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PAPER
Sébastien Rigali *et al.*
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PAPER

Altered desferrioxamine-mediated iron utilization is a common trait of bald mutants of *Streptomyces coelicolor*†

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Stéphany Lambert,‡[a] Matthew F. Traxler,‡[b] Matthias Craig,[a] Marta Maciejewska,[a] Marc Ongena,[c] Gilles P. van Wezel,[d] Roberto Kolter[b] and Sébastien Rigali*[a]

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Streptomyces coelicolor is an important model organism for developmental studies of filamentous GC-rich actinobacteria. The genetic characterization of mutants of *S. coelicolor* blocked at the vegetative mycelium stage, the so-called bald (*bld*) mutants that are unable to erect spore-forming aerial hyphae, has opened the way to discovering the molecular basis of development in actinomycetes. Desferrioxamine (DFO) production and import of ferrioxamines (FO; iron-complexed DFO) are key to triggering morphogenesis of *S. coelicolor* and we show here that growth of *S. coelicolor* on the reference medium for *Streptomyces* developmental studies is fully dependent on DFO biosynthesis. UPLC-ESI-MS analysis revealed that all *bld* mutants tested are affected in DFO biosynthesis, with *bldA*, *bldJ*, and *ptsH* mutants severely impaired in DFO production, while *bldF*, *bldK*, *crr* and *ptsI* mutants overproduce DFO. Morphogenesis of *bldK* and *bldJ* mutants was recovered by supplying exogenous iron. Transcript analysis showed that the *bldJ* mutant is impaired in expression of genes involved in the uptake of FO, whereas transcription of genes involved in both DFO biosynthesis and FO uptake is increased in *bldK* mutants. Our study allows proposing altered DFO production and/or FO uptake as a novel phenotypic marker of many *S. coelicolor bld* mutants, and strengthens the role of siderophores and iron acquisition in morphological development of actinomycetes.

Introduction

The great oxygenation event (GOE) which occurred around 2.4 billion years ago is a major episode of Earth's redox history.[1] This event had many irreversible consequences, and key among these was the global-scale oxidation of iron from its ferrous form (Fe^{2+}) to its poorly soluble ferric form (Fe^{3+}). This redox transition greatly decreased the bioavailability of iron and as Fe^{2+} is essential for many biological processes, GOE-surviving microorganisms had to develop strategies for acquiring Fe^{3+}. Many microbes have genes for the biosynthesis of iron-chelating metabolites called siderophores, the main function of which is to capture poorly soluble environmental Fe^{3+} thus facilitating its internalization, subsequent reduction into Fe^{2+}, and utilization for biochemical reactions. The role played by siderophores in nature is believed to be so fundamental that the inability of microorganisms to produce such compounds may be a major cause of microbial uncultivability.[2] This suggests that the survival of a large percentage of microorganisms on Earth may depend on neighbouring siderophore-producing species.[2]

Iron plays essential roles in house-keeping functions such as DNA replication, protein synthesis and respiration, and siderophore-mediated iron uptake is specifically associated with many other biological processes such as fungal sexual development[3] and germination,[4] plant growth promotion,[5] pilus formation,[6] and pathogen virulence.[7,8] In a study by Ueda and colleagues on terrestrial antibiotic-producing streptomycetes, it was reported that morphological development (*e.g.* formation of aerial hyphae and spores) and secondary metabolite production was triggered in certain species when they were grown in proximity to isolates producing siderophores (more precisely desferrioxamine E, DFOE).[9] This was the first observation suggesting that in *Streptomyces* species, these iron-chelating compounds might have a much more complex and

[a] *Centre for Protein Engineering, University of Liège, Institut de Chimie B6a, B-4000 Liège, Belgium. E-mail: srigali@ulg.ac.be*
[b] *Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA*
[c] *Walloon Centre for Industrial Biology, University of Liège/Gembloux Agro-Bio Tech, Gembloux B-5030, Belgium*
[d] *Molecular Biotechnology, Institute of Biology Leiden, Leiden University, Sylviusweg 72, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

† Electronic supplementary information (ESI) available: Fig. S1: phenotype of *S. coelicolor* M145 grown on R2YE with increasing concentrations of $FeCl_3$; Fig. S2: production of thick aerial hyphae of the *bldJ* mutant grown in R2YE with $FeCl_3$ supply. See DOI: 10.1039/c4mt00068d

‡ SL and MT contributed equally to this work.

subtle role in triggering a wide range of cellular processes.[6,9] In line with these findings, Tierrafria and collaborators showed that the disruption of the desferrioxamine-binding receptor encoded by *desE* resulted in loss of sporulation in *S. coelicolor*.[10] Recently, co-culture experiments demonstrated that iron competition through siderophore piracy between actinomycetes prevented *S. coelicolor* from entering its sporulation program.[11]

Pathways leading to the onset of sporulation are numerous and diverse among bacteria and *Streptomyces* are no exception.[12] However, from the plethora of genes required for morphological differentiation, it is important to distinguish between genes that are required for spore formation regardless of culturing conditions, and those that are only required for sporulation on specific media. Many of the so-called *bld* (bald) mutants in *Streptomyces* spp.,[13] which are unable to produce the 'hairy' aerial hyphae and therefore blocked at the vegetative mycelium stage, are clear examples of conditional mutants as they are able to produce aerial hyphae when grown on media containing non-repressing carbon sources.[14] R2YE medium is used as reference medium for *Streptomyces* developmental studies and is a complex, glucose-rich medium. Pope and collaborators showed that all *bld* mutants isolated when using R2YE medium were also altered in glucose-mediated carbon catabolite repression.[14] This result led the authors to propose that the 'baldness' of many *bld* mutants was a secondary consequence of their altered capacity to properly utilize glucose, and not, as previously suggested, due to their failure to directly activate genes of the developmental program.[14] Interestingly, deletion of the gene for glucose kinase which is required for carbon catabolite repression (CCR), allows *bldA* mutants to sporulate on glucose-containing media, showing that it is indeed CCR that suppresses their development.[15]

Given recent findings suggesting that siderophores and iron acquisition influence development in *Streptomyces*,[6,9–11] we investigated the possibility that some of the classical *bld* mutants and other *S. coelicolor* mutants unable to trigger morphogenesis are affected in siderophore-mediated iron uptake and/or assimilation.

Results and discussion

Desferrioxamines are essential for *S. coelicolor* growth on iron-limited medium R2YE

The R2YE medium used as a reference medium for *Streptomyces* developmental studies is not devoid of iron but the total concentration of $FeCl_3$ is estimated at $\sim$1.8 μM.[16] This medium is therefore considered iron-limited and, indeed, we recently showed that it strongly stimulates the production of the siderophores coelichelin (CCLIN) and desferrioxamines (DFO).[17] In order to assess the importance of the different types of siderophores for the growth of *S. coelicolor* on R2YE medium, we compared the growth of *S. coelicolor* M145 (wild-type) and that of its mutants W4 (Δ*cchH*),[18] and JMG1 (Δ*desA*)[19] which are unable to produce CCLIN and DFO, respectively.

