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Purification of an Extracellular Signaling Molecule Involved in Production of Aerial Mycelium by *Streptomyces coelicolor*

JUSTIN R. NODWELL AND RICHARD LOSICK*

Department of Molecular and Cellular Biology, Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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We have extensively purified a factor from conditioned medium that restores aerial mycelium formation to a mutant of *Streptomyces coelicolor* that is defective in morphological differentiation. Response to this factor is shown to depend on the presence of the BldK oligopeptide import system. We suggest that this substance acts at the first step in a putative cascade of developmental regulatory signals.

Multicellular organisms use intercellular signaling molecules to control many developmental processes (5). The filamentous, fungus-like bacterium *Streptomyces coelicolor* has a multicellular lifestyle in which extensive cell-cell signaling controls the formation of a spore-forming cell type called the aerial hyphae (11). The life cycle of *S. coelicolor* commences with spore germination and the formation of a branched network of vegetative hyphae called the substrate mycelium. Because cell division occurs only sporadically in the substrate mycelium, individual filaments typically contain tens or hundreds of chromosomes. Twenty-four to 36 h after germination, the colony begins to erect the aerial hyphae, in which a massive round of synchronous cell division leads to the formation of uninucleoidal spores over the next few days. A small hydrophobic molecule called SapB coats the aerial filaments and evidently contributes to their capacity to break with surface tension and stand up into the air (9a, 10). The resulting white fuzzy layer of aerial hyphae on the surface of the colony acts like a fruiting body, allowing the dispersal of the mature spores, thereby completing the life cycle (2).

The *bld* (from "bald") genes (1, 6, 7, 11) are believed to be involved, at least in part, in a cascade of extracellular signals that controls events like SapB synthesis and that leads to aerial mycelium formation (11). Mutations in *bld261*, for example, appear to block the production of the first signal of the cascade (signal 1 [8]). Cells containing mutations in *bldK*, which encodes an oligopeptide importer, are believed to be blocked after the release of, but before the response to, signal 1. It has been proposed that wild-type colonies produce and export signal 1, perhaps constitutively, and that after a period during which signal 1 accumulates extracellularly, import it through the BldK oligopeptide permease, triggering the next step in the cascade. Cells containing the mutations *bldA*, *-H*, *-G*, *-C*, and *-D* are believed to be blocked at later steps in this cascade (8, 11).

Many of the *bld* mutants show a marked defect in the catabolite repression of metabolic operons by rich carbon sources that is a characteristic of wild-type *S. coelicolor* physiology (9). This and the fact that the developmental phenotype of some of the *bld* mutants can be suppressed by growth on

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or features	Background	Source
Streptomyces coelicolor			
HU261	*bld261 hisA1 uraA1 strA1* NF SCP2*		J. Willey
LS17	*bldA39 hisA1 uraA1 strA1 Pgl*$^-$		K. Chater
NS17	*bldK1*::*aadA*		Lab collection
NS40	*bldK1*::*aad hisA1 uraA1 strA1*		This study
NS47	*bld261 hisA1 uraA1 strA1* NF SCP2* pJRM10		This study
NS48	*bld261 hisA1 uraA1 strA1* NF SCP2* pbldK22		This study
NS49	*bld261 bldK1*::*aadA hisA1 uraA1 strA1* NF SCP2* pJRM10		This study
NS50	*bld261 bldK1*::*aadA hisA1 uraA1 strA1* NF SCP2* pbldK22		This study
Escherichia coli			
DH5α	*supE44* Δ*lacU169* (ϕ*80lacZ*Δ*M15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1*		Lab collection
ER-2	*F′ lacI*^{q} *leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL136* (Str^r) *xyl-5 mtl-1 dam13*::*Tn9*(Cm^r) dcm-6 mcrB1 mcrA hsdR2(r_K^- m_K^+)		J. McCormick
Plasmids			
pJRM10	*bla tsr*	pIISK+ and pIJ911	Lab collection
pbldK22	*bldKABCDE tsr*	pJRM-10	Lab collection

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, Biological Laboratories, 16 Divinity Ave., Harvard University, Cambridge, MA 02138. Phone: (617) 495-1774. Fax: (617) 496-4642. E-mail: losick@biosun.harvard.edu.

