Extracellular Complementation and the Identification of Additional Genes Involved in Aerial Mycelium Formation in *Streptomyces coelicolor*

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ABSTRACT

Morphogenesis in the bacterium *Streptomyces coelicolor* involves the formation of a lawn of hair-like aerial hyphae on the colony surface that stands up in the air and differentiates into chains of spores. *bld* mutants are defective in the formation of this aerial mycelium and grow as smooth, hairless colonies. When certain pairs of *bld* mutants are grown close to one another on rich sporulation medium, they exhibit extracellular complementation such that one mutant restores aerial mycelium formation to the other. The extracellular complementation relationships of most of the previously isolated *bld* mutants placed them in a hierarchy of extracellular complementation groups. We have screened for further *bld* mutants with precautions intended to maximize the discovery of additional genes. Most of the 50 newly isolated mutant strains occupy one of three of the previously described positions in the hierarchy, behaving like *bldK*, *bldC*, or *bldD* mutants. We show that the mutations in some of the strains that behave like *bldK* are *bldK* alleles but that others fall in a cluster at a position on the chromosome distinct from that of any known *bld* gene. We name this locus *bldL*. By introducing cloned genes into the strains that exhibit *bldC* or *bldD*-like extracellular complementation phenotypes, we show that most of these strains are likely to contain mutations in genes other than *bldC* or *bldD*. These results indicate that the genetic control of aerial mycelium formation is more complex than previously recognized and support the idea that a high proportion of *bld* genes are directly or indirectly involved in the production of substances that are exchanged between cells during morphological differentiation.

THE filamentous bacterium *Streptomyces coelicolor* sporulates in specialized cells called aerial hyphae. These cells are produced during the course of a life cycle of several days' duration that, at least superficially, is more like that of some of the eukaryotic fungi than other prokaryotes (Adams *et al.* 1998). The first cells produced after the germination of *S. coelicolor* spores are the substrate hyphae. These hyphae are subdivided by crosswalls at relatively infrequent intervals and grow by elongating and branching, giving rise to long substrate filaments that can contain dozens of chromosomes. Within 48 hr of germination aerial hyphae begin to appear in the developing colony. These filaments stand up in the air, forming a white layer of fuzz on the colony surface called the aerial mycelium. Unlike the substrate hyphae, the aerial hyphae undergo extensive cell division that divides them into chains of uninucleoidal compartments. Each of these compartments then matures into a spore. While septation and spore formation are taking place in the aerial mycelium, the substrate mycelium produces antibiotics, including the blue- and red-pigmented compounds actinorhodin and undecylprodigiosin. The life cycle of *S. coelicolor* can therefore be thought of as a cooperative enterprise in which substrate hyphae surround the developing colony with a protective moat of antibiotic compounds and raise the reproductive hyphae of the aerial mycelium into the air, allowing the dispersal of the spores that will form the next generation (Chater 1993).

Two classes of developmental genes are required for the morphogenetic events of the *S. coelicolor* life cycle. The *whi* genes, which are not directly relevant to the work reported here, are required for cell division and spore maturation in the aerial hyphae (Chater 1972). Mutations in the second class of developmental genes block the life cycle before the production of the aerial mycelium (Merrick 1976; Champness 1988; Willey *et al.* 1993; Nodwell *et al.* 1996). These mutants lack the characteristic fuzzy appearance of the wild type and are therefore called *bld* (from *Bald*) mutants. Ten classes of *bld* mutants have been characterized: *bld261*, *bldA*, *bldB*, *bldC*, *bldD*, *bldF*, *bldG*, *bldH*, *bldI*, and *bldK*. In addition to their inability to produce aerial mycelium, many of these mutants exhibit a marked defect in the synthesis of antibiotics and therefore lack the characteristic pigmentation of wild-type substrate hyphae. This suggests that the synthesis of antibiotics and the production of aerial hyphae share some important regulatory links. Strains containing mutations in *bldK*, however, exhibit little or no defect in pigmentation, suggesting that the

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roles of BldK and perhaps other yet-to-be-discovered *bld* genes are restricted to morphogenesis (Nodwell *et al.* 1996).

The *bld* mutants are unable to produce a small hydrophobic molecule called SapB and this may account in part for their defect in aerial mycelium formation (Willey *et al.* 1991; Richter *et al.* 1998). When *bld* mutants are grown close to wild-type cells, SapB can diffuse through the growth medium and stimulate the hyphae of the mutant to stand up in the air. Indeed, when purified SapB is applied to *bld* mutant colonies, a white layer of fuzz that is suggestive of aerial hyphae appears on the colony surface. This effect is not a true rescue of the developmental phenotype, however, as these aerial filaments do not undergo the morphological alterations and massive septation of developing aerial hyphae and do not produce chains of spores (Tillotson *et al.* 1998).

This "aerializing" property of SapB is similar to the activity of the hydrophobins of the Deuteromycete fungi. Hydrophobins stimulate the formation of the aerial structures in which spore formation takes place but are not the developmental triggers for spore formation itself. Indeed, the hydrophobin SC3 produced by *Schizophyllum commune* can mimic the activity of SapB and induce aerial growth of the substrate hyphae of *S. coelicolor bld* mutants (Tillotson *et al.* 1998). Furthermore, streptofactin, a *S. tendae* analogue of SapB, can similarly substitute for SC3 in the formation of the *S. commune* aerial fruiting structures (J. Willey, personal communication; Richter *et al.* 1998; Tillotson *et al.* 1998). A corollary of this extraordinary example of convergent evolution is that SapB's role is a purely structural one. The absence of SapB in *bld* mutants, therefore, is only part of the explanation for the morphogenetic defect of these mutants: the *bld* gene products must do more than simply direct the synthesis of SapB.

In addition to the extracellular complementation of *bld* mutants by wild-type cells, a second form of extracellular complementation has been demonstrated between pairs of *bld* mutants during growth on rich media such as R2YE (Willey *et al.* 1993). These relationships are unidirectional: one mutant acts as donor and the other as recipient of what are believed to be extracellular molecule(s) that restore aerial mycelium formation in the recipient only. In at least some cases of extracellular complementation, a good example being the extracellular complementation of *bld261* by *bldK* mutants, the restoration is apparently complete and the recipient goes on to produce morphologically mature aerial hyphae, viable spores, and the full complement of pigmented antibiotics (J. Nodwell, unpublished observations).

The donor/recipient relationships between many of these mutants fall naturally into the hierarchy *bld261* < *bldK* < *bldA/H* < *bldG* < *bldC* < *bldD* (Willey *et al.* 1993; Nodwell *et al.* 1996). The mutants that fit into this scheme can extracellularly complement all of the mutants that occupy positions lower in the hierarchy (or to the left as shown above) but none of the mutants higher in the hierarchy (to the right above). Thus, a *bldD* mutant can extracellularly complement all of *bld261*, *bldK*, *bldA*, *bldH*, *bldG*, and *bldC* but cannot itself be extracellularly complemented by any of the other *bld* mutants. At the opposite extreme is the *bld261* mutant, which is unable to act as a donor but can be extracellularly complemented by all other *bld* mutants. An interpretation that could explain the extracellular complementation hierarchy is that a cascade of intercellular signals regulates aerial mycelium formation. According to this view, the positions in the hierarchy reflect the inability of mutants to complete specific steps in the signaling cascade. The *bld261* mutant then would be blocked at the beginning of the cascade and make none of the signals. The *bldD* mutant would be blocked late in this cascade, producing all of the signals but failing to couple this to aerial mycelium formation (Willey *et al.* 1993).

Molecular evidence that supports a role for intercellular signaling in aerial mycelium formation comes from work on *bld261* and *bldK*. The *bldK* gene encodes an ABC transporter that is required to import oligopeptides into cells (Nodwell *et al.* 1996). This suggests that the developmental block of this mutant is due to its inability to import a developmental signaling peptide. Indeed, a molecule has been purified that restores aerial mycelium formation to the *bld261* mutant, in a manner that depends on the ability of this mutant to import oligopeptides (Nodwell and Losick 1998). If the signaling hypothesis of extracellular complementation is correct, then this molecule might be the first signal in the cascade.

bld genes may also be involved in sensing the nutritional state of cells. Most of the *bld* mutants can be partially or in some cases completely restored in aerial mycelium formation when they are grown on poor carbon sources such as mannitol (Merrick 1976; Champness 1988). Furthermore, *bldA*, *bldB*, *bldC*, *bldD*, and *bldG* mutants, and perhaps others, are defective in the catabolite repression of metabolic operons that occurs in wild-type cells. Specifically, these mutants activate the *gal* operon when grown in the presence of glucose as sole carbon source, conditions under which this operon is normally repressed (Pope *et al.* 1996). This property of *bld* mutants is consistent with the idea that a shift in the nutritional state or metabolic activity of the colony is associated with the onset of aerial mycelium formation (Karandikar *et al.* 1997).