Fig. 1 reveals that CCLIN is not essential for *S. coelicolor* growth on R2YE since mutant W4 only shows limited growth

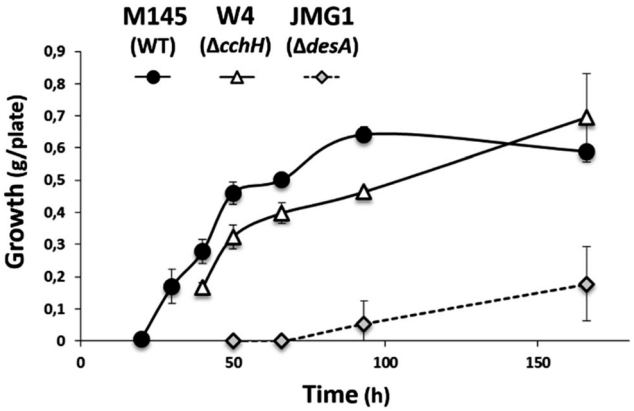


Fig. 1 Impaired growth of *S. coelicolor* JMG1 (Δ*desA*) on R2YE. *desA* mutants fail to produce DFOs and as a consequence have problems with growing on R2YE. Note that coelichelin production is less important for growth on R2YE. Growth is expressed in grams of fresh weight of mycelium collected per plate. Each point is the mean of three independent experiments (error bars represent the standard deviation).

retardation and biomass accumulation reaches similar levels. In contrast, the *desA* null mutant displays a strong growth delay as compared to M145, with the first colonies emerging from the agar plate after two to three days of incubation. Importantly, growth of the *desA* mutant was only observed when inoculated with a dense spore suspension (10^8–10^9 spores). At lower spore densities, *desA* mutant colonies of different shapes and sizes occasionally appeared which we believe could be spontaneous suppressors (not shown).

Given that the Δ*desA* strain cannot make DFO, we tested the ability of DFOB to restore growth to the Δ*desA* strain on R2YE medium. To do so, we streaked mutant JMG1 on R2YE medium and provided DFOB on paper discs placed on the agar. As expected, DFOB supplementation was sufficient to restore the growth and development of JMG1 (Fig. 2A). Finally, iron supplementation alone was also sufficient to restore viability of the *desA* mutant confirming that, under these culturing conditions, DFO-mediated iron uptake is vital for *S. coelicolor* (Fig. 2B).

S. coelicolor bld mutants are affected in siderophore biosynthesis

Based on the importance of DFO for the growth and development of *S. coelicolor*, we hypothesized that at least some classical *bld* mutants might be defective for DFO biosynthesis and/or uptake. We also include in our study mutants of the general enzymes of the sugar phosphotransferase system (PTS) of *S. coelicolor* which also lost the ability to produce aerial hyphae when grown on R2YE medium.[20] In order to preliminarily assess the ability of the selected bald mutants (Table 1) to produce siderophores, we applied a CAS agar solution overlay onto three-day-old colonies grown on R2YE agar plates. As shown in Fig. 3, a dense orange halo due to CCLIN and DFOs production was observed around the wild-type colonies of *S. coelicolor* M145. Colonies of *bldF*, *bldK* and the *pts* mutants BAP1 (Δ*ptsH*), BAP2 (Δ*crr*) and BAP3 (Δ*ptsI*), also display a dense halo indicative of the biosynthesis of siderophores.

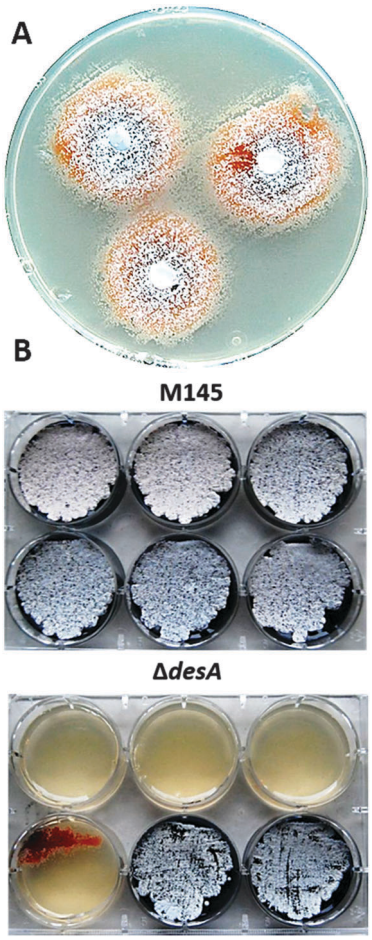


Fig. 2 Desferrioxamines are essential for growth of *S. coelicolor* on R2YE agar plates. (A) Exogenous supply of desferrioxamine B restores growth of the *desA* mutant. Note the growth of strain JMG1 (Δ*desA*) around the paper discs containing DFOB (7 μl of DFOB at 75 nM). (B) Exogenous supply of iron restores viability to *desA* mutants. Plates were inoculated with 4×10^6 spores of *S. coelicolor* M145 (wild-type) and its *desA* null mutant. From left to right and top to bottom, R2YE plates were supplemented with 0, 1, 10, 25, 35, and 50 μM of $FeCl_3$.

Table 1 *S. coelicolor* strains used in this study

Strains	Genotype	Ref.
M145	$SCP1^-$, and $SCP2^-$	16
J1700 (Δ*bldA*)	*bldA39 hisA1 uraA1 strA1*	32
161 (Δ*bldF*)	*bldF hisD3 pheA1 strA1*	46
HU61 (Δ*bldJ*)	*bldJ261 hisA1 uraA1 strA1*	40
NS17 (Δ*bldK*)	*bldK*::*aadA*	39
BAP1 (Δ*ptsH*)	*ptsH*::*aacC4*	47–49
BAP2 (Δ*crr*)	*crr*::*aacC4*	47–49
BAP3 (Δ*ptsI*)	*ptsI*::*aacC4*	47–49
W4 (Δ*cchH*)	*cchH*::*vph*	18
JMG1 (Δ*desA*)	*desA*::*aacC4*	19

However, *bldA* mutants failed to produce siderophores as was apparent from the lack of a halo around the colonies, while the minute zone around the *bldJ* mutant was indicative of severe reduction of its ability to chelate extracellular iron.

