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Genetics of Actinomycin C Production in *Streptomyces chrysomallus*

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Three distinct classes of mutations affecting the biosynthesis of actinomycin have been established in *Streptomyces chrysomallus* by crossing various actinomycin-nonproducing mutants with each other by protoplast fusion. In crosses between members of different classes of mutations, actinomycin-producing recombinant progeny arose, whereas in crosses between members of the same class, no actinomycin-producing recombinants were seen. Biochemical examination of a number of mutants revealed that the expression of all actinomycin synthetases was reduced by about 1 order of magnitude in mutants belonging to class II. In mutants of class I, the specific activities of the actinomycin synthetases were comparable with those measured in their actinomycin-producing parents. Feeding experiments with 4-methyl-3-hydroxyanthranilic acid (4-MHA), the biosynthetic precursor of the chromophore moiety of actinomycin, with representative mutants of the three genetic classes revealed formation of actinomycin in minute amounts by mutants of class I. It is suggested that mutants belonging to class I are mutated at a genetic locus involved in the biosynthesis of 4-MHA. Mutants belonging to class II appear to carry mutations at a locus involved in the regulation of the expression of the actinomycin synthetases. The role of the locus in class III mutations could not be assigned. Mapping studies in *S. chrysomallus* based on conjugal matings revealed the chromosomal linkage of all three loci. Mutations belonging to classes I and III were closely linked. Their genetic loci could be localized in a map interval of the chromosomal linkage group which is significantly distant from the gene locus represented by mutations belonging to class II.

Streptomyces chrysomallus is one of several streptomycetes which produce actinomycins. Previous work has established a genetic recombination system in this organism based on conjugation which allows the mapping of genes on its genome (14) and hence facilitates investigation of the genetics of actinomycin production in *S. chrysomallus*. Such studies will complement biochemical studies, which have already established the major features of the biosynthetic pathway; actinomycins are formed by the oxidative condensation of two actinomycin half-molecules, the 4-methyl-3-hydroxyanthranilic acid (4-MHA) pentapeptide lactones (Fig. 1) (8, 11). Two multifunctional enzymes called actinomycin synthetases II and III are responsible for the assembly of the five amino acids in the peptide lactone chains (12). Actinomycin synthetase II activates threonine and valine found in positions 1 and 2 (Fig. 1) as thioester, whereas actinomycin synthetase III activates the remaining three amino acids, proline, glycine, and valine (positions 3, 4, and 5). In addition, the latter enzyme has the methyltransferase function(s) involved in the N methylation of thioesterified glycine and valine to give sarcosine and *N*-methyl-L-valine. The two enzymes consist of single polypeptide chains of M_r 225,000 and 280,000, respectively. Actinomycin synthetase I is a 4-MHA activating enzyme which is involved in some way in the attachment of the aromatic carbonic acid to the pentapeptide chain (13). It is a single polypeptide chain of M_r 47,000 (U. Keller, manuscript in preparation). Besides this considerable number of different enzyme activities of actinomycin biosynthesis, which would be specified by a total of about 16 kilobases of coding DNA, there are enzymes which participate in the formation of 4-MHA. 4-MHA is a metabolite of tryptophan (26), and it is most likely that enzymes such as tryptophan pyrrolase (1), kynurenine formidase (2), and hydroxykynureninase (9) belong to this metabolic pathway. In addition, the involvement of kynurenine hydroxylase and of a C-methyltransferase, which methylates the aromatic nucleus, is quite evident. Phenoxazinone synthase is an enzyme catalyzing phenoxazinone synthesis from 4-MHA or 4-MHA peptides in vitro and is involved at least in *Streptomyces antibioticus* in actinomycin biosynthesis (9, 17). It is a tetramer of an 88-kilodalton protein, the gene of which has been cloned in *Streptomyces lividans* (3, 7). Interestingly, this enzyme is not detectable in *S. chrysomallus*, indicating a mechanism of phenoxazine synthesis which differs from that in *S. antibioticus* (9). In summary, the whole set of genes of actinomycin biosynthesis may comprise more than 20 kilobases of DNA and it is of interest to know how the biosynthetic determinants are ordered on the genome of actinomycin-producing organisms. The present report describes some characteristics and the organization of actinomycin biosynthesis genes in *S. chrysomallus*.

MATERIALS AND METHODS

Strains. Strains of *S. chrysomallus* and *Streptomyces parvulus* used in this study are listed in Table 1. *Bacillus subtilis* ATCC 6633 was from the American Type Culture Collection.

Media. Minimal medium and complete medium (CM) were as previously described (14). R2YE (6) was used containing either 0.25 or 0.3 M sucrose. Glutamate-histidine-fructose medium was as previously described (27). Medium S was as previously described (19). Liquid CM was CM without agar.

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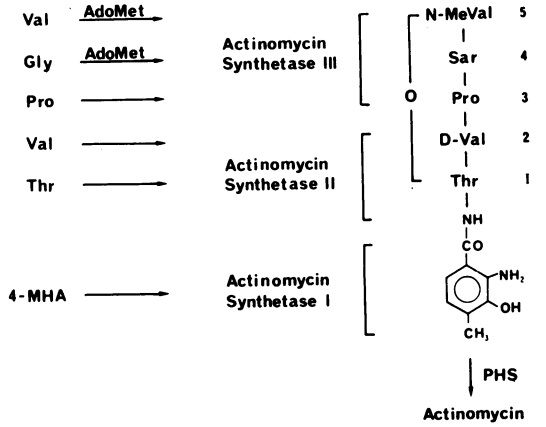


FIG. 1. Scheme of pentapeptide lactone biosynthesis. The actinomycin synthetases were identified by their ability to activate the various amino acids as adenylates and thioesters.

Galactose-glutamate-mineral salts medium (GGM) was as previously described (10). Soft Nutrient Agar was Difco Nutrient Agar (Difco Laboratories, Detroit, Mich.) diluted with Difco Nutrient Broth in a ratio of 1:1 (vol/vol).

Chemicals. 4-MHA was prepared as previously described (13). 4-Methyl-3-hydroxybenzoic acid (4-MHB) was purchased from Fluka (Liestal, Switzerland). 3-Hydroxyanthranilic acid and Silica Gel 60 plastic sheets were from Merck (Darmstadt, Federal Republic of Germany). Kynurenine and hydroxykynurenine were from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of the highest purity commercially available.

