GENE-ENZYME RELATIONS AFFECTING TRYPTOPHAN BIOSYNTHESIS IN *STREPTOMYCES COELICOLOR* A3(2)

by

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INTRODUCTION

The biosynthesis of tryptophan from chorismic acid, a common intermediate in the biosynthesis of tyrosine, phenylalanine and tryptophan, is presented in Figure 1. Genes affecting tryptophan synthesis in S. coelicolor were mapped, and enzymatic defects in several mutants were identified. These studies provide a basis for comparing tryptophan gene-enzyme relations in this morphologically complex prokaryote with other microorganisms. Considerable diversity, discussed later in the section on REVIEW OF LITERATURE, with respect to the organization and regulation of tryptophan genes and enzymes is known to occur in other microorganisms (Hutter and DeMoss 1967a; Pittard and Gibson 1970; Hutter 1973; Truffa-Bachi and Cohen 1973).

Engel (1973) studied twenty Trp (tryptophan-requiring) mutants of S. coelicolor. The mutants studied by him were divided into two classes based on their ability to utilize indole for tryptophan synthesis in vivo. Genetic analysis (Engel 1973) of these mutants indicated that the indole-utilizing mutants map between proA and hisC on the linkage map (Figure 2 and Table 1). Trp mutants unable to utilize indole map between hisC and ammA (P.P. Engel, personal communication).

The indole utilizers investigated by Engel (1973) were not mapped with respect to their closest known flanking markers, and eight unmapped indole utilizers (Trp-e8, e17, e18, e19, e20, e21, e22 and e23) were added to the collection of Trp mutants in the course of this investigation. My studies, to locate more precisely the map position of each indole-utilizing trp mutant, demonstrated that eighteen of the

twenty available indole utilizers map between rifB and thiC. Two other indole utilizers, Trp-e8 and e22, map between hisC and ammA, lack indoleglycerol phosphate synthase activity and fail to complement each other in vivo. Mutants Trp-e8 and e22 complement Trp mutants unable to utilize indole. Mutations affecting tryptophan synthase are closely linked to trp mutations in Trp-e8 and e22. Mutants lacking phosphoribosyl transferase were found to map between rifB and thiC on the linkage map of S. coelicolor.

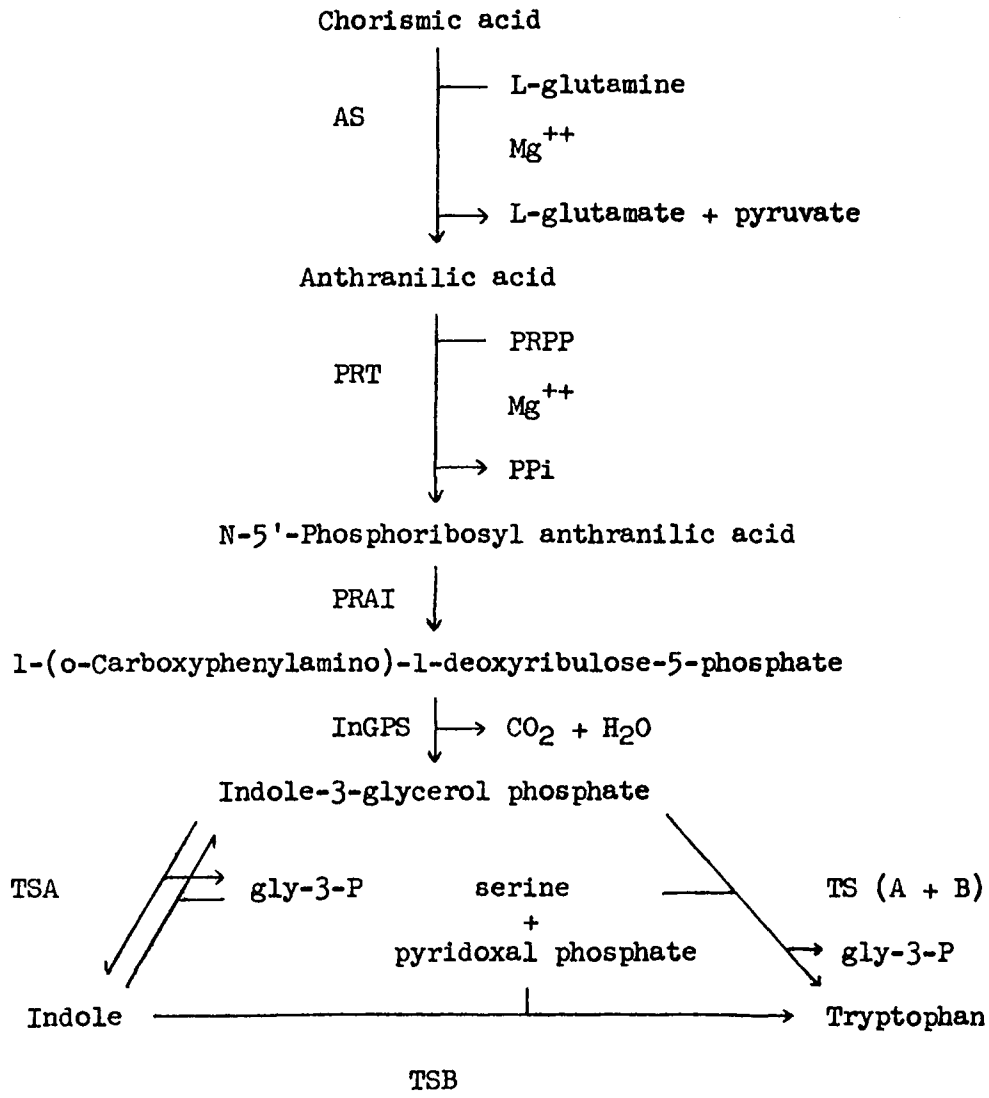


Figure 1. Biosynthesis of tryptophan from chorismic acid. Abbreviations: AS, anthranilate synthase; PRPP, 5-phosphoribosyl-1-pyrophosphate; PPi, inorganic pyrophosphate; PRT, anthranilate-PRPP phosphoribosyltransferase; PRAI, N-5'-phosphoribosylanthranilate isomerase; InGPS, indole-3-glycerol phosphate synthase; TSA, tryptophan synthase A component; TSB, tryptophan synthase B component; TS (A + B), tryptophan synthase A and B components; gly-3-P, glyceraldehyde-3-phosphate.

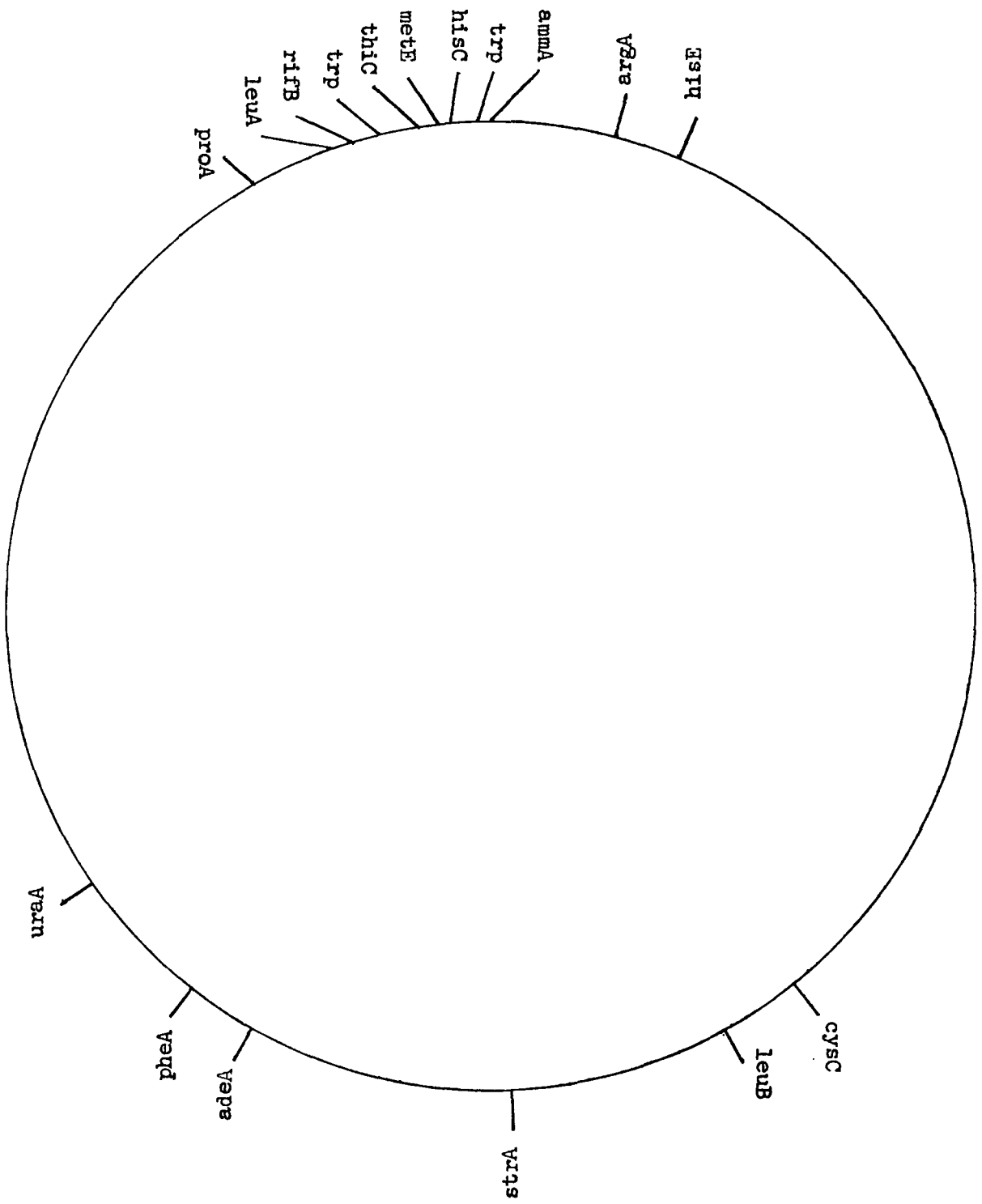


Figure 2. Relevant genetic markers on the chromosome of Streptomyces coelicolor A3(2).