There are several reasons for thinking that many of the genes required for the formation of the aerial mycelium have not yet been discovered. First of all, previous screens for developmental mutants were carried out before the discovery of the extracellular complementation of *bld* mutants by wild-type cells. Plating mutagen-

ized colonies in these screens at high density may have resulted in the masking of the phenotypes of important mutants. Second, two *bld* genes, *bldB* and *bldD*, encode what are likely to be DNA-binding proteins (Elliot *et al.* 1998; Pope *et al.* 1998) and a third, *bldA*, encodes a tRNA (Lawlor *et al.* 1987). If, as has been suggested, BldB and BldD are transcriptional regulators and the *bldA*-encoded tRNA is a translational regulator, then there ought to be regulons of developmental genes under their control. Third, the existence of *bld* mutants such as *bldK* that have nearly normal pigmentation suggests that there might be other genes that are dedicated to the formation of aerial mycelium having little or no impact on the synthesis of antibiotics. Finally, regardless of whether the *bld* genes are involved in signaling, metabolism, or both, there simply are not enough genes represented among the current collection to explain the formation of the aerial mycelium. For example, none of the genes that direct the synthesis of SapB or monitor the metabolic state of colonies is known.

In this work, therefore, we have screened for *bld* mutants among mutagenized cells plated on rich medium at relatively low densities. We report the discovery of 50 new mutants, all of which can take part in extracellular complementation and most of which can be clearly positioned in the extracellular complementation hierarchy: 10 in the same step as *bldK* mutants, 3 in the same step as *bldC* mutants, and 31 in the same step as *bldD* mutants. An additional group of 6 mutants, like some previously identified *bld* mutants, could not be positioned in the extracellular complementation hierarchy and reveal an extraordinary diversity in the repertoire of extracellular complementation phenotypes. We further show that a subset of the *bldK*-like group defines a novel genetic locus, which we map to a position near the *uraA* gene. By using cloned copies of a wild-type *bldC* and *bldD* gene we show that most of the new mutant strains are likely to contain mutations in previously unknown genes. Thus, our data provide evidence for the existence of at least three new developmental genes in *S. coelicolor*.

MATERIALS AND METHODS

Materials: Bialaphos was kindly provided by H. Anzai of Meiji Seika Kaisha Ltd. and by Mike Manson of the University of Texas. Enzymes and antibiotics were purchased from Sigma (St. Louis).

Strains, media, and growth conditions: Strains, bacteriophages, and plasmids used in this work are listed in Table 1. *S. coelicolor* was grown on R2YE agar (Hopwood *et al.* 1985) for mutant isolation, extracellular complementation, and the regeneration of transformed protoplasts. Yeast extract-malt extract media were used for liquid growth. Minimal media (MM) supplemented with glucose (1% w/v) were used for analyzing nutritional markers (Hopwood *et al.* 1985) and for assessing sensitivity to bialaphos (Nodwell *et al.* 1996). Streptomycin, chloramphenicol, kanamycin, thiostrepton, hygromycin, and lincomycin were added to R2YE plates at 10 μg/ml, 10 μg/ml, 200 μg/ml, 50 μg/ml, 50 μg/ml, and 40 μg/ml, respectively, as indicated. Bialaphos (Bayer *et al.* 1972) was added to MM glucose plates at concentrations ranging from 0.1 to 20 μg/ml. Cultures were grown at 30° in both liquid and plate culture. Spores were harvested and stored in 20% glycerol at −80° (Hopwood *et al.* 1985).

Mutagenesis of *S. coelicolor* spores: Spores of wild-type, prototrophic *S. coelicolor A(3)2* (strain 1147) were suspended in 0.05 m Tris + maleic acid (TM) pH 7.0 buffer and divided into aliquots of 0.1 ml containing 2×10^6 spores. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) dissolved in TM buffer was added to the spores at concentrations of 0.1, 0.2, 0.4, 0.8, and 1.6 mg/ml and incubated at 30° for 1 hr. To stop mutagenesis, the suspensions were centrifuged (5 min), the supernatants decanted, and the spores washed 2 times with 20% glycerol. By comparison of the number of viable spores in each mutagenized stock to the starting titre of the spore stock the following proportions of spores were shown to have survived each NTG treatment: 0.1 mg/ml, 94%; 0.2 mg/ml, 24%; 0.4 mg/ml, 21%; 0.8 mg/ml, 20.5%; 1.6 mg/ml, 6.5%. Each stock was plated on R2YE so that 100–200 colonies grew up, and plates were examined for the presence of mutants after 2–4 days of growth. Plating mutagenized spores under these conditions resulted in an average spacing of ~3 mm between colonies. Under these conditions there was relatively little extracellular complementation between colonies, allowing us to efficiently isolate *Bld* mutant strains. At each concentration of NTG, most plates contained at least one developmentally impaired mutant colony. We isolated 36 mutants from the 0.2 mg/ml mutagenesis, 85 from the 0.4 mg/ml mutagenesis, 88 from the 0.8 mg/ml mutagenesis, and 379 from the 1.6 mg/ml mutagenesis. Each mutant was single-colony purified 2 times and stored in 20% glycerol at −80°.

Conjugational mapping and statistical analysis: Crosses were conducted between *bld* mutants and strains containing arrays of selectable markers as described previously (Chater 1972; Hopwood *et al.* 1973, 1985; Merrick 1976; Champness 1988). Exconjugants (20/plate) were patched onto the medium used in the primary selection medium and allowed to grow for at least 4 days. These master plates were then replica plated to various defined media to determine the genotype of each exconjugant, and to R2YE to assess each colony's *Bld* phenotype. The frequency of cosegregation of the bld^+ and bld^- alleles with each marker was calculated. The significance of this cosegregation was tested statistically by a χ^2 test (Suzuki *et al.* 1981).

Correction of the K-, C-, and D-group mutants with cloned genes: To determine if the phenotypes of the K-group mutants could be corrected by a wild-type copy of the *bldK* gene, each of them was mated with the auxotrophoic strain NS71, containing the mobile plasmid pbldK22 (Nodwell *et al.* 1996) parallel to the *bldK1* mutant NS17. The desired exconjugants were selected by plating the products of the cross on MM containing thiostrepton. The cosmids D17 and D25 that have been shown to contain the wild-type allele of *bldC* (Redenbach *et al.* 1994; G. Kelemen, personal communication) were transformed into the C-group strains and the *bldC* mutant LS19. A total of 2 μl of 1 n NaOH was added to 8 μl of a 0.2 mg/ml solution of DNA propagated in the *dam- dcm- E. coli* strain and incubated for 10 min at 37°. A total of 2 μl of 1 n HCl was added to the denatured DNA solution, which was then stored on ice. Protoplasts of the C-group mutants and LS19 were then transformed with the denatured DNA (Hopwood *et al.* 1985; Oh and Chater 1997) and recombinants were selected that were resistant to 200 mg/ml kanamycin. High titre stocks (5 μl) of the bacteriophages KC741 and KC742 were spotted on patches of the K-group or D-group mutants, respectively, growing on R2YE. The patches were allowed to grow for 1 wk and then cells were harvested, macerated in

TABLE 1

Strains used in this work

Strains / plasmids	Description	Source / reference
	Streptomyces coelicolor	
1147	Prototroph	Losick strain collection
A332	*pheA1 NF SCP2**	Keith Chater
J701	*bldB15 mthB2 agaA7 NF*	Jacqueline Piret
J703	*bldI homB agaA7*	Jacqueline Piret
KS1	*bld173 hisA1 uraA1 strA1 NF::tsrR*	This work
KS2	*bld587 hisA1 uraA1 strA1 NF::tsrR*	This work
LS17	*bldA39 hisA1 uraA1 strA1 NF::tsrR*	Losick strain collection
LS19	*bldC13 mthB2 cysD18 NF SCP2**	Losick strain collection
C103	*bldG103 hisA1 uraA1 strA1 Pg1-*	Champness (1988)
C109	*bldH109 hisA1 uraA1 strA1 Pg1-*	Champness (1988)
LS59	*proA1 pheA1 hisA1 strA1 NF*	Losick strain collection
LS90	*bldD53 hisA1 strA1 NF SCP2* Pg1-*	Losick strain collection
LS92	*bld261 hisA1 strA1 NF SCP2**	Willey *et al.* (1993)
LS115	*argA1 hisD3 adeA3 NF*	Losick strain collection
NS17	*bldK::aadA*	Nodwell *et al.* (1996)
NS33	*bldK::aadA pbldK22*	Nodwell *et al.* (1996)
NS37	*bld173 proA1 hisA1 cysD18 uraA1 strA1*	This work
NS38	*bld587 proA1 hisA1 cysD18 uraA1 strA1*	This work
NS41	*bld261 bldK::aadA hisA1 uraA1 NF SCP2**	Nodwell and Losick (1996)
NS71	*hisA1 uraA1 argA1 proA1 strA1 pbldK22*	This work
NS135	*bld398 proA1 uraA1 NF::tsrR*	This work
YU105	*proA1 argA1 act::ermE whiE::hygR*	David Hopwood
	Streptomyces lividans	
LS39	Prototroph	Losick strain collection
	Bacteriophages	
KC741 KC703::bldG		Brenda Leskiw
KC742 KC703::bldD		Brenda Leskiw
	Plasmids	
pbldK22	*bldK* cloned into pJRM10	Nodwell *et al.* (1996)
D17	*bldC* locus of chromosome in Supercos	Redenbach *et al.* (1994)
D25	*bldC* locus of chromosome in Supercos	Redenbach *et al.* (1994)

0.85% saline solution, and spread on R2YE plates containing thiostrepton. Resistant clones were colony purified on the same medium used for selection and then compared with the untransformed strain for the presence of aerial mycelium during growth in the absence of selection.

