GENETIC AND PHENOTYPIC ANALYSES OF AUXOTROPHS REQUIRING AROMATIC AMINO ACIDS AND VITAMINS IN *STREPTOMYCES COELICOLOR*

by

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INTRODUCTION

The general outline of the aromatic biosynthetic pathway from glucose to the aromatic amino acids and vitamins is shown in Figure 1. The common portion of the aromatic pathway from phosphoenolpyruvic acid and erythrose 4-phosphate to chorismic acid is given in greater detail in Figure 2. Auxotrophic mutants of Streptomyces coelicolor A3(2) requiring the aromatic amino acids tryptophan, tyrosine, and phenylalanine, and the aromatic vitamin para-aminobenzoic acid are presumably blocked in the common aromatic biosynthetic pathway.

The abbreviation "Aro" (Demerec, Adelberg, Clark, and Hartman 1966) is used to designate (i) a mutant whose auxotrophic phenotype is the requirement for tryptophan, tyrosine, phenylalanine, and para-aminobenzoic acid, though the latter requirement may not be demonstrable, depending on the individual mutant; and (ii) a mutant whose auxotrophic phenotype is the requirement for phenylalanine and tyrosine. Two Aro mutants, requiring all three amino acids, were induced and characterized by this investigator. In addition, three Aro mutants, two obtained from R. A. Jensen and one provided by E. J. Friend and D. A. Hopwood, were analyzed in this study. The precise chromosomal position of the five Aro mutants, relative to closely linked genetic markers, was determined. Phenotypic analyses included complementation studies to determine whether these mutants affect the same gene, and growth response to aromatic vitamins other than para-aminobenzoic acid. These vitamins included 2, 3 dihydroxybenzoic acid, vitamins K_1 and K_3, and para-hydroxybenzoic acid.

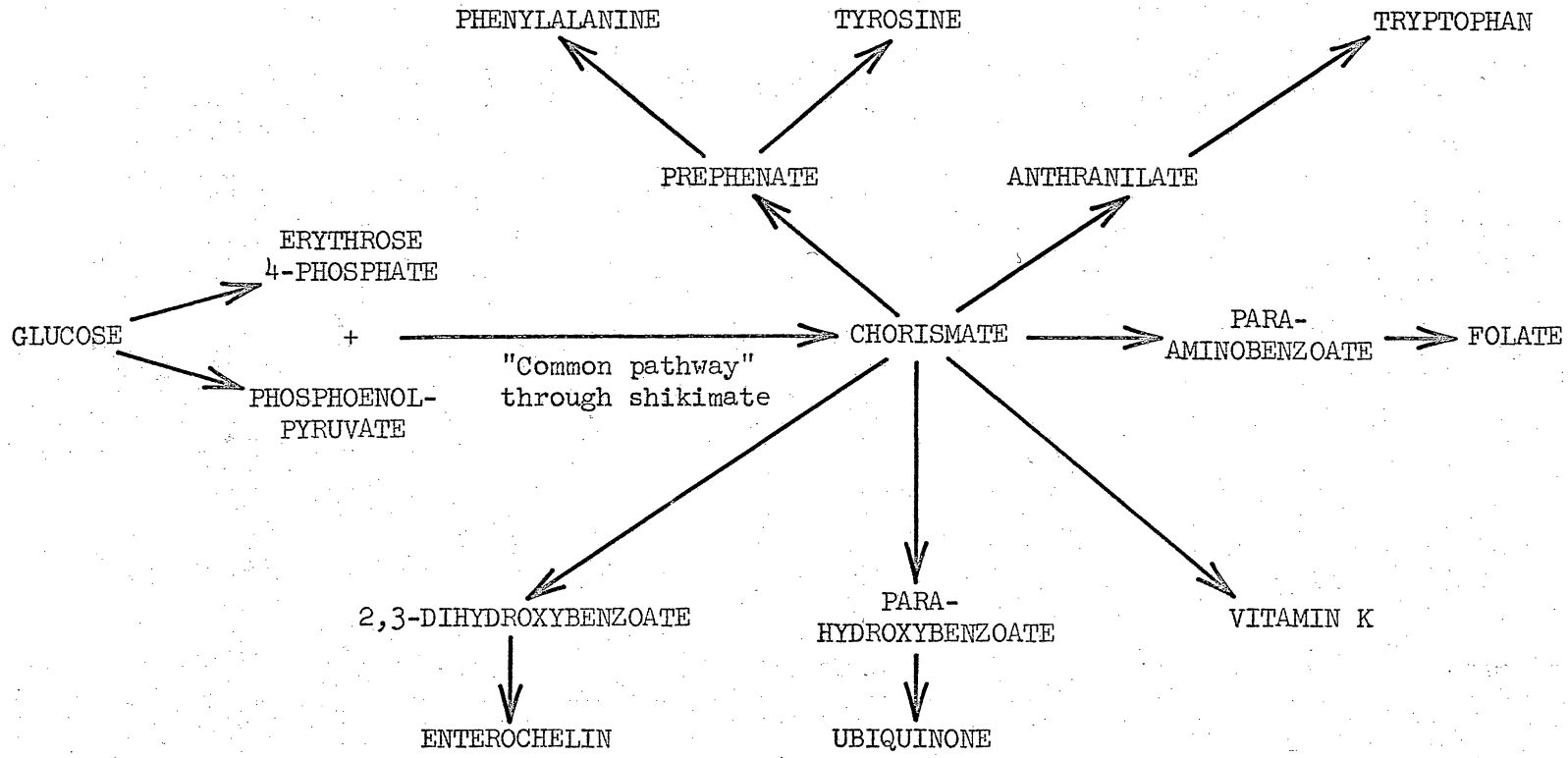


Fig. 1. General pathways for the formation of aromatic amino acids and vitamins in Escherichia coli and several other bacteria and fungi (Pittard and Gibson 1970; Ahmed and Giles 1969).

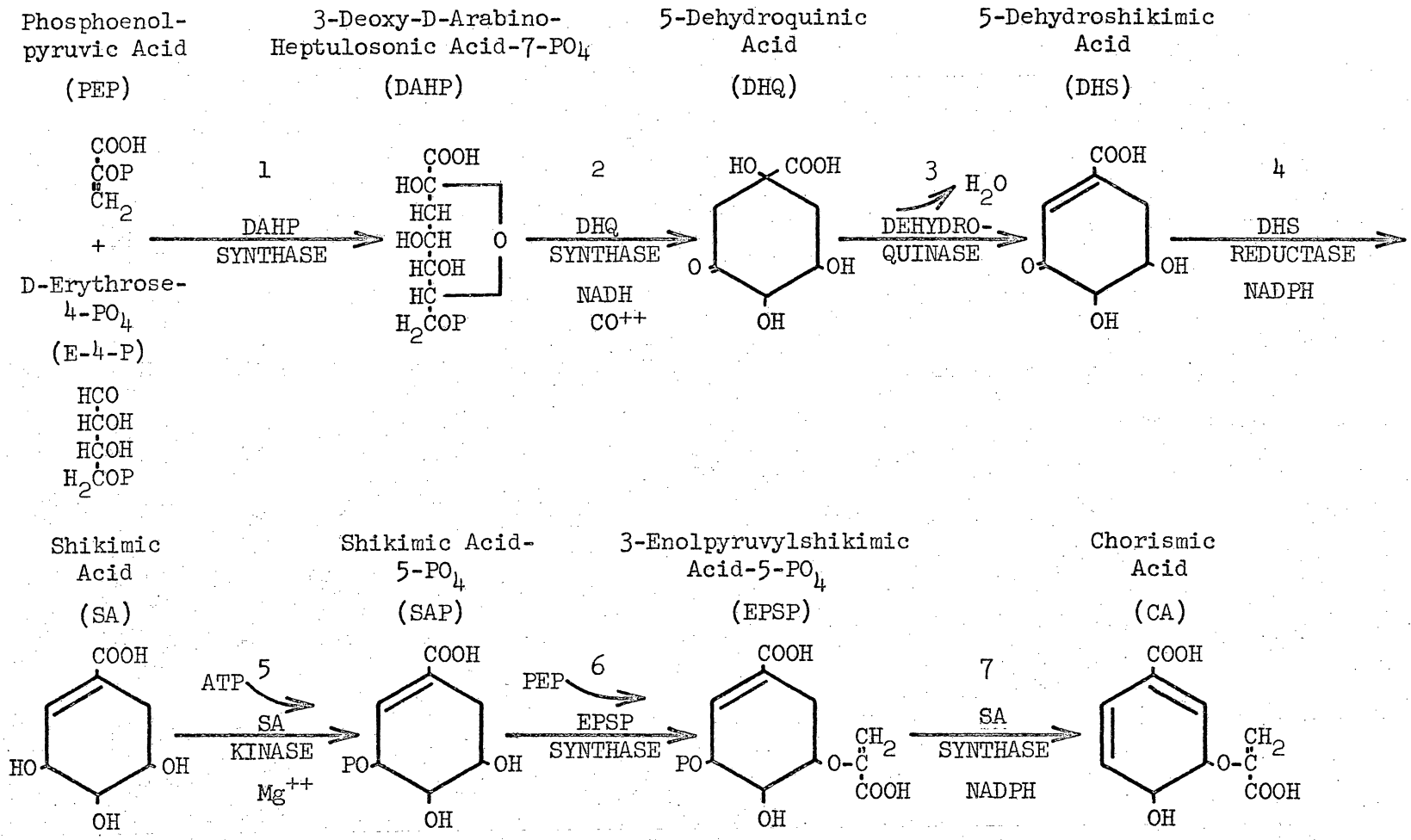


Fig. 2. Intermediates in the common pathway of aromatic biosynthesis based on the work of Gibson and Gibson (1964).

Since only one Aro mutant, mapped by E. J. Friend and D. A. Hopwood (Hopwood, personal communication), was investigated prior to this work, the induction and genetic analysis of additional mutants was considered significant in several respects:

(i) Aro mutants may be of value in determining the permeability of S. coelicolor to intermediates of the tryptophan, tyrosine, and phenylalanine biosynthetic pathways. Specifically, no tryptophan auxotrophs of S. coelicolor able to utilize anthranilic acid (ANTH) have been found in spite of efforts that will be described later. Such a mutant would presumably be defective in the conversion of chorismic acid to ANTH, a reaction catalyzed by anthranilate synthase (ASase). Mutants of S. coelicolor presumably defective in all steps of tryptophan biosynthesis except that catalyzed by ASase have been isolated (Engel, personal communication). The impermeability of S. coelicolor to ANTH was considered a possible explanation of the failure to obtain ANTH-utilizing tryptophan auxotrophs. The ability of Aro mutants to utilize ANTH in place of tryptophan would be an indication that failure to obtain ANTH-utilizing tryptophan auxotrophs is not due to the impermeability of S. coelicolor to ANTH. Results to be presented later show that Aro mutants utilize ANTH in place of tryptophan.

