STREPTOMYCES MORPHOGENESIS

What is currently (not) known about Streptomyces morphogenesis regulation

Good afternoon, ladies and gentlemen.
At least some of you *should* know my today's topic – Streptomyces morphogenesis regulation – generally better than I do.
So please tolerate minor inaccuracies, and if you see significant important gaps – please add them after the presentation.

Outline

- Importance of morphogenesis studies
- What studies have demonstrated so far
- What is still not known

We'll start with a brief refresh of actinobacteria morphogenesis and why people study it.
Then we'll look at the information collected over the years about the regulation of morphogenesis.
Missing pieces of information will be obvious along the way, but I will then briefly summarize the key unknowns.

WHY?

Why people study
Streptomyces morphogenesis regulation?

Why people research Streptomyces morphogenesis regulation?

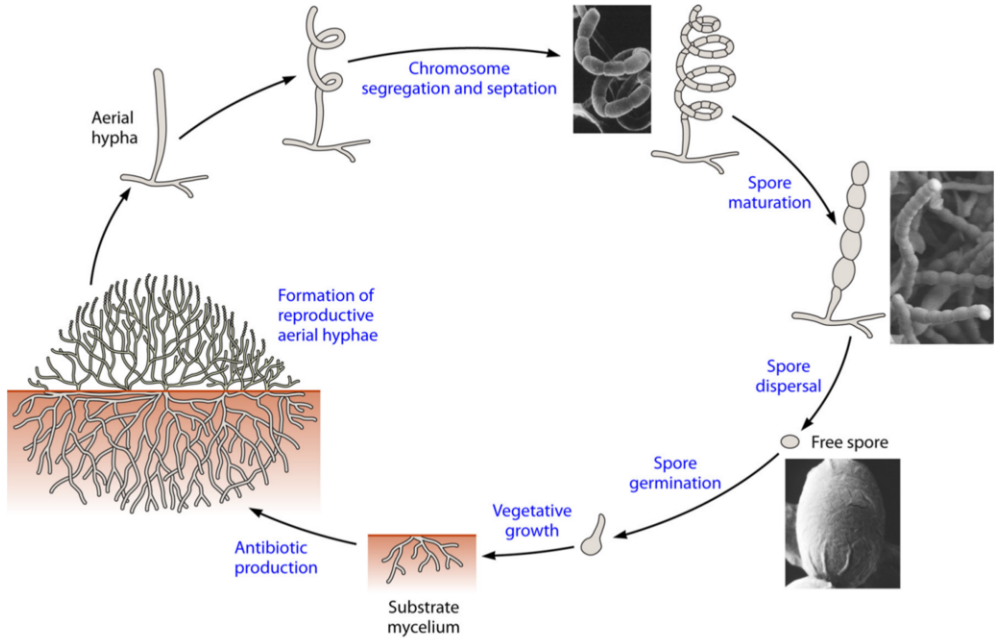
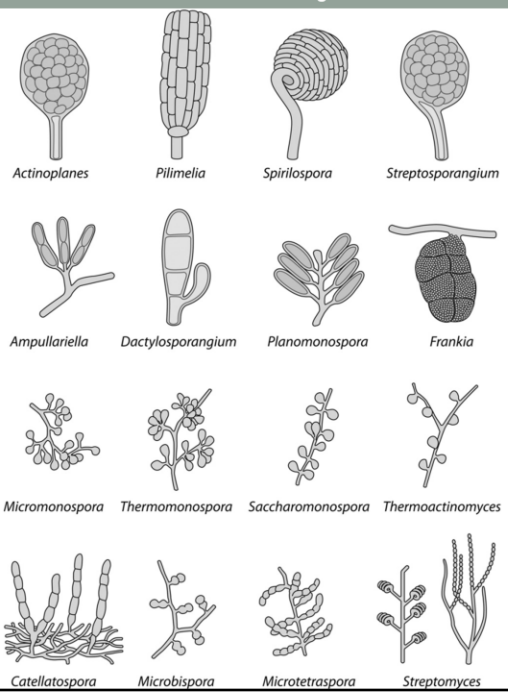


FIG 1 Schematic representation of the life cycle of sporulating actinomycetes.

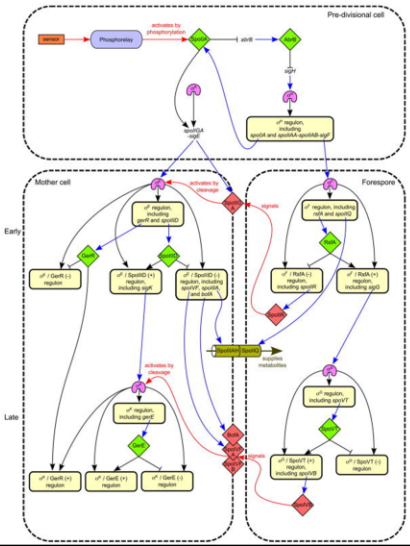
By morphogenesis, I am referring to this life cycle diagram.
If we start with a free Actinobacteria spore, then when it finds itself in a favourable environment – good temperature, humidity, nutrients concentrations – it will activate and start growing.
This initial phase is known as vegetative growth, which leads to a formation of substrate mycelium.
Vegetative growth is a process focused on consuming as much nutrients and as quickly as possible.
Under adverse conditions, such as nutrient depletion, the vegetative mycelium differentiates to form erected sporogenic structures called aerial hyphae.
The focus of aerial growth is to survive – namely, produce as many as possible viable free spores that we started with.
I'll be talking mostly about the regulation and transition from vegetative to aerial growth, all the way up to spore formation.
As you can see, antibiotic production is mentioned at this transition stage.
And this is the secondary reason to study this phenomenon.
The primary reason is, of course, curiosity.



Aerial hyphae are quite diverse across Actinobacteria, but the basic life cycle is very similar for all of them.

Intermediate summary

- Survival strategies are induced in low-nutrient conditions (starvation).
- Sporulation is a survival strategy.
- Secondary metabolite production is also a survival strategy.
- In *Streptomyces*, both strategies seem to be co-regulated.
- *Bacillus subtilis* has the best-studied sporulation regulation →
- Sporulation differs in *Streptomyces*.



To summarize this part:

- antibiotics production and decision to sporulate in Streptomyces are both the components of survival strategy in the low-nutrient, starvation conditions, and

- these two strategies appear to be co-regulated.

Of all the bacteria, Bacillus subtilis has the best studied sporulation, but it doesn't help us, because Streptomyces sporulation mechanisms are different from Bacillus subtilis mechanisms.

WHAT IS KNOWN

The Grand Scheme of Sporulation

Now let's try to form a holistic understanding of sporulation regulation.

Model Streptomyces

- 3 model species have provided nearly all the experimental information about morphological development of streptomycetes.
- The most widely studied is *S. coelicolor* A3(2)
- *S. griseus* has been studied for its production of a hormone-like developmental signaling molecule, A-factor.
- *S. venezuelae* sporulates rapidly, synchronously and comprehensively in submerged culture

Three model species have provided nearly all the experimental information about morphological development of Streptomycetes.
Historically, the most widely studied is *S. coelicolor*.
S. griseus has been studied for its production of a hormone-like developmental signaling molecule called A-factor.
Finally, in the recent years a lot of new information was gained from S. venezuelae, because this species can sporulate in a liquid medium, in a submerged culture.
It also sporulates rapidly, synchronously, and comprehensively.

Key regulators

- Bald, “hairless” phenotype mutants lacking fluffy aerial hyphae: ***bld*** genes (and mutants)
- Lack of common (but not universal) gray spore pigment: ***whi*** genes (and mutants)
- Rapid aerial mycelium formation (*S. lividans*): ***ram*** genes
- Complementation-resolved *S. griseus* sporulation mutants: *ssgA*-like genes

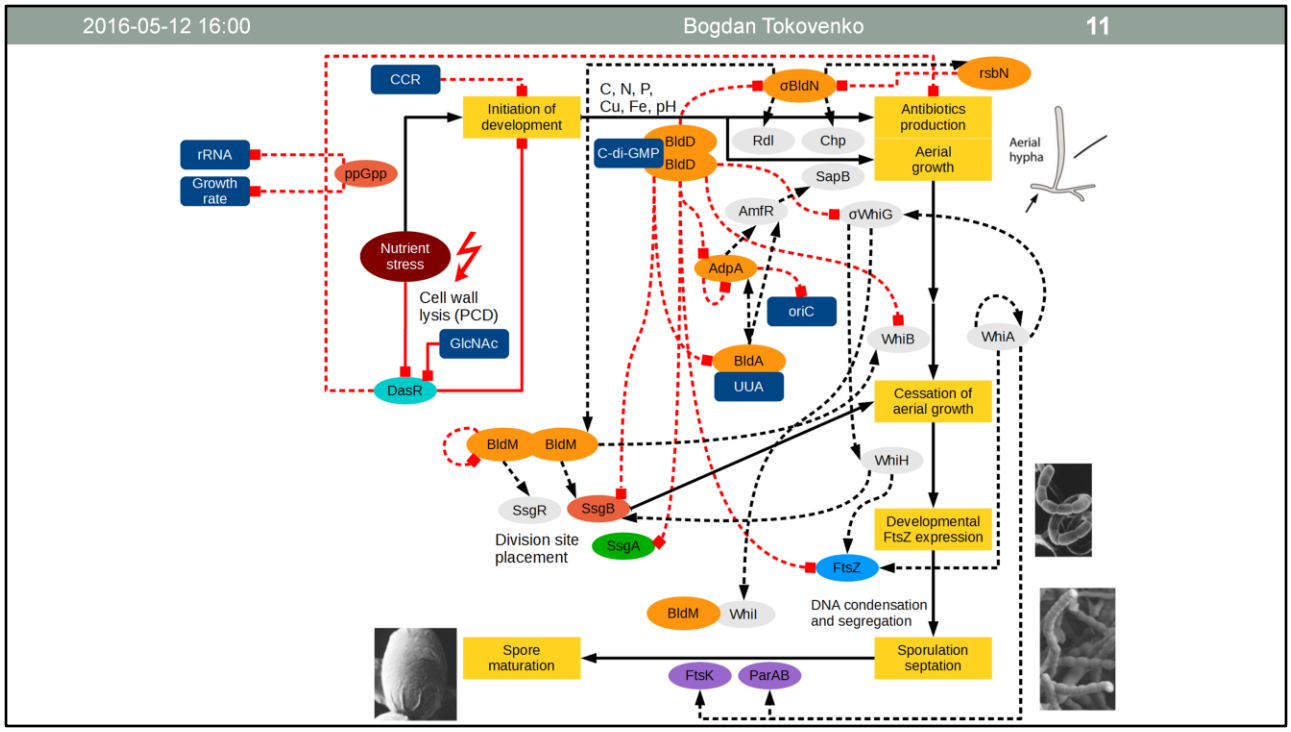
Genes which are believed to be the key regulators of sporulation were discovered based on specific phenotypes and physiology of mutants strains.
These genes are:

