Biochemical and Genetic Characterization of *Streptomyces rimosus* Mutants Impaired in Oxytetracycline Biosynthesis

By P. M. RHODES,* N. WINSKILL, E. J. FRIEND AND M. WARREN
Pfizer Central Research, Sandwich, Kent CT13 9NJ

(Received 22 September 1980)

Antibiotic non-producing mutants were isolated from an oxytetracycline (OTC) producing strain of *Streptomyces rimosus*. Cosynthesis tests and feeding known intermediates of the OTC pathway allowed the classification of the mutants into several groups. The biosynthetic lesion was determined for several of the mutants. Some of the mutants deficient in the reduction of 5a,11a-dehydrooxytetracycline, the final step in the pathway, were unable to synthesize a cofactor (CSF1) essential for this reaction. Mutations apparently in genes for enzymes of the main OTC pathway were found for three of a possible four steps between 4-aminodimethylaminoanhydrotetracycline (4-amino-ATC) and OTC. Mutations affecting three other steps of a possible five before 4-amino-ATC were found, but unambiguous identification of these was not possible. The genes for OTC production, including those for CSF1 synthesis, were located in two diametrically opposite clusters on the *S. rimosus* chromosomal linkage map. No evidence for plasmid-borne genes was obtained.

INTRODUCTION

Despite the extensive use of blocked mutants for the elucidation of the terminal steps of the biosynthesis of the antibiotic chlortetracycline by *Streptomyces aureofaciens* (McCormick, 1966, 1968), the lack of efficient methods for genetic analysis has prevented the study of gene-enzyme relationships in this pathway (Blumauerova *et al.*, 1972). In contrast, a well developed genetic system exists in *Streptomyces rimosus* which produces the related compound oxytetracycline (OTC) (Friend & Hopwood, 1971; Alačević *et al.*, 1973). Although mutants impaired in OTC production have been reported (Delić *et al.*, 1969; Alikhanian *et al.*, 1961) and preliminary gene mapping has been described (Boronin & Mindlin, 1971; Alačević, 1976), the biochemical nature of the lesions has not been studied.

In this paper we describe the isolation of OTC non-producing mutants, the investigation of the biosynthetic blocks, and the position of the genes involved on the *S. rimosus* linkage map. This is the first combined study of the biochemistry and genetics of OTC production.

METHODS

Strains. All the strains of *Streptomyces rimosus* (Table 1) were derived from a prototrophic strain used commercially for production of OTC (Friend & Hopwood, 1971). *Streptomyces aureofaciens* ATCC 13190 (Miller *et al.*, 1960) was used as a source of CSF1. A strain of *Bacillus subtilis* from the Pfizer collection was used in bioassay procedures for antibiotic detection.

Media. Complete medium (CM) and minimal medium (MM) were as described by Friend *et al.* (1978). Liquid complete medium (LM) contained, per litre: D-glucose, 10 g; yeast extract, 5 g; lactalbumin hydrolysate, 15 g; sucrose, 2.8 g; calcium carbonate, 1 g.

Mutagenesis. Three mutagens were used for the production of OTC-negative mutants: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Delić *et al.*, 1970); ultraviolet light of wavelength 254 nm; ultraviolet light of wavelength 365 nm after sensitization with 8-methoxypsoralen (Townsend *et al.*, 1971).

Table 1. *Streptomyces rimosus* strains

Strain no.	OTC mutation no.	Genotype
R260	OTC producing	<i>adeA2 hisA1</i>
R261	OTC producing	<i>argA1 lysB2</i>
R828	OTC producing	<i>proA3 uraA8</i>
R425	OTC producing	<i>proA3 lysB2 metB2</i>
K1378	4	<i>argA1 lysB2 otcD4</i>
R902	4	<i>ribB1 cysD3 otcD4</i>
K1581	19	<i>adeA2 hisA1 csfA19</i>
K1573	20	<i>adeA2 hisA1 otcX20</i>
K1591	25	<i>hisA1 otcA25</i>
K1626	56	<i>adeA2 hisA1 csfB56</i>
K1645	75	<i>adeA2 hisA1 otcC75</i>
K1660	90	<i>adeA2 hisA1 otcY90</i>
K1772	151	<i>argA1 lysB2 otcZ151</i>
K1727	155	<i>csfC155</i>

Isolation of OTC non-producing mutants. Mutagen-treated spore suspensions were spread on the surface of CM plates and incubated at 28 °C until colonies were just visible to the naked eye (about 2 d). Colonies, each on a cylinder of agar, were removed from the plates using a sterile cork borer of 4 mm diameter. Incubation was continued in a humid environment for a further 5 d. The plugs were then placed on the surface of antibiotic assay agar (Difco) seeded with *Bacillus subtilis*. After overnight incubation at 37 °C parental colonies produced an inhibition zone of diameter 25 mm.