(ii) Genetic analysis of Aro mutants, prior to enzymatic studies to identify these mutants with enzymes in the common aromatic pathway, should provide several advantages (eg. in the construction of appropriate experimental and control strains) for subsequent studies of genetic regulation of the aromatic pathway in S. coelicolor.

(iii) S. coelicolor is one of the few higher bacteria amenable to genetic analysis, thus providing experimental material for comparative studies of genetic organization and regulation. This research represents the initial effort to compare the molecular genetics of aromatic biosynthesis in S. coelicolor with other organisms in which biochemical and genetic analysis of the same pathway has been done. Salient features of genetic organization and regulation of aromatic biosynthesis revealed by analysis of Aro mutants in the bacteria Escherichia coli (Pittard and Wallace 1966), Salmonella typhimurium (Nishioka, Demerec, and Eisenstark 1967), and Bacillus subtilis (Nasser and Nester 1967) and in the fungus Neurospora crassa (Giles, Case, Partridge, and Ahmed 1967) are discussed in the following section.

REVIEW OF LITERATURE

Intermediates in the common aromatic biosynthetic pathway. Davis (1951) first established that aromatic amino acids have a common origin. He observed that certain mutants of E. coli requiring tryptophan, tyrosine, phenylalanine, and para-aminobenzoic acid were able to utilize shikimic acid (SA) in place of the quadruple aromatic supplement. Further study of cyclic compounds related to SA quickly led to the discovery and identification of 5-dehydroquinic acid (Salamin and Davis 1953), 5-dehydroshikimic acid (Weiss, Davis, and Mingioli 1953), and shikimic acid-5-phosphate (Davis and Mingioli 1953) as intermediates of the common aromatic biosynthetic pathway. The noncyclic precursors of SA were later shown to be phosphoenolpyruvic acid and erythrose-4-phosphate (Srinivasan, Shigeura, Sprecher, Sprinson, and Davis 1956) which condense to form the last noncyclic intermediate in the pathway 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (Srinivasan, Rothschild, and Sprinson 1963). The branch point and last compound of the common pathway is chorismic acid (Gibson and Gibson 1964) which is formed from 3-enolpyruvylshikimic acid-5-phosphate (Rivera and Srinivasan 1963). The same pathway is found in all bacteria, fungi, and higher plants examined with one exception (Sinha 1967). This is the dermatophyte Trichophyton rubrum in which the biosynthesis of aromatic amino acids does not involve SA (Zussman, Vicher, and Lyon 1970). S. coelicolor, based on analysis of reactions catalyzed by cell-free extracts from wild-type cells, has been shown to have the SA pathway (Berlyn and Giles 1969).

Control of aromatic biosynthesis. A branched pathway presents two unique regulatory dilemmas. The first problem is that the cell must synthesize several endproducts differentially from a common precursor. In the bacteria studied, this is accommodated by having the three aromatic amino acids control the synthesis and activity of one or more enzymes in each of the terminal pathways from chorismic acid to tryptophan, tyrosine, and phenylalanine (Gibson and Pittard 1968). The second problem is that total feedback inhibition of the common pathway by a single endproduct will inhibit the production of the other endproducts. Regulation of the common aromatic pathway is accomplished in a variety of ways in the bacteria examined.

Control of aromatic biosynthesis in E. coli. In E. coli, control of the common aromatic biosynthetic pathway is exerted primarily on the enzyme 3-deoxy-D-arabino heptulosonic acid-7-phosphate (DAHP) synthase which catalyzes the condensation of phosphoenolpyruvic acid and D-erythrose-4-phosphate. Three isozymes of DAHP synthase have been found (Brown and Doy 1963). $DAHP_{(tyr)}$ synthase is repressed and its activity inhibited by tyrosine; a mixture of phenylalanine and tryptophan inhibits $DAHP_{(tyr)}$ activity to a lesser degree. The activity of $DAHP_{(phe)}$ synthase is inhibited by phenylalanine and repressed multivalently by phenylalanine and tryptophan (Brown and Somerville 1971). $DAHP_{(trp)}$ synthase is repressed but not inhibited by tryptophan (Brown and Doy 1963). Control of the common pathway is effected by two regulatory genes, trpR and tyrR. TrpR regulates

$DAHP_{(trp)}$ synthase; tyrR regulates $DAHP_{(tyr)}$ and $DAHP_{(phe)}$ synthases (Brown and Somerville 1971). A fourth isozyme of DAHP synthase has been suggested for the synthesis of aromatic vitamins, but none has been found (Wallace and Pittard 1967). $DAHP_{(trp)}$ synthase, repressible by tryptophan, and $DAHP_{(phe)}$, inhibitable by phenylalanine, have been shown to contribute to vitamin production (Wallace and Pittard 1969). Repression of DAHP synthase is never complete so that enough chorismic acid is formed for aromatic vitamin synthesis even in the presence of high concentrations of tryptophan, tyrosine, and phenylalanine. The aromatic vitamins have no known function in control of the common pathway (Gibson and Pittard 1968).

Very little is known about control of the last six enzymes of the common pathway in E. coli. Mutants have been isolated which are defective in each of the six steps except the one catalyzed by shikimate kinase. The failure to obtain shikimate kinase mutants is thought to be a consequence of the existence of two isozymes of the enzyme making it highly unlikely that both would be inactivated simultaneously (Berlyn and Giles 1969). Dehydroquinic synthase and dehydroquinase have been found to be insensitive to a mixture of aromatic amino acids and their levels unaffected by mutation of tyrR (Brown and Somerville 1971). No information is available on repression of the other common pathway enzymes in E. coli.

Control of aromatic biosynthesis in B. subtilis. In B. subtilis, control is exerted by sequential feedback inhibition. Two isozymes of shikimic acid kinase have been found but all other enzymes in-

cluding DAHP synthase have only one form of each enzyme (Jensen and Nester 1966). DAHP synthase and shikimic acid kinase are not significantly inhibited by the aromatic amino acids but are inhibited by the intermediary metabolites prephenic acid and chorismic acid (Jensen and Nester 1965). In addition, the enzymes DAHP synthase, dehydroquinic synthase, and shikimic acid kinase are repressed by an aromatic amino acid mixture (Nasser and Nester 1967).

Control of aromatic biosynthesis in other organisms. Much less is known about control in other organisms. Salmonella typhimurium (Gollub, Zalkin, and Sprinson 1967), Saccharomyces cerevisiae (Lingens, Goebel, and Uesseler 1966), and Claviceps paspalis (Lingens, Goebel, and Uesseler 1967) resemble E. coli in that they have isozymes of DAHP synthase, each isozyme differentially inhibitable by the three aromatic amino acids. Various strains of Staphylococcus, Gaffkya, Flavobacterium, Achromobacter, and Alcaligenes resemble B. subtilis in that they show sequential feedback inhibition of DAHP synthase by either prephenic acid or chorismic acid (Jensen, Nasser, and Nester 1967).

The chromosomal organization of genes concerned with aromatic biosynthesis. Demerec (1964), in S. typhimurium, first demonstrated the clustering of genetic loci concerned with the same biosynthetic pathway. Often clustered genes occur in the same sequence as the reactions for which they code enzymes. The tryptophan cluster of E. coli is an example of this (Brown and Sommerville 1971). Although this phenomenon is well substantiated, the physiological significance

is unclear. A possible explanation was given by the operon hypothesis which suggested that clustering accomodates co-ordinated regulation. An operon is here defined as a genetic region whose expression is co-ordinately (or semi-co-ordinately) regulated. It has been demonstrated that certain groups of closely linked genes are transcribed onto a single mRNA molecule (Jacob, Perrin, Sanchez, and Monod 1960); however, this proposal fails to explain certain evidence. For example, the isoleucine-valine genes of E. coli, although tightly linked, constitute three distinct operons (Ramakrishnan and Adelberg 1965). Another suggestion is that since genetic transfer in bacteria is usually only partial, those genes whose functions are metabolically co-ordinated can be transferred as a unit (Hedges 1971). This hypothesis is supported by evidence that functionally related genes which are clustered as operons in certain bacteria are usually scattered in eucaryotic genomes (Horowitz 1965).

Organization of genes involved in aromatic biosynthesis. The chromosomal location of genes concerned with aromatic biosynthesis in E. coli, B. subtilis, and S. typhimurium is sufficiently known to make comparisons of gene organization with respect to sequence and distribution. The approximate chromosomal positions of aromatic (aro) genes in these bacteria are shown in Figure 3. Table 1 provides a key to the designation of genetic loci used in Figure 3.

The chromosomal sequence of aro genes. As shown in Figure 3, the sequence of aro genes in S. typhimurium and E. coli is identical while little gene sequence homology is seen between these bacteria

Table 1. Locus designations used in Figure 3.

Enzyme	Designation in Figure 3[a]	Genotypic designation		
		S. typhimurium	E. coli	B. subtilis
DAHP synthase$_{(trp)}$	aro-1$_{(trp)}$	aro-H	aro-H	aro-A[b]
DAHP synthase$_{(tyr)}$	aro-1$_{(tyr)}$	aro-F	aro-F	b
DAHP synthase$_{(phe)}$	aro-1$_{(phe)}$	aro-G	aro-G	b
DHQ synthase	aro-2	aro-B	aro-B	aro-B
Dehydroquinase	aro-3	aro-E	aro-D	aro-C
DHS reductase	aro-4	aro-C	aro-E	aro-D
SA kinase	aro-5	c	c	c
EPSP synthase	aro-6	aro-A	aro-A	aro-E
CA synthase	aro-7	aro-D	aro-C	aro-F
CA mutase	aro-8	c	c	aro-G & aro-H[d]
Aro permease	aro-P	aro-P	aro-P	c

[a] Number indicates numerical order of reaction catalyzed.

[b] Only one form of DAHP synthase, which is inhibitable by chorismic acid or prephenic acid, has been found in B. subtilis.