Table 1. Key to the designation of genetic loci or alleles.

locus or allele	phenotype[a]
adeA	requirement for adenine
ammA	unable to utilize nitrate
argA	requirement for arginine
cysC	requirement for cysteine
hisC, *hisE*	requirement for histidine
leuA, *leuB*	requirement for leucine
met-e40	requirement for methionine
pheA	requirement for phenylalanine
proA	requirement for proline
rifB	resistant to rifampin
strA	resistant to streptomycin
thiC	requirement for thiamine
trp	requirement for tryptophan
uraA	requirement for uracil

[a] (Hopwood *et al.* 1973).

REVIEW OF LITERATURE

Tryptophan gene-enzyme relationships and regulation in organisms other than S. coelicolor. Although tryptophan biosynthesis has been studied in many organisms, the following discussion is limited to those organisms amenable to genetic analysis. Thus comparisons can be made with respect to the regulation and organization of genes and enzymes involved in the biosynthesis of tryptophan.

Gene-enzyme relations and regulation in Escherichia coli. The structural genes involved in tryptophan biosynthesis in E. coli are clustered (Yanofsky and Lennox 1959; Rose and Yanofsky 1971). The gene sequence is trpE-trpD-trpC-trpB-trpA (Yanofsky and Lennox 1959). The product of trpE, anthranilate synthase, and trpD, phosphoribosyl transferase, form an enzyme complex. In the absence of phosphoribosyl transferase, anthranilate synthase requires ammonia rather than glutamine for the synthesis of anthranilate from chorismate. L-tryptophan inhibits anthranilate synthase. Phosphoribosyl transferase activity is inhibited by L-tryptophan only when complexed with anthranilate synthase (Ito and Yanofsky 1969; Ito, Cox and Yanofsky 1969). The trpC gene codes for a single polypeptide chain possessing phosphoribosyl anthranilate isomerase and indoleglycerol phosphate synthase activities (Creighton and Yanofsky 1966; Creighton 1970). Tryptophan synthase is a protein aggregate composed of the alpha and beta polypeptides specified, respectively, by the tryptophan synthase A and B genes (Crawford and Yanofsky 1958).

That the tryptophan structural genes of E. coli constitute an operon is based on the following observations. (1) The tryptophan biosynthetic enzymes are repressed in cells grown in excess tryptophan and derepressed

coordinately when tryptophan becomes limiting (Ito and Crawford 1965; Matsushiro et al. 1965). (2) Near the first structural gene of the tryptophan gene cluster there is an operator region involved in the expression of the structural genes (Mutsushiro et al. 1965; Hiraga 1969; Rose et al. 1973). (3) Extensive characterization of nonsense mutations affecting tryptophan genes has shown that such mutations affect the levels of enzymes coded for by genes distal to the genes in which the nonsense mutations occurred (Yanofsky and Ito 1966; Yanofsky et al. 1971). (4) RNA-DNA hybridization studies suggest that the tryptophan gene cluster is transcribed in vivo as a single multicistronic messenger RNA beginning near the first structural gene (Imamoto et al. 1965; Imamoto et al. 1966; Imamoto and Yanofsky 1967; Imamoto 1968). (5) TrpR, the tryptophan repressor locus (Cohen and Jacob 1959), codes for a repressor protein (Morse and Yanofsky 1969) which is involved in repression of transcription of the tryptophan structural genes in vivo (Imamoto and Yanofsky 1967) and in vitro (Rose et al. 1973; Shimiza et al. 1973).

Gene-enzyme relations and regulation in Salmonella typhimurium. The tryptophan structural genes of S. typhimurium are clustered and occur in the same sequence as the tryptophan genes of E. coli (Blume and Balbinder 1966). Anthranilate synthase and phosphoribosyl transferase compose an enzyme complex (Bauerle and Margolin 1966a; Nagana and Zalkin 1970). Both activities of the complex are inhibited by L-tryptophan (Bauerle and Margolin 1966a). The tryptophan structural genes are probably regulated as an operon under the control of a tryptophan repressor gene (Bauerle and Margolin 1966b) which is separate from the

structural genes (Stuttard 1972) and an operator region located near the anthranilate synthase gene (Cordoro and Balbinder 1971). Nonsense mutations in the anthranilate synthase gene have been shown to exert a polar affect on the synthesis of the remaining enzymes (Balbinder et al. 1968).

In E. coli (Morse and Yanofsky 1968) and S. typhimurium (Baurle and Margolin 1966b) two promotor-like elements, P1 and P2, have been proposed to explain observations of semi-coordinate synthesis of tryptophan biosynthetic enzymes. P1 is presumably located near the operator and P2 is located within the operon. P2 allows low-level expression of the last three genes in repressed cultures of both organisms. The physiological function of P2 is not known.

Gene-enzyme relations and regulation in Bacillus subtilis. The tryptophan structural genes of B. subtilis are closely linked and probably contiguous (Carlton and Whitt 1969). The gene order is trpE (anthranilate synthase)-trpD (phosphoribosyl transferase)-trpC (indoleglycerol phosphate synthase)-trpF (phosphoribosyl anthranilate isomerase) -trpB (tryptophan synthase B)-trpA (tryptophan synthase A) (Carlton and Whitt 1969; Hock et al. 1969). Another locus, trpX, has been identified (Kane and Jensen 1970a) and is genetically separate from the structural-gene cluster (Kane et al. 1972). The product of trpX is relatively small (mol. wt. about 16,000), repressible by tryptophan, associated with anthranilate synthase and confers upon anthranilate synthase the ability to use glutamine in the synthesis of anthranilate (Kane and Jensen 1970a). Based on Sephadex gel filtration, phosphoribosyl transferase, phosphoribosyl anthranilate isomerase and indoleglycerol phosphate

synthetase are not involved in enzyme aggregates (Hock et al. 1969). Tryptophan synthase is separable into components having tryptophan synthase A and B activities (Schwartz and Bonner 1964; Meduski and Zamenof 1969).

Regulation of the tryptophan biosynthetic pathway in B. subtilis is not completely understood. Anthranilate synthase activity is inhibited by L-tryptophan and activated by histidine (Kane and Jensen 1970b). Anthranilate synthase, phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, indoleglycerol phosphate synthase and tryptophan synthase B levels increase coordinately in cultures of a trpA mutant starved for tryptophan (Hock et al. 1969). Mutations resulting in resistance to 5-methyltryptophan are closely linked but not adjacent to the structural gene cluster (Hock 1974). Methyltryptophan-resistant (Mtr) mutants produce the tryptophan enzymes constitutively and have a partial requirement for phenylalanine. The phenylalanine bradytrophism is probably the result of the accumulation of tryptophan which inhibits prephenate dehydratase, an enzyme of phenylalanine biosynthesis (Nester and Jensen 1966; Hock et al. 1971). Derepression of the tryptophan genes in Mtr mutants or in tryptophan auxotrophs starved for tryptophan results in the coordinate formation of imidazolylacetolphosphate: L-glutamine aminotransferase (IAP aminotransferase) and prephenate dehydrogenase, enzymes of histidine and tyrosine synthesis respectively. IAP transaminase is derepressed when the concentration of histidine in the growth medium is limited provided the concentration of tyrosine is not excessive. An excessive concentration of either tyrosine or histidine, but not tryptophan,

prevents histidine-mediated derepression of IAP transaminase. Tyrosine limitation is capable of derepressing prephenate dehydrogenase synthesis in the presence of excess tryptophan and histidine. Tryptophan synthase B levels are not influenced by either tyrosine or histidine in the growth medium (Roth and Nester 1971). Since the structural genes for IAP transaminase and prephenate dehydrogenase are closely linked to the tryptophan gene cluster, Roth and Nester (1971) proposed that these genes are members of a supraoperon containing multiple operator and promotor sites.

Gene-enzyme relations and regulation in Acinetobacter calcoaceticus. Structural genes involved in tryptophan biosynthesis in A. calcoaceticus are located in three separate chromosomal regions. TrpE (anthranilate synthase) mutants map in one region. Mutants lacking tryptophan synthase B (TrpB), tryptophan synthase A (TrpA) and phosphoribosyl anthranilate isomerase (TrpF) map in a second region. Genes for phosphoribosyl transferase (trpD) and indoleglycerol phosphate synthase (trpC) are located in a third chromosomal region. Closely linked to trpC and trpD mutations are the trpG mutations which cause a requirement for p-aminobenzoate or folate in addition to tryptophan, anthranilate or indole for growth. Presumably, trpG mutants lack a functional component of anthranilate synthase and p-aminobenzoate synthase necessary for the utilization of glutamine in reactions catalyzed by these enzymes (Sawula and Crawford 1972). Enzyme aggregates were not detected by Twarog and Liggins (1970) using Sephadex gel filtration and sucrose gradient centrifugation. A complex between anthranilate synthase and the trpG gene function would not have been revealed in the experiments

by Twarog and Liggins (1970).

Both anthranilate synthase and phosphoribosyl transferase are feedback inhibited by tryptophan. Anthranilate synthase is the only tryptophan enzyme in A. calcoaceticus known to be repressible by tryptophan (Twarog and Liggins 1970).

Gene-enzyme relations and regulation in Staphylococcus aureus. The tryptophan structural genes are closely linked in S. aureus. The gene order is trpE (anthranilate synthase)-trpD (phosphoribosyl transferase)-trpC (indoleglycerol phosphate synthase)-trpF (phosphoribosyl anthranilate isomerase)-trpB (tryptophan synthase B)-trpA (tryptophan synthase A) (Proctor and Kloos 1970; Proctor and Kloos 1973). Enzyme aggregates, based on evidence from Sephadex gel filtration, involving anthranilate synthase, phosphoribosyl transferase, indoleglycerol phosphate synthase and phosphoribosyl anthranilate isomerase activities were not found.

Regulation of tryptophan synthesis is S. aureus is at least partially achieved by feedback inhibition of anthranilate synthase and repression, by tryptophan, of the synthesis of each enzyme in the tryptophan pathway (Proctor and Kloos 1973).

Gene-enzyme relations in Micrococcus luteus. Genetic mapping, based on transformation, of Trp mutants of M. luteus indicates that the genes involved in tryptophan biosynthesis are located in two separate genetic regions. Based on growth response and accumulation of intermediates in the biosynthesis of tryptophan, the genes for phosphoribosyl transferase and/or phosphoribosyl anthranilate isomerase are closely linked but some distance from a region containing the genes for

anthranilate synthase, indoleglycerol phosphate synthase, tryptophan synthase A and tryptophan synthase B (Kloos and Rose 1970).