Cosynthesis. Three different procedures were used.

(1) *Agar mixed culture test.* Spore suspensions (10^6 – 10^7 spores ml⁻¹) of two strains were mixed on the surface of 4 mm diameter cylinders of CM and incubated at 28 °C in a humid chamber for 5 d. Antibiotic was assayed by placing the cylinder on antibiotic assay agar (Difco) seeded with *B. subtilis* and incubating overnight at 37 °C. A halo of inhibited growth indicated cosynthesis.

(2) *Agar strip test.* This was an adaptation of the procedure of Delić *et al.* (1969). Spore suspensions of two strains were separately spread on each half of a Petri dish containing CM, with a 3 mm wide uninoculated strip between them. After incubation at 28 °C for 5 d a 10 mm wide strip of agar was cut from the plate, perpendicularly to the uninoculated strip. The agar strip was placed on *B. subtilis* agar and antibiotic production was indicated by a halo of inhibited growth around one of the strains on the strip. That strain was assumed to have produced OTC by cosynthesis.

(3) *Liquid culture test.* Spore suspensions of two strains were inoculated into 45 ml LM in a 300 ml Erlenmeyer flask. The concentration of each strain in the medium was 10^6 spores ml⁻¹. Flasks were shaken at 220 rev. min⁻¹ on a rotary shaker with a 4 cm stroke, at 28 °C. After incubation for 4 d the culture was acidified with 1 M-HCl to pH 2 and filtered through Whatman no. 1 paper. The filtrate was assayed for OTC by high performance liquid chromatography as described below.

Genetic mapping of OTC-negative mutations. The location of OTC-negative mutations relative to previously mapped auxotrophic markers was determined by haploid recombinant analysis (Hopwood, 1967*b*). Crosses between an OTC non-producing mutant and an OTC producing parent carrying complementary auxotrophic markers were plated on several different selective media to recover recombinants. Samples of the recombinants were purified by streaking twice on to the same selective medium to exclude contamination with parental spores. Each purified recombinant colony was inoculated as a patch on a master plate and on a plug of CM. Master plates were incubated for 3–4 d and then replicated to diagnostic media; agar plugs were incubated for 5 d and assayed for antibiotic activity against *B. subtilis* as described above. Each recombinant was classified for inheritance of auxotrophic markers and production of antibiotic. The size of the inhibitory zones produced by recombinants depended on the particular combination of markers inherited. However, scoring recombinants on the criterion of no inhibition versus some inhibition was unambiguous. Consideration of allele frequencies amongst recombinants gave two possible map locations for each OTC mutation (in either arc between the selected markers). The 'correct' alternative was that which minimized the crossovers required to produce the observed progeny of recombinants.

Chromatography. High performance liquid chromatography (HPLC) of OTC-containing extracts was carried out using a Waters Associates model ALC 200 chromatograph under the following conditions: column, Waters μ Bondapak C₁₈ (250 mm $\times$ 4 mm i.d.); mobile phase, acetonitrile/water (1:4) containing 0.1 M-perchloric acid; flow rate, 2.5 ml min⁻¹; detector, absorbance at 254 nm.

Chemicals. Oxytetracycline, anhydrotetracycline (ATC), 4-aminodimethylaminoanhydrotetracycline (4-amino-ATC), and 7-chloro-5a(11a)-dehydrotetracycline (7-chloro-DHTC) were Pfizer laboratory reference compounds.

RESULTS

Isolation of OTC-negative mutants

The choice of parental strains for the isolation of antibiotic-negative mutants was made to facilitate subsequent genetic analysis. A pair of strains (R260 and R261, see Table 1) carrying complementary auxotrophic mutations of known positions on the circular chromosome (Friend & Hopwood, 1971) was selected; none of the auxotrophies prevented the production of OTC under the test conditions.

