

# Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*

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The formation of an aerial mycelium by the filamentous bacterium *Streptomyces coelicolor* is determined in part by a small morphogenetic protein called SapB. A collection of representative bald (*bld*) mutants, which are blocked in aerial mycelium formation, are all defective in the production of this protein and regain the capacity to undergo morphological differentiation when SapB is supplied exogenously. We now report that most of the *bld* mutants are rescued for SapB production and aerial mycelium formation when grown near certain other *bld* mutants. Extracellular complementation experiments of this kind indicate that morphological differentiation is governed by a hierarchical cascade of at least four kinds of intercellular signals. At least one such signal is present in conditioned medium. It is resistant to boiling and protease treatment, and it remains effective even when diluted up to eightfold in fresh medium.

[Key Words: *Streptomyces coelicolor*; morphogenetic protein; aerial mycelium formation; morphological differentiation]

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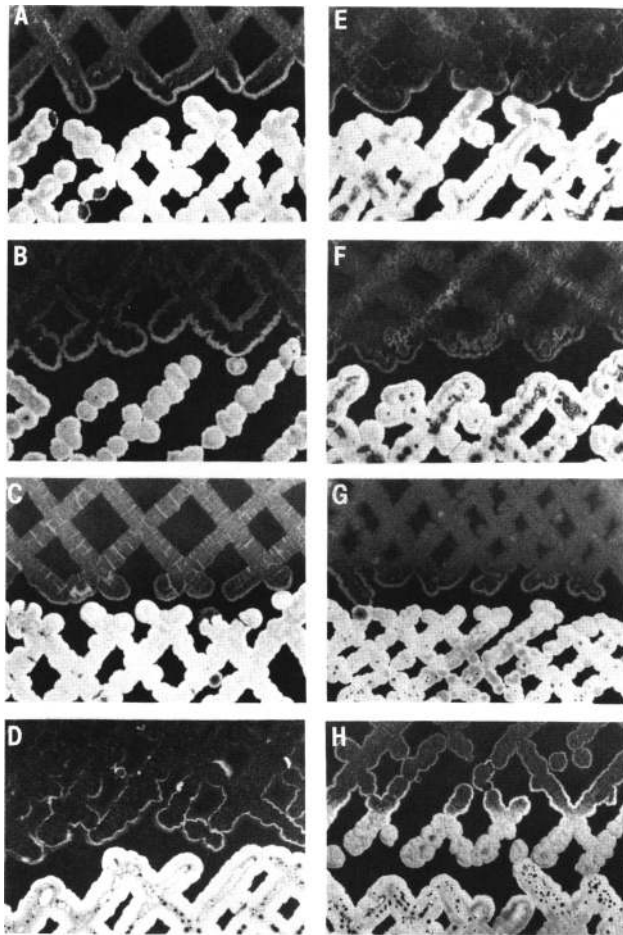
A striking feature of differentiation in filamentous microorganisms of several kinds is the formation of a complex structure known as the aerial mycelium. The aerial mycelium consists of hyphae that grow away from the substrate mycelium to impart a fuzzy white appearance to the colony surface. In the filamentous bacterium *Streptomyces coelicolor*, the formation of the aerial mycelium is governed by so-called bald (*bld*) genes; mutations in *bld* genes cause the formation of colonies consisting only of substrate mycelium (Chater 1989). Little is known about how the products of *bld* genes participate in morphological differentiation, but a possible clue was provided by the recent identification of a small protein of unusual structure called SapB (Guijarro et al. 1988), which appears to be directly involved in erecting aerial hyphae (Willey et al. 1991). SapB is secreted from colonies at the onset of differentiation and is believed to coat hyphae at the colony surface, thereby helping the hyphae to escape from the aqueous environment of the colony and to grow into the air. Production of SapB is impaired by mutations in various *bld* genes, and the capacity of one such *bld* mutant to differentiate is restored by the application of the protein in extracellular supplementation experiments (Willey et al. 1991). We now report that the restoration of aerial mycelium formation by SapB supplementation is a general feature of *bld* mutants and that certain pairs of *bld* mutants exhibit "cross feeding" (extracellular complementation) in which their ca-

capacity to form aerial hyphae, and, concomitantly, their ability to produce SapB, is restored. We conclude that intercellular communication may serve a central role in the formation of aerial hyphae and that *bld* genes directly or indirectly govern the production of extracellular signals that trigger the production of SapB.

## Results

### *SapB restores aerial mycelium formation to bld mutants*

Several *bld* mutants are known, the best characterized of which are called *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, *bldH*, and *bldI* (Merrick 1976; Champness 1988; Harasym et al. 1990; for review, see Chater 1989). We asked whether these mutants or a newly identified *bld* mutant called *bld261* (see Materials and methods) could be induced to form aerial mycelium by growth near colonies of SapB-producing bacteria or by the application of the purified protein. The SapB-producing bacterium used was the *whiF* sporulation mutant C99 (Chater 1972), as earlier work had shown that C99 is a particularly rich source of SapB (Willey et al. 1991). (C99 is impaired in the formation of normal gray spore chains from aerial hyphae and may conceivably idle at the stage of SapB secretion.) Figure 1 shows that all eight *bld* mutants developed a distinct white fringe of aerial mycelium in those colonies