Gene-enzyme relations and regulation in Pseudomonas putida. Anthranilate synthase, phosphoribosyl transferase and indoleglycerol phosphate synthase are repressed by tryptophan (Crawford and Gunsalus 1966; Queener and Gunsalus 1970). Phosphoribosyl anthranilate isomerase levels vary little with respect to tryptophan concentration in the growth medium. Tryptophan synthase A and B are induced by indoleglycerol phosphate (Crawford and Gunsalus 1966). Anthranilate synthase is feedback inhibited by L-tryptophan and is a two component enzyme. Both components, but not phosphoribosyl transferase, are required for the synthesis of anthranilate from chorismate using glutamine as the amino donor (Queener and Gunsalus 1970). Enzyme aggregates involving phosphoribosyl transferase, phosphoribosyl anthranilate isomerase and indoleglycerol phosphate synthase have not been detected using Sephadex gel filtration and sucrose gradient centrifugation. Tryptophan synthase is separable into tryptophan synthase A and B activities (Enatsu and Crawford 1967).

The loci affecting tryptophan biosynthesis are located in at least three different regions in the chromosome. The genes for anthranilate synthase (trpA), phosphoribosyl transferase (trpB) and indoleglycerol phosphate synthase (trpD) are closely linked but not linked to the genes controlling tryptophan synthase (trpE and trpF). Mutations affecting the phosphoribosyl anthranilate isomerase gene, trpC, are not linked to any of the other tryptophan loci (Chakrabarty et al. 1968). TrpX mutations, which result in constitutive synthesis

of tryptophan synthase, map near or in the trpEF cluster (Gunsalus et al. 1968). Mutations resulting in overproduction of the enzymes of the trpABD cluster have been studied but not mapped (Maurer and Crawford 1971).

Gene-enzyme relations and regulation in Saccharomyces cerevisiae. The tryptophan structural genes are unlinked in S. cerevisiae, and five genetic loci affecting tryptophan biosynthesis have been found (Manney, 1964). Trp-1, Trp-4 and Trp-5 mutants lack phosphoribosyl anthranilate isomerase, phosphoribosyl transferase and tryptophan synthase respectively. Trp-2 mutants lack anthranilate synthase and Trp-3 mutants lack anthranilate synthase or anthranilate synthase and indoleglycerol phosphate synthase (DeMoss 1965a; Doy and Cooper 1966). Anthranilate synthase and indoleglycerol phosphate synthase sediment together during zone centrifugation suggesting a complex of these two enzymes (DeMoss 1965a). Anthranilate synthase but not indoleglycerol phosphate synthase is inhibited by L-tryptophan (Doy and Cooper 1966). Doy and Cooper (1966) assayed anthranilate synthase in dialyzed extracts of cells grown in various concentrations of L-tryptophan. They concluded from their studies that the enzyme is repressible by tryptophan.

Gene-enzyme relations and regulation in Aspergillus nidulans. Five classes of Trp mutants have been identified in A. nidulans. TrpA (anthranilate synthase) and trpD (phosphoribosyl transferase) are linked to each other but are not linked to the other tryptophan genes. TrpC, trpB and trpE genes are unlinked (Roberts 1967). TrpB mutations affect tryptophan synthase. TrpE mutants, isolated from strains requiring biotin and nicotinic acid, have all the enzymatic activities involved

in the conversion of chorismate to tryptophan, although tryptophan is required for their growth (Hutter and DeMoss 1967b; Roberts 1967). Quinic acid, phenylalanine and tyrosine together, but not alone, replace the tryptophan requirement in TrpE mutants (Roberts 1967). TrpC mutants usually show a pleiotropic loss of anthranilate synthase, indoleglycerol phosphate synthase and phosphoribosyl anthranilate isomerase (Hutter and DeMoss 1967b). Roberts (1967) and Hutter and DeMoss (1967b) reported that one TrpC mutant studied lacked only indoleglycerol phosphate synthase. Anthranilate synthase, indoleglycerol phosphate synthase and phosphoribosyl anthranilate isomerase sediment coincidently during zone centrifugation with a sedimentation coefficient of about 10.5S. Thus, the enzymes may compose an enzyme aggregate (Hutter and DeMoss 1967b).

Gene-enzyme relations and regulation in Neurospora crassa. Four unlinked genetic loci, trp-1, trp-2, trp-3 and trp-4 have been identified as the chromosomal sites for structural genes involved in tryptophan biosynthesis (Ahmed and Catcheside 1960; DeMoss and Wegman 1965; Wegman and DeMoss 1965). Trp-3 mutants lack tryptophan synthase (Kaplan, Suyama and Bonner 1963) which is an enzyme separable into at least two different polypeptide chains (Carsiotis et al. 1965). Phosphoribosyl transferase mutants map at the trp-4 locus (Wegman and DeMoss 1965). Trp-1 and Trp-2 mutants are complex since their enzyme products are involved in an aggregate composed of anthranilate synthase, indoleglycerol phosphate synthase and phosphoribosyl anthranilate isomerase (DeMoss and Wegman 1965). Trp-2 mutants characteristically lack anthranilate synthase only and complement in vivo Trp-1 mutants

which have been known to lose one, two or all three enzyme activities associated with the enzyme complex (DeMoss and Wegman 1965; DeMoss, Jackson and Chalmers 1967). Based on genetic analysis of Trp-1 and Trp-2 mutants and biochemical characterization of the enzyme complex which has been purified 2,000-fold, it appears that trp-2 is the gene for anthranilate synthase and trp-1 is the gene or genes for phosphoribosyl anthranilate isomerase and indoleglycerol phosphate synthase (DeMoss, Jackson and Chalmers 1967; Gaertner and DeMoss 1969). Dissociation of the purified enzyme complex results in a loss of anthranilate synthase activity but not indoleglycerol phosphate synthase or phosphoribosyl anthranilate isomerase activities. The latter two enzymes have not been separated and may exist within a single polypeptide chain (Gaertner and DeMoss 1969).

Anthranilate synthase (DeMoss 1965b) and possibly phosphoribosyl transferase (Lester 1968) are inhibited by L-tryptophan. The tryptophan enzymes are repressed by tryptophan (Matchett and DeMoss 1962; Lester 1968, 1971) or perhaps by tryptophanyl-tRNA (Nazario, Kinsey and Ahmad 1971). Depression of the tryptophan enzymes is coordinate (Carsiotis et al. 1970). The accumulation of indoleglycerol and/or indoleglycerol phosphate induces the formation of tryptophan synthase B (Turner and Matchett 1968; Lester 1971). Coordinate derepression of the tryptophan enzymes of N. crassa has been observed in histidine auxotrophs starved for histidine. Excess tryptophan does not prevent histidine-mediated derepression in histidine auxotrophs. Furthermore, excess histidine does not prevent indoleglycerol (phosphate)-mediated induction of tryptophan synthase or tryptophan-mediated derepression of indole-

glycerol phosphate synthase in tryptophan synthase A mutants starved for tryptophan (Carsiotis et al. 1970). Although no data were given, Carsiotis et al. (1970) reported that either histidine or tryptophan starvation resulted in derepression of two arginine enzymes. Also, derepression of three histidine enzymes has been reported to occur as a result of tryptophan limitation (R.F. Jones and M. Carsiotis, Bacteriol. Proc., p. 136, 1968).

Genetic analysis in S. coelicolor. Crosses between two strains of S. coelicolor are done by mixing the mycelia and spores of each strain on slants of complete medium and incubating the mixed culture for several days at 30°C. At the end of the incubation period the spores are harvested and plated on a medium that prevents growth of parental-type cells, that is, on a medium that selects for at least one genetic marker from each parent. Those colonies which grow on the selective medium are transferred to master plates of the same composition as the selective medium. Following colony development on the master plates, the master plates are replicated to diagnostic plates to detect non-selected markers among the progeny.

Two types of colonies, haploids and heteroclones, can be recognized during the course of analysis. All the spores of a haploid colony have the same genotype. Heteroclones are mixed colonies which contain spores of different genotypes. When the spores of a single heteroclone are subcultured and analyzed a genetically heterogeneous array of haploid as well as heteroclone colonies are found (Hopwood 1967).

Origin of haploids and heteroclones. Chromosomal transfer in

S. coelicolor is probably mediated by cell contact although conjugation has never been demonstrated (Hopwood 1967, Hopwood et al. 1973). It is known that sizeable chromosomal fragments are transferred during mating (Hopwood 1967), and that certain markers can perhaps be excluded from the zygote by physically interrupting mating (Sermonti et al. 1971). The result of chromosomal transfer is a zygote which is usually partially diploid for variable lengths of the chromosome. A partially diploid zygote is called a merozygote. The genome of a merozygote could consist of two incomplete chromosomes or a complete chromosome accompanied by a chromosomal fragment. Genetic evidence favoring the latter hypothesis was discussed by Hopwood (1967). The most compelling evidence for the nature of merozygotes is based on genetic analysis of haploid progeny originating from individual heteroclones (Hopwood et al. 1963; Hopwood 1966, 1967). The extent of diploidy in the merozygote, that produced the heteroclone being analyzed, is indicated by the segregation of members of allelic pairs into the haploid progeny of a heteroclone (Hopwood et al. 1963). Analysis (Hopwood 1966) of different heteroclones has shown that the chromosomal fragments of different merozygotes vary in length, are not interrupted (i.e., there is one continuous fragment per merozygote) and lack constant ends. Furthermore, all markers that were not heterozygous in the merozygote were inherited from the same parent. These observations support the hypothesis that a merozygote is composed of a complete chromosome and a chromosomal fragment.

Chromosome circularity. Hopwood (1965) demonstrated that the mode of selection applied to a cross influenced the segregation of

widely spaced non-selected alleles. This indicated that these alleles are members of a single linkage group. These observations, in addition to observations, discussed earlier, on the nature of merozygotes, suggest that the chromosome of S. coelicolor is either circular or circularly permuted or in equilibrium between closed and open states (Hopwood 1965, 1966).