Colonies giving significantly smaller antibiotic inhibition zones than the parent strain were found at an average frequency of 0.5% after mutagenesis. These were isolated, purified and retested. After the elimination of leaky mutants and those sensitive to growth inhibition by tetracycline antibiotics, 57 mutants were selected out of about 40 000 colonies examined (0.14%). None of the selected mutants were grossly abnormal in colony size or morphology.

The OTC producing ability of the 57 mutants in liquid culture was tested by HPLC. An undetectable amount (less than $1 \mu\text{g ml}^{-1}$) of OTC was produced by 32 of the mutants. The remaining mutants produced 10–20 $\mu\text{g ml}^{-1}$ under the test conditions, compared with the parent strains which produced 1000–2000 $\mu\text{g ml}^{-1}$.

Cosynthesis between OTC-negative mutants

The classification of the mutants impaired in OTC biosynthesis was first attempted using the principle of cosynthesis (McCormick *et al.*, 1960). This process is the counterpart in non-essential metabolism of the phenomenon of syntrophism between auxotrophic mutants. When two OTC non-producing mutants of *S. rimosus* are grown together in close association and produce OTC, they are said to show cosynthesis. The underlying mechanism of this co-operative biosynthesis could theoretically result from (i) genetic repair via recombination, (ii) transfer of an enzyme or coenzyme from one culture to the other, or (iii) transfer of a biosynthetic precursor of OTC from one strain to the other (McCormick, 1966). If the final explanation is correct, the method affords an opportunity of deciding if two mutants are blocked at the same or different steps in the OTC pathway; cosynthesis then indicates blocks at different points, while lack of cosynthesis may indicate blockage at the same point. Alternatively, a failure to observe cosynthesis could result from the failure of a mutant to excrete the intermediate preceding the block, perhaps because of impermeability of the cell to the compound, or to chemical instability of the compound. Three different experimental procedures to demonstrate cosynthesis between OTC non-producing mutants were used in the present study, and the results obtained depended on the procedure used.

In the first procedure, two OTC non-producing mutants were grown together on the surface of an agar plug. Several hundred such tests were carried out, involving all 57 mutants, and the results showed that at least nine different types of mutant were present. Representative mutants of each type (*otc-4*, -19, -20, -25, -56, -75, -90, -151 and -155) were selected for more detailed examination. These cosynthesized in all possible pair combinations, confirming that nine different OTC non-producing mutations were present. The second procedure, the agar strip method, was designed to elucidate the sequence of the biosynthetic blocks. The nine representative mutants were tested in all possible pair combinations. The results were similar to those obtained previously in *S. rimosus* by Delić *et al.* (1969). The mutants could be divided into two classes on the basis of the cosynthetic patterns. Class 1 mutants (*otc-19*, -56 and -155) rarely demonstrated cosynthesis when grown with each other; positive results were equivocal and non-reproducible. Class 2 mutants (*otc-4*, -20, -25, -75, -90 and -151) also showed only infrequent and non-reproducible positive results. In contrast,

Table 2. ^{14}C content of OTC produced by cosynthesis

Mutant combinations	OTC produced ($\mu\text{g ml}^{-1}$)	Total activity in OTC (μCi)	Specific activity of OTC ($\mu\text{Ci mg}^{-1}$)
(A) <i>otc-4</i> * + <i>otc-151</i>	220	1.23×10^{-2}	1.60×10^{-3}
(B) <i>otc-4</i> + <i>otc-151</i> *	150	3.10×10^{-3}	5.74×10^{-4}
Ratio (A/B)		3.97	2.81

* Indicates the ^{14}C -labelled partner.

No OTC was produced in controls with mutant combinations *otc-4** + *otc-4* and *otc-151** + *otc-151*.

when a class 1 mutant was grown with a class 2 mutant, the agar strip test always gave cosynthesis of OTC, the class 1 strain always acting as the converter and the class 2 strain always as the secretor. Thus, we were not able to use the agar strip procedure to determine a simple consistent sequence for the blocks in OTC biosynthesis, and the results obtained with this procedure disagreed with those obtained in mixed culture. It seemed possible that the failure to observe cosynthesis in the agar strip method in some cases must be due to the failure of a compound to cross the 2–3 mm gap between the two strains when grown separately in this way.

