

Control of Differentiation in Streptomyces: Involvement of Extrachromosomal Deoxyribonucleic Acid and Glucose Repression in Aerial Mycelia Development

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When *Streptomyces alboniger* spores were grown in Hickey-Tresner broth containing 5 μ M ethidium bromide, a high frequency of permanently cured aerial mycelia-negative (am^-) colonies was recovered. The appearance of am^- colonies was time dependent: a very low frequency (0.3%) at zero time, a maximum (9 to 21%) after 2 to 5 days of growth, and a decline again to low frequencies later in the growth cycle. On agar, cured am^- colonies of *S. alboniger* still produced puromycin. The development of aerial mycelia in *S. alboniger*, *S. scabies*, and *S. coelicolor* was also sensitive to glucose repression. Colonies grown on Hickey-Tresner agar containing 2% glucose remained phenotypically am^- throughout the observation period. Adenine (2.5 mM or greater), and to a lesser extent adenosine and guanosine, specifically reversed the repression. The accumulation of undissociated organic acids appears to be involved in glucose repression of aerial mycelia formation. However, this does not appear to be the case with puromycin production in *S. alboniger*; glucose repression was observed over the pH range 5.0 to 7.5

One example of differentiation in a prokaryotic organism is the transition from substrate to aerial mycelia in the streptomycetes (13). When spores are placed on a suitable agar medium, they germinate, outgrow, and form a colony consisting of substrate mycelia; the colony appears smooth, waxy, and translucent. As the colony matures, aerial mycelia develop from the substrate mycelia and the colony becomes powdery and opaque. At a later time, spores develop in the aerial mycelia. Events involved in triggering this transition of substrate to aerial mycelia are not known. In this communication, we report evidence for the involvement of extrachromosomal deoxyribonucleic acid (DNA) and glucose repression in the control of aerial mycelia development in *Streptomyces*.

MATERIALS AND METHODS

Organisms. Strains used in these studies were *S. alboniger* ATCC 12461, *S. scabies* PA10, tye⁺ (from K. F. Gregory), *S. coelicolor* ATCC 3355, and *Staphylococcus aureus* ATCC 6538P.

Chemicals. Ethidium bromide (EB) was purchased from Calbiochem, uracil from Eastman, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Aldrich, and tryptic soy agar from Difco. All other special biochemicals were from Sigma, and chemicals used were of analytical grade.

Curing protocol. Spore-rich preparations of *S.*

alboniger were harvested by scraping cultures grown in Hickey-Tresner (HT) agar (11) (final pH of 7.3) in petri dishes for 10 days at 28 C. The cells were suspended in 0.1% Tween 80 and vortexed for 9 min at room temperature. This treatment ruptured all hyphae but left spores intact. The suspension was filtered through glass wool-Whatman no. 1 filter paper. Mycelia and debris remained trapped in the glass wool; spores passed through the glass wool-filter paper into a sterile tube. The filtrate was centrifuged, the supernatant was decanted, and the spore pellet was resuspended in 0.1% Tween 80. This suspension contained single spores, as judged by Gram staining.

Spores (5×10^7 to 7.5×10^7) were diluted into 25 ml of HT medium (control) or 25 ml of medium containing the appropriate concentration of EB or other dye. Cultures were grown at 28 C on a rotary shaker; at 24-h intervals, they were sampled, diluted, and plated on HT agar. After 3 to 4 days of growth at 28 C, the number of aerial mycelia negative (am^-) colonies was determined. For each experimental point, 350 to 1,500 colonies were scored.

These am^- colonies and appropriate controls were individually transferred to HT agar plates using sterile toothpicks, each colony spaced about 1 inch (2.54 cm) apart, and incubated for 6 days at 28 C. Each colony was scored for aerial mycelia development and puromycin production.

Antibiotic assay. An agar plug (6-mm diameter) of each colony was transferred to a tryptic soy agar plate (150 by 15 mm) swabbed with *S. aureus*. The plates were kept at 4 C for 2 h prior to incubation at 37 C

overnight. The zone of inhibition for each colony was measured and compared with puromycin standards.

