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Genetic Analysis of Erythromycin Production in *Streptomyces erythreus*

J. MARK WEBER,† CONSTANCE K. WIERMAN, AND C. RICHARD HUTCHINSON*

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706

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Streptomyces erythreus **produces the 14-membered macrolide antibiotic erythromycin A. The properties of erythromycin A nonproducing mutants and their genetic linkage to chromosomal markers were used to establish the rudiments of genetic organization of antibiotic production. Thirty-three Ery$^-$ mutants, produced by mutagenesis of *S. erythreus* NRRL 2338 and affecting the formation of the macrolactone and deoxysugar intermediates of erythromycin A biosynthesis, were classified into four phenotypically different groups based on their cosynthesis behavior, the type of biosynthetic intermediate accumulated, and their ability to biotransform known biochemical intermediates of erythromycin A. Demonstration of the occurrence of natural genetic recombination during conjugal mating in *S. erythreus* enabled comparison of the genetic linkage relationships of three different *ery* mutations with seven other markers on a simple chromosome map. This established a chromosomal location for the *ery* mutations, which appear to be located in at least two positions within one interval of the map.**

The gram-positive *Streptomyces* spp. produce about 75% of the commercially important and medically useful antibiotics (14). We are interested in the biochemistry of antibiotic formation by this genus, particularly macrolide and polyether antibiotics whose assembly processes resemble the more well-understood fatty acid metabolic pathways (20). The availability of the antibiotic production genes for use in producing biosynthetic pathway enzymes in the amounts needed for detailed mechanistic and regulatory studies and in explorations of the molecular biology underlying the regulation of antibiotic formation would be very advantageous to this work. Their study in other contexts, moreover, should reveal information about the relationship of antibiotic production to other developmentally regulated processes in *Streptomyces* spp. (6).

We therefore chose to develop a system for cloning antibiotic production genes from *S. erythreus*, which produces erythromycin A, the prototype of macrolide antibiotics (E. T. Seno and C. R. Hutchinson, *in* L. E. Day and S. W. Queener, ed., *The Bacteria: a Treatise on Structure and Function. IX.*, in press) and a clinically valuable antiinfective agent (14). To do this requires knowledge about the biosynthesis of erythromycin A, strains of *S. erythreus* having mutations specifically affecting erythromycin A formation, and suitable gene cloning methods. The first and third requirements have been met in fact (9) or in principle (8). Thus, we turned our attention to the second, and in this report we describe the generation and properties of mutants of *S. erythreus* NRRL 2338, including those defective in erythromycin A biosynthesis, and the development of a genetic linkage map containing information about the location and organization of the erythromycin biosynthesis genes.

* Corresponding author.
† Present address: Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All of the mutant strains were derived from *S. erythreus* NRRL 2338 and assigned the UW22 strain number in our collection. This strain is described in a U.S. patent issued to R. L. Bunch and J. M. McGuire (Number 2,653,899; September 29, 1953) as the one from which erythromycin A was first isolated and therefore is directly related to the Lilly M5-12559 strain (24). *Staphylococcus aureus* 340 was obtained from J. E. Davies.

Chemicals, growth media constituents, and antibiotics. We used materials available commercially from Sigma Chemical Co., St. Louis, Mo., or Difco Laboratories, Detroit, Mich., except for the following. Chloramphenicol sodium succinate was obtained from Parke Davis Co., Ann Arbor, Mich.; lipiarmycin was a gift from Bruno Cavalleri, Gruppo Lepetit, Milan, Italy; and erythromycin A base, 6-deoxyerythronolide B, erythronolide B, and 3α-mycarosylerythronolide B were gifts from Leonard Katz, Abbott Laboratories, North Chicago, Ill.

Media. For sporulation medium (R2T), the following ingredients were dissolved in 860 ml of distilled water and sterilized at 15 lb of pressure per in^2 and 121°C for 25 min: sucrose, 103 g; K_2SO_4, 0.25 g; yeast extract, 6.5 g; peptone, 4 g; tryptone, 5 g; and Bacto-Agar (Difco), 22 g. The following ingredients then were added to the base medium as separately sterilized solutions: 50% (wt/vol) glucose in water, 20 ml; 2 M Trizma base (pH 7.0), 12.5 ml; 0.5% KH_2PO_4, 5 ml; 1 N NaOH, 2.5 ml; 1 M $CaCl_2$, 50 ml; 1 M $MgCl_2$, 50 ml; and trace elements, 2 ml. Th .race elements consisted of the following (milligrams/liter): $ZnCl_2$, 40; $FeCl_3 \cdot 6H_2O$, 200; $CuCl_2 \cdot 2H_2O$, 9; $MnCl_2 \cdot 4H_2O$, 9; $Na_2B_4O_7 \cdot 10H_2O$, 9; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 9. Minimal medium (R2MM) was R2T medium with tryptone, peptone, and yeast extract replaced by L-asparagine (0.5 g). For liquid growth medium (YEME/S broth), we used the YEME–34% sucrose–5 mM Mg^{2+} medium of Chater et al. (8). The liquid antibiotic production medium was tryptic soy broth (TSB), and the