Model for the origin of haploids and heteroclones. Hopwood (1967) proposed a model for the origin of haploids and heteroclones based on the hypothesis that a merozygote contains a complete chromosome from one parent and a chromosomal fragment from the other parent. An even number of crossovers between the complete chromosome and the chromosomal fragment, with at least one of these crossovers occuring in the interval between selected markers, generates a haploid chromosome which becomes included in a spore during sporulation in the mixed culture. An odd number of crossovers between the complete chromosome and the chromosome fragment gives rise to a terminally redundant chromosome which may remain in the mycelium or become included in a spore. During growth of the colony and replication of the terminally redundant chromosome further crossing over occurs within different disomic regions of the terminally redundant chromosome; this gives rise to genetically different haploid progeny, as well as progeny capable of again giving rise to heteroclones. Colonies growing on the selective medium are either haploids or two different kinds of heteroclones. Heteroclones of the first kind arise from merozygotes in which an odd number of crossovers has occurred between the selected markers. Heteroclones of the second kind

arise from merozygotes in which no crossing over has occurred between selected markers, indicating that complementation occurred with respect to selected markers.

Fertility types in S. coelicolor. Three fertility types, classes of strains which differ in their capacity to produce recombinant progeny when mated with other strains, have been recognized in S. coelicolor A3(2) (Vivian and Hopwood 1970). The fertility types have been designated NF (normal fertility) (Hopwood et al. 1969), IF (initial fertility) (Vivian and Hopwood 1970) and UF (ultra-fertile) (Hopwood et al. 1969). Characterization of these fertility strains (Hopwood et al. 1969; Vivian and Hopwood, 1970; Vivian 1971; Puglia et al. 1973) does not adequately account for mating and recombination (Hopwood et al. 1973) in S. coelicolor.

MATERIALS AND METHODS

Bacterial strains. All strains of S. coelicolor used in this research were derived from the wild-type strain, S. coelicolor A3(2) (Hopwood 1959), by mutagenesis or recombination. These strains and their characteristics are presented in Table 2, Table 3 and Figure 2.

Three tryptophan-requiring mutants of Escherichia coli were obtained from Dr. Charles Yanofsky and used in my research. The E. coli strains used were 9778, 9830 and 9941. E. coli 9778 is a trpD mutant which lacks phosphoribosyl transferase activity. E. coli 9830, a trpC mutant, lacks phosphoribosyl anthranilate isomerase. E. coli 9941 is a trpC mutant which lacks indoleglycerol phosphate synthase activity. E. coli T8 was obtained from Dr. Bruce C. Carlton. E. coli T8 (trpA2/F' trpA2) is a tryptophan synthase A mutant which bears the indoleglycerol phosphate synthase gene on both the chromosome and the F-prime (Baker and Crawford 1966).

Media. The complete and minimal media of Hopwood (1967), solidified with 1.5% agar, were used for maintenance of strains and genetic analysis of S. coelicolor. Solid complete medium was supplemented with 50 µg L-tryptophan per ml to assure adequate growth of Trp mutants. Sensitivity to rifampin was determined on plates of minimal medium containing 50 µg rifampin per ml (K.F. Chater, personal communication).

Mutagenesis. Prof. D.A. Hopwood supplied the tryptophan-requiring mutants Trp-5, 7 and 8 which were induced with ultraviolet light as described by Hopwood and Sermonti (1962). Dr. P.P. Engel provided the tryptophan-requiring mutants Trp-e1, e2, e4, e5, e6, e7, e8, e9, e10, e12, e13, e14, e16, e17, e18, e19, e20, e21, e22. Mutants

Table 2. Characteristics of tryptophan-requiring strains.

Strain[a]					
P13	trp-e1	pheA1	argA1	cysC3	strA1
P14	trp-e1	pheA1	proA1	cysC3	strA1
P79	trp-e2	proA1	cysC3	strA1	
P86	trp-e2	pheA1	argA1	cysC3	strA1
P45	trp-e4	pheA1	argA1	cysC3	strA1
P46	trp-e4	pheA1	proA1	cysC3	strA1
P44	trp-e5	pheA1	proA1	cysC3	strA1
P182	trp-e5	argA1	strA1		
P119	trp-5	pheA1	argA1	strA1	
P120	trp-5	pheA1	proA1	strA1	
P59	trp-e6	pheA1	argA1	cysC3	strA1
P67	trp-e6	proA1	cysC3	strA1	
P3	trp-7		proA1	cysC3	strA1
P10	trp-7	argA1	cysC3	strA1	
P153	trp-e7	pheA1	proA1	strA1	
P185	trp-e7	argA1	strA1		
P143	trp-e8	pheA1	proA1	met-e1	strA1
P144	trp-e8	pheA1	argA1	met-e1	strA1
P18	trp-e9	pheA1	proA1	cysC3	strA1
P20	trp-e9	pheA1	argA1	cysC3	strA1
P147	trp-e10	proA1	strA1		
P183	trp-e10	argA1	strA1		
P164	trp-e12	pheA1	argA1	cysC3	strA1

Table 2. — Continued

P165	trp-e12	pheA1	proA1	cysC3	strA1
P184	trp-e13	argA1	strA1	strA1	
P186	trp-e13	pheA1	proA1	strA1	
P24	trp-e14	pheA1	argA1	strA1	
P38	trp-e14	pheA1	proA1	cysC3	strA1
P181	trp-e16	pheA1	proA1	strA1	
P188	trp-e16		argA1	strA1	
P130	trp-e17	pheA1	proA1	met-e1	strA1
P132	trp-e17	pheA1	met-e1	argA1	strA1
P146	trp-e18	pheA1	met-e1	argA1	strA1
P150	trp-e18	proA1	met-e1		
P195	trp-e19	pheA1	proA1	met-e1	strA1
P114	trp-e19	pheA1	met-e1	argA1	strA1
P92	trp-e20	pheA1	met-e1	argA1	strA1
P107	trp-e20	pheA1	proA1	met-e1	strA1
P152	trp-e21	pheA1	proA1	met-e1	strA1
P154	trp-e21	pheA1	met-e1	argA1	strA1
P159	trp-e22	pheA1	met-e1	argA1	strA1
P160	trp-e22	pheA1	proA1	strA1	
P191	trp-e23	adeA3	proA1	met-e1	strA1
P192	trp-e23	adeA3	met-e1	argA1	strA1

[a] All strains except P191 and P192 were obtained by Dr. P.P. Engel from crosses between trp mutants and strain 876. P191 and P192 are progeny of a cross between trp-e23 and P87.

Table 3. Characteristics of strains that do not require tryptophan.

Strain	Genotype					
A3(2)	wild-type prototroph					
P4	pheA1	proA1	hisC9	cysC3	strA1	
P8	pheA1	hisC9	argA1	cysC3	strA1	
P9	ammA5	argA1				
P80	pheA1	proA1	ammA5	cysC3	strA1	
P87	adeA3	proA1	hisA1	argA1	strA1	
P189	proA1	leuA1	rifB37	thiC2	strA1	
P190	pheA1	leuA1	rifB37	thiC2	argA1	strA1
P204	uraA1	proA1	argA1	hisE6	leuB5	strA1
876	pheA1	proA1	hisC9	argA1	cysC3	strA1

Trp-e2, e3, e4, e5, e6, e7, e9, e10, e11 and e12 were induced in wild-type cells treated with ethyl methane sulfonate using the procedure of Freese (1963). Mutants Trp-e1, e13, e14, and e16 were derived from wild-type cells treated with N-methyl-N'-nitro-N-nitrosoguanidine. The tryptophan-requiring mutants Trp-e8, e17, e18, e19, e20, e21, e22 and e-23 were isolated from cultures of a nitrosoguanidine-induced methionine auxotroph, Met-e1, following mutagenesis with nitrosoguanidine. The procedure described by Delic et al. (1970) was used for nitrosoguanidine mutagenesis.

Indole-utilization studies. Dr. P.P. Engel determined the response of each tryptophan mutant to indole, anthranilic acid and tryptophan by replica plating to appropriately supplemented minimal media. No anthranilate-utilizing mutants were found. Tryptophan-requiring mutants Trp-e1, e2, e4, e5, 5, e6, e7, 7, e8, e9, e10, e12, e16, e17, e18, e19, e20, e21, e22 and e23 are able to utilize indole to satisfy their tryptophan requirement (P.P. Engel, personal communication). Trp-8 is a leaky indole utilizer and mutants Trp-e13 and Trp-e14 are unable to utilize indole (Engel 1973).

Genetic analysis in S. coelicolor. The procedures used in the genetic analysis of S. coelicolor A3(2) have been described in detail by Hopwood (1967) and were briefly discussed in the LITERATURE REVIEW, pp. 6-19, of this report.

Preparation of crude extracts for enzyme assays. Since S. coelicolor grows very poorly in liquid minimal medium, extracts for assays of tryptophan biosynthetic enzymes were prepared from cells grown in liquid complete medium. Starter cultures were prepared by

suspending a small inoculum from a complete medium slant in 50 ml liquid complete medium containing 1% glucose and 50 µg tryptophan per ml. The starter cultures were allowed to grow statically at 30°C for about three days. Aliquots of the starter culture were agitated on a Vortex mixer and used as inocula for the preparation of cultures to be assayed for tryptophan enzymes.

Cultures to be used in enzyme assays were prepared as follows: Two-liter flasks containing 10 ml starter culture, 4.0 gm glucose, 4.0 mg L-tryptophan and single-strength complete medium in a final volume of 400 ml were incubated at 30°C on a gyrotory shaker (New Brunswick Scientific Co. Inc., New Brunswick N.J.) operating at 300 revolutions per minute. Glucose and filter-sterilized tryptophan were added after sterilization of the complete medium. Tryptophan was not added to cultures of tryptophan prototrophs (wild type). After 16 to 17 hours of incubation cultures were chilled on ice and centrifuged at 10,400 x g for 20 minutes at 4°C in a Sorvall RC2-B superspeed centrifuge (Ivan Sorvall Inc., Newtown, Conn.). The pellets were washed in about 10 times their wet weight (grams) of extraction buffer and centrifuged at 10,400 x g for 20 minutes at 4°C. Crude extracts were prepared by suspending the washed pellets in approximately twice their wet weight (grams) of cold extraction buffer and sonicating the mixture on ice for 30 to 60 seconds in 15 second intervals. A Virsonic Cell Disrupter, Model 150, (The Virtis Co. Inc. Gardiner, N.Y.) operating at 150 watts was used for sonicating the cells. The sonicate was centrifuged at 39,100 x g for 25 minutes. The supernatent was stored at -90°C and routinely used in enzyme assays without further purifi-

cation or concentration of activity. The extraction buffer used for washing and extracting cells of S. coelicolor contained 0.1 M potassium phosphate buffer, pH 7.8, 0.8 M sucrose, 0.1 mM ethylenediaminetetraacetic acid, 0.5 M KCl, 6.0 mM 2-mercaptoethanol and deionized water.