[c] No such mutant has been found.

[d] Mutants for two isozymes of CA mutase have been found in B. subtilis.

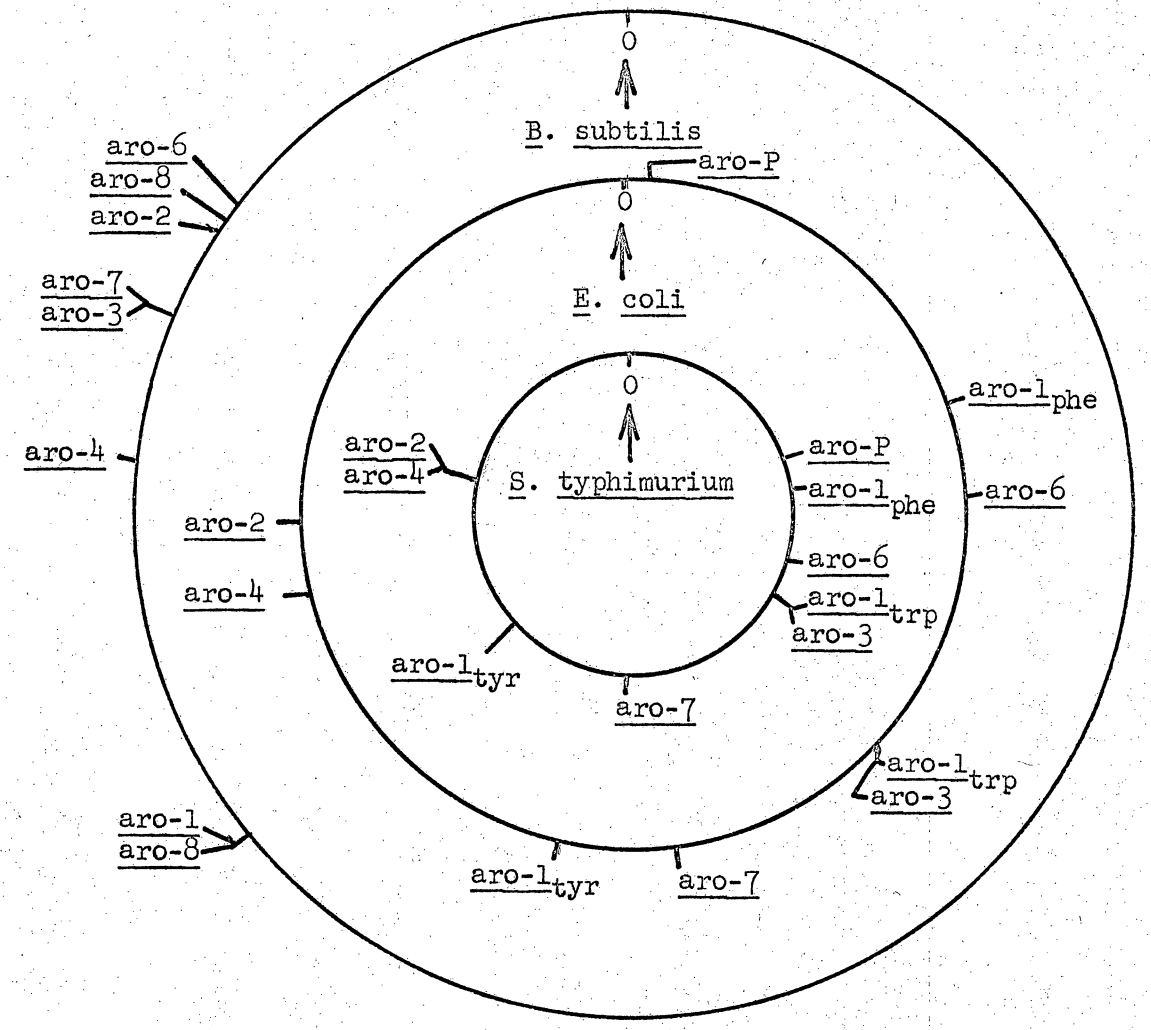


Fig. 3. The chromosomal organization of genes concerned with aromatic biosynthesis in S. typhimurium (Sanderson 1970), E. coli (Brown and Somerville 1971), and B. subtilis (Dubnau 1970).

and B. subtilis. Only aro-3 and aro-7 occur in the same relative sequence in all three bacteria. Comparisons of the sequence of all known genes in E. coli with those of S. typhimurium (Sanderson 1970; Taylor 1970), and of all known genes of S. coelicolor with those of Streptomyces rimosus (Friend and Hopwood 1971) and Streptomyces bikiniensis (Coats and Roeser 1971), show a high degree of similarity in these closely related bacteria, suggesting that there is limited stability of gene arrangement on procaryotic genomes. The degree to which the chromosomal sequence of homologous genes in different bacteria have changed or been conserved should be a valid taxonomic measurement (Friend and Hopwood 1971).

The distribution of aro genes. The aro genes are widely scattered in S. typhimurium and E. coli and less scattered in B. subtilis, being confined to a fourth of the genome in the latter bacterium. Aro-1$_{(trp)}$ is clustered with aro-3 in both S. typhimurium and E. coli. Aro-2 is with aro-4 in S. typhimurium only; while aro-7 is with aro-3 and aro-1 with aro-8 in B. subtilis only. The distribution of aro genes in these three bacteria is in contrast to the situation found in two eucaryotes, S. cerevisiae and N. crassa. In S. cerevisiae, aro-3 through aro-6 occur in a cluster (de Leeuw 1968). In N. crassa, aro-2 through aro-6 occur in a cluster; furthermore, this cluster of five aro genes has certain operon-like characteristics. Pleiotropic nonsense mutations which have a polarized effect occur in the aro cluster of N. crassa (Giles, et al. 1967). The organization of aro genes in the bacteria and eucaryotes studied is therefore in contrast to the observation of Horowitz (1965) that functionally related genes

which are clustered as operons in bacteria are usually scattered in eucaryotic genomes. Whether the organization of aro genes in a higher bacterium, such as S. coelicolor, resembles the situation in other bacteria or in eucaryotes is an interesting question.

Mating and formation of merozygotes in S. coelicolor. Chromosome transfer in S. coelicolor is probably mediated by cell fusion. Experiments to provide understanding of sexual processes in this bacterium have been described (Hopwood 1967, 1970; Spada-Sermonti and Sermonti 1970). These experiments were designed to distinguish between two alternative hypotheses: (i) prezygotic exclusion, only part of a chromosome is transferred, thus only part of the chromosome is available for integration by recombination; or (ii) postzygotic exclusion, an entire chromosome is transferred but only part of the chromosome is integrated in the formation of recombinants. In summary, none of the experiments clearly established whether the segment of chromosome derived from one parent is determined by prezygotic or postzygotic exclusion. Thus the initial events in the formation of the zygote are not known. Nevertheless, experiments primarily designed to determine genome structure (Hopwood 1966; 1967) permit inferences concerning the sequence of events in the formation of the zygote.

Genetic data, primarily from analysis of heteroclones to be discussed in more detail later, indicate that one parent in a cross contributes the major portion of the chromosome of recombinants. Only a segment of chromosome, variable in size, is contributed by

the other parent. Initial stages in the formation of the zygote may be represented by a circle, the genome of one parent, with an arc, the partial genome contributed by the other parent, closely associated to form a disomic region. The next step in the formation of a zygote presumably involves a crossover (or an odd number of crossovers) between the circle and the arc generating a terminally redundant genome--the merozygote--which may bear heterozygous alleles, if the parents were heterozygous for alleles in the disomic region.

Formation of haploid and heterozygous colonies from merozygotes. A second crossover (or an odd number of crossovers) in the disomic region of a merozygote generates a genome which is not redundant and a genome which should be covalently closed. Such cells should be haploid, and thus homozygous for all alleles borne in the genome. This second crossover almost certainly occurs before the genome is included in a spore.

Merozygotes may be included in spores and presumably replicate when the spore germinates. A second crossover (or some odd number of crossovers) may occur in different segments of the disomic region in different descendants of the original merozygote; thus giving rise to a colony with haploid cells whose genomes are genetically different. Such a colony is called a heteroclone. Heteroclones are detected by demonstrating that cells in a colony are genetically different.

In practice, haploid colonies are most suitable for mapping while heteroclones are of value in certain other studies such as complementation analysis and analysis of genome structure. Based on the type of analysis that is to be done, it is possible to design a cross in such a way that either mostly haploid colonies or mostly heteroclones will be obtained. Figure 4 shows an example of each type of cross. In model (a) the selected markers $\underline{c}^+$ and $\underline{h}$ (a resistance mutation) are not closely linked and most of the progeny recovered from such a cross are haploid. In model (b) the selected markers $\underline{g}^+$ and $\underline{h}$ are very closely linked so that the probability of a crossover between them is very small and most of the merozygotes are formed by a single crossover such as the one shown. Such a merozygote would give rise to heteroclones heterozygous for markers $\underline{f}$, $\underline{g}$, and $\underline{h}$. Other markers might also be heterozygous from this cross depending on the extent of the variable disomic region.