TABLE 1. Strains of *S. erythreus* used in this study

Strain	Genotype
UW6	*aro-6*
UW15	*arg-2 eryA23*
UW19	*ade-19 eryD24*
UW22	wild type[a]
UW23	*eryA23*
UW24	*eryD24*
UW25	*eryB25*
UW26	*eryB26*
UW34	*eryA34*
UW43	*pyr-43 eryC143*
UW60	*arg-60 aro-6*
UW62	*arg-60 aro-6 rif-62*
UW71	*aro-6 eryD24*
UW90	*aro-6 eryA34*
UW92	*his-67 arg-60 rif-62*
UW110	*leu-18 met-4 rif-63*
UW139	*arg-14 met-4 rif-63*
UW147	*leu-18 eryA34*
UW165	*leu-18 eryB25*
UW169	*met-4 eryB25*
UW174	*leu-18 eryD24*
UW176	*met-4 eryD24*
UW184	*met-4 rif-63 arg-14 eryA34*
UW203	*leu-3*
UW218	*cym-6*
UW219	*phe-25*
UW228	*leu-15*
UW234	*leu-18*
UW243	*met-4*
UW254	*eryC160*
UW261	*his-67 eryA34*
UW267	*arg-6 eryB25*
UW275	*ade-2 eryB25*
UW280	*arg-8 eryD24*
UW310	*pro-7 eryA34*
UW314	*ade-5 eryA41*
UW352	*arg-14 eryA34*
UW362	*ade-9 eryA23*
UW365	*ade-10 eryA44*
UW431	*pro-3 eryA16*
UW432	*pro-3 eryA62*

[a] NRRL 2338 strain.

solid antibiotic production medium was the same medium plus 1.5% Bacto-Agar. Antibiotic bioassay medium (ML) was LB medium (10) with NaCl (10 g/l). A 4% aqueous solution of triphenyltetrazolium chloride (5 ml) was added separately after autoclave sterilization.

Growth of strains. All strains were grown at 30°C on solid or in liquid cultures. The latter were grown in test tubes or baffled Erlenmeyer flasks in a G25 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.).

Mutagenic treatments. The general procedure was to treat 10^9 spores with a mutagen for the period of time which gave 0.1 to 1.0% survivors when grown on R2T medium; then single colonies were picked from plates of the desired survivor population for further analysis. Specific procedures for each mutagen were as follows. Spores were irradiated in UV light for 20 min with a UV flux at the level of the top of the petri dish of 8 nW/cm^2 (Blak-Ray UV meter; Ultra-violet Products, San Gabriel, Calif.). Spores were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described by Delic and co-workers (12). Spores were germinated and grown in liquid TSB with 0.15 to 1.0 μg of ethidium bromide. Spores were added to YEME/S broth (50 ml) and incubated at 30°C at 300 rpm for 18 h to produce freshly germinated spores. The cells then were washed twice with 1 M Tris buffer (pH 7) and suspended in this buffer (9.7 ml) containing 0.3 ml of either diethyl sulfate or ethyl methanesulfonate. This suspension was transferred to a 125-ml baffled Erlenmeyer flask and incubated further with samples taken at 30-, 40-, 50-, and 60-min intervals.

Isolation of drug-resistant mutants. Spontaneously generated drug-resistant mutants were selected by plating 10^9 spores on R2T plates containing approximately tenfold more drug than was necessary to inhibit background growth. If the drug was insoluble in water, it was added as a solution in an organic solvent. Final concentrations of 3% ethanol and 1% dimethyl sulfoxide were tolerated by *S. erythreus*.

Isolation of auxotrophic mutants. Survivors from mutagenic treatment were replicated to minimal medium (R2MM) and to R2T plates as a control. Colonies which did not grow (or grew noticeably less well than the other colonies) on R2MM but grew normally on R2T were chosen for further study.

Characterization of auxotrophic mutants followed the procedure described by Davis et al. (10). Medium supplements were used at the following concentrations: amino acids and nucleic acid bases, 60 μg/ml; vitamins, 6 μg/ml.

Protoplast formation and regeneration. A procedure similar to the one described by Chater et al. (8) was used with protoplast regeneration on R2T plates freshly dried to 85% of their original weight in a laminar flow hood.

Bioassay for erythromycin production. Agar plugs containing individual colonies of *S. erythreus* mutants or filter paper discs impregnated with broth from their liquid cultures were placed directly onto the surface of ML agar seeded with an overnight ML broth culture of *S. aureus* 340. Following incubation overnight at 37°C, the presence of erythromycin in the sample was indicated by the appearance of a clear zone of growth inhibition of *S. aureus* in the agar around the sample.

Detection of mutants in erythromycin biosynthesis. Survivors of mutagenic treatments were plated on R2T for single colonies and transferred with sterile toothpicks to microtiter plates (25861; Corning Glass Works, Corning, N.Y.) whose 96 wells were filled with TSB agar medium and to R2T master plates and incubated for at least 6 days. The agar plugs were removed from the microtiter plates and assayed for antibiotic production as described above. Erythromycin A-nonproducing (Ery$^-$) mutants had no zone of growth inhibition, whereas the wild-type strain (UW22) produced inhibition zones between 20 and 30 mm in diameter. This method ensured that each Ery$^-$ colony was an independently derived mutant (17) but prevented the isolation of mutants having metabolic blocks in the last two steps of the erythromycin A biosynthetic pathway, since erythromycin D, C, and B exhibit significant antibiotic activity (23).

Stability of the Ery$^-$ mutants. The most practical method involved sequential transfer of liquid cultures of Ery$^-$ strains to fresh medium. Spores from an Ery$^-$ mutant were inoculated into 5 ml of liquid TSB in a sterile, capped (KAP-UTS; Bellco Glass Co., Vineland, N.J.) test tube (16 by 150 mm) and incubated at a 45° slant while shaking. Samples (0.2 ml) of the culture were transferred to new test tube cultures at 4-day intervals. Each tube was removed from the shaker after incubating for a total of 7 days and then stored at −20°C until the broth was assayed for antibiotic activity as described above. If the culture broth did not become antibiotic

positive by the twentieth transfer, the Ery$^-$ mutant was considered to be stable.