The third method consisted of growing pairs of mutants together in liquid medium in shaken flasks. The observations on mutants grown together in mixed culture on agar plugs were confirmed, and it was also confirmed that the antibiotically active product was OTC.

Transfer of ^{14}C -labelled OTC precursors

In an attempt to provide direct evidence that OTC precursor exchange was taking place in cosynthesizing mixed cultures, and to decide on the sequence of blocks in the OTC pathway, a radiotracer method of analysis was devised. Radiolabelled acetate, a precursor of OTC, was added to the growth medium of one strain in order to label the intracellular pool of OTC precursors. The mycelium was harvested, washed free of excess acetate by centrifugation and inoculated into fresh medium which contained unlabelled mycelium of the other strain. After further incubation, the OTC produced was recovered from the medium and its radiochemical content was determined. If cosynthesis was occurring by transfer of a biosynthetic intermediate, then labelled OTC should be formed when the secretor strain was labelled, but not when the converter strain had been labelled.

The results shown in Table 2 demonstrate this phenomenon in a pair of class 2 mutants. The specific radioactivity of the OTC produced when the labelled strain was *otc-4* was 2.8 times greater than when *otc-151* was labelled, showing that *otc-4* was blocked later in the OTC pathway. The [^{14}C]OTC formed when *otc-151* was labelled could have arisen from secretion of a labelled early intermediate by *otc-151*, partial conversion to a later intermediate by *otc-4* and then terminal reaction(s) being completed by *otc-151*. Other pairs of mutants tested in this type of experiment failed to cosynthesize, perhaps because of damage to the mycelium during the washing procedure. Improvements in technique may be required to extend the usefulness of this new method.

Response of OTC-negative mutants to cosynthetic factor 1

Miller *et al.* (1960) showed that certain mutants of *S. aureofaciens* were unable to produce chlortetracycline because they lacked a flavin-like cofactor, which they termed cosynthetic factor 1 (CSF1), required in catalytic amounts for the final step in the biosynthetic pathway. Since an identical step occurs in OTC biosynthesis (Miller *et al.*, 1965), we determined if any of our *S. rimosus* mutants also lacked this cofactor.

Fig. 1. Biosynthesis of oxytetracycline, indicating the steps at which various OTC-negative mutants are blocked.

Culture filtrate of the CSF1 producing strain *S. aureofaciens* ATCC 13190 (Miller *et al.*, 1960) was supplied to each OTC mutant. All three class 1 mutants (*otc-19*, *-56*, *-155*), but no class 2 mutant, responded by producing OTC. The requirement for a catalytic quantity of active substance was shown by demonstrating the same effect with a 1000-fold diluted culture filtrate of *S. aureofaciens* ATCC 13190.

Class 1 mutants failed to stimulate the production of chlortetracycline by the CSF1 non-producing mutant *S. aureofaciens* ATCC 12748 (McCormick *et al.*, 1960), and so they evidently did not excrete CSF1. Conversely, all six class 2 mutants (*otc-4*, *-20*, *-25*, *-75*, *-90* and *-151*) produced CSF1 as indicated by this test.

All these results are consistent with the hypothesis that the class 1 mutants are each blocked in the CSF1 biosynthetic pathway, at various points. CSF1 is known to be chemically stable and can be transferred from cell to cell by diffusion. Some CSF1 non-producing mutants, e.g. *otc-155* and ATCC 12748, were found to be auxotrophic for riboflavin suggesting the existence of one or more common biosynthetic steps. The three CSF1 non-producing mutants *otc-19*, *-56* and *-155* were assigned to genes *csfA19*, *csfB56* and *csfC155*, respectively.

Conversion of precursors to OTC

Having established that the class 1 mutants were deficient in CSF1, attention was turned to the biochemical nature of the lesions in OTC biosynthesis in each representative class 2 mutant. Cosynthesis results in mixed cultures had indicated at least six different groups of such mutants.