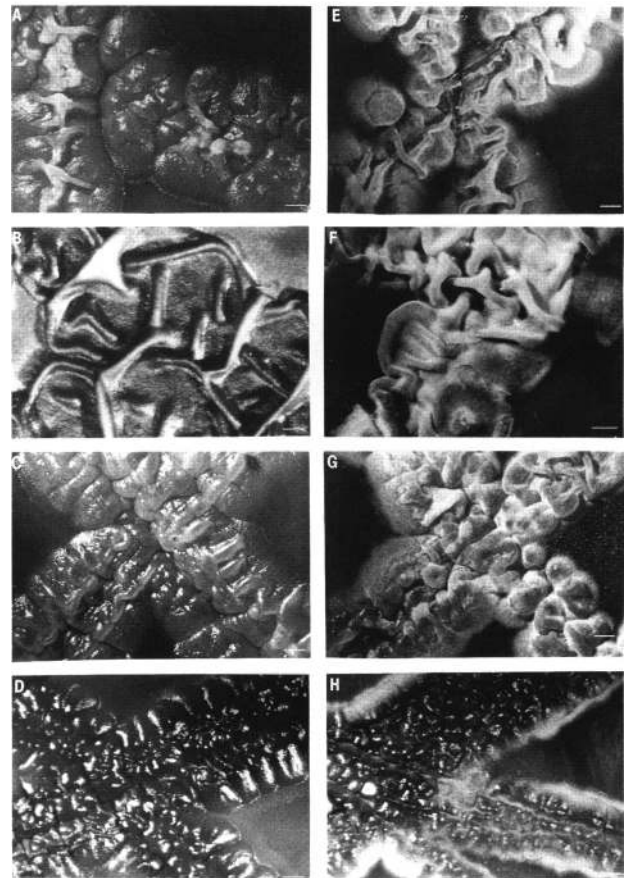


**Figure 1.** Extracellular complementation of *bld* mutants by SapB-producing colonies. The lower half of each sporulation (R2YE) agar plate was streaked with the SapB-producing strain C99 (*whiF99*) and the upper half with a *bld* mutant. Aerial mycelium from extracellular complementation of the *bld* mutants is seen as the white fringes along the edges of the colonies closest to the C99 colonies. (A) J1700 (*bldA39*); (B) J669 (*bldB43*) (similar results were obtained when the *bldB* mutant HU67 was tested); (C) J660 (*bldC18*); (D) HU66 (*bldD53*); (E) C103 (*bldG103*); (F) C109 (*bldH109*); (G) C249 (*bldI249*); and (H) HU261 (*bld261*).

nearest the SapB-producing strain C99, although the magnitude of the response varied from mutant to mutant. The most dramatic response was observed with *bld261* (Fig. 1H), but aerial hyphae were also seen clearly with *bldA*, *bldB*, *bldD*, *bldG*, and *bldI* (Fig. 1A, B, D, E, and G, respectively). The *bldC* (Fig. 1C) and *bldH* (Fig. 1F) mutants produce colonies with a sculpted surface that appears white when photographed, but visual and microscopic inspection confirmed that aerial hyphae were confined to the edges of colonies near C99. Upon continued incubation, the fringes of aerial mycelium on the colonies of all eight *bld* mutants eventually sporulated. Thus, *bld* mutants are not blocked in spore formation per se but, rather, simply for the formation of the

precursor structure (the aerial mycelium) within which the spores are produced, an inference in accord with conclusions from the results of earlier work (see, e.g., Chater et al. 1989).

The defect in morphological differentiation by all of the *bld* mutants could also be corrected by the direct application of the purified SapB protein (1–2  $\mu$ g) to colonies growing on agar plates (Fig. 2). Because the level of response was not as great as during growth near SapB-producing colonies, we used higher magnification to demonstrate the presence of aerial hyphae, which can be seen as white fringes at the edges of colonies of *bldB* (not shown) and *bld261* (Fig. 2H) mutants and as fuzziness on the surfaces of colonies of *bldA* (Fig. 2E), *bldD* (Fig. 2F), and *bldH* (Fig. 2G) and *bldC* and *bldI* (not shown) mutants. In contrast to extracellular complementation, the



**Figure 2.** Restoration of aerial mycelium formation in *bld* mutants by the application of purified SapB. The *bld* mutants were grown on sporulation agar (R2YE) for 2 days at 30°C, at which time 1.5  $\mu$ g of gel-purified SapB (E–H) or an equal volume of PBS (A–D) was added directly to the colonies [see Materials and methods]. Following an additional 2 days of incubation, photographs were taken through a Zeiss SR dissecting microscope with a Nikon FX-35 camera. The mutants were *bldA* in A and E, *bldD* in B and F, *bldH* in C and G, and *bld261* in D and H. The mutant strains were as described in the legend to Fig. 1. Bars, 500  $\mu$ m.

restoration of aerial mycelium formation by the addition of purified protein was transient; aerial hyphae were most readily seen 2–3 days after the application of SapB but disappeared by the sixth day. Nonetheless, the two sets of experiments strongly implicate SapB in the process of aerial mycelium formation and suggest that the inability of the *bld* mutants to undergo morphological differentiation is largely, if not exclusively, the result of their failure to produce the morphogenetic protein. Failure to produce SapB is not, however, the only consequence of *bld* mutations. Many *bld* mutations are pleiotropic, not only impairing aerial mycelium formation but also the production of pigmented antibiotics, such as actinorhodin (Merrick 1976; Champness 1988; Chater 1989). Because the addition of purified SapB to *bld* mutants restored only aerial mycelium formation, at least certain *bld* genes evidently have a separate function in antibiotic production.

#### Extracellular complementation between *bld* mutants

An unexpected finding of the present investigation was the discovery of extracellular complementation between certain pairs of *bld* mutants, although the level of complementation was weaker and slower to develop than that observed with the SapB-producing strain C99. Specifically, when colonies of two different *bld* mutants were grown near each other for 7–10 days on rich sporulation medium (R2YE), aerial mycelium grew on one particular member of the pair (Fig. 3; data not shown). Only certain pairs of mutants exhibited this phenomenon. Thus, *bld261* was complemented by all of the other mutants but did not complement any other mutant; *bldA* and *bldH* did not complement each other but were complemented by all of the other *bld* mutants except *bld261*; *bldG* was complemented by only by *bldC* and *bldD*; and, finally, *bldC* was complemented only by *bldD*. These *bld* mutants thus fit into a hierarchical set of extracellular complementation groups with *bld261* at the