Analysis of cosynthesis properties of Ery$^-$ mutants. Two Ery$^-$ mutants were streaked perpendicularly, but not touching, on TSB plates and grown at 30°C for 60 to 80 h. Antibiotic production was visualized by overlaying the plate with ML agar seeded with *S. aureus* 340.

Thin-layer chromatography of growth medium extracts from Ery$^-$ mutants. Ery$^-$ strains were grown in lawns on TSB agar plates for 7 to 10 days. Six agar plugs 12 mm in diameter were removed from each lawn and melted in a steam bath. One drop of 10 N NaOH was added, and the liquefied mixture was extracted with 2 ml of ethyl acetate. The extraction was repeated twice, and the combined extracts were concentrated to 100 μl in a stream of nitrogen. Portions (10 μl) of the extracts were spotted on silica gel thin-layer chromatography plates, and the plates were developed in chloroform-95% ethanol (10:1). After air drying briefly, the plates were sprayed with anisaldehyde-sulfuric acid-95% ethanol (1:1:9) and heated at 100°C for 5 min for visualization of the separated compounds. The isolated erythromycin biosynthetic intermediates were identified by comparison of R_f and color with known standards.

Detection of bioconversion of erythromycin biosynthetic intermediates. The following is a variation of the method described by Delic et al. (13). A TSB plate was divided into four sectors by removing a thin slice of agar along an X shape in the plate so that diffusion of intermediates could not occur between sectors. Spores of an Ery$^-$ strain were spread over each agar sector, and the plate was incubated for 48 h. Paper strips saturated with a 1-mg/ml aqueous or ethanolic solution of the biosynthetic intermediate were placed on the surface of the agar sectors, and the plates were incubated for a further 48 h. The agar sectors then were removed from the plate and placed on the surface of an *S. aureus* bioassay plate. Conversion of a particular intermediate to erythromycin was indicated by the appearance of a zone of growth inhibition around the agar sector.

Mating experiments. These were performed by suitable minor variations of the methods described by Hopwood (16). We used R2T and R2MM for the complete and minimal media, respectively, and crosses or recombinant strains were incubated for 5 to 7 days, the time usually required for good sporulation, before analysis. A microcomputer program (available on request from Weber and Bownds, Madison, Wis.) written in UCSD Pascal by collaboration with M. Deric Bownds, University of Wisconsin-Madison, for Apple II microcomputers was used for data compilation and analysis of four-factor crosses.

RESULTS

Mutagenic treatments. *S. erythreus* auxotrophs were isolated from UV- and NTG-treated spores at about the same frequency (0.3 to 1.7%), but at a substantially lower level of kill with NTG (Tables 2 and 3). NTG was a more potent mutagen at a basic pH (8.7) than at an acidic pH (5.5), as reported for NTG treatment of *S. coelicolor* spores (12). Diethyl sulfate, ethyl methanesulfonate, and ethidium bromide were mutagenic to *S. erythreus* mycelia, but not to spores. The auxotrophic and drug resistance mutations generated by these procedures are listed in Tables 4 and 5.

TABLE 2. Mutation of *S. erythreus* strains by UV irradiation [a]

Strain	Survivor fraction (%)	No. of survivors screened	No. of auxotrophs found	Auxotroph frequency (%)
UW228	0.1 – 1.0	634	2	0.3
UW218	0.1 – 1.0	1,751	12	0.7
UW219	0.1 – 1.0	1,350	16	1.2
UW234	0.1 – 1.0	893	15	1.7
UW243	0.1 – 1.0	544	5	0.9

[a] Conditions are described in the text.

TABLE 3. Mutation of *S. erythreus* strains by NTG treatment[a]

pH	NTG concn (mg/ml)	Treatment time (min)	Survivor fraction (%)	Mutant/survivor ratio[b]	Auxotroph frequency (%)
5.5	0	40	1.00	1.8×10^{-8}	0.01
5.5	3.0	40	0.58	2.8×10^{-6}	1.1
8.7	0.3	40	0.58	3.0×10^{-6}	1.2
8.7	3.0	20	0.0013	2.0×10^{-4}	8.7[c]

[a] Strain UW110 was used.
[b] Ratio of the number of *leu*$^+$ revertants to the number of survivors of mutagenesis.
[c] Many of these auxotrophs appeared defective in growth on complete medium.

Mutations in erythromycin A biosynthesis. Thirty-three Ery$^-$ mutants from four different parts of the erythromycin A biosynthetic pathway (Fig. 1) were found from screening about 12,500 survivors of various forms of mutagenic treatment. Of the mutants, 67% (EryA) affected formulation of the macrolactone intermediate 6-deoxyerythronolide B; 15% (EryB) affected the formation of mycarose or its attachment to erythronolide B; 15% (EryC) affected the formation of desosamine or its attachment to 3α-mycarosylerythronolide B; and 3% (one mutant; EryD) affected the formation or attachment of both sugar moieties. Two different kinds of *eryC* mutation were found: regular *eryC1*, which conferred no other phenotypes, and *eryC2*, which conferred

