since other nutrients and metabolites of the primary metabolism and their regulation may distort results further, as examplified by the supression of the *relA*$^-$ mutations under phosphate-limited growth conditions (chapter 3.3.5.2; Chakraburtty and Bibb, 1997; Kang et al., 1999). Assuming that many such interactions may not be known at present, interpretation of developmental phenotypes remains a formidable challenge.

3.3.6 Phosphate and Sulfur

The effect of phosphate concentration on secondary metabolism has been demonstrated in several studies to be highly dependent on the strain, the secondary metabolite and the basic composition of the medium. The production of chlorotetracycline by *S. aureofaciens* and that of oxytetracycline by *S. rimosus* is repressed in the presence of inorganic phosphate (Doskoèil et al., 1959; McDowall et al., 1999). Low initial phosphate concentrations also supported higher Act production in *S. coelicolor* (Liao et al., 1995).

The best characterized phosphate-controlled system is the *pho* system of *E. coli,* which encodes a set of genes required for phosphate uptake (Wanner, 1996 and references therein). Under conditions of low phosphate PhoB, a transcriptional activator similar to the SARPs mentioned in chapter 3.2.1, is phosphorylated by PhoR, a sensor kinase, and then binds to the respective promoters to activate transcription. Interestingly, in the absence of functional PhoR, PhoB can be phosphorylated by other sensor kinases, thus integrating the process of phosphate assimilation into the central pathways for carbon and energy metabolism (Wanner, 1996). The pathway-specific activators for secondary metabolite synthesis in *Streptomyces* (discussed in chapter 3.2.1.) might be, similar to PhoB, susceptible to more than one activation signal. In support of this hypothesis are data from physiological studies on the Act production by *S. coelicolor* (Hobbs et al., 1990). Under carbon-limited growth condition, Act production was completely suppressed by as little as 24 mM phosphate, but Red was still produced at these phosphate concentrations in the medium.

The possibility that sulfur exerts regulatory effects on secondary metabolism has been summarized by Martin and Demain (1980). Mostly induction-like stimulation was observed for cephalosporin C biosynthesis by *Cephalosporium acremonium*. Methionine, a precursor, and norleucine, the non-sulfur analog of methionine, are both inducing cephalosporin C biosynthesis. It remains to be shown, whether a similar regulation by sulfur exists in streptomycetes.

3.4 Other Signals that Trigger Differentiation

A large body of research has accumulated over recent years that describes various parameter that change during growth and that may be implicated in triggering the developmental program in *Streptomyces*. Some of these parameters are discussed in the following paragraphs.

3.4.1 cAMP

Cyclic AMP (cAMP) is one of the most common low-molecular-weight signaling molecules in both eucaryotic and procaryotic microbes. It can serve either as an intracellular or as an excreted intercellular signaling molecule. In *E. coli*, cAMP is known to relieve glucose repression (see chapter 3.3.2.). Interestingly, about 98% of the cAMP is found in the medium, yet the biological significance of this phenomenon in unknown (Crenon and Ullmann, 1984). Increased cAMP levels have been reported to enhance morphological development and the production of secondary metabolites in *Streptomyces*. For example, mycelial growth in *S. hygroscopicus* appears to be cAMP-regulated by increasing the rate of synthesis of protein, DNA, RNA, and by reversing the phosphate-induced inhibition of turimycin biosynthesis (Gersch, 1979). Similar effects were reported for *S. alboniger* during morphogenesis (Surowitz and Pfister, 1985b). In *S. fradiae*, the addition of chloroquine, a drug that stimulates adenylate cyclase activity and hence increases cAMP level, has been shown to significantly increase the rate of synthesis of tylosin and the cellular level of DNA (Colombo et al., 1982; Tata and Menawat, 1994). But again, the observed effects greatly depend on the nutrients provided with the medium.

Stimulation of secondary metabolism could result from increased levels of cAMP relieving repression of enzymes and pathways. A more detailed study on effects of cAMP in *S. coelicolor* showed that cAMP levels peak during germination and later when aerial mycelium and actinorhodin are being produced (Süsstrunk et al., 1998). Spores of the adenylate cyclase (*cya*) deficient mutant

BZ1 had a delayed germ-tube emergence and an overall very low germination efficiency. Further defects in later development and Act production were result of an irreversible acidification of the medium that, however, could be reversed by addition of exogenous cAMP or by growing the mutant in close proximity of a wild-type colony (Süsstrunk et al., 1998). Bld mutants (chapter 3.1) that also irreversibly acidify the medium could not be rescued with exogenous cAMP. Interestingly, exogenous cAMP repressed the production of Red in *S. coelicolor*. In an earlier study Redshaw and coworker (1976) showed that in *S. alboniger* the addition of adenine rather than cAMP reduced acid production of glucose-grown cultures which otherwise repress aerial mycelium formation (see chapter 3.3.3.).

A recent study with *S. griseus* (Kang et al., 1999) shows that exogenous addition of cAMP to wild-type strain had no effect on differentiation, however, that addition of moderate amounts of exogenous cAMP (5 µmol) to an A-factor deficient and *arpA* defective mutant resulted in restored aerial mycel and streptomycin formation. In contrast, overexpression of the adenylate cyclase (CyaA) or addition of high concentrations of exogenous cAMP (10-15 µmol) suppressed aerial mycel and antibiotic formation as well as tyrosine phosphorylation.

Our current knowledge suggests that in *Streptomyces* cAMP is implicated in switching metabolic routes in response to changing requirements during germination and later during differentiation (Süsstrunk et al., 1998).

3.4.2 Redox

Several examples for redox-dependent gene expression have been found in bacteria. Recent examples for redox-dependent gene expression include that of the dimethyl sulfoxyde reductase (*dor*) (Mouncey and Kaplan, 1998a/b) and the cbb3 cytochrome oxidase induction under limited-oxygen conditions in *Rhodobacter spheroides* (Mouncey and Kaplan, 1998c). There are reports about similar oxygen or redox-sensing circuits that govern gene expression in streptomycetes (Zou

et al., 1999) yet it remains to be shown whether this redox potential does influence differentiation and secondary metabolism. Such a system might, for example, respond to the changed demand on reduced cofactors for polyketide biosynthesis.

3.4.3 Stress - Osmotic / Heat Shock / Other

Analysis of heat shock response throughout the growth of a differentiating culture of *S. coelicolor* showed that each growth phase has a specific heat shock response, demonstrating that cells are in distinct physiological stages. In addition, during the growth phase in which actinorhodin production starts many heat shock proteins (HSP) could be induced either developmentally or by thermal stress, implying that induction of these stress regulons share common control elements (Puglia et al., 1995). Furthermore, a more detailed study in this strain on the effect of stress induced by heat, cold, salt, ethanol, or antibiotic shock revealed that each stress condition invoked a distinct set of HSPs, suggesting an independent control by the different physiological stresses (Vohradsky et al., 2000). A model for stress response in *Streptomyces coelicolor* has been proposed (Figure 3.8.): Stress stimulons contain unique nonoverlapping groups of proteins (Stimulons), presumably reflecting the role of independent regulators. Subsets of heat, salt, and cold shock proteins had similar patterns of synthesis as a function of development, suggesting that corresponding stress regulators were functional during growth. In addition, the observation that many members of two different stress stimulons (heat and salt shock) had related patterns of synthesis during development suggested that their corresponding stress-specific regulators were under the control of a common signal from a higher-level regulator. The existence of this regulatory cascade involving stress stimulons suggests that corresponding regulatory systems may play a role in mediating developmental switches. There may also be alternative developmental pathways that bypass these stress circuits. The proposed common regulatory elements are still unknown, as are the reasons why some stress responses are more similar to the developmentally induced responses than others.

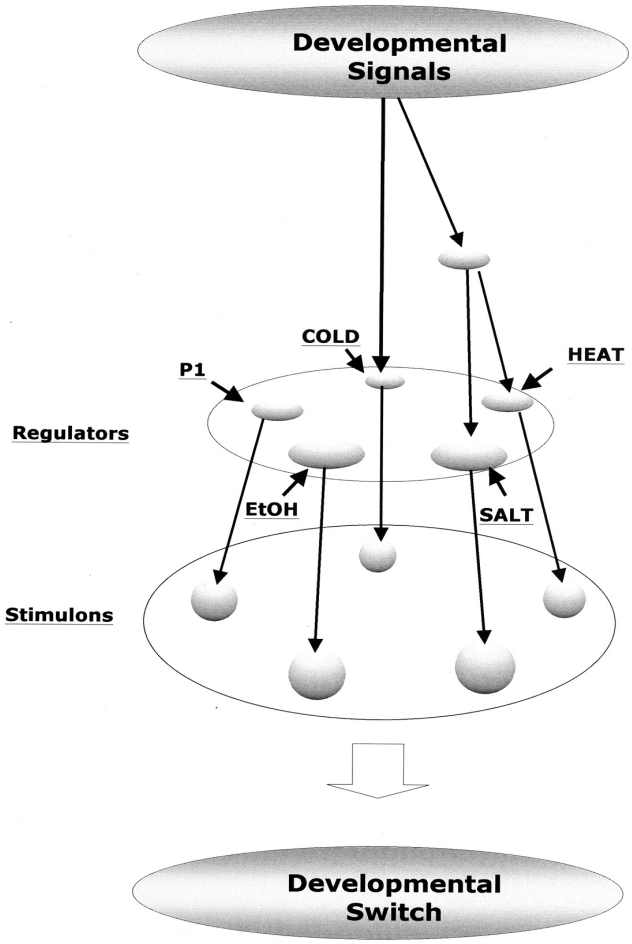


Figure 3.8. The model for an integrated network of developmental and stress-induced proteins in *S. coelicolor*. 2D gel patterns indicated that *S. coelicolor* cultures underwent a developmental program involving four stages. Developmental signals controlled the family of proteins which were also induced by stress treatments (heat, NaCl, cold, ethanol [EtoH], or P1 shock) (short arrows). See text for details. (source: Vohradsky et al., 2000)

A gene, *sgaA* (**s**upression of **g**rowth disturbance caused by **A**-factor at high concentration and high osmolarity during early growth), has recently been identified in *S. griseus*. *sgaA* is required for normal growth at high osmolarity (Ando et al., 1997b). Strain HH1 carries a large deletion that includs *afsA* (A-factor synthesis). HH1 requires A-factor for normal morphological and physiological development, supplied either as addition to the medium or by cloning and expression of *afsA* gene. Growth defects were observed when this strain was grown on media containing high concentrations of sucrose, sorbitol, mannitol, KCl, or NaCl, which led to the identification of *sgaA*. These growth disturbances were not detected in ArpA muntants, suggesting that ArpA may mediate the effects of osmolarity. Interestingly, even though A-factor in *S. griseus* controls both,

morphological and physiological development, no alterations in streptomycin production were observed in response to A-factor supplementation on strain HH1 grown on high osmolarity medium (Ando et al., 1997b). The molecular function of SgaA remains to be elucidated.

3.4.4 Post-Transcriptional Regulation of Differentiation

Two genes, encoding for RNases have been mentioned earlier. OrnA of *S. griseus* represents a AdpA-dependently expressed oligoribonuclease (chapter 3.2.2.1) that is needed for vegetative growth and the initiation of differentiation (Ohnishi et al., 2000), while AbsB, identified in *S. coelicolor*, represents an RNase III homologue that may be regulating RNA translation (chapter 3.2.2.2; Price et al., 1999).

The ribosome itself can be implicated in regulation at the level of translation. For example, *S. lividans* TK21 is impaired in the production of secondary metabolites. The steptomycin-resistant mutant strain, TK24, however, is capable of producing actinorhodin. Genetic analysis revealed that a point mutation (*str-6*) in the *rpsL* gene, encoding the ribosomal protein S12, changing Lys^{88} to Glu, had rendered the strain streptomycin resistant and conferred the ability to make actinorhodin (Shima et al., 1996b). In addition, this mutation also suppressed the $RelA^-$ phenotype. The authors suggest that binding of streptomycin changes the ribosome structure which induces translation rather than on misreading of mRNA codons, a well characterized effect of streptomycin (Ruusala and Kurland, 1984).

4. Biotechnological Aspects of Secondary Metabolite Production

Biotech and pharma industries struggle to find new bioactive molecules to treat an increasing number of multidrug-resistant pathogens or to find treatments for diseases for which adequate

treatment is not yet available (e.g. Huang et al., 1999). The classical routes are based on either the isolation of new organisms (for review Hunter-Cevera and Belt, 1999) and to analyze their products, or on semi-synthetic drugs based on biologically produced synthons that are further modified chemically. However, in recent years a paradigm shift is taking place from the traditional approaches to new approaches that are driven by the advances in the field of bioinformatics. Databases for DNA, RNA and proteins enable reverse genetics approaches for the isolation of genes encoding interesting functions, for the identification of new targets for drug screening and design, and for studying gene expression and physiology. Databases such as EcoCyc (ecocyc.PangeaSystems.com/ecocyc/ecocyc.html) or KEGG (www.genome.ad.jp/kegg/) can provide information for putative pathways in secondary metabolism that can lead to improved bioprocesses. Several examples are discussed in more detail in a recent review by Bull and coauthors (2000).