E. coli Trp mutants were grown in the minimal medium of Vogel and Bonner (1956) supplemented with 0.05% acid-hydrolyzed casein, 0.5% glucose and 5 µg L-tryptophan per ml (Creighton and Yanofsky 1970). Cultures containing 400 ml of medium in two-liter flasks were incubated on a gyrotory shaker (300 rpm) at 37°C for 15 to 16 hours. At the end of the incubation period, cultures were chilled on ice, centrifuged and washed in extraction buffer. The extraction buffer for preparation of E. coli extracts contained 0.1 M potassium phosphate, pH 7.0, 1.0 mM ethylenediaminetetraacetic acid and 1.0 mM dithioerythritol (Creighton and Yanofsky 1970). Crude extracts of E. coli were prepared by suspending 1 gram of the washed pellet in 1 ml of extraction buffer and sonicating at 150 watts for 15 to 30 seconds in 15 second intervals on ice.

Enzyme assays. Anthranilate synthase and phosphoribosyl transferase were assayed by the method of Kane and Jensen (1970b) using a Farrand fluorometer, model A4, (Farrand Optical Co., Inc., Mt. Vernon, N.Y.) and a Gilford recorder, model 242, (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The primary filter was a 313 nm interference filter (Farrand Optical Co. Inc.) and the secondary filter (Corning Glass Works, Corning, N.Y., filter CSO-51) gives maximum transmission at 393 nm. Reaction mixtures in both assays were pre-

incubated at 37°C and 0.1 ml of cold crude extract was added to 0.9 ml of reaction mixture to begin the reaction. The change in temperature following addition of crude extract was not controlled. The appearance of anthranilic acid, detected as an increase in fluorescence, in the anthranilate synthase assay and the PRPP-dependent disappearance, detected as a decrease in fluorescence, in the phosphoribosyl transferase assay were monitored continuously. An anthranilic acid standard was routinely used to determine the anthranilic acid concentration in reaction mixtures and controls. For both assays, the aperture and range controls were set at 3 and 30 respectively. Fluorescence in each assay was determined at an excitation wavelength of 313 nm and an emission wavelength of 393 nm.

The reaction mixture used for measuring anthranilate synthase contained 50 μmoles tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5, 10 μmoles $MgCl_2$, 20 μmoles L-glutamine, 100 nmoles chorismic acid and crude extract in a final volume of 1 ml. This is a modification of the reaction mixture described by Kane and Jensen (1970b) which contained 200 nmoles potassium chorismate. The reaction rate was linear for at least five minutes.

Phosphoribosyl transferase activity was assayed in a 1 ml reaction mixture containing 50 μmoles Tris-HCl buffer, pH 7.5, 10 μmoles $MgCl_2$, 300 nmoles 5-phosphoribosyl-1-pyrophosphate (PRPP), 2 nmoles anthranilic acid and crude extract (Kane and Jensen 1970). The reaction rate was linear for at least 1.5 minutes. The data for phosphoribosyl transferase presented in Table 12 are based on a coupled assay using E. coli 9778 crude extract which lacks phosphoribosyl

transferase activity. The reaction mixture contained 30 µmoles potassium phosphate buffer, pH 7.8, 2 µmoles $MgSO_4$, 300 nmoles PRPP, 120 µmoles sucrose, 150 nmoles anthranilic acid, water and crude extract in a final volume of 0.5 ml. The reaction mixture was incubated for 20 minutes at 37°C and indoleglycerol phosphate was measured by the method of Gibson and Yanofsky (1960).

Phosphoribosyl anthranilate isomerase was assayed in a coupled assay using E. coli 9830 which lacks phosphoribosyl anthranilate isomerase. The reaction mixture contained water, 150 nmoles anthranilic acid, 300 nmoles PRPP, 2 µmoles $MgSO_4$, 30 µmoles potassium phosphate buffer, pH 7.8, 120 µmoles sucrose and crude extract in a final volume of 0.5 ml. The reaction was allowed to proceed for 20 minutes at 37°C and indoleglycerol phosphate was measured by the method of Gibson and Yanofsky (1960). Since the quantity of E. coli crude extract in the phosphoribosyl anthranilate isomerase assays was uncontrolled, the results obtained using this assay may not be comparative. Controls were always included to insure that failure to detect phosphoribosyl anthranilate isomerase activity was not a consequence of the quantity of E. coli extract used. Controls were not included to insure that activity in this assay resulted from complementation between otherwise defective enzymes in crude extracts of E. coli 9830 and S. coelicolor.

Indoleglycerol phosphate synthase was assayed in a 0.5 ml reaction mixture containing 30 µmoles potassium phosphate buffer, pH 7.8, 120 µmoles sucrose, approximately 700 nmoles 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CdRP), water and crude extract. This is a modification of the reaction mixture described by Hock et al. (1969).

The reaction mixture of Hock et al. (1969) contained 2 µmoles hydroxylamine, pH 7.0, and 900 nmoles CdRP. Indoleglycerol phosphate was assayed at the end of a 20-minute incubation period at 37°C by the procedure of Gibson and Yanofsky (1960).

Tryptophan synthase A and B activities were assayed by following the appearance and disappearance of indole respectively. Indole was determined by the method of Yanofsky (1955). The tryptophan synthase A reaction mixture contained water, 100 µmoles potassium phosphate buffer, pH 7.8, 120 µmoles sucrose, 250 µmoles freshly prepared hydroxylamine hydrochloride, pH 7.0, 200 nmoles indoleglycerol phosphate and crude extract in a final volume of 0.5 ml.

Tryptophan synthase B was assayed in a 0.5 ml reaction mixture containing water, 50 µmoles potassium phosphate buffer, pH 7.8, 200 nmoles indole, 120 µmoles sucrose, 15 µmoles L-serine, 20 nmoles pyridoxal phosphate and crude extract. The TSB reaction mixture is similar to that described by Hock et al. (1969). Hock et al. (1969) used KCl but not sucrose in their reaction mixture. The tryptophan synthase A and B reaction mixtures were incubated at 37°C for 20 minutes except where noted in the RESULTS section, pp. 31-49, of this report.

Specific activities in the continuous assays for anthranilate synthase and phosphoribosyl transferase are expressed as nmoles of substrate utilized or product formed per minute per milligram of protein. Specific activities in the discontinuous assays for phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, indoleglycerol phosphate synthase, tryptophan synthase A and tryptophan synthase B are presented as nmoles of substrate utilized or product formed per 20

minutes per milligram of protein. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Chemicals. 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate was synthesized by the method of Creighton and Yanofsky (1970) and stored under ethyl acetate at -14°C. The concentration of the chemically synthesized CdRP was estimated enzymatically using crude extracts of E. coli T8 as a source of indoleglycerol phosphate synthase. Indoleglycerol phosphate was prepared enzymatically by the method of Wegman and Crawford (1968) using E. coli T8 crude extract. The concentration of indoleglycerol phosphate was estimated by metaperiodate oxidation (Gibson and Yanofsky 1960) and enzymatically by conversion to indole in the presence of E. coli 9941 crude extract. Chorismic acid was prepared by the method of Gibson (1964). All other chemicals were obtained from commercial sources.

RESULTS

Preliminary crosses to locate the position of trp mutations on the linkage map of S. coelicolor. Twelve of the twenty indole-utilizing Trp mutants were known to map between proA and hisC (Figure 2) on the linkage map of S. coelicolor (Engel 1973). Eight indole utilizers had never been mapped, and it was necessary to obtain a rough estimation of their map position. The eight unmapped Trp mutants were crossed with a multiply marked strain, P204 (Figure 3). Progeny which inherited the streptomycin-resistance allele (strA1) from strain P204 and the prototrophic arginine marker (argA1$^+$) from the Trp mutant were selected on plates of minimal medium lacking arginine and containing streptomycin, tryptophan, methionine, proline, histidine, leucine and uracil. Randomly chosen StrA ArgA$^+$ progeny were analyzed for their unselected alleles by replica plating master plates to appropriately supplemented minimal media. The selected alleles, strA and argA$^+$, divide the linkage map into two arcs. An odd number of crossovers in each arc of each chromosome is required for the production of StrA Arg$^+$ haploid progeny. Figure 3 illustrates the data from a preliminary cross between a Trp mutant, Trp-e18 Met-e40, and strain P204. The frequency of each unselected allele among the selected progeny was determined, these frequencies (in percent) are given in parentheses in Figure 3. The result is a gradient of allele frequencies which decrease in clockwise and counter-clockwise directions from the selected alleles on each chromosome. Hopwood (1967) discussed the theorectical basis to account for an allele-frequency gradient. The position of the trp-e18 mutation is inferred