TABLE 4. Auxotrophic mutants of *S. erythreus*

Genotype	Nutritional requirement[a]	No. found	Sporulation phenotype
ade	Adenine	16	+
arg	Arginine	19	+
aro	Aromatic amino acids[b]	3	+
cym	Cysteine and methionine	13	+
his	Histidine	6	+
ile	Isoleucine	10	+
leu	Leucine	24	+
met	Methionine	23	+
phe	Phenylalanine	4	+
pro	Proline	12	+
pyr	Pyridoxine	3	−[c]
ser	Serine	3	−[d]
thr	Threonine	2	+
trp	Tryptophan	14	+
ura	Uracil	1	−[c]
urg	Uracil and arginine	1	−[c]
val	Valine	1	+
vit	Vitamin	4	+
yex	Yeast extract	10	+

[a] Determined as described in the text.
[b] Tryptophan, phenylalanine, and tyrosine.
[c] No aerial mycelium or spores formed on R2T medium.
[d] Aerial mycelium, but no spores, formed on R2T medium.

TABLE 5. Resistance mutants of *S. erythreus*

Genotype	Drug	Selection level (μg/ml)[a]	Sensitivity level (μg/ml)[a]
rif	Rifamycin	10	0.5
str	Streptomycin	500	50
lip	Lipiarmycin	1	0.1
cam	Chloramphenicol	25	5

[a] Determined on R2T medium.

pleiotropic morphological and nutritional phenotypes as well as the Ery⁻ phenotype.

The properties of the Ery⁻ mutants are listed in Table 6. None exhibited obvious pleiotropic phenotypes, and all were stable, except for the *eryC2* mutation. EryC2 mutants simultaneously resumed antibiotic production, sporulation, and prototrophic growth by the second or third subculture. All of the Ery⁻ mutants were resistant to erythromycin A.

Formation and regeneration of protoplasts affected the antibiotic production phenotype at low frequency compared with some other antibiotic-producing *Streptomyces* spp. in which this has been studied (18). From a total of 4,600 colonies regenerated from protoplasts, only 4 were Ery⁻: two EryA, one EryB, and one EryC1. The Ery⁻ phenotype was not observed in a screen of an equal number of colonies not subjected to protoplast treatment.

Locations of mutational blocks in erythromycin A biosynthesis. The most probable location of an *ery* mutation was determined from the results of three tests: cosynthesis behavior, identification of erythromycin A precursors accumulating in growth media, and in vivo conversion of these precursors to erythromycin A.

The cosynthesis behavior (13) and type of erythromycin biosynthetic intermediates accumulated (Table 6) suggested that the erythromycin biosynthetic pathway was blocked at the locations shown in Fig. 1. The EryA mutants, which were universal converters and did not accumulate any of the known intermediates in detectable quantities, were blocked in the formation of 6-deoxyerythronolide B; the EryB and EryD mutants, which accumulated erythronolide B but could be distinguished by their different cosynthesis properties, were blocked in the formation of 3α-mycarosylerythronolide B; and the EryC mutants, which accumulated erythronolide B and 3α-mycarosylerythronolide B and were secretors for two other types of mutant, were blocked in the formation of erythromycin D.

The results of bioconversion experiments (Table 6) confirmed the locations of the biochemical blocks determined from the above data and showed that the *ery* mutations had the following biochemical phenotypes. The *eryA* mutation must lie earliest in the biosynthetic pathway, between propionate and 6-deoxyerythronolide B, because EryA mutants were able to convert 6-deoxyerythronolide B, erythronolide B, and 3α-mycarosylerythronolide B to erythromycin A.

The *eryB* mutation did not allow conversion of 6-deoxyerythronolide B or erythronolide B to erythromycin A. It did, however, allow conversion of 3α-mycarosylerythronolide B to erythromycin A, indicating that the mutation lies between erythronolide B and 3α-mycarosylerythronolide B in the pathway. Consequently, the *eryB* mutation prevents the biosynthesis of mycarose or its attachment to the macrolactone. (EryB mutants did not make erythromycin when fed mycarose.)

The *eryC* mutation did not allow conversion of any of the biosynthetic intermediates tested (erythromycin D was not available). This result, together with other data in Table 4, indicates that the EryC mutants are blocked beyond 3α-mycarosylerythronolide B, probably in the biosynthesis of desosamine or its attachment to the macrolactone ring.