- bld, for bald, hairless phenotype;
- Whi, for white, or lacking the gray sporulation pigment – if the wild type has this pigment, which, for example, is not the case for the *S. albus*;
- Ram, for rapid aerial mycelium formation in *S. lividans*;
- ssgA-like, for sporulation of *Streptomyces griseus*

Some of regulators and effectors

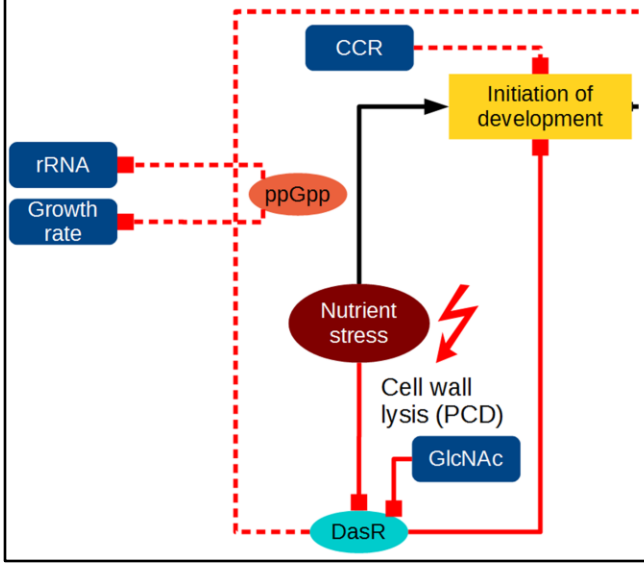
- DasR
- Bld: B, C, D, G, H, J, K, M, N
- Whi: A, B, D, G, H, I, J;
- WhiB-like (Wbl): A, C, E
- SsgA, SsgB, SsgR
- AmfR
- Chp: long (A, B, C), short (D, E, F, G, H)
- Rdl: A, B
- Sap: B, T; AmfS
- Fts: Z, K
- Par: A, B

This is an incomplete list of regulators and effector genes involved in sporulation. Believe it or not, but by the time I finish, you will have a basic understanding of most of them.



Here is a strongly simplified grand overview scheme.
We will now have a look at parts of this scheme, and then later come back to the full view once again.
We'll go through initiation of development, aerial growth and cessation of aerial growth, FtsZ expression and septation, and then just mention spore maturation.

Onset of development



- Guanosine tetraphosphate (ppGpp), amino acids alarmone
- Cell wall derived N-acetyl-glucosamine (GlcNAc) => DasR
- Some bald mutants sporulate on minimal media: carbon catabolite repression

Let us start with how a decision to grow aerial mycelium is made – after a nutrient stress hits hard.

One of the sensor systems is the guanosine tetraphosphate alarmone, which (indirectly) responds to the increase of uncharged tRNAs – which, in turn, happens if there is a shortage of amino acids.
Guanosine tetraphosphate directly represses ribosomal RNA promoters, and changes RNA polymerase promoter preferences in a specific way, decreasing growth rate and protein synthesis in order to save amino acids.

When nutrient depletion occurs, the vegetative mycelium is autolytically degraded by a programmed cell death (PCD)-like mechanism to acquire the building blocks needed to erect a second mass of (aerial) mycelium.
PCD results in the accumulation of amino acids, aminosugars, nucleotides, and lipids around the lysing substrate mycelium, which inevitably attract motile competing microbes in the habitat.
It is logical to assume that antibiotics are produced at this time to protect the pool of nutrients.

Cell wall lysis releases N-acetyl-glucosamine, which is a ligand of the GntR family regulator DasR.
DasR normally prevents development initiation, and antibiotics synthesis.

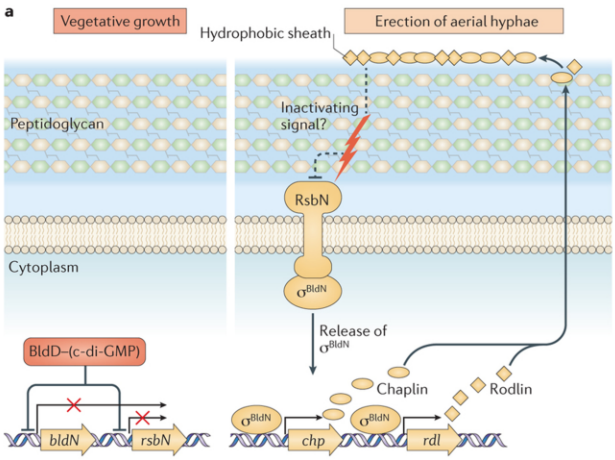
Adding GlcNAc removes this roadblock, and bacteria initiate both development and antibiotics synthesis.

Many bald mutants have a conditional bald phenotype.
They are able to produce at least some aerial hyphae and spores on minimal media with nonrepressive carbon sources, such as mannitol (192, 198, 227).
A logical assumption is that this is the result of carbon catabolite repression (CCR), whereby favorable carbon sources such as glucose signal the presence of abundant food, thus favoring growth over development and antibiotic production (232, 233).

This also works for humans: poor humans on minimal media usually have lots of hair, while humans on more reach media usually have less hair.

Thus, antibiotic biosynthesis and secretion could be induced by adding GlcNAc to minimal medium with a non-repressive carbon source.

Early hyphae growth

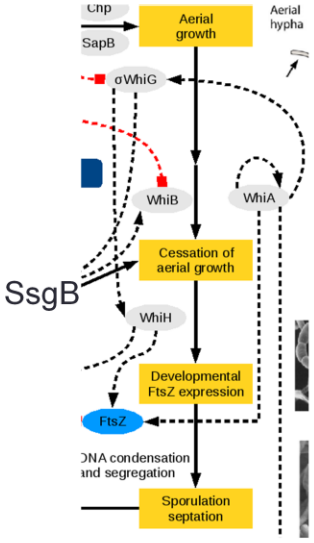


- BldD is a repressor of *bldN*
- BldN is a sigma factor controlling chaplins and rodlins
- RsbN is a BldN "repressor", BldN is RsbN activator
- SapB (*ramS*, *amfS*)

As I've said before, bld genes control the hairy phenotype, and we see two of such genes here – bdlD and bldN.
There are more bld genes, and together they seem to form a kind of a signal transduction cascade, as shown by complementation studies.

bldD is a repressor, and we'll talk more about it later, but here it represses bldN and rsbN.
bldN controls the expression of chaplins and rodlins – proteins which form a hydrophobic sheath around the growing hypha.
They faciliate hypha erection from the moist soil.
There are 2 rodlins and 8 chaplins, but just 3 chaplins were found to be sufficient for hyphal growth.
Apparently, this may depend on the specific growth environment.
BldN is also controlled by RsbN – a membrane-bound protein, which appears to keep BldN away from its DNA targets.
RsbN responds to a yet unknown signal, upon which it releases BldN.
One possibility is that chaplins and rodlins themselves can be this signal, to form a positive feedback loop.
Another hyphal growth protein is SapB, which is a surfactant released by the bacteria into the environment to faciliate hyphae growth.

Cessation of growth and septation



- Unknown signal => *whiA*/*whiB*; *ssgB*
- *whiA*$^{-}$/*whiB*$^{-}$: long hypercoiling hyphae
- *ssgB*$^{-}$: large aerial biomass
- *whiA*: syntenic with 3 genes of glycolysis/gluconeogenesis and 1 protein secretion gene
- Developmental FtsZ expression
- FtsZ overrides *whi* mutant phenotypes

After the hyphae have grown for a while, they need to stop and undergo septation into individual spores.
The signal for growth cessation is not yet known but likely relates to the Whi regulatory proteins WhiA and WhiB, as well as the cell division activator SsgB.
Mutations of whiA and whiB produce identical phenotypes, with hypercoiling and very long aerial hyphae that fail to initiate cell division (23, 174, 274).
SsgB mutations produce a large colony phenotype, forming an extremely large aerial biomass (275).

whiA across actinobacteria is syntenic with 3 genes of glycolisis/gluconeogenesis pathways, and 1 gene of the protein secretion system.
This observation might be linked to what kind of signal whiA and whiB may receive.

The next step is developmental FtsZ expression.
FtsZ is a tubulin homolog, and it is expressed also during vegetative growth.
However, during vegetative growth FtsZ expression does not fully isolate fragments of the mycelium – this is why, to highlight the difference, this step is called Developmental FtsZ expression.

It had been shown that constitutive FtsZ expression restores sporulation in the whi mutants.
This finding suggests that Whi genes are the only controllers of FtsZ expression.