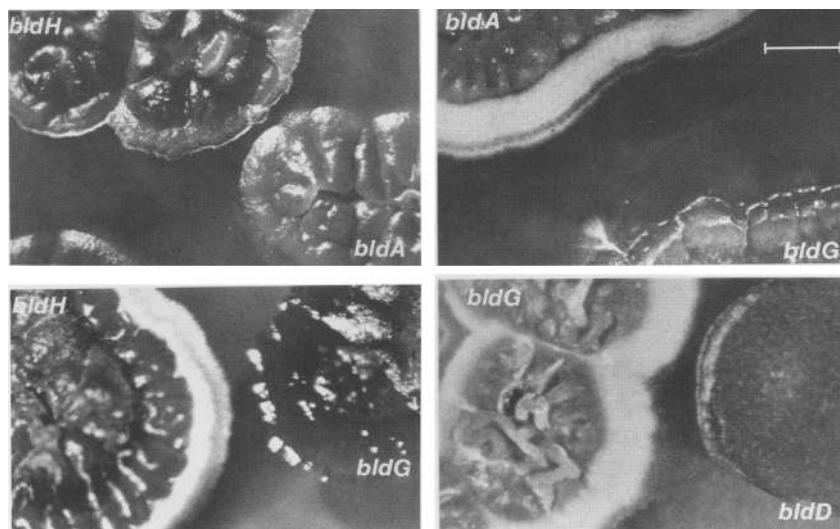
bottom of the hierarchy and *bldD* at the top: [*bld261*] < [*bldA*, *bldH*] < [*bldG*] < [*bldC*] < [*bldD*]. Only the second complementation group from the bottom has more than one member.

In contrast to these results, the results obtained with *bldB* and *bldI* did not fit neatly into this hierarchy. Thus, *bldB* appeared to be in the same extracellular complementation group as *bldC* except that it failed to complement *bldA* and *bldH*; and *bldI* appeared to be a member of the *bldAH* complementation group except that it was not complemented by *bldC*.

#### Extracellular complementation between *bld* mutants restores production of SapB

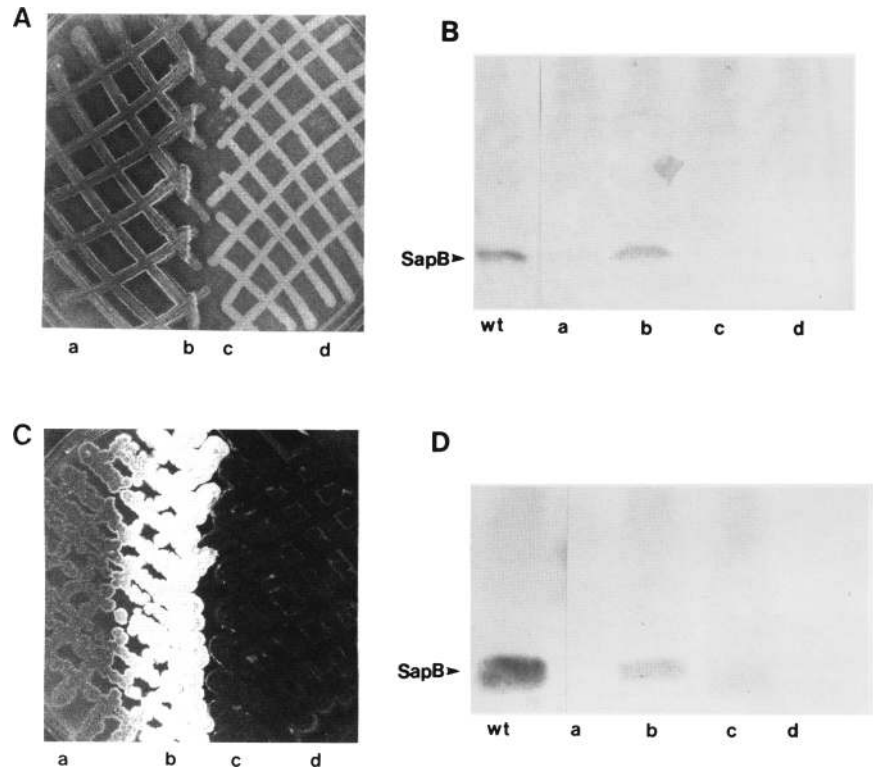
Because the *bld* mutants are all impaired in SapB production when grown alone, complementation between them cannot be attributable to diffusion of SapB itself from one mutant to another. Rather, the restoration of aerial mycelium formation must be attributable to other kinds of molecules that are (or can be) exchanged between cells by diffusion. If so, and if aerial mycelium formation depends on the production of SapB, then these molecules must be involved in, or somehow govern, the synthesis of SapB, its stability, or its release from cells.

To test this prediction, we investigated whether production of SapB was restored in the zone of extracellular complementation of one *bld* mutant by another, that is, in the region in which aerial hyphae are present. The Western blot experiment of Figure 4 shows that SapB was present in the narrow zone of aerial mycelium formed by growth of a *bldG* mutant near a *bldC* mutant and in the broad band of aerial mycelium formed by growth of a *bld261* mutant near a *bldD* mutant. Similar experiments (data not shown) demonstrated that *bldA* restored SapB production to *bld261*. Thus, extracellular complementation restores the capacity of the *bld* mutants tested to produce SapB. Because SapB production was confined to regions in which the formation of aerial



**Figure 3.** Extracellular complementation between pairs of *bld* mutants. The indicated *bld* mutants, which are listed in the legend to Fig. 1, were streaked onto R2YE agar plates and grown for 8 days at 30°C. Bar, 500  $\mu$ m.