Like the *eryC* mutation, the *eryD* mutation prevented

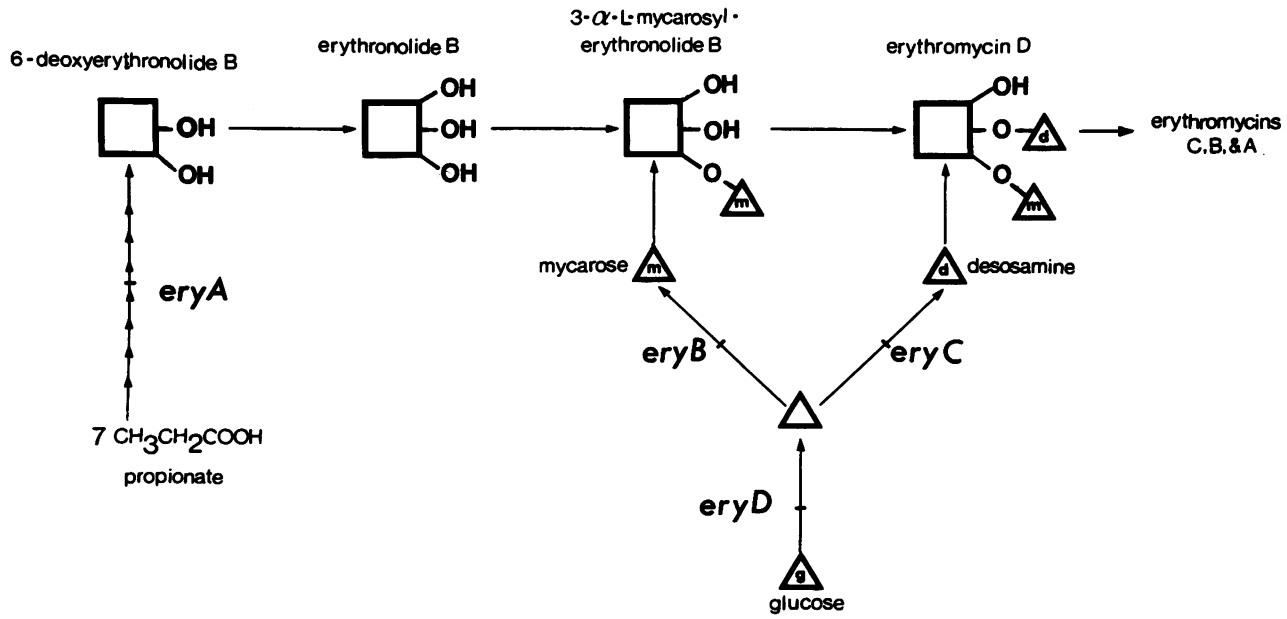


FIG. 1. The biosynthetic pathway for erythromycin A showing the probable locations of four different mutations affecting the formation of erythromycin D. The known intermediates are represented stylistically to represent the known (9) sequence of biochemical transformation, beginning with propionic acid. The four types of *ery* mutation correspond to those described in Table 6.

TABLE 6. Properties of Ery⁻ mutants

Property	Ery⁻ mutant type or characteristic			
	A	B	C	D
Cosynthesis:[b]				
Acted as convertor with mutant types	B, C, D	C	None	None
Acted as secretor with mutant types	None	A	A, B	A
Did not cosynthesize with mutant types	A	B, D	C, D	B, C, D
Biosynthetic intermediate accumulated[b]	None detected	EB[a]	3A[a]	EB
Intermediates bioconverted to erythromycin A[b]	6D[a], EB, 3A	3A	None	None
Probable position of the metabolic block	Propionate to 6D	EB to 3A	3A to EmD[a]	Early sugar pathway
Sporulation	Normal	Normal	3 Bald, 2 normal	Normal
Stability of Ery⁻ phenotype[b]	Stable	Stable	3 Bald, unstable; 2 normal, stable	Stable
Pleiotropic phenotypes	None	None	Baldness, *pyr* auxotrophy	None
Resistance to erythromycin A[c]	Normal	Normal	Normal	Normal

[a] EB, Erythronolide B; 3A, 3α-mycarosylerythronolide B; 6D, 6-deoxyerythronolide B; EmD, erythromycin D.
[b] Assayed as described in the text.
[c] Vegetative mycelia exhibited a normal level of resistance to 2 mg of erythromycin A per ml when grown on TSB agar.

bioconversion of any of the intermediates tested; however, it did not allow the production of 3α-mycarosylerythronolide B. This indicates that the *eryD* mutation is in a step affecting the biosynthesis or attachment of both mycarose and desosamine.

Cosynthesis and bioconversion results indicate that the *ery* mutations we chose for further study affected antibiotic biosynthesis at specific steps in the pathway rather than through pleiotropic interactions affecting secondary metabolic or developmental functions in general.

Genetic recombination. Progeny from mixed cultures of singly auxotrophic *S. erythreus* mutants consisted of a much higher proportion of prototrophs than could be explained by reversion of the mutant alleles, indicating the occurrence of genetic recombination. The results of five crosses of this type (Table 7) show that the recombination frequencies varied between 10^{-3} and 10^{-5} and were 2 or more orders of magnitude higher than the background reversion frequencies of the genetic markers. The majority of prototrophic progeny grew normally and stably when replica plated to fresh minimal medium. A small proportion of unstable prototrophs were found in cross 3, which presumably were heterokaryons (16) because they reverted to the parental phenotypes on subculturing. Higher recombination frequencies have been observed in crosses between mutants of industrial strains of *S. erythreus* and were attributed to the operation of a fertility factor, SEP1 (J. DeWitt, Am. Soc. Microbiol. Conf. Genet. and Mol. Biol. Industr. Microorg., abstr. no. 206 1984). These data confirm earlier, limited observations of genetic recombination in *S. erythreus* (11, 21).

Chromosome mapping. Four-factor crosses were performed and analyzed by the method of Hopwood (16) to generate a genetic linkage map. The results of a representative cross (Table 8) indicated a circular linkage map having the four markers in the sequence shown in Fig. 2. The data show that recombinant genotypes appearing on more than one selective medium did so at comparable frequencies, indicating the absence of interference in the analysis owing to selective growth disadvantages. Complementary recombinant genotypes did not always appear at equal frequencies; however, this did not significantly interfere with marker sequence analysis.

Chi-square analysis (16) of the data from cross 6a (Table 9) indicated nonindependent segregation, and therefore adjacent positions, for the unselected markers on each medium. A similar analysis of the data (not shown) from the reciprocal cross, 6b (UW147 × UW139), confirmed the former results; moreover, the data indicated independent segregation of the nonadjacent alleles *arg-14 rif-63* and *leu-18 met-4*, as predicted from the results of cross 6a.