Glucose repression studies. HT agar was the basic medium used in these studies. Glucose and glycerol were autoclaved separately and added to HT agar at a final concentration of 2%. Adenine, guanine, MES (6), MOPS (6), and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were added to HT broth at desired concentrations, and the pH was adjusted before adding agar and autoclaving. The other compounds tested were filter sterilized and pipetted into sterile petri plates. Cooled HT agar was then added to each plate.

Spores from plates of the appropriate organism were inoculated by sterile-toothpick transfer. Colony morphology was observed for 5 to 14 days of growth at 28 C, depending upon the organism.

RESULTS

Curing of aerial mycelia formation in *S. alboniger*. Preliminary studies to demonstrate the involvement of extrachromosomal DNA in aerial mycelia and puromycin production in *S. alboniger* were stimulated by the following observations. (i) Gregory and Huang (8) and Okanishi et al. (15) reported significant "curing" of tyrosinase, antibiotic, and aerial mycelia formation in *S. scabies*, *S. venezuelae*, and *S. kasugaensis* after treatment with acridine dyes or growth at elevated temperature. The involvement of extrachromosomal DNA in the case of tyrosinase transfer was supported by genetic evidence (7). (ii) Marked differential inhibition of *S*-adenosyl-*L*-methionine:*O*-demethyl-puromycin *O*-methyltransferase formation by EB was observed in *S. alboniger* grown in liquid culture under conditions in which there was no effect on overall growth (16). (iii) Continued low levels of *O*-methyltransferase even after several transfers of EB-treated cells in the absence of dye were also observed (17). In our first studies, large numbers of colonies grown under different conditions in the presence of varying EB concentrations were surveyed by either plating hyphae or isolated spores, but only one permanently cured am^- and puromycin-negative colony was isolated. In a second approach using fragmented hyphae (8, 15) from EB-treated cultures, 3 out of 300 colonies were am^- and produced lower levels (20%) of antibiotic. No spontaneous loss of wild-type characteristics was ever seen in controls.

A more successful approach was based on a technique used by Redshaw with *Saccharomyces cerevisiae* (P. A. Redshaw, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, G54, p. 29), which was found to yield high frequencies of cytoplasmic petite mutants from ascospores. Single spores of *S. alboniger*, isolated by Tween treatment and

filtration, were allowed to germinate in the presence of EB and acridine dyes. In these studies, EB was more effective as a "curing" agent than either acridine orange or acriflavine. Results obtained by growing spores in 5 μ M EB at 28 C are presented in Fig. 1 and 2. The frequency of occurrence of am^- colonies (Fig. 1) showed a time-dependent pattern throughout spore germination, outgrowth, and further vegetative growth. This frequency remained low for day 1, began increasing after 2 days, peaked by 5 days, and then decreased sharply. In some experiments, a second round of increased curing was observed. Ninety percent of the primary am^- isolates were found to be permanently cured of their ability to form aerial mycelia. In Fig. 2, a primary plating is shown of *S. alboniger* treated 5 days with 5 μ M EB; colonies cured of aerial mycelia appear smooth, waxy, and translucent (bald [13]), whereas those having aerial mycelia are white, opaque, and powdery (hairy). All am^- colonies tested did not produce the black pigment characteristic of the wild type. Tests of large numbers of cured colonies on HT agar have shown only a small mean reduction (a maximum of 50% in the experiment summarized in Fig. 1) in the amount of puromycin produced.