Another approach, that in part complements the classical strategies, is to redesign pathways for the production of secondary metabolites in order to generate novel compounds *in vivo*. The tool for genetic engineering approaches are available (Baltz and Hosted, 1996). While this approach has been demonstrated to work for aromatic polyketides, macrolides, and non-ribosomal peptide antibiotics, exemplified by the work by Khosla and coworker (McDaniel et al., 1993, Fu et al., 1996), (McDaniel et al., 1999), and other groups (Schauwecker et al., 2000; Mootz et al., 2000), respectively, a pharmacologically useful compound designed by these technologies has yet to emerge.

In respect to new natural isolates, major obstacles are encountered that include the normally very low titers of secondary metabolites and the production of more than one secondary metabolite. Hence, this route of discovery largely depends on massive parallel cultivations and traditional strain improvement programs (for review, Vinci and Byng, 1999). In the first steps, it is necessary to gain insight into the physiology of production and to obtain enough material for initial tests and structure determination. Modern molecular biology is needed to supply the genetic information on the biosynthetic pathways, which will open up the possibility to modify the primary metabolism as

needed. Alternatively, potential precursor, for example propanol or propionate in case of the erythromycin production, may by supplied (R. Schwartz, personal communication; Minas et al., 1998). An example for an altered precursor supply and its effect on secondary metabolism, is the production of avermectins by *S. avermitilis* (Denoya et al., 1995). Deleting the *bkdF* gene (brached-chain α-keto acid dehydrogenase, BCDH) rendered the strain unable to grow on medium containing isoleucine, leucine, and valine as sole carbon source. Furthermore, since BCDH provides the α–branched chain fatty acid starter units for avermectin biosynthesis, the mutant strain was unable to produce the natural avermectins unless S(+)-α-methylbutyrate, and isobutyrate were supplied. Supplementing the medium with either or both of natural precursors for the starter units, restored the production of the respective avermectin(s), supplementing the medium with alternative fatty acids yielded novel molecules (Denoya et al., 1995).

As discussed earlier, the production of secondary metabolites also depends on the general composition of the medium. Cultivation parameters such as pH, dissolved oxygen tension and others may alter the product specifications made (Bystrykh et al., 1996; Clark et al., 1995; Wilson and Bushell, 1995).

Several routine approaches have been developed for process optimizations including the downstream processing (for review see Strobel and Sullivan, 1999; Dahod, 1999). Determination of the proper balance between, and sources of C, N, and P is the starting point, but additional bottlenecks may be encountered during scale-up. Oxygen saturation and oxygen transfer are critical parameter in large scale fermentations, especially as production of most secondary metabolites requires sufficient or even controlled oxygen saturation throughout the cultivation (Okabe et al., 1992; Yegneswaran et al., 1991), which is particular difficult to achieve when cell pellets are formed and the culture viscosity is high. On the process site, reactor and stirrer design can help to overcome this problem (Roman and Gavrilescu, 1994), but the biological system may need adaptation as well. We have recently successfully utilized a genetic approach to increase oxygen

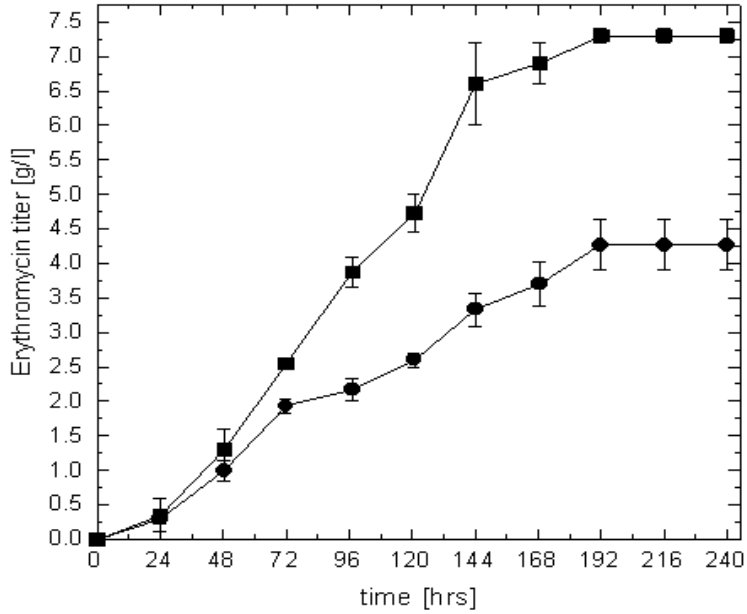


Figure 4.1. Erythromycin production by *Saccharopolyspora erythraea* in 20-L scale in complex production medium. Only the production phase is presented. Means from replicates of three cultivations of the original strain (circles) and two of the *vhb*-expressing recombinant strain (squares) are represented (source: Minas et al., 1998).

availability to the cells in an oxygen-demanding industrial bioprocess. The bacterial hemoglobin gene from *Vitreoscilla* (*vhb*), which has previously been shown to enhance protein production and growth under oxygen-limited conditions (Khosla and Bailey, 1988), was cloned into an industrial strain of *Saccharopolyspora erythraea*, the producer of erythromycin (Brünker et al., 1998; Minas et al., 1998). Compared to the original strain without the hemoglobin gene, the recombinant, *vhb*-expressing strain had an over 70% increased erythromycin titer (7.5 g/l). Furthermore, the production was faster increasing the space-time yield over 100% (Figure 4.1). Opimizing the culture conditions further increased titers to about 8.5 g/l. Interestingly, the genetic manipulation of the strain did not alter the ratio of erythromycin A:B:C (Minas, unpublished results).

By now, genetic manipulation of actinomycetes has become a routine in many labs. However, genetic engineering of industrial strains is much more difficult in many respects and often limited to self-cloning to avoid the legal hurdles in dealing with genetically modified organisms (GMO) in large scale and avoid the expenses associated with the need for declarations of a products that is derived from a GMO. Nevertheless, targeted genetic manipulations can help speeding up the

development of an industrial strain and help to reduce production costs.

The fast diversity of potentially useful, mostly secondary, metabolites made by actinomycetes was mentioned in the beginning. Even though great expectations were placed on combinatorial chemistry and recombinant DNA technology, success has largely been limited to the areas of recombinant therapeutic antibodies and other small peptides. More promising are approaches for a targeted and very specific *in vitro* or *in vivo* modifications of a natural product. Nevertheless, the isolation of novel natural products and pathways that will feed into the pipelines for modifications will remain important.

Based on our current knowledge, the great diversity in secondary metabolites made is accompanied by numerous species specific regulatory systems that control primary and secondary metabolism. As of writing this discussion, no generalized rules have been developed that would allow to predict an optimized production process, but as more data become available and the genetic analysis of the genes and loci described progresses, a better educated guess about parameters that regulate production of secondary metabolites will be possible.

5. Conclusions and Perspectives

After reviewing the current state of the regulatory aspects in actinomycetes, it became clear that due to the complexity of the regulatory networks and the differences between even relatively closely related strains, no general model can be developed so far. Already regulation one level above the pathway specific activation of secondary metabolism is largely unknown. An attempt to combine the more general regulatory interactions between primary and secondary metabolism and stress responses in a graphic representation is shown in Figure 5.1. Looking at this network, it will be of great importance to find some central nodes that integrate the various potential signals sensing environmental factors and nutritional states of the cell. AdpA may be one such node that receives a

signal, A-factor, and sends out a signal that activates several cellular responses needed for morphological and physiological differentiation (chapter 3.2.2.1.). But the reverse must exist too, a receiver that can be triggered by different signals, individually or in combination, and then invoking always the same response, such as switching on a pathway for secondary metabolites. PhoB, the regulator of the *E. coli* phosphate uptake system, represents such a node (chapter 3.3.6.). Some pathway specific regulators (chapter 3.2.2.2.) may also possess such qualities.

Related to the regulation of secondary metabolism is the question of the function of secondary metabolites in nature. Removal of surplus primary metabolites, e.g. proline into undecylprodigiosine, or the role in defending the mycelium against attacks by other organisms have been discussed. Spore pigments may protect against light induced damage, while other metabolites may play a role in signaling presence of symbiotic partners (Demain, 1989). These information should help us understanding extracellular aspects of regulation that are not obviously related to nutrition.

Any regulatory network for cell development will remain incomplete if the networks from both, the primary and the secondary metabolism are not cointegrated. Currently only fragments of various signaling pathways have been elucidated, best understood is the A-factor mediated regulation of differentiation in *S. griseus* (chapter 3.2.2.1.), but parallel routes controlling morphogenesis exist (*ram* and *amf*, chapter 3.2.2.2.) that have not been integrated into the models. For most of the well established *bld*-signal cascade, the genetic and/or molecular mechanism is unknown and yet less is known about ligands and targets of the numerous sensor kinases and response regulator pairs and other genes.

In addition, our current understanding of the primary metabolism of actinomycetes is similarly rudimentary. Regulation of only a limited number of key enzymes has been studied and the data available hardly suffice to deduce a regulatory network for the main routes of the central metabolism.

However, it can be expected that the DNA sequence information derived from the *S.*

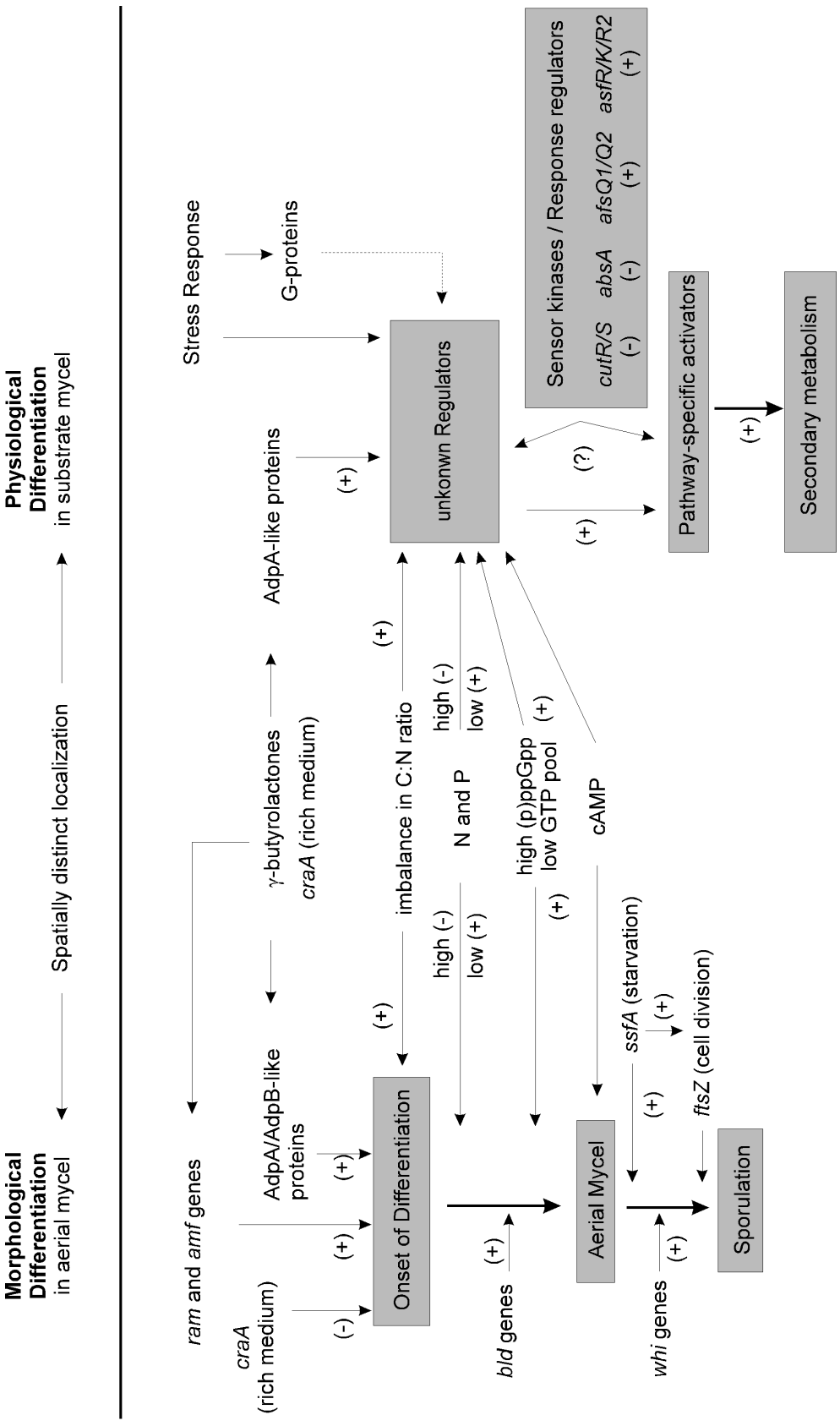


Figure 5.1. Graphical overview of the known and more general regulatory signals and pathways that are involved in the differentiation process in *Streptomyces*. (+) and (-) indicate positive and negative regulation, plain arrows indicate required components, dashed arrows hypothetical signals

coelicolor sequencing project will provide some long awaited answers. With the DNA sequence available, a new era for *Streptomyces* research will commence, in which one will have to revoke the traditional way of looking at *Bacillus* to find answers on newly discovered genes in *Streptomyces*. Already many examples exist in which genes of a *Streptomyces* were more similar to homologes found in eucaryotes than to those found in bacteria; some of the two-component systems are one example. It will also be important to remember that actinomycetes are soil inhabiting saprophytic organisms that are exposed to foreign DNA of diverse origin which may be acquired and integrated into their genome. This might explain and/or provide further insight into their diversity.

