N-Acetylglucosamine-Mediated Inhibition of Siderophore Biosynthesis Counteracts the Suicidal Tendencies of *Streptomyces coelicolor*

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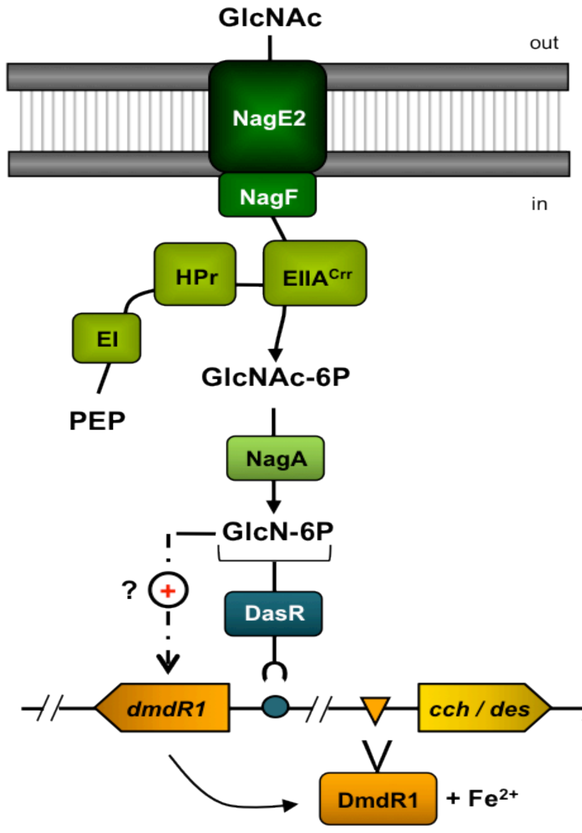
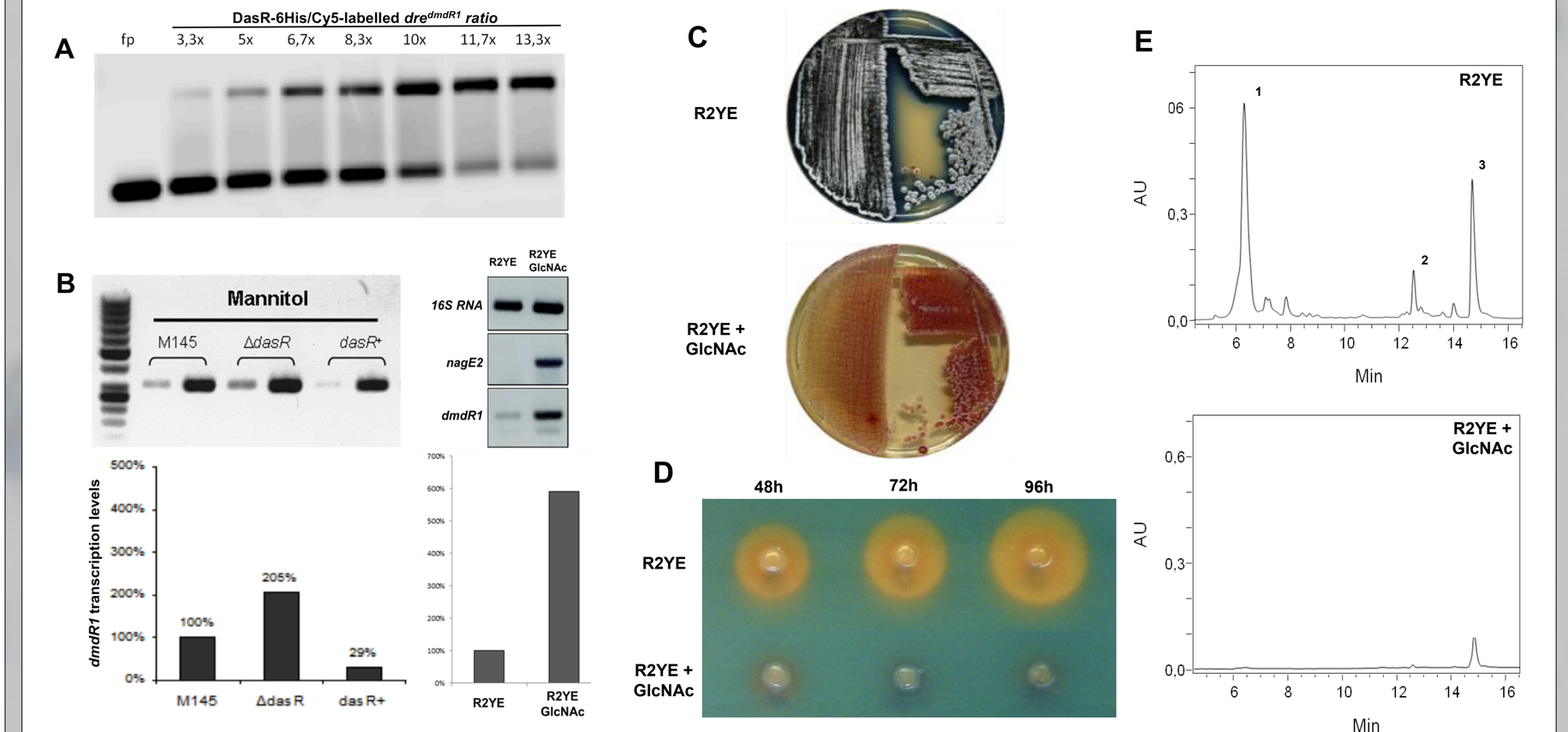
Introduction

