Genetic Analysis of A-factor Synthesis in Streptomyces coelicolor A3(2) and Streptomyces griseus

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A-factor is a potent pleiotropic effector produced by Streptomyces griseus and is essential for streptomycin production and spore formation in this organism. Its production is widely distributed among various actinomycetes including Streptomyces coelicolor A3(2). Genetic analysis of A-factor production was carried out with S. coelicolor A3(2), and two closely linked loci for A-factor mutations (afsA and B) were identified between cysD and leuB on the chromosomal linkage map. In contrast, genetic crosses of A-factor-negative mutants of S. griseus, using a protoplast fusion technique, failed to give a fixed locus for A-factor gene(s) and suggested involvement of an extrachromosomal or transposable genetic element in A-factor synthesis in this organism.

INTRODUCTION

A-factor, 2-S-isocapryloyl-3-S-hydroxyxymethyl-γ-butyrolactone, found in Streptomyces griseus and Streptomyces bikiniensis (Khokhlov et al., 1973), is a potent autoregulating factor essential for both streptomycin biosynthesis and spore formation in these organisms (Khokhlov et al., 1973; Khokhlov, 1980; Hara & Beppu, 1982a). Furthermore, recent work from this laboratory has revealed that A-factor is also involved in streptomycin resistance in these organisms through its inducible effect on the synthesis of streptomycin-6-phosphotransferase (Hara & Beppu, 1982b). Mutants lacking A-factor simultaneously lose streptomycin production capability, the ability to form spores and a large degree of streptomycin resistance. Exogenous supplementation of A-factor to the culture of these mutants restores all these phenotypes. A-factor-negative mutants of S. griseus and S. bikiniensis were easily obtained at high frequency by treatment with acridine dyes or by incubation at high temperature, which suggested involvement of an extrachromosomal genetic determinant in the biosynthesis of A-factor in these organisms.

A-factor productivity has been found in various species of actinomycetes including Streptomyces coelicolor A3(2) (Efremenkova et al., 1979; Hara & Beppu, 1982a), but in contrast to S. griseus and S. bikiniensis, no A-factor-negative mutants of S. coelicolor A3(2) were obtained by the curing treatments. In this report, we describe genetic analyses of A-factor genes of S. coelicolor A3(2) and S. griseus, using conjugation and protoplast fusion techniques, respectively. The results clearly indicate that A-factor gene(s) of S. coelicolor A3(2) are located at a fixed position on the chromosomal linkage map, while in S. griseus, they appear to be extrachromosomal.

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Abbreviations: NTG, N-methyl-N’-nitro-N-nitrosoguanidine; QCO, quadruple crossover.

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Table 1. *Streptomyces* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genetic markers</th>
<th>Plasmid status</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. griseus</em> IFO 13189</td>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>pro leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>arg trp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>pro leu A-factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>arg trp A-factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. coelicolor</em> A3(2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A317</td>
<td>hisAI uraAI strAI</td>
<td>NF SCP2*</td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>hisAI uraAI strAI</td>
<td>SCP1- SCP2+</td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>hisAI uraAI strAI</td>
<td>SCP1- SCP2-</td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>hisAI uraAI strAI</td>
<td>SCP1- SCP2-</td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>hisAI uraAI strAI</td>
<td>SCP1- SCP2-</td>
<td></td>
</tr>
<tr>
<td>B369</td>
<td>leuB5 mthB2 guaA1 act-118</td>
<td>SCP1- SCP2*</td>
<td></td>
</tr>
<tr>
<td>BH2*</td>
<td>proA1 argA1 cysD18 A-factor</td>
<td>SCP1- SCP2*</td>
<td></td>
</tr>
<tr>
<td>BH5*</td>
<td>proA1 argA1 cysD18 A-factor</td>
<td>SCP1- SCP2*</td>
<td></td>
</tr>
<tr>
<td>BH10†</td>
<td>proA1 argA1 cysD18 A-factor</td>
<td>SCP1- SCP2*</td>
<td></td>
</tr>
</tbody>
</table>

† Mutants BH2 to BH10 were all derived from strain A700 by independent treatment with NTG.

**METHODS**

*Strains.* The strains used are listed in Table 1. Parental *S. griseus* IFO 13189 was obtained from the Institute for Fermentation, Osaka, Japan and auxotrophic derivatives of *S. coelicolor* A3(2) were supplied by Dr D. A. Hopwood, John Innes Institute, Norwich, U.K. Mutagenesis was performed by UV irradiation of spores, giving 1-0-0.1% survival, or by a treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as described by Hopwood *et al.* (1973). Several A-factor-negative mutants of *S. griseus* were derived by cultivation at high temperature (Hara & Beppu, 1982a). Double auxotrophic mutants of *S. griseus*, i.e. strain 105 (*pro leu*) and strain 109 (*arg trp*), were derived by UV irradiation; each auxotrophic character was a result of a separate mutagenic treatment. A-factor-negative mutants of *S. griseus* FT-1 and *Bacillus subtilis* ATCC 6633 were used as indicator organisms for bioassay of A-factor.