RESULTS

Isolation of *bld* mutants under conditions of low colony density: To identify new genes involved in morphogenesis in *S. coelicolor* we treated wild-type spores with NTG and screened for mutants that were defective in the formation of aerial mycelium during growth on the rich medium R2YE. To minimize extracellular complementation by neighboring colonies, fewer than 200 colonies were grown on each plate and *bld* mutants were isolated as soon as their developmental defects became apparent. The majority of the *bld* mutants that arose as a result of our mutagenesis exhibited what appeared to be normal pigmentation; mutants defective in both morphogenesis and pigmentation were very rare. Overall, we screened 43,025 colonies and isolated 588 strains having defects in aerial mycelium formation.

More than 300 of the mutants from the primary screen were capable of producing aerial hyphae, although with a delay relative to the wild type. These were not studied further. Another group of the original set of mutants grew very slowly, produced irregularly shaped colonies, and were also not pursued further. The remaining mutants were all resistant to 10 μg / ml chloramphenicol, a feature of wild-type *S. coelicolor*, and therefore could not have resulted from the well-known high-frequency class of chloramphenicol-sensitive mutations that are associated with chromosomal rearrangements (Flett and Cullum 1987; Romero and Palacios 1997). Many of the final collection of mutants (which were all bald on minimal medium with glucose as the carbon source) produced substantial amounts of aerial hyphae when grown on mannitol minimal medium. This property, which is called carbon source suppression, is a feature of most of the previously identified *bld*

TABLE 2

Properties of the K-group mutants

Strain	Bialaphos phenotype	Correction of Bld phenotype by pbldK22?	Correction of Bld phenotype by KC741?
MY151	S	−	−
MY173	S	−	−
MY203	R	−	NT
MY225	R	−	NT
MY243	R	+	NT
MY314	R	−	NT
MY398	S	−	−
MY525	R	−	NT
MY557	R	+	NT
MY587	S	−	−
NS17	R	+	NT
LS21	S	n	+

S, sensitive to bialaphos; R, resistant to bialaphos; −, Bld phenotype not rescued by plasmid or bacteriophage; +, Bld phenotype restored to wild type by plasmid or phage; NT, not tested.

mutants (Merrick *et al.* 1976; Champness *et al.* 1988). None of them, however, was fully restored to wild-type development under these conditions, and we have not studied this property further.

Classification of *bld* mutants by extracellular complementation: Part of the motivation for seeking *bld* mutants in *S. coelicolor* was to discover additional genes in the extracellular complementation hierarchy. We were particularly curious to know whether and how newly identified mutants would fall into the hierarchical pattern of extracellular complementation exhibited by previously identified *bld* mutants. We therefore screened all 50 of the new mutants for extracellular complementation with strains containing the mutations *bld261*, *bldK1*, *bldA39*, *bldH109*, *bldG103*, *bldC18*, and *bldD53*. We found that all the new mutants took part in unidirectional extracellular complementation either as donor or as recipient. Moreover, most of the new mutants could be unambiguously positioned at one of the steps in the extracellular complementation hierarchy. To facilitate discussion of the salient mutations in these strains, we designated the mutant genes in each of them using the MY strain number of each mutant and the three-letter term *bld*. Thus, for example, we will refer to strains MY173 and MY382 as containing mutant alleles of the developmental genes *bld173* and *bld382*, respectively.

New members of the K group of extracellular complementation mutants: A subset of the new mutants exhibited the same pattern of extracellular complementation as strains containing defective alleles of *bldK* (Table 2). One such mutant was MY173. Like NS17, which harbors a deletion/insertion mutation (*bldK1*) in *bldK* (Figure 1e), MY173 was induced to produce aerial mycelium when it was grown close to colonies of a *bldA* mutant (Figure 1a) or to colonies of *bldH*, *bldG*, *bldC*, and *bldD* mutants (data not shown). Conversely, when MY173 was grown close to a *bld261* mutant (Figure 1b), it acted as a donor to restore aerial mycelium formation to the *bld261* recipient, as did the *bldK* mutant (Figure 1f). Finally, when MY173 was grown next to the *bldK* mutant NS17, neither mutant was able to extracellularly complement the other (Figure 1c). These data place MY173 in the same extracellular complementation group as the *bldK* mutant.

For a *bld261* mutant to be extracellularly complemented by a *bldK* mutant, it must itself have an active BldK oligopeptide importer (Nodwell and Losick 1998; see Figure 1, f and g), suggesting that this phenotypic restoration is mediated by a diffusible substance that is probably an oligopeptide. We found this to be true for extracellular complementation by MY173 as well. While MY173 was able to extracellularly complement a *bld261* mutant, when it was grown adjacent to the strain NS41 containing both *bld261* and *bldK1* mutations, neither strain was able to restore the capacity of the other to produce aerial mycelium (Figure 1d). This finding reinforces the view that MY173 is a member of the same extracellular complementation group as the *bldK* mutant NS17.

In addition to MY173, 9 mutants (MY151, MY203, MY225, MY243, MY314, MY398, MY525, MY557, and MY587) exhibited the same pattern of extracellular complementation as NS17. These 10 mutants, along with the two known *bldK* mutations *bldK1* and *bldK29*, are therefore assigned to the same extracellular complementation group, which we refer to as the K group. We show below that while some of the new strains in this group contain defective alleles of *bldK*, others are fully competent for BldK-mediated oligopeptide import and contain mutations that define a distinct genetic locus.

All members of the K group, including the *bldK1* insertion/deletion mutant NS17, appeared to produce the red- and blue-pigmented antibiotics at amounts similar to that of the wild type, although there appeared to be a delay in the onset of actinorhodin accumulation. Pigment production by K-group mutants is evident in Figure 1, for example, where the substrate mycelia of MY173 and NS17 appear darker than those of the *bld261* and *bldA* mutants, which are blocked in pigment production. We conclude that members of the K group of mutants are defective in aerial mycelium formation but not in the synthesis of secondary metabolites. An interesting observation that we have not pursued is that, whereas K-group mutants were unable to restore aerial mycelium formation to the *bld261 bldK1* double mutant, NS17, but not MY173, stimulated the production of the red-colored antibiotic undecylprodigiosin by the double mutant.

New members of the C group of extracellular complementation mutants: Another group of mutants was found to exhibit the same pattern of extracellular com-

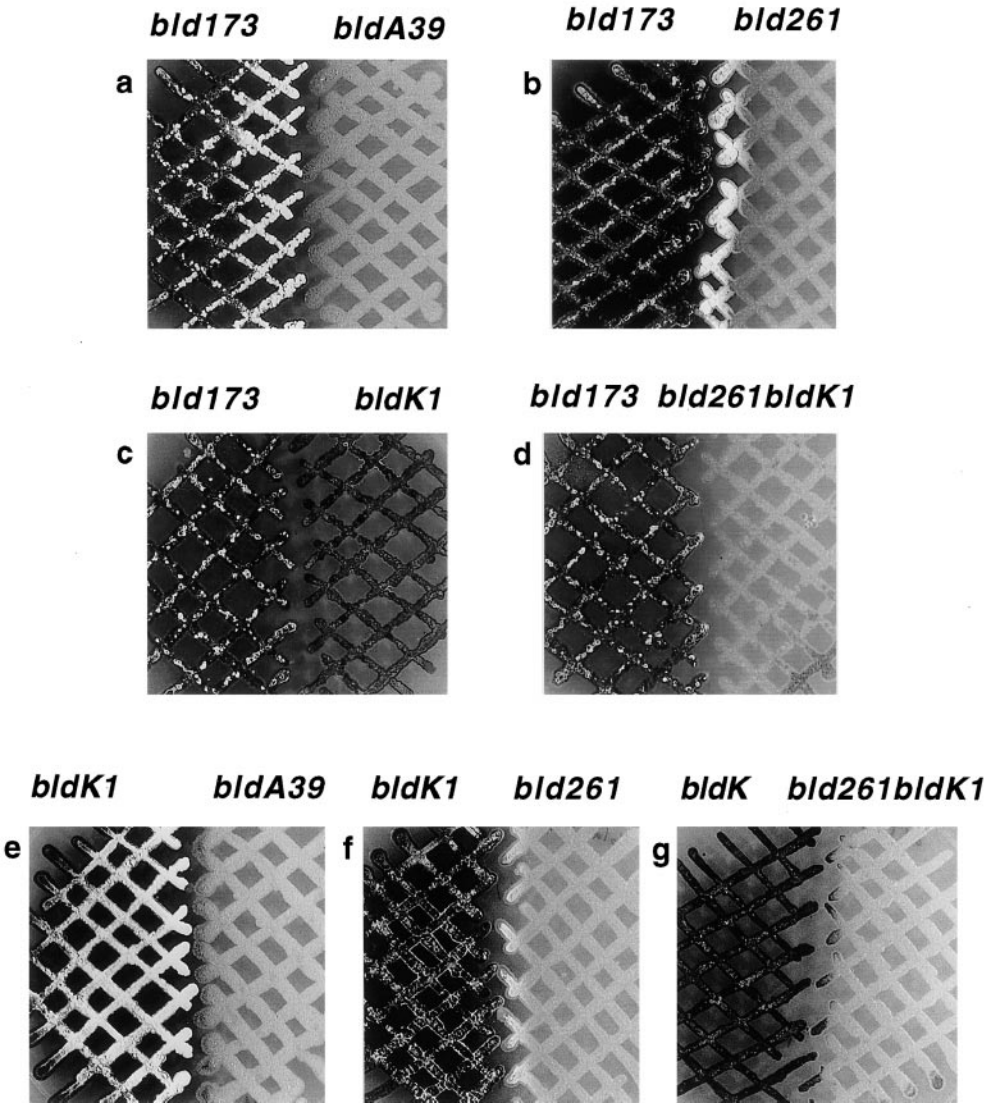


Figure 1.—Extracellular complementation by K-group mutants. Pairs of *bld* strains are shown highlighting the extracellular complementation relationships of mutants in (a) *bld173* and *bldA39*, (b) *bld261* and *bld173*, (c) *bld173* and *bldK1*, (d) *bld173* and *bld261 bldK1*, (e) *bldK1* and *bldA39*, (f) *bld261* and *bldK1*, and (g) *bldK1* and *bld261 bldK1*. Note that in *bldK* complemented the *bld261 bldK* double mutant for antibiotic synthesis but not aerial mycelium formation.