In addition to the genetic information, more detailed physiological studies are needed. Even though the current paradigm for the cell entering differentiation is an imbalance in nutrient supply (C:N:P ratio), the involvement of regulation by stringent response, phosphate and/or nitrogen remains largely circumstantial. It would be particularly useful if studies were conducted under conditions where distinct effects can be studied. For example, phosphate, ammonium and glucose have pronounced effects on gene expression, mediate catabolite repression and influence secondary metabolism. Eliminating the effect of one or better two components would permit a better understanding of regulation by the other(s). It would certainly help if strains of a similar genetic background could be used. Currently, a wide range of fairly diverse strains are analyzed for their metabolic and regulatory properties, which certainly slows down progress in understanding the global aspects on metabolism and regulation.

For our contribution to the ongoing efforts to understand regulatory aspects of secondary metabolism, we use a strain, *S. coelicolor* WM2303, derived from strain CH999, and a chemically defined medium that permit a detailed analysis of the broth to monitor changes in the environment and to analyze Act production. A protocol for reproducible cultivations, even in a micro-scale (Minas et al., 2001) has been developed for parallel cultivations evaluating putative trigger signals. Since glutamate is used as sole source of carbon and nitrogen, we largely avoid the combination of effects from glucose, ammonium, and phosphate repression.

With the *S. coelicolor* genome sequenced, it remains to be seen, if ORFmer arrays will become available for transcriptom analysis which would certainly be a great asset for future studies.

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7. References

Aceti, D. J., and W. C. Champness. 1998. Transcriptional regulation of *Streptomyces coelicolor* pathway-specific antibiotic regulators by the *absA* and *absB* loci. J Bacteriol **180**(12)**:**3100-3106.

Aharonowitz, Y., and A. L. Demain. 1979. Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus*. Can J Microbiol **25**(1)**:**61-67.

Ahmed, Z. U., S. Shapiro, and L. C. Vining. 1984. Excretion of α-keto acids by strains of *Streptomyces venezuelae*. Can J Microbiol **30:**1014-1021.

Ahnn, J., P. E. March, H. E. Takiff, and M. Inouye. 1986. A GTP-binding protein of *Escherichia coli* has homology to yeast RAS proteins. Proc Natl Acad Sci U S A **83**(23)**:**8849-8853.

Aida, K., K. Chiabata, K. Nakayama, K. Takinami, and H. Yamada. 1986. Biotechnology of amino acid production. Elsevier, Amsterdam.

Aínsa, J. A., H. D. Parry, and K. F. Chater. 1999. A response regulator-like protein that functions at an intermediate stage of sporulation in *Streptomyces coelicolor* A3(2). Mol Microbiol **34**(3)**:**607-619.

Aínsa, J. A., N. J. Ryding, N. Hartley, K. C. Findlay, C. J. Bruton, and K. F. Chater. 2000. WhiA, a protein of unknown function conserved among gram-positive bacteria, is essential for sporulation in *Streptomyces coelicolor* A3(2). J Bacteriol **182**(19)**:**5470-5478.

Alexander, D. C., and S. E. Jensen. 1998. Investigation of the *Streptomyces clavuligerus* cephamycin C gene cluster and its regulation by the CcaR protein. J Bacteriol **180**(16)**:**4068-4079.

Alves, A. M., W. G. Meijer, J. W. Vrijbloed, and L. Dijkhuizen. 1996. Characterization and phylogeny of the *pfp* gene of *Amycolatopsis methanolica* encoding PP_i-dependent phosphofructokinase. J Bacteriol **178**(1)**:**149-155.

Alves, A. M., G. J. Euverink, M. J. Bibb, and L. Dijkhuizen. 1997. Identification of ATP-dependent phosphofructokinase as a regulatory step in the glycolytic pathway of the actinomycete *Streptomyces coelicolor* A3(2). Appl Environ Microbiol **63**(3)**:**956-961.

Anderson, T., P. Brian, P. Riggle, R. Kong, and W. Champness. 1999. Genetic suppression analysis of non-antibiotic-producing mutants of the *Streptomyces coelicolor absA* locus. Microbiology **145**(Pt 9)**:**2343-2353.

Ando, N., N. Matsumori, S. Sakuda, T. Beppu, and S. Horinouchi. 1997a. Involvement of *afsA* in A-factor biosynthesis as a key enzyme. J Antibiot **50**(10)**:**847-852.

Ando, N., K. Ueda, and S. Horinouchi. 1997b. A *Streptomyces griseus* gene (*sgaA*) suppresses the growth disturbance caused by high osmolality and a high concentration of A-factor during early growth. Microbiology **143**(Pt 8)**:**2715-2723.

Angell, S., C. G. Lewis, M. J. Buttner, and M. J. Bibb. 1994. Glucose repression in *Streptomyces coelicolor* A3(2): a likely regulatory role for glucose kinase. Mol Gen Genet **244**(2)**:**135-143.

Anisova, L. N., I. N. Blinova, O. V. Efremenkova, P. Koz'min Iu, and V. V. Onoprienko. 1984. Regulators of development in Streptomyces coelicolor A3(2). Izv Akad Nauk SSSR [Biol](1)**:**98-108.

Anzai, H., T. Murakami, S. Imai, A. Satoh, K. Nagaoka, and C. J. Thompson. 1987. Transcriptional regulation of bialaphos biosynthesis in *Streptomyces hygroscopicus*. J Bacteriol **169**(8)**:**3482-3488.

Arias, P., M. A. Fernández-Moreno, and F. Malpartida. 1999. Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. J Bacteriol **181**(22)**:**6958-6968.

Babcock, M. J., and K. E. Kendrick. 1990. Transcriptional and translational features of a

sporulation gene of *Streptomyces griseus*. Gene **95**(1)**:**57-63.

Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. Salmond, G. S. Stewart, and P. Williams. 1992. *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem J **288**(Pt 3)**:**997-1004.

Baltz, R. H., and T. J. Hosted. 1996. Molecular genetic methods for improving secondary-metabolite production in actinomycetes. Trends Biotechnol **14**(7)**:**245-250.

Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Multiple signalling systems controlling expression of luminescence in Vibrio harveyi: sequence and function of genes encoding a second sensory pathway. Mol Microbiol **13**(2)**:**273-286.

Beall, B., and J. Lutkenhaus. 1991. FtsZ in *Bacillus subtilis* is required for vegetative septation and for asymmetric septation during sporulation. Genes Dev **5**(3)**:**447-455.

Bibb, M. J., V. Molle, and M. J. Buttner. 2000. σ^{BldN}, an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2). J Bacteriol **182**(16)**:**4606-4616.

Birch, A., A. Hausler, and R. Hutter. 1990. Genome rearrangement and genetic instability in *Streptomyces* spp. J Bacteriol **172**(8)**:**4138-4142.

Birkó, Z., A. Sumegi, A. Vinnai, G. van Wezel, F. Szeszák, S. Vitális, P. T. Szabó, Z. Kele, T. Janáky, and S. Biró. 1999. Characterization of the gene for factor C, an extracellular signal protein involved in morphological differentiation of *Streptomyces griseus*. Microbiology **145**(Pt 9)**:**2245-2253.

Bormann, E. J., and R. Herrmann. 1968. Zur Pyruvat- und α-Ketoglutaratausscheidung durch *Streptomyces rimosus*. Arch Mikrobiol **63**(1)**:**41-52.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349**(6305)**:**117-127.

Braña, A. F., C. Méndez, L. A. Díaz, M. B. Manzanal, and C. Hardisson. 1986. Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus*. J Gen Microbiol **132**(Pt 5)**:**1319-1326.

Braña, A. F., and A. L. Demain. 1988. Nitrogen control of antibiotic biosynthesis in actinomycetes, p. 99-119. *In* S.-E. S. (ed.), Nitrogen source control of microbial processes. CRC Press, Boca Raton, FL.

Brawner, M. E., S. G. Mattern, M. J. Babcock, and J. Westpheling. 1997. The *Streptomyces galP1* promoter has a novel RNA polymerase recognition sequence and is transcribed by a new form of RNA polymerase in vitro. J Bacteriol **179**(10)**:**3222-3231.

Brian, P., P. J. Riggle, R. A. Santos, and W. C. Champness. 1996. Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative signal transduction system. J Bacteriol **178**(11)**:**3221-3231.

Brown, D., M. J. Hitchcock, and E. Katz. 1986. Purification and characterization of kynurenine formamidase activities from *Streptomyces parvulus*. Can J Microbiol **32**(6)**:**465-472.

Bruton, C. J., K. A. Plaskitt, and K. F. Chater. 1995. Tissue-specific glycogen branching isoenzymes in a multicellular prokaryote, *Streptomyces coelicolor* A3(2). Mol Microbiol **18**(1)**:**89-99.

Brünker, P., W. Minas, P. T. Kallio, and J. E. Bailey. 1998. Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the *Vitreoscilla* haemoglobin gene (*vhb*). Microbiology **144**(Pt 9)**:**2441-2448.

Bu'Lock. 1961. Intermediary metabolism and antibiotic synthesis. Adv Appl Microbiol **3:**293-342.

Bull, A. T., A. C. Ward, and M. Goodfellow. 2000. Search and discovery strategies for biotechnology: the paradigm shift. Microbiol Mol Biol Rev **64**(3)**:**573-606.

Burbulys, D., K. A. Trach, and J. A. Hoch. 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64**(3)**:**545-552.

Buttner, M. J., A. M. Smith, and M. J. Bibb. 1988. At least three different RNA polymerase

holoenzymes direct transcription of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2). Cell **52**(4)**:**599-607.

Byrne, K. M., and M. Greenstein. 1986. Nitrogen repression of gilvocarcin V production in *Streptomyces arenae* 2064. J Antibiot **39**(4)**:**594-600.

Bystrykh, L. V., M. A. Fernández-Moreno, J. K. Herrema, F. Malpartida, D. A. Hopwood, and L. Dijkhuizen. 1996. Production of actinorhodin-related "blue pigments" by *Streptomyces coelicolor* A3(2). J Bacteriol **178**(8)**:**2238-2244.

Castro, J. M., P. Liras, J. Cortés, and J. F. Martin. 1985. Regulation of α-aminoadipyl-cysteinyl-valine, isopenicillin N synthase, isopenicillin N isomerase, and deacetoxycephalosporin C synthase by nitrogen sources in *Streptomyces lactamdurans*. Appl Microliol Biotechnol **21:**32-.

Chakraburtty, R., and M. Bibb. 1997. The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. J Bacteriol **179**(18)**:**5854-5861.

Champness, W. C. 1988. New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. J Bacteriol **170**(3)**:**1168-1174.

Champness, W. 1999. Cloning and Aanalysis of regulatory genes involved in streptomycete secondary metabolite biosynthesis, p. 725-739. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.

Champness, W. C., and K. F. Chater. 1994. Regulation and integration of antibiotic production and morphological differentiation in *Streptomyces* spp., p. 61-93. *In* P. Piggot, C. P. Moran jr., and P. Youngman (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D.C.

Champness, W., P. Riggle, T. Adamidis, and P. Vandervere. 1992. Identification of *Streptomyces coelicolor* genes involved in regulation of antibiotic synthesis. Gene **115**(1-2)**:**55-60.

Chang, P. C., and S. N. Cohen. 1994. Bidirectional replication from an internal origin in a linear streptomyces plasmid. Science **265**(5174)**:**952-954.

Chang, H. M., M. Y. Chen, Y. T. Shieh, M. J. Bibb, and C. W. Chen. 1996. The *cutRS* signal transduction system of *Streptomyces lividans* represses the biosynthesis of the polyketide antibiotic actinorhodin. Mol Microbiol **21**(5)**:**1075-1085.

Chater, K. F. 1972. A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor*. J Gen Microbiol **72**(1)**:**9-28.

Chater, K. F. 1975. Construction and phenotypes of double sporulation deficient mutants in *Streptomyces coelicolor* A3(2). J Gen Microbiol **87**(2)**:**312-325.

Chater, K. F. 1989a. Multilevel regulation of *Streptomyces* differentiation. Trends Genet **5**(11)**:**372-377.

Chater, K. F. 1989b. Aspects of multicellular Differentiation in *Streptomyces coelicolor* A3(2), p. 99-107. *In* C. L. Hershberger, S. W. Queener, and Hegemann (ed.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, D.C.

Chater, K. F. 1992. *Streptomyces coelicolor*: a mycelial, spore-bearing procaryote, p. 61-74. *In* V. A. Russo, S. Brody, D. Cove, and S. Ottolenghi (ed.), Molecular genetics of development. Blackwell, Oxford.

Chater, K. F. 1993. Genetics of differentiation in *Streptomyces*. Annu Rev Microbiol **47:**685-713.

Chater, K. F., and C. J. Bruton. 1985. Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. Embo J **4**(7)**:**1893-1897.