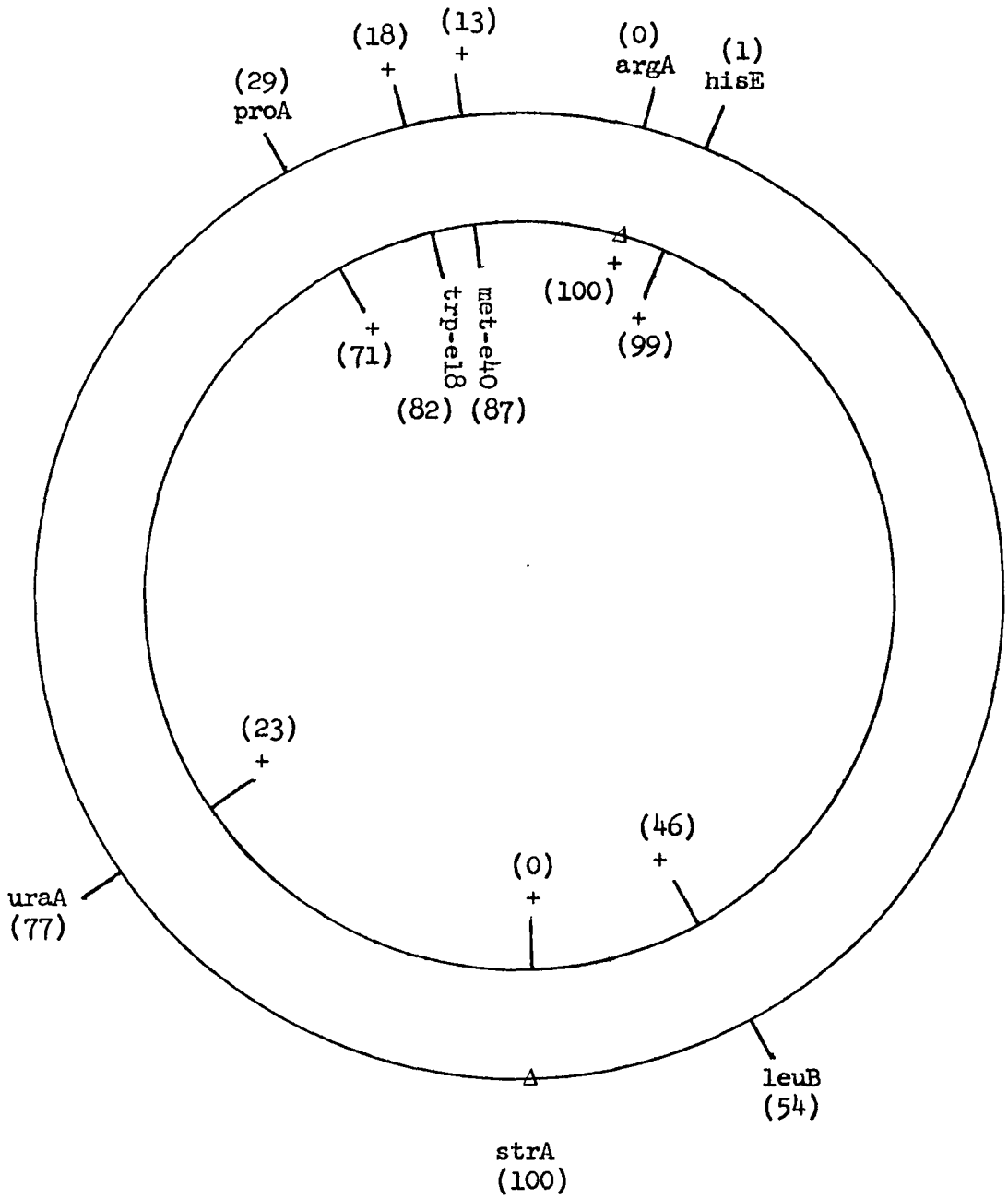


Figure 3. Preliminary cross to locate the position of trp-e18 on the chromosome of S. coelicolor A3(2). The inner circle represents the chromosome of the Trp mutant and the outer circle represents the chromosome of strain P204. Numbers in parentheses are allele frequencies given in percent. Triangles indicate selected alleles.

from the allele-frequency gradient. Thus, the trp-e18 allele, in Figure 3, has a frequency of 82% and is inferred to be either between met-e40 and proA or between hisE and leuB.

A summary of the data from preliminary crosses involving all eight Trp mutants is presented in Table 4. As can be seen in Table 4, it is difficult to assign a map position to an unmapped trp mutation based on these preliminary crosses. However, it is clear that these trp mutations do not map between proA and strA.

Six-point reciprocal crosses to locate trp mutations relative to leuA, rifB and thiC. Engel (1973) showed that all indole utilizers mapped by him occur between proA and hisC on the linkage map. Reference to Figure 2 shows that leuA, rifB and thiC are also located between proA and hisC.

The twenty indole-utilizing Trp mutants listed in the MATERIALS AND METHODS section, pp.20-30, were mapped relative to leuA, rifB and thiC in a series of six-factor reciprocal crosses in which $proA^+$ and $argA^+$ were the selected markers. The rationale of these six-factor reciprocal crosses is presented in Figure 4, and the genetic techniques are as follows. Strains bearing proA trp and argA trp, representing each of the twenty Trp mutants, were obtained from preliminary crosses performed by myself or Dr. Engel. Each proA trp strain was crossed with strain P190 which bears the markers argA, leuB, rifB and thiC. In the reciprocal cross, each argA trp strain was crossed with strain P189 which bears the genetic markers proA, leuB, rifB and thiC. Among the selected Pro^+ Arg^+ progeny from each cross, the frequencies of Trp^+ Thi^+, Trp^+ Leu^+ and Trp Rif recombinants

Table 4. Location of *trp* mutant sites on the chromosome of strain P204 based on allele frequencies among progeny from crosses of Trp mutants X P204.

Trp mutant[b]	No. progeny	Allele frequencies[a] (%)							
		str	*ura*	*pro*	*met*$^+$	*arg* = 0 *trp*$^+$	*his*	*leu*	*str*
e8	144	100	93	40	25	23	0	21	100
e17	148	100	91	43	37	38	2	21	100
e18	154	100	77	29	13	18	1	54	100
e19	134	100	84	43	26	27	1	25	100
e20	144	100	96	38	26	30	2	20	100
e21	142	100	88	42	20	25	1	22	100
e22	221	100	81	24	23	23	3	33	100
e23	177	100	93	35	21	22	1	20	100

[a] Selected markers were *strA1* and *argA1*$^+$. Dotted lines indicate the possible locations of *trp* mutant sites.

[b] All these Trp mutants require methionine; these Trp mutants were obtained by mutagenesis of *Met-e40*.

	Cross I						Cross II					
(1)	pro	trp	+	+	+	+	pro	+	leu	rif	thi	+
	+	+	leu	rif	thi	arg	+	trp	+	+	+	arg
(2)	pro	+	trp	+	+	+	pro	leu	+	rif	thi	+
	+	leu	+	rif	thi	arg	+	+	trp	+	+	arg
(3)	pro	+	+	trp	+	+	pro	leu	rif	+	thi	+
	+	leu	rif	+	thi	arg	+	+	+	trp	+	arg
(4)	pro	+	+	+	trp	+	pro	leu	rif	thi	+	+
	+	leu	rif	thi	+	arg	+	+	+	+	trp	arg

Figure 4. Alternative locations of *trp* mutant sites. The selected markers are *pro*$^+$ and *arg*$^+$. Predictions: (1) If the order is *pro trp leu rif thi arg*, both *trp*$^+$ *leu*$^+$ and *trp*$^+$ *thi*$^+$ recombinants will be more frequent in cross I and *trp rif* recombinants will be more frequent in cross II. (2) If the order is *pro leu trp rif thi arg*, both *trp*$^+$ *leu*$^+$ and *trp rif* recombinants will be more frequent in cross II and *trp*$^+$ *thi*$^+$ recombinants will be more frequent in cross I. (3) If the order is *pro leu rif trp thi arg*, both *trp*$^+$ *thi*$^+$ and *trp rif* recombinants will be more frequent in cross I and *trp*$^+$ *leu*$^+$ recombinants will be more frequent in cross II. (4) If the order is *pro leu rif thi trp arg*, both *trp*$^+$ *leu*$^+$ and *trp*$^+$ *thi*$^+$ recombinants will be more frequent in cross II and *trp rif* recombinants will be more frequent in cross I. Wild-type alleles are designated as +. *Rif* indicates resistance to rifampin, *rif*$^+$ indicates rifampin sensitivity.

were determined by replica plating on diagnostic media. Haploid Trp^+ Thi^+ and Trp^+ Leu^+ colonies were differentiated from mixed colonies (heteroclones) composed of Trp^+ Leu and Trp Leu^+ or Trp^+ Thi and Trp Thi^+ progeny by replication to minimal media not supplemented with tryptophan and leucine or tryptophan and thiamine respectively.

The position of each trp mutation relative to the unselected markers, leuB, rifB and thiC, was then determined based on the predictions set forth in Figure 4. The predictions in Figure 4 assume that a single crossover between $proA^+$ and $argA^+$ is more probable than multiple crossovers between the selected markers. The data obtained from six-factor reciprocal crosses involving all twenty indole utilizers are given in Table 5. The data in Table 5 indicate that eighteen of the twenty indole utilizers investigated map between rifB and thiC on the linkage map. Mutants trp-e8 and e22 map to the right of thiC based on the data in Table 5.

Location of mutations trp-e8 and e22 on the linkage map. The only other region in which trp mutations are known to map is located between hisC and ammA on the linkage map. This region was shown to be occupied by those mutations resulting in an impaired ability to utilize indole for tryptophan biosynthesis (P.P. Engel, personal communication). Although Trp-e8 and e22 utilize indole, it seemed appropriate to determine whether trp-e8 and e22 indeed map between hisC and ammA. Four-point reciprocal crosses were performed to locate the trp-e8 and e22 mutations relative to hisC and ammA.

The data in Table 6 show that, when $ProA^+$ $ArgA^+$ progeny were selected, the frequencies of unselected $HisC^+$ Trp^+ progeny were

Table 5. Location of *trp* mutant sites with respect to nonselected markers *leuA1*, *rifB37* and *thiC2*.