Further evidence for plasmid involvement in aerial mycelia formation was found with penicillin-resistant (pen^r) mutants isolated from am^- cultures of *S. alboniger* after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In preliminary experiments, crosses of wild type ($am^+ pen^s$) with two $am^- pen^r$ strains have yielded only $am^+ pen^r$ and no $am^- pen^r$ colonies. All individual colonies were allowed to sporulate, were treated with Tween 80 (see

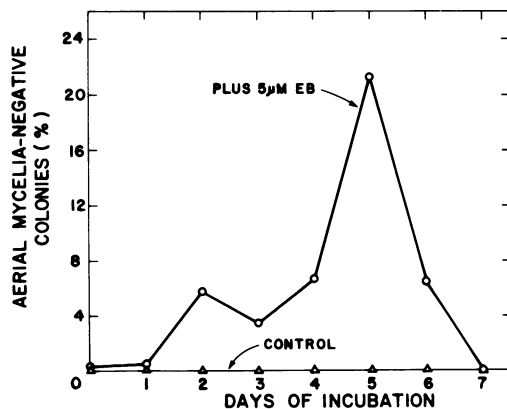


FIG. 1. Frequency of am^- colonies of *S. alboniger* after growth on HT medium \pm EB. In this experiment, the maximum spontaneous am^- frequency was 0.25% (day 0).

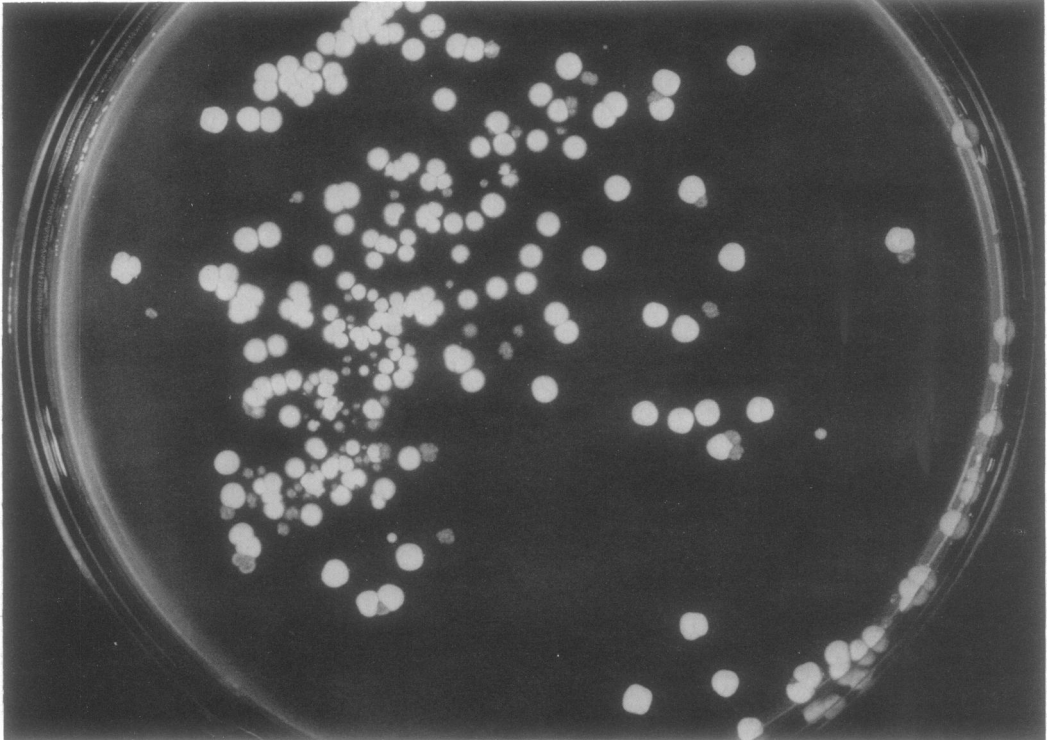


FIG. 2. *S. alboniger* colonies on HT agar after 5 days of growth in HT medium containing $5 \mu\text{M}$ EB. Wild-type colonies are white and powdery; am^- colonies appear smooth, waxy, and translucent. $\times 1.6$.

above) to obtain uninucleate haploid spores (12), and were replated before scoring.