Chater, K. F., and D. A. Hopwood. 1993. Streptomyces, p. 83-99. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), Bacillus subtilis and other gram-positive bacteria. American

Society for Microbiology, Washington, D.C.
Chater, K. F., and R. Losick. 1996. The mycelial-life style of *Streptomyces coelicolor*A3(2) and its relatives, p. 149-182. *In* H. J. Shapiro and M. Dworkin (ed.), Bacterial as multicellular organisms. Oxford university Press, New York.
Chater, K. F., and M. J. Merrick. 1979. *Streptomyces*, p. 93-114. *In* J. H. Parish (ed.), Developmental biology of procaryotes. Blackwell, Oxford.
Chater, K. F., C. J. Bruton, K. A. Plaskitt, M. J. Buttner, C. Mendez, and J. D. Helmann. 1989. The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*. Cell **59**(1)**:**133-143.
Chatterjee, S., and L. C. Vining. 1981. Nutrient utilization in actinomycetes. Induction of α-glucosidases in *Streptomyces venezuelae*. Can J Microbiol **27**(7)**:**639-645.
Chatterjee, S., and L. C. Vining. 1982. Catabolite repression in *Streptomyces venezuelae*. Induction of β- galactosidase, chloramphenicol production, and intracellular cyclic adenosine 3',5'-monophosphate concentrations. Can J Microbiol **28**(3)**:**311-317.
Chopade, B. A., S. Shankar, G. W. Sundin, S. Mukhopadhyay, and A. M. Chakrabarty. 1997. Characterization of membrane-associated *Pseudomonas aeruginosa* Ras-like protein Pra, a GTP-binding protein that forms complexes with truncated nucleoside diphosphate kinase and pyruvate kinase to modulate GTP synthesis. J Bacteriol **179**(7)**:**2181-2188.
Clark, G. J., D. Langley, and M. E. Bushell. 1995. Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor culture. Microbiol **141:**663-669.
Cochrane, V. W. 1961. Physiology of actinomycetes. Ann Rev Microbiol **15:**1-26.
Colombo, A. C., N. Crespi-Perellino, and S. Micalizio. 1982. Relationships between growth, cyclic AMP and tylosin production in two mutants of *Streptomyces fradiea*. Biotechnol Lett **4:**447-452.
Court, D. 1993. RNA processing and degradation by RNase III, p. 71-116. *In* J. G. Belasco and G. Brawerman (ed.), Control of messenger RNA stability. Academic Press, San Diego.
Crenon, I., and A. Ullmann. 1984. The role of cyclic AMP excretion in the regulation of enzymesynthesis in *Escherichia coli*. FEMS Microbiol Lett **22:**47-51.
Cullum, J., F. Flett, and W. Piendl. 1988. Genetic instability in streptomycetes. Microbiol Sci **5**(8)**:**233-235.
Dahod, S. K. 1999. Raw material selection and medium development for industrial fermentation processes, p. 213-220. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.
Dai, K., and J. Lutkenhaus. 1991. *ftsZ* is an essential cell division gene in *Escherichia coli*. J Bacteriol **173**(11)**:**3500-3506.
Davis, N. K., and K. F. Chater. 1992. The *Streptomyces coelicolor whiB* gene encodes a small transcription factor-like protein dispensable for growth but essential for sporulation. Mol Gen Genet **232**(3)**:**351-358.
Dekleva, M. L., J. A. Titus, and W. R. Strohl. 1985. Nutrient effects on anthracycline production by *Streptomyces peucetius* in a defined medium. Can J Microbiol **31**(3)**:**287-294.
Delic, I., P. Robbins, and J. Westpheling. 1992. Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. Proc Natl Acad Sci U S A **89**(5)**:**1885-1889.
Demain, A. L. 1989. Function of secondary metabolites, p. 1-11. *In* C. L. Hershberger, S. W. Queener, and Hegemann (ed.), Genetics and molecular biology of industrial microorganisms. American Society for Microbiology, Washington, D.C.
Denoya, C. D., R. W. Fedechko, E. W. Hafner, H. A. McArthur, M. R. Morgenstern, D. D. Skinner, K. Stutzman-Engwall, R. G. Wax, and W. C. Wernau. 1995. A second branched-chain alpha-keto acid dehydrogenase gene cluster (*bkdFGH*) from *Streptomyces*

avermitilis: its relationship to avermectin biosynthesis and the construction of a *bkdF* mutant suitable for the production of novel antiparasitic avermectins. J Bacteriol **177**(12)**:**3504-3511.

Dharmatilake, A. J., and K. E. Kendrick. 1994. Expression of the division-controlling gene *ftsZ* during growth and sporulation of the filamentous bacterium *Streptomyces griseus*. Gene **147**(1)**:**21-28.

Donadio, S., K. Mansouri, M. McAlpine, P. J. Staver, P. J. Sheldon, M. Jackson, S. J. Swanson, E. Wendt-Pienkowski, W. Yi-Guang, B. Jarvis, C. R. Hutchinson, and L. Katz. 1993. Recent developments in the genetics of erythromycin formation, p. 257-265. *In* R. H. Baltz, Hegeman, G.D. and Skatrud, P.L. (eds.) (ed.), Industrial microorganisms: Basic and applied molecular genetics. American Society for Microbiology, Washington, D.C.

Doskoèil, J. C. T. S. C., Z. Hostalek, J. Kasparova, J. Zajicek, and M. Herold. 1959. Development of *Streptomyces aureofaciens* in submerged culture. J Biochem Microbiol Tech Eng **1:**261-271.

Doull, J. L., and L. C. Vining. 1989. Culture conditions promoting dispersed growth and biphasic production of actinorhodin in shaken cultures of *Streptomyces coelicolor* A3(2). FEMS Microbiol Lett **65:**265-268.

Dunlap, P. V. 1996. *N*-acyl-L-homoserine lactone autoinducers in bacteria, p. 69-106. *In* S. a. Dworkin (ed.), Bacteria as mlticellular organisms. Oxford University Press, Oxford.

Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20**(9)**:**2444-2449.

Eberl, L., M. K. Winson, C. Sternberg, G. S. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov. 1996. Involvement of *N*-acyl-L-hormoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol Microbiol **20**(1)**:**127-136.

Elbein, A. D. 1967a. Carbohydrate metabolism in *Streptomyces hygroscopicus*. I. Enzymatic synthesis of trehalose phosphate from guanosine diphosphate D-glucose- ^{14}C. J Biol Chem **242**(3)**:**403-406.

Elbein, A. D. 1967b. Carbohydrate metabolism in streptomycetes. II. Isolation and enzymatic synthesis of trehalose. J Bacteriol **94**(5)**:**1520-1524.

Elliot, M., F. Damji, R. Passantino, K. Chater, and B. Leskiw. 1998. The *bldD* gene of *Streptomyces coelicolor* A3(2): a regulatory gene involved in morphogenesis and antibiotic production. J Bacteriol **180**(6)**:**1549-1555.

Elliot, M. A., and B. K. Leskiw. 1999. The BldD protein from *Streptomyces coelicolor* is a DNA-binding protein. J Bacteriol **181**(21)**:**6832-6835.

Fernández-Moreno, M. A., A. J. Martin-Triana, E. Martínez, J. Niemi, H. M. Kieser, D. A. Hopwood, and F. Malpartida. 1992. *abaA*, a new pleiotropic regulatory locus for antibiotic production in *Streptomyces coelicolor*. J Bacteriol **174**(9)**:**2958-2967.

Fink, D., D. Falke, W. Wohlleben, and A. Engels. 1999. Nitrogen metabolism in *Streptomyces coelicolor* A3(2): modification of glutamine synthetase I by an adenylyltransferase. Microbiology **145**(Pt 9)**:**2313-2322.

Fisher, S. H., and A. L. Sonenshein. 1991. Control of carbon and nitrogen metabolism in *Bacillus subtilis*. Annu Rev Microbiol **45:**107-135.

Fisher, S. H. 1992. Glutamine synthesis in *Streptomyces*--a review. Gene **115**(1-2)**:**13-17.

Flärdh, K., K. C. Findlay, and K. F. Chater. 1999. Association of early sporulation genes with suggested developmental decision points in *Streptomyces coelicolor* A3(2). Microbiology **145**(Pt 9)**:**2229-2243.

Flores, M. E., and S. Sanches. 1985. Nitrogen regulation of erythromycin formation in *Streptomyces erythreus*. FEMS Microbiol Lett **26:**191-.

Floriano, B., and M. Bibb. 1996. *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). Mol Microbiol **21**(2)**:**385-396.

Fu, H., M. A. Alvarez, C. Khosla, and J. E. Bailey. 1996. Engineered biosynthesis of novel polyketides: regiospecific methylation of an unnatural substrate by the *tcmO* O-methyltransferase. Biochemistry **35**(21)**:**6527-6532.

Fuqua, C., and S. C. Winans. 1996. Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J Bacteriol **178**(2)**:**435-440.

Furuya, K., and C. R. Hutchinson. 1996. The *DnrN* protein of *Streptomyces peucetius*, a pseudo-response regulator, is a DNA-binding protein involved in the regulation of daunorubicin biosynthesis. J Bacteriol **178**(21)**:**6310-6318.

Gaal, T., M. S. Bartlett, W. Ross, C. L. Turnbough, Jr., and R. L. Gourse. 1997. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria [see comments]. Science **278**(5346)**:**2092-2097.

Gagnat, J., H. Chouayekh, C. Gerbaud, F. Francou, and M. J. Virolle. 1999. Disruption of *sblA* in *Streptomyces lividans* permits expression of a heterologous α-amylase gene in the presence of glucose. Microbiology **145**(Pt 9)**:**2303-2312.

Garcia-Dominguez, M., J. F. Martin, and P. Liras. 1989. Characterization of sugar uptake in wild-type *Streptomyces clavuligerus*, which is impaired in glucose uptake, and in a glucose-utilizing mutant. J Bacteriol **171**(12)**:**6808-6814.

Geistlich, M., R. Losick, J. R. Turner, and R. N. Rao. 1992. Characterization of a novel regulatory gene governing the expression of a polyketide synthase gene in *Streptomyces ambofaciens*. Mol Microbiol **6**(14)**:**2019-2029.

Gersch, D. 1979. Dependence of macromolecule synthesis on cyclic AMP in antibiotica producing strains of *Streptomyce hygroscopicus*. FEMS Micobiol Lett **6:**343-347.

Gil, J. A., G. Naharro, J. R. Villanueva, and J. F. Martin. 1985. Characterization and regulation of *p*-aminobenzoic acid synthase from *Streptomyces griseus*. J Gen Microbiol **131**(Pt 6)**:**1279-1287.

Gram, L., A. B. Christensen, L. Ravn, S. Molin, and M. Givskov. 1999. Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. Appl Environ Microbiol **65**(8)**:**3458-3463.

Gramajo, H. C., E. Takano, and M. J. Bibb. 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol Microbiol **7**(6)**:**837-845.

Granozzi, C., R. Billetta, R. Passantino, M. Sollazzo, and A. M. Puglia. 1990. A breakdown in macromolecular synthesis preceding differentiation in *Streptomyces coelicolor* A3(2). J Gen Microbiol **136**(Pt 4)**:**713-716.

Gräfe, U., H. Bocker, G. Reinhardt, and H. Thrum. 1975. Induzierbare Akkumulation von a-Ketoglutarsäure in Kulturen eines Makrolidantibioticum-bildenden Stammes von *Streptomyces hygroscopicus* JA6599. Z Allg Mikrobiol **15**(8)**:**575-583.

Gräfe, U., H. Bocker, and H. Thrum. 1977. Regulative influence of *o*-aminobenzoic acid on the biosynthesis of nourseothricin in cultures of *Streptomyces noursei* JA 3890b. II. Regulation of glutamine synthetase and the role of the glutamine synthetase/glutamate synthase pathway. Z Allg Mikrobiol **17**(3)**:**201-209.

Gräfe, U., W. Schade, I. Eritt, W. F. Fleck, and L. Radics. 1982. A new inducer of anthracycline biosynthesis from *Streptomyces viridochromogenes*. J Antibiot **35**(12)**:**1722-1723.

Gräfe, U., G. Reinhardt, W. Schade, I. Eritt, W. F. Fleck, and L. Radics. 1983. Interspecific inducers of cytodifferentiation and anthracycline biosynthesis from *Streptomyces bikinensis* and *S. cyaneofuscatus*. Biotech Lett **5**(9)**:**591-596.

Gui, L., A. Sunnarborg, B. Pan, and D. C. LaPorte. 1996. Autoregulation of *iclR*, the gene

encoding the repressor of the glyoxylate bypass operon. J Bacteriol **178**(1)**:**321-324.

Guijarro, J., R. Santamaria, A. Schauer, and R. Losick. 1988. Promoter determining the timing and spatial localization of transcription of a cloned *Streptomyces coelicolor* gene encoding a spore- associated polypeptide. J Bacteriol **170**(4)**:**1895-1901.

Guthrie, E. P., C. S. Flaxman, J. White, D. A. Hodgson, M. J. Bibb, and K. F. Chater. 1998. A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket [published erratum appears in Microbiology 1998 Jul;144(Pt 7):2007]. Microbiology **144**(Pt 3)**:**727-738.