Radioisotopes. L-[U-^{14}C]threonine (232 Ci/mol), L-[U-^{14}C]valine (285 Ci/mol), and L-[U-^{14}C]proline (290 Ci/mol) were from Amersham International. [U-^{14}C]glycine (100 Ci/mol) was obtained from CEA (Gif-sur-Yvette, France), and tetrasodium [^{32}P]pyrophosphate (2.9 Ci/mol) was from New England Nuclear Corp. (Dreieich, Federal Republic of Germany).

Growth of organisms. For the production of mycelium suitable for the preparation of protoplasts, strains of *S. chrysomallus* and *S. parvulus* were grown in 250-ml Erlenmeyer baffled flasks containing 25 ml of medium S with 1% glycine. The baffles were steel springs (as described in reference 6). Incubation was in a New Brunswick Environmental Shaker (model G25; New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C and 220 rpm for 48 h.

The antibiotic production medium was liquid CM for both *S. chrysomallus* and *S. parvulus* in 250-ml Erlenmeyer flasks containing 100 ml of medium with and without baffles, respectively. Incubation time was 3 days.

Antibiotic production by strains of *S. chrysomallus* and *S. parvulus* was also monitored in GGM and glutamate-histidine-fructose medium, respectively. The appropriately supplemented media were used in 100-ml volumes in 250-ml Erlenmeyer flasks without steel springs (*S. chrysomallus*) and in 50-ml volumes with steel springs (*S. parvulus*) for up to 8 days. On solid media, strains were incubated at 28 to 30°C for 3 days.

Mutagenic treatment. Previously described procedures were used for mutagenesis of spore suspensions with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (5; survival rate, 50 to 90%), and for UV mutagenesis in the presence of 8-methoxypsoralen (23) to give 99.9% killing. Mutagenesis with acriflavine was done as follows. Samples (2 ml) of a 2-day-old culture of *S. chrysomallus* strains in medium S were inoculated into 25 ml of medium S in 250-ml Erlenmeyer flasks, with steel springs, containing 5, 10, 15, and 20 μg of acriflavine per ml. After 3 days of growth, 2 ml of each culture was transferred to fresh flasks containing medium

TABLE 1. Strains of *S. chrysomallus* and *S. parvulus* used in this study

Strain	Genotype (class of *acm* allele)	Origin or reference
S. chrysomallus		
Wild type		ATCC 11523
X2		14
N2080	*his-1 ura-1*	14
560	*arg-1 ade-1*	14
E1	*arg-8 ser-1*	14
N2	*his-1*	14
N8	*leu-1*	14
R20	*arg-2 his-1 thr-3*	14
R36	*ade-1 phe-1 pdx-1*	14
R57	*ura-1 phe-1 leu-1*	14
R58	*cys-1 leu-1 ade-1*	14
R81	*phe-1 leu-1 ade-1*	14
R82	*arg-1 leu-1 met-4*	14
R81-32	*phe-1 leu-1 met-4 ura-2*	14
R81-90	*phe-1 leu-1 met-4 his-8*	14
R81-133a	*phe-1 leu-1 met-4 trp-3*	14
R88	*phe-1 leu-1*	14
R89	*pro-3 thr-3*	14
R116	*his-1 thr-3*	14
R117	*ura-1 thr-3*	14
201	*his-1 ura-1 acm-13*[a] (I)	2080/UV
202	*his-1 ura-1 acm-14* (I)	2080/NTG
206	*his-1 ura-1 acm-3* (I)	2080/acriflavine[b]
207	*his-1 ura-1 acm-4* (I)	2080/acriflavine[b]
4/5	*ade-1 arg-1 acm-5* (II)	560/acriflavine[b]
25/1	*ade-1 arg-1 acm-6* (II)	560/acriflavine[b]
18/6	*ade-1 arg-1 acm-7* (II)	560/acriflavine[b]
MOP46	*arg-8 ser-1 acm-10* (II)	E1/8-MOP-UV[c]
NTG45	*arg-8 ser-1 acm-11* (II)	E1/NTG
MOP4	*arg-8 ser-1 acm-9* (–)[d]	E1/8-MOP-UV
810	*arg-2 leu-1 acm-2* (I)	N8/NTG
2033	*his-1 pdx-1 acm-8* (I)	N2/NTG
X2-1	*acm-17* (I)	X2/NTG
A1	*arg-8 acm-9e* (III)	Cross MOP4 × R57
A2	*arg-8 acm-9b* (I)	Cross MOP4 × R57
A5	*arg-8 acm-9c* (I)	Cross MOP4 × R57
A9	*arg-8 met-4 acm-9a* (II)	Cross MOP4 × R81-32
A13	*arg-8 phe-1 acm-9d* (I)	Cross MOP4 × R57
A15	*his-1 leu-1 acm-9e* (III)	Cross A1 × R81-90
A22	*his-1 phe-1 acm-9e* (III)	Cross A15 × R36
B4	*ura-1 ade-1 acm-4* (I)	Cross 207 × R58
B7	*arg-2 leu-1 acm-3* (I)	Cross 206 × R82
B12	*leu-1 trp-3 acm-10* (II)	Cross MOP46 × R81-133a
B13	*arg-8 leu-1 trp-3 acm-10* (II)	Cross MOP46 × R81-133a
B23	*pro-3 ade-1 acm-4* (I)	Cross B4 × R89
S. parvulus		
Wild type		ATCC 12434
4305	*his-1 str-1*	14
4305-2	*his-2 str-1 acm-2* (–)[e]	4305/NTG

[a] *acm* denotes actinomycin-defective allele.
[b] For mutagenesis, intact mycelium was used.
[c] 8-MOP, 8-methoxypsoralen.
[d] MOP4 is a mutant carrying at least three mutations, one at each of the three genetic loci of actinomycin biosynthesis.
[e] 4305-2 carries mutations at the genetic loci of classes II and III.

with the same concentration of mutagen. After incubation for another 3 days, the cultures were harvested by centrifugation and the mycelium was converted to protoplasts as described previously (15). Protoplasts were then spread in dilutions onto R2YE (containing 0.25 M sucrose), and after regeneration, single colonies were isolated and tested for antibiotic production as described below.