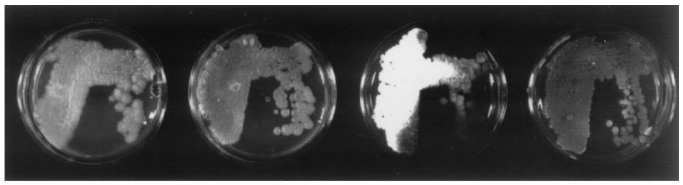

FIG. 1. Stimulation of aerial mycelium formation in a *bld261* mutant by conditioned media. *bld261* recipient cells were grown on control medium or a 1:4 mixture of *bld261*-, *bldK*-, or *bldA*-conditioned medium and fresh R2YE agar (4) as indicated.

poor carbon sources have led to the suggestion that a role of the *bld* gene products, and perhaps therefore the signaling cascade, is to couple morphological differentiation to the nutritional state of the substrate mycelium (1, 7, 9).

To explore this putative cell-cell signaling mechanism, we used *bld* mutant strains of *S. coelicolor* (Table 1) to condition *Streptomyces* growth medium and examined the effects of this conditioning on the developmental phenotype of the *bld261* mutant strain HU261. These experiments were carried out by using a previously described procedure (11) with the modification that recipient cells were grown directly on conditioned medium rather than on filter discs. When equivalent amounts of medium conditioned by either a *bldK* mutant (NS40) or a *bldA* mutant (LS17) were added to fresh R2YE growth medium (4), both stimulated some degree of aerial mycelium formation in HU261, indicating that both strains produced a signal molecule(s) whose production is blocked by the *bld261* mutation. Whereas medium conditioned by the *bldK* mutant induced HU261 to produce an abundant lawn of aerial mycelium (reminiscent of wild-type development), medium conditioned by the *bldA* mutant stimulated only a thin layer of aerial mycelium that was not visible unless viewed under magnification (Fig. 1). Likewise, medium conditioned by mutants believed to be blocked at later steps in the signaling cascade (*bldH*, *bldG*, *bldC*, or *bldD*) was able to stimulate only a small amount of aerial mycelium formation in HU261 (data not shown). We interpret this to mean that a signaling molecule(s) that induces aerial mycelium formation in a *bld261* mutant is produced by the *bldA*, *bldH*, *bldG*, *bldC*, and *bldD* mutants but that most of it is then imported into the donor cells by the BldK oligopeptide importer. In contrast, because the *bldK* mutant is defective in oligopeptide import, the signaling molecule accumulates to a higher concentration in medium conditioned by this mutant than in medium conditioned by the mutants that are *bldK*$^+$. The stimulation of aerial mycelium formation in HU261 was not due to a spurious effect of growing the cells on partially depleted medium because HU261-conditioned medium did not elicit any effect on the any of the *bld* mutants (Fig. 1 and data not shown).

To determine whether the capacity of a *bld261* mutant to respond to the factor present in conditioned medium requires the BldK oligopeptide importer, we created strains doubly mutant for *bld261* and *bldK*. NS47 and NS48 contain the *bld261* mutation and, respectively, the control plasmid pJRM10 and the BldK-expressing plasmid pbldK22. NS49 and NS50 contain the *bld261* mutation, the *bldK1* mutation, and plasmids pJRM10 and pbldK22, respectively. When these strains were grown on medium conditioned by a *bldK* mutant, only those *bld261* mutant cells with an active BldK oligopeptide importer were able to produce the aerial mycelium (Table 2). This suggests that the signaling molecule present in this medium might be an oligopeptide and that it must be imported into cells through the BldK oligopeptide importer to bring about aerial mycelium formation. The dependence of the activity of this factor on uptake by BldK further suggests that it is the first signal of the cascade that has previously been designated signal 1 (8).