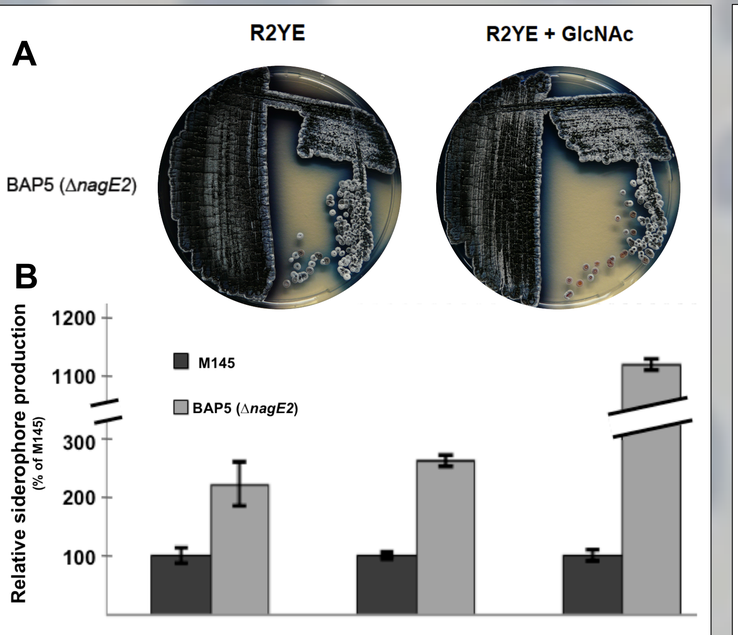
Iron is an essential element for all organisms as it is required for vital biological processes. To circumvent the low bioavailability of iron in the environment, microorganisms biosynthesize and excrete high-affinity iron chelators, known as siderophores, to scavenge iron by forming soluble complexes that can be imported into the cell. Because iron overload is toxic, siderophore synthesis is tightly regulated in order to maintain strict homeostasis[1]. In this work, we challenge the authoritative dogma according to which siderophore biosynthesis is inextricably tied to iron availability. Indeed, our *in silico* predictions suggest the existence of a regulatory mechanism independent of environmental iron concentration, in which siderophore-mediated iron uptake would be repressed by the peptidoglycan subunit N-acetylglucosamine (GlcNAc) in *Streptomyces coelicolor*. Furthermore, we propose an evolutionary-oriented explanation for this regulatory mechanism that might at first seem fortuitous. This approach could, *in fine*, bring to light alternative biological functions exerted by coelichelin and desferrioxamine, the siderophores of *S. coelicolor*[2].