Circularity of the S. coelicolor genome. The preceding arguments were based on the assumption that the genome is either (i) a covalently closed structure, or (ii) a linear structure which is circularly permuted, or (iii) both a circular structure and a circularly permuted linear structure in equilibrium. Although all known markers of S. coelicolor are linked on a circular genetic map (Hopwood 1967; Chater 1972), this does not prove the physical circularity of the genome, or chromosome. Since haploids arise from merozygotes only by an even number of crossovers the analysis of recombinants would indicate a circular map even if the genome were open with ends in

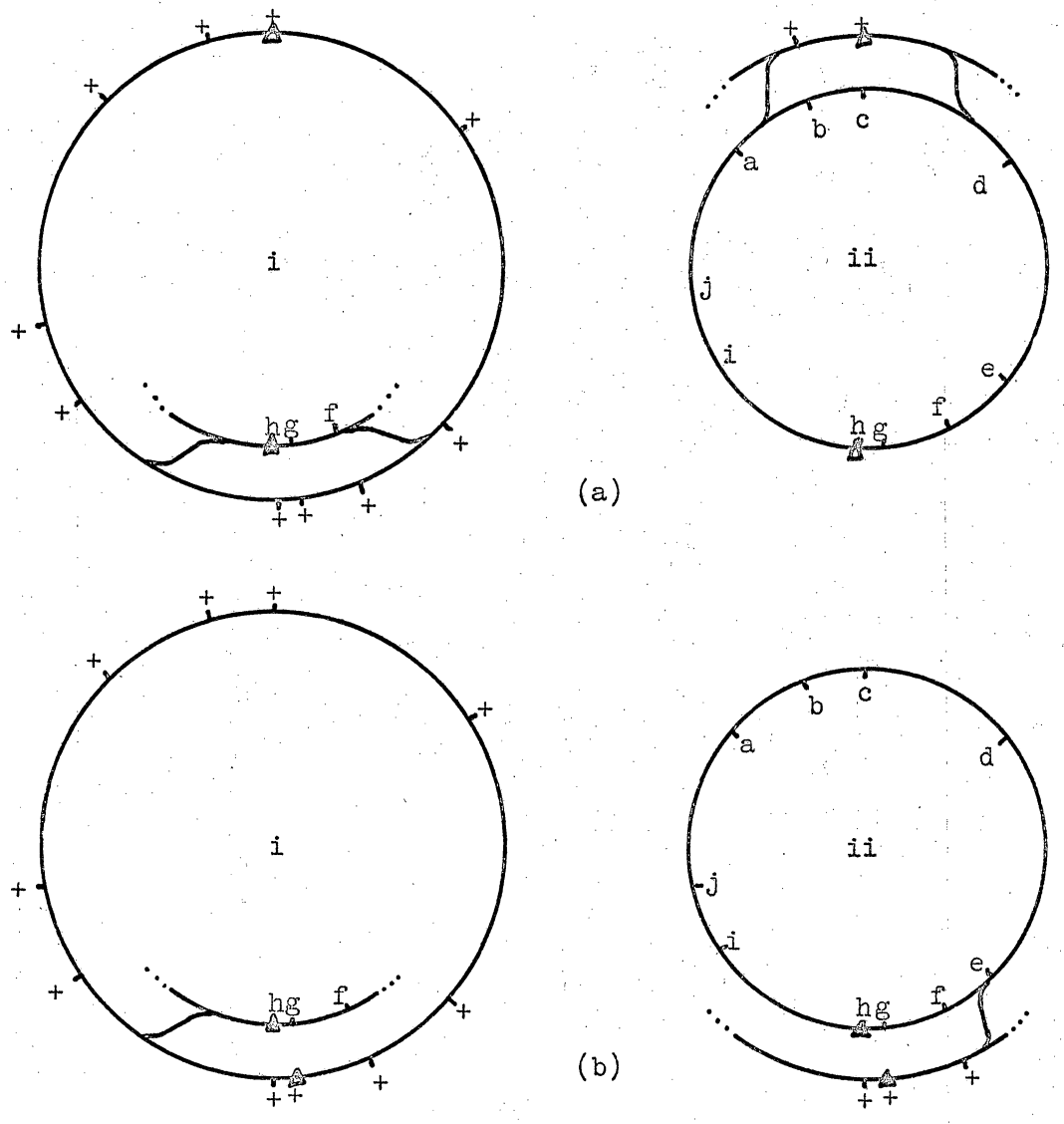


Fig. 4. Two types of crosses illustrating the hypothetical origin of haploid and heterozygous colonies. In (a) the selected markers are not closely linked and haploid colonies are recovered. In (b) the selected markers are closely linked and the occurrence of a single crossover gives rise to heteroclones. The two reciprocal illustrations (i and ii) indicate first one parent, and then the other, contributing the major portion of the chromosome. Triangles indicate selected markers.

fixed positions. Hopwood (1966) has mapped overlapping disomic regions, obtained from different heteroclones, to account for the entire circular map; thus eliminating the possibility that the genome is open with fixed ends. Hopwood (1967) has concluded, therefore, that the genome of S. coelicolor is either circular or circularly permuted, or there may be an equilibrium between these states.

Although there is no direct physical evidence for a covalently closed chromosome, this model is favored in light of direct evidence in other bacteria such as E. coli (Cairns 1963) and Mycoplasma hominis (Bode and Morowitz 1967).

Genetic analysis in S. coelicolor--locating the chromosomal position of a mutant site based on the number of recombinant phenotypes. Only a brief discussion of this topic is given since a more detailed consideration is given in the RESULTS section; pp. 30-33. In the initial mapping of a mutant, a cross such as the one shown in model (a) of Figure 4 is done. Assume the unmapped mutation is present in the otherwise wild-type parent (the parent designated by +). Since the donor fragment is variable in length but always contains the selected marker h, the unselected marker closest to h, in this case g, will appear in recombinants at a higher frequency than any other marker. The frequency of the unselected markers among recombinants form a continuous gradient along two opposite arcs; from zero frequency for c, the counter-selected allele, to a frequency of 100% for h, the selected allele. An example of the results of such a cross is shown in Figure 6. The frequency of the mutant allele generally fits into the

gradient at only one positon on both arcs. Usually one of these two alternative positions may be eliminated by analysis of the frequency with which certain combinations of unselected markers appear among the progeny. This latter point is discussed in the following section.

Genetic analysis in S. coelicolor--locating the chromosomal position of a mutant site based on minimum number of crossovers. Haploid colonies may arise by any even number of crossovers; however, four crossovers occur much less frequently than two and much more frequently than six. The preferred configuration of markers is the one in which the recombinants obtained may occur by a minimum number of crossovers. A mutant site may be ordered with respect to virtually any other marker by a similiar minimum crossover analysis of four-point reciprocal crosses such as those to be described later. Further discussion of this topic is deferred to the section on RESULTS; pp. 34-42.

Complementation analysis in S. coelicolor. Once the position of a mutant has been determined it is possible to determine whether the mutant affects the same gene as mutations previously mapped at the same chromosomal position. If the mutations affect different genes they are said to complement each other. Complementation analysis is accomplished by crossing the two closely linked mutants and monitoring the heterozygosity of the region with unrelated genetic markers. If two closely linked mutations are complementary, recombinants containing both mutations heterozygously are able to grow on media which is selective against both mutations. Further discussion of this topic is deferred to the section on RESULTS; pp. 42-49.

MATERIALS AND METHODS

General. Amino acids, vitamins, and antibiotics were purchased from Sigma Chemical Co. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and 2,3-dihydroxybenzoic acid were purchased from Aldrich Chemical Co.

The media and culture conditions were the same as those described by Hopwood (1967), except the concentration of some supplements in media had to be altered to achieve optimal growth and diagnosis of strains. These exceptions are noted in the following sections. Complete medium (CM), used for crosses and maintenance of strains, was supplemented with L-tryptophan (TRP), L-tyrosine (TYR), and L-phenylalanine (PHE) at 50 µg/ml for each of these three amino acids. CM, not supplemented with these three amino acids, did not support the growth of tightly blocked Aro mutants. Minimal medium (MM), used for the detection of auxotrophic mutants and the diagnosis of strains, in addition to the supplementation schedule of Hopwood, was supplemented with 0.25 µg/ml of para-aminobenzoic acid (PABA) and 5 µg/ml of yeast extract (YE).

Strains. All strains were derivatives of S. coelicolor A3(2) (Hopwood 1959) and were obtained either by mutagenesis or recombination. These strains are listed in Table 2. The linkage relations of their genetic markers are shown in Figure 5. Table 3 is a key to the genotypic designations of these strains.

Induction of mutants. Before attempting mutagenesis, efforts were made to develop an enrichment method for auxotrophs; no such

Table 2. Genotype of relevant strains.

Strain	Genotype
A3(2)	wild type
P42	*thiC2*, *strA1*
P196	*met-e1*, *strA1*
Aro-1	*aro-1*
Aro-2	*aro-2*
Aro-f1	*aro-f1*
Aro-w1	*aro-w1*, *thiC2*, *strA1*
Aro-w3	*aro-w3*, *met-e1*, *strA1*
C6 [a]	*aro-w1*, *thiC2*
C7 [a]	*aro-w3*, *met-e1*
C36	*proA1*, *argA1*, *hisE6*, *leuB5*, *strA1*, *uraA1*

[a] This strain was used in place of the original Aro mutant in preliminary crosses.

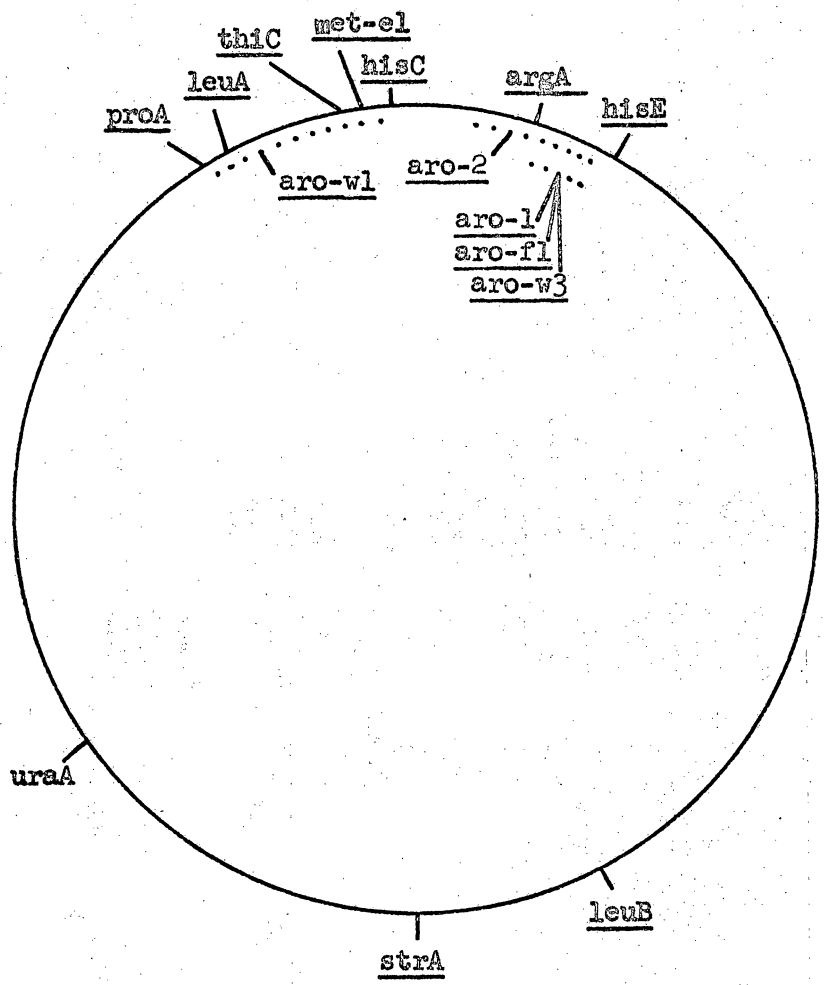


Figure 5. Chromosomal location of relevant genetic markers (Chater 1972).