To isolate the signal 1 molecule, NS17-conditioned medium was converted to a form in which it could be applied to a C_{18} column. This involved melting the medium at 80°C for 1 h and spinning it at 35,000 rpm and 15°C, allowing the agar to resolidify and form a pellet. In preparative-scale experiments, 4 liters of signal-containing supernatant was applied to a series of C_{18} columns and eluted with increasingly shallow gradients of acetonitrile (Table 3). Fractions collected from the eluates were lyophilized, resuspended in 0.01 Tris (pH 7.0)–0.01 M NH_4Cl, added to fresh R2YE growth medium, and assayed for aerial mycelium-stimulating activity by using strain HU261. Interestingly, during the second chromatographic step of the purification, factors having the capacity to induce aerial mycelium formation in the *bld261* mutant were detected in two discrete peaks (peaks A and B) (Fig. 2), both of which eluted later than the majority of the UV-absorbing material that had been loaded on the column. Fractions containing the factors corresponding to peak A (fractions 38 to 41) and peak B (fractions 46 to 48) were pooled separately and subjected to high-performance liquid chromatography with a C_{18} column, where they eluted at 28 and 42%, respectively. It proved impossible to isolate sufficient amounts of the factor corresponding to peak A for further chromatographic or biophysical analysis. The factor present in peak B was purified to apparent homogeneity in one further chromatography step (Fig. 3). Each of the UV-absorbing peaks in this final elution profile was assayed for the stimulation of aerial mycelium formation in HU261. Only the peak indicated in Fig. 3, eluting at 40% acetonitrile, showed any such activity. Fractions flanking either side of this peak showed no activity. It was concluded that this fraction contained an essentially pure signaling molecule.

We anticipated that this signaling molecule would be an

TABLE 2. Requirement of *bldK* for signal response

Assay strain	Genotype	Aerial mycelium in response to *bldK*-conditioned medium[a]
NS47	*bld261*(pJRM10)	+
NS48	*bld261*(pbldK22)	+
NS49	*bld261 bldK*(pJRM10)	−
NS50	*bld261 bldK*(pbldK22)	+

[a] Aerial mycelium was (+) or was not (−) produced.

TABLE 3. Purification of peak A and peak B signal molecules

Step	Column	Flow rate (ml/min)	Elution method	Comment
1	150-ml C_{18} Waters 125 A bulk packing material[a]	8	200 ml of 80% acetonitrile	
2	50-ml C_{18} Waters 125 A bulk packing material[a]	1	0 to 80% acetonitrile gradient; 0.8%/ml over 100 ml	Two activity peaks
3A	4.6- by 250-mm Vydac 218TP C_{18} (peak A)[a]	0.5	20 to 34% acetonitrile gradient; 0.18%/min over 75 min	Eluted at 28% acetonitrile
3B	4.6- by 250-mm Vydac 218TP C_{18} (peak B)[a]	0.5	35 to 48% acetonitrile gradient; 0.18%/min over 75 min	Eluted at 42% acetonitrile
4	1- by 150-mm Zorbax C_{18} (peak B only)[b]	0.05	0 to 100% acetonitrile gradient; 0.25%/min over 400 min	Eluted at 40% acetonitrile

[a] Run and eluted in the presence of 0.02 M phosphate buffer at pH 7.
[b] Run and eluted in the presence of 0.1% trifluoroacetic acid.

oligopeptide since it rescues the phenotype of *bld261* mutants in a manner that depends on active oligopeptide uptake. Mass spectrometry and amino acid composition analyses suggested that the purified factor from peak B contains a molecule with a mass of 655 Da that contains serine and glycine, properties that would be consistent with import by an oligopeptide importer such as BldK (3). Unfortunately, however, repeated attempts at obtaining an amino acid sequence by sequential Edman degradation were unsuccessful, suggesting that if this factor is a peptide, its amino terminus is blocked by some sort of covalent modification.

Notably, very small amounts of the factors in peaks A and B (we presume nanomolar concentrations on the basis of UV absorbance) were able to reproducibly induce a large amount of aerial mycelium in *bld261* mutants, supporting the idea that these are bona fide components of signal 1. Finally, there is at present no explanation for the existence of two factors, peaks A and B, having the activity of signal 1, and it remains possible that one of these molecules is a biologically active fragment of the other. We anticipate that future molecular genetic studies, including especially the cloning and sequencing of the *bld261* locus, will shed further light on the nature of signal 1.