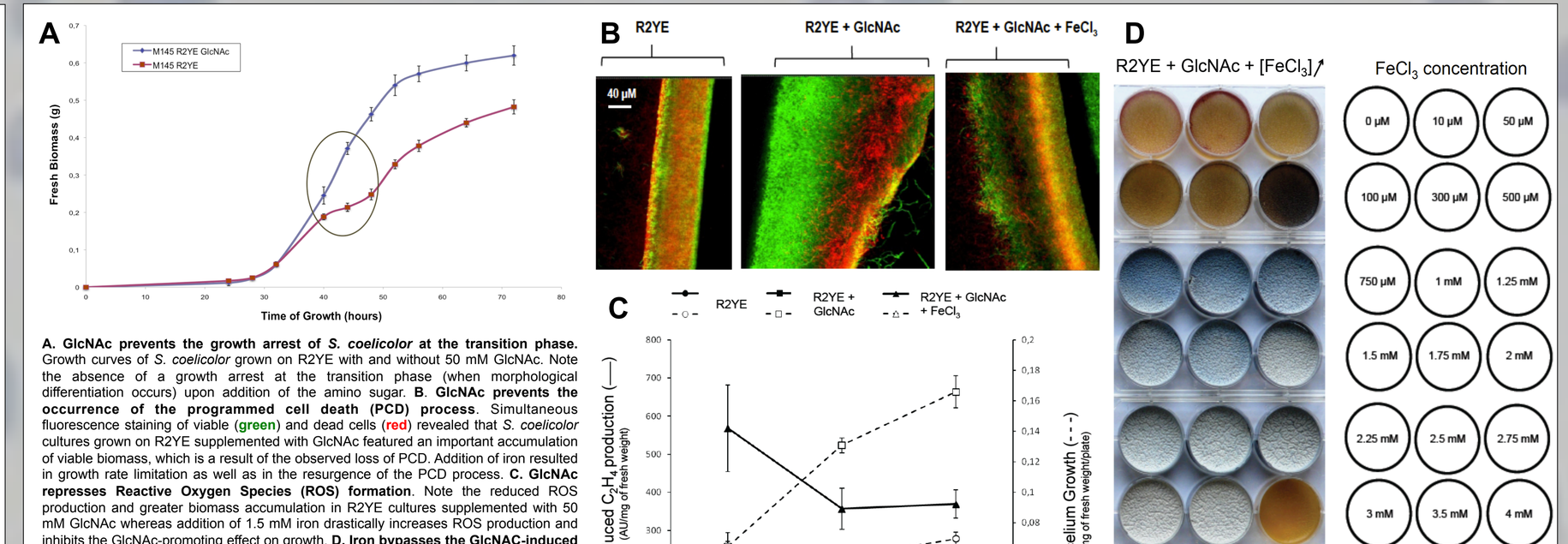
Predicted signaling cascade from GlcNAc transport to siderophore biosynthesis inhibition in streptomycetes. GlcNAc is transported and phosphorylated by the phosphotransferase system (PTS). The intracellular GlcNAc-6P is then deacetylated by NagA leading to GlcN-6P, the allosteric effector of the GlcNAc utilization regulator DasR. Once complexed to GlcN-6P, DasR interaction with the *dre* upstream of *dmdR1* (blue circle) is weakened, which leads to increased *dmdR1* expression. The over expression of *dmdR1* results in an increased transcriptional repression of siderophore biosynthetic clusters (*cch* and *des* for coelichelin and desferrioxamine biosynthesis, respectively) due to DmdR1-Fe^{2+} binding to 'iron boxes' identified upstream of *cch* and *des* clusters (yellow triangle).



A. DasR binds *dre*^{dmdR1}. EMSA performed with 15 pmol of Cy5-labelled *dre*^{dmdR1} probe (fp, free probe) and increasing concentrations of pure DasR-6His. **B. *dmdR1* expression is induced by N-acetylglucosamine and repressed by DasR.** Left panel: RNA samples were collected from *S. coelicolor* M145, the Δ*dasR* mutant and the *dasR* multicopy mutant (*dasR*$^+$) on MM mannitol agar plates with or without 50 mM GlcNAc. Non-saturated PCR products obtained after 27 and 31 cycles are presented in the first and second wells for each strain. Right panel: RNA samples were collected from *S. coelicolor* M145 on R2YE agar plates with or without 50 mM GlcNAc. The *16S RNA* and *nagE2* genes were used as positive and negative controls, respectively. **C. GlcNAc blocks *S. coelicolor* development at the vegetative mycelium stage and inhibits antibiotic production.** Phenotypes of *S. coelicolor* M145 grown for 96h on R2YE supplemented or not with 50 mM GlcNAc. **GlcNAc represses siderophore production.** Siderophores were extracted from M512 (Δ*actII-4/ΔredD* mutant) cultures grown on R2YE, with or without 50 mM GlcNAc, for 48, 72 and 96 hours. **D. CAS assay.** Wells made in the CAS medium were filled with the siderophore extracts. The increasing diameter of the halos corresponding to the R2YE cultures attest to the increase in siderophore production with time. Almost no iron chelating activity was observed in the extracts stemming from cultures containing GlcNAc. **E. HPLC analysis.** The R2YE and R2YE + GlcNAc samples extracted after 96 hours of growth were analyzed by HPLC. The three main peaks have been identified as ferricoelichelin **(1)** and ferrioxamines B **(2)** and E **(3)** by LC-MS analysis. Only a small amount of ferrioxamine E was detected in the R2YE + GlcNAc extract, while the other two iron chelators were produced at a basal level in the presence of N-acetylglucosamine.