Cross I[a]						Cross II[a]					
pro + + trp + +						pro leu rif + thi +					
+ leu rif + thi arg						+ + + trp + arg					
			Frequency (%)[b]						Frequency (%)[b]		
P190 x trp strain		No. progeny analyzed	trp+ thi+	trp+ leu+	trp rif	P189 x trp strain		No. progeny analyzed	trp+ thi+	trp+ leu+	trp rif
P14	(e1)	121	4	1	11	P13	(e1)	128		63	
P79	(e2)	125	4	1	4	P86	(e2)	141		72	
P46	(e4)	133	5	1	6	P45	(e4)	132		73	
P120	(5)	128	5	2	4	P119	(5)	145		89	
P44	(e5)	122	8	1	5	P182	(e5)	121		82	
P67	(e6)	138	5	1	2	P59	(e6)	147		68	1
P3	(7)	118	8		3	P10	(7)	128		66	
P153	(e7)	107	5	1	4	P185	(e7)	137	1	79	
P143	(e8)	133	1		11	P144	(e8)	143	4	81	

Table 5. — Continued

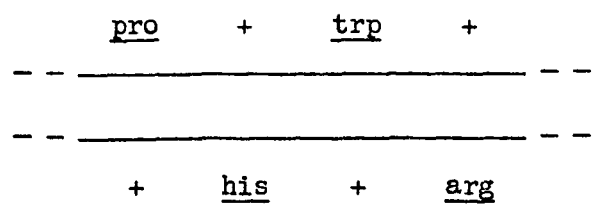
Cross I[a]						Cross II[a]					
			Frequency (%)[b]						Frequency (%)[b]		
P190 x trp strain		No. progeny analyzed	trp+ thi+	trp+ leu+	trp rif	P189 x trp strain		No. progeny analyzed	trp+ thi+	trp+ leu+	trp rif
P18	(e9)	123	2	2	10	P20	(e9)	132		57	
P147	(e10)	134	8	2	5	P183	(e10)	123	2	63	
P165	(e12)	122	5		7	P164	(e12)	138		57	
P181	(e16)	130	5		12	P188	(e16)	119		56	1
P130	(e17)	101	4		3	P132	(e17)	142	1	86	
P150	(e18)	115	7	1	9	P146	(e18)	146		79	1
P195	(e19)	112	6	2	4	P114	(e19)	144		91	
P107	(e20)	93	4		11	P92	(e20)	141		65	
P152	(e21)	103	3		4	P154	(e21)	149	1	76	
P160	(e22)	86			7	P159	(e22)	125	6	81	1
P191	(e23)	142	6	7	2	P192	(e23)	144		93	

[a] Selected markers were proA1+ and argA1+.

[b] No recombinants where frequencies are not given.

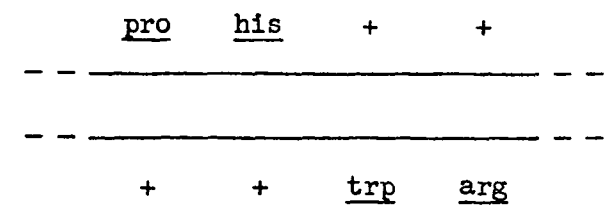
Table 6. Location of trp mutant sites with respect to nonselected marker hisC9.

Cross I[a]



P8 x trp strain	No. progeny analyzed	Frequency[b] his+ trp+ progeny (%)
P143 (e8)	104	
P160 (e22)	108	1

Cross II[a]



P4 x trp strain	No. progeny analyzed	Frequency[b] his+ trp+ progeny (%)
P144 (e8)	101	4
P159 (e22)	101	6

[a] Selected markers were proA1+ and argA1+.

[b] No recombinants where frequencies are not given.

higher in Cross II (P4 x ArgA Trp) than in Cross I (P8 x ProA Trp). The results of HisC$^+$ Trp$^+$ selection are given in Table 7 where it can be seen that ProA$^+$ ArgA$^+$ progeny are more frequent in Cross II (P4 x ArgA Trp) than in the reciprocal cross. Taken together, the data in Tables 6 and 7 indicate that trp-e8 and e22 map clockwise of hisC on the linkage map.

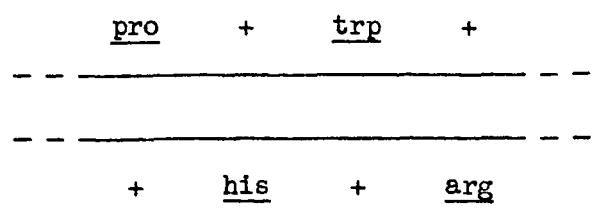
The locations of trp-e8 and e22 relative to ammA were determined by reciprocal crosses. A comparison (Table 8) of the frequencies of unselected AmmA$^+$ Trp$^+$ progeny among selected ProA$^+$ ArgA$^+$ progeny from each cross indicates that trp-e8 and e22 are located counter-clockwise of ammA.

Complementation analysis of trp-e8 and e22. Complementation tests may suggest whether two mutations affect the same gene or different genes. Gene is here defined as a unit of genetic material that determines the primary structure of a nucleic acid or a polypeptide.

In S. coelicolor, the presence of heteroclones from a cross between two mutants is considered evidence that the mutations in question affect different genes. As an example applicable to the data in Tables 9 and 10, spores from a cross between two strains bearing trp mutations were plated on a medium selecting Trp$^+$ progeny and on a medium selecting ProA$^+$ ArgA$^+$ progeny. Colonies from the Trp$^+$ selection were transferred to master plates and analyzed for Trp$^+$ haploid progeny and heteroclones. In this case, heteroclones were recognized as mixed colonies containing spores of different genotypes with respect to the nonselected markers proA$^+$, proA, argA$^+$ and argA. The detection of heteroclones from the Trp$^+$ selection is consid-

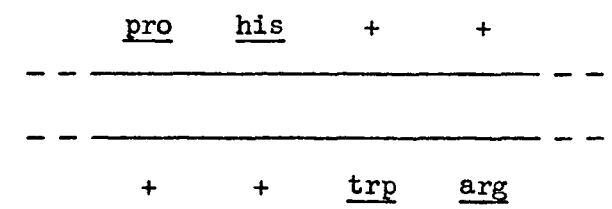
Table 7. Location of *trp* mutant sites with respect to *hisC9*.

Cross I[a]



P8 x *trp* strain	No. progeny analyzed	Frequency *pro*$^+$ *arg*$^+$ progeny (%)
P143 (e8)	55	11
P160 (e22)	51	10

Cross II[a]

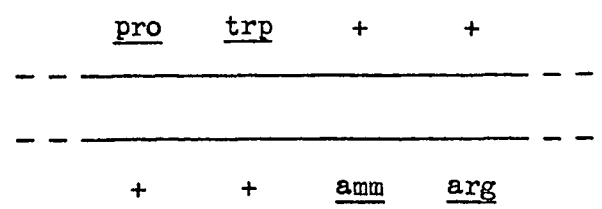


P4 x *trp* strain	No. progeny analyzed	Frequency *pro*$^+$ *arg*$^+$ progeny (%)
P144 (e8)	45	50
P159 (e22)	49	43

[a] Selected markers were *hisC9*$^+$ and *trp*$^+$.

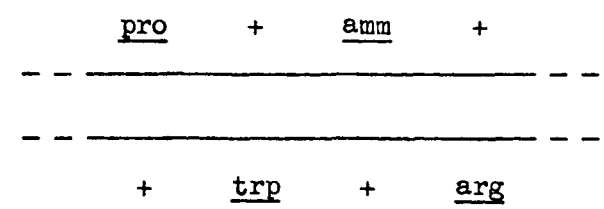
Table 8. Location of trp mutant sites with respect to nonselected marker ammA5.

Cross I[a]



P9 x trp strain	No. progeny analyzed	Frequency amm+ trp+ progeny (%)
P143 (e8)	134	2
P160 (e22)	114	3

Cross II[a]



P80 x trp strain	No. progeny analyzed	Frequency[b] amm+ trp+ progeny (%)
P144 (e8)	98	
P159 (e22)	84	

[a] Selected markers were proA1+ and argA1+.

[b] No recombinants where frequencies are not given.

ered evidence of complementation between the trp mutations in the initial merozygote. In those cases where heteroclones were not detected, the presence of Trp^+ haploid progeny in either the Trp^+ or $ProA^+$ $ArgA^+$ selections would indicate that heterozygosity of the regions in which the trp mutations occurred was possible.

Reciprocal crosses between Trp-e8 and e22 failed to yield colonies in the Trp^+ selection although Trp^+ progeny were recovered in the Pro^+ Arg^+ selection (Table 9). In crosses between Trp-e8 and Trp-el3 and between Trp-e22 and Trp-el4 heteroclones were detected (Table 10). This was expected since Trp-el3 and el4 are unable to utilize indole and map between hisC and ammA (Engel 1973).

Enzymatic analysis of tryptophan enzymes in Trp mutants. Several Trp mutants were assayed for the known tryptophan biosynthetic enzymes as described in MATERIALS AND METHODS, pp. 20-30.

The map positions of trp-e8 and e22 suggested that these mutants might lack tryptophan synthase A activity since the tryptophan synthase A and B genes are closely linked in other organisms (Hutter and DeMoss 1967a). The results presented in Tables 11 and 12 suggest that crude extracts of Trp-e8 and e22 lack only indoleglycerol phosphate synthase activity. Trp-8, a feeble indole utilizer (Engel 1973), has no detectable tryptophan synthase A activity and relatively weak tryptophan synthase B activity. Crude extracts of Trp-el4 have extremely weak tryptophan synthase B activity. Phosphoribosyl transferase activity was not found in crude extracts of Trp-e6 and Trp-el0. It is not likely that the failure to detect enzyme activities in these experiments resulted from an inhibitor in the extracts for the following

Table 9. Complementation test between Trp-e8 and Trp-e22.

Cross	Colonies per plate[a] when selected progeny were Pro^+ Arg^+	Trp^+	No. Pro^+ Arg^+ progeny analyzed for Trp^+	Frequency (%)[b] Trp^+ among Pro^+ Arg^+ Progeny
P143 (Trp-e8) X P159 (Trp-e22)	71		275	
P160 (Trp-e22) X P144 (Trp-e8)	30		69	1.5

[a] 0.1 ml of a 1:10 dilution of the spore suspension was plated per plate. No colonies detected in the Trp^+ selection.

[b] No recombinants where frequencies are not given.

Table 10. Complementation between Trp-e8 and Trp-el3, also between Trp-e22 and Trp-el4.

Cross	Colonies per plate[a] when selected progeny were Pro$^+$ Arg$^+$	Trp$^+$	No. colonies analyzed for heteroclones	Heteroclones[b] (%)
P160 (Trp-e22) X P24 (Trp-el4)	37	8	118	
P159 (Trp-e22) X P38 (Trp-el4)	220	74	33	15
P143 (Trp-e8) X P184 (Trp-el3)	80	22	88	35
P144 (Trp-e8) X P186 (Trp-el3)	197	119	62	33

[a] 0.1 ml of a 1:10 dilution of the spore suspension was plated per plate.

[b] No heteroclones where frequencies are not given.

Table 11. Specific activities of tryptophan enzymes in wild type and Trp mutants of S. coelicolor.

