A GENETIC AND BIOCHEMICAL STUDY OF HISTIDINE BIOSYNTHESIS IN *MICROCOCCUS LUTEUS*[1]

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ABSTRACT

Histidine auxotrophs of *Micrococcus luteus* strain ATCC 27141 were induced by treatment of the parent strain with N-methyl-N'-nitro-N-nitrosoguanidine. Auxotrophs were biochemically characterized by examining culture accumulations of histidine intermediates, using paper chromatography and the Bratton-Marshall test, and growth responses to L-histidinol. *his(IG)* mutants failed to accumulate Pauly-positive imidazoles; *his(EAHF)* mutants accumulated 5-amino-1-ribosyl-4-imidazole carboxamide; *hisB* mutants accumulated imidazoleglycerol; *hisC* mutants accumulated imidazoleacetol; *hisD* mutants accumulated histidinol. L-histidinol failed to stimulate the growth of *hisD* mutants, but did stimulate all other histidine mutants, blocked at earlier steps in the biosynthetic pathway. In addition, imidazoleglycerol phosphate dehydrase activity was assayed in representative mutants of each class. *hisB* mutants lacked activity for this enzyme.—Two-point, three-point, and cotransformation analyses resolved linkage relationships of histidine genes and in two gene clusters aided in determining their sequences. Histidine biosynthetic genes exist in at least four separate, unlinked regions of the chromosome. One histidine gene cluster is closely linked to a tryptophan gene cluster and appears to be contiguous in the sequence *his(IG)–his(EAHF)–trpE–trpC–trpB–trpA*. A second and unlinked histidine cluster has the tentative gene sequence *his(EAHF)–hisB–hisC–his(EAHF)*. The *hisD* gene and an unclassified mutant site *his-94* are not linked to any of the other histidine genes examined in this study or to each other.

THE genetics and biochemistry of histidine biosynthesis have been investigated in a variety of microorganisms. Eukaryotic organisms studied include *Neurospora crassa* (AHMED, CASE and GILES 1964; CATCHESIDE 1965), *Saccharomyces cerevisiae* (FINK 1964, 1966), and *Aspergillus nidulans* (BERLYN 1967). The histidine biosynthetic genes were carried on several different chromosomes in all three organisms, but there was one gene cluster containing three genes (for steps 3, 2, and 10 of the histidine biosynthetic pathway) that was common to each organism.

Prokaryotic organisms also showed diversity in their gene arrangements. In *Pseudomonas aeruginosa*, the histidine genes are apparently located in at least

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five unlinked groups (MEE and LEE 1967). *Streptomyces coelicolor* (RUSSI *et al.* 1966) has five histidine genes located in a cluster and three other genes at separate sites. *Bacillus subtilis* (CHAPMAN and NESTER 1969) has histidine genes located in at least three separate unlinked regions of the chromosome, and in two of these regions, histidine genes are located in clusters. *Salmonella typhimurium* (AMES and HARTMAN 1963; LOPER *et al.* 1964), *Escherichia coli* (GARRICK-SILVERSMITH and HARTMAN 1970), and *Staphylococcus aureus* (KLOOS and PATTEE 1965a, 1965b) appear to have histidine genes arranged in a single cluster (or operon) in a similar sequence. The linkage relationships of histidine genes in these organisms are shown in Figure 1.

Biochemical studies with *S. typhimurium* have revealed that the biosynthesis of L-histidine involves ten enzymatic steps, beginning with the condensation of 5-phosphoribosyl-1-pyrophosphate (PRPP) with adenosine triphosphate (ATP) to form N-1-(5′phosphoribosyl) adenosine triphosphate (PR-ATP) (AMES, MARTIN and GARRY 1961; AMES and HARTMAN 1962; LOPER *et al.* 1964; SMITH and AMES 1965). The enzymatic steps, pathway intermediates, and genes related to histidine biosynthesis are outlined in Figure 2. Histidine biosynthesis appears to follow similar steps in other microorganisms; however, in most, only parts of the pathway have been studied in detail.