**Figure 4.** Restoration of SapB synthesis by extracellular complementation between *bld* mutants. Sporulation agar plates were streaked with *bld* mutants capable of extracellular complementation and incubated for 10 days at 30°C. Mycelium from four separate regions on each plate, *a–d*, was harvested, and protein was extracted and subjected to SDS-PAGE and Western blot analysis using anti-SapB antibodies as described previously (Willey et al. 1991). [A] Complementation of *bldG* by *bldC*; [B] the corresponding Western blot; [C] complementation of *bld261* by *bldD*; [D] the corresponding Western blot. In both Western blots, lanes *a–d* are proteins extracted from mycelium harvested from regions *a–d* of the corresponding agar plate. (wt) The corresponding wild-type parent of the complemented *bld* mutant [B, J1501; D, J1508]. (D) The material just below the position of SapB in lane *c* is attributable to a violet pigment that was extracted from the cells and was present on the filter before the addition of the antibodies.



mycelium had been restored and because the addition of purified SapB to *bld* mutants was sufficient to restore aerial mycelium formation (above), we infer that extracellular complementation restored the capacity of the mutant cells to produce SapB, which in turn allowed aerial hyphae to be formed.

#### *Synthesis of SapB is resistant to chloramphenicol*

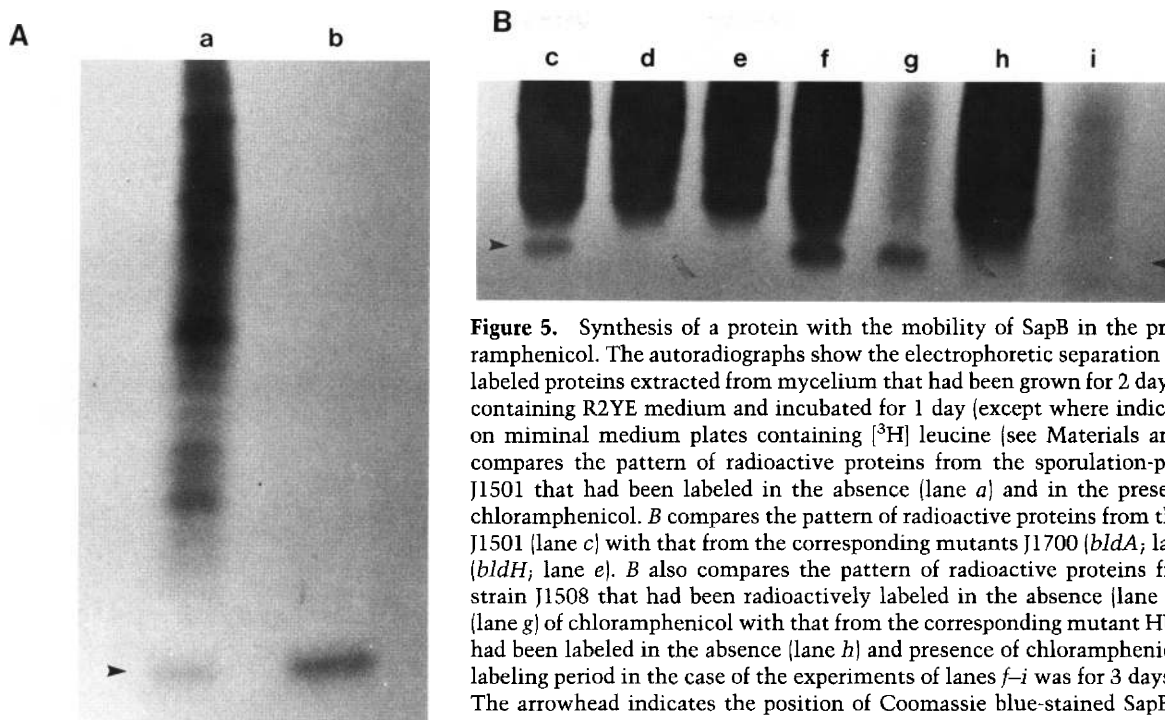
Because of the small size (2 kD) and unusual structure of SapB (Willey et al. 1991), we wondered whether SapB is a DNA-encoded protein or whether it is synthesized by a nonribosomal mechanism. Certain kinds of peptide antibiotics, for example, have unusual amino acids and are known to be produced by large multidomain enzyme complexes (peptide synthetases) rather than on ribosomes (Kleinkauf and von Dohren 1990; Marahiel 1992). To distinguish between these possibilities, we investigated whether the synthesis of SapB was sensitive to the ribosomal inhibitor chloramphenicol. Developing cells were radioactively labeled with [<sup>3</sup>H] leucine [an abundant amino acid in SapB (Willey et al. 1991)] in the presence and absence of the protein synthesis inhibitor. Radioactively labeled proteins were then separated by SDS-PAGE. Figure 5A shows that in the absence of the drug, radioactivity was incorporated into a wide spectrum of protein species, but that in the presence of the drug incorporation was restricted to a single species with the mobility of SapB.

As expected, incorporation of radioactivity into SapB (in the absence of the drug) was much reduced in *bldA*,

*bldH*, and *bldD* mutants (Fig. 5B). The *bldA* and *bldH* mutants are, however, not completely blocked in SapB production because incorporation into a protein with the mobility of SapB was observed with a long labeling period (up to 10 days) or by labeling in the presence of chloramphenicol (not shown). In contrast, no incorporation of radioactivity into SapB was detected in a *bldD* mutant with a long labeling period (not shown) or by labeling in the presence of chloramphenicol (Fig. 5B).