Table 3. Key to the designation of genotypes.

Marker	Phenotype
argA	requirement for arginine
aro	requirement for aromatic amino acids
hisC	requirement for histidine
hisE	requirement for histidine and a purine
leuA and *leuB*	requirement for leucine
met-el	requirement for methionine
proA	requirement for proline
strA	resistance to streptomycin
thiC	requirement for thiamine
uraA	requirement for uracil

method presently exists for S. coelicolor. Wild-type cells and auxotrophic cells were mixed, and their relative proportion determined by plate counts. The relative proportion of auxotrophs was again determined after the following treatments which were all preceded by one hour of incubation in MM: (i) growth for 0, 1, 4, and 16 hours in MM containing 10, 50, and 200 units bacitracin per ml; (ii) growth for 0, 1, 4, and 16 hours in MM containing 50, 250, and 1000 µg D-cycloserine per ml; (iii) growth for 0, 1, 4, and 16 hours in MM followed by filtration through a 9 µ milipore filter. None of the procedures led to any detectable increase in proportion of auxotrophs.

Since efforts to enrich for auxotrophic mutants failed, the mutants isolated in this investigation were initially recognized as auxotrophs by their failure to grow on replica plates containing diagnostic media. Cells from the wild-type strain, strain P42, and strain P196, in separate experiments, were treated with NTG, using the procedure of Delić, Hopwood, and Friend (1970), to induce mutants. Spores suspended in 0.05 M Tris-HCl buffer, pH 9, containing 3 mg NTG per ml were incubated at 30° for 30 minutes. The cells were then concentrated by centrifugation and washed with sterile distilled water.

The cells were then plated on MM containing either: (i) 50 µg/ml quinic acid; (ii) 50 µg/ml shikimic acid; (iii) 50 µg/ml TRP, TYR, PHE, and 1 µg/ml PABA, para-hydroxybenzoic acid (POBA), 2,3-dihydroxybenzoic acid (DOBA) and menadione, also known as vitamin

K_3 ($VITK_3$). After three days incubation at 30°, colonies which appeared on plates containing either quinic acid or shikimic acid were replica plated to MM and control plates with MM containing either quinic acid or shikimic acid. Colonies which appeared after three days, on plates containing the aromatic amino acids and vitamins were replica plated to MM containing PABA, POBA, DOBA, and $VITK_3$ and to control plates of MM supplemented with the aromatic amino acids and vitamins. Colonies that failed to grow after two days on the diagnostic media were isolated; their phenotype was then more precisely determined.

RESULTS

Isolation of mutants. Approximately two percent of colony forming units survived mutagenesis as determined by plate counts. Approximately 45,000 colonies were examined. No auxotrophs were isolated from plates containing quinic acid or shikimic acid. Thirty auxotrophs were isolated from plates containing aromatic amino acids and vitamins. Nineteen of these auxotrophs required TRP, six required TYR, and three required PHE. Only two of the thirty auxotrophs required all three aromatic amino acids; these two mutants are designated Aro-w1 and Aro-w3.

Phenotypic analysis of aromatic mutants. The growth response of the five Aro mutants (Aro-1 and Aro-2 were obtained from R. A. Jensen; Aro-f1 was obtained from D. A. Hopwood) used in this research is shown in Table 4. All data are based on subjective analysis of colony growth after replication to appropriate diagnostic media. Aro-w1 and Aro-w3 require some substance, or substances, which is satisfied by 5 µg YE per ml. The relatively low concentration of substance, or substances, contained in YE which satisfies the undetermined growth requirement, or requirements, suggests this may be a vitamin, or vitamins.

Folic acid, vitamin K_1 and two ubiquinones, coenzyme Q_6 and coenzyme Q_{10}, were tested in concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, and 5 µg/ml, in addition to PABA, POBA, DOBA, and VITK$_3$. PABA was the only vitamin found to have a stimulatory effect. Stimulation by PABA was maximal at approximately 0.25 µg/ml and inhibition was noted at 2.5 µg/ml.

Table 4. Growth response of aromatic mutants.

	Growth response of aromatic mutants				
Supplements in MM	Aro-1	Aro-2	Aro-f1	Aro-w1	Aro-w3
TRP, TYR, PHE, YE	++	++	++	++	++
TRP, TYR, PHE, PABA, POBA, DOBA, $VITK_3$	++	++	++	+	+
TRP, TYR, PHE, PABA	++	++	++	+	+
TRP, TYR, PHE	++	+	++	-	-
PABA	-	-	-	-	-
TRP, TYR, PABA	+	-	+	-	-
TRP, PHE, PABA	+	-	+	-	-
TYR, PHE, PABA	+	-	++	-	-
Indole, TYR, PHE, PABA	++	++	++	+	+
ANTH, TYR, PHE, PABA	++	++	++	+	+
Quinic Acid	-	-	-	-	-
Shikimic Acid	-	-	-	-	-

Growth response is indicated: good growth as ++; poor growth as +; no growth as -.

Although Aro-f1 requires PHE and TYR, no TRP requirement could be demonstrated for this mutant. This may be a consequence of "leakiness". A mutant is considered leaky if the mutant phenotype is barely perceptible; the enzyme altered by mutation presumably has sufficient activity to provide enough endproduct for growth, or the development of a nearly normal phenotype. There are degrees of leakiness, and Aro-f1 is the leakiest of the five Aro mutants. Another possibility is that Aro-f1 requires only PHE and TYR and is deficient in the conversion of chorismic acid to prephenic acid--the reaction catalyzed by chorismate mutase.

All five mutants are unable to utilize either quinic acid or shikimic acid (SA). This suggests that either S. coelicolor is impermeable to these acids and/or the mutants are blocked in one, or more, of the three terminal reactions in the common aromatic pathway--one, or more, of the three reactions involved in the conversion of SA to chorismic acid. In an effort to decide between impermeability versus mutational block, the wild-type strain, A3(2), was tested for ability to utilize SA as sole carbon source. Tubes of liquid MM containing various concentrations of SA or glucose were innoculated from a two-day-old culture grown on 0.1% glucose and incubated two days on a shaker at 30°. Sodium nitrate (0.05%) was used in place of L-asparagine as sole nitrogen source in all tubes. Results from these experiments are shown in Table 5. Good growth was seen at 0.5% SA while no growth was seen in a control containing no carbon source. Inhibition of growth by 1% SA was noted. A second

Table 5. Growth response of S. coelicolor A3(2) utilizing several different concentrations of glucose or shikimic acid as sole carbon source.

Carbon source	Percent concentration	Growth response
None	-	-
Glucose	.1	+
Glucose	.2	++
Glucose	.5	++
Glucose	1.0	++
Glucose	2.0	++
SA	.1	-
SA	.2	++
SA	.5	++
SA	1.0	+
SA	2.0	-

Growth response is indicated: good growth as ++; poor growth as +; no growth as -.

tube of 0.5% SA was inoculated from the first tube (0.5% SA) and good growth was again observed in two days.

Preliminary mapping of aromatic mutants. In the preliminary crosses each Aro mutant was crossed with the multiply marked, streptomycin-resistant strain C36. Spore progeny from the crosses were plated on a medium containing streptomycin and lacking arginine. Only those recombinants that had inherited the *argA*$^{+}$ allele from the Aro parent and the *strA*^{R} allele (streptomycin resistance) from the C36 parent were able to grow on the selective medium. The remaining growth requirements of C36 (HIS + PUR, PRO, URA, LEU) and the Aro parent (PHE, TRP, TYR, PABA) were satisfied by appropriate supplementation of the medium. Thus recombinants segregated unselected markers (*his*$^{+}$ and *his*, *pro*$^{+}$ and *pro*, etc.). The various recombinant phenotypes were determined by replica plating to appropriate diagnostic media. Recombinant phenotypes for all five preliminary crosses are shown in Table 6. Figure 6 gives an example of how the number of recombinant phenotypes from a preliminary cross is used in determining the position of a mutant site. The frequency of each unselected allele in all five preliminary crosses was calculated and is shown in Table 7. In Table 7, most probable positions of the *aro* mutant sites are indicated by broken lines.

Further analysis of the same crosses was based on consideration of the number of crossovers required to generate the different recombinant classes. This analysis involves placing a mutant site in all six marked regions of the genetic map and determining the number

Table 6. Genotypes of progeny recovered from preliminary crosses of Aro mutants with strain C36 in which *arg*$^+$ and *str*^{R} were selected markers.

Genotypes of progeny[a]	Number of progeny				
	Aro-1	Aro-2	Aro-f1	Aro-w1	Aro-w3
str, *ura*	2	0	0	0	0
str, *pro*	0	0	0	1	0
str, *leu*	0	0	0	2	0
str, *aro*	24	11	15	2	2
str, *ura*, *pro*	0	0	0	3	0
str, *ura*, *aro*	54	41	269	25	4
str, *pro*, *leu*	0	0	0	4	0
str, *pro*, *aro*	2	2	0	0	0
str, *leu*, *aro*	6	4	0	30	36
str, *ura*, *pro*, *leu*	0	0	0	17	0
str, *ura*, *pro*, *aro*	67	55	10	4	0
str, *ura*, *leu*, *aro*	19	84	4	86	99
str, *pro*, *leu*, *aro*	0	1	0	2	1
str, *ura*, *pro*, *his*, *leu*	0	0	0	0	1
str, *ura*, *pro*, *leu*, *aro*	23	2	0	9	56
str, *ura*, *his*, *leu*, *aro*	0	0	0	1	0
Total progeny	197	200	298	186	199

[a] Wild-type alleles are omitted.