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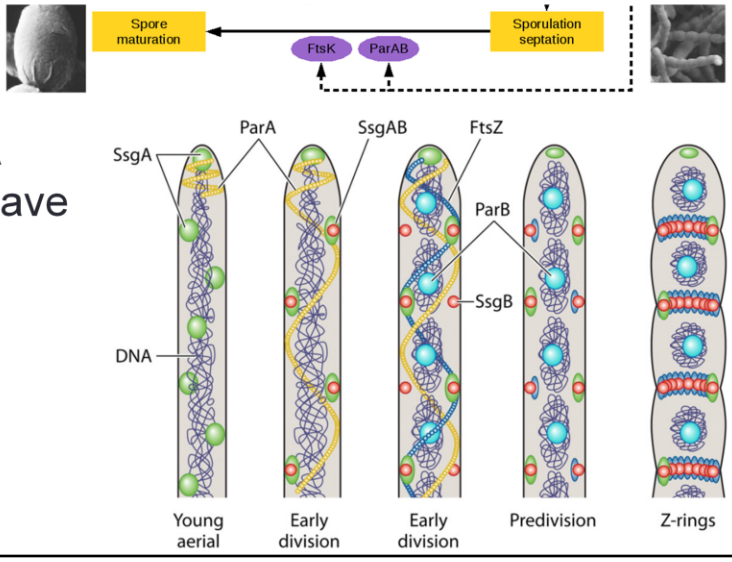
There is also conspicuous synteny on the other side of whiA in actinobacteria (but this does not extend to B. subtilis): three genes for steps in glycolysis/gluconeogenesis , glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and triose phosphate isomerase, are always found next to whiA (or separated from it by one or two genes in some streptomycetes), along with secG, encoding part of the protein secretion system.
If the notion of 'guilt by association' is applied to whiA, we may guess that it operates in the context of a physiological transition resulting from nutritional limitation, such that assimilated nutrients are redirected via gluconeogenesis to generate glucose-6-phosphate, which may then be converted into N-acetyl glucosamine for cell wall synthesis during aerial growth (perhaps also feeding into mycothiol biosynthesis, see below). This model does not account for all the conserved genetic linkage of whiA, but it is consistent with the apparent inability of aerial hyphae of whiA mutants of streptomycetes to stop growing and switch to sporulation (Chater, 1972).

Sporulation-specific cell division

- SsgA, SsgB, FtsZ: septation sites
- ParA, ParB, FtsK: DNA segregation (mutants have incomplete spore chromosomes)
- Other proteins: SmeA, SffA, HupS, sIHF, Smc, Dps



Now that we have mentioned FtsZ, let's have a brief look at how septation and DNA segregation are believed to work.
SsgA, SsgB and FtsZ all together define septation sites along the hypha, and then participate in septa formation.
ParA, ParB, and FtsK assist with proper DNA segregation. Mutants lacking these genes have chromosome fragments in spores.
There are more proteins involved in the process, but we are not going to look into them right now.

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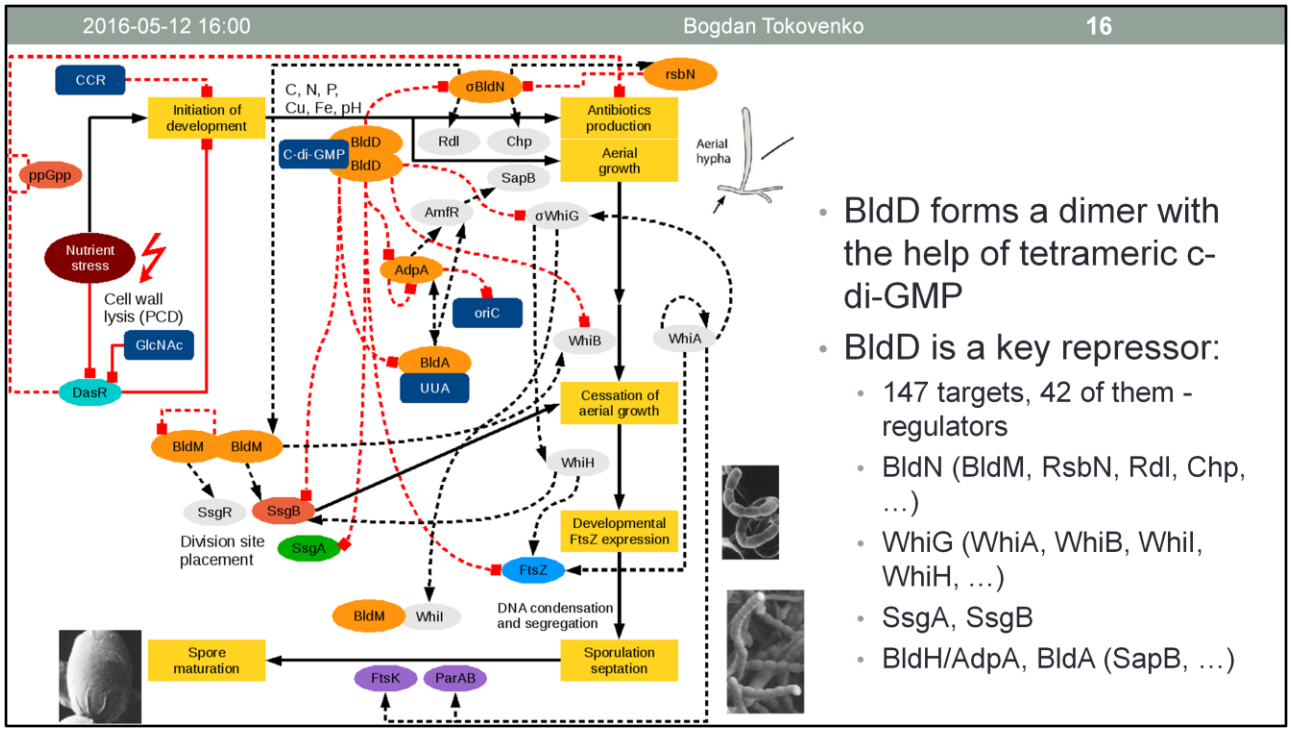
It is therefore unclear how streptomycetes avoid DNA damage in the multinucleoid hyphae. Elegant work on DNA partitioning revealed the important role of the ParAB proteins in DNA segregation during growth and development

FtsK helps to avoid "guillotining" of the DNA by pumping chromosomes into the spore compartments prior to septum closure, and ftsK mutants frequently generate spores with incomplete chromosomes (302–304). Other proteins that should be considered are SmeA and SffA, which play key roles in DNA translocation during sporulation (302), and also the DNA-packaging proteins HupS (305), sIHF (306, 307), Smc (308), and Dps (309).

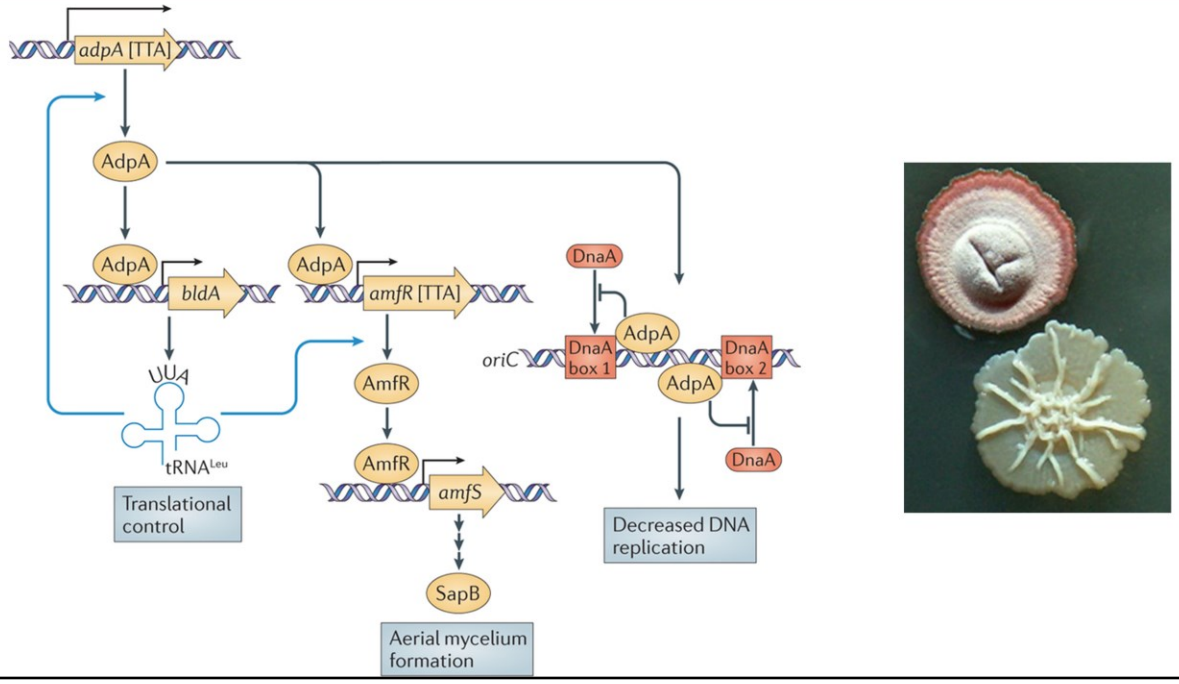
Model for the control of sporulation-specific cell division in Streptomyces.