Detection of mutants impaired in the biosynthesis of actinomycin. Survivors of mutagenic treatments (300 to 5,000 for each experiment) were plated on CM (or, in the case of protoplasts, on R2YE plus 0.25 M sucrose). Where strains with high actinomycin production were mutagenized, prescreening by observing yellow, characteristic of actinomycin, on the reverse side of the colonies was possible. Colonies which were not yellow were transferred onto plugs of CM. After incubation at 30°C for 5 days, the plugs were bioassayed for actinomycin production.

Bioassay for actinomycin production. Agar plugs containing individual colonies of mutants or recombinants of *S. chrysomallus* or *S. parvulus* were placed on Soft Nutrient Agar containing 1% (wt/vol) glucose and 0.5% of an overnight culture of *B. subtilis*. After incubation at 30°C overnight, the presence of actinomycin in the plug was indicated by the appearance of a clear zone of growth inhibition of *B. subtilis* around the plugs. For bioautography, thin-layer chromatograms were overlaid with CM agar. A portion (1 ml) of an overnight culture of *B. subtilis* in Difco Nutrient Broth was spread onto its surface. Overlaid plates were incubated in a humid chamber at 30°C overnight.

Methods of analysis. Actinomycin was extracted from liquid cultures with ethylacetate (11). Extraction of 4-MHA or anthranilic acid from cultures was done with ethyl acetate at pH 2 (18, 23). Thin-layer chromatography of actinomycins and acyl pentapeptide lactones was done as previously described (11). 4-MHA and anthranilic acid were chromatographed as previously described (13). Actinomycin formed in the regeneration plates of protoplast fusions was isolated by cutting the agar from one plate into small pieces and leaching the agar with 100 ml of water for 1 h with stirring at room temperature. After this period, the agar was filtered off and the aqueous phase was extracted as mentioned above. 4-MHB pentapeptide lactones were visualized on thin-layer chromatography plates by spraying with Pauli's reagent (4). All analytical procedures concerning the actinomycin synthetases were performed as described previously (12).

Enzymatic analysis of mutants. Kynureninase and hydroxykynureninase activities were isolated from strains of *S. chrysomallus* by a modification of the procedure of Troost et al. (24). Some 5 g of freshly harvested wet mycelium grown for 2 to 3 days in GGM or liquid CM was suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7)–0.5 mM dithioerythritol (all operations were carried out at 4°C). The suspension was passed through a French pressure cell (Aminco) at 5,000 lb/in^2. After addition of 100 μl of 1 M $MgCl_2$ and 500 μg of DNase (grade II; Sigma), the suspension was gently stirred for 90 min. After centrifugation of the sample at 10,000 rpm (SS34 rotor, Sorvall RC-2B centrifuge) for 30 min, saturated $(NH_4)_2SO_4$ solution was added to the supernatant until 60% saturation was reached. The mixture was left on ice for 30 min and then centrifuged for 20 min as mentioned above. The pelleted precipitate was finally taken up in 1 ml of the same buffer as mentioned above and applied onto an Ultrogel AcA 44 column (30 by 2 cm) which had been equilibrated previously with the same buffer. Fractions (1.8 ml) were collected and assayed for the presence of kynureninase and hydroxykynureninase essentially as previously described (24). Partially purified actinomycin synthetase II and III were prepared according to the method of Keller (12), with the exception that *S. chrysomallus* strains were grown in liquid CM. The propylagarose fraction containing both enzymes was used for the assay, which was done by measurement of thioester formation of threonine, proline, glycine, and valine. Actinomycin synthetase I was isolated and assayed as described previously (13).

Crosses. Conjugal matings between *Streptomyces* strains were performed on fully supplemented minimal medium in petri dishes as described previously (14). The results of multifactor crosses were evaluated as described previously. Protoplast fusions between strains of *S. chrysomallus* were performed as described previously (15) with the exception that in the regeneration media 0.5% yeast extract was present. Fusion cultures between strains of *S. chrysomallus* and *S. parvulus* were regenerated on R2YE in 5 ml of top layers consisting of a mixture of R2YE and medium P (6) in a ratio of 1:1.

Feeding experiments. Feeding experiments with strains of *S. chrysomallus* were generally performed in cultures growing in GGM. Samples from stock solutions of 4-MHA (10 mM), 4-MHB (0.1 M), kynurenine (10 mM), and hydroxykynurenine (10 mM) were added after 48 h in daily intervals to give a 50 μM increase in concentration per day. After 1 week, the cultures were extracted with ethyl acetate and subjected to chromatographic analysis.

RESULTS

Mutants impaired in the formation of actinomycins. Most of the mutants of *S. chrysomallus* impaired in the biosynthesis of actinomycin were prepared by treating spores of various genetically marked strains and strain X2 with NTG (Table 1). With this mutagen, antibiotic nonproducers and auxotrophs were obtained at comparable frequencies in each experiment (0.2 to 3% of survivors). The frequencies depended on the concentration of mutagen and the strain. Furthermore, several of the mutants were obtained by growing mycelium in the presence of acriflavine (0.6%). Treatment of spores with UV light at 254 or 366 nm in the presence of 8-methoxypsoralen was rather ineffective (0.1 and 0.02% nonproducers, respectively). We also observed strains where in one mutagenic step with NTG, lesions in both antibiotic synthesis and nutritional genes appeared simultaneously. Two of these mutants, designated 810 and 2033, are listed in Table 1. The various actinomycin-nonproducing mutants and recombinants of *S. chrysomallus* did not differ conspicuously in morphology from the actinomycin-producing strains. *S. chrysomallus* sporulates poorly when compared with *S. parvulus*, and this was also the case with a considerable number of the actinomycin-nonproducers. However, all of their *acm* alleles after matings with actinomycin producers could be found unaltered in well-sporulating recombinants. Therefore, the lack of actinomycin synthesis in *S. chrysomallus* was apparently unconnected with the extent of formation of aerial mycelium or spores.