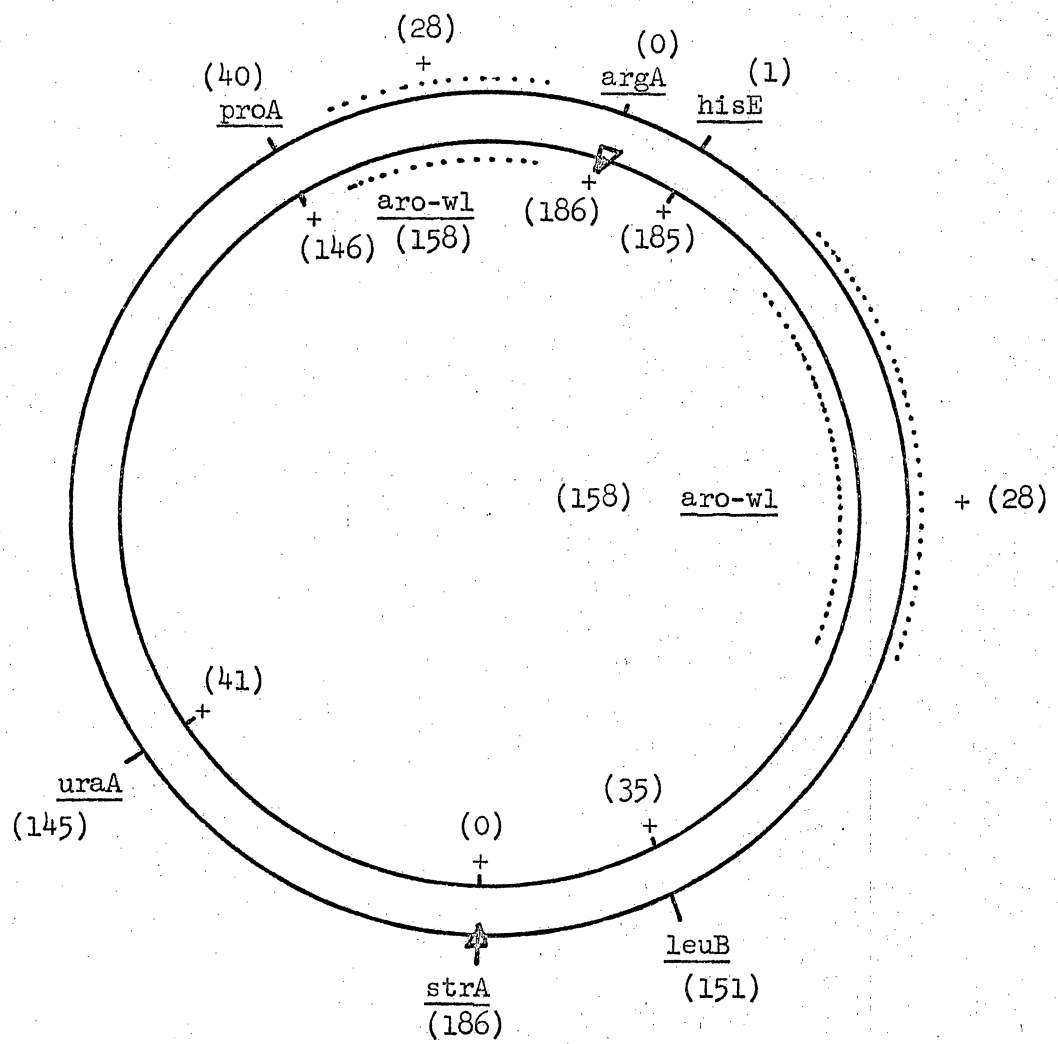


Figure 6. Representation of progeny recovered from a cross of Aro-wl with C36. Number of progeny bearing a particular allele is shown in parenthesis. Selected markers are indicated with triangles. Alternative positions of the aro-wl mutant site, based on position in gradient, are indicated by broken lines.

Table 7. Frequency of alleles scored among progeny resulting from a cross between Aro mutants and C36 in which *arg*$^+$ and *str*^{R} were the selected markers.

Mutant	Number of Progeny	Allele frequency (%)						
		str^R	*ura*$^-$	*pro*$^-$	*arg*$^-$	*his*$^-$	*leu*$^-$	*str*^{R}
Aro-1	197	100	84	47	0	0	24	100
					aro-1$^+$ (1)			
Aro-2	200	100	91	30	0	0	46	100
					aro-2$^+$ (0)			
Aro-f1	298	100	95	3.4	0	0	1.3	100
					aro-f1$^+$ (0)			
Aro-w1	186	100	78	21	0	0.5	65	100
					aro-w1$^+$ (15)			
Aro-w3	199	100	33	29	0	0.5	97	100
					aro-w3$^+$ (1)			

The indicated position of each *aro* mutant locus is shown by a broken line.

of crossovers required to generate the observed progeny. One assumes the mutant site occurs in that region which requires the fewest crossovers to generate the different recombinant classes. Figure 7 shows the six alternative positions of an aro mutant site with intervals between each marker designated by letters a to g. Table 8 shows intervals in which crossovers would be required in alternative marker configurations for all progeny obtained. Total crossovers required in each configuration are shown for all five preliminary crosses in Table 9.

The preliminary crosses indicate that aro-1, aro-2, aro-f1, and aro-w3 are located clockwise of proA and counterclockwise of leuB on the genetic map. Aro-w1 is probably clockwise of proA and counterclockwise of argA or clockwise of hisE and counterclockwise of leuB. The location of these mutant sites was more precisely determined relative to previously mapped genetic markers on the basis of four-point reciprocal crosses.

Four-point reciprocal crosses to determine the position of aro mutant sites relative to hisC. The position of all five aro mutant sites was determined relative to hisC by the following method. In the crosses shown in Table 10, aro is first linked with the outside selected marker argA; hisC, the marker serving as the reference point for mapping aro, is linked to the outside selected marker proA. In the reciprocal cross, the linkage relationship of aro and hisC with respect to the outside selected markers is reversed. Thus, it is possible to determine whether the aro mutant site is left or right of hisC by comparing the frequency of his^+ aro^+ colonies generated in

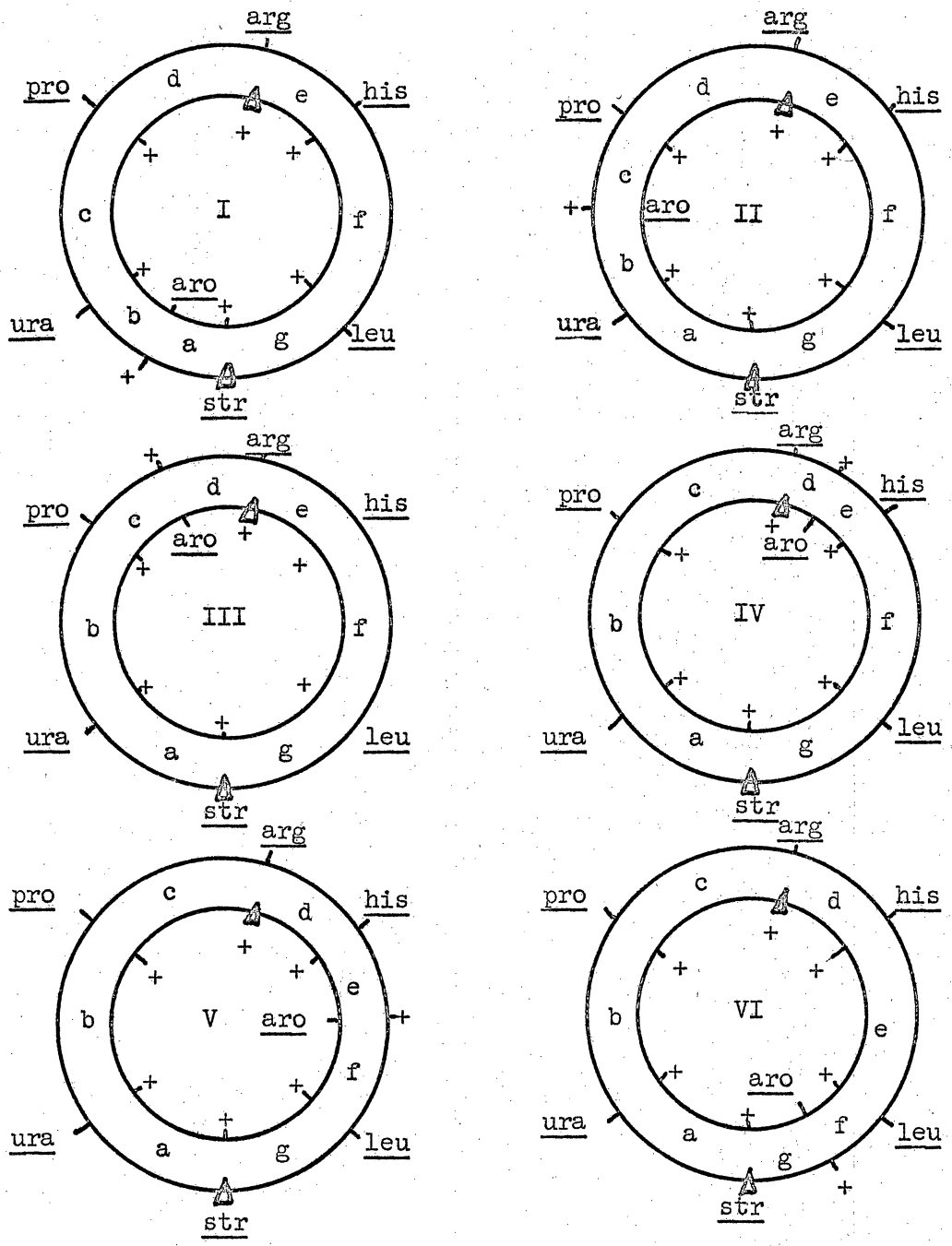


Figure 7. Representations of all possible configurations of aro loci in a cross with C36 in which arg^{+} and str^{R} were selected markers. Intervals between markers are designated with letters a to g. The outer circle represents the chromosome of strain C36, the inner circle the chromosome of the Aro parent.

Table 8. Intervals in which nonselected crossovers occurred when all possible locations of aro mutant sites are considered.