Glucose repression of aerial mycelia formation. In previous studies, L. Sankaran had noted that the morphological characteristics of *S. alboniger* on HT-glucose (HTG) agar were very similar to those of the am^- colonies obtained after EB treatment. We confirmed this observation and found that colonies of *S. alboniger*, *S. scabies*, and *S. coelicolor* grown on HTG agar did not develop aerial mycelia (Fig. 3 [top view] and Fig. 4 [cross section]). Addition of glycerol to HT agar had no effect on aerial mycelia formation. Although colonies grown in the presence of glucose developed only substrate mycelia, microscope examination showed the eventual formation of spores in all cases. Unexpectedly, the glucose repression of aerial mycelia formation was reversed most effectively by adenine (2.5 mM or higher) (Fig. 3), whereas cyclic adenosine 3',5'-monophosphate (cAMP) had no effect. *S. scabies* showed the best reversal with 2.5 mM adenine; 5 mM levels worked well with *S. alboniger* and *S. coelicolor*. With all three organisms, adenosine and guanosine reversed glucose repression but not as

effectively as adenine. The following compounds (2.5 or 5 mM) had no influence on glucose repression: cAMP or its $N^6, O^{2'}$ -dibutyl derivative, cyclic guanosine 3,5'-monophosphate (cGMP) or its $N^2, O^{2'}$ -dibutyl derivative, 5'-AMP, 5'-GMP, cytidine, uridine, thymidine, thymine, uracil, cytosine, or guanine.

Recent studies by Haavik have implicated the accumulation of undissociated organic acids (acetic and pyruvic) in the catabolite repression of bacitracin formation in *Bacillus licheniformis* (9, 10). When the pH of agar surrounding *S. alboniger* colonies was tested with phenol red after 2 days of growth, a decrease from pH 7.3 to < 6 was noticed in the case of HTG agar-grown colonies; no change of pH occurred around colonies grown on HT or HTG agar containing 5 mM adenine. HT agar containing Good (6) buffers (50 mM MES [pH 5.0 to 6.5], MOPS [pH 6.5 to 8.0], and HEPES [pH 6.5 to 8.0]) was used to study more definitively the influence of pH on aerial mycelia development. Adequate growth of the colonies, although slower at more acid pH's, was found over the pH range used. Glucose repression was complete (or

