Regulation of 'Conditional' Aerial Mycelium Mutants of Streptomyces

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Hickey–Tresner agar was originally developed to yield large amounts of aerial mycelium and spores with a variety of *Streptomyces* strains. Two mutant strains, *S. coelicolor* M110 and *S. alboniger* 504, behaved unusually when grown on this medium in that no aerial mycelium was formed. With the former strain, abundant formation of aerial mycelium and spores was found on other media. These variants have been defined as being aerial mycelium-conditional, because the absence or presence of wild-type morphology is dependent on the type of growth medium. This defect is due to the formation and excretion of excess acid and can be reversed by raising the pH above 7 either by growing certain aerial mycelium-negative strains of streptomyces or *Sarcina lutea* near these variants, by raising the pH with a suitable buffer or, in the case of *S. coelicolor* M110, by adding exogenous adenine.

INTRODUCTION

We describe in this paper a novel group of morphological mutants of streptomyces and discuss some of the physiological abnormalities which occur in these variants. These strains have been defined as being aerial-mycelium 'conditional', because the formation of wild-type aerial mycelium (Amy⁺) is dependent on the type of growth medium used. Our original observation of this unusual behaviour was made when we crossed two *Streptomyces coelicolor* strains by protoplast fusion, and selected for the Amy⁺ character on a medium (Hickey–Tresner agar) originally formulated by Hickey & Tresner (1952) to yield large numbers of spores with a variety of streptomyces species. *S. coelicolor* M110 behaved unusually when grown on this medium in that no aerial mycelium appeared, although there was abundant aerial mycelium and spore formation on other media. In the fusion experiment between *S. coelicolor* M110 and an Amy[–] strain, large numbers of Amy⁺ colonies appeared which were found to be Amy[–] on further purification. Another observation, namely that M110 colonies growing near Amy[–] colonies were Amy⁺, prompted us to investigate the factors involved in producing these morphological changes.

METHODS

Organisms. Strains of *Streptomyces* used in this study are listed in Table 1. The details of isolation and characteristics of Amy[–] strains were described by Redshaw *et al.* (1979). *S. coelicolor* M110 was kindly provided by M. J. Bibb, John Innes Institute, Norwich, UK.

Chemicals. Polyethylene glycol (PEG) 1000 was from Fisher Scientific Co., Fairlawn, New Jersey, USA. All other special biochemicals were from Sigma and chemicals used were of analytical grade.

Media. Recipes are for 1 litre of medium. The components marked * were sterilized separately and added after autoclaving. MR agar: sucrose, 150 g; glucose, 10 g; KCl, 0.25 g; MgSO₄·7H₂O, 1 g; Difco Casamino acids, 1 g; trace element solution (per litre: ZnCl₂, 40 mg; FeCl₃·6H₂O, 200 mg; CuCl₂·2H₂O, 10 mg; MnCl₂·4H₂O,

Abbreviation: HTA, Hickey–Tresner agar.

Table 1. *Characteristics of Streptomyces strains*

Strain	Phenotype/genotype
<i>S. coelicolor</i>	
M110	Amy (conditional) <i>hisA1 uraA1 strA1 SCP2*</i>
1098/4	Amy ⁻ <i>argG pheA1</i>
1098/9A	Amy ⁻ <i>argG pheA1</i>
<i>S. alboniger</i>	
12461	Amy ⁺
12461/5	Amy ⁻ <i>argG</i>
504	Amy (conditional) Ura ⁻

10 mg; Na₂B₄O₇·10H₂O, 10 mg; (NH₄)₆Mo₇O₂₄·4H₂O, 10 mg), 2 ml; *KH₂PO₄ (0.5%), 10 ml; *CaCl₂·2H₂O (7.37%, w/v), 100 ml; *L-proline (20%, w/v), 15 ml; *TES buffer (0.25 M, pH 7.2), 100 ml; Difco agar, 20 g. Hickey-Tresner agar (HTA) (Hickey & Tresner, 1952) was adjusted to a final pH of 7.4. Buffered HTA contained 50 mM concentrations of MES, final pH 6.0; MOPS, final pH 7.4; or HEPES, final pH 8.0.

pH estimation. The pH of the agar plates was measured with a Beckman Zeromatic IV pH meter, using either a Beckman flat bulb combination electrode or a Sigma combination microelectrode.