Actinomycin-nonproducers of *S. parvulus* were obtained by treatment of spores of strain 4305 (*his-1 str-1*) with NTG (1 to 2% of nonproducers). One of these strains, 4305-2, was used in this study for analyzing the various *acm* alleles in *S. chrysomallus* by interspecific crosses (see below).

The various strains carrying actinomycin-negative alleles listed in Table 1 are nonproducers. During the mutagenesis experiments with both *S. chrysomallus* and *S. parvulus*, it was noted that with all mutagens a considerable proportion

of colonies (5 to 15%) produced reduced zones of inhibition in the bioassay. These isolates were not characterized further.

Genetic characterization of actinomycin-nonproducing mutants. Initially, all actinomycin-nonproducing strains of *S. chrysomallus* listed in Table 1 were tested in crossfeeding experiments for biochemical complementation. However, none was observed on solid medium. Therefore, various mutants were crossed pairwise with each other via protoplast fusion to see whether actinomycin-producing recombinants appeared in the recombinant progeny. Because of the very high frequency of recombination in protoplast fusions between strains of *S. chrysomallus* (15), it was expected that recombination should take place even between closely linked gene loci and that the "rare" recombinants could be detected.

Interestingly, we observed strong actinomycin synthesis on the regeneration plates of several fusion cultures. In other fusions, this was not the case. Analysis of spores from actinomycin-producing fusion cultures in which selection for recombinants was possible showed that, regardless of the selected interval, more than 10% (up to 50%) of the stable recombinant progeny always produced actinomycin. This proved that actinomycin-producing cultures arose by recombination rather than by some cooperative effect of mixed growth. From fusion cultures in which actinomycin production was not detected, fewer than 1% (i.e., none out of 100) of the selected recombinants produced actinomycin.

A set of 12 mutants (201, 202, 206, 207, 810, 2033, 4/5, 25/1, 18/6, MOP46, NTG45, MOP4) was classified by means of these crosses into genetic classes designated class I (201, 202, 206, 207, 810, 2033) and class II (4/5, 25/1, 18/6, MOP46, NTG45) (Table 1). Members of each class in protoplast fusions gave rise to actinomycin-producing progeny only when fused with a member of the other class. The twelfth mutant, designated MOP4, did not yield actinomycin-producing recombinants in fusions with members of either class and was tentatively considered to carry mutations in both of the loci represented by the two genetic classes of actinomycin-nonproducers.

The ease with which it was possible to detect recombination between the two loci of actinomycin synthesis in protoplast fusions without selecting for recombinants made it possible to classify other actinomycin-producing mutants of *S. chrysomallus* and *S. parvulus* in fusions by using 206, 2033, MOP46, and NTG45 as tester strains (Table 1). Most of the mutants fell into classes I or II, with the exception of strains carrying the *acm-9e* allele and *S. parvulus* 4305-2 (see below).

Work in this laboratory had indicated that transfer of chromosomal markers in matings between strains of *S. chrysomallus* and *S. parvulus* took place at frequencies of 10^{-6} to 10^{-5}. We therefore expected that in interspecific fusion crosses between actinomycin-nonproducing mutants of these two organisms, actinomycin-producing recombinants would appear provided that the various mutations were nonallelic. The results of protoplast fusions between various strains of *S. chrysomallus* with *S. parvulus* strain 4305-2 are shown in Table 2. The frequencies of prototrophic recombinants in these interspecific crosses were between 10^{-5} and 10^{-2}. Phenotypically, nearly all of these recombinants resembled *S. chrysomallus* (reduced aerial mycelium formation, soft substrate mycelium), which was not surprising because the regeneration conditions favored the regeneration of *S. chrysomallus*. Actinomycin-producing recombinants were obtained in crosses with either of the test strains 206 or MOP46. Strain 4305-2, therefore, contains a mutation which differs from class I and class II mutations in *S. chrysomallus*.

TABLE 2. Proportion (%) of actinomycin producers among prototrophic recombinants obtained from interspecific protoplast fusions between *S. parvulus* 4503-2 and various actinomycin-nonproducing strains of *S. chrysomallus*

S. chrysomallus strain (allele)	Genetic class(es)	% Actinomycin producers
206 (*acm-3*)	I	98
MOP46 (*acm-10*)	II	98
A13 (*acm-9d*)	I	80
A9 (*acm-9a*)	II	0
MOP4 (*acm-9*)		0
A1 (*acm-9e*)	III	0

Surprisingly, no actinomycin-producing recombinants were obtained in the fusion between strains *S. chrysomallus* MOP4 and *S. parvulus* 4305-2. This finding indicated that strain MOP4, already thought to be a double mutant, apparently was a triple mutant, since it failed to give actinomycin-producing strains in crosses with representatives of each of the three classes.

To construct recombinants carrying only one of its supposed three mutant alleles, strain MOP4 was crossed in conjugal matings with the actinomycin-producing strains R57 and R81-32 of *S. chrysomallus*. We obtained 29 actinomycin-nonproducing recombinants, of which 17 were tested in fusions for allelism of their *acm* alleles with mutant alleles of classes I and II. The tester strains were 2033, 206, NTG45, and MOP46. The recombinants A2 (*acm-9b*), A5 (*acm-9c*), and A13 (*acm-9d*) were classified as belonging to class I, whereas strain A9 (*acm-9a*) belonged to class II. Most important was that strain A1 (*acm-9e*) yielded actinomycin-producing progeny when fused to class I or class II mutants and therefore was considered to carry an expected third type of mutation in *S. chrysomallus* (class III). Fusion crosses between A1 and A13 or A9 yielded actinomycin-producing progeny and confirmed the above-mentioned conclusions.

However, when A1, A9, and A13 were each fused with *S. parvulus* 4305-2, actinomycin-producing progeny were formed only in the cross with strain A13 (Table 2). The most plausible explanation for this result is that *S. parvulus* strain 4305-2 carries mutations at two loci which correspond to classes II and III in *S. chrysomallus*. The interspecific crosses also revealed a previously unsuspected heterogenity in class II mutants, since MOP46, unlike A9, gave actinomycin-producing recombinants. This may indicate the existence of subclasses of mutations within class II.