UPLC-ESI-MS analyses were performed to semi-quantitatively assess the production levels of the different types of siderophores synthesized by the *S. coelicolor* M145 and the *bld* mutants, which were all grown as lawns on cellophane overlays for 68 h on R2YE agar plates (Fig. 3). At the time of collection, the cellophane with biomass was removed, and the spent agar was placed in water to extract siderophores. The collection time was chosen just prior to the onset of development of M145 in order to compare strains that are at the same morphological stage of their life cycle, *i.e.* vegetative growth. This confirmed our observation that *bldA* and *bldJ* mutants produce no or very low levels of siderophores. CCLIN could not be detected in either mutants, and DFOB and DFOE production was drastically reduced. Similarly, the *ptsH* mutant also failed to produce CCLIN and showed 60%, and 85% reduction of DFOB and DFOE production, respectively. Mutants that lack *bldF*, *bldK*, *crr* or *ptsI* instead overproduced DFO, with *crr* null mutant (BAP2) producing an 11-, and 8-fold excess of DFOB and DFOE, respectively (Fig. 3). However, UPLC-ESI-MS and CAS overlay analyses did not show strictly similar siderophore production patterns (see the *ptsH* mutant), suggesting that the siderophore-producing capacity of single colonies of the *bld* mutants differs from that of mutants grown as confluent lawns.

In order to identify the mutants possibly impaired in sporulation due to altered iron utilization, we streaked the bald mutants onto R2YE agar plates supplemented with different concentration of $FeCl_3$. To our surprise, at a concentration of 1 mM, $FeCl_3$ restored development to the *bldJ* mutant, which produced the WhiE grey pigment associated with the latest stage of the maturation of spores chains[21] (Fig. 4). Similarly, at a lower concentration of 0.1 mM, $FeCl_3$ restored development to *bldK* mutants (Fig. 4). Scanning electron microscopy (SEM) confirmed that indeed the *bldJ* and *bldK* mutants produced abundant aerial hyphae when grown with iron (Fig. 5). Fully mature chains of spores were occasionally observed for the *bldK* mutant though the majority of aerial hyphae did not show signs of differentiation (Fig. 5), as evidenced by the lack of constriction that is indicative of initiation of sporulation-specific cell division.[22] It has to be noted that the *bldJ* mutant needed prolonged incubation (>one week) to observe spores of different sizes and shapes. Earlier, many aerial hyphae where thicker or were often found stuck one to each other and did not display septa (Fig. S2, ESI†).

In contrast, the life cycle of *bldA*, *bldF*, *ptsH*, *crr*, and *ptsI* mutants was still blocked at the vegetative growth phase. Interestingly, the *bldA* mutant could recover the ability to produce the red-pigmented prodiginines but not biosynthesis of the blue-pigmented antibiotic actinorhodin (Fig. 4). However, *bldA*, *crr*, and *ptsI* mutants were still viable when streaked onto R2YE medium supplemented with 5 mM $FeCl_3$ (Fig. 4), a concentration lethal for wild-type *S. coelicolor* (Fig. S1, ESI†). The viability of *crr* and *ptsI* mutants could be attributed to their overproduction of DFOB and DFOE (Fig. 3), which might allow the mutants to detoxify a large portion of the excess $FeCl_3$.

Additionally, we also tested the effect of added iron on *S. coelicolor* M145 grown on R2YE with *N*-acetylglucosamine (GlcNAc). The aminosugar GlcNAc is known to block *Streptomyces* development in the vegetative growth phase when added to the R2YE medium, thus leading to a nonsporulating (bald)

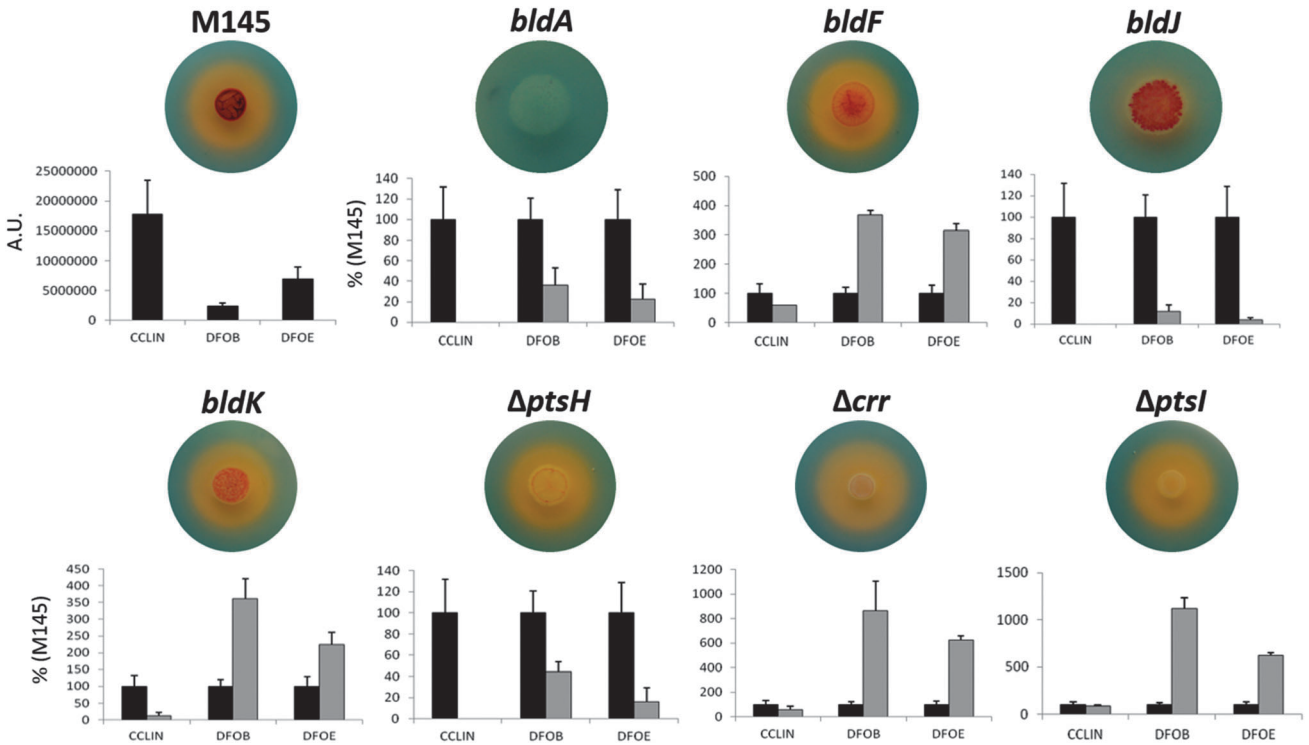


Fig. 3 Siderophore production is altered in bald mutants. Top: details of bald mutant colonies overlaid with a CAS agar solution to detect the extracellular production of iron-chelating molecules (orange halos). The diameter of the colonies varies between 1 and 0.5 cm. Bottom: histograms displaying coelichelin (CCLIN), desferrioxamine B (DFOB) and desferrioxamine E (DFOE) production quantified by UPLC-ESI-MS analysis. Relative amounts of siderophores produced by the mutants (Grey bars) are expressed as a percentage of levels produced by *S. coelicolor* M145 (wild-type strain, Black bars). A.U., arbitrary units.