When sporulation starts, SsgA localizes dynamically in young aerial hyphae, while SsgB and FtsZ are still diffuse at this stge.
At this point, ParA is constrained to the hyphal tip.
During early cell division, SsgA and SsgB colocalize temporarily at either side of the aerial hyphae, with ParA extending downward as filaments along the aerial hypha.
ParB complexes are then formed over the uncondensed chromosomes, while FtsZ assembles in spiral-like filaments.
Subsequently, FtsZ and SsgB colocalize and stay together until FtsZ disperses, whereby SsgB recruits FtsZ and stimulate its polymerization into protofilaments.
The way the SsgB-FtsZ complex is tethered to the membrane in the absence of a membrane domain in either protein is unclear, but a likely role is played by the SepG protein (SCO2078 in S. coelicolor) encoded by a gene upstream of divIVA (L. Zhang, J. Willemse, D. Claessen, and G. P. Van Wezel, unpublished data).
Z-rings are then formed at the sporulation stage, followed by chromosome condensation and segregation and the production of sporulation septa.
SsgA eventually marks the future germination sites. The figure was adapted from references 277 and 316.

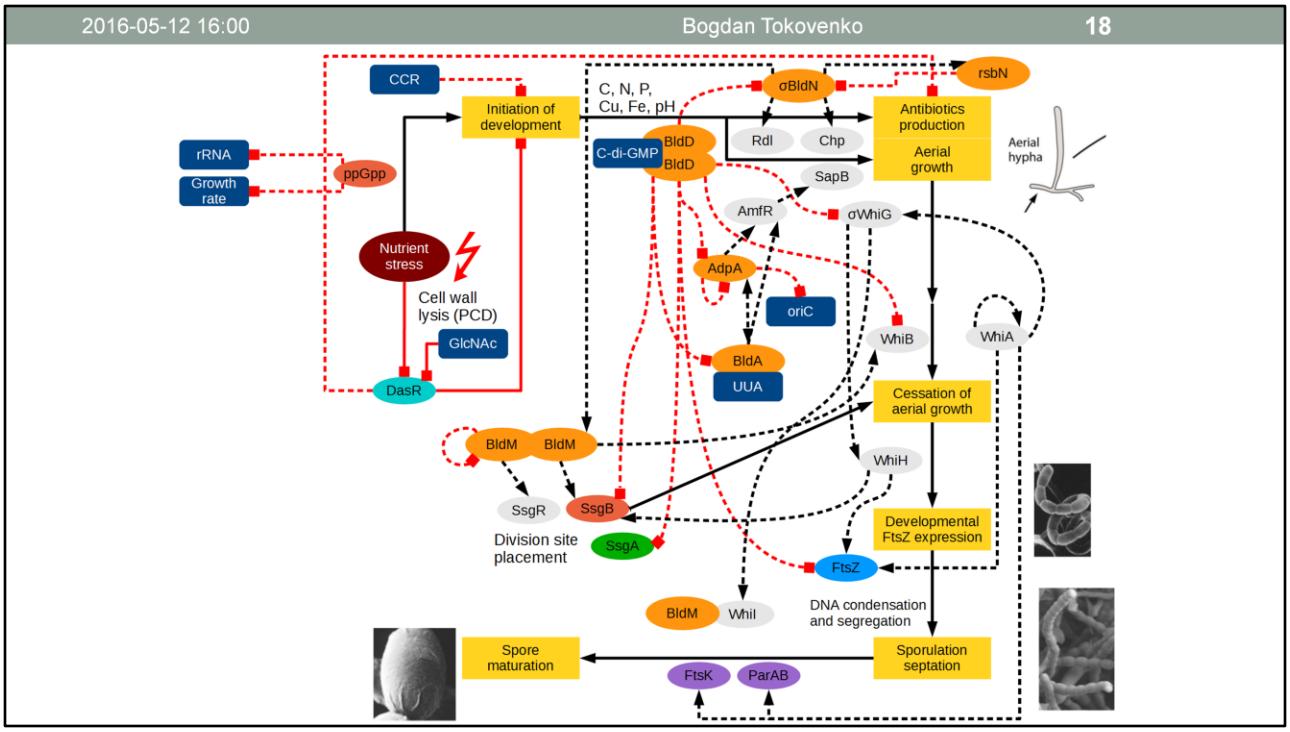


We are almost done now.
We have looked at all the development steps and their details, and now we are going to look at the center of regulation.
And here we see a prominent key repressor, BldD.
It is only functional as a homodimer formed with the help of secondary messenger tetrameric cyclic di-guanylate.
It has over 147 targets, of them 42 are regulators themselves.
It reppresses BldN, and thus rodlins, chaplins, but also BldM, which we'll talk about in a minute.
It represses sigma factor WhiG and also WhiB, which means the entire Whi cascade – down to FtsZ, and partitioning proteins – are also repressed.
It represses SsgA and SsgB.
And finally, it represses both BldA and BldH.

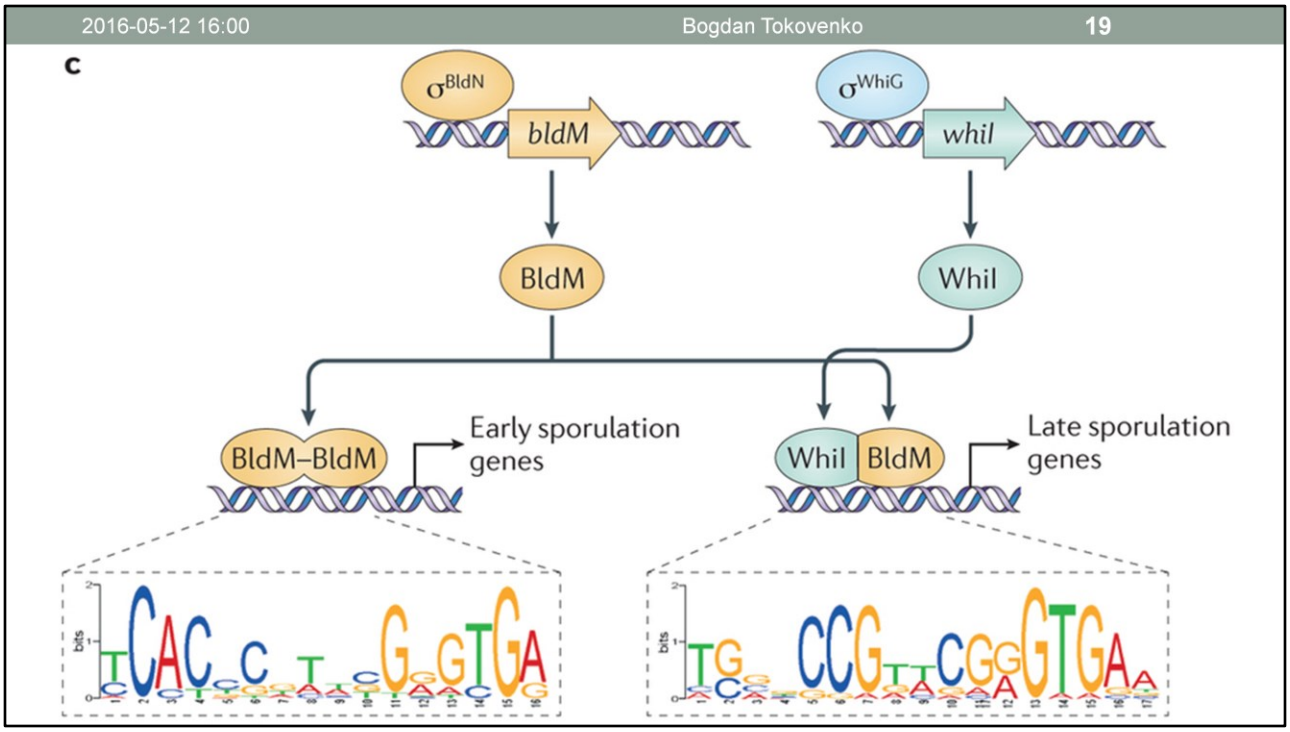
// cyclic di-guanylate



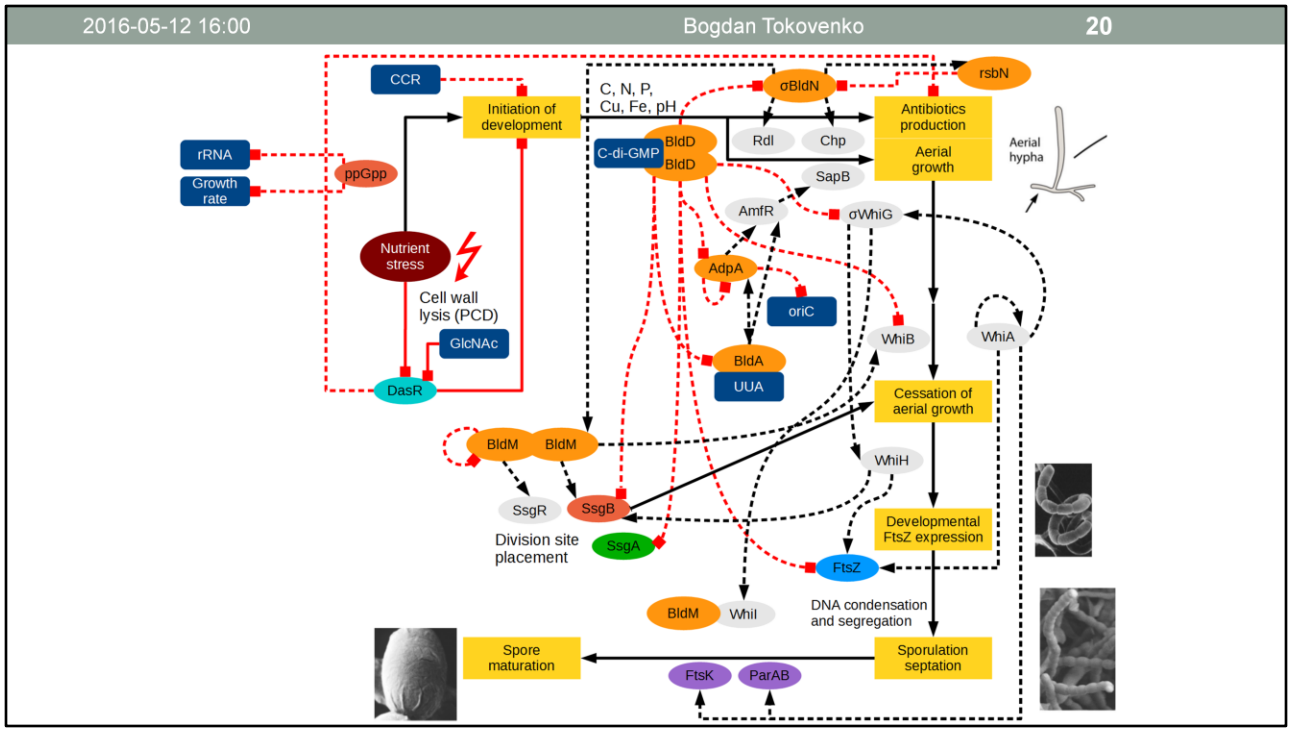
BldH is also known as AdpA.
It controls BldA, and is controlled by BldA.
BldA encodes a Leucine tRNA for a TTA codon, and thus translationally controls 147 genes in S. coelicolor.
bldA-minus coelicolor mutants also have bald phenotype
But the most prominent, the most consereved target is AdpA.
AdpA is an activator of SapB protein production, and also an inhibitor of DNA replication.