Biochemical characterization of actinomycin-nonproducing mutants of *S. chrysomallus*. Examination of all actinomycin-nonproducers of *S. chrysomallus* and *S. parvulus* revealed that none formed 4-MHA in liquid culture. Therefore, feeding experiments with 4-MHA were carried out by adding 50 μM 4-MHA to cultures of the various *S. chrysomallus* strains in GGM at daily intervals for a period of 1 week. (Daily replenishment of 4-MHA was necessary because it is extremely susceptible to oxidation. On the other hand, 4-MHA inhibits actinomycin synthesis in vivo [11] and the conditions were therefore chosen to keep the concentration of the compound as low as possible.)

In these conditions, strains 206 and 207 (class I) formed a small but significant amount of actinomycin (Fig. 2). Members of class II, such as MOP46 or NTG45, did not respond to 4-MHA. Interestingly, strain 206 also produced actinomy-

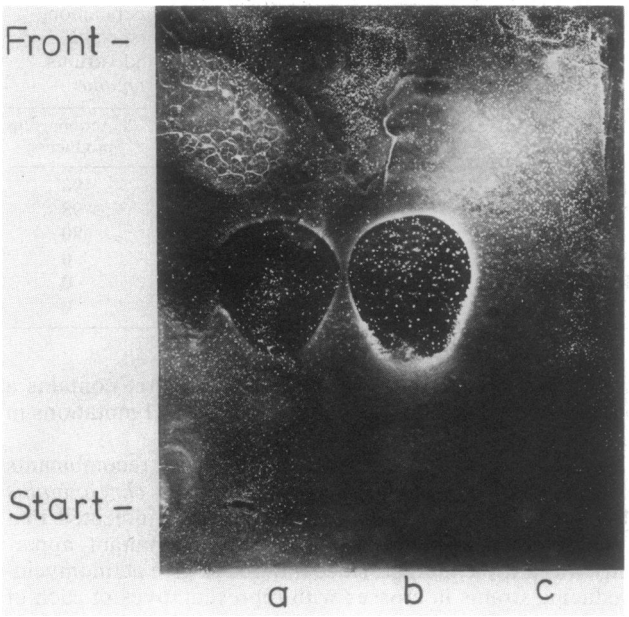


FIG. 2. Actinomycin production in a culture of strain 207 after feeding with 4-MHA. Lanes: a, authentic actinomycin C; b, actinomycin formed after addition of 4-MHA; c, control without addition of 4-MHA. Cultures of strain 207 were grown in GGM with and without 4-MHA. After 7 days, 600 ml of culture from each experiment was extracted with ethyl acetate. After evaporation to dryness, the residues of extracts were chromatographed on thin-layer chromatography silica gel plates by using ethyl acetate-methanol-water (100:5:5, vol/vol/vol) as solvent system and overlaid with agar for bioautography. The inhibition zone in lane b clearly reveals the formation of actinomycin by strain 207 after addition of 4-MHA.

cin when fed with 3-HA (data not shown). Feeding with 4-MHB led to the formation of traces of 4-MHB pentapeptide lactone in cultures of 201, 206, 207, and 2033 (class I) but not of MOP46, 4/5, and NTG45 (class II). Thus, class I mutants still possess the actinomycin synthetases. However, the mutants are apparently unable to synthesize 4-MHA, so they are probably mutated in one or more of the genes involved in the biosynthesis of this compound.

In *S. parvulus*, distinct forms of kynurenine formamidase have been detected; moreover, as well as an inducible kynureninase, it possesses a hydroxykynureninase whose specific activity is correlated with the production of actinomycin during the course of cultivation (23). Kynurenine formamidase and hydroxykynureninase are believed to participate in actinomycin biosynthesis (2, 23). Cultivating all the actinomycin-nonproducers and the wild-type *S. chrysomallus* in the presence of 0.6 mM tryptophan or 0.1 mM kynurenine on solid or in liquid medium resulted in the excretion of large amounts of anthranilic acid. The lack of actinomycin synthesis in these strains was therefore not due to the absence of tryptophan pyrrolase, kynurenine formamidase, or kynureninase (Fig. 3).

Like *S. parvulus*, *S. chrysomallus* harbors a kynureninase and a hydroxykynureninase which can be separated from each other by gel filtration on Ultrogel AcA 44. Qualitative tests revealed the presence of a hydroxykynureninase activity in strains MOP46, NTG45, 206, 207, and MOP4. Quantitative measurements with extracts from strains 206 (class I) and NTG45 (class II) showed that the specific activity of enzyme in both strains was significantly lower than in the

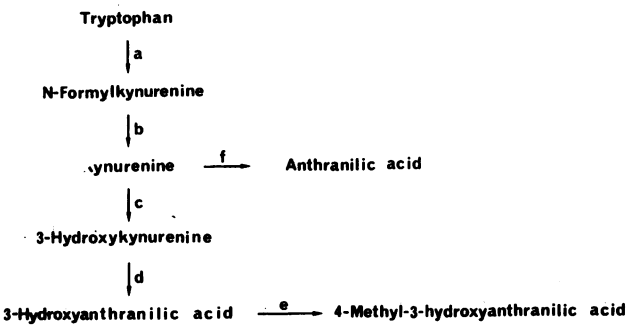


FIG. 3. Scheme of 4-MHA biosynthesis. The various steps are catalyzed by the following enzymes: a, tryptophan pyrrolase; b, kynurenine formamidase; c, kynurenine hydroxylase; d, hydroxykynureninase; e, C-methylating enzyme; f, kynureninase.

wild type (30 and 45%, respectively). In view of the fluctuations of enzyme level in the wild type, the difference between the levels of enzyme in the two mutants does not appear to be significant. By elimination, therefore, class I mutants must be defective in either kynurenine hydroxylase or the C-methylating enzyme. Attempts to isolate kynurenine hydroxylase from *S. chrysomallus* wild type have been unsuccessful.

Actinomycin synthetase I was present in the wild type and three representative class I mutants at specific activities between 10 and 30 pkat of protein per mg (Table 3). Strains MOP46 and A9 (class II) had significantly reduced levels, as was the case with A1 (class III). The triple mutant, MOP4, showed a nearly undetectable level of actinomycin synthetase I.