#### *An extracellular signal produced by bldD mutant cells is resistant to boiling, protease treatment, and dilution*

Finally, in an effort to characterize one or more of the newly discovered signaling molecules, we attempted to detect the presence in medium that had been conditioned by growth of the *bldD* mutant (a universal donor in the hierarchy) of a substance that would restore aerial mycelium formation to the *bld261* mutant (a universal responder). R2YE medium was conditioned by the growth of *bldD* mutant cells on an agar plate that had been covered by a disc of water-permeable cellophane. As a control, medium was similarly conditioned by the growth of *bld261* cells over cellophane-covered agar. After growth for 6 days the cells were removed by peeling off the discs of cellophane. We then applied discs of cellophane on which *bld261* cells had been grown. When the *bld261* cells were applied to the *bldD*-conditioned medium, little or no response was observed. However, when the *bld261* cells were applied to the *bldD*-condi-



**Figure 5.** Synthesis of a protein with the mobility of SapB in the presence of chloramphenicol. The autoradiographs show the electrophoretic separation of radioactively labeled proteins extracted from mycelium that had been grown for 2 days on agar plates containing R2YE medium and incubated for 1 day (except where indicated otherwise) on minimal medium plates containing [ $^3$ H] leucine (see Materials and methods). *A* compares the pattern of radioactive proteins from the sporulation-proficient strain J1501 that had been labeled in the absence (lane *a*) and in the presence (lane *b*) of chloramphenicol. *B* compares the pattern of radioactive proteins from the parent strain J1501 (lane *c*) with that from the corresponding mutants J1700 (*bldA*; lane *d*) and C109 (*bldH*; lane *e*). *B* also compares the pattern of radioactive proteins from the parent strain J1508 that had been radioactively labeled in the absence (lane *f*) and presence (lane *g*) of chloramphenicol with that from the corresponding mutant HU66 (*bldD*) that had been labeled in the absence (lane *h*) and presence of chloramphenicol (lane *i*). The labeling period in the case of the experiments of lanes *f*–*i* was for 3 days, rather than 1. The arrowhead indicates the position of Coomassie blue-stained SapB that had been subjected to electrophoresis on the same gel.

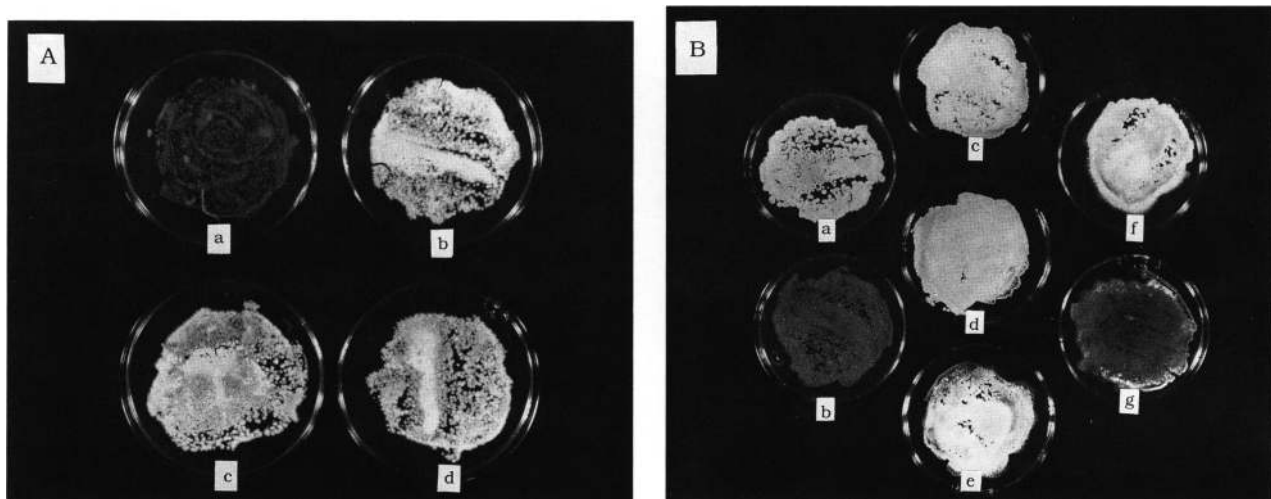
tioned medium, abundant growth of aerial mycelium was observed, fully covering the substrate mycelium within 3–5 days of incubation. We interpret this to indicate that *bldD* had released into the agar a substance or substances that could restore aerial mycelium formation to *bld261* cells. (The alternative possibility that *bldD* depletes the agar of an inhibitor of aerial mycelium formation is addressed below.)

To facilitate the characterization of the signaling substance, we prepared R2YE medium with low-melt-point agarose. The *bldD*-conditioned agarose medium could then be removed from the plate, melted at 65°C, and resolidified. The resolidified agarose medium showed no loss in its capacity to support aerial mycelium formation by *bld261*. We then tested the activity of conditioned agarose medium after each of the following three treatments (Fig. 6A). First, the agarose was heated at 100°C for 10 min. This caused no observable loss of potency in restoring aerial mycelium formation. In the second procedure, the melted agarose was incubated with 100  $\mu$ g/ml of proteinase K at 50°C for 10 min. Once again, no loss of potency was observed. As a positive control for the effectiveness of the protease treatment,  $\beta$ -galactosidase was added to the melted agarose and was shown to be completely destroyed in a protease-dependent fashion. Finally, the melted agarose was diluted to various extents with melted agarose medium that had not been conditioned (Fig. 6B). The conditioned medium was found to retain its effectiveness even when diluted up to eightfold. This finding strongly supports the view that *bldD* introduced into the medium a heat- and protease-resistant signaling molecule that was capable of restor-

ing aerial mycelium formation to the *bld261* cells. The results of the dilution experiment are difficult to reconcile with the alternative view that *bldD* depletes from the medium an inhibitor of aerial mycelium formation.