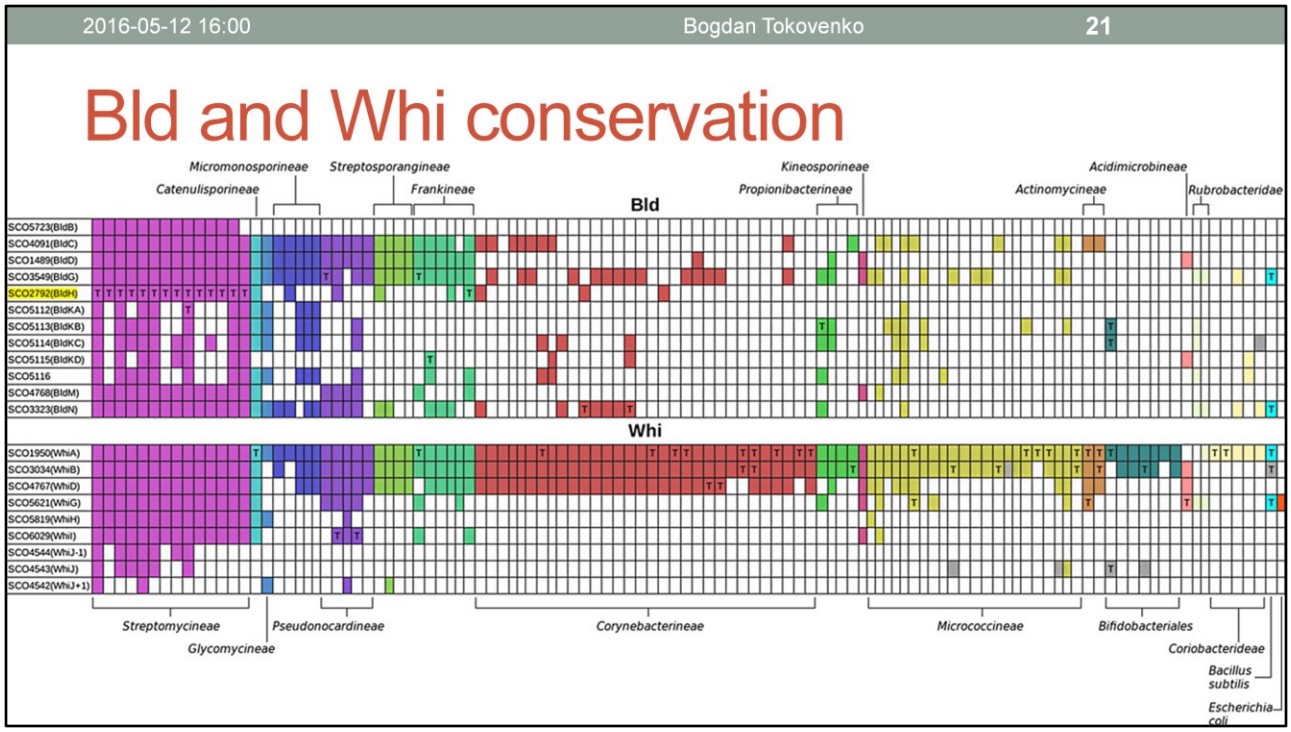
The only part we haven't looked at is BldM here and BldM here.



This is believed to be an overlap of two signaling cascades, bld and whi.
BldM homodimer is believed to control the expression of SsgA and B – at the step of developmental FtsZ expression.
Heterodimer of BldM and WhiI controls later stages of sporulation – when actual septation occurs and spore matures.



You have to keep in mind, that this diagram is compiled from experimental data for all three model organisms.
There are clearly common elements, but there are also parts which differ.



To illustrate the differences, here is a plot of Bld and Whi genes across many Actinobacteria.
Bld system is well-conserved only in Streptomyces, while the Whi system (specifically A, B and D), despite being apparently less important, is conserved across many more Genii.

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Distribution of probable orthologues of Bld and Whi proteins of Streptomyces coelicolor encoded in more than 100 actinobacterial genomes, as detected by reciprocal BLASTP best hits. Each column represents one genome, and the genomes are grouped and coloured to indicate subgroup relationships (e.g. Corynebacterineae columns, including Mycobacterium, Nocardia and Corynebacterium, were coloured Indian red). Grey boxes indicate reciprocal hits falling below the minimal criteria adopted for orthology. White boxes indicate the absence of a reciprocal hit. The yellow highlighted SCO genes contain a TTA codon, and the presence of TTA codons in apparent orthologues is indicated by a T in the coloured box. A similar display of reciprocal BLASTP analysis of the entire S. coelicolor genome against the 111 genomes, with links to StrepDB, is available at http://streptomyces.org.uk/actinoblast/. The tables at that site allow clicking onto any coloured box to show the gene identifier together with minimal annotation, as well as information about the length of the overlap and the percentage identity. The sources of genomes are listed in Table 1 of Gao & Gupta (2012). Organisms were as

follows (in order across the tabulation). Magenta: Streptomycineae, S. Lividans TK24, S. viridochromogenes DSM 40736, S. scabiei 87.22, S. sviceus ATCC 29083, S. avermitilis MA-4680, S. griseoflavus Tu4000, S. Venezuelae ATCC 10712, S. griseus subsp. griseus NBRC 13350, S. hygroscopicus ATCC 53653, S. pristinaespiralis ATCC 25486, S. roseosporus NRRL 15998, S. albus G J1074, S. clavuligerus ATCC 27064, Kitasatospora setae KM-6054. Turquoise: Catenulispora acidiphila DSM 44928. Light blue: Stackebrandtia nassauensis DSM 44728. Dark blue: Salinispora, S. tropica CNB-440, S. arenicola CNS-205; Micromonospora, M. sp. L5, M. sp. ATCC39149, M. aurantiaca ATCC 27029. Purple: Saccharomonospora viridis DSM 43017; Saccharopolyspora erythraea NRRL 2338; Amycolatopsis mediterranei U32; Actinosynnema mirum DSM 43827; Thermobispora bispora DSM 43833. Yellow green: Streptosporangiumroseum DSM 43021; Thermomonospora curvata DSM 43183; Thermobifida fusca YX; Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111. Blue green: Acidothermus cellulolyticus 11B; Frankia, F. sp. EAN1pec, F. sp. CcI3, F. alni ACN14a; Geodermatophilus obscurus DSM 43160; Nakamurella multipartita DSM 44233. Rust red: Gordonia bronchialis DSM 43247; Nocardia farcinica IFM 10152; Segniliparus rotundus DSM 44985; Tsukamurella paurometabola DSM 20162; Rhodococcus, R. opacus B4, R. jostii RHA1, R. erythropolis PR4, R. equi 103S; Mycobacterium, M. vanbaalenii PYR-1, M. ulcerans Agy99, M. sp. Spyr1, M. sp. MCS, M. sp. KMS, M. sp. JLS, M. smegmatis str. MC 2 155, M. marinum M, M. leprae Br4923, M. gilvum PYR-GCK, M. abscessus ATCC 19977, M. avium subsp. paratuberculosis K-10, M. avium 104, M. Tuberculosis H37Rv, M. bovis AF2122/97; Corynebacterium, C. urealyticum DSM 7109, C. pseudotuberculosis FRC41, C. kroppenstedtii DSM 44385, C. jeikeium K411, C. glutamicum ATCC 13032 2, C. efficiens YS-314, C. diphtheriae NCTC 13129, C. aurimucosum ATCC 700975. Bright green: Nocardioides sp. JS614; Kribbella flavida DSM 17836; Propionibacterium, P. freudenreichii subsp. shermanii CIRM-BIA1, P. acnes KPA171202. Plum: Kineococcus radiotolerans SRS30216. Olive yellow: Beutenbergia cavernae DSM 12333; Cellulomonas flavigena DSM 20109; Brachybacterium faecium DSM 4810; Kytococcus sedentarius DSM 20547; Intrasporangium calvum DSM 43043; Jonesia denitrificans DSM 20603; Clavibacter michiganensis subsp. michiganensis NCPPB 382; Leifsonia xyli subsp. xyli str. CTCB07; Microbacterium testaceum StLB037; Arthrobacter, A. sp. FB24, A. phenanthrenivorans Sphe3, A. chlorophenolicus A6, A. aurescens TC1, A. arilaitensis Re117; Kocuria rhizophila DC2201; Micrococcus luteus NCTC 2665; Renibacterium salmoninarum ATCC 33209; Rothias, R. mucilaginosa DY-18, R. dentocariosa ATCC 17931; Xylanimonas cellulosilytica DSM 15894; Sanguibacter keddieii DSM 10542; Tropheryma whipplei str. Twist. Brown: Mobiluncus curtisii ATCC 43063; Arcanobacterium haemolyticum DSM 20595. Cyan: Gardnerella vaginalis ATCC 14019; Bifidobacterium, B. longum NCC2705, B. longum DJO10A, B. dentium Bd1, B. bifidum PRL2010, B. animalis subsp. lactis Bl-04, B. adolescentis ATCC 15703. Pink: Acidimicrobium ferrooxidans DSM10331. Pale grey green: Conexibacter woesii DSM14684; Rubrobacter xylanophilus DSM9941. Beige: Atopobium parvulum DSM

20469; Cryptobacterium curtum DSM 15641; Eggerthella lenta DSM 2243; Olsenella uli DSM 7084; Slackia heliotrinireducens DSM 20476.