plementation as a *bldC* mutant. For example, proximity to *bldD* mutant cells induced MY159 to produce aerial mycelium along the colony edge that was closest to the *bldD* donor (Figure 2b). On the other hand, when plated next to a *bldG* mutant, MY159 was not extracellularly complemented, but induced aerial mycelium formation in the *bldG* recipient (Figure 2a). This behavior was identical to that of the *bldC* mutant strain LS19 (Figure 2, d and e). Also, like LS19, MY159 restored aerial mycelium formation to colonies of *bld261*, *bldK*, *bldA*, *bldH*, and *bldG* (data not shown). Finally, when MY159 and a *bldC* mutant were plated side by side, neither mutant showed any capacity to extracellularly complement the other (Figure 2c). Two other strains in the collection of new *bld* mutants (MY152 and MY321) exhibited this *bldC*-like pattern of extracellular complementation and these mutants, along with LS19, which harbors the previously known *bldC* mutation *bldC18*, are therefore assigned to extracellular complementation group C. We show below that at least two of the C-group strains are likely to contain mutations in novel developmental genes.

New members of the D group of extracellular complementation mutants: The largest group of mutants exhibited the same extracellular complementation pattern as strains containing the previously identified mutation *bldD53*. MY382, for example, could not be extracellularly complemented by any of the other *bld* mutants but was induced to produce an aerial mycelium when grown next to wild-type cells (not shown). However, when LS90 or MY382 were grown adjacent to a strain containing a *bldC18* mutation they induced the *bldC* mutant to produce a fringe of aerial mycelium (Figure 3a). We found that this extracellular complementation was rather weak and resulted only in the induction of a small amount of aerial hyphae along the edge of the *bldC* colonies that was closest to the MY382 donor strain. This fringe of aerial mycelium did not expand with longer incubation time and never extended very far beyond the donor-proximal edge of the *bldC* mutant. Also, like LS90,

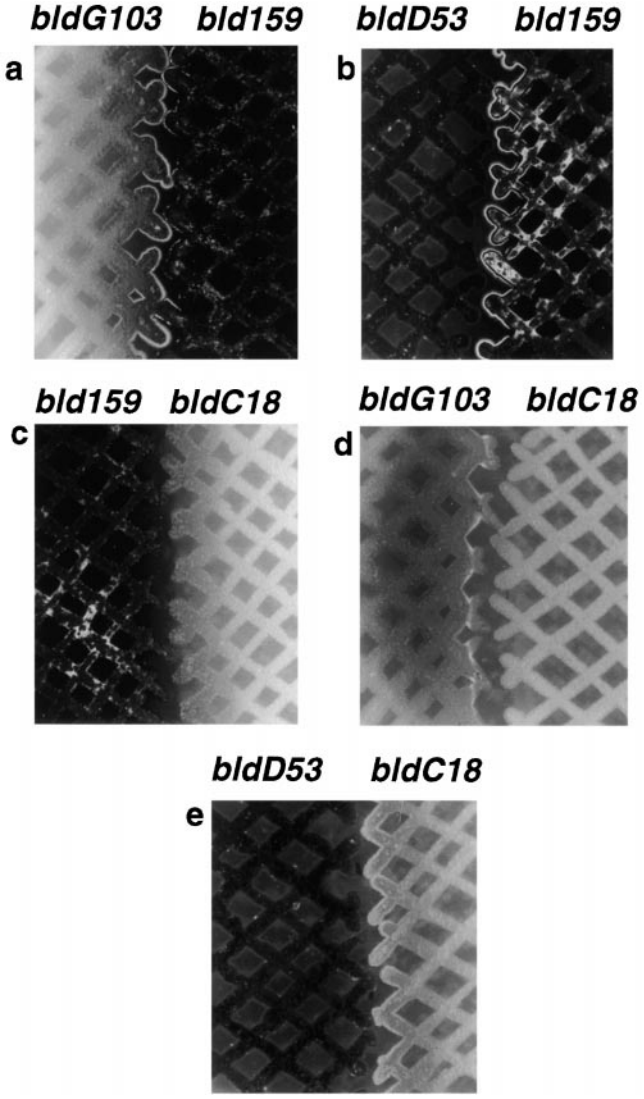


Figure 2.—Extracellular complementation by C-group mutants. Pairs of *bld* strains are shown highlighting the extracellular complementation relationships of mutants in (a) *bldG103* and *bld159*, (b) *bldD53* and *bld159*, (c) *bld159* and *bldC18*, (d) *bldG103* and *bldC18*, and (e) *bldD53* and *bldC18*.

MY382 was able to extracellularly complement strains containing *bld261*, *bldK*, *bldA39*, *bldH109*, or *bldG103* mutations, but usually more strongly than it complemented the *bldC* mutant (data not shown). Finally, neither MY382 nor the *bldD* mutant LS90 extracellularly complemented each other (Figure 3b). This *bldD*-like constellation of properties was shared by a large majority of the new *bld* mutants, including MY61, MY73, MY83, MY96, MY118, MY125, MY128, MY139, MY170, MY202, MY207, MY262, MY304, MY308, MY315, MY331, MY337, MY343, MY352, MY361, MY382, MY405, MY466, MY486, MY533, MY536, MY537, MY539, MY572, MY574, and MY588. These 31 mutants are therefore assigned to extracellular complementation group D.

Mutants that do not fall in a single step in the extracellular complementation hierarchy: A few of the previously characterized *bld* mutants, including *bldB* and *bldI*, do not occupy a clear position in the extracellular complementation hierarchy. *bldB* mutants, for example, can act as donors by extracellularly complementing *bld261* and *bldG* mutants and as recipients from *bldD* mutants. This behavior would be consistent with that of a C-group mutant but, unlike *bldC* mutant strains or MY152, MY159, or MY321, *bldB* mutants neither complement nor are complemented by strains containing *bldK*, *bldA*, or *bldH* mutations (Willey *et al.* 1993). The *bldI* mutant, on the other hand, can act as a donor to *bld261* but neither complements nor is complemented by any of the *bldK*, *bldA*, *bldH*, *bldG*, *bldC*, or *bldD* mutants. These data suggest that some *bld* gene products impinge on more than one signaling step. We therefore expected that some of our new *bld* mutants would have similar unclassifiable extracellular complementation relationships with the test mutants. Indeed, six of the new *bld* mutants, MY292, MY304, MY343, MY512, MY533, and MY590, showed extracellular complementation properties that did not fit the hierarchy. Strikingly, none of these showed the same pattern of extracellular complementation as either *bldB* or *bldI* mutants or each other (Table 3).

An intriguing example of unclassifiable extracellular complementation was shown by the mutant MY292. This strain acted as donor by extracellularly complementing *bld261*, *bldK*, and *bldG* mutants. However, unlike all of the other mutants that do this, MY292 was not able to extracellularly complement either *bldA* or *bldH* mutants, as if the phenotype of this strain "skips a step" in extracellular complementation. Furthermore, MY292 could act as a recipient, being extracellularly complemented by both *bldC* and *bldD* mutants. This is also unexpected because none of the classical *bld* mutants acts as recipient from *bldC* and donor to *bldG* mutants. MY512 shares this property of being extracellularly complemented by a *bldC* mutant and extracellularly complementing a *bldG* mutant but differs from MY292 in its relationship with *bldA* and *bldH* mutants. The mutant MY304 behaves similarly to MY292 in its extracellular complementation relationships with *bld261*, *bldK*, *bldA*, *bldH*, and *bldG* but, unlike this mutant, was not extracellularly complemented by either of the *bldC* or *bldD* mutants. MY343, MY361, and MY537 (Table 3) also exhibited unique patterns of extracellular complementation.

Only two common themes emerged in the behavior of these mutants: all of them (like all of the *bld* mutants isolated to date) could extracellularly complement a *bld261* mutant and, second, all of them behaved in the same way toward *bldA* and *bldH*, which are themselves in the same extracellular complementation group.

The K group of mutants defines at least two genes: The strains that make up the K group were of particular interest because we believed that these might contain novel alleles of *bldK* or, more importantly, mutations in genes encoding additional components of the peptide

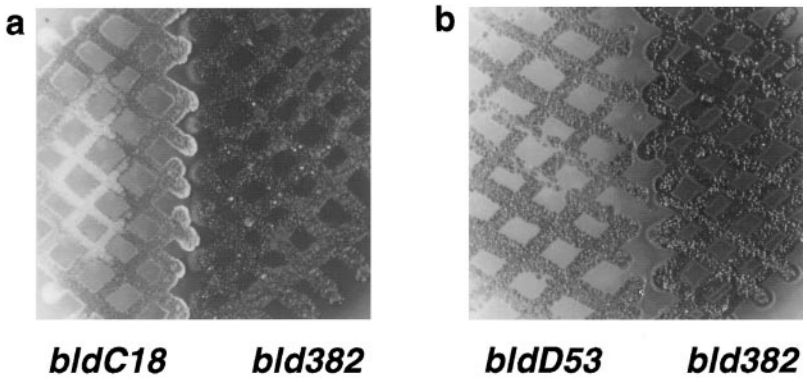


Figure 3.—Extracellular complementation by D-group mutants. Pairs of strains are shown highlighting the extracellular complementation relationships of the mutants (a) *bldC18* and *bld382* and (b) *bldD53* and *bld382*.

signaling step that we believe is mediated by the BldK oligopeptide importer. We therefore carried out two experiments to determine whether any of these strains contained mutant alleles of *bldK*.