Protoplast fusion. The original procedures described by Okanishi *et al.* (1974) and Hopwood *et al.* (1977) had to be modified in order to get significant regeneration of protoplasts prepared from Amy⁻ strains of *S. coelicolor*. The major changes included adding sucrose to the initial growth medium and bovine serum albumin (BSA) (Gabor & Hotchkiss, 1979) to medium P, and changing the composition of medium R (H. Akagawa & M. Suter, unpublished results). With this modified procedure, regeneration of *S. coelicolor* 1098/4 protoplasts is routinely 3–10%.

Preparation of protoplasts. Mycelium grown on HTA was inoculated into 20 ml Bacto tryptic soy broth (TSB; Difco), containing 10% (w/v) sucrose, in a 150 ml flask, and incubated for 2 d at 28 °C in a gyrotatory shaker. The seed culture was transferred [10% to 20 ml TSB containing 10% sucrose and glycine (1% for strain 1098/4 and 0.8% for strain M110)] and incubated for 24 h. The mycelium was harvested by centrifugation at 4 °C, washed twice with medium P containing 1% BSA (P-BSA) and resuspended and homogenized in 3 ml lysozyme (2 mg ml⁻¹ in P-BSA). After incubation with shaking for 3 h at 32 °C, 4 ml P-BSA was added and the suspension was pipetted up and down 10 times with a 5 ml pipette to aid the release of the protoplasts from the still largely coherent mycelial mass. The protoplasts were then filtered through a 8 µm Nucleopore membrane with a prefilter. After centrifugation at 4 °C, the protoplasts were resuspended by gentle shaking in 3 ml P-BSA medium and the number of protoplasts counted with a haemocytometer. Each preparation was adjusted to a concentration of 10⁸ protoplasts ml⁻¹.

S. coelicolor M110 and 1098/4 protoplasts (0.5 ml of each) were mixed together and 48% (w/v) PEG 1000 in P-BSA added to a final concentration of 16%. After 1 min the concentration of PEG 1000 was increased to 24%. After 3 min more, the protoplasts were centrifuged, washed twice with P-BSA, resuspended in P-BSA, and serial dilutions plated on MR agar and incubated at 28 °C.

RESULTS

Transfer of Amy⁺ character by protoplast fusion

Before attempting to clone the genes required for the expression of the Amy⁺ character in Amy⁻ strains, we first wanted to see if stable Amy⁺ recombinants could be obtained by protoplast fusion. Such a successful transfer was achieved between *S. coelicolor* strains M110 (conditional Amy⁺) and 1098/4 (Amy⁻). After PEG-induced protoplast fusion, a primary selection was carried out for the Amy⁺ character on HTA, on which neither of the parent strains was Amy⁺ (Fig. 1a). Each Amy⁺ colony was purified by three cycles of treatment with Tween 80 (Redshaw *et al.*, 1979) and the genotype then determined using suitable diagnostic plates. Various recombinants were obtained including Phe⁻, His⁻, Phe⁻His⁻ and prototrophic variants. Interestingly, in all cases the Amy⁺ isolates were now Arg⁺, and, in addition, some variation in morphology and pigment formation was seen (Fig. 1a).

Co-metabolism is involved in conversion of strain M110 to Amy⁺ morphology by strain 1098/4

An unusual observation in these crosses was the finding that a large number of the Amy⁺ isolates became Amy⁻ after purification. We suspected that a possible complementation might be occurring between the two parent colonies, and this proved to be correct. When strain 1098/4

Fig. 1. Variation in morphology on HTA of different *S. coelicolor* strains and protoplast fusion recombinants. Plates incubated for 6 days. (a) Upper left, *S. coelicolor* 1098/4; upper right, *S. coelicolor* M110. Recombinants obtained after protoplast fusion of these strains: lower left, *Amy*⁺ *Phe*⁻ isolate; lower right, *Amy*⁺ prototroph. (b) and (c) Upper culture, *S. coelicolor* M110; lower culture, *S. coelicolor* 1098/4.

was grown close to a M110 culture, strain M110 became *Amy*⁺ (Fig. 1*b*). Growing strain 1098/4 at a greater distance from M110 did not affect morphology of the latter (Fig. 1*c*). Strain M110 again appeared *Amy*⁻ when regrown in the absence of strain 1098/4.