OTC precursors were supplied to liquid cultures of all class 2 mutants, and their conversion, or lack of conversion, to OTC was determined by HPLC and by bioassay. When

Table 3. *Conversion of precursors to OTC (or 7-chloro-OTC) by class 2 mutants*

Mutant	Percentage conversion of precursor to 7-chloro-OTC or OTC		
	7-Chloro-DHTC*	ATC	4-Amino-ATC
<i>otc-20</i>	+	30	44
<i>otc-90</i>	+	49	36
<i>otc-151</i>	+	86	4
<i>otc-4</i>	+	105	0
<i>otc-75</i>	+	0	0
<i>otc-25</i>	—	0	0

* Production of 7-chloro-OTC was estimated by bioassay only: +, production; —, no detectable production.

the supplied compound was converted to OTC, the mutant was assumed to be blocked at a step in the OTC biosynthetic sequence prior to that intermediate. An outline of the sequence, incorporating the results of Miller *et al.* (1965), is given in Fig. 1. The earlier steps in OTC biosynthesis were assumed to be analogous to the equivalent steps in chlortetracycline synthesis, although this has never been formally demonstrated. Available intermediates were fed to the six representative class 2 mutants (Table 3).

The final intermediate before OTC, DHOTC, has apparently never been isolated and therefore was not available for test. The penultimate intermediate, DHTC, is unstable and therefore also was not available for testing. The 7-chloro derivative, however, is stable and is converted by *S. rimosus* into 7-chloro-OTC (Martin *et al.*, 1966). The presence of 7-chloro-OTC in fermentation broths was established by the following criteria: antibiotic potency, ultraviolet spectrum, and HPLC behaviour. Mutants *otc-4*, *-20*, *-75*, *-90* and *-151* were able to carry out this conversion. Mutant *otc-25*, which could not carry out this conversion, might have been blocked either between OTC and DHOTC or between DHTC and DHOTC (Fig. 1), or might have been blocked at more than one step, perhaps through a regulatory defect causing repression of the whole OTC pathway. If the mutant were defective in the conversion of DHTC to DHOTC, then it should produce an antibiotically active substance, tetracycline; this was not the case. The third possible reason for OTC non-production by *otc-25* is eliminated because of its ability to cosynthesize with mutants blocked earlier in the OTC pathway (such as *otc-75*, see below). This shows that *otc-25* is capable of generating an OTC precursor such as DHTC or DHOTC (Fig. 1) and cannot therefore be deficient in any step before DHTC. Thus, *otc-25* can only be blocked between DHOTC and OTC; it was assigned to gene *otcA*.

ATC was converted to OTC by all the class 2 mutants tested except *otc-25* and *-75*. Mutant *otc-75* could convert 7-chloro-DHTC to 7-chloro-OTC and must therefore be blocked between ATC and DHTC. Mutant *otc-75* was assigned to gene *otcC*.

4-Amino-ATC was converted to OTC by all the class 2 mutants tested except *otc-25*, *-75* and *-4*. Since *otc-4* could convert ATC and 7-chloro-DHTC to OTC and 7-chloro-OTC, respectively, it must be blocked between 4-amino-ATC and ATC. Mutant *otc-4* was assigned to gene *otcD*.

None of the mutants apparently blocked before 4-amino-ATC was able to convert the earlier precursors 4-hydroxy-6MPT or 6MPT to OTC. Since a negative result might have been due to deficiencies in experimental method, no definite conclusion could be drawn. However, cosynthesis data showed that three different blocks were present, and so the mutants *otc-20*, *-90* and *-151* were assigned to genes *otcX*, *otcY* and *otcZ*, respectively. Further work is required to clarify the nature of the deficiencies in these mutants.

Fig. 2. The location of OTC-negative mutations on the genetic map of *S. rimosus*. The loci are arbitrarily spaced at equal intervals. The dashed line indicates the region of the *otc* genes reported by Pigac *et al.* (1974).

Table 4. Location of *otcD4* in a cross of R902 (*proA3⁺ ribB1⁺ uraA8⁺ cysD3⁺ otcD4⁻*) with R828 (*proA3⁺ ribB1⁺ uraA8⁺ cysD3⁺ otcD4⁺*) (selected markers *uraA8⁺/ribB1⁺*)

Recombinant genotype	No.	Crossing over in intervals	
		Position I	Position II
<i>cys pro otc</i>	9	a, d	a, c, d, e
<i>cys pro +</i>	3	b, d	a, c
<i>+ pro otc</i>	0	a, b, c, d	b, c, d, e
<i>+ pro +</i>	20	c, d	b, d
<i>cys + otc</i>	14	a, e	a, e
<i>cys + +</i>	5	b, e	a, d
<i>+ + otc</i>	0	a, b, c, e	b, e
<i>+ + +</i>	9	c, e	b, d
Total	60		

The two map locations for *otc-4* indicated (I and II) are consistent with the observed allele ratio *otc-4/otc-4⁺*. Position I is chosen because this location requires only two crossovers to generate all recombinant classes, including the nine *cys pro otc* recombinants. This recombinant class would require four crossovers if position II were correct.