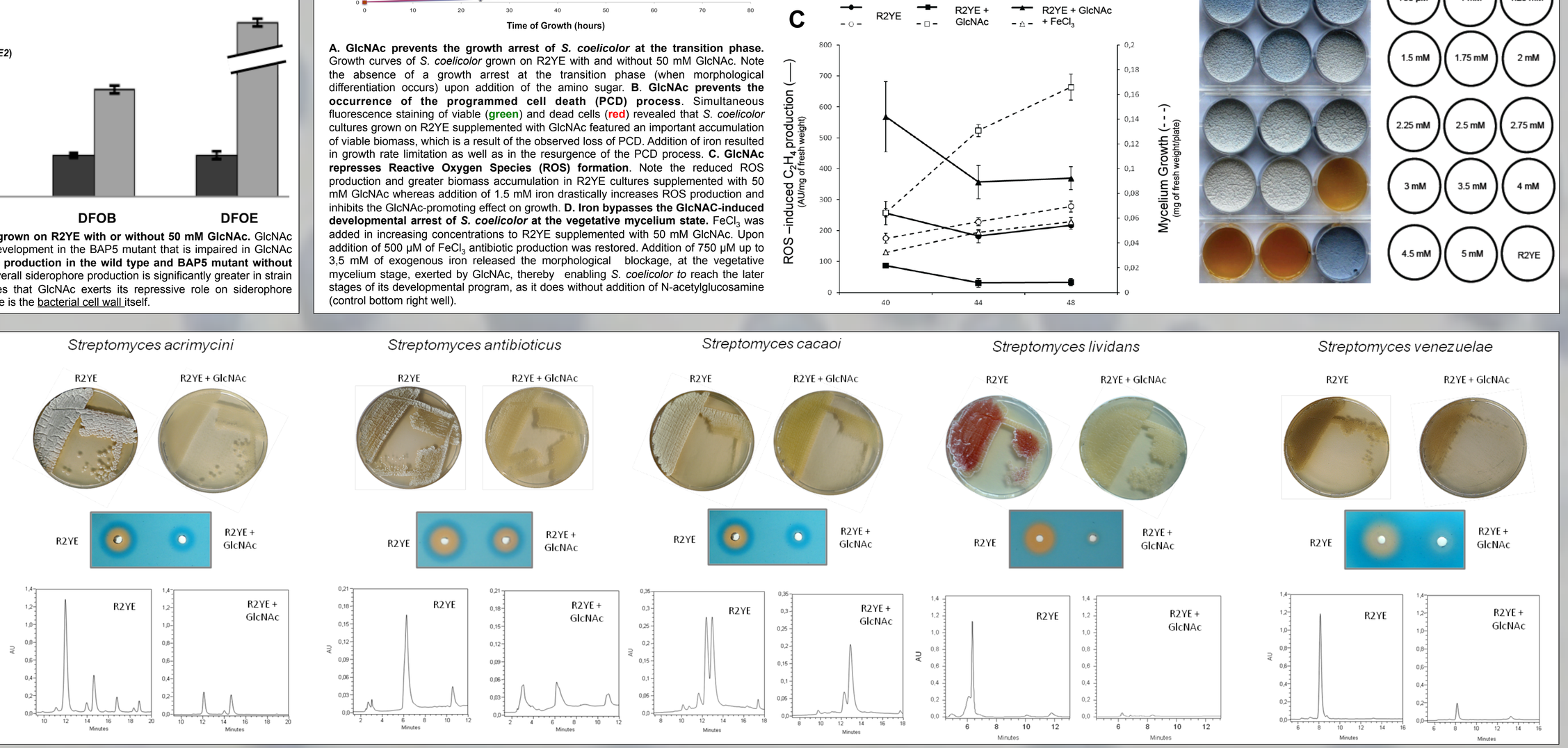
A. Phenotypes of the BAP5 mutant grown on R2YE with or without 50 mM GlcNAc. GlcNAc does not exert its blocking effect on development in the BAP5 mutant that is impaired in GlcNAc uptake. **B. Histogram of siderophore production in the wild type and BAP5 mutant without exogenous supply of GlcNAc.** The overall siderophore production is significantly greater in strain BAP5 compared to M145. This implies that GlcNAc exerts its repressive role on siderophore production when its sole possible source is the bacterial cell wall itself.