Measurements of the thioester formation catalyzed by actinomycin synthetases II and III in partially purified enzyme extracts from various mutants revealed that in strain MOP4 the two synthetases were absent (Table 4). By contrast, mutants of class I such as 201, 206, and B7 contained the two enzymes at specific activities fairly comparable to those of their actinomycin-producing parent strain N2080. Class II mutants MOP46 and NTG45 contained less enzyme, whereas strain A1 (class III) had a higher level of the two synthetases than the two class II mutants did.

From the data in Table 4 it can be seen that there are considerable fluctuations in the specific activities within each group, especially when one considers those of proline and glycine activation. Work in this laboratory (A. Stindl and U. Keller, unpublished data) indicates that this is most probably due to the fact that the synthetases are charged to

TABLE 3. Specific activities of actinomycin synthetase I in various actinomycin-nonproducing mutants of *S. chrysomallus*

Strain (allele)	Genetic class	Sp act[a] (pkat/mg)
201 (*acm-13*)	I	13.7 (15.8)
A13 (*acm-9d*)	I	23.5
A5 (*acm-9c*)	I	12.6
MOP46 (*acm-10*)	II	2.7 (3.2)
A9 (*acm-9a*)	II	4.8
A1 (*acm-9e*)	III	4.9 (5.4)
MOP4 (*acm-9*)		0.3 (0, 0.2, 0.1)

[a] Enzyme was purified from 2-day-old mycelium by DEAE-cellulose chromatography and gel filtration on Ultrogel AcA 44 as described previously (13). The values are each from double determinations. The values in parentheses represent results from repeated determinations.

TABLE 4. Thioester formation from various amino acids catalyzed by actinomycin synthetases II and III in partially purified protein extracts derived from various *acm* mutants of *S. chrysomallus*

Strain (allele)	Genetic class	Amino acid (U/mg of protein)[a]			
		L-Thr	L-Val	L-Pro	Gly
N2080		27.09	12.60	0.72	1.53
201 (*acm-13*)	I	8.91 (4.6)	7.49 (5.23)	1.08 (0.67)	1.35 (1.06)
206 (*acm-3*)	I	9.0	9.36	8.7	5.22
B7 (*acm-4*)	I	10.8	15.75	0.45	8.55
X2-1 (*acm-17*)	I	2.93	38.7	5.08	5.1
NTG45 (*acm-11*)	II	1.17 (0.94)	0.81 (0.75)	0.27 (0)	1.17 (0.2)
MOP46 (*acm-10*)	II	0.9 (0.42)	3.69 (1.29)	0 (0)	0 (0)
A1 (*acm-9e*)	III	3.6 (4.5)	5.67 (5.22)	6.39 (2.22)	20.43 (8.35)
MOP4 (*acm-9*)		0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)

[a] One unit is defined as the amount of enzyme which covalently binds 1 pmol of amino acid per 15 min. The enzyme extracts were purified from 2-day-old mycelium grown in liquid CM as described previously (12). The determinations were performed with the pooled and concentrated propylagarose fraction containing both enzymes. Numbers in parentheses are from determinations with independently prepared enzyme extracts. Preparations from strains 2033, A13 (class I), and A9 (class II) showed comparable activities with those measured in extracts of mutants of the respective genetic classes (data not given).

varying extents with amino acids in vivo and that this depends on the physiological conditions in the cells. Detailed investigations in protein extracts of strain X2 grown in the chemically defined GGM revealed that both actinomycin synthetases II and III had nearly the same specific activities (based on threonine and glycine thioester formation) (12). However, when the same strain was grown in liquid CM, actinomycin synthetase III was charged with glycine to a much lesser extent (10 to 15%) than actinomycin synthetase II with threonine. On the other hand, analysis of the protein fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that in the latter case both synthetases were present in approximately equal amounts as judged from the intensities of the stained bands.

Therefore, protein extracts from several mutants carrying various *acm* alleles were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). The high molecular weight of the synthetases allow their easy detection in the gels. In cell extracts of class I mutants 2033 and A13, the two bands corresponding to the two actinomycin synthetases, though faint, are clearly visible. In extracts of members of class II, such as MOP46 and A9, they are much fainter though probably still present, whereas in the extract of strain A15 (class III) they appear to be more intense than those of the class II mutants. The coordinate reduction in the levels of the two synthetase proteins in the various mutants suggests that the mutations in class II have affected the regulation of synthetase synthesis rather than the intrinsic properties of the enzymes.

Mapping of actinomycin biosynthesis genes. Various crosses based on conjugal matings between actinomycin-nonproducing mutants and genetically marked actinomycin-producing strains of *S. chrysomallus* were performed. Three of these crosses involving the *thr-3* marker and the *acm* alleles *acm-4*, *acm-10*, and *acm-9e*, representing mutations of class I, II, and III, respectively, are shown in Fig. 5. After scoring of recombinant genotypes, the frequency of each segregating allele among the recombinants was calculated, and the values were inserted in diagrammatic representations of the crosses. The frequency of an allele is related to the closeness of its linkage to the selected marker with which it was coupled in the parent strain, so that the frequency of the *acm* mutation in each cross indicated the closeness of its linkage to thr^+ compared with that of other markers in the cross. The *acm* mutation was thus given two alternative locations, one in each of the two arcs between the selected markers. The selections were performed such that in each arc was one pair of nonselected alleles, which enables one to decide between the two possible locations by tabulating the segregation of the *acm* mutation with that of the nonselected markers. The consideration of potential multiple crossover classes (marked in each cross by an asterisk) clearly indi-