WHAT IS NOT KNOWN?

Remaining unknowns

- Many gaps in the grand scheme
- Lack of coherence and unresolved complexity
- Signals controlling BldD? (other than c-di-GMP)
- BldD is a repressor…
  - but bldD^{-} mutant is… bald!
  - Possibly because of RbsN-BldN cascade?

If by now you feel that you understand all the parts,
but do not quite understand how it all works together, then I have succeeded,
as this is a correct reflection of the current state of sporulation regulation knowledge.

The grand scheme has lots of gaps.
It is also not sufficiently clear, and constructed from 3 model species.
One of the mysteries is that BldD is a pleiotropic repressor, but BldD mutants are also bald!
A possible explanation is that de-repression of RbsN anti-sigma factor blocks BldN and thus prevents sporulation.

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The rsbN gene of S. venezuelae has its own promoter, which is BldN-dependent, and is also a BldD target (den Hengst et al., 2010; Bibb et al., 2012). As a bldD mutant might therefore be expected to overexpress rsbN, the resulting increase in anti-BldN activity might interfere with the expression of BldN-dependent genes and contribute significantly to the bald phenotype of bldD mutants.

References

- Taxonomy, Physiology, and Natural Products of Actinobacteria
- Synthesis of the spore envelope in the developmental life cycle of Streptomyces coelicolor
- Developmental biology of Streptomyces from the perspective of 100 actinobacterial genome sequences
- c-di-GMP signalling and the regulation of developmental transitions in streptomycetes

I have mostly used these sources to prepare the presentation.
I hope you found it useful,

THANK YOU FOR YOUR ATTENTION

And thank you for your attention.

Additional material

bldC

- bldC encodes an apparently single-domain small protein with a helix-turn-helix of the MerR type (Hunt et al., 2005)
- implied to control the Whi-genes cascade

bldD

- highly pleiotropic transcription factor that controls hundreds of development-related genes
- bldD encodes a protein distantly related to SinR, a transition state regulator of B. subtilis (Elliot et al., 1998)
- BldD orthologues always show high conservation and local synteny
- implied to control the Whi-genes cascade

Let's look at the individual regulators.
bldC orthologues are somewhat less highly conserved and are located among less extensively conserved genes

bldD

- The BldD regulon has been subjected to detailed analysis by immunoprecipitation of in vivo BldD–DNA complexes, which showed that BldD directly targets about 147 transcription units in vegetative, liquid-grown S. coelicolor (den Hengst et al., 2010).
- These include 42 regulatory genes, several of which are developmental (bldA, bldC, bldD, bldH, bldM, bldN, whiB, whiG). These are all repressed by BldD.

These are all repressed by BldD. Based on a consensus sequence derived from these ChIP-chip data, BldD recognition sequences were found upstream of many of the same genes not only in other streptomycetes, but also in other sporulating actinobacteria (den Hengst et al., 2010). Such species included Saccharopolyspora erythraea, an organism in which a constructed bldD mutant had a bald colony phenotype (Chng et al., 2008). Thus, BldD orthologues appear to coordinate development in diverse sporulating actinomycetes, perhaps preventing the expression of genes for morphological differentiation and antibiotic production during vegetative growth and connecting the regulons of other regulators of these processes (den Hengst et al., 2010). BldD orthologues in simpler actinomycetes might well have roles both during growth, to repress functions associated with entry into stationary phase, and in stationary phase, in coordinating the expression of different stationary phase regulatory genes.

bldD

- Despite the extensive characterisation of BldD and its regulon, it is not understood why, if BldD represses developmental functions, bldD mutants are bald rather than hypersporulating (but see the slides on BldN below)
- there is no information about any signals that BldD might respond to (an initial search for possible proteins interacting with BldD was reported to have had negative results: den Hengst et al., 2010)

It has been suggested that an interaction of BldD with another sporulation regulatory protein, BldB, could determine the rate of turnover of BldD (McCormick & Fl€ ardh, 2012); but BldB is confined to streptomycetes, so it could not fulfil such a role in other complex actinomycetes such as Sac. erythraea

bldD

- BldD represses the sporulation-specific promoter of ftsZ (den Hengst et al., 2010).

bldG

- A member of the highly-paralogous family of anti-anti-sigma factors.
- Indeed, BldG influences the activity of the stress-responsive sigma factor SigH in S. Coelicolor (Sevcikova et al., 2010; Takano et al., 2011), and the anti-anti-sigma/antisigma/sigma interactions of this general type have considerable potential for promiscuity in Streptomyces (Kim et al., 2008b; Sevcikova et al., 2010; Takano et al., 2011).

Some BldD-regulated bld genes of S. coelicolor belong to classes of genes that are widespread and often represented by multiple paralogues in any one genome. For such genes, it can be difficult to be confident that reciprocal BLASTP hits between genomes are meaningful, particularly when the extent of amino acid identity falls well below
levels that are typically seen for conserved housekeeping genes. For example, the general kind of anti-anti-sigma factor to which the BldG protein belongs is almost universally found among both Gram-positive firmicutes and actinobacteria; so the presence in some actinobacteria of BldG reciprocal best hits with identities only in the 20–
40% range is relatively uninformative (in fact, such low-scoring hits did not show the local synteny seen with those having higher identities). Evidently, anti-anti-sigmas (and corresponding antisigmas) of this general class were present in the ur-actinobacterium, giving rise to the possibility of subtle control of sigma factor activity by signals
that might include morphological checkpoints

bldN

- RNA polymerase sigma factor
- which control early events during sporulation (although bldN is also strongly transcribed during aerial growth)
- One of about 50 S. coelicolor ECF sigma factors (Bibb et al., 2000; den Hengst et al., 2010).
- A direct target of BldD.
- At least in S. venezuelae, BldN is a direct activator of the genes for chaplins (and their associated rodlins)
- This emergence into the air has been suggested as a trigger for the sporulation pathway controlled by the whi genes (Claessen et al., 2006).

rsbN

- in S. venezuelae, an anti-sigma factor controlling BldN is encoded by the adjacent gene, termed rsbN (= SCO3324 in S.coelicolor; Bibb et al., 2012).
- In BLASTP analysis, a reciprocal best hit to rsbN is found next to nearly all bldN orthologues in actinomycete genomes;
- but, strikingly, the RsbN-like proteins are much more divergent than their BldN target or most other families of orthologous proteins of actinobacteria

It has been demonstrated that, in S. venezuelae, an anti-sigma factor controlling BldN is encoded by the adjacent gene, termed rsbN (= SCO3324 in S.coelicolor; Bibb et al., 2012). In BLASTP analysis, a reciprocal best hit to rsbN is found next to nearly all bldN orthologues in actinomycete genomes; but, strikingly, the RsbN-like proteins are much more divergent than their BldN target or most other families of orthologous proteins of actinobacteria (Fig 6). We speculate that this may imply differences in the signal responsiveness of different RsbN proteins, thereby contributing to the differences between different organisms in the interplay of ecology and development: in other words, they may be potential agents of speciation.

rsbN

- The rsbN gene of S. venezuelae has its own promoter, which is BldN-dependent, and is also a BldD target (den Hengst et al., 2010; Bibb et al., 2012). As a bldD mutant might therefore be expected to overexpress rsbN, the resulting increase in anti-BldN activity might interfere with the expression of BldN-dependent genes and contribute significantly to the bald phenotype of bldD mutants.

bldM

- Encodes an orphan response regulator (Molle & Buttner, 2000)
- Target of BldN.
- The distribution of convincing reciprocal hits to bldM is closely similar to that of bldN hits, suggesting that the BldN to BldM regulatory step was established very early in the evolution of actinomycete complexity.

The distribution of BldM was even more closely similar to that of orthologues of another developmental orphan response regulator, WhiI (Fig. 3).

whiG

- RNA polymerase sigma factor, which controls early events during sporulation
- an orthologue of an ancient sigma factor, regulates more recently acquired regulatory genes specific to aerial sporulation
- none is more widespread across the bacterial kingdom than whiG
- WhiG protein is a sigma factor critically involved in the decision of aerial hyphae to sporulate, and in its absence, colonies develop long, thin aerial hyphae and entirely fail to sporulate

It is orthologous with the extensively studied FliA of E. coli and SigD of B. subtilis, which are involved in regulating genes important for motility and chemotaxis, adhesion and invasion, some aspect(s) of cell wall remodelling and cyclic di-AMP hydrolysis (Helmann, 1991; Claret et al., 2007; Luo & Helmann, 2012).

whiG targets

- RNA polymerase containing WhiG sigma directly activates two regulatory genes involved in slightly later stages in sporulation (whiH, Ryding et al., 1998; whiI, Ainsa et al., 1999).
- The other WhiG target regulatory gene, whiH, encodes an autoregulating GntR-like protein (Ryding et al., 1998; Persson et al., 2013) confined to streptomycetes and their closest relatives (Catenulispora and Kitasatospora).