We introduced the *bldK*-expressing plasmid pbldK22 (Nodwell *et al.* 1996) into each of the K-group strains to determine if they could be genetically complemented by a wild-type copy of the *bldK* gene. As expected, pbldK22 caused full genetic complementation of the *bldK* mutant NS17 (Figure 4a). Similarly, when we introduced pbldK22 into MY557 it was restored to wild-type aerial mycelium formation (Figure 4b). MY173, however, retained its block in aerial mycelium formation whether or not it contained pbldK22 (Figure 4c). In all, only two of the K-group strains, MY243 and MY557, were genetically complemented by pbldK22. The rest of the K-group mutants were not complemented by this plasmid, suggesting either that these strains contain dominant *bldK* alleles or that they contain mutations in other genes (Table 2).

As a second criterion for identifying mutations in *bldK* we screened the K-group mutants for sensitivity or resistance to bialaphos. This toxic peptide is imported into bacterial cells through ABC oligopeptide importers like BldK (Nodwell *et al.* 1996) and we have previously shown that bialaphos kills *bldK*$^+$ but not *bldK*$^-$ *S. coelicolor* (Nodwell *et al.* 1996). As observed previously, bialaphos killed wild-type cells but not the *bldK1* deletion mutant NS17 (Figure 5, Table 2). The K-group strain MY557 showed the same resistance to bialaphos as the *bldK* deletion mutant (Figure 5), as did five of the other K-group strains, MY203, MY225, MY243, MY314, and MY525 (Table 2). This suggests that these strains are all defective in importing bialaphos and therefore that *bld203*, *bld225*, *bld243*, *bld314*, *bld525*, and *bld557* are all defective alleles of *bldK*. Because pbldK22 restored aerial mycelium formation to only MY243 and MY557, we suspect that *bld203*, *bld225*, *bld314*, and *bld525* are dominant alleles of *bldK*, although it is also possible that these strains contain mutations in both *bldK* and another K-group *bld* gene. Conversely, the growth of MY173 was completely inhibited by bialaphos (Figure 5), showing that this strain has a functional BldK oligopeptide import system. Three other K-group strains (MY151, MY398, and MY587) were also sensitive to bialaphos, and none of the MY151, MY173, MY398, and MY587 mutants was restored to a Bld$^+$ phenotype by pbldK22, suggesting that *bld151*, *bld173*, *bld398*, and *bld587* are likely to be alleles of genes other than *bldK*.

Mapping an additional K-group gene(s): To test the hypothesis that *bld151*, *bld173*, *bld398*, and *bld587* are alleles of a gene(s) other than *bldK* and to determine their location, we mapped *bld173*, *bld398*, and *bld587* on the *S. coelicolor* chromosome by conjugation (Hopwood *et al.* 1973, 1985).

In five different crosses, the *bld173* mutation mapped

TABLE 3

***bld* mutants that do not fit into the extracellular complementation hierarchy**

MY292	MY304	MY343	MY512	MY537	MY590	J701	J703	Test mutants
Donor	Donor	Donor	Donor	Donor	Donor	Donor	Donor	*bld261*
Donor	Donor	Donor	Neither	Neither	Donor	Neither	Neither	*bldK*
Neither	Neither	Donor	Neither	Donor	Donor	Neither	Neither	*bldA/H*
Donor	Donor	Recipient	Donor	Donor	Donor	Donor	Neither	*bldG*
Recipient	Neither	Recipient	Recipient	Neither	Neither	Neither	Neither	*bldC*
Recipient	Neither	Neither	Recipient	Neither	Neither	Recipient	Neither	*bldD*

This table indicates whether the mutants in the row across the top act as donors, recipients, or neither in extracellular complementation experiments with the seven test mutants.

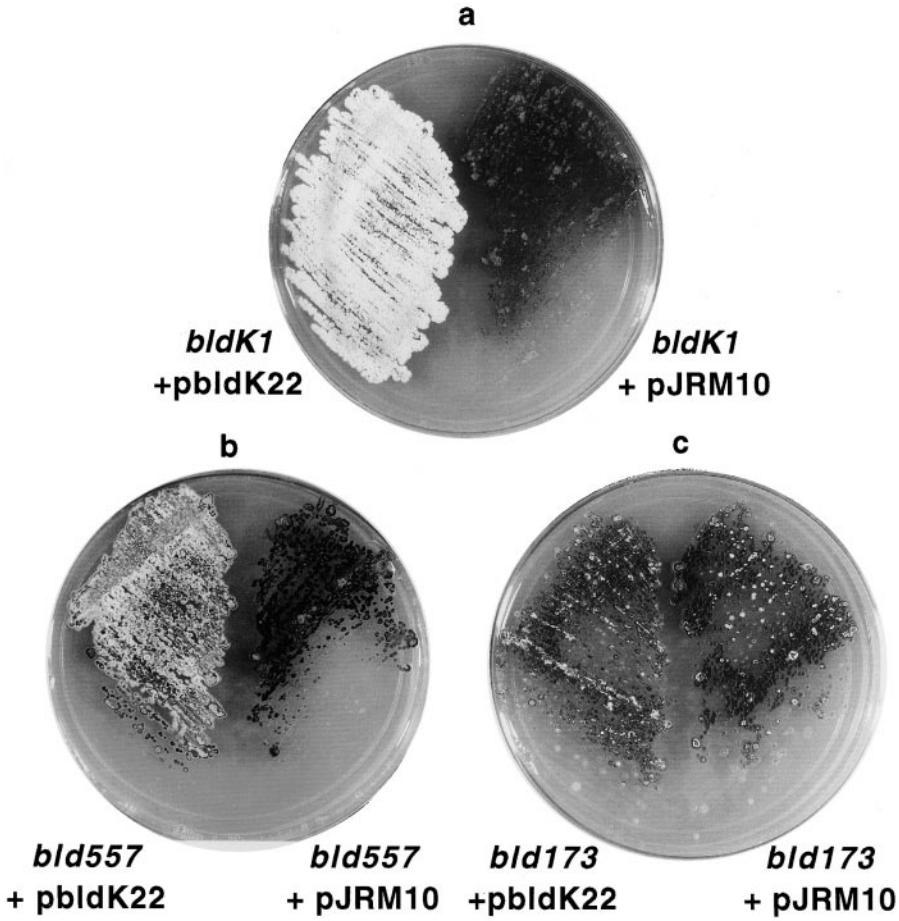


Figure 4.—The phenotypic effect of the expression of wild-type *bldK* in K-group mutants. The plasmids pbldK22 and pJRM10 (a vector control) were introduced into (a) the *bldK1* mutant NS17, (b) the K-group mutant MY557, and (c) the K-group mutant MY173.

to the interval between the NF fertility factor insertion site and the *pheA* locus at around 8 o'clock on the *S. coelicolor* chromosome. In the cross shown in Figure 6a, for example, the *bld173* containing strain KS1 was crossed with the strain YU105. We found that *bld173* cosegregated with the *uraA* allele in 87% of exconjugants, strongly suggesting genetic linkage of these two loci. Statistical analysis suggested that the probability that this cosegregation arose by chance (*P* value) was $<0.5\%$. In contrast, the *P* value for the apparent 42% cosegregation of *bld173* with *argA* was $>90\%$, suggesting that there was no linkage between these two loci. Indeed, the only marker in this cross that showed statistically meaningful cosegregation with *bld173* was *uraA1*. Furthermore, the relative proportion of Bld^+ and Bld^- exconjugants in this cross further suggested that the location of *bld173* was between *uraA* and the NF fertility factor insertion site (Figure 6a).