At first we suspected that the *Amy*⁻ culture might be releasing into the agar a diffusible substance which was inducing the morphological change in M110. Several such compounds of diverse structures, including A-factor (2*S*-isocaprolyl-3*R*-hydroxymethyl- γ -butyrolactone) (Khokhlov *et al.*, 1973), factor C, a polypeptide (Biro *et al.*, 1980), and pamamycin (McCann & Pogell, 1979), have been isolated. However, attempts to isolate a specific stimulator from cultures of 1098/4 grown on agar, or in liquid medium, gave negative results.

Further studies suggested that some sort of co-metabolism was essential for the observed change of strain M110 to *Amy*⁺. Thus, if strain 1098/4 was first grown for several days on HTA and then removed from the plate, strain M110 streaked on the agar remained *Amy*⁻ (Fig. 2*a*). However, if both strains were first grown and then removed, a new streak of strain M110 turned *Amy*⁺ (Fig. 2*b*). A control plate where neither original culture was removed gave similar results (Fig. 2*c*). Clearly some substance(s) released into or removed from the agar was responsible for the morphological reversal.

Other organisms can also alter the morphology of S. coelicolor M110 and another 'conditional' variant, S. alboniger 504

A survey of the *Streptomyces* mutants we had available showed that wild-type *S. coelicolor* 1098 (*Amy*⁺ on HTA) had no effect on the morphology of strain M110, whereas strain 1098/9A, another *Amy*⁻ *argG* mutant, changed strain M110 to *Amy*⁺. Similarly, wild-type *S. alboniger* 12461 had no effect on strain M110 (Fig. 3*b*), whereas an *Amy*⁻ *argG* derivative of it, strain 12461/5, had the same positive effect on strain M110 as did the *S. coelicolor* *Amy*⁻ strains (Fig. 3*a*). We found one other similar 'conditional' mutant in a group of *S. alboniger* *Amy*⁻ strains isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. This mutant (strain 504,

Fig. 2. Involvement of co-metabolism between *S. coelicolor* strains M110 and 1098/4 for conversion of strain M110 to *Amy*⁺ morphology. (a) Strain 1098/4 was grown on agar for 6 d, cut out and strain M110 streaked close to the removed region. (b) The two strains were grown parallel and close together for 6 d, both streaks cut out and a second streak of strain M110 made above the 1098/4 area. (c) Same as (b) except that neither culture was removed. Second incubations for 3 d.

Fig. 3. Effects on morphology of *S. coelicolor* M110 of interactions with different *S. alboniger* strains. Three different strains of *S. alboniger* (right side of dish) were streaked at angles to an M110 culture. (a) *S. alboniger* 12461/5 (*Amy*⁻); (b) *S. alboniger* 12461 (*Amy*⁺ wild-type); (c) *S. alboniger* 504 (conditional *Amy*⁺). Incubations for 6 d.

Amy⁻ *Ura*⁻) was converted to *Amy*⁺ morphology by growth near strain 12461/5, but not by growth near wild-type *S. alboniger* (Fig. 4*a, b*). However strains 504 and M110, both conditional variants, had no effect on each other's morphology (Fig. 3*c*).

A chance observation of the conversion of strain M110 to *Amy*⁺ on a contaminated plate led to the finding that the contaminating bacterium (a Gram-positive coccus), as well as a pure culture of *Sarcina lutea*, could produce the same changes as the *Amy*⁻ strains: the two 'conditional' variants were changed to *Amy*⁺, whereas the three *Amy*⁻ *argG* strains remained unchanged (Figs 5 and 4*c*).

Fig. 4. Effects on morphology of *S. alboniger* 504 (conditional *Amy*⁺) of interactions with *S. alboniger* *Amy*⁺ and *Amy*⁻ strains and *Sarcina lutea*. Cultures were streaked at angles to strain 504 (upper streak). (a) *S. alboniger* 12461/5 (*Amy*⁻); (b) *S. alboniger* 12461 (*Amy*⁺ wild-type); (c) *Sarcina lutea*. Incubations for 6 d.

Fig. 5. Effect of interaction with *Sarcina lutea* (central smear) on morphology of different *Streptomyces* strains: (a) *S. coelicolor* M110; (b) *S. coelicolor* 1098/9A; (c) *S. alboniger* 504; (d) *S. alboniger* 12461/5; (e) *S. coelicolor* 1098/4. Incubated for 4 d.