***ery* loci.** The *eryA34* allele was mapped to the *met-4 rif-63* interval by the strategy described by Hopwood (16). Two possible positions for *eryA34* were indicated by the allele frequencies of the markers in cross 6b (Fig. 2). The final position chosen was the one that required the least number of quadruple crossovers to explain the data (Table 10).

The *eryB25* and *eryD24* alleles, used in five factor crosses, also segregated as chromosomal markers. Their chromosomal locations could not be determined with the same certainty as for the *eryA34* allele, although the data generally were consistent with their location in the *met-4 rif-63* interval. Reciprocal crosses between *met-4* and *leu-18* auxotrophs carrying the *eryB25* and *eryD24* alleles produced no detectable ery^+ recombinants among randomly chosen samples of the selected prototrophic progeny (Table 11, crosses

TABLE 7. Genetic recombination in *S. erythreus*

Cross no.	Strains	Genotypes	Marker reversion frequencies (×10⁶)	Marker recombination frequency (×10⁶)	No. of heterokaryons/ recombinants
1	UW6	*aro-6*	0.11	180	0/92
	UW19	*ade-19*	0.64		
2	UW6	*aro-6*	0.11	100	NA[a]
	UW19	*ade-19*	0.64		
3	UW203	*leu-3*	<0.017	37	11/188
	UW15	*arg-2*	0.063		
4	UW6	*aro-6*	0.11	87	NA
	UW15	*arg-2*	0.063		
5	UW15	*arg-2*	0.063	3,300	0/190
	UW261	*his-67*	0.048		

[a] NA, Data not available.

TABLE 8. Cross 6a: UW352 (*arg-14 ery-34*) × UW110 (*leu-18 met-4 rif-63*)

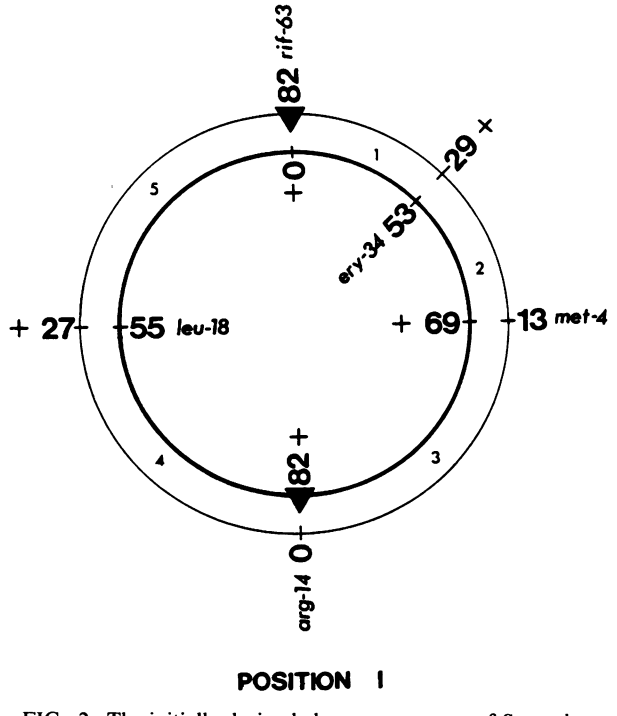
Class	Genotype[a]				No. of colonies on selective medium containing:[b]								Average relative frequency[c]
					Leucine		Arginine, methionine, rifampin		Methionine		Arginine, leucine, rifampin		
	arg	*leu*	*met*	*rif*	A	B	A	B	A	B	A	B	
1	+	+	+	+	85	192			42	163			178
2	−	+	+	−			46	18			12	24	21
3	+	−	+	−	41	93					31	61	77
4	+	+	−	−			30	12	3	12			12
5	+	+	+	−	1	2	0	0	0	0	0	0	1
	+	−	+	+	10	23							16
6	−	+	−	−			21	8					
	+	+	−	+					22	85			
7	−	−	+	−							9	18	52

[a] +, Wild type; −, mutation.
[b] A, Actual number of colonies counted; B, total number of colonies.
[c] Average of total colony counts.

7 and 8); therefore, *eryB25* and *eryD24* are likely to be very close to each other on the chromosome. In a similar pair of reciprocal crosses between *met-4* and *leu-18*, auxotrophs carrying the *eryA34* and *eryB25* mutations, segregation of the two *ery* alleles was observed (Table 11, crosses 9 and 10). Results from reciprocal crosses involving the *eryA34* and *eryD24* alleles (Table 11, crosses 11 and 12) were comparable to the results obtained in crosses 9 and 10 and consistent with *eryD24* and *eryA34* being at separate positions.

A series of 10 crosses between auxotrophs carrying different *eryA* mutations revealed comparatively much lower frequencies of antibiotic-producing recombinants (0 to 0.8%) among the samples of prototrophic progeny screened (data not shown). Even though more than one enzymatic function must be involved in the formation of 6-deoxyerythronolide B (9, 20), evidence for multiple *eryA* loci was not found. These results indicate the close proximity of the *eryA* alleles tested or reflect the requirement for quadruple crossover events for formation of ery^+ recombinants, or both. Consequently, it appears that the *ery* genes are located in at least two positions within the same interval on the chromosome: one location, *eryA*, for macrolactone biosynthesis, and the other, *eryB eryD*, for deoxysugar biosynthesis. The relative location of the *eryC1* mutation could not be determined because the crosses in which it was used were too infertile to allow genetic analysis.