Genotypes of progeny[a]	Intervals containing nonselected crossovers[b]					
	I	II	III	IV	V	VI
str, ura	cg	cg	bcdg	bdeg	befg	bf
str, pro	bcdg	abdg	abdg	abcdeg	abcefg	abcf
str, leu	bf	abcf	acdf	adef	ae	ae
str, aro	ag	ag	ag	ag	ag	ag
str, ura, pro	dg	dg	dg	cdeg	cefg	cf
str, ura, aro	abcg	bg	bg	bg	bg	bg
str, pro, leu	bcdf	abdf	abdf	abcdef	abce	abcf
str, pro, aro	acdg	acdg	abcg	abcg	abcg	abcg
str, leu, aro	af	af	af	af	af	aefg
str, ura, pro, leu	df	df	df	cdef	ce	ce
str, ura, pro, aro	abdg	bcdg	cg	cg	cg	cg
str, ura, leu, aro	abcf	bf	bf	bf	bf	befg
str, pro, leu, aro	acdf	acdf	abcf	abcf	abcf	abcefg
str, ura, pro, his, leu	de	de	de	ce	cd	cd
str, ura, pro, leu, aro	abdf	bcdf	cf	cf	cf	cefg
str, ura, his, leu, aro	abce	be	be	be	bdef	bdfg

[a] Wild-type alleles have been omitted.

[b] See Figure 7 for interval designations and alternative positions of aro mutant site.

Table 9. Total number of crossovers required to give rise to progeny obtained in preliminary crosses.

Aro mutant site	Number of progeny	Number of crossovers required[a]					
		I	II	III	IV	V	VI
aro-1	197	724	578	402	402	402	494
aro-2	200	770	520	410	410	410	592
aro-f1	298	1162	616	596	596	596	604
aro-w1	186	636	416	390	440	396	642
aro-w3	199	718	512	398	398	400	784

[a] See Figure 7 for alternative positions of aro mutant sites and Table 8 for a description of the crossovers required to give rise to progeny obtained in preliminary crosses.

the reciprocal crosses. In the first cross, with aro and argA linked, if aro is left of hisC, colonies which are his$^+$ aro$^+$ may arise by three crossovers between the selected markers. In the reciprocal cross, with aro and proA linked, only one crossover is required to generate his$^+$ aro$^+$ colonies if aro is left of hisC, while three crossovers are required if aro is right of hisC.

In summary, if aro is right of hisC, aro$^+$ his$^+$ colonies are recovered most frequently from the first cross in which aro and argA are linked; if aro is left of hisC, aro$^+$ his$^+$ colonies are recovered most frequently from the reciprocal cross in which aro and and proA are linked. Only aro$^+$ his$^+$ colonies are considered in this analysis since such colonies will grow on MM eliminating the possibility that a colony having a heterogeneous mixture of phenotypes could be incorrectly diagnosed. As shown in Table 10, aro-1, aro-2, aro-f1, and aro-w3 map right of hisC while aro-w1 maps left of hisC.

Four-point reciprocal crosses to determine the position of aro-w1 relative to proA. Data from reciprocal crosses shown in Table 11 demonstrate that aro-w1 is right of proA. The aro-w1 mutant site has therefore been found to map clockwise of proA and counterclockwise of hisC.

Four-point reciprocal crosses to determine the position of aro mutant sites relative to argA and hisE. Evidence from four-point reciprocal crosses shown in Table 12 demonstrate that aro-1, aro-2, aro-f1, and aro-w3 map left of hisE. Data from four-point reciprocal

Table 10. Four-point reciprocal crosses to determine the position of aro mutant sites relative to hisC.

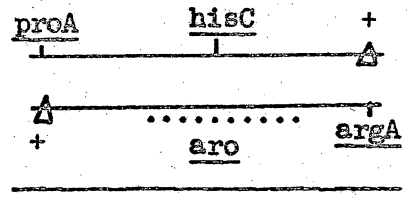
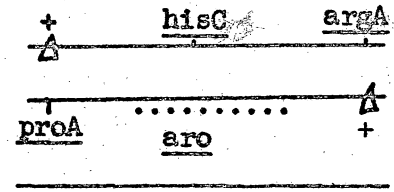
	Cross: 		Cross: 		
Aro mutant	Number of progeny	his^+ aro^+ (%)	Number of progeny	his^+ aro^+ (%)	Position relative to hisC
aro-1	50	36	95	3	Right
aro-2	50	52	49	0	Right
aro-f1	50	42	93	2	Right
aro-w1	133	0.75	147	18	Left
aro-w3	84	44	100	1	Right

Table 11. Four-point reciprocal crosses to determine the position of the aro-w1 mutant site relative to proA.

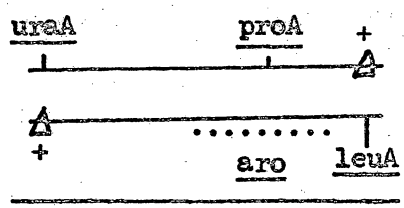
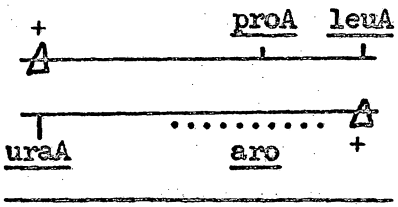
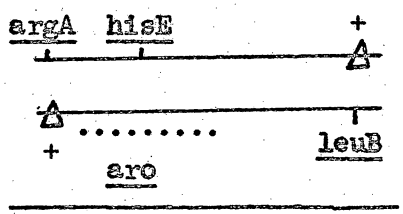
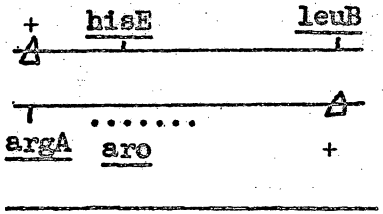
	Cross:			Cross:			
							
Aro mutant	Number of progeny	pro+ (%)	aro+	Number of progeny	pro+ (%)	aro+	Position relative to proA
aro-w1	196	1.5		200	0		Right

Table 12. Four-point reciprocal crosses to determine the position of aro-1, aro-2, aro-f1, and aro-w3 relative to hisE.

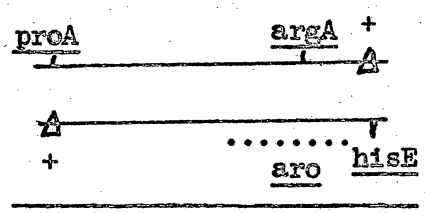
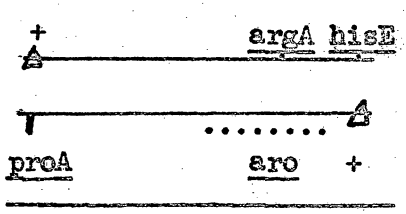
Aro mutant	Cross:  Number of progeny	$his E^+$ aro^+ (%)	Cross:  Number of progeny	$his E^+$ aro^+ (%)	Position relative to hisE
aro-1	295	0.6	193	26	Left
aro-2	260	0.7	187	38	Left
aro-f1	245	2.4	185	19	Left
aro-w3	121	0	150	16	Left

crosses shown in Table 13 demonstrate that aro-1, aro-f1, and aro-w3 map right of argA and are therefore clockwise of argA and counterclockwise of hisE. The data for aro-2 in Table 13 indicate that aro-2 is left of argA; however, this evidence is so weak, particularly when considered in light of complementation behavior to be presented later, that it seems higly unlikely that aro-2 could be left of argA. The position of the aro-2 mutant site relative to argA has not been determined with confidence; nevertheless, aro-2 is certainly closely linked to argA. A summary of aro mutant site positions is given in Figure 5.

Complementation analysis. Complementation reactions of all five Aro mutants were tested. Complementation is generally considered evidence that two mutant sites occur in different genes and thus affect different polypeptides. Furthermore, complementation response may indicate that a mutant is an operator-negative mutant. In S. coelicolor, the identification of heteroclones is considered sufficient to show that two mutants complement (Hopwood 1967). Heteroclones are colonies derived from crosses in which individual colonies bear haploid recombinants which are genetically different.

Failure to complement is evidence that two mutant sites occur in the same gene. As with other experiments in which failure to observe something is the basis of a conclusion, it is necessary to show that the failure is not attributable to some uncontrolled process. Failure to find heteroclones coupled with identification of haploid recombinants formed by crossover between the two mutant sites whose complementation reaction is being studied means that failure to com-

Table 13. Four-point reciprocal crosses to determine the position of aro-1, aro-2, aro-f1, and aro-w3 relative to argA.

	Cross: 		Cross: 		
Aro mutant	Number of progeny	$argA^+$ aro^+ (%)	Number of progeny	$argA^+$ aro^+ (%)	Position relative to argA
aro-1	204	2.5	87	0	Right
aro-2	197	0	214	0.9	Not determined
aro-f1	171	3.5	177	0.6	Right
aro-w3	200	7.0	113	1.8	Right

plement, i.e., to form heteroclones, is not a consequence of some incompatibility preventing the formation of merozygotes disomic for the region embracing both aro mutant sites--a necessary prior condition for formation of heteroclones. The complementation analysis reported here satisfies these criteria.

The complementation analysis was done as follows. Aro mutants linked with proA were crossed with Aro mutants linked with hisE. Serial dilutions of cells from the cross were plated on two types of media: (i) a medium containing PRO and HIS + PUR; and (ii) a medium containing aromatic amino acids, PABA, and YE. Figure 8 shows the selection effected by these media. After two to three days, colonies were counted, and the ratio of the number of colonies on the medium selecting hypothetical markers $aroX^+$ and $aroY^+$ (Figure 8) to the number of colonies on the medium selecting $proA^+$ and $hisE^+$ was determined. The colonies on the medium selecting markers $aroX^+$ and $aroY^+$ were streaked to the appropriate medium and diagnosed for heteroclones and haploid recombinants.