WhiI protein resembles response regulators,
many of which are part of two-component systems in which activity of the response regulator is determined by its phosphorylation by a partner sensor kinase. WhiI, however, does not have a known partner kinase, being one of 13 ‘orphan’ response regulators present in S. coelicolor (Hutchings, 2007), and lacks key residues normally required for phosphorylation (Tian et al., 2007). It occurs almost exclusively in developmentally complex WhiG-containing actinomycetes and is absent from WhiG-containing, morphologically simple, motile actinobacteria; but both WhiG and WhiI are absent from many mycelial actinomycetes whose sporulation does not involve the formation of chains of spores on long aerial hyphae (Frankia, Micromonospora, Salinispora, Thermobispora, Nocardiopsis, Thermobifida, Streptosporangium and Thermomonospora).

whiA

- WhiA orthologues are not confined to actinobacteria: one is present in most Gram-positive bacteria, including all actinobacteria except Acidimicrobium ferrooxidans.
- WhiA showed in vitro DNA binding to its own promoter and to a sporulation-activated promoter of the parAB operon (Kaiser & Stoddard, 2011), both of which are also WhiA-dependent in vivo (Jakimowicz et al., 2006).

whiA

- The whiA sporulation-specific promoter could be transcribed in vitro by WhiG-containing RNA polymerase (Kaiser & Stoddard, 2011), in contradiction of an earlier result (Ainsa et al., 2000).
- WhiA exerted a modest inhibitory effect on this transcription and showed some evidence of direct interaction with WhiG in a pull-down experiment involving the two purified proteins (Kaiser & Stoddard, 2011). Although not conclusive, this is the first suggestion of direct interplay between the WhiG- and WhiA-dependent parts of the sporulation regulatory cascade, previously thought to be separate (Chater, 1998; Fl€ ardh et al., 1999).

whiA

- whiA and the upstream three genes form a cluster that is highly conserved in actinobacteria and even in B. subtilis. This putative operon is probably responsible for a low level of whiA (SCO1950) expression during growth (Ainsa et al., 2000). The three upstream genes encode apparently unrelated deduced functions

whiB

- A phenotype identical to that of whiA mutants results from mutations in whiB (SCO3034)
- Mutation of the whiB orthologue (whmD) of Mycobacterium smegmatis indicated a likely role in cell division that could represent its core activity (Gomez & Bishai, 2000).
- There are strong two-way transcriptional influences (not necessarily direct) between whiA and whiB (Jakimowicz et al., 2006), but little is known about other possible WhiB targets.

whiH

- which controls the onset of sporulation-specific cell division

wblX (whiB-like)

- Orthologues of four other Wbl proteins (WblA, WblC, WhiD and WblE) occur in most actinomycetes (Figs 3 and 4), even though WblA and WhiD have developmental roles in S. coelicolor: WblA plays a key part in the transition of aerial hyphal initial branches to a sporulation-directed fate (wblA mutants have thin aerial hyphae often embedded in an extracellular matrix, with only occasional spore chains: Fowler-Goldsworthy et al., 2011); and mutants lacking WhiD have defects at a later stage, having thin-walled spores and uncontrolled sporulation septation (McVittie, 1974; Molle et al., 2000).

If MSNO was significant for early actinobacteria that emerged before the evolution of complex eukaryotes that produce NO as a defence and signalling molecule, NO may be an endogenous signal molecule in actinobacteria, which in Streptomyces fulfils roles in development (and in any other general physiological changes influenced by Wbl proteins).

whiJ

- SCO4543
- a deduced DNA-binding protein (Gehring et al., 2000; Ainsa et al., 2010).
- whiJ-like genes are widely present in complex actinobacteria, but they are absent from morphologically simple ones (corynebacteria, mycobacteria, rhodococci, propionibacteria and micrococci except Beutenbergia and Intrasporangium) and from nonactinobacterial bacteria.

Most mycelial actinomycetes have two or three WhiJ paralogues, but K. setae has five, and all streptomycetes have more than 10, sometimes more than 20.

whiJ-associated

- Two kinds of immediately neighbouring genes:
- one kind encoding very small DNA-binding proteins (i.e. like SCO4542), and the other
- encoding proteins with features like antisigma factors (e.g. SCO4544) (Gehring et al.,2000; Ainsa et al., 2010).

whiJ

- Certain mutations in whiJ gave rise to a white-colony appearance caused by a deficiency in sporulation, although the complete deletion of whiJ had no obvious phenotypic consequences (Ainsa et al., 2010).
- WhiJ acts mainly to repress reproductive development until a suitable signal has been perceived via the SCO4542 DNA-binding protein, which would then directly interact with WhiJ to relieve repression (Ainsa et al., 2010).

A mutant lacking the whiJ-neighbouring gene SCO4542, encoding a predicted small DNA-binding protein, had a bald colony phenotype and overproduced the pigmented antibiotic actinorhodin. This phenotype was entirely suppressed by the co-deletion of whiJ itself. Putting these observations together, it was suggested that WhiJ acts mainly to repress reproductive development until a suitable signal has been perceived via the SCO4542 DNA-binding protein, which would then directly interact with WhiJ to relieve repression (Ainsa et al., 2010). It is thought that WhiJ mediates its effects both on the emergence of aerial hyphae and, separately, on their further differentiation into spore chains. There is no information about the direct or indirect targets of WhiJ regulation or about the role of the antisigma-like protein (SCO4544).

bldB

- encodes a diverged member of the SCO4542 family, but is an ‘orphan’ lacking neighbouring whiJ- or SCO4544-like genes.
- bldB is the only classical bld gene to be confined to, yet universal among, streptomycetes (Fig. 3).
- We speculate that the bald phenotype of bldB mutants could imply a promiscuous interaction of BldB with WhiJ-like proteins encoded elsewhere in the genome and that this may be connected with the large numbers of such proteins found in streptomycetes.

bldH

- A major target of bldA-mediated translational control is bldH (adpA), which encodes an important global regulator of development and antibiotic production (212–215). Transcription of adpA is activated in response to the gamma-butyrolactone A-factor in S. griseus and to the related molecule SCB1 in S. Coelicolor (216–220).
- An interesting feedback loop exists whereby the translation of the adpA mRNA depends on BldA (221, 222), while AdpA in turn controls bldA transcription (223).

bldH

- Target of BldD (den Hengst et al., 2010).
- In S. griseus it is the agent of the effects of the hormone-like A-factor (Horinouchi, 2002).
- It comprises a structurally characterised C-terminal AraC/XylS-like DNA-binding domain (Yao et al., 2012) and an N-terminal domain that may sense adenine nucleotides (Wolanski et al., 2012; Liu et al., 2013a).

It plays a central role in the decisions leading to colony differentiation, notably affecting extracellular functions such as protease cascades, extracellular morphogenetic peptides and secondary metabolism (Akanuma et al., 2009; Chater et al., 2010; Higo et al., 2012), but also contributing to the regulation of DnaA-mediated chromosome replication initiation (Wolanski et al., 2012).

bldH

- The regulation of adpA in streptomycetes is remarkably complex (reviewed in detail in Liu et al., 2013a). It involves at least three levels of control:
- transcriptional [autorepression (Kato et al., 2005), repression by BldD (den Hengst et al., 2010), repression by gamma-butyrolactone-binding proteins (Horinouchi, 2007; Xu et al., 2009)];
- mRNA processing by RNaseE (Xu et al., 2010); and
- mRNA translation (Nguyen et al., 2003; Takano et al., 2003).

Translational regulation is via a very rare UUA codon in the adpA mRNA, falling between the segments encoding the two domains of AdpA. UUA is the only one of the six leucine codons to comprise only A and U residues, so the corresponding TTA codon is comparatively rare in GC-rich genomes – it occurs in only 147 chromosomal genes in S. coelicolor (Li et al., 2007). UUA codons have a special regulatory role in Streptomyces, as indicated by the finding that mutants (bldA) in the gene for the UUA-reading tRNA grow well, but fail to form aerial mycelium or some antibiotics (Merrick, 1976; Lawlor et al., 1987). adpA is the only gene that has a TTA codon in all the streptomycetes analysed (Fig. 3; Table 2; Chater & Chandra, 2008), a feature also found in the adpA orthologue in Kitasatospora setae.

TTA bldH

- The TTA codon in adpA was shown by mutagenesis to be the main (but not entire) cause of the Bld phenotype of bldA mutants of S. coelicolor (Nguyen et al., 2003; Takano et al., 2003).
- A study of S. griseus and S. coelicolor has shown that the abundance of bldA tRNA is important in determining whether AdpA reaches levels sufficient to activate development and, remarkably, that there is a mutual feedforward mechanism in which AdpA activates bldA transcription (Higo et al., 2011).

However, the adpA-like genes of other actinomycetes, including Catenulispora acidiphila (the closest genome-sequenced relative of Streptomyces and K. setae), are nearly all TTA-free (in the single exception, Nakamurella multipartita, the TTA codon is not located in the interdomain-coding region, but close to the 3'-end of the gene). Thus, bldA-adpA interplay was apparently established after node 7 (Fig. 2), branching to Catenulispora, but before the Streptomyces and Kitasatospora lines diverged (node 8).

TTA

- The broader developmental significance of bldA may not extend beyond Streptomycineae, as in non-Streptomycineae genomes TTA codons do not show the positional bias towards the start of genes that is observed in streptomycetes, and sometimes occur in conserved growth-associated genes (Chater & Chandra, 2008).
- Interestingly, there is a strong target for BldD binding within bldA (den Hengst et al., 2010).