phenotype.[20,23,24] We recently showed that GlcNAc supply drastically reduces siderophore biosynthesis through the direct transcriptional control of the gene for the iron utilization repressor DmdR1.[19,25–27] Specifically, we showed that repression of DmdR1 by DasR, the central regulator of the GlcNAc-metabolic regulon,[17] was relieved in the presence of GlcNAc. The resulting enhanced expression of DmdR1 then leads to increased repression of siderophore biosynthetic genes. In order to assess if the developmental arrest imposed by GlcNAc was, at least in part, due to the inhibition of siderophore biosynthesis, we streaked strain M145 onto the GlcNAc-containing R2YE medium supplemented with increasing concentrations of $FeCl_3$. Fig. 6 shows that at a concentration of 1 mM, $FeCl_3$ restored the biosynthesis of red- and blue-pigmented antibiotics prodiginines and actinorhodin, as well as the production of aerial hyphae. Analysis by SEM revealed that the aerial hyphae induced by iron in the presence of GlcNAc indeed matured into spores (Fig. 5). The iron-mediated restoration of development in GlcNAc-containing R2YE is another example that illustrates the phenomenon of how '*curing baldness activates antibiotic production*' as recently reported in *Streptomyces calvus*.[28,29] Taken together, these results demonstrate that inhibition of development and antibiotic production by physiological signalling can also be circumvented by potentiating siderophore-mediated iron uptake in *S. coelicolor*.[9–11]

Transcription of iron- and development-related genes in *bldA*, *bldJ* and *bldK* mutants

To provide a rational interpretation for the altered siderophore production and/or the partial restoration of development to *bldA*, *bldJ* and *bldK* mutants upon iron supplementation, we assessed the transcription of a series of genes associated with iron utilization. The quantification of mRNAs in cell lysates of *S. coelicolor* M145 and the *bld* mutants was performed *via* the Nanostring nCounter analysis system[30] as described previously.[11] We specifically examined individual transcripts for genes *cchF* and *cchB* (SCO0494 and SCO0498 involved in ferricoelichelin uptake and CCLIN biosynthesis, respectively), *desE* and *desA* (SCO2780 and SCO2782 involved in ferrioxamine uptake and DFO biosynthesis, respectively), and *cdtB* (SCO7399, an alternative transporter for ferricoelichelin and ferrioxamine B). We also included SCO5800 (*rhsC*), which together with SCO5801 and SCO799 is presumably involved in the synthesis of a rhizobactin-like cryptic siderophore.[31] Simultaneously, we also assessed the expression of 12 developmental genes involved in (i) regulating or signalling the onset of aerial hyphae formation and antibiotic production (*bldA*, *bldD*, *bldH*, *bldKB*, *bldN*, *sigN*) (ii) formation of the hydrophobic coat of aerial hyphae (*ramS*, *ramC*, *chpA*, and *rdlA*), spore chain maturation, (*whiG*), or production of WhiE spore pigment (*whiE*). The comparative expression of these 19 genes between

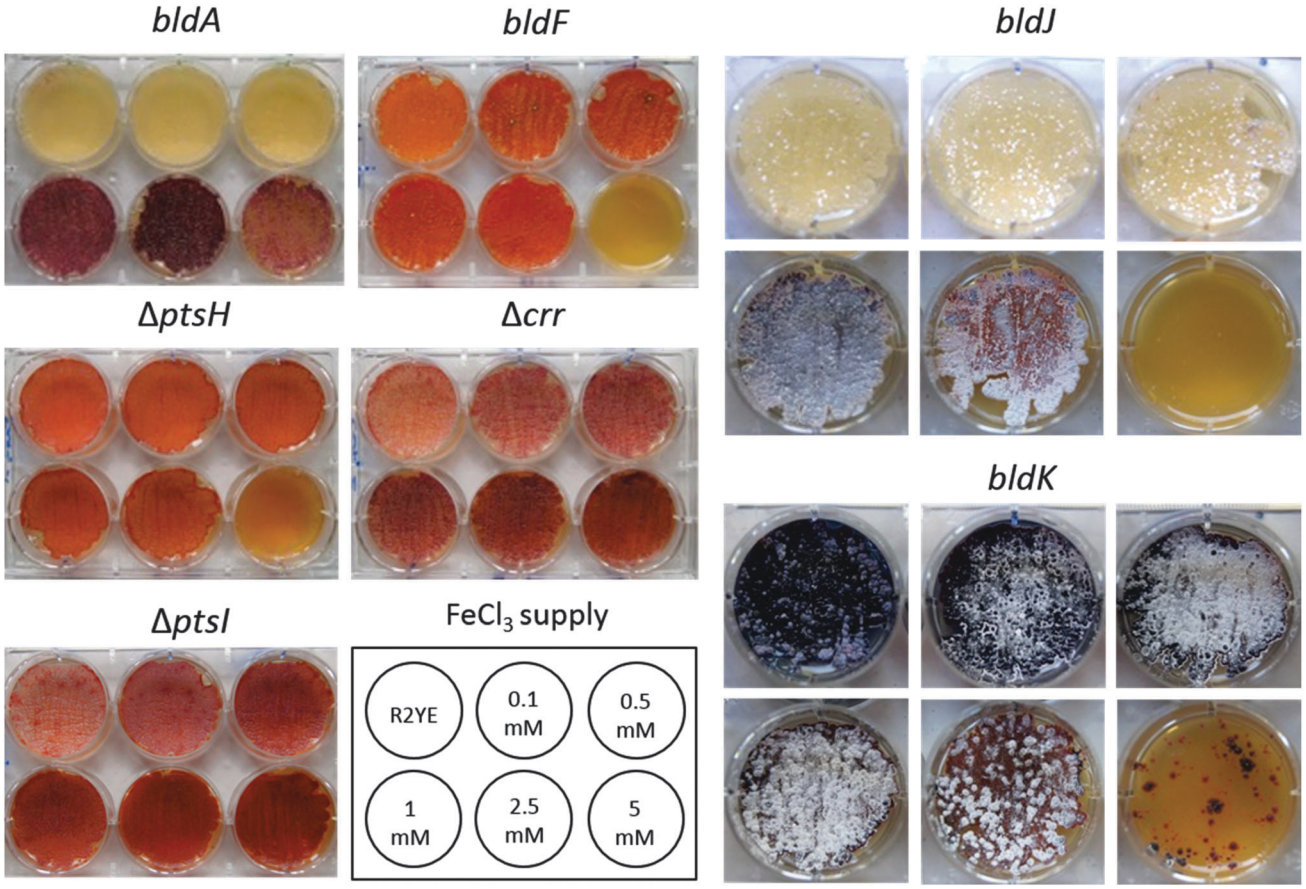


Fig. 4 Effect of iron on the development of bald mutants grown on R2YE agar plates. Note the restored production of the red-pigmented prodiginines (Red) to the *bldA* mutant by iron (1 mM $FeCl_3$) supply and the iron-dependent restoration of white/grey phenotypes associated with aerial structures in *bldJ* and *bldK* mutants. While Red production was restored to *bldA* mutants, development was not. Development was not restored in *bldF, crr, ptsH* and *ptsI* null mutants. The phenotype of the wild-type strain M145 grown on R2YE supplied with $FeCl_3$ is shown in Fig. S1 (ESI†).