Table 5. Location of *otcC75* in a cross of K1645 (*proA3⁺ adeA2 lysB2⁺ metB2⁺ hisA1 otcC75*) with R425 (*proA3⁺ adeA2⁺ lysB2 metB2 hisA1⁺ otcC75⁺*) (selected markers *adeA2⁺/lysB2⁺*)

Recombinant genotype	No.	Crossing over in intervals	
		Position I	Position II
<i>his met + otc</i>	20	a, c	b, d
<i>his met + +</i>	3	a, d	a, d
<i>+ met pro otc</i>	0	a, c, d, f	b, f
<i>+ met pro +</i>	5	a, f	a, f
<i>his + pro otc</i>	0	b, c, d, e	c, d
<i>his + pro +</i>	1	b, e	a, b, c, e
<i>his + + otc</i>	9	b, c	c, d
<i>his + + +</i>	1	b, d	a, b, c, d
<i>+ + pro otc</i>	0	b, c, d, f	a, b, c, f
<i>+ + pro +</i>	4	b, f	a, b, c, f
Total 43			

Position I is chosen as the map location of *otc-75* since it requires only two crossovers to generate all the observed recombinants, while position II would require four crossovers to generate six progeny belonging to three recombinant classes.

Genetic mapping

The position of *otc* and *csf* loci on the linkage map of *S. rimosus* was investigated by haploid recombinant analysis (Hopwood, 1967*b*). In preliminary experiments, an OTC non-producing mutant was crossed with an OTC producing strain carrying complementary genetic markers. In this way mutants in *otcD4*, *otcX20*, *otcY90* and *otcZ151* were positioned between *argA1* and *lysB2*, and *csfA19*, *csfB56* and *csfC155* were located between *hisA1* and *adeA2* (Fig. 2). A further series of crosses involving markers within these two broad regions of the chromosome enabled the same two groups of mutations to be allocated to shorter regions, namely *ribB1* to *cysD3* and *proA1* to *adeA2* (Fig. 2). The results for two of these crosses, which enabled the locations of *otcD4* and *otcC75* to be determined, are given in Tables 4 and 5, respectively.

The type of genetic analysis employed does not provide map distances, but the tendency for clustering of the genes involved in OTC and CSF1 biosynthesis was clearly demonstrated. No evidence for plasmid involvement in OTC production was obtained since every *otc* mutation studied showed clear evidence of chromosomal linkage.

DISCUSSION

From our collection of 57 OTC non-producing mutants, it has been possible to select individuals impaired in one of six different steps in the biosynthetic sequence leading to this antibiotic. The mutations are thought to be located in the structural genes of the enzymes responsible for these steps, since all the mutants were involved in specific cosynthetic reactions with other mutants, but definitive proof of this assumption would require the isolation and purification of the enzymes. The nature of the impaired step was defined in three mutants by determining their ability to convert known precursors into OTC. In this way, all the expected types of point-blocked mutants after 4-amino-ATC were found. No mutants which were unable to hydroxylate DHTC in the 5-position were found, but these would not have had the antibiotic-negative phenotype sought in the mutant screening procedure, since they would have produced the antibiotic tetracycline. Three other mutants, all blocked prior to 4-amino-ATC were shown to be blocked in different steps by their ability to cosynthesize with each other. Feeding experiments with early precursors failed to demonstrate conversion, and the blocked steps could not be determined, perhaps because the compounds failed to reach the required intracellular site.

Strong evidence has been presented that a number of mutants impaired in the final step in OTC biosynthesis lacked an essential cofactor (CSF1) for this reaction. At least three different blocks in CSF1 biosynthesis were demonstrated in mixed culture cosynthesis tests, or using the agar strip test of Delić *et al.* (1969). These workers, however, concluded that class 1 mutants were altered in 'structural genes involved in the main pathway of antibiotic synthesis'. It therefore seems possible that the OTC non-producing mutants subsequently studied by this group (Alačević, 1976; Pigac *et al.*, 1974) were in fact CSF1-deficient mutants. Conversely, class 2 mutants, which Delić *et al.* (1969) suggested might be altered in genes not directly involved in the main pathway of antibiotic synthesis, have been shown to be impaired at several different points in that pathway.