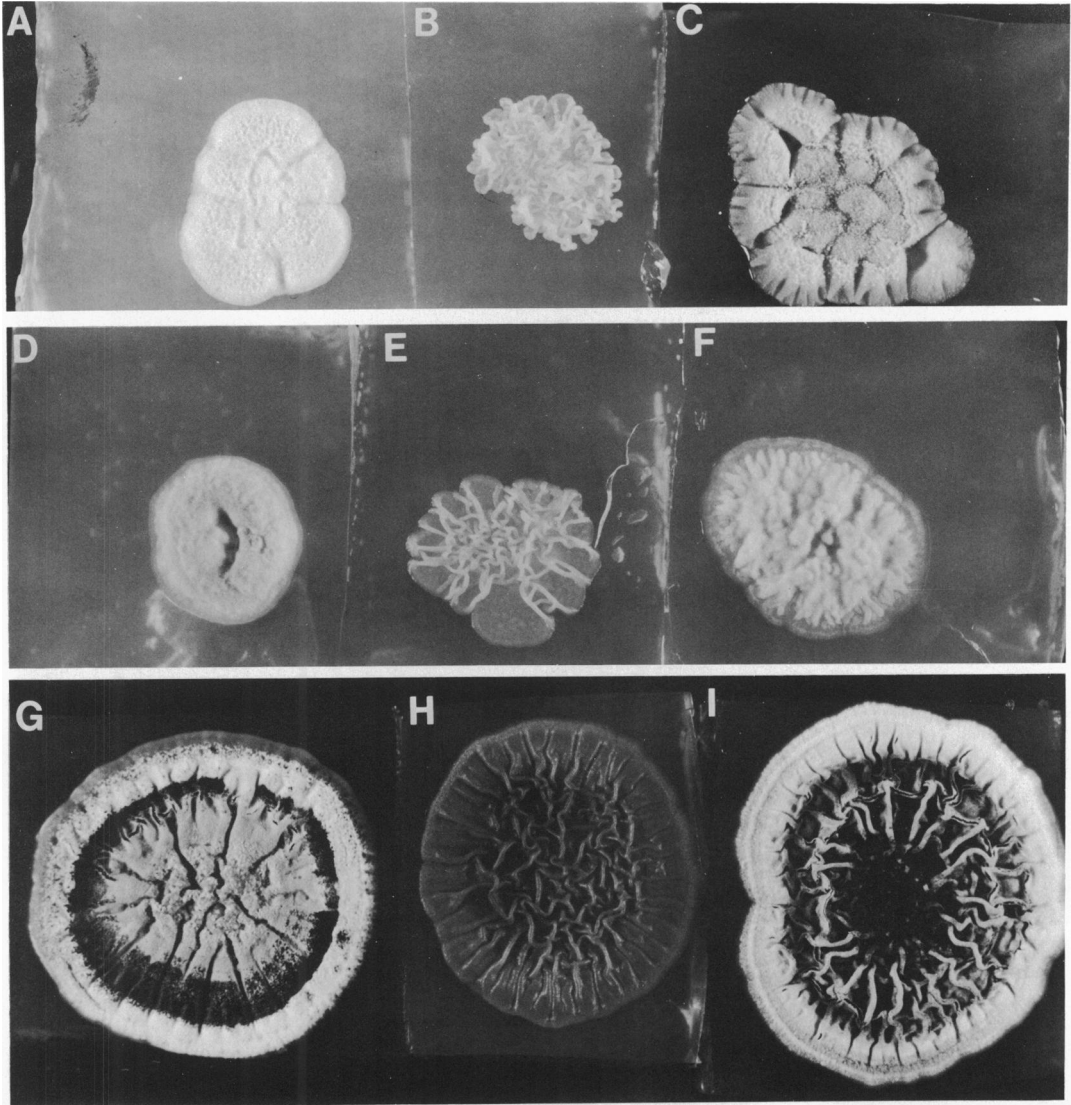


FIG. 3. Glucose repression and adenine reversal of aerial mycelia formation in *Streptomyces* (top view). Colonies were grown at 28 C for 5 days (*S. alboniger* and *S. scabies*) and 14 days (*S. coelicolor*). (A, B, and C) *S. alboniger*; (D, E, and F) *S. scabies*. (G, H, and I) *S. coelicolor* grown on HT-MES at pH 6.0; (A, D, and G) grown on HT agar; (B, E, and H) grown on HTG agar (HT + 2% glucose); (C, F, and I) grown on HTG + adenine (5 mM for *S. alboniger* and *S. coelicolor*; 2.5 mM for *S. scabies*). $\times 2.5$.

almost so) at the more acid pH's (5.0 to 5.5) with *S. alboniger* and *S. scabies* and only partial or zero at higher pH's. With *S. coelicolor*, glucose repression was evident over a wider pH range (5.0 to 7.0). Adenine significantly reversed glucose repression, except at pH 5.0, with *S. alboniger* and *S. coelicolor*. In all cases, the pH of the agar surrounding the colonies did not show any change when tested with appropriate indicator dyes (bromcresol

green, bromcresol purple, and phenol red). However, the colonies themselves (at pH's >5.5) always appeared more acidic in HTG agar, suggesting that accumulation of undissociated organic acids was occurring. We, therefore, think that a mechanism similar to that described by Haavik (10) was operative to inhibit aerial mycelia formation.