S. coelicolor M145 and *bldA*, *bldJ*, and *bldK* mutant colonies is presented in Fig. 7.

Impaired siderophore production and iron-mediated production of prodiginines in the *bldA* mutant

bldA is perhaps the best-characterized *bld* mutant of *S. coelicolor*. Mutations in *bldA* have been mapped to the gene specifying the Leucyl-tRNA BldA for translation of the rare UUA codon.[32] *bldA* mutations are expected to disrupt the tRNA structure and that the *bldA* phenotype would be linked to a non-functional tRNALeu UUA preventing translation of key developmental genes, such as AdpA. Consistent with its known role, we observed that the *bldA* mutant showed decreased expression of many genes in the developmental cascade including *adpA*, *bldN*, *ramC*, and *ramS*. In contrast, the *bldA* mutant did not exhibit significantly altered expression of genes for siderophore biosynthetic or binding proteins. Thus, the underlying cause of the lowered amount of siderophores produced by *bldA* mutant remains elusive (Fig. 7).

In contrast to *bldJ* and *bldK* mutants (see below), addition of exogenous iron could only restore the production of prodiginines to the *bldA* mutant, while actinorhodin production and sporulation could not be restored (Fig. 4). From the 145 genes that possess a TTA codon,[33] at least one, *adpA* (*bldH*), is required for sporulation on glucose-containing media.[34,35] This suggests that the ability of iron to promote development must act upstream of the *adpA* regulatory network. The failure of iron to recover actinorhodin production is readily explained by the presence of a TTA codon within the sequence of the pathway-specific activator *act*II-ORF4 gene which is required for activation of actinorhodin biosynthesis, as well as in *act*II-ORF2 for actinorhodin export.[36] Production of prodiginines involves two activator genes, of which *redZ* encodes an orphan response regulator that in turn activates expression of *redD*, for the final pathway-specific activator of the *red* cluster. Of these, *redZ* contains a TTA codon.[37] We therefore hypothesize that restoration of prodiginine production to *bldA* mutants by the addition of iron likely acts downstream of *redZ*, as it should bypass the *bldA*-mediated post-transcriptional control.

Down-regulation of siderophore uptake systems in the *bldJ* mutant

The *bldJ* mutant (also referred to as *bld261*) is one of the last developmental mutants of *S. coelicolor* in which the mutation

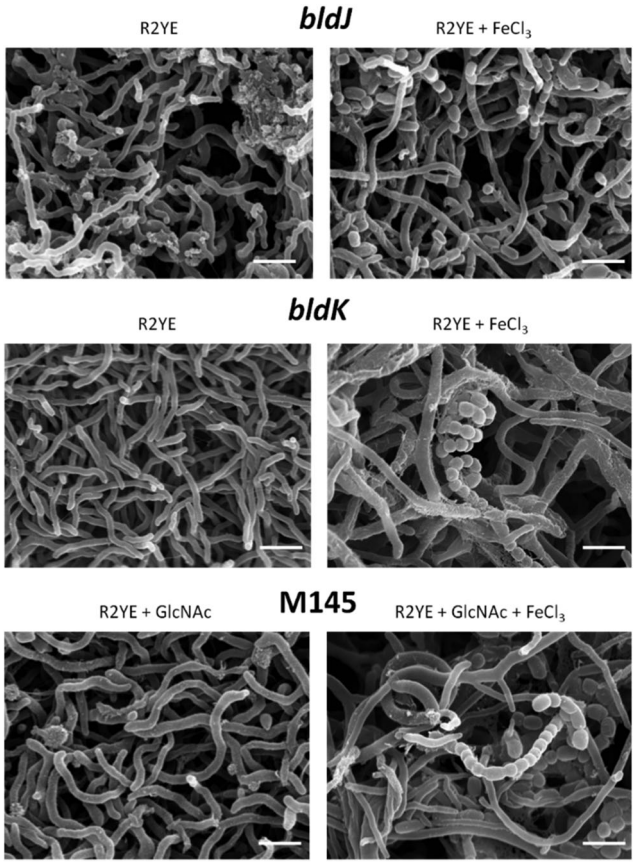


Fig. 5 Scanning electron micrographs of *S. coelicolor* M145 and *bldJ* and *bldK* mutants. Single colonies of *bldJ* and *bldK* mutants were grown in R2YE with or without iron supply. The concentration of $FeCl_3$ added is 1 and 1.5 mM for *bldJ* and *bldK*, respectively. Longer incubation (> one week) was required to observe fully maturated spore in the *bldJ* mutant grown in R2YE supplied with iron. The wild-type strain *S. coelicolor* M145 was grown in R2YE with 30 mM GlcNAc and $FeCl_3$ was added to a 2 mM final concentration. Bar, 2 μm.