## Discussion

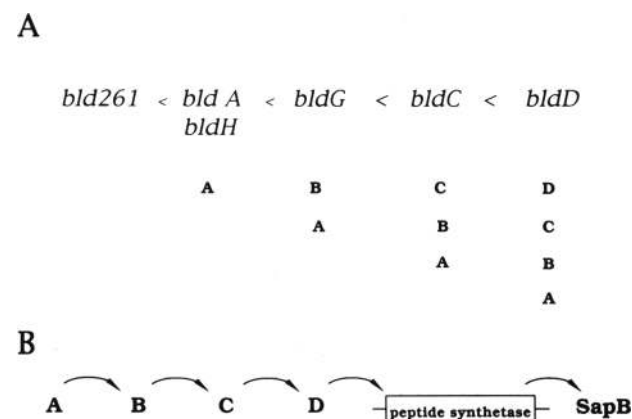
Our results indicate that the production of SapB and, hence, the formation of aerial hyphae, depends on a series of extracellular molecules (signals) that are (or can be) exchanged between cells by diffusion. We presume that these signals are the products of *bld* genes or are produced under their control. If each extracellular complementation group indicates the existence of one such signal, then extracellular complementation must involve the exchange of at least four kinds of signals (or four different modifications of the same signal molecule), which we designate A, B, C, and D [Fig. 7A; in analogy with the nomenclature of Kaiser (1989)]. We interpret our results to indicate that the *bldD* mutant, which is capable of complementing all of the other mutants, produces all four signals; that *bldC*, which complements *bld261*, *bldA*, *bldH*, and *bldG* but not *bldD*, is defective in D but produces A, B, and C; and so forth for the other mutants as depicted in Figure 7A. This is an oversimplification in that it does not take into account the exceptional cases of *bldB* and *bldI* (and perhaps other *bld* mutants yet to be identified). Nevertheless, a tentative model that accommodates our observations is that the four signals constitute a hierarchical cascade in which A induces the appearance of B, which in turn causes the appearance of C, which then allows D to ap-



**Figure 6.** Characterization of an extracellular signal in medium conditioned by a *bldD* mutant. The donor strain HU66 (*bldD*) was grown on top of cellophane on R2YE medium containing low-melt-point agarose for 6 days. The cellophane containing the mycelium was peeled off, and the underlying medium was melted and subjected to the treatments indicated below. A cellophane containing mycelium from the recipient strain, HU261 (*bld261*), which had been grown on R2YE for 1 day, was placed on top of the treated, HU66-conditioned medium. (A) The results 3 days after HU261 was placed on: medium that had been conditioned with HU261 instead of HU66 as a control (plate *a*); HU66-conditioned medium (plate *b*); HU66-conditioned medium that had been treated with proteinase K (plate *c*); and HU66-conditioned medium that had been boiled for 10 min (plate *d*). (B) The results 5 days after HU261 was placed in HU66-conditioned medium (plate *a*) and on HU261-conditioned medium that had not been melted (plate *b*). (B) also shows the results after HU261 was placed on: HU66-conditioned medium that had been melted and then diluted with melted fresh R2YE medium in the following proportions: 100 parts conditioned medium : 0 parts fresh medium (vol/vol) (plate *c*); 50 : 50 (plate *d*); 25 : 75 (plate *e*); 12.5 : 87.5 (plate *f*); and 100 : 0 (plate *g*).

pear (Fig. 7B). In a final step, D triggers the production of SapB (and perhaps other proteins yet to be identified that participate in erecting aerial hyphae), which mediates the formation of aerial hyphae.

At least one of the signals produced by the *bldD* mutant cells could be detected in conditioned medium. It



**Figure 7.** Intercellular signals govern the production of SapB. *A* summarizes the hierarchical pattern of extracellular complementation observed between the *bld* mutants and the inferred distribution of intercellular signals among the complementation groups (see text). *B* proposes that the signals constitute a hierarchical cascade that governs the production of SapB (see text).

was shown to be resistant to boiling and protease treatment, and it retained its effectiveness even when it was diluted up to eightfold in fresh medium. The chemical nature of the signal(s) will have to await its purification. Previous work has documented the existence of small molecules produced by other *Streptomyces* species that stimulate the formation of aerial hyphae. Examples are A-factor and factor-C in the species *Streptomyces griseus* and pamamycin in *Streptomyces alboniger* (Khokhlov et al. 1973; McCann and Pogell 1979; Biro et al. 1980; Khokhlov 1985; Beppu 1992). It is not known whether these species use a SapB-like molecule in erecting aerial hyphae and whether these molecules act by stimulating the production of such a morphogenetic protein.

Analogous extracellular complementation experiments have demonstrated the existence of multiple extracellular signals governing the formation of fruiting bodies by the social bacterium *Myxococcus xanthus* (Hagen et al. 1978; Kaiser 1989). For at least two of the *M. xanthus* signals (designated A and C), different complementation groups have been shown to correspond to different signal molecules by their chemical isolation (Kim and Kaiser 1990; Kuspa et al. 1992a,b).

Earlier work revealed unusual aspects to the structure of SapB, such as the apparent presence of vicinal hydroxyl groups and a block in its susceptibility to sequential Edman degradation at the fifth reaction cycle (Willey et al. 1991). Together with the small size of SapB, these findings raise the possibility that like certain peptide an-

tibiotics (which often contain unusual amino acids) SapB is not synthesized on ribosomes and, hence, is not DNA encoded (Kleinkauf and von Dohren 1990; Marahiel 1992). In support of this idea, our present results show that SapB synthesis is resistant to chloramphenicol. We cannot exclude that SapB synthesis escaped inhibition by the drug because of its small size (~18 residues; Willey et al. 1991) or because of some idiosyncratic feature of its mRNA, but the simplest explanation for our observations is that SapB is not ribosomally synthesized. If so, then as with many peptide antibiotics SapB can be expected to be synthesized by a large complex composed of one or more multidomain peptide synthetases (Kleinkauf and von Dohren 1990; Marahiel 1992). Given the position of *bldD* at the top of the hierarchy (Fig. 7A) and the failure of a *bldD* mutant to synthesize SapB even at a low level (Fig. 5B), *bldD* could be the structural gene for such a peptide synthetase or could be involved in its expression. In extension of our model the D signal could function in part by inducing the expression of this peptide synthetase gene (Fig. 7B).

So far only in the case of one *bld* gene has a function been assigned to its product. This is *bldA*, which encodes the tRNA for a rare leucine codon (Lawlor et al. 1987; Leskiw et al. 1991). We presume, therefore, that the function of the *bldA* gene in the production of an extracellular signal is indirect. Further understanding of the role of *bld* genes in the formation of aerial hyphae requires the isolation of the chemical signals that govern the synthesis of SapB and the elucidation of the roles of *bld* genes in their production.