Effect of pH on the morphology of 'conditional' strains

Since we could find no evidence for a specific effector that was altering morphology, another explanation had to be sought. Redshaw *et al.* (1976) had shown that at high glucose concentrations organic acids accumulated and inhibited aerial mycelium formation. Our measurements showed that after growth of both 'conditional' strains, the pH of HTA plates had fallen to near 5. Furthermore, with *S. coelicolor* M110, buffering the agar with either 50 mM-MOPS, pH 7.4, or 50 mM-HEPES, pH 8.0, caused the formation of normal wild-type aerial mycelium on these plates (Fig. 6). The pH of the agar was still neutral or alkaline after growth, and the pigments were now purple in colour, as expected at these pH values. Increasing the pH with these buffers had no effect on the morphology of *S. coelicolor* *Amy*⁻ *argG* strains. Similar results were obtained with *S. alboniger* 'conditional' strain 504. On HTA plus MOPS buffer, normal wild-type aerial mycelium was formed, significant amounts of geosmin could be detected, and the pH of the agar remained >7. However, no black pigment was excreted. Of interest was our finding that strain M110 also became *Amy*⁺ in the presence of 5 mM-adenine (Fig. 6b), whereas strain 504 did not. Previously adenine had been found to reverse the repression of aerial mycelium formation at high glucose concentrations (Redshaw *et al.*, 1976). None of a number of other *S. alboniger* *Amy*⁻ mutants tested, including three *Arg*⁻ strains, four other auxotrophs and three prototrophs, were altered in morphology by the addition of MOPS buffer or adenine to the media on which they were grown.

Fig. 6. Effects of pH and adenine on morphology of *S. coelicolor* M110. (a) HTA with no added buffer, final pH of agar after growth was 5. (b) HTA + 5 mM-adenine, final pH of 8. (c) HTA + 50 mM-MOPS, final pH of 7.3. (d) HTA + 50 mM-HEPES, final pH of 8. Incubations for 5 d.

DISCUSSION

The simplest interpretation of the effects of *Amy*⁻ strains and *Sarcina lutea* is that these organisms take up and metabolize the excess organic acids produced by the two 'conditional' *Streptomyces* strains, thus maintaining the pH of the growth medium and permitting normal aerial mycelium and spore formation. The requirement for co-metabolism of the excreted acids would explain why products excreted by the *Amy*⁻ strains do not alter the morphology of the conditional strains, and also why simply neutralizing the excess acids with buffers produces wild-type morphology. However, the metabolic processes and genetic alterations involved in producing these effects still remain unknown. We have reported (Liu *et al.*, 1983b, 1984) that large deletions, as big as 35 kb in the case of *S. cattleya*, occur in the *argG* gene region of the *Amy*⁻ *argG* strains, so the possibility of multiple gene losses is very likely. Effects on transport systems may be involved, since we have recently found a deficiency in arginine transport in *S. coelicolor* 1098/4 (unpublished observations).

Other correlations between morphological variation of streptomycetes and acid production have been reported. The addition of glucose to HTA resulted in acid production and repression of aerial mycelium formation in wild-type *S. alboniger*, *S. scabies* and *S. violaceus-ruber*; adding buffers to raise the pH resulted in normal *Amy*⁺ morphology (Redshaw *et al.*, 1976). Nakano & Ogawara (1980) reported that an *Amy*⁻ *argG* mutant of *S. lavendulae* produced more acid in liquid medium than the wild-type. They also described two groups of *Arg*⁻ mutants in *S. kasugaensis*: the first type was *Amy*⁻ and produced acid in liquid culture, while the other group produced aerial mycelium and maintained higher pH values in liquid culture (Nakano *et al.*, 1980).

Discoveries related to ours have been made for *S. sulfureus* by Meganathan and co-workers (Bentley & Meganathan, 1981; Meganathan & Coffell, 1984). Aerial mycelium, spores and geosmin were formed at 28 °C but not at 36 °C with this strain (an example of a temperature-sensitive *Streptomyces* mutant). Furthermore, the morphological and metabolic alterations were shown to be related to acid production: a pH of > 7 was required for production of the wild-type characteristics. A 'conditional' aerial mycelium mutant from *S. fradiae* has also been described (Vargha *et al.*, 1983). This *Arg*⁻ strain was defective in ornithine transcarbamylase and formed aerial mycelium in the presence of citrulline but not arginine. The effects of amino acids as both stimulators and inhibitors of aerial mycelium and spore formation have also been observed in *S. viridochromogenes* (Coleman & Ensign, 1982).

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