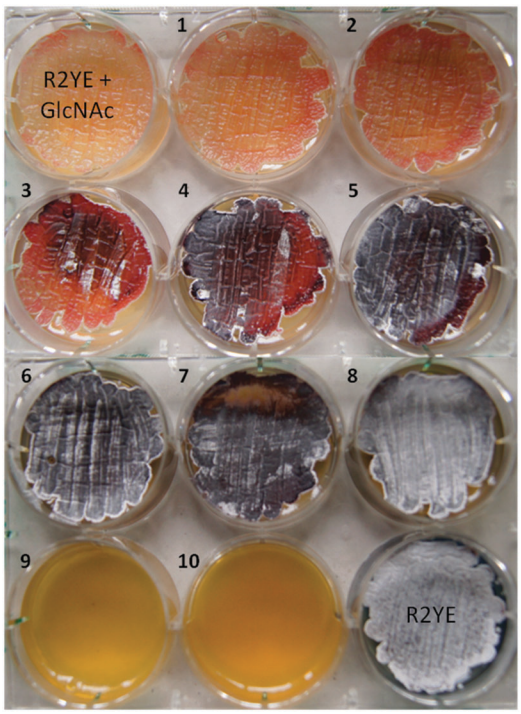


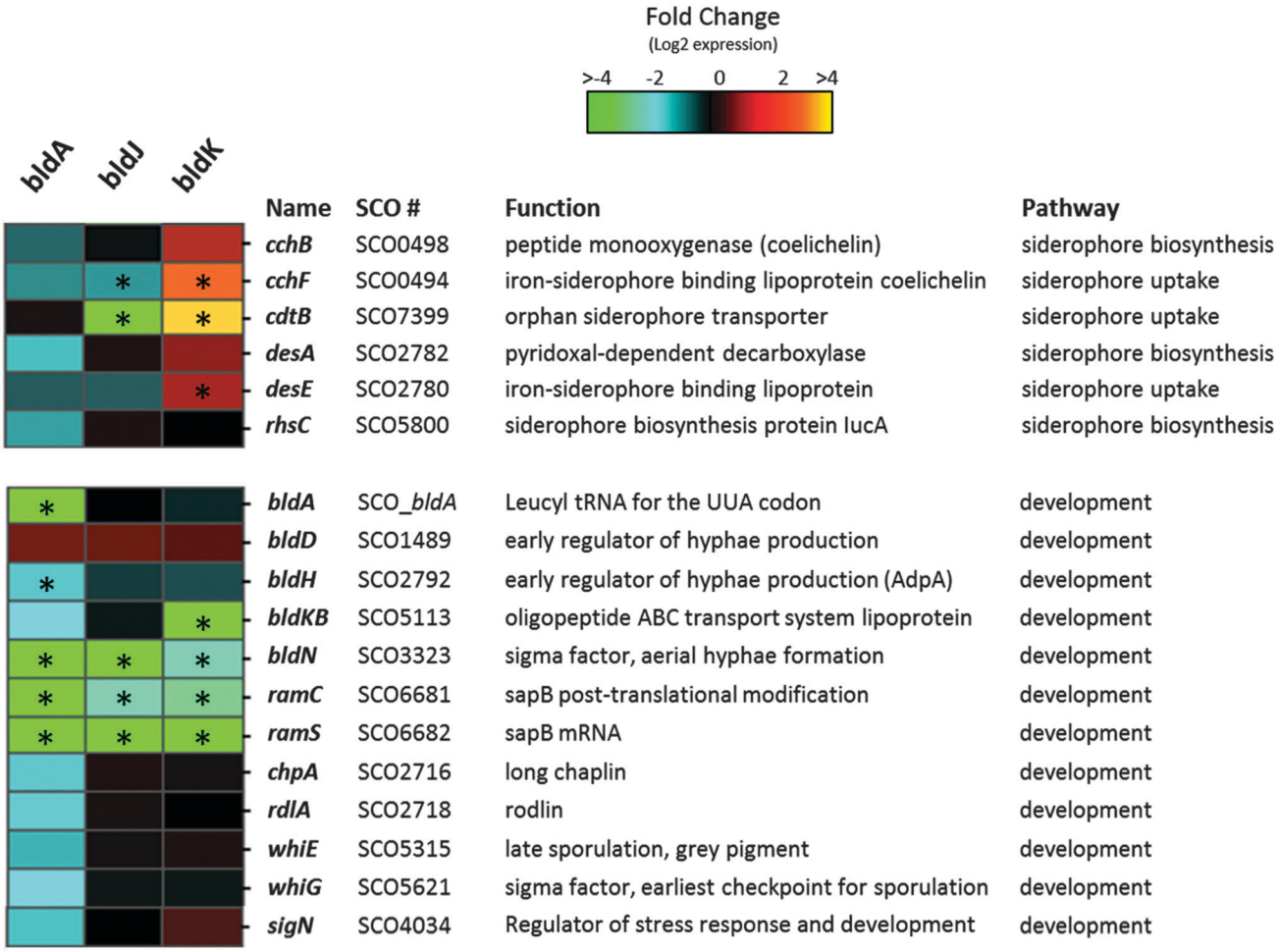
Fig. 6 Iron bypasses the blocking effect of *N*-acetylglucosamine on *S. coelicolor* development. Colonies were grown on R2YE with 30 mM GlcNAc, and in addition 0.01 0.1, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 mM of $FeCl_3$ (plates labelled 1–10; plates top left and bottom right have no added iron).

has not yet been mapped onto the *S. coelicolor* chromosome. The *bldJ* mutant is particularly interesting because it is predicted to be impaired in the production of the very first signal of the cascade that leads to the onset of *S. coelicolor* morphological differentiation.[38–40] The reduced siderophore production in *bldJ* mutants is unlikely to be the consequence of impaired expression of siderophore biosynthetic genes as *desA*, *cchB* and even SCO5800 for the cryptic siderophore were transcribed at similar levels as in the wild-type strain M145 (Fig. 7).

Surprisingly however, genes for ferricoelichelin and ferrioxamines (*cchF* and *cdtB*) uptake were significantly downregulated as compared to M145 transcription levels, respectively (Fig. 7). The uncoupled expression patterns of genes for siderophore biosynthesis and transport is unexpected as all *des* and *cch* clusters genes possess similar *cis*-acting elements (iron boxes) within their upstream region and are all under the transcriptional control of the iron utilization regulator DmdR1.[19,26,31] Our data suggest that an additional regulatory system is in place that differentially controls the expression of siderophore biosynthetic and uptake genes. If siderophore importer genes have their own transcription regulatory system, the same may be true for the exporter genes. The genes encoding the specific permeases for DFO secretion have not been identified in *Streptomyces*. Since transcript levels of *des* and *cch* clusters in the *bldJ* mutant are similar to those in the wild-type cells, a plausible explanation for the measured low amounts of extracellular siderophores could be attributed to an impaired expression of genes involved in siderophore export or to post-transcriptional control. However, the latter hypothesis should not imply the *bldA*-dependent translational control as expression of *bldA* is not affected in the *bldJ* mutant background (Fig. 7) and no TTA codon has been identified within the DmdR1-dependent iron utilization regulon.

Increased expression of genes associated with siderophore biosynthesis and uptake in the *bldK* mutant

The *bldKABCDE* locus corresponds to genes SCO5112 to SCO5116, which together encode an oligopeptide ATP-binding cassette (ABC) transporter.[39] The *bldK* mutant had enhanced transcription of genes involved in siderophore production and uptake overall. The increased production of siderophores in the *bldK* mutant correlates well with this observation (Fig. 7). High expression of genes involved in siderophore production/uptake even in the presence of abnormally high exogenous siderophore levels may indicate a problem in siderophore uptake in the *bldK* mutant. As the *bldK* locus itself encodes an oligopeptide uptake system, it is possible that the BldK proteins themselves may play a direct role in siderophore uptake. Alternatively, it is possible that the strong