*Media.* Complete medium (CM), minimal medium (MM) and general cultural techniques for *S. coelicolor* A3(2) (Hopwood, 1967) were used for most of the experiments. Minimal medium for *S. griseus* contained (g 1\(^{-1}\)): glucose, 10; urea, 1; KH\(_2\)PO\(_4\), 1; NaCl, 0.5; MgSO\(_4\), 7H\(_2\)O, 0.5; FeSO\(_4\), 7H\(_2\)O, 0.01; agar, 20 (pH 6.8). Three media, S, P and R1, for protoplast production and protoplast regeneration were those of Okanishi *et al.* (1974). Difco nutrient agar and Difco nutrient broth were used for assay of A-factor.

*Genetic analyses.* Conjugation and genetic analysis of *S. coelicolor* A3(2) were performed according to the method of Hopwood (1967). Genetic crosses between auxotrophic strains of *S. griseus* were achieved using a protoplast fusion technique and the standard conditions of Hopwood & Wright (1978) with following modifications: lytic enzyme no. 2 was omitted from the lytic mixture and 50% PEG 1000 was used for fusion. For the selection of recombinants, 0.1 ml of suitable dilutions of a mixed protoplast preparation was plated on to each selective regeneration medium, i.e. R1 medium lacking Casamino acids but containing appropriate supplements. After incubation at 28 °C for a week, colonies developing on a selective medium were transferred on to a suitable supplemented minimal medium with a toothpick and phenotypes of the recombinants were determined. Regeneration frequencies of protoplasts were about 10\(^{-4}\) per protoplast and recombination frequencies were about 10\(^{-3}\) per regenerated protoplast under these conditions. The mapping procedure in *S. griseus* was essentially the same as that described by Akagawa *et al.* (1975).

*Detection of A-factor production.* The mutagenized colonies or recombinant colonies were transferred with a toothpick on to complete medium agar pieces (5 mm diameter) which were separately placed in a petri dish. Petri dishes were incubated at 28 °C for 2 d. After the incubation, A-factor secreted in the agar piece was detected by the plate assay method as reported previously (Hara & Beppu, 1982a).

*Chemicals.* Synthetic A-factor provided by Dr K. Mori (Mori, 1981) of this department was used. PEG 1000 was purchased from Sigma.

**RESULTS**

Isolation of A-factor-negative mutants of *S. coelicolor* A3(2)

Since no A-factor-negative mutants were obtained from derivatives of *S. coelicolor* A3(2) by treatments with acridine orange and ethidium bromide or by incubation at high temperature, mutagenesis with NTG was necessary to obtain the mutants. Four mutants (BH2, 5, 6 and 10) ...
A-factor gene(s) in Streptomyces

were isolated out of 582 colonies tested from strain A700. Unlike the A-factor-negative mutants of *S. griseus* and *S. bikiniensis* which lose spore forming ability, similar mutants of *S. coelicolor* A3(2) showed normal morphological features in sporulation. However, of these four mutants, two, BH5 and BH6, lost the ability to produce the red pigments characteristic for this species. This simultaneous loss of A-factor and red pigments is discussed below. Any combination of crosses of these four mutants failed to show positive cosynthesis.

**Genetic analysis of A-factor-negative mutations of *S. coelicolor* A3(2)**

Using the A-factor-negative mutants described above, we carried out genetic crosses by the method of Hopwood (1967). A cross was made between NF strain A317 [*hisA1 uraA1 strA1*] and an A-factor-negative mutant BH10 (*proA1 argA1 cysD18 A-factor*), derived from strain A700, with selection for *argA*+ and *uraA*+. The result clearly indicated that the A-factor gene (termed A-factor) was closely linked to *cysD* and was located on the chromosome between *cysD* and *strA* (Fig. 1). Similar results were obtained from other crosses with BH2, BH5 and BH6. For more precise mapping, A-factor-negative mutants BH10 and BH2 were crossed with strain B369 (*leuB5 mthB2 guaA1 act-118*). In each cross, the allele ratio for *A-factor*+/A-factor was consistent with a map location between *cysD* and *leuB* as indicated by the preliminary cross (Fig. 2a, b). The position of A-factor in the *cysD–strA* segment was thus narrowed down to the short interval between *cysD* and *leuB* since only this location was compatible with the gradient of allele frequencies at the *cysD*, A-factor and *guaA* loci.