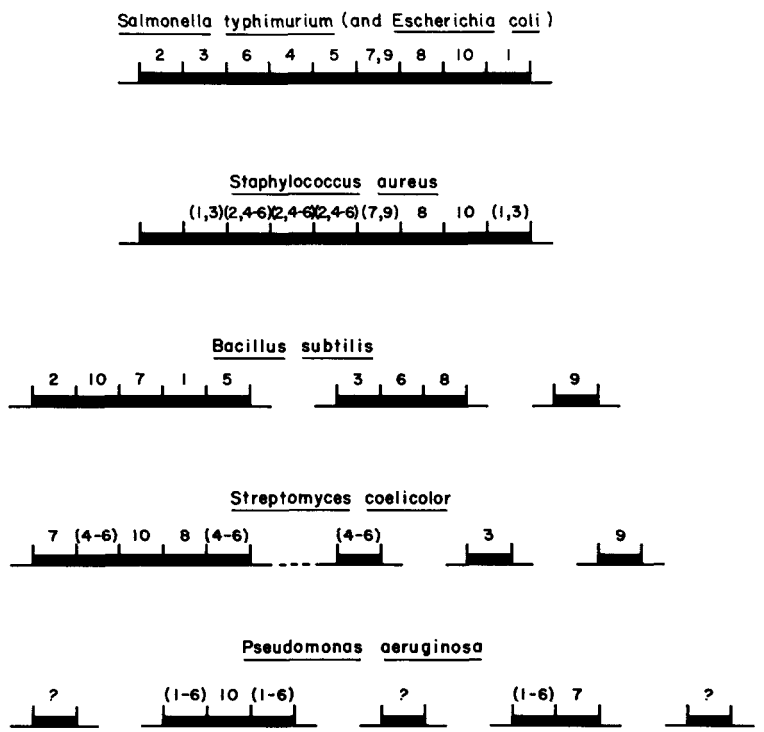

FIGURE 1.—Linkage groups of histidine genes in various bacteria. Genes are designated by the corresponding number of the step they control in the biosynthetic pathway. Parentheses indicate that the exact designation of the gene is not known.

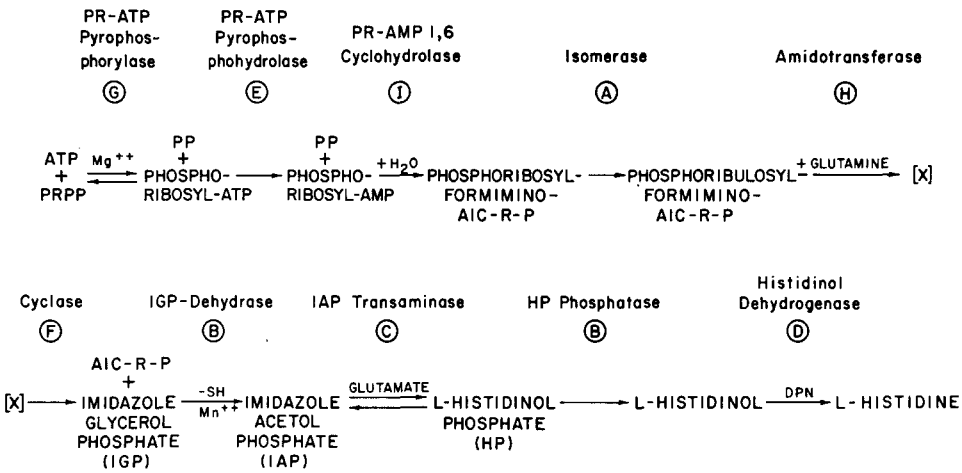

FIGURE 2.—Pathway of histidine biosynthesis, as determined in *Salmonella typhimurium* (AMES and HARTMAN 1963; SMITH and AMES 1965). The encircled letters represent the Salmonella genes coding the enzymes. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-AMP and PR-ATP, N-1-(5′-phosphoribosyl) adenosine mono- and triphosphate; AIC-R-P, 5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide.

Genetic and biochemical studies of *Micrococcus luteus* were undertaken to compare the chromosomal arrangement of histidine genes with those of other organisms and further to compare steps in histidine biosynthesis.