Puromycin production on agar by *S. alboniger* was repressed by glucose over the entire

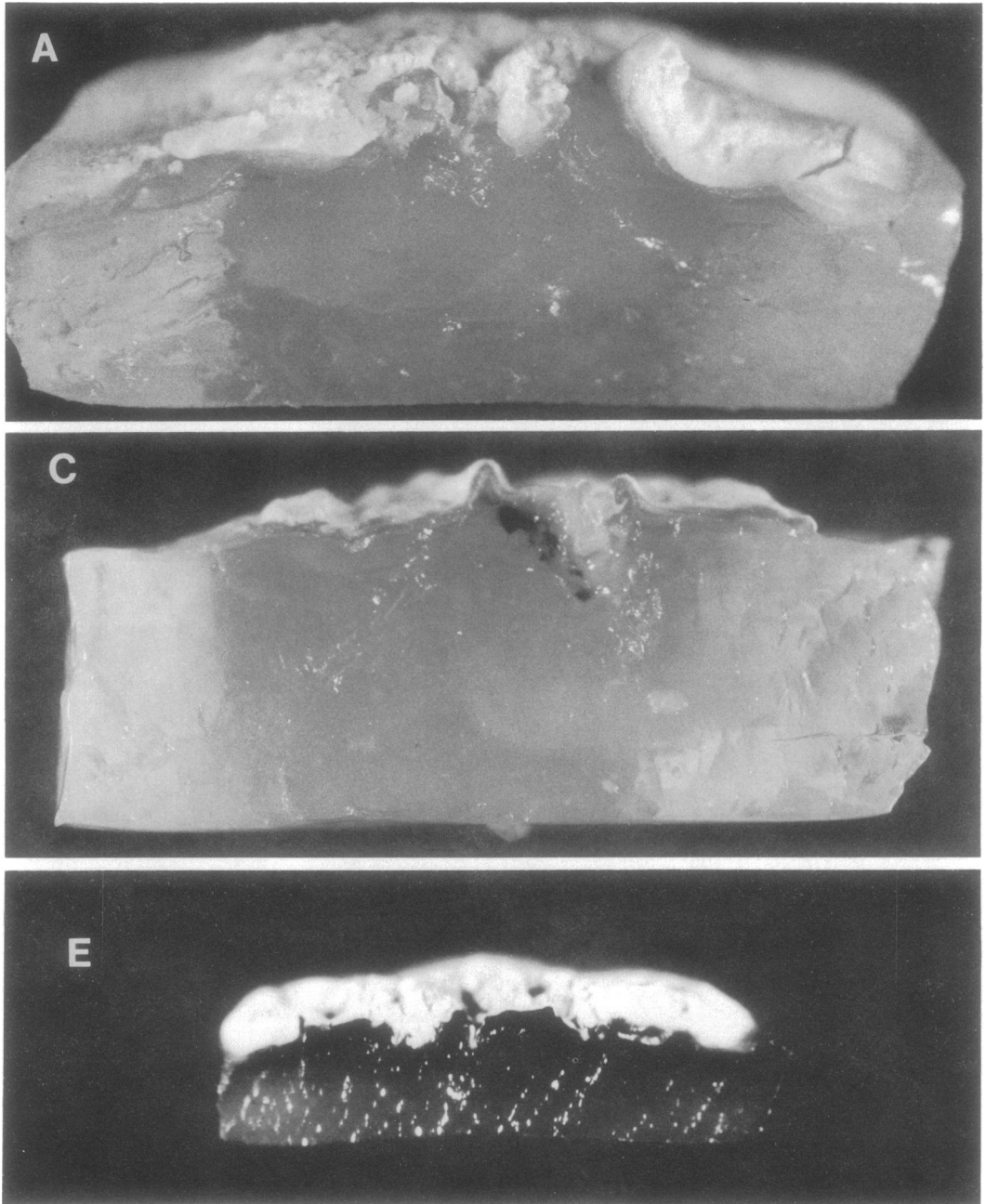


FIG. 4. Cross sections of *Streptomyces* colonies. *S. alboniger* (A, B) at 8 days of growth, $\times 12.6$; *S. scabies* (C, D) at 5 days of growth, $\times 12.6$; *S. coelicolor* (E, F) at 10 days of growth, $\times 8.1$. (A, C, and E) Grown on HT agar; (B, D, and F) grown on HT agar + 2% glucose (HTG).

pH range tested and, therefore, is probably regulated through a mechanism more related to classical catabolite repression. These results agreed with studies on the *O*-methyltransferase, which specifically catalyzes the formation of

puromycin, where glucose repression in liquid culture was found in media buffered with CaCO_3 to maintain the pH above 7 (17).