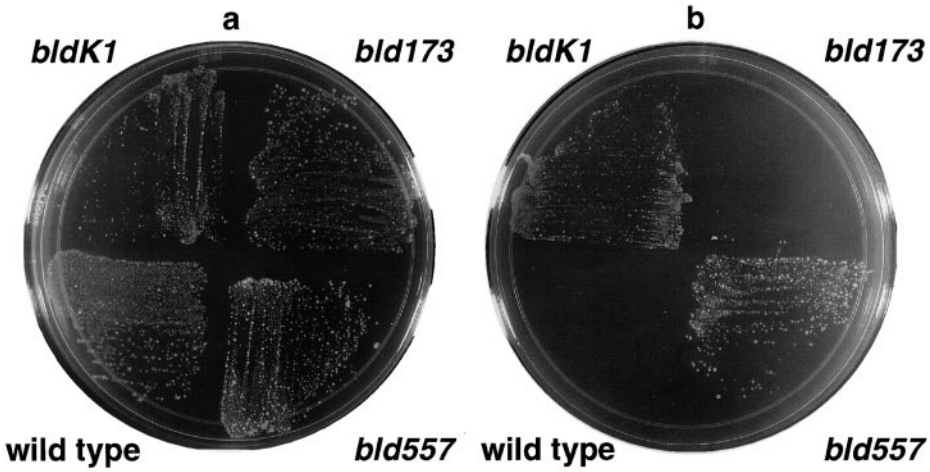
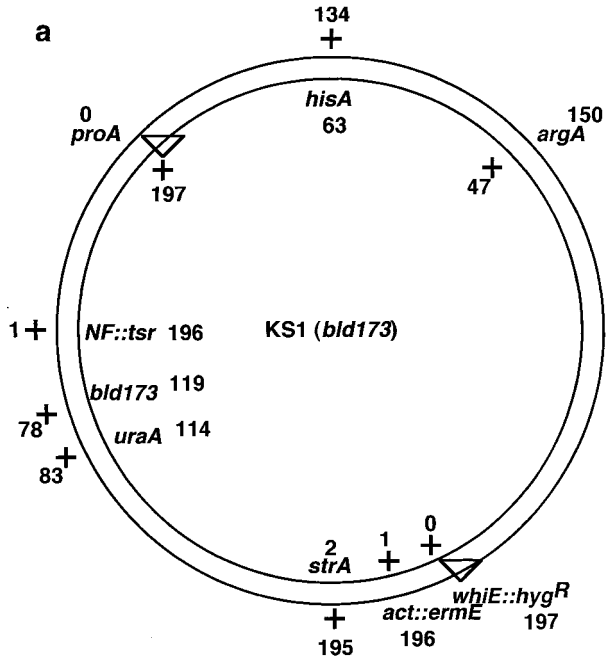


Figure 5.—Bialaphos resistance or sensitivity of K-group mutants. The strains NS17, M145, MY173, and MY557 were grown on (a) minimal glucose medium and (b) minimal glucose medium containing bialaphos.



	Bld⁻	Bld⁺
Ura⁻	104	10
Ura⁺	15	68

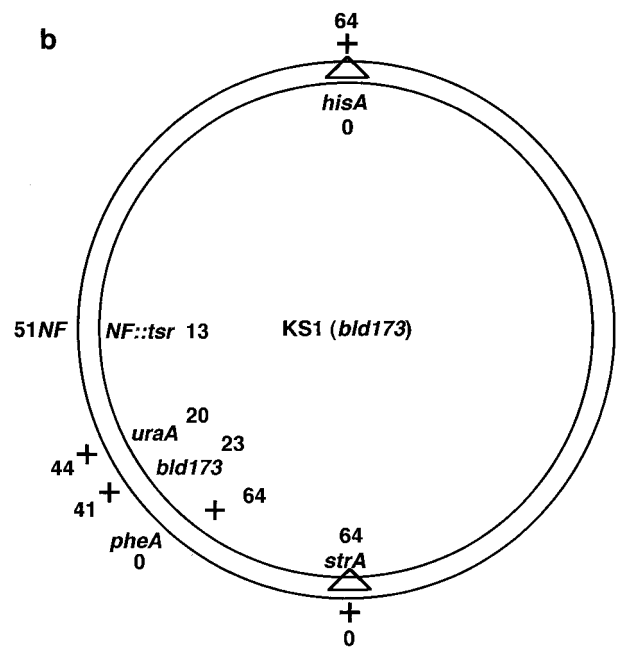
p<0.005

	Bld⁻	Bld⁺
Arg⁺	25	22
Arg⁻	93	57

p>0.9

	Bld⁻	Bld⁺
His⁻	41	22
His⁺	78	56

p>0.9



	Bld⁻	Bld⁺
Ura⁻	20	0
Ura⁺	3	41

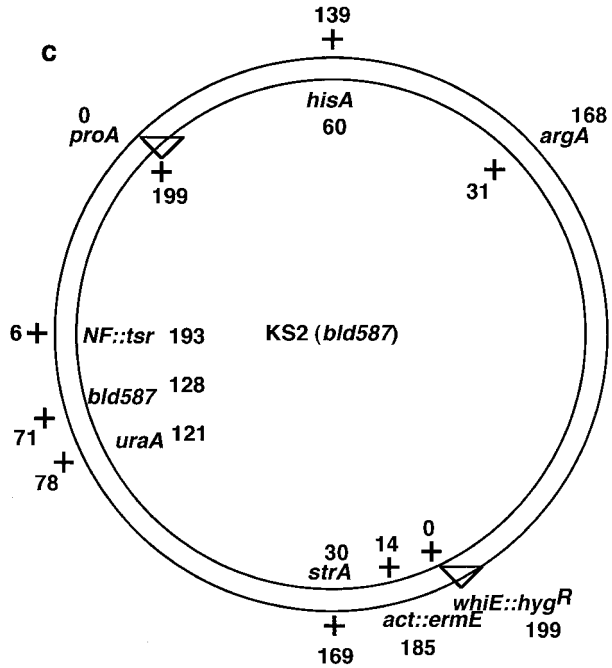
p<0.005

	Bld⁻	Bld⁺
TsrR	12	1
TsrS	11	40

p>0.01

	Bld⁻	Bld⁺
Phe⁺	41	23
Phe⁻	0	0

p=?



	Bld⁻	Bld⁺
Ura⁻	117	1
Ura⁺	11	70

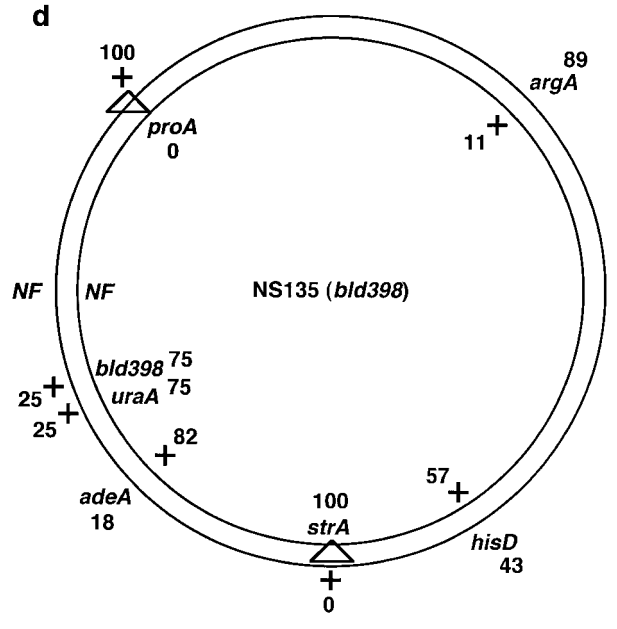
p<0.005

	Bld⁻	Bld⁺
Arg⁺	24	7
Arg⁻	104	64

p>0.9

	Bld⁻	Bld⁺
His⁻	43	17
His⁺	85	54

p>0.9



	Bld⁻	Bld⁺
Ura⁻	71	3
Ura⁺	4	22

p<0.005

	Bld⁻	Bld⁺
Arg⁺	9	1
Arg⁻	66	21

p>0.9

	Bld⁻	Bld⁺
His⁺	36	21
His⁻	39	4

p>0.5

Most of the crosses we conducted suggested that the *bld173* mutation is located between the NF site and the *uraA* locus. An exception to this, which nevertheless supported strong linkage of *bld173* to *uraA*, is shown in Figure 6b. Here, KS1 was crossed with the strain A332, and again *bld173* cosegregated with the *uraA* allele in 95% of the exconjugants ($P < 0.5\%$). Unlike the results of the cross shown in Figure 6a, however, the relative proportions of Bld^+ and Bld^- exconjugants suggested that *bld173* was on the other side of *uraA*, between *uraA* and the gene *pheA*. This ambiguity may reflect difficulties that we encountered in determining the Bld phenotype of some exconjugants. We found that most of the K-group mutations had differing degrees of severity when they were moved from one genetic background to another. It is possible, therefore, that some exconjugants that were assigned a Bld^- phenotype were in fact Bld^+, shifting the apparent location of the gene in this cross. Nevertheless, these results agree with those of Figure 6a, suggesting that *bld173* is located close to the *uraA* gene.

We also carried out several crosses with the mutants MY398 and MY587, and the results of these crosses suggested that the mutations in these two strains, like *bld173*, were located close to *uraA*. Figure 6c shows a cross of the strain KS2, which contains the *bld587* mutation, with the strain YU105. The results of this cross are strikingly similar to those in Figure 6a, showing that *bld587* cosegregated with *uraA1* in 94% of exconjugants ($P < 0.5\%$), while there was no statistically meaningful cosegregation of *bld587* with any of the other genetic markers. Furthermore, the relative proportions of Bld^+ and Bld^- exconjugants suggested that, like *bld173*, *bld587* was situated between the NF fertility factor insertion site and *uraA*.

Finally, we crossed the strains NS135 (containing the K-group mutation *bld398*) and LS115. In this cross *bld398* cosegregated with the *uraA* allele in 93% of the exconjugants, strongly suggesting that, like *bld173* and *bld587*, the *bld398* gene was closely linked to the *uraA* gene ($P < 0.5\%$). Furthermore, the relative proportions of Bld^+ and Bld^- exconjugants suggested that the location of this gene was between *uraA* and the NF fertility factor insertion site.