A cross between the A-factor-negative mutant BH6 and B369 indicated a similar location for the A-factor mutation of BH6, between *cysD* and *leuB* (Fig. 2c). However, the allele frequencies suggested a slightly different position for the BH6 mutation when compared with that in BH10 and BH2 described above. Analysis with BH5 gave similar results to those of BH6.

**Genetic analysis of A-factor-negative mutations of *S. griseus***

A-factor-negative mutants of *S. griseus* IFO 13189 with double auxotrophic markers were derived from strains 105 (*pro leu*) and 109 (*arg trp*) by cultivation in liquid medium at high temperature as previously described (Hara & Beppu, 1982a).
Fig. 2. Results of crosses of strain B369 (leuB5 mthB2 guaA1 act-118) with A-factor-negative mutants BH10, BH2 and BH6 (proA argA2 cysD18 A-factor). (a) BH10, (b) BH2, (c) BH6. Selection was made for cysD+ and guaA+ (indicated by triangles). Data for proA1 and act-118 markers have been omitted. The numbers of recombinants are given for each marker.

Table 2. Analysis of a cross between strains 109 and 115 of S. griseus by protoplast fusion

<table>
<thead>
<tr>
<th>Recombinant genotype</th>
<th>No. of recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-A-factor-leu-arg-trp-(pro)</td>
<td></td>
</tr>
<tr>
<td>+        +        +        +        +</td>
<td>79</td>
</tr>
<tr>
<td>+        +        +        +        trp</td>
<td>2</td>
</tr>
<tr>
<td>+        +        A-factor +        +        trp</td>
<td>3*</td>
</tr>
<tr>
<td>+        +        +        arg     +        +</td>
<td>3</td>
</tr>
<tr>
<td>+        +        +        leu     +        +        +</td>
<td>20</td>
</tr>
<tr>
<td>pro      +        +        +        +        +</td>
<td>12</td>
</tr>
<tr>
<td>+        A-factor +        leu     +        +        trp</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
</tr>
</tbody>
</table>

* Additional QCO recombinants when A-factor was placed in the sequence minimizing QCO.

The mutants 115 (pro leu A-factor) and 118 (arg trp A-factor) were used for genetic analysis of the A-factor gene in *S. griseus* by the protoplast fusion technique. In the cross between strains 109 and 115, 127 recombinants were obtained from four selection media (arginine + proline, arginine + leucine, tryptophan + proline, and tryptophan + leucine). As shown in Table 2, all recombinants obtained were then analysed for the unselected markers. A reciprocal cross between strains 105 and 118 was also carried out in the same way. Table 3 shows the analysis of 89 recombinants. There are three possible sequences of four markers on a circular chromosome. The frequencies of quadruple crossover (QCO) recombinants excluding A-factor are shown in Table 4. As the lowest QCO frequency was observed for the order pro-leu-arg-trp, this was the most probable sequence of markers.

The location of the A-factor gene was analysed in a similar way for the marker sequence pro-leu-arg-trp (Table 5). In the cross between strains 109 and 115, the lowest QCO frequency was obtained when A-factor was positioned between pro and leu. However, in the reciprocal cross between strains 105 and 118, the lowest QCO frequency was obtained when A-factor was located between trp and pro. The location of A-factor minimizing the QCO frequency in one cross resulted in a much higher QCO frequency in other crosses. For the order pro-leu-trp-arg, a
A-factor gene(s) in Streptomyces

Table 3. Analysis of a cross between strains 105 and 118 of S. griseus by protoplast fusion

<table>
<thead>
<tr>
<th>Recombinant genotype</th>
<th>No. of recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-leu-arg-trp-A-factor-(pro)</td>
<td></td>
</tr>
<tr>
<td>+ + + + +</td>
<td>49</td>
</tr>
<tr>
<td>+ + + A-factor</td>
<td>2</td>
</tr>
<tr>
<td>+ + + trp +</td>
<td>1*</td>
</tr>
<tr>
<td>+ + + trp A-factor</td>
<td>2</td>
</tr>
<tr>
<td>+ + arg + +</td>
<td>8</td>
</tr>
<tr>
<td>+ + arg + A-factor</td>
<td>1</td>
</tr>
<tr>
<td>+ leu + + +</td>
<td>12</td>
</tr>
<tr>
<td>+ leu + + A-factor</td>
<td>1</td>
</tr>
<tr>
<td>pro + + + +</td>
<td>10</td>
</tr>
<tr>
<td>+ leu arg + +</td>
<td>1†</td>
</tr>
<tr>
<td>pro + arg + +</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
</tr>
</tbody>
</table>

* Additional QCO recombinants when A-factor was placed in the sequence minimizing QCO.
† QCO recombinants omitting A-factor.