A. GlcNAc prevents the growth arrest of *S. coelicolor* at the transition phase. Growth curves of *S. coelicolor* grown on R2YE with and without 50 mM GlcNAc. Note the absence of a growth arrest at the transition phase (when morphological differentiation occurs) upon addition of the amino sugar. **B. GlcNAc prevents the occurrence of the programmed cell death (PCD) process**. Simultaneous fluorescence staining of viable (**green**) and dead cells (**red**) revealed that *S. coelicolor* cultures grown on R2YE supplemented with GlcNAc featured an important accumulation of viable biomass, which is a result of the observed loss of PCD. Addition of iron resulted in growth rate limitation as well as in the resurgence of the PCD process. **C. GlcNAc represses Reactive Oxygen Species (ROS) formation**. Note the reduced ROS production and greater biomass accumulation in R2YE cultures supplemented with 50 mM GlcNAc whereas addition of 1.5 mM iron drastically increases ROS production and inhibits the GlcNAc-promoting effect on growth. **D. Iron bypasses the GlcNAC-induced developmental arrest of *S. coelicolor* at the vegetative mycelium state.** $FeCl_3$ was added in increasing concentrations to R2YE supplemented with 50 mM GlcNAc. Upon addition of 500 µM of $FeCl_3$ antibiotic production was restored. Addition of 750 µM up to 3,5 mM of exogenous iron released the morphological blockage, at the vegetative mycelium stage, exerted by GlcNAc, thereby enabling *S. coelicolor to* reach the later stages of its developmental program, as it does without addition of N-acetylglucosamine (control bottom right well).

Conservation of the GlcNAc/ siderophore relationship in other streptomycetes. To further confirm the link between GlcNAc metabolism and iron homeostasis, several strains of streptomycetes were tested for their capacity to produce siderophores with GlcNAc as the main carbon and nitrogen source. Siderophores were extracted from these cultures and subjected to CAS assay and HPLC analysis. A strong reduction of iron-chelating molecules in all strains grown on R2YE supplemented with 50 mM GlcNAc was observed.

Interestingly, the addition of GlcNAc resulted in the blockage of the morphological development at the vegetative mycelium stage as well as in the inhibition of pigmented antibiotic synthesis for most strains tested.



Conclusion

In previous investigations, we demonstrated that actinorhodin and prodiginines, the pigmented metabolites produced by *S. coelicolor*, were under the control of DasR and GlcNAc via pathway specific regulators[3]. In this work, we provide evidence that biosynthesis of another category of microbial 'small molecules', the tris-hydroxamate siderophores coelichelin and desferrioxamine, is also controlled by the cell wall component GlcNAc. Our results demonstrate the existence of a siderophore production inhibitory pathway that is entirely independent of environmental iron availability[4] but is part of a genetically programmed cell death (PCD) mechanism associated with *Streptomyces* development.

We propose that the oxidative stress (ROS formation) generated by iron accumulation could initiate the PCD process. This hypothesis is strongly supported by the occurrence of siderophore biosynthetic proteins within PCD-induced dying cells but not in the surviving cells[5] as well as by the role played by siderophores in triggering *Streptomyces* development[6]. *Streptomyces* require both a programmed growth arrest - the transition phase - as a key step in morphological and physiological differentiation, as well as preset extensive cell lysis that liberates nutrients, thereby allowing the development of aerial hyphae. We propose that one of the strategies used by *S. coelicolor* to enter PCD could be auto-induced iron poisoning. The most likely evolutionary meaning of this relationship between GlcNAc utilization and siderophore biosynthesis is that it would enable streptomycetes to control the iron-mediated PCD. Indeed, the amino sugar GlcNAc released during autolytic cell-wall dismantling is a logical parameter for cell-wall integrity, with its extracellular concentration functioning as an indicator of the degree of cell lysis. The PCD process needs to be limited in order for *S. coelicolor* to reach the later stages of its developmental program. GlcNAc could play the role of PCD-limiting agent by reducing the biosynthesis of siderophores and, as a consequence, shutting down iron auto-poisoning.

The conservation of the GlcNAc/siderophore relationship in distantly related streptomycetes species provides strong validation for the siderophore regulatory pathway model we propose, and suggests that this regulatory event is the consequence of a successful evolutionary process. Investigations are underway to assess if the PCD/siderophore-mediated iron poisoning occurs in other streptomycete species.

References:

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