These mapping experiments suggested that the *bld173*, *bld398*, and *bld587* mutations mapped close to one another on the *S. coelicolor* chromosome. As further evidence that these three mutations (and the other K-group mutation *bld151*) fall in a linked cluster near the *uraA* gene we found that crosses of NS37 (*bld173*) and NS38 (*bld587*) with strains containing any of *bld151*, *bld173*, *bld398*, or *bld587* followed by selection for His^+ Str^R progeny yielded few, if any, wild-type exconjugants (data not shown). In addition to revealing the locations of *bld151*, *bld173*, *bld398*, and *bld587* these studies support our conclusion from the genetic complementation experiments (Figure 4) and bialaphos resistance (Figure 5) that these K-group mutations are alleles of a gene(s) different from *bldK*.

The position of these *bld* mutations is close to that of *bldG* on the *S. coelicolor* genetic map. We therefore created lysogens of each of MY151, MY173, MY398, and MY587 with the bacteriophage KC741, which contains a wild-type copy of the *bldG* gene (kindly provided by Brenda Leskiw). As expected, KC741 restored the developmental phenotype of a *bldG103* containing strain C103 to wild type. Consistent with the idea that *bld151*, *bld173*, *bld398*, and *bld587* are alleles of a developmental gene other than *bldG*, KC741 lysogens of these mutants remained blocked in aerial mycelium formation (Table 2).

The C and D groups of mutants define at least two additional genes: Extracellular complementation experiments with the mutants of the C and D groups suggested that these strains occupied positions in the extracellular complementation hierarchy that were higher than the mutants of the K group, or indeed than any of *bld261*, *bldA*, *bldH*, or *bldG*. Consistent with this, we found that all of the C- and D-group strains had the same sensitivity to the drug bialaphos as wild-type cells, demonstrating that they contain wild-type BldK oligopeptide importers (data not shown). We were therefore interested in determining whether these strains contained mutations in genes different from the previously described genes *bldC* and *bldD*.

The cosmids D17 and D25 (kindly provided by Helen Kieser and David Hopwood) contain overlapping chromosomal inserts that include a wild-type copy of the *bldC* locus (Redenbach *et al.* 1994). We therefore introduced these cosmids into the C-group mutants. We were able to transform both cosmids into MY159, MY321, and the *bldC18* mutant LS19 (Figure 7). As expected, the cosmid restored the *bldC* mutant to wild-type aerial mycelium formation and antibiotic synthesis. Both of the novel C-group strains MY159 and MY321, however, remained blocked in aerial mycelium formation even though they had integrated a wild-type copy of the *bldC* gene into their chromosomes. We obtained the same result following transformation of the mutant strains with the *bldC*

Figure 6.—Genetic mapping of *bld173*, *bld398*, and *bld587*. The results of crosses of the *bld173* containing strain KS1 with (a) YU105 and (b) A332; (c) the *bld587* containing strain KS2 with YU105 and (d) the *bld398* containing strain NS135 with the strain LS115. In the crosses in a and c, KS1 and KS2 served as the genetic donors as they were the only strains that contained the NF-inserted SCP1 fertility plasmid. In the crosses in b and d, however, both parents contained the NF insertion of SCP1 and therefore both served as donor and recipient of chromosomal DNA. In each cross the genetic markers used to select for exconjugates are indicated by the open wedges.

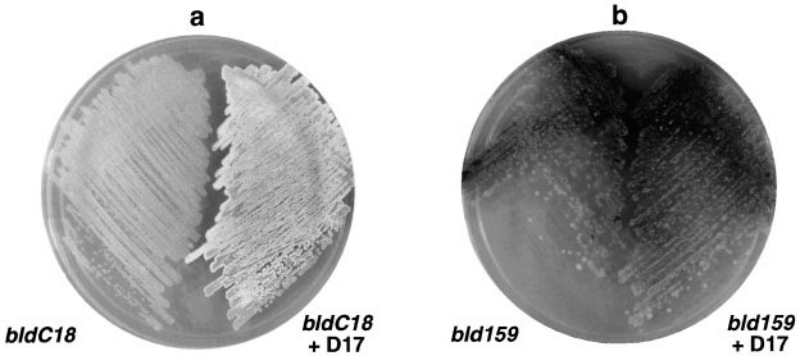


Figure 7.—The phenotypic effect of *bldC* containing cosmids on C-group mutants. The *bldC* containing cosmid D17 was introduced into (a) a *bldC18* containing strain and (b) a *bld159* containing strain.

containing cosmid D25: rescue of aerial mycelium formation in the *bldC* mutant but not of MY159 or MY321. These results suggest that these C-group strains are likely to contain mutations in genes other than *bldC*. Attempts to transform MY152 with the cosmids were unsuccessful because it grew poorly in liquid medium and yielded few viable protoplasts.

To determine whether the D-group strains contained mutations in the *bldD* gene or in novel genes we created lysogens of each of them with the bacteriophage KC742 (Elliot *et al.* 1998), which contains a wild-type copy of the *bldD* gene (kindly provided by Brenda Leskiw). As shown in Figure 8a, introducing this bacteriophage into the *bldD* mutant strain LS90 restored aerial mycelium formation to the mutant. Conversely, when we introduced the bacteriophage into the strain MY382 (Figure 8b), it had no effect on its capacity to produce aerial mycelium, suggesting that the developmental mutation in this strain is in a gene different from *bldD*. Lysogenization of MY405 and MY486 with KC742 caused a modest improvement in the ability of these strains to produce aerial mycelium so it is possible that these strains contain mutant alleles of *bldD*. However, this effect was quite weak, and even after prolonged incubations the two lysogens were still clearly impaired in development (data not shown). All of the other mutants in the D group remained as bald as their nonlysogen parents, suggesting that most or perhaps all of these strains contain mutations in genes other than *bldD*.

While it is possible that some of the C- and D-group strains contain dominant alleles of *bldC* and *bldD*, we do not believe that this could be the case in many of these strains. In particular, out of the 50 mutants described in this work, only 6 were in the relatively large (>6000 bp) *bldK* gene cluster. The *bldD* gene is less than a tenth the size of *bldK* (<550 bp), suggesting that we would have expected fewer than six *bldD* alleles among this group of strains. We believe, therefore, that the majority of the D group, and probably also of the C-group mutants, contain alleles of genes other than *bldC* and *bldD*.

DISCUSSION

Our strategy for discovering additional *bld* genes was based on the idea that mutants in these genes could be extracellularly complemented by wild-type colonies and therefore easily missed in screens. We also suspected that there were likely to be many *bld* mutations having little or no effect on the synthesis of antibiotics. The mutants we have discovered during the course of this screen bear this out: most of them have little or no defect in pigmentation and all of them can be induced to form aerial mycelium by neighboring colonies. In fact, we have shown that 44 of the 50 newly isolated *bld* mutants fell into previously identified extracellular complementation groups: 10 in the K group, 3 in the C group, and 31 in the D group. In addition to these 44 new hierarchy members we have identified 6 mutants

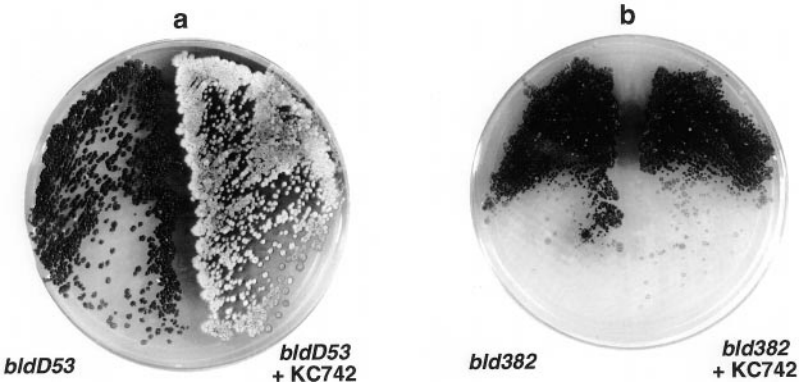


Figure 8.—The phenotypic effect of the *bldD* containing bacteriophage KC742 on D-group mutants. The *bldD* containing bacteriophage clone KC742 was introduced into (a) a *bldD53* strain (b) a *bld382* strain.

that, like the previously isolated *bldB* and *bldI* mutants, exhibit extracellular complementation phenotypes that are unique to themselves and that do not obey the rules of the extracellular complementation hierarchy. We have shown that 4 of the K-group, 2 of the C-group, and at least 29 of the D-group mutations could not be corrected by wild-type *bldK*, *bldC*, or *bldD* genes, respectively, suggesting that they reveal mutations in previously unrecognized *bld* genes. We confirmed that the 4 K-group mutations are in a previously unknown gene by mapping them on the *S. coelicolor* chromosome and showing that they are a linked cluster close to, but distinct from, *bldG*. Taken together, these results support our contention that there are many additional *bld* genes and reveal the existence of at least one additional gene in each of the K, C, and D groups.

The extracellular complementation, genetic complementation, and conjugational mapping data suggest that the four novel K-group mutations *bld151*, *bld173*, *bld398*, and *bld587* identify either a gene or a group of genes having related functions near *uraA* on the *S. coelicolor* chromosome. We therefore propose the name *bldL* for this additional *bld* locus. Similarly, previous genetic mapping experiments have shown that the *bld261* mutation identifies an additional *bld* gene positioned close to *cysD* (Willey *et al.* 1993), which we propose should henceforth be referred to as *bldJ*.

The cloning and characterization of the *bldA*, *bldB*, and *bldD* genes suggest that transcriptional and translational regulation of developmental genes is likely to play an important role in aerial mycelium formation (Lawlor *et al.* 1987; Elliot *et al.* 1998; Pope *et al.* 1998). If, as has been inferred from their sequences, these genes encode transcriptional and translational regulatory factors, then there is likely to be a distinct group of developmentally regulated genes under the control of each. It is possible, therefore, that some of the genes identified in our screen are BldA, BldB, or BldD targets. The genes identified by the 31 D-group mutations, for example, are reasonable candidates for targets of the BldD transcription factor.