Complementation response--evidence from ratio of colonies on two types of selective media. If aroX and aroY do not complement, colonies with only one type of haploid recombinant per colony may result from the $aroX^+$ and $aroY^+$ selection by a crossover in the very small interval between aroX and aroY. This type of colony should be infrequent if two closely linked mutants fail to complement. Thus a value less than one for the ratio: # of colonies when $aroX^+$ and $aroY^+$ selected/ # of colonies when $proA^+$ and $hisE^+$ selected is an indication

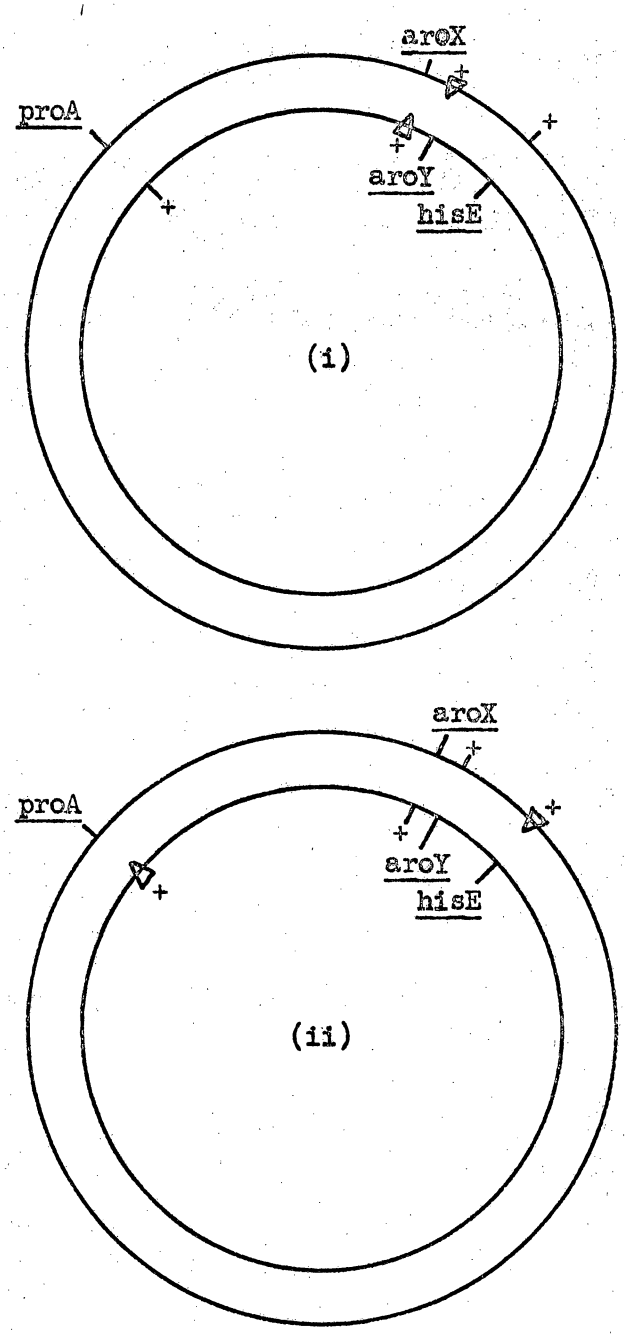


Figure 8. Representation of selections done in complementation tests. Selected markers are shown with triangles. AroX and aroY are closely linked hypothetical mutant sites whose complementation response is being determined.

whether complementation occured--if mutants complement there is no reason to suppose that they could not give rise to viable units capable of developing into colonies on a medium which lacks the supplement required by both mutants. A low value (less than one) for the ratio, by itself, is not sufficient evidence that complementation did not occur. One must show that only colonies bearing a single type of haploid recombinant per colony, i.e., no heteroclones, developed on the medium selecting aroX$^+$ and aroY$^+$. An inherent problem is that so few colonies may appear on the medium selecting aroX$^+$ and aroY$^+$ that analysis of a sufficient number of colonies to determine whether heteroclones are present is precluded. Since absence of evidence for complementation (few colonies on the medium selecting aroX$^+$ and aroY$^+$; and too few colonies occurring on this medium to show definitively that heteroclones are absent) could be attributed to the failure to form merozygotes disomic in the region embracing the aro mutant sites, it is necessary to have an internal control to determine whether merozygotes disomic for the region embracing the aro mutant sites were indeed formed. Identification of haploid recombinants formed by crossover between aroX and aroY thus means that failure to complement is not a consequence of some incompatibility preventing the formation of merozygotes disomic for the region embracing both aro mutant sites.

In summary, one may conclude that two mutants do not complement when the following syndrome is exhibited: a low ratio of colonies on the selective media, no heteroclones formed and haploid recombi-

nants formed by crossover between the two mutant sites. It is evident in Table 14 that aro-1 and aro-2 do not complement based on the observation of the syndrome just discussed.

Complementation response--evidence for complementation based on presence of heteroclones. Heteroclones are colonies derived from crosses in which a single colony bears haploid recombinants which are genetically different. Colonies appearing on the medium selecting markers aroX$^+$ and aroY$^+$ are streaked to the same kind of medium on which the colonies developed. After incubating for two to three days, the streak plates are then replicated to media which diagnose for heterozygosity of outside markers. In the example shown in Figure 8, heteroclones from the aroX$^+$ and aroY$^+$ selection will have cells which are his$^+$ and his and/or pro$^+$ and pro. This means that merozygotes disomic for the region embracing the aro mutant sites and hisE or proA were formed and replicated on medium lacking aromatic amino acids; meaning the reactions affected by complementing Aro mutants are not identical. Evidence presented in Table 14 indicates that all Aro mutants, so far tested, affect different genes, except Aro-1 and Aro-2 which fail to complement. The overall complementation response for these mutants is summarized in Table 15.

Table 14. Complementation analysis of Aro mutants.

Mutants	# colonies on aro^+-aro^+ selection / # colonies on pro^+-his^+ selection	# colonies from aro^+-aro^+ selection analyzed	Heteroclones (%)	Complementation
Aro-1 X Aro-2	0.01	7[a]	0	No
Aro-1 X Aro-w1	1.8	113	2.3	Yes
Aro-1 X Aro-w3	.33	80	26	Yes
Aro-2 X Aro-f1	1.4	58	21	Yes
Aro-2 X Aro-w1	1.0	120	2.5	Yes
Aro-2 X Aro-w3	1.5	99	33	Yes
Aro-w1 X Aro-w3	1.0	119	5	Yes

[a] These colonies were homozygous, that is, an individual colony never containeda mixture of pro^+ and pro cells or a mixture of his^+ and his cells. One colony had only pro^+ his^+ cells and presumably arose by recombination between the aro-1 and aro-2 mutant sites. This evidence eliminates the possibility that failure to recover heteroclones was due to an inability to form merozygotes disomic for the region embracing both aro mutant sites.

Table 15. Summary of complementation response of Aro mutants.

	Aro-1	Aro-2	Aro-f1	Aro-w1
Aro-2	0			
Aro-f1	NT	+		
Aro-w1	+	+	NT	
Aro-w3	+	+	NT	+

Responses are indicated: no complementation as 0; complementation as +; not tested as NT.

DISCUSSION AND CONCLUSIONS

All five Aro mutants described are probably the result of single mutational blocks. This assumption is based on the following: (i) Simultaneous mutations blocking the TRP, TYR, PHE, and PABA pathways, in the case of Aro-2, Aro-w1, and Aro-w3, or even of only the TYR and PHE pathways, in the case of Aro-f1, would be an exceedingly unlikely event; (ii) Cultures of Aro-2 have spontaneously reverted to the wild-type; and (iii) The various aromatic requirements never segregate during crosses.

The undetermined requirement for a component of YE in Aro-w1 and Aro-w3 is probably an aromatic vitamin. This is indicated by the relatively low concentration of YE required (5 µg/ml) and also by the failure of the YE requirement to segregate from the other aromatic requirements. The absence of a requirement for YE in Aro-1 and Aro-2 or for PABA in Aro-1 is probably due to leakiness of the mutational blocks which allows chorismic acid to be formed in quantities sufficient to serve as a precursor of vitamin production but not for amino acid production.

The ability of wild-type cells to utilize shikimic acid as a sole carbon source is an indication that the failure of Aro mutants to utilize shikimic acid in place of aromatic amino acids is due to their being blocked in post-shikimic acid reactions. Unfortunately the experiment described was not definitive since it did not eliminate the possibility that shikimic acid was being broken down in the media before getting into cells. It should be pointed out; however, that permeability to shikimic acid is not a problem in other bacteria

and fungi in which the pathway has been studied including Trichophyton rubrum in which shikimic acid-U-C^{14} was taken up even though this organism does not synthesize or utilize this compound for amino acid production.

Based on mapping and complementation analysis the five aro mutations have been shown to occur in at least three genes. These are (i) the gene defined by aro-w1 which is here designated aroB; (ii) the gene in which the noncomplementing mutations aro-1 and aro-2 occur which is here designated aroC; (iii) the gene in which aro-w3 occurs. Aro-f1 defines the gene aroA which is possibly the same gene in which aro-w3 occurs; however, further complementation analysis is necessary to determine this. The mapping of aro-f1 between argA and hisE in this research supports the findings of Hopwood and Friend (personal communication).

The ability of four Aro mutants having a TRP requirement to utilize anthranilic acid in place of TRP is the first indication that failure to obtain mutants of S. coelicolor able to utilize anthranilic acid in place of TRP, and presumably defecient in anthranilate synthase activity, is not due to impermeability of cells to anthranilate. The failure to isolate anthranilate utilizing TRP auxotrophs thus remains an unresolved problem. A possible explanation of the failure to find anthranilate utilizing TRP auxotrophs is that the gene for anthranilate synthase also codes for a step between anthranilate and indole. Two activities for a single polypeptide is a well documented phenomenon (Creighton and Yanofsky 1966).

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GENETIC AND PHENOTYPIC ANALYSES OF AUXOTROPHS REQUIRING AROMATIC AMINO ACIDS AND VIATMINS IN STREPTOMYCES COELICOLOR

by

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(ABSTRACT)

Five mutants of Streptomyces coelicolor having an auxotrophic requirement for aromatic amino acids were mapped relative to closely linked genetic markers. Based on four-point reciprocal crosses mutants were found to map in at least two locations. One mutant mapped between proA and hisC while three fell between argA and hisE. Another mutant mapped between hisC and hisE; however, its position relative to argA has not been determined.

Phenotypic analysis of the Aro mutants was undertaken. One mutant has a requirement for tyrosine and phenylalanine; one mutant has a requirement for all three aromatic amino acids, tryptophan, tyrosine, and phenylalanine; one mutant has a requirement for aromatic amino acids and para-aminobenzoic acid; and two mutants have a requirement for aromatic amino acids, para-aminobenzoic acid, and an undetermined requirement satisfied by 5 µg/ml of yeast extract. None of the mutants were able to utilize quinic acid or shikimic acid in place of the aromatic requirements; however, the wild-type strain is able to utilize shikimic acid as a sole carbon source. Mutants having a tryptophan requirement were able to utilize anthranilic acid or indole in place of tryptophan.

Complementation tests based on analysis of heteroclones have shown that the five mutants affect at least three different genes. Two genes have been designated aroB and aroC; mutants in these genes did not complement aro-f1 which defines aroA.