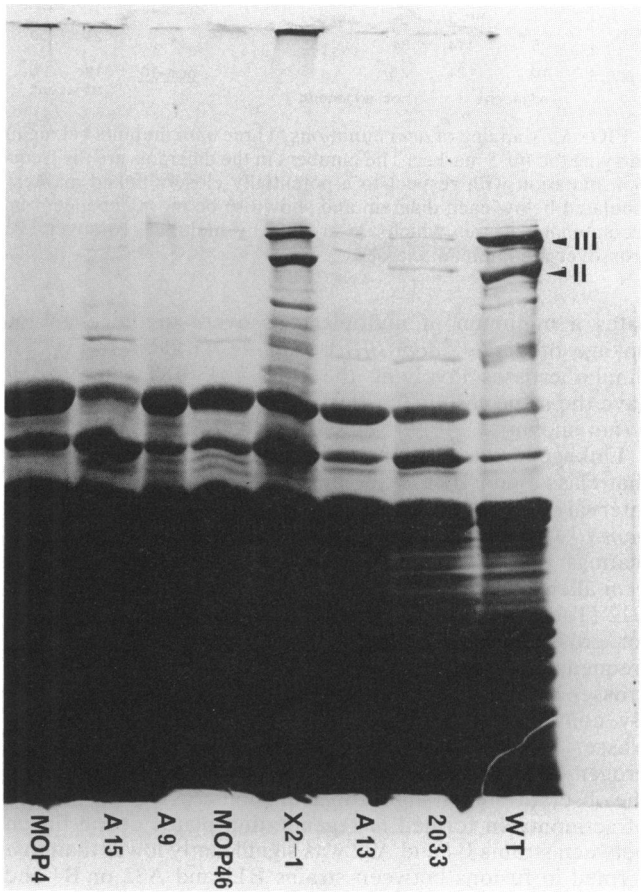
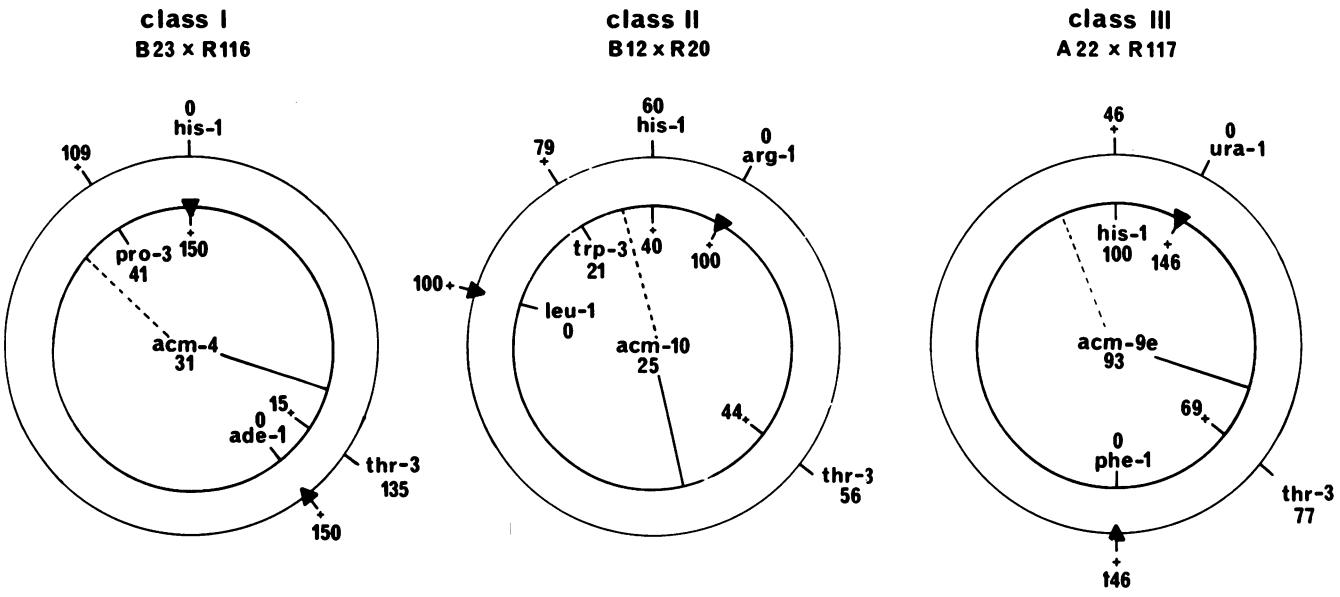


FIG. 4. Separation of ammonium sulfate (55% saturation)-fractionated protein extracts of various strains of *S. chrysomallus* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. About 800 μg of protein in a total of 25 μl of protein extract was loaded on each slot of a 5% slab gel. The staining was with silver nitrate. III, Actinomycin synthetase III; II, actinomycin synthetase II. WT, Wild type.



	+	thr-3	+	pro-3
+	5*	114	84	35
acm-4	10	21	25*	6
	adjacent		not adjacent	

	+	thr-3	+	his-1	+	trp-3
+	25	50	37	38	54	21*
acm-10	19	6*	3	22*	25	0
	adjacent		not adjacent		not adjacent	

	+	thr-3	+	his-1
+	8*	45	25	28
acm-9e	61	32	21*	72
	adjacent		not adjacent	

FIG. 5. Mapping of *acm* mutations. Three *acm* mutants belonging to classes I, II, and III were crossed with actinomycin-producing strains carrying the *thr-3* marker. The numbers in the diagrams are the frequencies of alleles among the recombinant progeny. The segregation of each *acm* mutation with respect to a potentially closely linked marker from each arc between the selected markers (indicated by triangles) is tabulated below each diagram and shown to be more dependent upon one marker than on the other. In the case of linkage, the number of recombinant classes which are created by multiple crossover events are rarer than in the case of independent segregation. *, Multiple crossover recombinant classes.

cates a minimum of multiple crossovers in the event of linkage of *acm-4*, *acm-10*, or *acm-9e* with *thr-3* (Fig. 5). Similar crosses involving the pairs 2033/R89 and A9/R89 gave the same results for class I and II mutations as in Fig. 5 (not shown).

Linkage of classes I, II, and III. From Fig. 5 it is evident that class I and III mutations (*acm-4*, *acm-9e*) fell into the interval counterclockwise of *thr-3*, whereas class II mutation *acm-10* was found to lie clockwise of *thr-3*. Furthermore, matings were performed between strains carrying different *acm* alleles, such as B4 and A22, B12 and A22, and B4 and B12 (Table 5). The data clearly reveal that under selection for crossover in the largest intervals in each cross, the frequencies of actinomycin-producing progeny are large in crosses between class II mutants and class I or III mutants. By contrast, in the cross between mutants belonging to classes I and III, the frequency of actinomycin-producing progeny was very low. This result was already indicated by the observation that in protoplast fusion crosses, the amount of actinomycin formed in regeneration plates of the fusion between strains B4 and A22 was significantly lower than that formed in fusions between strains B12 and A22 or B4 and B12. The data strongly suggest that class I and III mutations are closely linked, whereas the class II locus lies some distance apart from the other two loci. This is a further confirmation of the data given in Fig. 5. In addition, the analysis of the various genotypes produced in these crosses clearly revealed the same order of loci given in Fig. 5 (not shown).