Genetic mapping of *otc* and *csf* genes has revealed two chromosomal locations, in agreement with the early work of Boronin & Mindlin (1971), who were, however, unable to locate their two regions on a general linkage map. Only a single location was reported by Pigac *et al.* (1974) for seven genes in a chromosomal region partially overlapping one of those determined here (Fig. 2), a result consistent with the hypothesis that these may in fact have been CSF1 genes. In the present study, mutations in the OTC pathway after ATC, and all CSF1 mutations, mapped in a single region of the chromosome between *proA3* and *adeA2*. Mutations before ATC mapped to a second region, diametrically opposite the first on the circular chromosome, between *cysD3* and *ribB1*. Clustering and 180° symmetry of biosynthetically related genes of primary metabolism has been observed in several *Streptomyces* species (Hopwood, 1967*a*; Friend & Hopwood, 1971) but only limited information is available on the location of antibiotic genes (Hopwood & Merrick, 1977). A single gene cluster was found for actinorhodin genes in *S. coelicolor* (Rudd & Hopwood, 1979). In the same organism the genes for methylenomycin A biosynthesis were located on a plasmid, SCP1 (Kirby & Hopwood, 1977).

The symmetry of gene clusters in *Streptomyces* may be a reflection of an evolutionary increase in chromosome size by duplication (Hopwood, 1967*b*). If this theory is correct, the foundations of the OTC pathway must have been laid prior to this event. If the OTC pathway has evolved in a similar manner to primary pathways, this seems to imply a function for OTC in the evolutionary survival of the *S. rimosus* species. This conclusion is also consistent with the fact that a fully antibiologically active compound is only formed in the final step of the complex OTC pathway.

It is hoped that basic studies in biochemical genetics of antibiotic synthesis will lead to an improved understanding of the regulation of these pathways, and of the evolutionary and functional significance of these so-called secondary metabolites. Such studies should also assist the application of recent techniques for genetic manipulation in *Streptomyces*, such as

co-mutation (Randazzo *et al.*, 1973), protoplast fusion (Hopwood & Wright, 1978) and gene cloning via transformation by plasmid DNA (Bibb *et al.*, 1978), to the construction of high-yielding industrial strains and strains producing novel compounds. Work along these lines is in progress in our laboratories.

We would like to thank Mr D. Baldock, Dr D. E. F. Harrison, Professor D. A. Hopwood and Professor R. Thomas for many useful discussions, and for their critical reading of the manuscript. We are also grateful to Mr G. Clements, Miss T. Frampton, Mr J. Tiley and Miss W. Tolley for skilled technical assistance.