	Specific activity[a]					
Strain	AS	PRT	PRAI	InGPS	TSA	TSB
A3(2)	0.1	0.4	4.7	32.5	2.1	18.4
Trp-e6	15.0	ND[b]	4.7	38.0	9.2	82.4
Trp-e10	13.2	ND	8.6	35.3	15.4	145.1
Trp-e8 Met-e40	9.5	1.7	27.0	ND	24.0	211.0[c]
Trp-e22 Met-e40	9.7	2.3	25.1	ND	15.9	165.4
Trp-8	16.4	4.1	19.6	43.8	ND	35.3
Trp-e14	13.3	3.3	6.1	46.9	27.8[d]	7.3

[a] Specific activities for anthranilate synthase (AS) and phosphoribosyl transferase (PRT) are expressed as nmoles substrate utilized or product formed per minute per milligram protein. Specific activities for phosphoribosyl anthranilate isomerase, (PRAI) indoleglycerol phosphate synthase (InGPS), tryptophan synthase A (TSA) and tryptophan synthase B (TSB) are expressed as nmoles substrate utilized or product formed per 20 minutes per milligram protein.

[b] ND, not detected.

[c] Tryptophan synthase B in crude extracts of Trp-e8 Met-e40 was assayed in a reaction mixture incubated for 10 minutes at 37°C.

[d] Tryptophan synthase A in crude extracts of Trp-e14 was assayed in a reaction mixture incubated at 37°C for 10 minutes.

Table 12. Specific activities of tryptophan enzymes in crude extracts of Trp-e8 Met-e40 grown in different concentrations of tryptophan.

µg tryptophan per ml CM[a]	Specific activity[b]				
	PRT[c]	PRAI	InGPS	TSA	TSB
10	48.1	27.0	ND[d]	24.0	211.0[e]
25	23.3	15.1	ND	13.6	88.4
50	ND	5.9	ND	1.4	9.8
100	ND	5.5	ND	0.6	2.0

[a] Cultures were grown in 400 ml complete medium (CM), supplemented with tryptophan, for 16 hours at 30°C on a gyrotary shaker (300 revolutions per minute).

[b] Specific activities for phosphoribosyl transferase (PRT) phosphoribosyl anthranilate isomerase (PRAI), indoleglycerol phosphate synthase (InGPS), tryptophan synthase A (TSA) and tryptophan synthase B (TSB) are expressed as nmoles substrate utilized or product formed in 20 minutes per milligram protein.

[c] Phosphoribosyl transferase was assayed in a reaction mixture containing 30 µmoles potassium phosphate buffer, pH 7.8, 2 µmoles $MgSO_4$, 300 nmoles PRPP, 120 µmoles sucrose, 150 nmoles anthranilic acid, *E. coli* 9778 crude extract and *S. coelicolor* crude extract. Following incubation of the reaction mixture at 37°C for 20 minutes, indoleglycerol phosphate was assayed by the method of Gibson and Yanofsky (1960).

[d] ND, not detected.

[e] The value of 211.0 in the TSB column resulted from a reaction mixture incubated for 10 minutes at 37°C.

reasons: (1) Missing activities were not restored following dialysis of 1.0 to 1.5 ml of crude extract in 150 ml of extraction buffer for four hours. (2) No inhibition of enzyme activity was observed when mutant and wild-type enzymes were mixed. (3) Indoleglycerol phosphate synthase activity was not detected in crude extracts of Trp-e8 using the phosphoribosyl anthranilate isomerase reaction mixture modified to contain E. coli 9941 crude extract instead of E. coli 9830 crude extract.

Regulation of tryptophan biosynthetic enzymes in S. coelicolor A3(2). The data in Tables 12 and 13 indicate that the concentration of tryptophan in the growth medium affects, with the exception of indoleglycerol phosphate synthase, the specific activities of the tryptophan enzymes. The exception, indoleglycerol phosphate synthase, does not vary much in crude extracts of Trp-e14 grown in different concentrations of tryptophan (Table 13). Also, the level of indoleglycerol phosphate synthase in mutants is comparable to the level found in crude extracts of wild-type cells (Table 11). The data in Tables 12 and 13 may suggest enzyme repression. The possibility of an inhibitor being present in higher concentration in crude extracts from cells grown in the presence of 100 µg L-tryptophan per ml than in crude extracts from cells grown in lower concentrations of tryptophan has not been eliminated.

Table 13. Specific activities of tryptophan synthase A and indoleglycerol phosphate synthase in crude extracts of Trp-el4 grown in different concentrations of tryptophan.

µg tryptophan per ml CM[a]	Specific activity[b]	
	TSA	InGPS
10	27.8	46.9
20	19.6	36.2
30	5.8	38.6
50	4.4	40.6
100	0.4	41.2

[a] Cultures were grown in 400 ml tryptophan-supplemented complete medium (CM) for 16 hours at 30°C on a gyrotary shaker (300 revolutions per minute).

[b] Tryptophan synthase A (TSA) reaction mixtures were incubated for 10 minutes at 37°C. Indoleglycerol phosphate synthase (InGPS) reaction mixtures were incubated for 20 minutes at 37°C. Specific activities in the TSA and InGPS assays are expressed as nmoles product formed in 20 minutes per milligram protein. Indole was the product assayed in the TSA reaction and indoleglycerol phosphate was the product assayed in the InGPS reaction.

DISCUSSION

Eighteen indole-utilizing mutants map between rifB and thiC. Two indole-utilizing mutants (Trp-e8 and e22) map between hisC and ammA. The location of mutations with respect to the closest known flanking markers was established. This assures that further progress in S. coelicolor genetics is based on precise observations.

The discovery that Trp-e8 and e22 lack indoleglycerol phosphate synthase activity and map in the same short region where all known mutants that do not utilize indole also map (Engel 1973) provides insight into the organization of genes affecting tryptophan biosynthesis. A mutant that does not utilize indole, Trp-e14, lacks tryptophan synthase B activity; another mutant, Trp8, grows poorly on indole, lacks tryptophan synthase A activity and has weak tryptophan synthase B activity. Assuming these mutations affect structural genes, it appears that the genes for indoleglycerol phosphate synthase, tryptophan synthase A and tryptophan synthase B are very near each other and perhaps even contiguous on the chromosome. This arrangement, in S. coelicolor, of genes for these three enzymes is quite different from the arrangement found in Pseudomonas putida (Chakrabarty et al. 1968) and Acinetobacter calcoaceticus (Sawula and Crawford 1972) where the genes for indoleglycerol phosphate synthase and tryptophan synthase A and B are located in separate chromosomal regions.

Indole-utilizing mutants Trp-e6 and e10 lack phosphoribosyl transferase activity and map between rifB and thiC. Sixteen other indole-utilizing mutants map in the region between rifB and thiC. Some of these mutants have been assayed for phosphoribosyl anthranilate

isomerase activity (P.P. Engel, personal communication) and apparently have this activity. Phosphoribosyl anthranilate isomerase activity is assayed by measuring indoleglycerol phosphate made from anthranilate. Cell-free extracts, to provide sufficient phosphoribosyl transferase and indoleglycerol phosphate synthase activities so the conversion of phosphoribosyl anthranilate to CdRP is the only possible rate limiting reaction, from E. coli 9830, a missense mutant lacking phosphoribosyl anthranilate isomerase activity (Yanofsky et al. 1971), are mixed with crude extracts from S. coelicolor to assay phosphoribosyl anthranilate isomerase activity. It is possible that in vitro complementation, manifested as phosphoribosyl anthranilate isomerase activity, between defective polypeptides from E. coli and S. coelicolor might explain the failure to find phosphoribosyl anthranilate isomerase mutants in S. coelicolor.

A class of Trp mutants, those that can utilize either anthranilate or indole or tryptophan for growth, that presubably lacks anthranilate synthase has not been found in S. coelicolor. Failure to find anthranilate-utilizing Trp mutants is probably not due to impermeability to anthranilate (Watkins 1972). Perhaps mutants lacking anthranilate synthase will be found among the mutants that have been mapped but whose enzymatic defects have not been identified.

Comprehensive comparisons between S. coelicolor and other prokaryotes regarding chromosomal arrangements of trp genes are not possible at this time since anthranilate synthase and phosphoribosyl anthranilate isomerase mutants have not been found in S. coelicolor. Nevertheless, it is certain that the organization of tryptophan genes in S. coelicolor

differs from that in E. coli (Yanofsky and Lennox 1959), S. typhimurium (Blume and Balbinder 1966) and B. subtilis (Carlton and Whitt 1969) where trp genes are found in operons. In M. luteus, the genes controlling indoleglycerol phosphate synthase, tryptophan synthase and anthranilate synthase are closely linked and separate from the genes for phosphoribosyl transferase and phosphoribosyl anthranilate isomerase (Kloos and Rose 1970). Further studies might corroborate other similarities in arrangement of trp genes in M. luteus and S. coelicolor.

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GENE-ENZYME RELATIONS AFFECTING TRYPTOPHAN BIOSYNTHESIS IN *STREPTOMYCES COELICOLOR* A3(2)

by

Charles M. Smithers

(ABSTRACT)

Earlier investigations indicated that the genes involved in the biosynthesis of tryptophan from anthranilic acid were located in at least two separate regions on the chromosome of *Streptomyces coelicolor* A3(2). Tryptophan-requiring mutants capable of utilizing indole mapped counterclockwise of *hisC9* between *hisC9* and *proA1*. Trp mutants unable to use indole in place of tryptophan mapped clockwise of *hisC9* between *hisC9* and *ammA5*.

Other genetic markers, located between *proA1* and *hisC9*, include *leuA1*, *rifB37* and *thiC2*. Twenty indole-utilizing Trp mutants were mapped relative to *leuA1*, *rifB37* and *thiC2*. All but two recently isolated mutants, Trp-e8 and e22, mapped between *rifB37* and *thiC2*. Genetic and enzymatic analyses of Trp-e8 and e22 indicate that these mutants map between *hisC9* and *ammA5*, fail to complement each other *in vivo* and lack indoleglycerol phosphate synthase. Two mutants, Trp-e14 and 8, mapping between *hisC9* and *ammA5* were assayed for their tryptophan enzymes. Trp-e14 lacks tryptophan synthase B activity. Tryptophan synthase A activity was missing and tryptophan synthase B activity was relatively weak in crude extracts of Trp-8.

Anthranilate-PRPP phosphoribosyltransferase activity was not detected in crude extracts of two mutants, Trp-e6 and e10, mapping between *rifB37* and *thiC2*.