Name	SCO #	Function	Pathway
cchB	SCO0498	peptide monooxygenase (coelichelin)	siderophore biosynthesis
cchF	SCO0494	iron-siderophore binding lipoprotein coelichelin	siderophore uptake
cdtB	SCO7399	orphan siderophore transporter	siderophore uptake
desA	SCO2782	pyridoxal-dependent decarboxylase	siderophore biosynthesis
desE	SCO2780	iron-siderophore binding lipoprotein	siderophore uptake
rhsC	SCO5800	siderophore biosynthesis protein lucA	siderophore biosynthesis
bldA	SCO_*bldA*	Leucyl tRNA for the UUA codon	development
bldD	SCO1489	early regulator of hyphae production	development
bldH	SCO2792	early regulator of hyphae production (AdpA)	development
bldKB	SCO5113	oligopeptide ABC transport system lipoprotein	development
bldN	SCO3323	sigma factor, aerial hyphae formation	development
ramC	SCO6681	sapB post-translational modification	development
ramS	SCO6682	sapB mRNA	development
chpA	SCO2716	long chaplin	development
rdlA	SCO2718	rodlin	development
whiE	SCO5315	late sporulation, grey pigment	development
whiG	SCO5621	sigma factor, earliest checkpoint for sporulation	development
sigN	SCO4034	Regulator of stress response and development	development

Fig. 7 Transcription of genes for siderophore production and uptake in *S. coelicolor bldA*, *bldJ* and *bldK* mutant colonies. Gene names are shown at right of heat map; color legend for Log2 expression (fold change) is shown above. Note the strong downregulation of the iron-induced sigma factor BldN in *bldJ* and *bldK* mutants. Genes were considered significantly regulated if they had *P* values of <0.02 in Student's *T*-test (three replicates) when compared between wild-type and mutant strains. Genes meeting this criterion are noted with an asterisk.

production of DFO and CCLIN may result in lowering the local availability of iron nearby the colony. Indeed, CAS overlay assays showed that secreted siderophores diffuse far from the colony (Fig. 3), suggesting that a small proportion is re-imported into the cell. The suggested extracellular iron sequestration by the excess of siderophores produced by the *bldK* mutant would finally result in an iron depleted environment in the surrounding area of the colony and therefore in lower amounts of iron imported for housekeeping and developmental processes. We note, however, that genes encoding transporter systems of siderophores (*desE*, *cdtB*, and *cchF*) are also overexpressed in the *bldK* mutant (Fig. 7), which should also improve the uptake of both FOs and ferricoelichelin. This scenario could potentially lead to iron overload. However, we view this as unlikely, given that the bald phenotype of *bldK* is complemented upon iron supply.

The developmental transcription network is similarly affected in *bldJ* and *bldK* mutants

Interestingly, extracellular complementation assays showed that the *bldK* and *bldJ* mutants are inextricably linked.[38–40] In the proposed model for the developmental signalling cascade it has been shown that *S. coelicolor* produces and exports the very first signal of morphogenesis through the product of genes associated with the *bldJ* mutation. After extracellular accumulation of this signal, it is imported through the BldK oligopeptide permease, triggering the next step in the cascade.[38,39] Nodwell and Losick reported the isolation of an extracellular molecule able to restore sporulation of the *bldJ* mutant and which is particularly abundant in the extracellular medium of *bldK* mutant cultures.[38]

Our transcript analysis further supports the close linkage between *bldK* and *bldJ* mutants. Indeed, both mutants display very similar altered expression of the developmental genes analysed (Fig. 7 and 8). In each case we observed a strong reduction in transcript levels for *ramC* and *ramS* genes involved in the biosynthesis of the lantibiotic-like, surfactant peptide SapB, which is conditionally required for the onset of aerial growth.[41,42] The downregulation of *ramC* and *ramS* is most likely the consequence of decreased signalling through AdpA, the details of which are currently unknown. AdpA indirectly activates genes required for SapB production.[43,44]

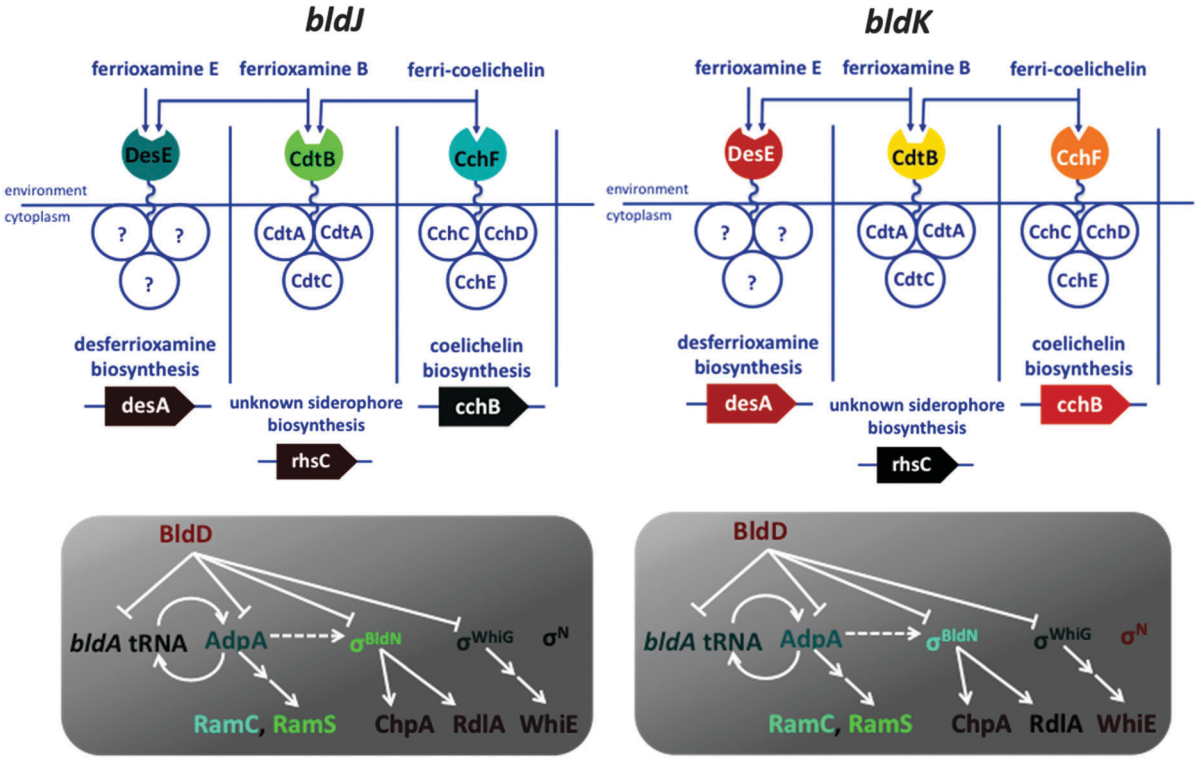


Fig. 8 Siderophore iron uptake and biosynthetic genes in context of the *S. coelicolor* developmental transcription network. Arrows represent positive or activating regulatory effects. Perpendicular lines represent negative or repressive regulatory effects. The dashed arrow represents a connection based on conflicting results. Double arrows indicate indirect regulation. Results shown are from a direct comparison of transcripts from *S. coelicolor* wild-type colonies and *bldJ* and *bldK* mutants grown 72 hours on the R2YE medium. Protein names or symbols are colored according to the expression scale shown in Fig. 7. Note the drastic down-regulation of the gene for the iron-induced sigma factor BldN in both mutants.