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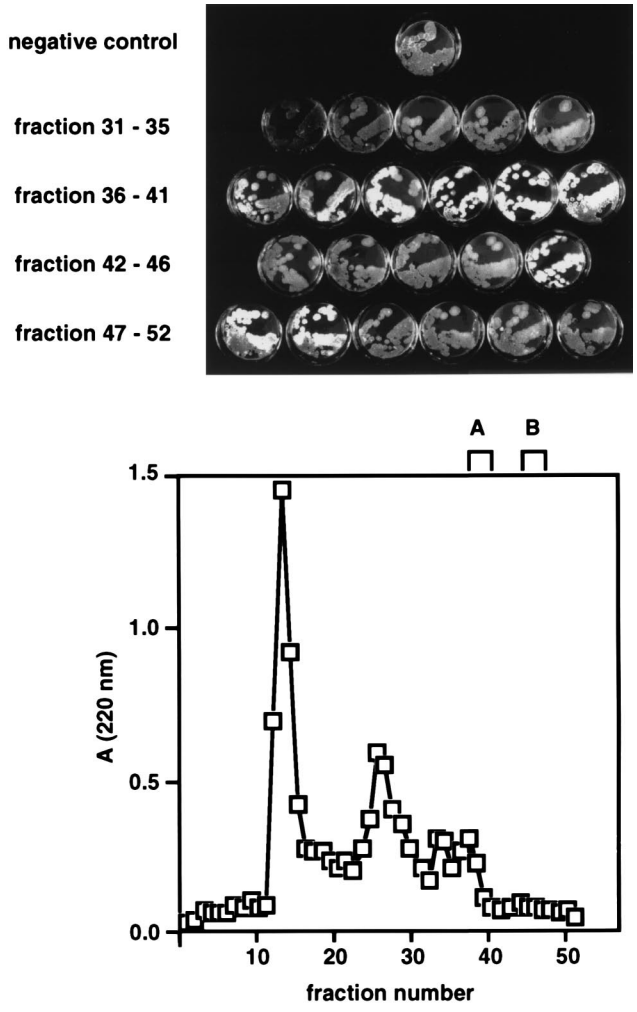

FIG. 2. C_{18} chromatography resolves two discrete factors in conditioned medium. Signal activity from the first step in the purification procedure was passed over a 50-ml C_{18} column and eluted with a gradient of acetonitrile. Fractions were assayed for developmental activity against *bld261* mutant recipient cells.

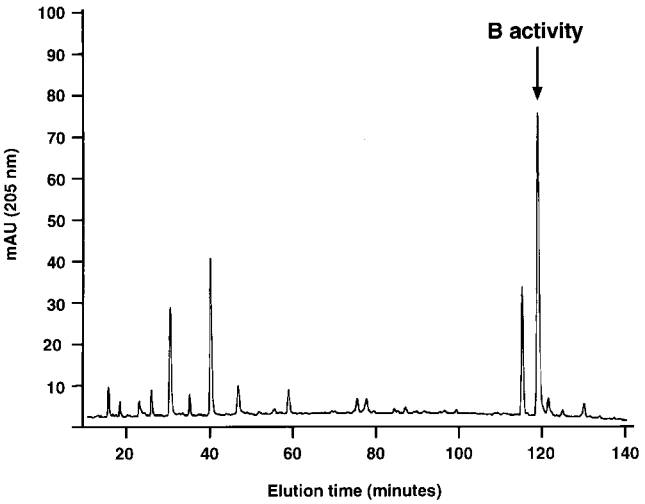

FIG. 3. Purification of the signaling molecule contained in peak B to homogeneity. The column eluate from the third chromatography step in the purification that was highly enriched for the peak B factor was passed over a high-performance liquid chromatography C_{18} column. Each UV-absorbing peak was assayed for the presence of a signal molecule that could induce aerial mycelium formation in *bld261* mutant recipient cells. mAU, milliabsorbance units.

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