Finally, we note that SapB and the *bld* genes that control its production evidently constitute only one of two or more alternative pathways for the formation of aerial hyphae. Our present experiments were carried out using the rich sporulation medium R2YE. It is known, however, that SapB is not made in detectable levels during sporulation on minimal medium containing mannitol or certain other carbon sources (Willey et al. 1991), conditions in which the phenotype of several of the *bld* mutants (including *bldA*, *bldD*, *bldG*, and *bldH*) is suppressed (Chater 1989). We speculate that *Streptomyces* may have evolved several different ways for erecting aerial hyphae because of the extreme importance of the aerial mycelium in the dispersal of spores. It will be interesting to determine whether alternative pathways for morphological differentiation are similarly mediated by intercellular signals.

## Materials and methods

### Bacterial strains and phages

The following bacterial and phage strains were provided by K. Chater: J1501 (*hisA1*, *uraA1*, *strA1*, *SCP1*<sup>-</sup>, *SCP2*<sup>-</sup>, *Pgl*<sup>-</sup>); J1508 (*hisA1*, *uraA1*, *dagA*, *strA1*, *Pgl*<sup>-</sup>, NF) (Chater et al. 1982); J1700 (*bldA39*, *hisA1*, *uraA1*, *strA1*, *SCP1*<sup>-</sup>, *SCP2*<sup>-</sup>, *Pgl*<sup>-</sup>) (Piret and Chater 1985); J669 (*bldB43*, *mthB2*, *cysD18*, *dagA*, NF, *SCP2*<sup>\*</sup>); J660 (*bldC18*, *mthB2*, *cysD18*, *dagA*, NF, *SCP2*<sup>\*</sup>); J169 (*bldD53*, *hisA1*, *mthB2*, *pheA1*, *strA1*, NF, *SCP2*<sup>\*</sup>) (Merrick 1976); C99 (*whiF*, *SCP1*<sup>+</sup>, *SCP2*<sup>+</sup>) (Chater 1972);  $\phi$ C31

KC603 (*c*<sup>+</sup>,  $\Delta$ *att*, *vph*, *bldA*<sup>+</sup> insert) (Piret and Chater 1985); and  $\phi$ C31 KC742 (*c*<sup>+</sup>, *att*<sup>+</sup>, *tsr*, *bldD*<sup>+</sup> insert; R. Passantino, B. Leskiw, and K. Chater, pers. comm.). The following bacterial and phage strains were provided by W. Champness: C103 (*bldG103*, *hisA1*, *uraA1*, *strA1*, *SCP1*<sup>-</sup>, *SCP2*<sup>-</sup>, *Pgl*<sup>-</sup>), C109 (*bldH109*, *hisA1*, *uraA1*, *strA1*, *SCP1*<sup>-</sup>, *SCP2*<sup>-</sup>, *Pgl*<sup>-</sup>) (Champness 1988); C249 (*bldI249*, *hisA1*, *uraA1*, *strA1*, *SCP1*<sup>-</sup>, *SCP2*<sup>-</sup>, *Pgl*<sup>-</sup>) (Champness 1988; Harasym et al. 1990); and  $\phi$ C31 KC741 (*c*<sup>+</sup>, *att*<sup>+</sup>, *tsr*, *bldG*<sup>+</sup> insert; R. Passantino, B. Leskiw, and K. Chater, pers. comm.). J. Piret provided strain J701 (*bldB15*, *mthB2*, *cysD18*, *dagA*, NF, *SCP2*<sup>\*</sup>) (Harasym et al. 1990).

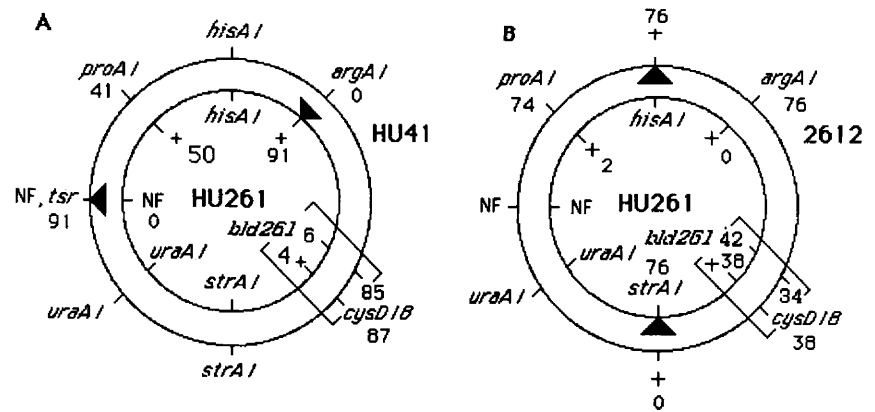
Because *bld* mutations are in several genetic backgrounds, we sought to minimize genetic variability by using *bld* mutations that were in the J1501 genetic background or the closely related J1508 background (which differs by the presence of the large linear plasmid *SCP1* integrated at the 9 o'clock position of the chromosome, denoted as NF). *bldA* (J1700), *bldG* (C103), *bldH* (C109), *bldI* (C249), and *bld261* (HU261; below) strains are already derivatives of J1501. HU66 (*bldD53*, *hisA1*, *strA1*, *Pgl*<sup>-</sup>, NF, *SCP2*<sup>\*</sup>), a *bldD* derivative of strain J1501, was constructed by mating J1508 with strain 1169. Similarly, strain HU67 (*bldB15*, *uraA1*, *mthB2*, NF, *SCP2*<sup>\*</sup>) was constructed by mating the *bldB* mutant J701 with J1508. We were unable to construct easily a J1501 derivative for *bldC* and used instead strain J660, which is a derivative of strain J650 (*mthB2*, *cysD18*, *dagA*, NF, *SCP2*<sup>\*</sup>) (Merrick 1976).