TTA

- As 27/29 of the TTA-containing genes/clusters were found only in Streptomycineae, we infer that these genes and their TTA codons have adaptive value to streptomycetes and not to other actinobacteria.

ftsZ

- Sufficient accumulation of FtsZ is required to support sporulation, and developmental ftsZ transcription is largely dependent on the “early” whi regulatory genes whiA, whiB, whiG, whiH, whiI, whiJ. Consistent with the notion that the control of ftsZ transcription may be a key event, at least in S. coelicolor, the non-sporulating phenotype of many of these early whi mutants could be overruled by constitutive expression of ftsZ during development. This also suggests that no other genes that are required for sporulation completely depend on these whi genes, at least not when FtsZ is overexpressed.

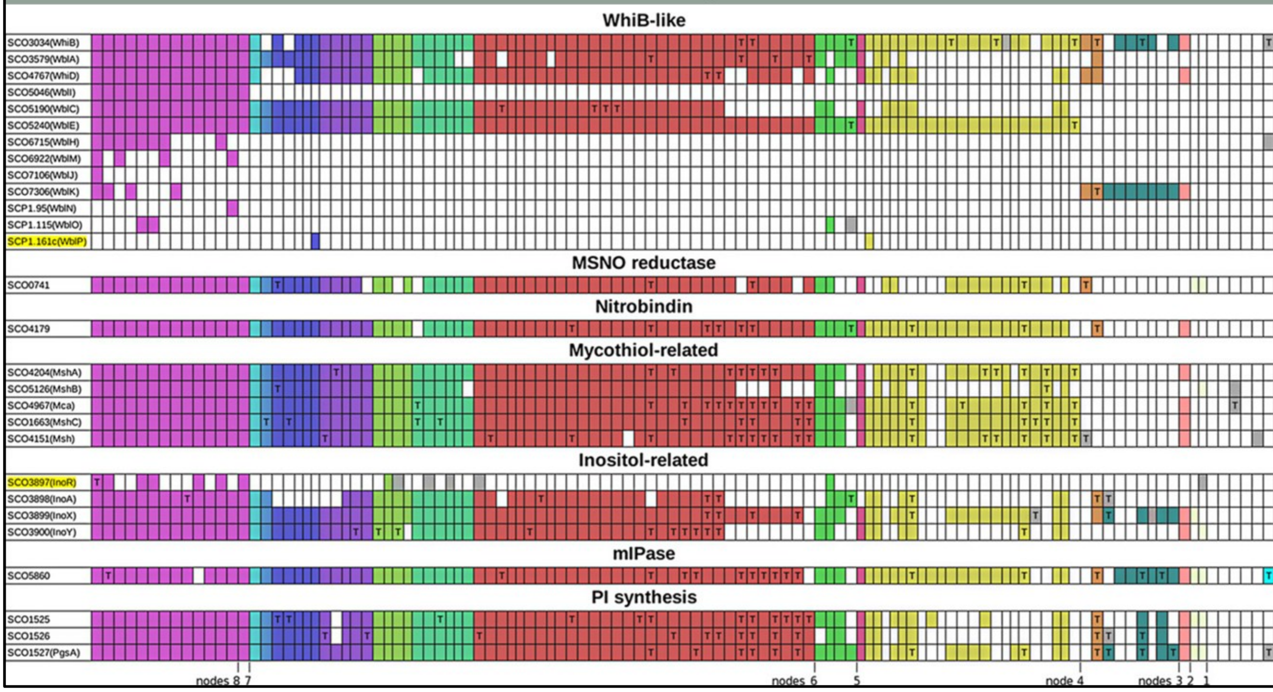
Constitutive FtsZ expression restores sporulation to a number of *whi* mutants, which suggests that *ftsZ* is transcriptionally regulated my some/several *whi* genes

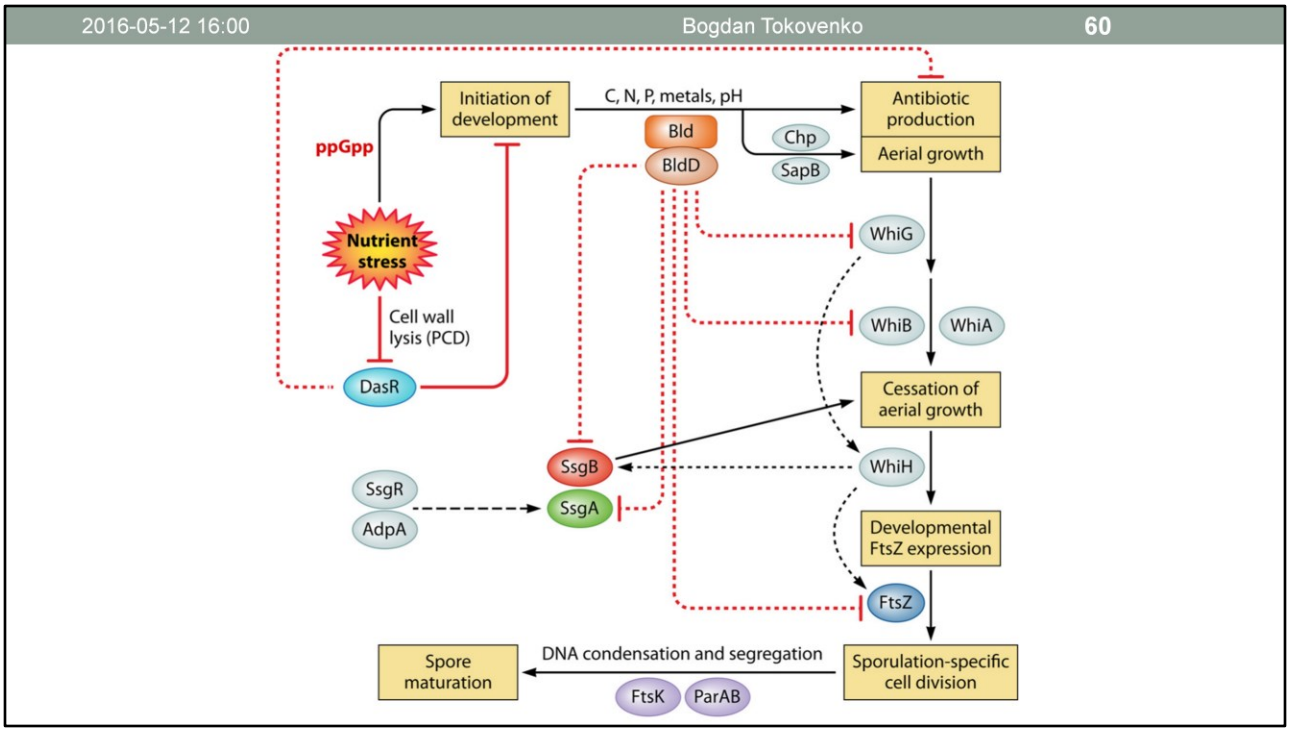
ssgA

- In terms of septum site localization, a key role is played by the SsgA-like proteins (SALPs), which only occur in sporulating actinobacteria (310, 311). SsgA activates sporulation-specific cell division (312, 313), and both ssgA and ssgB are required for sporulation (275, 314, 315).

ssgB

- The membrane associated SALP, SsgB, plays a prominent role in recruiting the tubulin homolog FtsZ to the division planes (Willemse et al., 2011). Formation of unigenomic spores from multi-genomic aerial hyphae requires the accurate and synchronized segregation of tens of chromosomes into pre-spore compartments. This process involves the partitioning system ParAB, the segregation protein FtsK and its homologue SffA together with the small SmeA protein which localizes SffA to the sporulation septa.


WhiB-like
SCO3034(WhiB)
SCO3579(WblA)
SCO4767(WhiD)
SCO5046(WblI)
SCO5190(WblC)
SCO5240(WblE)
SCO6715(WblH)
SCO6922(WblM)
SCO7106(WblJ)
SCO7306(WblK)
SCP1.95(WblN)
SCP1.115(WblO)
SCP1.161c(WblP)
MSNO reductase
SCO0741
Nitrobindin
SCO4179
Mycothiol-related
SCO4204(MshA)
SCO5126(MshB)
SCO4967(Mca)
SCO1663(MshC)
SCO4151(Msh)
Inositol-related
SCO3897(InoR)
SCO3898(InoA)
SCO3899(InoX)
SCO3900(InoY)
mIPase
SCO5860
PI synthesis
SCO1525
SCO1526
SCO1527(PgsA)
nodes 8 7
nodes 6
5
node 4
nodes 3 2 1



Major events during development of Streptomyces. Nutrient stress is a major trigger of development, leading to the accumulation of ppGpp, resulting in cessation of early growth and repression of the nutrient sensory DasR protein by cell wall-derived metabolites following PCD of the substrate mycelium. Bld proteins and environmental signals control the procession toward aerial growth and antibiotic production. The developmental master regulator BldD (when bound to tetrameric cyclic-di-GMP) represses the transcription of genes for many key developmental regulatory proteins, including WhiB, WhiG, SsgA, and SsgB, as well as FtsZ. Chaplins and SapB provide a supportive hydrophobic layer to allow aerial hyphae to become erect and break through the moist soil surface.

White proteins control aerial growth, whereby WhiAB and SsgB likely play a role in growth cessation. Eventually, FtsZ accumulates and localizes to septum sites in an SsgAB-dependent manner. Ladders of FtsZ are formed, which subsequently delimit the spore compartments. Chromosome condensation and segregation are followed by septum closure and spore maturation. The onset of antibiotic production typically correlates temporally to the transition from vegetative to aerial growth. Solid black arrows represent major transitions in development. Dark dotted lines indicate transcriptional control (arrows for activation, ovals for repression).