It is of interest that uric acid, adenine, and other purine bases have been reported to in-

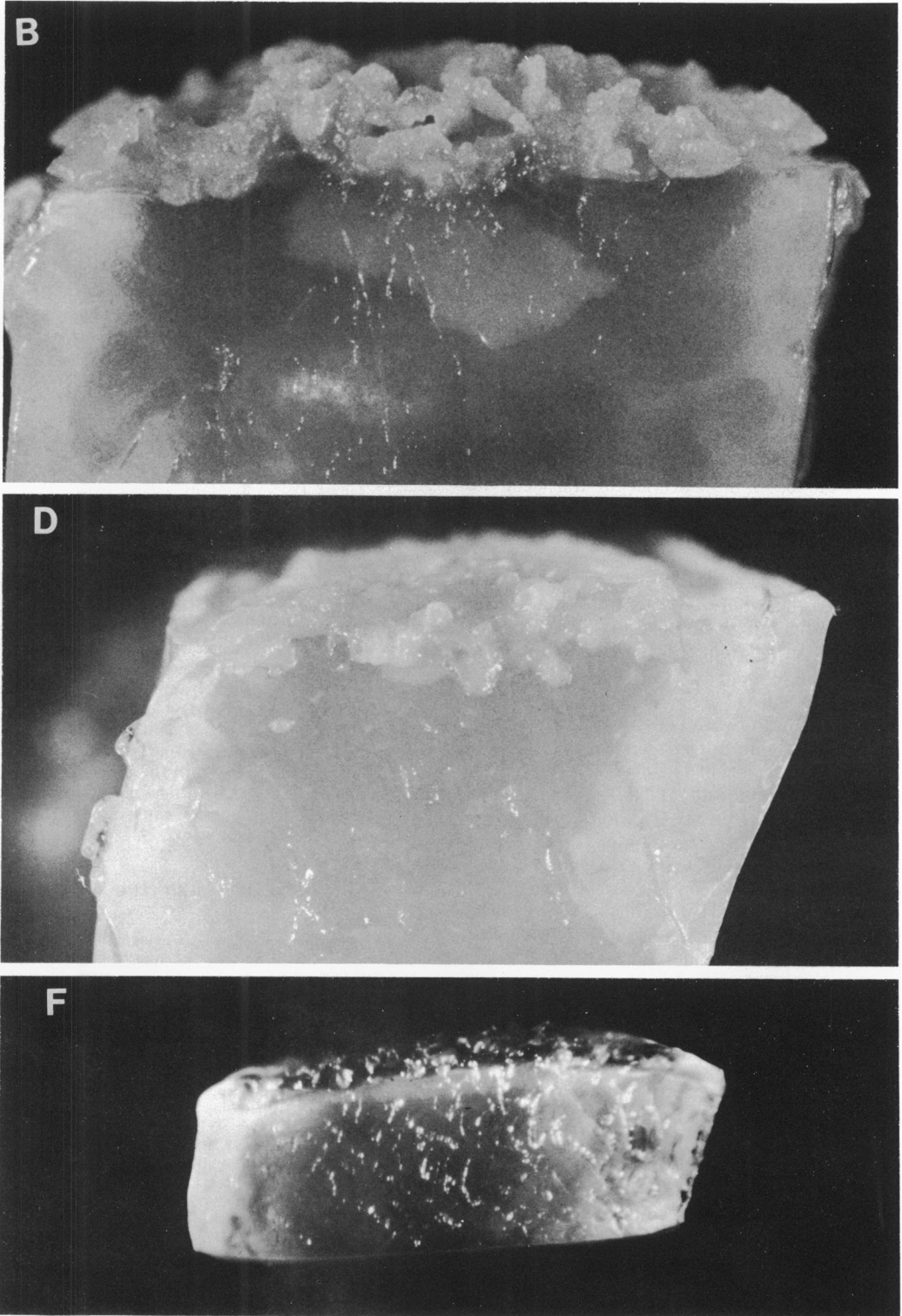


FIG. 4. B, D, F

crease puromycin yields in *S. alboniger* (S. A. Szumski and J. J. Goodman, U.S. patent no. 2,797,187; June 25, 1957). We found that adenine, and to a lesser extent adenosine, increased antibiotic production on HT agar containing glycerol and that higher levels (7.5 to 10 mM) of adenine reversed repression of puromycin formation. In *S. scabies* (tye⁺[8]), melanin formation was also repressed by glucose and reversed by 2.5 mM adenine.

DISCUSSION

Our studies have confirmed and extended the observations of Okanishi et al. (15) and a recent report by Kähler and Noack (14) on the "curing" of the am⁺ trait in streptomycetes. We have presumptive evidence for the involvement of extrachromosomal DNA in the formation of aerial mycelia in *S. alboniger*. The use of inocula of individual spores allowed us to follow the incidence of appearance of am⁻ colonies during spore germination and growth. The results showed that the frequency of am⁻ colonies was highly time dependent and suggest that a sensitive period for curing occurs, possibly at a time in growth where extrachromosomal DNA is being replicated or expressed. The later decrease in curing frequencies to base line levels might be explained by reintroduction of plasmids into cured cells as hyphal division, branching, and growth continue.

Although Schrempf et al. (18) have recently reported the isolation of covalently closed circles of extrachromosomal DNA from *S. coelicolor*, all of our attempts (E. Zelinkova, unpublished data) to physically demonstrate the presence of plasmid DNA in *S. alboniger* or *S. scabies* have so far been unsuccessful.

The molecular basis for glucose repression of aerial mycelia formation is not known. More unexpected and also not yet explained is the inability of cyclic nucleotides to reverse the repression and the relatively specific reversal by adenine and purine nucleosides. It is conceivable that these compounds somehow stimulate changes