Hafner, E. W., B. W. Holley, K. S. Holdom, S. E. Lee, R. G. Wax, D. Beck, H. A. McArthur, and W. C. Wernau. 1991. Branched-chain fatty acid requirement for avermectin production by a mutant of *Streptomyces avermitilis* lacking branched-chain 2-oxo acid dehydrogenase activity. J Antibiot **44**(3)**:**349-356.

Han, L., and K. A. Reynolds. 1997. A novel alternate anaplerotic pathway to the glyoxylate cycle in streptomycetes. J Bacteriol **179**(16)**:**5157-5164.

Harasym, M., L. H. Zhang, K. Chater, and J. Piret. 1990. The *Streptomyces coelicolor* A3(2) *bldB* region contains at least two genes involved in morphological development. J Gen Microbiol **136**(Pt 8)**:**1543-1550.

Hey, A. E., and A. D. Elbein. 1968. Partial prufication and properties of a trehalase from *Streptomyces hygroscopicus*. J Bacteriol **96**(1)**:**105-110.

Hey-Ferguson, A., M. Mitchell, and A. D. Elbein. 1973. Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. J Bacteriol **116**(2)**:**1084-1085.

Hindle, Z., and C. P. Smith. 1994. Substrate induction and catabolite repression of the *Streptomyces coelicolor* glycerol operon are mediated through the GylR protein. Mol Microbiol **12**(5)**:**737-745.

Hobbs, G., F. C.M., G. D.C.J., C. J.A., and O. S.G. 1989. Dispersed growth of *Streptomyces* in liquid culture. Appl Microbiol Biotechnol **31:**272-277.

Hobbs, G., F. C.M., G. D.C.J., F. F., and O. S.G. 1990. Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): Kinetice and the influence of nutrients. J Gen Microbiol **136:**2291-2296.

Hoch, J. A. 1993. Regulation of the phosphorelay and the initiation of sporulation in Bacillus subtilis. Annu Rev Microbiol **47:**441-365.

Hockenhull, D. J. D., K. H. Fantes, M. Herbert, and B. Whitehead. 1954. Glucose utilization by *Streptomyces griseus*. J Gen Microbiol **10:**353-370.

Hodgson, D. A. 1980. PhD Thesis. University of East Anglia, Norwich, UK.

Hodgson, D. A. 1982. Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. J Gen Microbiol **128:**2417-2430.

Hodgson, D. A. 2000. Primary metabolism and its control in streptomycetes: a most unusual group of bacteria. Adv Microb Physiol **42:**47-238.

Holt, T. G., C. Chang, C. Laurent-Winter, T. Murakami, J. I. Garrels, J. E. Davies, and C. J. Thompson. 1992. Global changes in gene expression related to antibiotic synthesis in *Streptomyces hygroscopicus*. Mol Microbiol **6**(8)**:**969-980.

Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Burton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf (ed.). 1985. Genetic manipulation of Streptomyces - a laboratory manual. The John Innes Foundation, Norwich, UK.

Horinouchi, S. 1996. *Streptomyces* genes in volved in aerial mycelium formation. FEMS Microbiol Lett **141:**1-9.

Horinouchi, S., and T. Beppu. 1984. Production in large quntities of actinorhodin and undecylprodigiosin induces by*afsB* in *Streptomyces lividans*. Agric Biol Chem **48**(8)**:**2131-2133.

Horinouchi, S., and T. Beppu. 1992. Autoregulatory factors and communication in actinomycetes. Annu Rev Microbiol **46:**377-398.

Horinouchi, S., and T. Beppu. 1994. A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. Mol Microbiol **12**(6)**:**859-864.

Horinouchi, S., O. Hara, and T. Beppu. 1983. Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J Bacteriol **155**(3)**:**1238-1248.

Horinouchi, S., H. Suzuki, and T. Beppu. 1986. Nucleotide sequence of *afsB*, a pleiotropic gene involved in secondary metabolism in *Streptomyces coelicolor* A3(2) and "*Streptomyces lividans*". J Bacteriol **168**(1)**:**257-269.

Horinouchi, S., M. Kito, M. Nishiyama, K. Furuya, S. K. Hong, K. Miyake, and T. Beppu. 1990. Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). Gene **95**(1)**:**49-56.

Hostalek, Z., I. Tobek, M. A. Bobyk, and I. S. Kulayev. 1976. Role of ATP-glucokinase and polyphosphate glucokinase in *Streptomyces aureofaciens*. Folia Microbiol **21**(2)**:**131-138.

Hsieh, C. J., and G. H. Jones. 1995. Nucleotide sequence, transcriptional analysis, and glucose regulation of the phenoxazinone synthase gene (*phsA*) from *Streptomyces antibioticus*. J Bacteriol **177**(20)**:**5740-5747.

Hsing, W., F. D. Russo, K. K. Bernd, and T. J. Silhavy. 1998. Mutations that alter the kinase and phosphatase activities of the two- component sensor EnvZ. J Bacteriol **180**(17)**:**4538-4546.

Huang, L., S. Steven-Miles, and R. B. Lingham. 1999. Screening for activities, p. 21-28. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.

Huisman, G. W., and R. Kolter. 1994. Sensing starvation: a homoserine lactone--dependent signaling pathway in *Escherichia coli*. Science **265**(5171)**:**537-539.

Hunter-Cevera, J. C., and A. Belt. 1999. Isolation of cultures, p. 3-20. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.

Ikeda, H., H. Kotaki, H. Tanaka, and S. Omura. 1988. Involvement of glucose catabolism in avermectin production by *Streptomyces avermitilis*. Antimicrob Agents Chemother **32**(2)**:**282-284.

Inbar, L., and A. Lapidot. 1988. Metabolic regulation in *Streptomyces parvulus* during actinomycin D synthesis, studied with ^{13}C- and ^{15}N-labeled precursors by ^{13}C and ^{15}N nuclear magnetic resonance spectroscopy and by gas chromatography-mass spectrometry. J Bacteriol **170**(9)**:**4055-4064.

Ingram, C., I. Delic, and J. Westpheling. 1995. *ccrA1*: a mutation in *Streptomyces coelicolor* that affects the control of catabolite repression. J Bacteriol **177**(12)**:**3579-3586.

Ishizuka, H., S. Horinouchi, H. M. Kieser, D. A. Hopwood, and T. Beppu. 1992. A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. J Bacteriol **174**(23)**:**7585-7594.

Itoh, M., A. Penyige, S. Okamoto, and K. Ochi. 1996. Proteins that interact with GTP in *Streptomyces griseus* and its possible implication in morphogenesis. FEMS Microbiol Lett **135**(2-3)**:**311-316.

Ji, G., R. C. Beavis, and R. P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci U S A **92**(26)**:**12055-12059.

Jiang, H., and K. E. Kendrick. 2000. Characterization of *ssfR* and *ssgA*, two genes involved in sporulation of *Streptomyces griseus*. J Bacteriol **182**(19)**:**5521-5529.

Jiao, R. S., Y. M. Chen, M. G. Wu, and G. W.L. 1979. Studies on the metabolic regulation of biosynthesis of rifamycin by *Nocardia mediterranei*. I. The stimulative effect of nitrate on

biosynthesis of rifamycin SV by *Nocardia mediterranei*. Acta Phytophysiol Sin **5:**402-.

Johnstone, B. H., A. A. Handler, D. K. Chao, V. Nguyen, M. Smith, S. Y. Ryu, E. L. Simons, P. E. Anderson, and R. W. Simons. 1999. The widely conserved Era G-protein contains an RNA-binding domain required for Era function *in vivo*. Mol Microbiol **33**(6)**:**1118-1131.

Kaiser, D. 1984. Regulation of multicellular development in myxobacteria, p. 197-218. *In* R. Losick and L. Shapiro (ed.), Microbial Development. Cold Spring Habor Laboratory, Cold Spring Habor, N.Y.

Kang, S. G., W. Jin, M. Bibb, and K. J. Lee. 1998. Actinorhodin and undecylprodigiosin production in wild-type and *relA* mutant strains of *Streptomyces coelicolor* A3(2) grown in continuous culture. FEMS Microbiol Lett **168**(2)**:**221-226.

Kang, D. K., X. M. Li, K. Ochi, and S. Horinouchi. 1999. Possible involvement of cAMP in aerial mycelium formation and secondary metabolism in *Streptomyces griseus*. Microbiology **145**(Pt 5)**:**1161-1172.

Kannan, L. V., and Z. Rehacek. 1970. Formation of poly-beta-hydroxybutyrate by *Actinomycetes*. Indian J Biochem **7**(2)**:**126-129.

Kawamoto, S., H. Watanabe, A. Hesketh, J. C. Ensign, and K. Ochi. 1997. Expression analysis of the *ssgA* gene product, associated with sporulation and cell division in *Streptomyces griseus*. Microbiology **143**(Pt 4)**:**1077-1086.

Keen, C. L., S. Mendelovitz, G. Cohen, Y. Aharonowitz, and K. L. Roy. 1988. Isolation and characterization of a linear DNA plasmid from *Streptomyces clavuligerus*. Mol Gen Genet **212**(1)**:**172-176.

Kelemen, G. H., P. Brian, K. Flärdh, L. Chamberlin, K. F. Chater, and M. J. Buttner. 1998. Developmental regulation of transcription of *whiE*, a locus specifying the polyketide spore pigment in *Streptomyces coelicolor* A3 (2). J Bacteriol **180**(9)**:**2515-2521.

Kelemen, G. H., and M. J. Buttner. 1998. Initiation of aerial mycelium formation in *Streptomyces* [published erratum appears in Curr Opin Microbiol 1999 Feb;2(1):106]. Curr Opin Microbiol **1**(6)**:**656-662.

Khosla, C., and J. E. Bailey. 1988. Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli*. Nature **331**(6157)**:**633-635.

Khosla, C., S. Ebert-Khosla, and D. A. Hopwood. 1992. Targeted gene replacements in a *Streptomyces* polyketide synthase gene cluster: role for the acyl carrier protein. Mol Microbiol **6**(21)**:**3237-3249.

Kieser, T., J. Bibb, M. J. Buttner, K. F. Chaiter, and D. A. Hopwood (ed.). 2000. Practial *Streptomyces* genetics. The John Innes Foundation, Norwich.

Kinoshita, H., H. Ipposhi, S. Okamoto, H. Nakano, T. Nihira, and Y. Yamada. 1997. Butyrolactone autoregulator receptor protein (BarA) as a transcriptional regulator in *Streptomyces virginiae*. J Bacteriol **179**(22)**:**6986-6993.

Kinoshita, H., T. Tsuji, H. Ipposhi, T. Nihira, and Y. Yamada. 1999. Characterization of binding sequences for butyrolactone autoregulator receptors in streptomycetes. J Bacteriol **181**(16)**:**5075-5080.

Kitani, S., H. Kinoshita, T. Nihira, and Y. Yamada. 1999. In vitro analysis of the butyrolactone autoregulator receptor protein (FarA) of *Streptomyces lavendulae* FRI-5 reveals that FarA acts as a DNA- binding transcriptional regulator that controls its own synthesis. J Bacteriol **181**(16)**:**5081-5084.

Kominek, L. A. 1972. Biosynthesis of novobiocin by *Streptomyces niveus*. Antimicrob Agents Chemother **1**(2)**:**123-134.

Kretschmer, S. 1991. Correlation between branching and elongation in germ tubes of *Streptomyces granaticolor*. J Basic Microbiol **31:**587-595.

Kroening, T. A., and K. E. Kendrick. 1987. In vivo regulation of histidine ammonia-lyase activity from *Streptomyces griseus*. J Bacteriol **169**(2)**:**823-829.

Kudo, N., M. Kimura, T. Beppu, and S. Horinouchi. 1995. Cloning and characterization of a gene involved in aerial mycelium formation in *Streptomyces griseus*. J Bacteriol **177**(22)**:**6401-6410.

Kwakman, J. H., and P. W. Postma. 1994. Glucose kinase has a regulatory role in carbon catabolite repression in *Streptomyces coelicolor*. J Bacteriol **176**(9)**:**2694-2698.

Lawlor, E. J., H. A. Baylis, and K. F. Chater. 1987. Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). Genes Dev **1**(10)**:**1305-1310.

Lazazzera, B. A., and A. D. Grossman. 1998. The ins and outs of peptide signaling. Trends Microbiol **6**(7)**:**288-294.

Lazazzera, B. A., I. G. Kurtser, R. S. McQuade, and A. D. Grossman. 1999. An autoregulatory circuit affecting peptide signaling in *Bacillus subtilis*. J Bacteriol **181**(17)**:**5193-5200.

Lee, M. S., and D. A. Morrison. 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. J Bacteriol **181**(16)**:**5004-5016.

Lengeler, J. W., and P. W. Postma. 1999. Global regulatory networks an signal transduction pathways, p. 491-523. *In* J. W. Lengeler, G. Drews, and H. G. Schlegel (ed.), Biology of the procaryotes. Thieme, Stuttgart.

Lerner, C. G., and M. Inouye. 1991. Pleiotropic changes resulting from depletion of Era, an essential GTP- binding protein in *Escherichia coli*. Mol Microbiol **5**(4)**:**951-957.