Table 4. QCO frequency in the ordering of four nutritional markers on a circular chromosome

<table>
<thead>
<tr>
<th>Order of loci</th>
<th>QCO frequency (%) in cross:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>109 x 115</td>
</tr>
<tr>
<td></td>
<td>105 x 118</td>
</tr>
<tr>
<td>pro-leu-arg-trp-(pro)</td>
<td>0</td>
</tr>
<tr>
<td>pro-leu-trp-arg-(pro)</td>
<td>0.8</td>
</tr>
<tr>
<td>pro-arg-leu-trp-(pro)</td>
<td>67.7</td>
</tr>
</tbody>
</table>

Table 5. Frequency of A-factor* recombinants and frequency of QCO recombinants for the sequence with A-factor between each pair of markers

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Total no. of recombinants</th>
<th>Percentage of A-factor* recombinants</th>
<th>Percentage of QCO recombinants for the sequence minus A-factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Percentage of QCO recombinants for different locations of A-factor in the sequence: pro-leu-arg-trp-(pro)*</td>
<td></td>
</tr>
<tr>
<td>109 x 115</td>
<td>127</td>
<td>91</td>
<td>7.9 16 87 12</td>
</tr>
<tr>
<td>105 x 118</td>
<td>89</td>
<td>93</td>
<td>66 13 4.5 2.2</td>
</tr>
</tbody>
</table>

* Locations of A-factor are represented by dashes between the genetic determinants.

similar result was also obtained. These results suggest that the A-factor gene has no fixed position on the chromosomal linkage map. Furthermore, almost all of the recombinants obtained from both of these crosses (91–93%) were found to be A-factor-positive. All these results suggest that the A-factor gene(s) of S. griseus IFO 13189 is extrachromosomal in nature.

**DISCUSSION**

From the genetic studies described in this paper, we conclude that the A-factor gene(s) of S. coelicolor A3(2) is located at a fixed position on the chromosome while that of S. griseus is not. Furthermore, in genetic crosses with S. griseus using protoplast fusion, almost all fusants between A-factor-negative and A-factor-positive strains became A-factor-positive. This suggests that the A-factor gene in S. griseus is located on a highly transferable transposon or plasmid. These conclusions are also consistent with the observations that A-factor-negative mutations in S. griseus occur at high frequency by treatment with curing agents or by cultivation at high temperature, while no such mutants of S. coelicolor A3(2) are obtained by these treat-
ments. In *S. griseus*, A-factor acts as a pleiotropic effector on streptomycin biosynthesis, streptomycin resistance, and spore formation. It is of interest that a gene concerned with A-factor synthesis is extrachromosomal, as it is well known that various phenotypic characters such as antibiotic production and resistance, formation of aerial mycelium, melanoid pigment production and nutrient auxotrophy, are simultaneously lost by pleiotropic mutations in actinomycetes (Okanishi *et al.*, 1970; Shaw & Piwowarski, 1977; Redshaw *et al.*, 1979). The extrachromosomal A-factor gene may be one of many examples involving such pleiotropic gene expression in actinomycetes.

A-factor productivity is widely distributed among actinomycetes (Hara & Beppu, 1982a) and it is possible that a common A-factor gene has spread due to its intrinsic transferable characteristics, like many drug resistance genes. However, it is still not clear whether the extrachromosomal A-factor gene of *S. griseus* is identical in structure and function with the mapped A-factor gene(s) of *S. coelicolor* A3(2). Although no cosynthesis of A-factor was observed between any A-factor-negative mutants of *S. griseus* and *S. coelicolor* A3(2), several different types of the mutant could be present among them. Mapping data from the mutants, BH2, 5, 6, and 10 of *S. coelicolor* A3(2) indicated a slightly different map position for BH10 and BH2, and another for BH5 and BH6. These two groups were also discriminated by whether or not they retained the ability to produce red pigment, and it seems possible that two distinct loci are involved, one of which (exemplified by mutants BH5 and 6) might be a regulatory gene with a pleiotropic function. Recent cloning experiments, which will be reported elsewhere, are consistent with this proposal. These two loci in *S. coelicolor* A3(2) will tentatively be designated by *afs* (A-factor synthesis): *afsA* for the mutants BH10 and 2 and *afsB* for BH5 and 6.

We would like to thank Dr D. A. Hopwood for providing *S. coelicolor* A3(2) strains. We are also grateful to Dr T. Ishikawa, Meiji Seika Fermentation Technology Laboratories for helpful discussions.

**REFERENCES**