in intracellular cyclic nucleotides. Possible permeability barriers to the entrance of cyclic and other nucleotides were not tested. cGMP seems to be involved in the control of growth and sporulation in *B. licheniformis* (1). cAMP has been implicated in the induction of differentiation in some organisms, including the transition of *Dictyostelium discoideum* from the amoeboid to multicellular aggregate stage (5), formation of predivisional cell forms from elongated stalk cells in *Caulobacter crescentus* (19), and inhibition of uniform conidia formation and

stimulation of mycelia growth and aerial hyphae formation in *Neurospora crassa* (20). However, adenine was inactive under conditions in which cAMP stimulated formation of tyrosinase in *N. crassa* (4) and fruiting bodies in *Myxococcus xanthus* (2). Also, adenine had no effect in human astrocytoma cells, where adenosine and adenine nucleotides increased intracellular cAMP levels (3).

Morphological differences between substrate and aerial mycelia in streptomycetes have been reported (13), but little is known about possible biochemical differences. Elucidation of the molecular events involved in triggering development of aerial mycelia in *Streptomyces* offers an exciting approach to the study of differentiation in a prokaryote.

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ADDENDUM

Recently, O. Durieu-Trautmann and J. Tavlitzki (J. Cell Biol. 66:102-113, 1975) described similar reversible and permanent effects on the morphology of the fungus *Ustilago cynodontis*. On 4% glucose medium, the organism grew in the mycelial form; however, when grown on nonfermentable carbon sources, glucose plus chloramphenicol, or glucose plus EB, yeastlike colonies developed. These changes were reversible in the case of nonfermentable carbon sources, but permanently cured yeastlike colonies were obtained after growth in the presence of chloramphenicol or EB. The authors suggested that a loss of mitochondrial function caused the permanent morphological alterations.

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