Where possible, to verify that the strains in our collection carried the designated *bld* mutations, we used  $\phi$ C31 derivatives carrying cloned copies of complementing insert DNA. Thus, the presence of the *bldA* mutation in J1700 and the *bldG* mutation in strain C103 was confirmed by complementation with the  $\phi$ C31 KC603 and  $\phi$ C31 KC741, respectively. The presence of the *bldD* mutation in strain HU66 was confirmed by complementation with a  $\phi$ C31 KC742. Control experiments in which these phages were introduced into other *bld* mutants failed to result in complementation of the Bld phenotype (e.g., KC742 failed to complement C103 and C109).

### Isolation and characterization of a *bld261* mutant

HU261 (*bld261*, *hisA1*, *uraA1*, *strA1*, *Pgl*<sup>-</sup>, NF, *SCP2*<sup>\*</sup>) is a mutant that arose (evidently spontaneously) when we mated J660 (*bldC18*) with J1508. Because 85% of the recombinants were sporulation proficient after backcrossing HU261 with J660, we concluded that *bld261* is a distinct locus from *bldC18*. To determine the chromosomal location of the *bld261* mutation, strain HU261 was first crossed with strain 1514 (*nicA1*, *proA1*, *cysA1*, *argA1*, *uraA1*, NF) (Champness 1988). Recombinants that were His<sup>+</sup> and Str<sup>R</sup> were selected. Because the *bld261* allele failed to segregate with the *nicA1*, *proA1*, and *cysA1* alleles and its segregation relative to the *argA1* allele was inconclusive, we deduced that the *bld261* mutation mapped on the right half of the chromosome. HU261 was therefore crossed with strain HU41 (*argA1*, *cysD18*, *proA1*, *hisA1*, *uraA1*, *strA1*, *tsr*, NF, *SCP2*<sup>\*</sup>; J. McCormick and R. Losick, unpubl.) and Arg<sup>+</sup>, Thio<sup>R</sup> recombinants were selected. The results of this cross suggested that *bld261* was linked to *cysD18* as shown in Figure 8A. This was confirmed by a second cross (Fig. 8B) in which HU261 was mated with strain 2612 (*proA1*, *argA1*, *cysD18*, NF, *SCP2*<sup>\*</sup>; a gift of S. Fisher) and His<sup>+</sup>, Str<sup>R</sup> recombinants were selected. A random collection of Bld<sup>-</sup> recombinants from these two crosses were tested for extracellular complementation by other *bld* mutants. In all cases, the patterns of complementation were found to be the same as observed with the parent strain (i.e., recombinants were extracellularly complemented by

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**Figure 8.** Mapping *bld261*. Results are shown of conjugational crosses between HU261 (inner circles) and strains HU41 (A; outer circle) and 2612 (B; outer circle). Exconjugants were obtained by selection for the markers indicated with the arrowheads. The numbers along the circles indicate allele frequencies among the scored recombinants. Probability values (*p*) for segregation of *bld261* relative to the indicated markers were determined as described (Hopwood et al. 1985). The brackets indicated uncertainty in the order of *bld261* and *cysD18*.

	<i>bld</i> <sup>+</sup>	<i>bld</i> <sup>-</sup>		<i>bld</i> <sup>+</sup>	<i>bld</i> <sup>-</sup>
<i>pro</i> <sup>+</sup>	45	5		1	1
<i>pro</i> <sup>-</sup>	40	1		33	41
	<i>p</i> ~0.1			<i>p</i> ~0.85	
	<i>bld</i> <sup>+</sup>	<i>bld</i> <sup>-</sup>		<i>bld</i> <sup>+</sup>	<i>bld</i> <sup>-</sup>
<i>cys</i> <sup>+</sup>	0	4		1	37
<i>cys</i> <sup>-</sup>	85	2		33	5
	<i>p</i> <0.001			<i>p</i> <0.001	

all other *bld* strains), indicating that the capacity for complementation and the Bld phenotype were tightly linked. These mapping experiments were performed as described previously (Willey et al. 1991).

HU261 produces pigmented antibiotics but lacks aerial mycelium when grown on rich sporulation agar or minimal medium supplemented with 1% glucose. As with many other *bld* mutants (e.g., *bldA*, *bldD*, *bldG*, *bldH*), the Bld phenotype of HU261 was suppressed by growth on minimal medium supplemented with carbon sources other than glucose, such as mannitol, arabinose, maltose, and galactose (1% final concentration).

#### Medium and growth conditions

Rich sporulation medium (R2YE) and incubation conditions were as described (Willey et al. 1991).

#### Application of purified SapB to colonies

Protein purification and application to *bld* mutant colonies growing on R2YE agar was performed as described (Willey et al. 1991).

#### Immunoblotting

Gel electrophoresis and Western blot analysis was done as described previously (Willey et al. 1990).

#### In vivo protein labeling with [<sup>3</sup>H] leucine

Cells were streaked on nitrocellulose filters that had been overlaid on R2YE agar and incubated for 2 days at 30°C. Colonies were then transferred by moving the filter on which they were growing to minimal medium supplemented with 1% glucose, amino acids (40 μg/ml) when required, and [<sup>3</sup>H]leucine (Amersham, sp. act. 135 Ci/mmol; ~300 μCi added per 25-ml plate). Where indicated, the minimal medium was supplemented with chloramphenicol to a final concentration of 500 μg/ml, the minimum concentration required to inhibit growth of *S. coelicolor* strain J1501 on overlaid filters (100 μg/ml is sufficient to inhibit growth when cells are plated directly on agar). Following additional incubation for 1 or 3 days as indicated at 30°C, mycelium was harvested and proteins extracted and separated electrophoretically as described previously (Guijarro et al. 1988). Gels were treated with the radioactive fluor Entensify (New England Nuclear Research Products) before exposure to X-ray film.

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## Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*.

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