An additional cross (not shown here) between MOP46 (*arg-8 ser-1 acm-10*) and R81-133a (*phe-1 leu-1 met-4 trp-3 acm*$^+$) revealed a more precise map location for the class II mutation between *thr-3* and *met-4*. The results of all the mapping data including those not shown here are summarized in Fig. 6.

DISCUSSION

Three distinct types of mutations affecting the biosynthesis of actinomycin were identified in *S. chrysomallus* by a combination of genetic and biochemical criteria, even though their classification by cosynthesis reactions was not

TABLE 5. Frequencies of actinomycin producers among recombinants in crosses based on conjugal matings between various actinomycin-nonproducing strains of *S. chrysomallus*

Cross (class) and selection	Frequency of actinomycin producers (%)
B4 (I) × A22 (III)	
ura$^+$/phe$^+$	0
ade$^+$/his$^+$	1
B12 (II) × A22 (III)	
leu$^+$/phe$^+$	13
trp$^+$/phe$^+$	11
B4 (I) × B12 (II)	
leu/ade$^+$	35
trp$^+$/ade$^+$	21

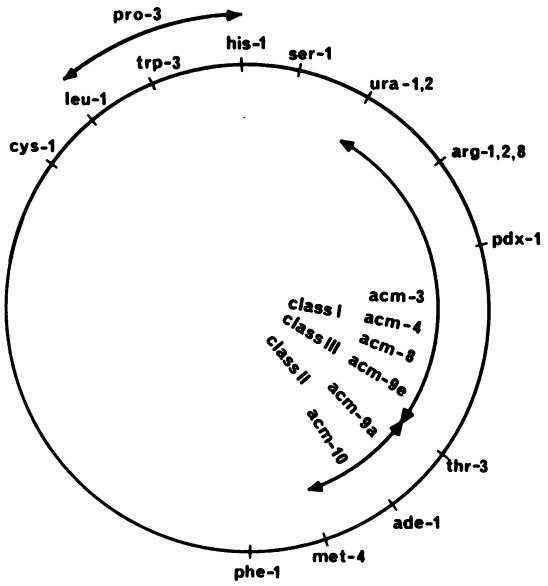


FIG. 6. Genetic map of the markers of the mutants of *S. chrysomallus* listed in Table 1. The relative distances between the markers and their positions came from the multifactor crosses described in reference 14.

possible. It is known that the intermediates of enzymatic peptide synthesis are covalently bound to the multifunctional enzymes, and this most probably prevents the diffusion of these intermediates (16). Similar observations have been made with various *eryA* mutants of *Streptomyces erythreus* blocked in the formation of the macrolactone moiety of erythromycin, which also did not crossfeed each other (25).

Class I mutants of *S. chrysomallus* produced small amounts of actinomycin or acyl pentapeptide lactones when fed with 4-MHA or 4-MHB, and it is therefore most likely that these mutants are impaired in the biosynthesis of 4-MHA. In addition, mutants of this class contained all of the actinomycin synthetases at an appreciable level, whereas mutants belonging to class II contained much lower levels. Furthermore, mutants of the latter class do not respond to 4-MHA or 4-MHB by forming the corresponding acyl peptide lactones.

At present, we have no detailed knowledge of the actinomycin synthetases which, besides the principal amino acid activation steps, possess numerous catalytic activities, and it is therefore not possible to decide whether the various class II mutants possess defective actinomycin synthetases which arise by mutations in the respective structural genes. The results with the mutants belonging to class II indicate that they are affected in the regulation of synthesis of the actinomycin synthetases I, II, and III, because in the strains examined the level of the synthetases is significantly lower than in actinomycin-producing strains. By contrast, mutants of class I and III displayed a significantly higher activity in the principal activation reactions catalyzed by actinomycin synthetases II and III. With respect to actinomycin synthetase I, classes I and III differ from each other by a significantly reduced specific activity of this enzyme in strains belonging to class III. Thus, the mutation of class III affects in some way the expression of actinomycin synthetase I.

All three actinomycin synthetases were nearly undetectable in the mutant MOP4, which carries at least three mutations. Thus, it may be concluded that in this strain the simultaneous presence of class II and III mutations in *S. chrysomallus* abolishes the expression of the three synthetases, whereas enzymes thought to be involved in the biosynthesis of 4-MHA, such as tryptophan pyrrolase, kynurenine formamidase, and hydroxykynureninase, are still present. In the case of hydroxykynureninase, it was found that actinomycin-nonproducing mutants of both classes I and II contained significantly reduced levels of this enzyme when compared with the wild type. It therefore seems unlikely that all the mutants described here possess mutations in the structural genes of this or the other two enzymes. However, it has not been excluded that class I mutants may be defective in kynurenine hydroxylase or the C-methyltransferase involved in aromatic C methylation.

The mapping data clearly reveal a chromosomal linkage of the alleles represented by the three genetic classes. Interestingly, the three genetic loci are not organized in one continuous cluster as is the case for the genes of a number of *Streptomyces* antibiotics such as actinorhodin or undecylprodigiosin in *Streptomyces coelicolor* (21, 22). The gene loci of classes I and III fell into the interval *thr-3* to *pdx-1*, whereas the gene locus for class II was mapped into the interval *met-4* to *thr-3* (Fig. 6). A further example of a noncontinuous cluster of antibiotic biosynthesis genes is that of the genes for oxytetracycline biosynthesis in *Streptomyces rimosus* (20), in which two gene loci were mapped on opposite sides of the chromosomal map. The data presented here do not reveal in which gene locus the structural genes of the actinomycin synthetases lie. It is expected that cloning of the actinomycin biosynthesis genes will help to answer this and other as yet unsolved questions in the genetics of peptide lactone biosynthesis.

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