***S. erythreus* linkage map.** Six additional crosses were performed to produce the linkage map shown in Fig. 3. The



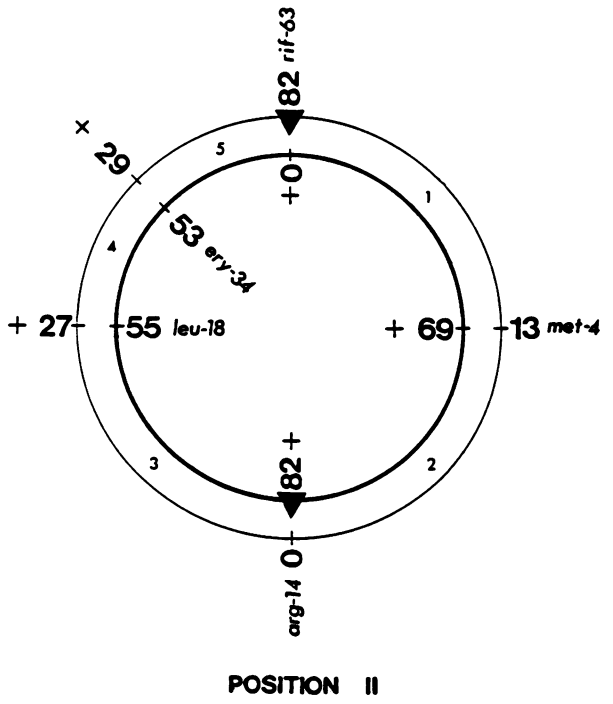


FIG. 2. The initially derived chromosome map of *S. erythreus* showing its circularity by the linkage-relationships of four different markers and two possible positions for the *eryA34* mutation.

TABLE 9. Chi-square analysis of cross 6a

Genotype	No. of colonies with indicated gene combination on selective medium containing:							
	Leucine[a]		Arginine, methionine, rifampin[a]		Methionine[b]		Arginine, leucine, rifampin[a]	
	leu$^+$	*leu*$^-$	*arg*$^+$	*arg*$^-$	*met*$^+$	*met*$^-$	*arg*$^+$	*arg*$^-$
rif$^+$	85	10			42	22		
rif$^-$	1	41			0	3		
met$^+$			0	46				
met$^-$			30	21				
leu$^+$							0	12
leu$^-$							31	9

[a] $P < 0.001$.
[b] Sample size too small for chi-square analysis.

placement of the other markers is meant to indicate their sequence on the map and not map distances, although some markers, e.g., *his-67* and *aro-6*, appeared to be very closely linked in two reciprocal crosses and are therefore drawn more closely to one another than to neighboring markers. The *eryA16* allele is placed in the same interval as *eryA34*, but there is some uncertainty in this location, as indicated by the dashed line in Fig. 3, since it was mapped by using a strain with the *pro-3* mutation as one marker.

DISCUSSION

We constructed the first simple genetic linkage map for *S. erythreus* which includes positions of representative mutations influencing the erythromycin A biosynthetic pathway. Three types of these *ery* mutations appear to have metabolic blocks similar to those described by Martin and co-workers (as reviewed by Queener et al. [26]), but the *eryD* mutation appears not to have been described previously.

It is interesting that analysis by cosynthetic reactions identified only four different types of *ery* mutation in the biosynthetic pathway preceding erythromycin D. Because the number of steps required for erythromycin D biosynthesis greatly exceeds the number of cosynthetic groups found, we assume that diffusion of many erythromycin biosynthetic intermediates between cells does not occur. This is most notable for the cosynthetic properties of EryA mutants and is consistent with the hypothesis that formation of the macrolactone is similar to formation of saturated fatty acids by the fatty acid synthetase complexes of bacteria and yeasts (28). In both systems, covalent attachment of precursors to an enzyme complex or multicatalytic-site protein presumably prevents diffusion of intermediates, thereby preventing cosynthesis in the macrolactone pathway and making intragenic complementation of yeast fatty acid synthetase mutants a rare observation (28). It is likely that diffusion of deoxysugar precursors also does not occur, because cosynthesis was not observed between different mutants blocked in the formation of the same deoxysugar.

Assuming random distribution of *ery* mutations, the predominance of *eryA* mutations (67% of all Ery$^-$ mutants isolated) suggests that macrolactone formation genes constitute the largest portion of DNA for erythromycin biosynthesis. This represents another parallel with studies on fatty acid biosynthesis, in which a large number of mutations confer similar phenotypes (28). Our failure to find one other expected cosynthetic mutant, one blocked in the hydroxylation of 6-deoxyerythronolide B to erythronolide B, suggests that the DNA target for this step may be relatively small by comparison.

Mutants analogous to those reported here have been obtained in a study of *Streptomyces fradiae* mutants blocked in production of tylosin, a 16-membered ring macrolide

TABLE 10. Location of *eryA34* allele by least- quadruple-crossover analysis[a]

Genotype[b]	No. of colonies with the specified genotype	Crossover intervals for position:	
		I	II
leu-18 + +	11	2, 5	1, 4
leu-18 + *eryA34*	37	1, 5	1, 5
+ *met-4* +	3	3, 4	2, 3
+ *met-4 eryA34*	3	1, 2, 3, 4	2, 3, 4, 5
+ + +	8	2, 4	1, 3
+ + *eryA34*	13	1, 4	1, 3, 4, 5
leu-18 met-4 +	7	3, 5	2, 4
leu-18 met-4 eryA34	0	1, 2, 3, 4	2, 5

[a] The intervals in which crossover is required to generate each phenotype in the first column are listed under the third and fourth columns, which represent the two possible positions for placement of the *eryA34* allele. The number of each genotype found in cross 6b is listed in the second column and was used to calculate the total number of quadruple-crossover recombinants for the two possible positions, which were 3 and 16 for position I and II, respectively.
[b] +, Wild type.