Finally, the *bldK* and *bldJ* mutants both displayed significantly lowered expression of *bldN* (*p* values of 0.0093 and 0.0008 respectively) (Fig. 7). BldN is an extracytoplasmic function subfamily RNA polymerase sigma factor necessary to form aerial mycelium *via* the control of genes encoding chaplin and rodlin proteins, which are major components of the hydrophobic sheath that coats the aerial hyphae and spores[45] (Fig. 7 and 8). Excitingly, *bldN* expression is known to be activated by iron.[11] We therefore speculate that iron complementation in the *bldJ* and *bldK* mutants restores the expression of *bldN* to levels sufficient to trigger the synthesis of proteins required for aerial hyphae formation.

Conclusions

The phenotypes of most *bld* mutants of *S. coelicolor* are defined as 'conditional' since many of the bald phenotypes of these mutants can be reversed depending on the growth medium. Many *bld* mutants were isolated on R2YE medium, where glucose is the principal carbon source. Pope and collaborators suggested in 1996 that the bald phenotype of certain mutants could be a secondary consequence of impaired glucose utilization. Inspired by this previous study, we questioned the paradigm of conditional *bld* mutants of *S. coelicolor* based on an entirely different characteristic of the R2YE medium, namely the low levels of available iron. We found that the 'conditional' phenotype of at least some of the *bld* mutants could be attributed to altered DFO-mediated iron utilization. The data presented here shows that all the mutants we investigated displayed either increased or reduced DFO production patterns compared to the wild-type strain *S. coelicolor* M145.

In a previous investigation on the control of iron homeostasis in *Streptomyces*, we showed that the *dasR* mutant of *S. lividans*, which also displayed a bald phenotype, produced less siderophore than the wild-type strain. We found that this lowered siderophore production was a consequence of the loss of direct repression by the regulator DasR of *dmdR1*, which encodes the repressor of DFO and CCLIN biosynthetic genes.[17] In the course of our investigation we also observed that other bald mutants investigated (*bldB*, *bldC*, *bldD*, *dasA*, *adpA*) also produced different levels of siderophores (not shown). However, they were not included in this study because they either did not show a robust phenotype or exhibited too severe growth defect for proper comparative study with the wild-type strain M145. We therefore propose that a more accurate definition of the 'conditional' phenotype of *bld* mutants should be expanded to included impaired iron utilization.

We propose altered production of DFOs as a novel phenotypic marker of *S. coelicolor bld* mutants. As several *bld* mutants suffer from problems with iron acquisition, this early signal may be – or may depend on – a molecule able to chelate metal-ions.

The iron-utilization conditional phenotype is best exemplified by the early signalling *bldJ* and *bldK* mutants, which partially recovered development when the medium was supplemented with large amounts of iron. *bldJ* and *bldK* mutants are involved in the earliest steps of the regulatory cascade that leads to development of *S. coelicolor*.[38–40] Unravelling the nature of the *bldJ* mutant as well as the oligopeptide transported by BldK, will provide important new leads towards understanding the pathways that control siderophore-mediated iron utilization and developmental control in *Streptomyces*.

Experimental section

Bacterial strains and culture conditions

The *S. coelicolor* strains used in this study are listed in Table 1. R2YE medium was used for siderophore production assays and phenotype studies. All media were prepared according to the 'Practical *Streptomyces* Genetics' manual.[16] Growth curve were made by inoculating R2YE plates covered with cellophane membranes (GE Osmonics Labstore, Ref K01CP09030) with 500 μl of a 10^8 spore suspension. Growth is expressed in grams of fresh weight of mycelium collected per plate.

Siderophore production and extraction

Siderophore extraction was performed as described previously.[17] After 68 h of incubation at 28 °C, mycelia were collected from the cellophane membrane and weighed for fresh biomass determination. Siderophores were extracted from the spent agar with an equal volume of MilliQ water, lyophilized, and finally resuspended in MilliQ water according to the measured biomass (fresh weight) for further comparative analyses.

UPLC-ESI-MS analysis of siderophores

Siderophore extracts were analysed with a UPLC (Acquity H-class, Waters s.a., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD mass analyzer) on a ACQUITY UPLC® BEH C_{18} 1.7 μm column. Ten μl were injected and elution was performed at 40 °C with a constant flow rate of 0.6 mL min^{-1} using a gradient of ACN in water both acidified with 0.1% formic acid as follows: 0.5 min at 0%, from 0% to 35% in 2.5 min, from 35% to 100% in 0.2 min and maintained at 100% for 1.8 min. Compounds were detected as protonated molecular ions detected in electrospray positive ion mode (scan in the mass range *m*/*z* 550–700) by setting SQD parameters as follows: source temperature 130 °C; desolvation temperature 300 °C, and desolvation/cone nitrogen flow: 1000/50 L h^{-1}. For optimal detection, the cone voltage was set at 40 V.

CAS assay for siderophore detection

Chrome azurol S medium was prepared according to Schwyn and J. B. Neilands,[50] without the addition of nutrients. The medium for 1 l of CAS was as follows: CAS 60.5 mg, hexadecyltrimethyl ammonium bromide (HDTMA) 72.9 mg, piperazine-1,4-bis-(2-ethanesulfonic acid) (PIPES) 30.24 g and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 ml. Agarose (0.9 w/v) was used as gelling agent. The CAS agar solution was overlaid onto colonies of solid *Streptomyces* cultures.

Transcriptomic analysis

Expression of genes of *S. coelicolor* wild-type and bald mutant strains was monitored as described previously.[11] The wild-type and three mutants were quadrant streaked on R2YE and allowed to grow for 68 h. Whole colonies were collected with a razor blade and immediately ground in liquid nitrogen using a mortar and pestle. After the liquid nitrogen had evaporated, 150 μl of buffer RLT (Qiagen) was added to the mortar and the biomass was further pulverized to yield a crude cell lysate. 1 μl of this lysate was subjected to probe hybridization and processing with the Nanostring nCounter Prep Station and Digital Analyzer according to the manufacture's instructions. Raw code counts were analyzed using the nSolver software package; briefly, total transcript counts were normalized using internal controls with background subtraction. Transcript counts for three genes (*hrdB*, *folB*, and *def*) were used for geometric mean normalization to correct for differences in total mRNA concentration. All data were collected from three biological replicates and gene expression was considered significantly altered if mutant counts had a p value < 0.02 in a student's T-test when compared with the wild-type.

Scanning electron microscopy

The morphological study of surface-grown vegetative and aerial hyphae of *S. coelicolor* M145, *bldJ*, and *bldK* mutants by cryo-scanning electron microscopy (JEOL JSM-840A) was performed as previously described.[51]

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