Leskiw, B. K., M. J. Bibb, and K. F. Chater. 1991. The use of a rare codon specifically during development? Mol Microbiol **5**(12)**:**2861-2867.

Lezhava, A., T. Mizukami, T. Kajitani, D. Kameoka, M. Redenbach, H. Shinkawa, O. Nimi, and H. Kinashi. 1995. Physical map of the linear chromosome of *Streptomyces griseus*. J Bacteriol **177**(22)**:**6492-6498.

Li, Y., and W. R. Strohl. 1996. Cloning, purification, and properties of a phosphotyrosine protein phosphatase from *Streptomyces coelicolor* A3(2). J Bacteriol **178**(1)**:**136-142.

Liao, X., L. C. Vining, and J. L. Doull. 1995. Physiological control of trophophase-idiophase separation in streptomycete cultures producing secondary metabolites. Can J Microbiol **41**(4-5)**:**309-315.

Lilley, G., A. E. Clark, and G. C. Lawrence. 1981. Control of the production of cephamycin C and thienamycin by *Streptomyces cattleya* NRRL 8057. J Chem Tech Biotechnol **31:**127-.

Lin, Y. S., H. M. Kieser, D. A. Hopwood, and C. W. Chen. 1993. The chromosomal DNA of *Streptomyces lividans* 66 is linear. Mol Microbiol **10**(5)**:**923-933.

Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov. 1998. *N*-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. J Bacteriol **180**(23)**:**6384-6388.

Lounès, A., A. Lebrihi, C. Benslimane, G. Lefebvre, and P. Germain. 1995. Glycerol effect on spiramycin production and valine catabolism in *Streptomyces ambofaciens*. Curr Microbiol **31**(5)**:**304-311.

Lutkenhaus, J. 1993. *Escherichia coli* cell division. Curr Opin Genet Dev **3**(5)**:**783-788.

Ma, H., and K. Kendall. 1994. Cloning and analysis of a gene cluster from *Streptomyces coelicolor* that causes accelerated aerial mycelium formation in *Streptomyces lividans*. J Bacteriol **176**(12)**:**3800-3811.

Madden, T., J. M. Ward, and A. P. Ison. 1996. Organic acid excretion by *Streptomyces lividans* TK24 during growth on defined carbon and nitrogen sources. Microbiol **142:**3181-3185.

Madduri, K., and C. R. Hutchinson. 1995. Functional characterization and transcriptional analysis of the $dnrR_1$ locus, which controls daunorubicin biosynthesis in *Streptomyces peucetius*. J Bacteriol **177**(5)**:**1208-1215.

Magasanik, B. 1996. Regulation of nitrogen utilization. I. Cellular and molecular biology, p. 1344-1356. *In* F. C. Neidhard, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*, 2nd ed. Americam Society of Microbiology, Washington, D.C.

Magnolo, S. K., D. L. Leenutaphong, J. A. DeModena, J. E. Curtis, J. E. Bailey, J. L. Galazzo, and D. E. Hughes. 1991. Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin. Biotechnology (N Y) **9**(5)**:**473-476.

Malpartida, F., and D. A. Hopwood. 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature **309**(5967)**:**462-464.

Martin, J. F., and A. L. Demain. 1980. Control of antibiotic biosynthesis. Microbiol Rev **44**(2)**:**230-251.

Martínez-Costa, O. H., P. Arias, N. M. Romero, V. Parro, R. P. Mellado, and F. Malpartida. 1996. A *relA*/*spoT* homologous gene from *Streptomyces coelicolor* A3(2) controls antibiotic biosynthetic genes. J Biol Chem **271**(18)**:**10627-10634.

Martínez-Costa, O. H., M. A. Fernández-Moreno, and F. Malpartida. 1998. The *relA*/*spoT*-homologous gene in *Streptomyces coelicolor* encodes both ribosome-dependent (p)ppGpp-synthesizing and -degrading activities. J Bacteriol **180**(16)**:**4123-4132.

Martínez-Costa, O. H., A. J. Martín-Triana, E. Martínez, M. A. Fernández-Moreno, and F. Malpartida. 1999. An additional regulatory gene for actinorhodin production in *Streptomyces lividans* involves a LysR-type transcriptional regulator. J Bacteriol **181**(14)**:**4353-4564.

Masuma, R., Y. Tanaka, and S. Omura. 1983. Ammonium-ion depressed fermentation of tylosin by the use of a neutral zeolite and its significance in the study of biosynthetic regulation of the antibiotic. J Ferment Technol **61:**607-.

Matsumoto, A., S. K. Hong, H. Ishizuka, S. Horinouchi, and T. Beppu. 1994. Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. Gene **146**(1)**:**47-56.

Mattern, S. G., M. E. Brawner, and J. Westpheling. 1993. Identification of a complex operator for *galP1*, the glucose-sensitive, galactose-dependent promoter of the *Streptomyces* galactose operon. J Bacteriol **175**(5)**:**1213-1220.

McCormick, J. R., E. P. Su, A. Driks, and R. Losick. 1994. Growth and viability of *Streptomyces coelicolor* mutant for the cell division gene *ftsZ*. Mol Microbiol **14**(2)**:**243-254.

McCormick, J. R., and R. Losick. 1996. Cell division gene *ftsQ* is required for efficient sporulation but not growth and viability in *Streptomyces coelicolor* A3(2). J Bacteriol **178**(17)**:**5295-5301.

McCue, L. A., J. Kwak, J. Wang, and K. E. Kendrick. 1996. Analysis of a gene that suppresses the morphological defect of bald mutants of *Streptomyces griseus*. J Bacteriol **178**(10)**:**2867-2875.

McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla. 1993. Engineered biosynthesis of novel polyketides. Science **262**(5139)**:**1546-1550.

McDaniel, R., A. Thamchaipenet, C. Gustafsson, H. Fu, M. Betlach, and G. Ashley. 1999. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products [published erratum appears in Proc Natl Acad Sci U S A 1999 May 11;96(10):5890]. Proc Natl Acad Sci U S A **96**(5)**:**1846-1851.

McDowall, K. J., A. Thamchaipenet, and I. S. Hunter. 1999. Phosphate control of oxytetracycline production by *Streptomyces rimosus* is at the level of transcription from promoters overlapped by tandem repeats similar to those of the DNA-binding sites of the OmpR family. J Bacteriol **181**(10)**:**3025-3032.

McVittie, A. M. 1974. Ultrastructural studies on sporulation in wild-type and white colony mutants of *Streptomyces coelicolor*. J Gen Microbiol **81:**291-302.

Merrick, M. J. 1976. A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. J Gen Microbiol **96**(2)**:**299-315.

Miguélez, E. M., M. García, C. Hardisson, and M. B. Manzanal. 1994. Autoradiographic study of hyphal growth during aerial mycelium development in *Streptomyces antibioticus*. J Bacteriol **176**(7)**:**2105-2107.

Miguélez, E. M., C. Hardisson, and M. B. Manzanal. 1999. Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. J Cell Biol **145**(3)**:**515-525.

Minas, W., P. Brünker, P. T. Kallio, and J. E. Bailey. 1998. Improved erythromycin production in a genetically engineered industrial strain of *Saccharopolyspora erythraea*. Biotechnol Prog **14**(4)**:**561-566.

Minas, W. 2003 (submitted). Glucose-independent excretion of organic acids precedes actinorhodin production in Streptomyces coelicolor *liquid cultivations*.

Minas, W., J. E. Bailey, and W. Duetz. in Press. Streptomycetes in micro-cultures: Growth, production of secondary metabolites, and storage in the 96-well format. Ant. Leuven. 79:235-245.

Miyake, K., S. Horinouchi, M. Yoshida, N. Chiba, K. Mori, N. Nogawa, N. Morikawa, and T. Beppu. 1989. Detection and properties of A-factor-binding protein from *Streptomyces griseus*. J Bacteriol **171**(8)**:**4298-4302.

Mizuno, K., S. Sakuda, T. Nihira, and Y. Yamada. 1996. Enzymatic resolution of 2-acyl-3-hydroxymethyl-4-butanolide and preparation of optically active IM-2, the autoregulator of *Streptomyces* sp. FRI-5. Tetrahedron Lett **50:**10849-10858.

Molle, V., and M. J. Buttner. 2000. Different alleles of the response regulator gene *bldM* arrest *Streptomyces coelicolor* development at distinct stages. Mol Microbiol **36**(6)**:**1265-1278.

Mootz, H. D., D. Schwarzer, and M. A. Marahiel. 2000. Construction of hybrid peptide synthetases by module and domain fusions. Proc Natl Acad Sci U S A **97**(11)**:**5848-5853.

Morita, R. Y. 1985. Starvation and miniaturisation of heterotrophes, with special emphasis on maintenance of the starved viable state, p. 111-130. *In* M. Fletscher and G. D. Flodgate (ed.), Bacteriain their natural environments. Academic Press, London.

Mouncey, N. J., and S. Kaplan. 1998a. Redox-dependent gene regulation in Rhodobacter sphaeroides 2.4.1(T): effects on dimethyl sulfoxide reductase (dor) gene expression. J Bacteriol **180**(21)**:**5612-5618.

Mouncey, N. J., and S. Kaplan. 1998b. Cascade regulation of dimethyl sulfoxide reductase (dor) gene expression in the facultative phototroph Rhodobacter sphaeroides 2.4.1T. J Bacteriol **180**(11)**:**2924-2930.

Mouncey, N. J., and S. Kaplan. 1998c. Oxygen regulation of the ccoN gene encoding a component of the cbb3 oxidase in Rhodobacter sphaeroides 2.4.1T: involvement of the FnrL protein. J Bacteriol **180**(8)**:**2228-2231.

Nádvorník, R., T. Vomastek, J. Janecek, Z. Techniková, and P. Branny. 1999. Pkg2, a novel transmembrane protein Ser/Thr kinase of *Streptomyces granaticolor*. J Bacteriol **181**(1)**:**15-23.

Nealson, K. H., T. Platt, and J. W. Hastings. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. J Bacteriol **104**(1)**:**313-322.

Nguyen, J., F. Francou, M. J. Virolle, and M. Guérineau. 1997. Amylase and chitinase genes in *Streptomyces lividans* are regulated by *reg1*, a pleiotropic regulatory gene. J Bacteriol **179**(20)**:**6383-6390.

Nguyen, J. 1999. The regulatory protein Reg1 of *Streptomyces lividans* binds the promoter region of several genes repressed by glucose. FEMS Microbiol Lett **175:**51-58.

Ni, X., and J. Westpheling. 1997. Direct repeat sequences in the *Streptomyces* chitinase-63 promoter direct both glucose repression and chitin induction. Proc Natl Acad Sci U S A **94**(24)**:**13116-13121.

Nicieza, R. G., J. Huergo, B. A. Connolly, and J. Sanchez. 1999. Purification, characterization, and role of nucleases and serine proteases in *Streptomyces* differentiation. Analogies with the biochemical processes described in late steps of eukaryotic apoptosis. J Biol Chem **274**(29)**:**20366-20375.

Nihira, T., Y. Shimizu, H. S. Kim, and Y. Yamada. 1988. Structure-activity relationships of virginiae butanolide C, an inducer of virginiamycin production in *Streptomyces virginiae*. J Antibiot **41**(12)**:**1828-1837.

Nodwell, J. R., K. McGovern, and R. Losick. 1996. An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. Mol Microbiol **22**(5)**:**881-893.

Nodwell, J. R., and R. Losick. 1998. Purification of extracellular signalling molecule incolved in production of aerial mycelium by *Streptomyces coelicolor*. J Bacteriol **180**(5)**:**1334-1337.

Nodwell, J. R., M. Yang, D. Kuo, and R. Losick. 1999. Extracellular complementation and the identification of additional genes involved in aerial mycelium formation in *Streptomyces coelicolor*. Genetics **151**(2)**:**569-584.

Novotná, J., and Z. Hostálek. 1985. Phosphorylation of hexoses in *Streptomyces aureofaciens*: evidence that the phosphoenolpyruvate:phosphotransferase system is not operative. FEMS Microbiol Lett **28:**347-350.

Obanye, A. I. C., G. Hobbs, D. C. J. Gardner, and S. G. Oliver. 1996. Correlation between carbon flux through the pntose phosphate pathway and production of the antibiotic methylenomycin in *Streptomyces coelicolor* A3(2). Microbiol. **142:**133-137.

Ochi, K. 1986. Occurrence of the stringent response in *Streptomyces sp.* and its significance for the initiation of morphological and physiological differentiation. J Gen Microbiol **132**(Pt 9)**:**2621-2631.

Ochi, K. 1987a. A *rel* mutation abolishes the enzyme induction needed for actinomycin synthesis in *Streptomyces antibioticus*. Agric Biol Chem **51:**829-835.

Ochi, K. 1987b. Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent response (ppGpp) and GTP content in relation to A factor. J Bacteriol **169**(8)**:**3608-3616.

Ochi, K. 1988. Nucleotide pools and stringent response in regulation of*Streptmyces* differentiation, p. 330-337. *In* Y. Okami, T. Beppu, and H. Ogawara (ed.), Boiology of cactinomycetes '88. Scientific Societies Press, Tokyo.

Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Lino. 1992. A novel transcriptional regulation mechanism in the flagellar regulon of Salmonella typhimurium: an antisigma factor inhibits the activity of the flagellum-specific sigma factor, sigma F. Mol Microbiol **6**(21)**:**3149-3157.

Ohnishi, Y., S. Kameyama, H. Onaka, and S. Horinouchi. 1999. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. Mol Microbiol **34**(1)**:**102-111.

Ohnishi, Y., Y. Nishiyama, R. Sato, S. Kameyama, and S. Horinouchi. 2000. An oligoribonuclease gene in *Streptomyces griseus*. J Bacteriol **182**(16)**:**4647-4653.

Okabe, M., T. Kuwajima, M. Satoh, K. Kimura, K. Okamura, and R. Okamoto. 1992. Preferential and high-yield production of a cephamycin C by dissolved oxygen controlled fermentation. J Ferment Bioeng **73**(4)**:**292-296.

Okamoto, S., M. Itoh, and K. Ochi. 1997. Molecular cloning and characterization of the obg gene of *Streptomyces griseus* in relation to the onset of morphological differentiation. J Bacteriol **179**(1)**:**170-179.

Okamoto, S., and K. Ochi. 1998. An essential GTP-binding protein functions as a regulator for

differentiation in *Streptomyces coelicolor*. Mol Microbiol **30**(1)**:**107-119.
Olukoshi, E. R., and N. M. Packter. 1994. Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. Microbiology **140**(Pt 4)**:**931-943.
Omura, S., Y. Tanaka, C. Kitao, H. Tanaka, and Y. Iwai. 1980a. Stimulation of leucomycin production by magnesium phosphate and its relevance to nitrogen catabolite regulation. Antimicrob Agents Chemother **18**(5)**:**691-695.
Omura, S., Y. Tanaka, H. Tanaka, Y. Takahashi, and Y. Iwai. 1980b. Stimulation of the production of macrolide antibiotics by magnesium phosphate and related insoluble materials. J Antibiot **33**(12)**:**1568-1569.
Omura, S., and Y. Tanaka. 1984. Control of ammonium ion level in antibiotic fermentation, p. 367-. *In* L. Ortiz-Ortiz, L. F. Bojalil, and V. Yakoleff (ed.), Biological, biochemical, and biomedical aspects of actinomycetes. Academic Press, Orlando, FL.
Onaka, H., and S. Horinouchi. 1997. DNA-binding activity of the A-factor receptor protein and its recognition DNA sequences. Mol Microbiol **24**(5)**:**991-1000.
Onaka, H., T. Nakagawa, and S. Horinouchi. 1998. Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. Mol Microbiol **28**(4)**:**743-753.
Packter, N. M., and S. Flatman. 1983. Characterization of acetoacetyl-CoA reductase (3-oxoreductase) from *Streptomyces coelicolor*: Its possilble role in polyhydroxybutyrate biosynthesis. Biochem Soc Trans **11:**598-599.
Paget, M. S., J. G. Kang, J. H. Roe, and M. J. Buttner. 1998. σ^R, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). Embo J **17**(19)**:**5776-5782.
Paget, M. S. B., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner. 1999. Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J Bacteriol **181**(1)**:**204-211.
Palková, Z., B. Janderová, J. Gabriel, B. Zikánová, M. Pospísek, and J. Forstová. 1997. Ammonia mediates communication between yeast colonies. Nature **390**(6659)**:**532-536.
Pan, B., I. Unnikrishnan, and D. C. LaPorte. 1996. The binding site of the IclR repressor protein overlaps the promoter of *aceBAK*. J Bacteriol **178**(13)**:**3982-3984.
Pandza, S., M. Baetens, C. H. Park, T. Au, M. Keyhan, and A. Matin. 2000. The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. Mol Microbiol **36**(2)**:**414-423.
Parkinson, J. 1995. Genetic approaches for signaling pathways and proteins. *In* J. A. Hoch and T. J. Silhavy (ed.), 1995. American Society for Microbiology, Washington, D.C.
Passantino, R., A.-M. Puglia, and K. F. Chater. 1991. Additional copies of the *act*II regulatory gene induce actinorhodin production in pleiotropic *bld* mutants of *Streptomyces coelicolor* A3(2). J Gen Microbiol **137:**2059-2064.
Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol **181**(4)**:**1203-1210.
Perlman, D., and G. H. Wagman. 1952. Studies on the utilization of lipids by *Streptomyces griseus*. J. Bacteriol. **63:**253-262.
Petersen, C., and L. B. Moller. 2000. Invariance of the nucleoside triphosphate pools of *Escherichia coli* with growth rate. J Biol Chem **275**(6)**:**3931-3935.
Pérez-Llarena, F. J., P. Liras, A. Rodriguez-Garcia, and J. F. Martin. 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both beta-lactam compounds. J Bacteriol **179**(6)**:**2053-2059.
Pierson, L. S., III, D. W. Wood, and E. A. Pierson. 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annu Rev Phytopathol **36**(207-225).

Pillutla, R. C., J. D. Sharer, P. S. Gulati, E. Wu, Y. Yamashita, C. G. Lerner, M. Inouye, and P. E. March. 1995. Cross-species complementation of the indispensable *Escherichia coli era* gene highlights amino acid regions essential for activity. J Bacteriol **177**(8)**:**2194-2196.

Plaskitt, K. A., and K. F. Chater. 1995. Influences of developmental genes on localized glycogen deposition in colonies of a mycelial procaryote, *Streptomyces coelicolor* A3(2): a possible interface between metabolism and morphogenesis. Philos Trans R Soc Lond Biol Sci **347:**105-121.

Pope, M. K., B. D. Green, and J. Westpheling. 1996. The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell--cell signalling. Mol Microbiol **19**(4)**:**747-756.

Pope, M. K., B. Green, and J. Westpheling. 1998. The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production, and catabolite control in *Streptomyces coelicolo*r. J Bacteriol **180**(6)**:**1556-1562.

Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev **57**(3)**:**543-594.

Potúèková, L., G. H. Kelemen, K. C. Findlay, M. A. Lonetto, M. J. Buttner, and J. Kormanec. 1995. A new RNA polymerase sigma factor, σF, is required for the late stages of morphological differentiation in *Streptomyces* spp. Mol Microbiol **17**(1)**:**37-48.

Price, B., T. Adamidis, R. Kong, and W. Champness. 1999. A *Streptomyces coelicolor* antibiotic regulatory gene, *absB*, encodes an RNase III homolog. J Bacteriol **181**(19)**:**6142-6151.

Prosser, J. I., and A. J. Tough. 1991. Growth Mechanisms and growth kinetics of filamentous microorganisms. Crit Rev Biotechnol **10**(4)**:**253-274.

Puglia, A. M., and E. Cappelletti. 1984. A bald superfertile U.V.-resistant strain in *Streptomyces coelicolor* A3(2). Microbiologica **7**(3)**:**263-266.

Puglia, A. M., J. Vohradsky, and C. J. Thompson. 1995. Developmental control of the heat-shock stress regulon in *Streptomyces coelicolor*. Mol Microbiol **17**(4)**:**737-746.

Raibaud, A., M. Zalacain, T. G. Holt, R. Tizard, and C. J. Thompson. 1991. Nucleotide sequence analysis reveals linked N-acetyl hydrolase, thioesterase, transport, and regulatory genes encoded by the bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus*. J Bacteriol **173**(14)**:**4454-4463.

Ranade, N., and L. C. Vining. 1993. Accumulation of intracellular carbon reserves in relation to chloramphenicol biosynthesis by *Streptomyces venezuelae*. Can J Microbiol **39**(4)**:**377-383.

Redenbach, M., H. M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, and D. A. Hopwood. 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol Microbiol **21**(1)**:**77-96.

Redshaw, P. A., P. A. McCann, L. Sankaran, and B. M. Pogell. 1976. Control of differentiation in streptomycetes: involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development. J Bacteriol **125**(2)**:**698-705.

Richardson, M. A., S. Kuhstoss, M. L. Huber, L. Ford, O. Godfrey, J. R. Turner, and R. N. Rao. 1990. Cloning of spiramycin biosynthetic genes and their use in constructing *Streptomyces ambofaciens* mutants defective in spiramycin biosynthesis. J Bacteriol **172**(7)**:**3790-3798.

Roman, R. V., and M. Gavrilescu. 1994. Oxygen transfer efficiency in the biosynthesis of antibiotics in bioreactors with a modified Rushton turbine agitator. Acta Biotechnol **14**(2)**:**181-192.

Romero, J., P. Liras, and J. F. Martin. 1984. Dissociation of chephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol **20**(318-).

Romero, N. M., and R. P. Mellado. 1995. Activation of the actinorhodin biosynthetic pathway in *Streptomyces lividans*. FEMS Microbiol Lett **127**(1-2)**:**79-84.

Roth, M., B. Schwalenberg, R. Reiche, D. Noack, R. Geuther, and I. Eritt. 1982. Genetic

segregation in a high-yielding streptomycin-producing strain of *Streptomyces griseus*. Z Allg Mikrobiol **22**(8)**:**557-563.

Rudd, B. A., and D. A. Hopwood. 1979. Genetics of actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2). J Gen Microbiol **114**(1)**:**35-43.

Ruusala, T., and C. G. Kurland. 1984. Streptomycin preferentially perturbs ribosomal proofreading. Mol Gen Genet **198**(1)**:**100-104.

Ryding, N. J., M. J. Bibb, V. Molle, K. C. Findlay, K. F. Chater, and M. J. Buttner. 1999. New sporulation locus in *Streptomyces coelicolor* A3(2). J Bacteriol **181**(17)**:**5419-5425.

Sakuda, S., and Y. Yamada. 1991. Sereochemistry of butyrolacone autoregulators from *Streptomyces*. Tetrahedron Lett **32:**1817-1820.

Salas, J. A., L. M. Quiros, and C. Hardisson. 1984. Pathways of glucose catabolism during germination of *Streptomyces* spores. FEMS Microbiol Lett **22:**229-233.

Sato, K., T. Nihira, S. Sakuda, M. Yanagimoto, and Y. Yamada. 1991. Isolation and structure of a new butyrolactone autoregulator from *Streptomyces* sp. FRI-5. J Ferment Bioeng **68:**170-173.

Schauwecker, F., F. Pfennig, N. Grammel, and U. Keller. 2000. Construction and in vitro analysis of a new bi-modular polypeptide synthetase for synthesis of N-methylated acyl peptides. Chem Biol **7**(4)**:**287-297.

Scheu, A. K., E. Martinez, J. Soliveri, and F. Malpartida. 1997. *abaB*, a putative regulator for secondary metabolism in *Streptomyces*. FEMS Microbiol Lett **147**(1)**:**29-36.

Schimmel, T. G., A. D. Coffman, and S. J. Parsons. 1998. Effect of butyrolactone I on the producing fungus, *Aspergillus terreus*. Appl Environ Microbiol **64**(10)**:**3707-3712.

Schneider, D., C. J. Bruton, and K. F. Chater. 1996. Characterization of *spaA*, a *Streptomyces coelicolor* gene homologous to a gene involved in sensing starvation in *Escherichia coli*. Gene **177**(1-2)**:**243-251.

Schneider, D., C. J. Bruton, and K. F. Chater. 2000. Duplicated gene clusters suggest an interplay of glycogen and trehalose metabolism during sequential stages of aerial mycelium development in *Streptomyces coelicolor* A3(2). Mol Gen Genet **263**(3)**:**543-553.

Schreier, H. J. 1993. Biosynthesis of glutamine and glutamate and the assimilation of ammonia, p. 281-298. *In* L. A. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other Gram-positive bacteria. American Society for MIcrobiology, Washington, D.C.

Schwartz, D., S. Kaspar, G. Kienzlen, K. Muschko, and W. Wohlleben. 1999. Inactivation of the tricarboxylic acid cycle aconitase gene from *Streptomyces viridochromogenes* Tu494 impairs morphological and physiological differentiation. J Bacteriol **181**(22)**:**7131-7135.

Schwecke, T., J. F. Aparicio, I. Molnar, A. Konig, L. E. Khaw, S. F. Haydock, M. Oliynyk, P. Caffrey, J. Cortes, J. B. Lester, and et al. 1995. The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc Natl Acad Sci U S A **92**(17)**:**7839-7843.

Seed, P. C., L. Passador, and B. H. Iglewski. 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by *LasR* and the *Pseudomonas autoinducer* PAI: an autoinduction regulatory hierarchy. J Bacteriol **177**(3)**:**654-659.

Sekurova, O., H. Sletta, T. E. Ellingsen, S. Valla, and S. Zotchev. 1999. Molecular cloning and analysis of a pleiotropic regulatory gene locus from the nystatin producer *Streptomyces noursei* ATCC11455. FEMS Microbiol Lett **177**(2)**:**297-304.

Seno, E. T., and K. F. Chater. 1983. Glycerol catabolic enzymes and their regulation in wild-type and mutant strains of *Streptomyces coelicolor* A3(2). J Gen Microbiol **129**(Pt 5)**:**1403-1413.