TABLE 11. Results from crosses between different Ery mutants

Cross no.	Strains	Markers	Prototroph formation frequency	Proportion of Ery$^+$ prototrophs[a]
7	UW165 UW176	*leu-18 eryB25* *met-4 eryD24*	7.6×10^{-3}	0/186 (0)
8	UW169 UW174	*met4 eryB25* *leu-18 eryD24*	4.8×10^{-3}	0/186 (0)
9	UW147 UW169	*leu-18 eryA34* *met-4 eryB25*	7.9×10^{-3}	11/184 (6)
10	UW165 UW184	*leu-18 eryB25* *met-4 eryA34*	1.3×10^{-4}	30/184 (16)
11	UW147 UW176	*leu18 eryA34* *met-4 eryD24*	3.1×10^{-6}	14/92 (15)
12	UW184 UW174	*met-4 eryA34* *leu-18 eryD24*	1.3×10^{-3}	11/69 (16)

[a] The percentage of antibiotic-producing colonies among a randomly chosen group of prototrophs is shown in parentheses.

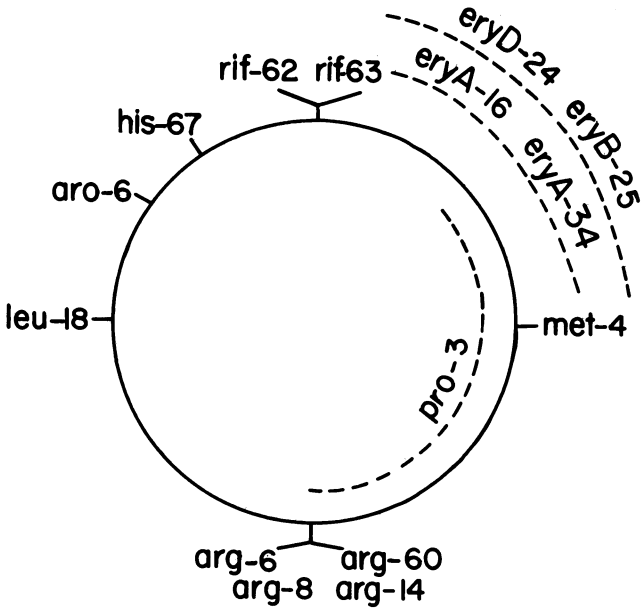


FIG. 3. The rudimentary chromosome map of *S. erythreus* showing the genetic linkages of mutations and the placement of four different *ery* mutations within one map interval. Mutation abbreviations are defined in Tables 4, 5, and 6.

antibiotic with three deoxysugar moieties (2, 4). The frequency and properties of tylactone formation mutants (TylG) closely resemble those of the EryA mutants. The TylA mutant corresponds to the EryD mutant, and the properties of these two mutants indicate that formation of a common sugar precursor was affected, although we cannot rule out that these phenotypes are due to a regulatory mutation. This finding, and results from earlier in vitro studies of the enzymology of thymidine diphospho-L-mycarose synthesis (25), suggest that this common precursor is thymidine diphospho-4-keto-6-deoxy-D-glucose, which lies at the branching point for the biosynthesis of several deoxysugars found in antibiotic structures (15).

The results of conjugal mating experiments provide evidence for the existence of a circular chromosome in *S. erythreus* and demonstrate that genetic markers can be mapped in sequence, which are characteristics of other streptomycetes (7). That the *ery* genes appear to reside in at least two separate positions is not surprising, since the biochemistry of erythromycin formation involves two quite different metabolic pathways. We do not know, however, if the observed recombination frequencies between *ery* genes reflect a significant physical separation of the genes on the chromosome. For other antibiotic biosynthetic genes that have been mapped, the general pattern is that they reside in a single cluster on the chromosome (7, 9, 29). Two notable exceptions to this rule are the oxytetracycline genes that map to two positions on the *S. rimosus* chromosome (27) and the plasmid-borne methylenomycin A genes (22).

Interestingly, our results indicate significant differences between the genetics of macrolide antibiotic biosynthesis in the tylosin and erythromycin systems. In *S. fradiae*, high-frequency transfer of tylosin-related genes was observed in the absence of detectable recombination between chromosomal markers (3). Furthermore, 20 to 70% of the recombinants analyzed in crosses of *S. fradiae* strains by protoplast fusion lost the tylosin production characteristic (1). These results suggest the possibility of plasmid involvement in tylosin biosynthesis, in contrast with the situation for erythromycin biosynthesis, in which similar indications of plasmid involvement were not found in the NRRL 2338 strain. Plasmids have been physically identified in this strain of *S. erythreus* (30), however, and their existence is inferred in other strains from the appearance of pocks and genetic evidence for a fertility factor (Dewitt, Am. Soc. Microbiol. Conf. Genet. and Mol. Biol. Industr. Microorg. 1984).

Future work on *S. erythreus* will focus on studies of the biochemical genetics of erythromycin A formation. This now is feasible, since we recently developed a transformation system for *S. erythreus* protoplasts by using one of the plasmid cloning vectors developed by Hopwood and coworkers (5; H. Yamamoto, K. H. Maurer, C. R. Hutchinson, and D. A. Hopwood, unpublished results). Thus, the framework for such work has been established, and the way is clear for investigations of the molecular biology of antibiotic formation by this organism.

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