REFERENCES

- ALAČEVIĆ, M. (1976). Recent advances in *Streptomyces rimosus* genetics. In *Second International Symposium on the Genetics of Industrial Microorganisms*, pp. 513–519. Edited by K. D. Macdonald. London: Academic Press.
- ALAČEVIĆ, M., STRAŠEK-VEŠLIGAJ, M. & SERMONTI, G. (1973). The circular linkage map of *Streptomyces rimosus*. *Journal of General Microbiology* **77**, 173–185.
- ALIKHANIAN, S. I., ORLOVA, N. V., MINDLIN, S. Z. & ZEITSEVA, Z. M. (1961). Genetic control of oxytetracycline biosynthesis. *Nature, London* **189**, 939–940.
- BIBB, M. J., WARD, J. M. & HOPWOOD, D. A. (1978). Transformation of plasmid DNA into *Streptomyces* at high frequency. *Nature, London* **274**, 398–400.
- BLUMAUEVA, M., HOSTALEK, Z. & VANEK, Z. (1972). Biosynthesis of tetracyclines: problems and perspectives in genetic analysis. In *Fermentation Technology Today*, pp. 223–232. Edited by G. Terui. Osaka: Osaka Society of Fermentation Technology.
- BORONIN, A. M. & MINDLIN, S. Z. (1971). Genetic analysis of *Actinomyces rimosus* mutants with impaired synthesis of antibiotic. *Genetika* **8**, 125–131 (in Russian).
- DELIĆ, V., PIGAC, J. & SERMONTI, G. (1969). Detection and study of cosynthesis of tetracycline antibiotics by an agar method. *Journal of General Microbiology* **55**, 103–108.
- DELIĆ, V., HOPWOOD, D. A. & FRIEND, E. J. (1970). Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Streptomyces coelicolor*. *Mutation Research* **9**, 167–182.
- FRIEND, E. J. & HOPWOOD, D. A. (1971). The linkage map of *Streptomyces rimosus*. *Journal of General Microbiology* **68**, 187–197.
- FRIEND, E. J., WARREN, M. & HOPWOOD, D. A. (1978). Genetic evidence for a plasmid controlling fertility in an industrial strain of *Streptomyces rimosus*. *Journal of General Microbiology* **106**, 201–206.
- HOPWOOD, D. A. (1967*a*). In discussion to F. W. Stahl's paper: Circular genetic maps. *Journal of Cellular Physiology* **70**, Suppl. 1, 1–12.
- HOPWOOD, D. A. (1967*b*). Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriological Reviews* **31**, 373–403.
- HOPWOOD, D. A. & MERRICK, M. J. (1977). Genetics of antibiotic production. *Bacteriological Reviews* **41**, 595–635.
- HOPWOOD, D. A. & WRIGHT, H. M. (1978). Bacterial protoplast fusion: recombination in fused proto-
- plasts of *Streptomyces coelicolor*. *Molecular and General Genetics* **162**, 307–317.
- KIRBY, R. & HOPWOOD, D. A. (1977). Genetic determination of methylenomycin synthesis by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **98**, 239–252.
- MARTIN, J. H., MITSCHER, L. A., MILLER, P. A., SHU, P. & BOHONOS, N. (1966). 5-Hydroxy-7-chlorotetracycline. I. Preparation, isolation and physicochemical properties. *Antimicrobial Agents and Chemotherapy*, 563–567.
- MCCORMICK, J. R. D. (1966). Biosynthesis of the tetracyclines: an integrated biosynthetic scheme. In *Antibiotics—Advances in Research, Production and Clinical Use*, pp. 556–573. Edited by M. Herold & Z. Gabriel. Prague: Czechoslovak Medical Press.
- MCCORMICK, J. R. D. (1968). Point blocked mutants and the biogenesis of tetracyclines. In *Genetics and Breeding of Streptomyces*, pp. 163–176. Edited by G. Sermonti & M. Alačević. Zagreb: Yugoslav Academy of Sciences and Arts.
- MCCORMICK, J. R. D., HIRSCH, U., SJOLANDER, N. O. & DOERSCHUK, A. P. (1960). Cosynthesis of tetracyclines by pairs of *Streptomyces aureofaciens* mutants. *Journal of the American Chemical Society* **82**, 5006–5007.
- MILLER, P. A., SJOLANDER, N. O., NALESYNYK, S., ARNOLD, N., JOHNSON, S., DOERSCHUK, A. P. & MCCORMICK, J. R. D. (1960). Cosynthetic factor 1, a factor in hydrogen-transfer in *Streptomyces aureofaciens*. *Journal of the American Chemical Society* **82**, 5002–5003.
- MILLER, P. A., HASH, J. H., LINCKS, M. & BOHONOS, N. (1965). Biosynthesis of 5-hydroxytetracycline. *Biochemical and Biophysical Research Communications* **18**, 325–331.
- PIGAC, J., VEŠLIGAJ, M. & DELIĆ, V. (1974). Mapping of oxytetracycline genes in *Streptomyces rimosus*. In *Genetics of Industrial Microorganisms, Abstracts of the Second International Symposium*, p. 105. London: Academic Press.
- RANDAZZO, R., SERMONTI, G., CARERE, A. & BIGNAMI, M. (1973). Comutation in *Streptomyces*. *Journal of Bacteriology* **113**, 500–501.
- RUDD, B. A. M. & HOPWOOD, D. A. (1979). Genetics of actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **114**, 35–43.
- TOWNSEND, M. E., WRIGHT, H. M. & HOPWOOD, D. A. (1971). Efficient mutagenesis by near ultraviolet light in the presence of 8-methoxypsoralen in *Streptomyces*. *Journal of Applied Bacteriology* **34**, 799–801.