Shapiro, S., and L. C. Vining. 1984. Suppression of nitrate utilization by ammonium and its relationship to chloramphenicol production in *Streptomyces venezuelae*. Can J Microbiol **30**(6)**:**798-804.

Shima, J., A. Penyige, and K. Ochi. 1996a. Changes in patterns of ADP-ribosylated proteins during differentiation of *Streptomyces coelicolor* A3(2) and its development mutants. J

Bacteriol **178**(13)**:**3785-3790.

Shima, J., A. Hesketh, S. Okamoto, S. Kawamoto, and K. Ochi. 1996b. Induction of actinorhodin production by *rpsL* (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). J Bacteriol **178**(24)**:**7276-7284.

Soliveri, J. A., J. Gomez, W. R. Bishai, and K. F. Chater. 2000. Multiple paralogous genes related to the *Streptomyces coelicolor* developmental regulatory gene *whiB* are present in *Streptomyces* and other actinomycetes. Microbiology **146**(Pt 2)**:**333-343.

Stein, D., and S. N. Cohen. 1989. A cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. J Bacteriol **171**(4)**:**2258-2261.

Stock, J., M. Surette, M. Levit, and P. Park. 1995. Two-component signal transduction systems: structure-function relationships and mechanismus for catalysis. *In* J. A. Hoch and T. J. Silhavy (ed.), 1995. American Society for Microbiology, Washington, D.C.

Strauch, E., E. Takano, H. A. Baylis, and M. J. Bibb. 1991. The stringent response in *Streptomyces coelicolor* A3(2). Mol Microbiol **5**(2)**:**289-298.

Strobel, R. J., and G. R. Sullivan. 1999. Experimental design for improvement of fermentations, p. 80-102. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.

Stuart, F., and I. S. Hunter. 1993. Purification and characterization of 3-deoxy-D-arabino-heptulosonate-7- phosphate synthase from *Streptomyces rimosus*. Biochim Biophys Acta **1161**(2-3)**:**209-215.

Sugiyama, M., H. Onaka, T. Nakagawa, and S. Horinouchi. 1998. Site-directed mutagenesis of the A-factor receptor protein: Val-41 important for DNA-binding and Trp-119 important for ligand-binding. Gene **222**(1)**:**133-144.

Surowitz, K. G., and R. M. Pfister. 1985a. Glucose metabolism and pyruvate excretion by *Streptomyces alboniger*. Can J Microbiol **31:**702-706.

Surowitz, K. G., and R. M. Pfister. 1985b. Variations in levels of cAMP and RNA in *Streptomyces albniger* under conditions of aerial mycelium formation and repression. FEMS Microbiol Lett **26:**1-4.

Süsstrunk, U., J. Pidoux, S. Taubert, A. Ullmann, and C. J. Thompson. 1998. Pleiotropic effects of cAMP on germination, antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. Mol Microbiol **30**(1)**:**33-46.

Takano, E., H. C. Gramajo, E. Strauch, N. Andres, J. White, and M. J. Bibb. 1992. Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor A3(2)*. Mol Microbiol **6**(19)**:**2797-2804.

Tanaka, Y., R. Masuma, and S. Omura. 1984. Control of ammonium ion level for efficient nanaomycin production. J Antibiot **37**(11)**:**1370-1375.

Tang, L., A. Grimm, Y. X. Zhang, and C. R. Hutchinson. 1996. Purification and characterization of the DNA-binding protein *DnrI*, a transcriptional factor of daunorubicin biosynthesis in *Streptomyces peucetius*. Mol Microbiol **22**(5)**:**801-813.

Tata, M., and A. S. Menawat. 1994. Cyclic AMP regulation of tylosin biosynthesis and secondary metabolism in *Streptomyces fradiae*. Biotechnol Bioeng **44:**283-290.

Tillotson, R. D., H. A. Wosten, M. Richter, and J. M. Willey. 1998. A surface active protein involved in aerial hyphae formation in the filamentous fungus *Schizophillum commune* restores the capacity of a bald mutant of the filamentous bacterium *Streptomyces coelicolor* to erect aerial structures. Mol Microbiol **30**(3)**:**595-602.

Titgemeyer, F., J. Walkenhorst, J. Reizer, M. H. Stuiver, X. Cui, and M. H. Saier, Jr. 1995. Identification and characterization of phosphoenolpyruvate:fructose phosphotransferase

systems in three *Streptomyces* species. Microbiology **141**(Pt 1)**:**51-58.

Ueda, K., K. Miyake, S. Horinouchi, and T. Beppu. 1993. A gene cluster involved in aerial mycelium formation in *Streptomyces griseus* encodes proteins similar to the response regulators of two- component regulatory systems and membrane translocators. J Bacteriol **175**(7)**:**2006-2016.

Ueda, K., C.-W. Hsheh, T. Tosaki, H. Shinkawa, T. Beppu, and S. Horinouchi. 1998. Characterization af an A-factor responsive repressor for *amfR* essential for onset of aerial mycelium formation in *Streptomyces griseus*. J Bacteriol **180**(19)**:**5085-5093.

Ueda, K., K. Matsuda, H. Takano, and T. Beppu. 1999. A putative regulatory element for carbon-source-dependent differentiation in *Streptomyces griseus*. Microbiology **145**(Pt 9)**:**2265-2271.

Ueda, K., S. Kawai, H.-O. Ogawa, A. Kiyama, T. Kubota, H. Kawanobe, and T. Beppu. 2000. Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among *Streptomyces* species. J Antibiot **53**(9)**:**979-982.

Umeyama, T., P. C. Lee, K. Ueda, and S. Horinouchi. 1999. An AfsK/AfsR system involved in the response of aerial mycelium formation to glucose in *Streptomyces griseus*. Microbiology **145**(Pt 9)**:**2281-2292.

van Wezel, G. P., J. White, P. Young, P. W. Postma, and M. J. Bibb. 1997a. Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malR*, a member of the *lacl-galR* family of regulatory genes. Mol Microbiol **23**(3)**:**537-549.

van Wezel, G. P., J. White, M. J. Bibb, and P. W. Postma. 1997b. The *malEFG* gene cluster of *Streptomyces coelicolor* A3(2): characterization, disruption and transcriptional analysis. Mol Gen Genet **254**(5)**:**604-608.

van Wezel, G. P., J. van Der Meulen, S. Kawamoto, R. G. Luiten, H. K. Koerten, and B. Kraal. 2000. *ssgA* Is Essential for Sporulation of *Streptomyces coelicolor* A3(2) and Affects Hyphal Development by Stimulating Septum Formation. J Bacteriol **182**(20)**:**5653-5662.

Vidwans, S. J., K. Ireton, and A. D. Grossman. 1995. Possible role for the essential GTP-binding protein Obg in regulating the initiation of sporulation in *Bacillus subtilis*. J Bacteriol **177**(11)**:**3308-3311.

Vinci, V. A., and G. Byng. 1999. Strain improvements by nonrecombinant methods, p. 103-113. *In* A. L. Demain and J. E. Davies (ed.), Manual of the industrial microbiology and biotechnology (2nd edition). ASM Press, Washington, D.C.

Viollier, P. H., W. Minas, G. E. Dale, M. Folcher, and C. J. Thompson. 2001a submitted. The role of acid metabolism in *Streptomyces coelicolor* morphological differentiation and antibiotic biosynthesis. J Bacteriol 183:3184-3192.

Viollier, P. H., K. T. Nguyen, W. Minas, M. Folcher, G. E. Dale, and C. J. Thompson. 2001b submitted. The role of aconitase in growth, metabolism, and morphological differentiation of *Streptomyces coelicolor*. J Bacteriol 183:3193-3203.

Virolle, M. J., and J. Gagnat. 1994. Sequences involved in growth-phase-dependent expression and glucose repression of a *Streptomyces* α-amylase gene. Microbiology **140** (Pt 5)**:**1059-1067.

Vohradsky, J., X. Li, G. Dale, M. Folcher, L. Nguyen, P. H. Viollier, and C. J. Thompson. 2000. Developmental control of stress stimulons in *Streptomyces coelicolor* revealed by statistical analyses of global gene expression patterns. J Bacteriol **182**(17)**:**4979-4986.

Vomastek, T., R. Nádvorník, J. Janecek, Z. Techniková, J. Weiser, and P. Branny. 1998. Characterisation of two putative protein Ser/Thr kinases from actinomycete *Streptomyces granaticolor* both endowed with different properties. Eur J Biochem **257**(1)**:**55-61.

Vögtli, M., P. C. Chang, and S. N. Cohen. 1994. *afsR2*: a previously undetected gene encoding a 63-amino-acid protein that stimulates antibiotic production in *Streptomyces lividans*. Mol

Microbiol **14**(4)**:**643-653.

Waki, M., T. Nihira, and Y. Yamada. 1997. Cloning and characterization of the gene (*farA*) encoding the receptor for an extracellular regulatory factor (IM-2) from *Streptomyces* sp. strain FRI-5. J Bacteriol **179**(16)**:**5131-5137.

Walker, J. B., and M. S. Walker. 1982. Enzymatic synthesis of streptomycin as a model system for study of the reglation and evolution of antibiotic biosynthetic pathways, p. 271-. *In* V. Krumphanzl, B. Sikyta, and Z. Vanek (ed.), Overproduction of microbial products. Academic Press, London.

Wanner, B. L. 1996. Phosphorous assimilation and control of thephosphate regulon, p. 1357-1381. *In* F. C. Neidhard, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*, 2nd ed. Americam Society of Microbiology, Washington, D.C.

Warr, S. R. C., Box, S.J., Burbidge, C., Edwards, H., and Jones, J.J. 1994. Milbemycin production by *Streptomyces* sp.: the effect of carbohydrates. J Industr Microbiol **13:**43-48.

Weißschuh, N., D. Fink, S. Vierling, M. J. Bibb, W. Wohlleben, and A. Engels. 2000. Transcriptional analysis of the gene for the glutamate synthetase II and two upstream genes in *Streptomyces coelicolor* A3(2). Mol Gen Genet **264**(4):461-469.

Welsh, K. M., K. A. Trach, C. Folger, and J. A. Hoch. 1994. Biochemical characterization of the essential GTP-binding protein Obg of *Bacillus subtilis*. J Bacteriol **176**(23)**:**7161-7168.

Whitaker, A. 1992. Actinomyces in submerged culture. Appl Biochem Biotechnol **32:**23-35.

White, J., and M. Bibb. 1997. bldA dependence of undecylprodigiosin production in Streptomyces coelicolor A3(2) involves a pathway-specific regulatory cascade. J Bacteriol **179**(3)**:**627-633.

Wietzorrek, A., and M. Bibb. 1997. A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold [letter]. Mol Microbiol **25**(6)**:**1181-1184.

Wildermuth, H. 1970. Development and organization of the aerial mycelium in *Streptomyces coelicolor*. J Gen Microbiol **60**(1)**:**43-50.

Willey, J., R. Santamaria, J. Guijarro, M. Geistlich, and R. Losick. 1991. Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. Cell **65**(4)**:**641-650.

Wilson, G. C., and M. E. Bushell. 1995. The induction of antibiotic synthesis in *Saccharopolyspora erythraea* and *Streptomyces hygroscopicus* by growth rate decrease is accompanied by a down-regulation of protein synthesis rate. FEMS Microbiol Lett **129:**89-96.

Wray, L. V., Jr., A. E. Ferson, K. Rohrer, and S. H. Fisher. 1996. TnrA, a transcription factor required for global nitrogen regulation in *Bacillus subtilis*. Proc Natl Acad Sci U S A **93**(17)**:**8841-8845.

Wu, J., M. I. Cho, and H. K. Kuramitsu. 1995. Expression, purification, and characterization of a novel G protein, SGP, from *Streptococcus mutans*. Infect Immun **63**(7)**:**2516-2521.

Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2000. An A-factor-dependent extracytoplasmic function sigma factor (σ^{AdsA}) that is essential for morphological development in *Streptomyces griseus*. J Bacteriol **182**(16)**:**4596-4605.

Yegneswaran, P. K., M. R. Gray, and B. G. Thompson. 1991. Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. Biotechnol Prog **7**(3)**:**246-250.

Yonekawa, T., Y. Ohnishi, and S. Horinouchi. 1999. Involvement of *amfC* in physiological and morphological development in *Streptomyces coelicolor* A3(2). Microbiology **145**(Pt 9)**:**2273-2280.

Young, M. D., L. L. Kempe, and F. G. Bader. 1985. Effects of phosphate, glucose, and

ammonium on cell growth and linomycin production by *Streptomyces lincolnensis* in chemically defined medium. Biotechnol Bioeng **27:**327-.

Zhang, K., and A. W. Nicholson. 1997. Regulation of ribonuclease III processing by double-helical sequence antideterminants. Proc Natl Acad Sci U S A **94**(25)**:**13437-13441.

Zhu, J., J. W. Beaber, M. I. Moré, C. Fuqua, A. Eberhard, and S. C. Winans. 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J Bacteriol **180**(20)**:**5398-5405.

Zou, P., I. Borovok, D. Ortiz de Orué Lucana, D. Müller, and H. Schrempf. 1999. The mycelium-associated *Streptomyces reticuli* catalase-peroxidase, its gene and regulation by FurS. Microbiology **145**(Pt 3)**:**549-559.