Prior to this screen the only position in the extracellular complementation hierarchy held by more than one mutant was that of *bldA* and *bldH*. Thus it was unclear whether different steps would be discovered in this work, and the biological relevance of the known ones was rather tenuous. The fact that we were able to correctly identify new alleles of *bldK* (*bld203*, *bld225*, *bld243*, *bld314*, *bld525*, and *bld557*) among a large random set of new *bld* mutants using only extracellular complementation supports the idea that this is a meaningful way to catalog mutants that are defective in aerial mycelium formation. More importantly, we have not identified any additional steps in the hierarchy and have found many more members for the K, C, and D groups, lending credence to the idea that the extracellular complementation phenotypes of each group might represent true developmental intermediates in the process of aerial mycelium formation.

Curiously, we have not identified additional mutants that fall into any of the *J*, *A*/*H*, or *G* positions of the extracellular complementation hierarchy. One noteworthy feature of most of the mutants we have isolated is that their substrate mycelia have apparently normal pigmentation, suggesting that they are defective in aerial mycelium formation but not antibiotic synthesis, unlike most of the previously identified *bld* mutants. One explanation for our failure to identify *bldJ*-, *bldA*/*H*-, or *bldG*-like mutants, therefore, might be simply that all mutations in these groups disrupt antibiotic synthesis as well as aerial mycelium formation and that, by focusing on *bld* mutants having wild-type pigmentation, we introduced a bias against these groups into our screen.

Accounting for the 50 new mutants that we have isolated, the positions of the extracellular complementation hierarchy now consist mostly of groups of mutants, as follows:

$$bldJ < \text{K group} < \text{A/H} < bldG < \text{C group} < \text{D group}.$$

A hypothesis that we have advanced in the past to explain this hierarchy (Willey *et al.* 1993; Nodwell *et al.* 1996; Nodwell and Losick 1998) posits that each group of mutants is blocked in the completion of one step in a cascade of five intercellular signals. According to this scheme the *bldJ* mutant is blocked at the earliest step in signaling, before the release of the first signal (signal 1). The K group, represented now by mutations in *bldK* and *bldL*, produces and secretes signal 1 but fails to respond appropriately to it by producing the next signal, signal 2. Mutants in *bldA* and *bldH* can produce both signals 1 and 2 but cannot proceed onward in the cascade to produce signal 3. Extending this hypothesis to all of the extracellular complementation groups, the G, C, and D groups of mutants therefore would be blocked at subsequent steps in signaling, after the release of signals 3, 4, and 5, respectively. While it is not yet clear whether this hypothesis is correct in detail, there have been several demonstrations that intercellular signaling is an important aspect of the life cycles of the streptomycetes. For example, in *S. griseus* a butyrolactone signaling molecule known as A factor is required to trigger the synthesis of streptomycin and the production of aerial hyphae (Horinouchi *et al.* 1994). Whether other signaling molecules contribute to this process is not known. In *S. clavuligerus* it has been shown that the synthesis of the antibiotics cephamycin D and clavulinic acid may depend on an extracellular signaling molecule that acts in a quorum sensing-like mechanism (Sanchez and Brana 1996). Finally, in *S. coelicolor*, intercellular signaling by γ-butyrolactones is believed to regulate the synthesis of antibiotics and aerial mycelium formation (Bibb 1995; Onaka *et al.* 1998).

Particularly strong evidence that intercellular signaling plays an important role in the formation of aerial

mycelium by *S. coelicolor* comes from the cloning of *bldK* (Nodwell *et al.* 1996). This cluster of five genes encodes an oligopeptide importer of the ABC transporter family. In *Bacillus subtilis* a similar ABC transporter encoded by the *spo0K* operon is required for the initiation of endospore formation and the acquisition of genetic competence (Perego *et al.* 1991; Rudner *et al.* 1991). In this bacterium secreted peptide pheromones are imported into cells, triggering the accumulation of the phosphorylated forms of the transcription factors Spo0A and ComA (Perego *et al.* 1994, 1996; Solomon *et al.* 1995; Solomon *et al.* 1996; Perego and Hoch 1996; Lazzazera *et al.* 1997). These then activate genes involved in spore formation and genetic competence, respectively (Grossman 1995). We have shown that BldK similarly imports at least one signaling molecule (signal 1; Nodwell *et al.* 1996) and have purified a compound having the activity of this signal. In support of the importance of BldK-mediated oligopeptide import to aerial mycelium formation, we have isolated six new alleles of *bldK* in this work, all of which confer resistance to the toxic tripeptide bialaphos, demonstrating strong defects in oligopeptide uptake.

According to the reasoning we have applied to the extracellular complementation hierarchy, the four K-group mutants we have isolated in the *bldL* locus are able to produce signal 1 but not signals 2, 3, 4, or 5. They are able, however, to carry out BldK-mediated oligopeptide import, as demonstrated by their sensitivity to the toxic peptide bialaphos. This suggests that they not only produce and secrete signal 1 but, unlike the *bldK* mutants, should be able to import it as well. Their defect in signaling therefore might reflect an inability to respond to the importation of signal 1 by producing signal 2, a phenotype consistent with a defect in a signal 1 receptor or transduction apparatus. In *B. subtilis* the response to the Spo0K-imported pheromones occurs at the level of a three-protein phosphorelay (Hoch 1994). The Spo0F protein, the first component in the phosphorelay, is the target of sporulation kinases. Phospho-Spo0F passes its phosphate to Spo0B, which in turn phosphorulates the transcription factor Spo0A. Phospho-Spo0A then activates sporulation gene expression (Grimsley *et al.* 1994). After their import by Spo0K, the oligopeptide pheromones are believed to repress the synthesis or action of Phospho-Spo0F-specific phosphatases, ultimately enhancing the accumulation of phospho-Spo0A. While there is no evidence for a phosphorelay controlling aerial mycelium formation in *S. coelicolor*, the behavior of the *bldL* mutants could be consistent with one.

As we have noted previously (Nodwell *et al.* 1996), the signaling hypothesis of extracellular complementation does not predict that all of the *bld* genes must encode signal molecules or receptors; indeed, we know that *bldA*, *bldB*, and *bldD* do not. For example, the mutant MY159, one of the additional C-group mutants, extracellularly complements *bldJ*, *bldK*, *bldA*, *bldH*, and *bldG* and is extracellularly complemented by a *bldD* mutant, behavior consistent with production of signals 1, 2, 3, and 4 and a failure to respond to signal 4 by producing signal 5. While this could suggest that *bld159* encodes the signal 4 receptor, there are many other potential explanations for this phenotype. An instructive example is the activation of the forespore-specific sigma factor σ^G of *B. subtilis*. For σ^G to become active a (currently unknown) signal must be sent from the mother cell and presumably be sensed by a receptor in the forespore. The *bld159* gene product therefore could play a role similar to this hypothetical forespore receptor in *B. subtilis*. However, mutations in genes such as *spoIID*, *M*, *P*, or *Q*, which are involved in sporangium morphogenesis and have nothing to do with intercellular signaling *per se*, nevertheless block the post-translational activation of σ^G. The *bld159* mutation therefore could be an analog of one of these morphogenetic genes, playing no direct role in signaling but blocking some other process upon which the completion of the signaling step depends. This caveat can be applied to all of the *bld* mutations in this collection.

A noteworthy feature of this collection of *bld* mutants is that there are so many D-group mutants, the majority of which appear to be in genes other than *bldD*. According to the signaling hypothesis these mutants run through all of the steps of the signaling cascade but fail to then produce aerial mycelium. This could be consistent with an inability to produce the morphogenetic compound SapB. It is believed that SapB, which is at least partially peptidic, is produced nonribosomally because inhibitors of translation did not prevent its synthesis (Willey *et al.* 1991). Nonribosomal polypeptide synthesis is usually carried out by large multienzyme complexes (see, for example, Galli *et al.* 1994) that are encoded by gene clusters that span tens of thousands of base pairs. We suggest, therefore, the possibility that some of the D-group mutants could identify genes encoding proteins governing the synthesis of SapB. Mutants defective in SapB synthesis might indeed have the D-group extracellular complementation phenotype but have a normal capacity to produce the pigmented antibiotics.

Regardless of whether the signaling hypothesis is correct as we envision it here, this work certainly supports the idea that intercellular signaling could be as important for morphogenesis in *S. coelicolor* as it is for antibiotic synthesis (Bibb 1996). The idea that alternating rounds of intercellular signaling and gene activation are central to bacterial development is not unique to *S. coelicolor*. In *B. subtilis*, for example, following the appearance of phospho-Spo0A, at least three signals are passed back and forth between the compartments of the developing sporangium coordinating the activity of compartment-specific σ-factors. A less well-understood, but probably equally complex, cascade of intercellular signaling regulates the chemotactic and morphogenetic events that

lead to myxospore production in the bacterium *Myxococcus xanthus* (Kaiser and Losick 1993). It remains to be seen whether multiple signal molecules dependent on the *bldJ*, K, A/H, G, C, and D groups of *bld* mutants similarly conspire with BldB and BldD to coordinate aerial mycelium formation but, as additional *bld* genes are cloned and studied in molecular detail, we anticipate that a definitive answer to this question will be forthcoming.

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