MATERIALS AND METHODS

Bacterial strains: All *Micrococcus luteus* histidine and tryptophan auxotrophs used in this study were derived from an adenine-independent mutant *(ade -1)* of the high transforming strain *ATCC 27141*. The tryptophan auxotrophs have been previously described (KLOOS and ROSE 1970). Mutant auxotrophs were induced by treatment of the parent strain with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (300 μg/ml) (ADELBERG, MANDEL and CHEN 1965).

Histidine auxotrophs *hisB456*, *hisB578*, *hisC2*, *hisD108*, *hisD494*, *hisG1302*, and *hisE11* of *Salmonella typhimurium* (HARTMAN, LOPER and SERMAN 1960) were used as sources of histidine intermediates and references in enzyme assays. They were obtained from DR. R. TWAROG (University of North Carolina, Chapel Hill, North Carolina) and DR. C. ATKINS (Ohio University, Athens, Ohio).

Media: The complex broth (P broth) and agar (P agar) media used to propagate *M. luteus* have been previously described by NAYLOR and BURGI (1956). The defined broth and agar media have been described by KLOOS (1969a) and KLOOS and SCHULTES (1969), respectively. The defined agar medium was prepared by adding autoclaved defined broth to a separately autoclaved solution of liquefied Special Noble Agar (Difco). Supplementation concentrations of L-histidine, L-histidinol-HCl, or L-tryptophan added to defined media varied depending upon experimental requirements. The glucose-salts Medium E of VOGEL and BONNER (1956) was used for culturing *S. typhimurium* histidine auxotrophs.

Growth conditions: All inocula of *M. luteus* and *S. typhimurium* for experiments came from 18-hr P agar slope cultures. *M. luteus* was cultured at 34° while *S. typhimurium* was cultured at 37°. Broth cultures were shaken on either a Burrell Wrist-Action Shaker (Burrell Corporation, Pittsburgh, Pennsylvania) with arms mounted over water baths, a Fermentation Design Rotary Shaker (Fermentation Design Incorporated, Allentown, Pennsylvania), or in a Controlled

Environment Incubator Shaker (New Brunswick Scientific Company, New Brunswick, New Jersey). Cultures were stored on P agar slopes at 4°.

Biochemical characterization of histidine auxotrophs: All histidine auxotrophs were tested for their ability to utilize exogenous L-histidine and L-histidinol by two procedures. In the first procedure, about 10^7 cocci of each mutant strain were spread over the surface of a defined agar medium supplemented with 20 μg/ml L-histidine or L-histidinol-HCl. In the second procedure, auxotrophs were spread onto unsupplemented defined agar media and several crystals of L-histidine and L-histidinol-HCl were then placed on opposite sides of each agar plate. Growth responses were scored after incubation for 48 hr.

Histidine auxotrophs were further characterized according to their accumulation of histidine precursors and/or their derivatives, which were identified by paper chromatography (AMES and MITCHELL 1952; KLOOS and PATTEE 1965a) and the Bratton-Marshall test (as modified by AMES, MARTIN and GARRY 1961). *M. luteus* mutants were propagated for accumulations in the defined broth medium described above with the omission of L-tyrosine and NH_4Cl, both of which interfered with chromatogram interpretation. Separate tubes containing 5 ml of the modified defined broth supplemented with 20 μg/ml L-histidine were inoculated with each mutant and were shaken at 300 rpm on a rotary shaker for 48 rh. For paper chromatography of Pauly-positive imidazoles, culture supernatant fluids were lyophilized to dryness and then rehydrated with 0.2 ml deionized water for use in spotting. For detection of Bratton-Marshall-positive compounds, a 1-ml sample of the above culture was used to inoculate 20 ml of modified defined broth supplemented with 1 μg/ml L-histidine in Erlenmeyer flasks. Flask cultures were shaken at 300 rpm on a rotary shaker for 48 hr. This second propagation step, in the presence of low levels of L-histidine, was necessary for obtaining maximum accumulations of these compounds, but not for other imidazoles. The supernatant fluids were collected and chilled to 4° prior to use in the Bratton-Marshall test or lyophilized to dryness and then rehydrated as above for use in paper chromatography.

Imidazoleglycerol phosphate dehydrase activity was assayed in various mutants by the method of AMES, GARRY and HERZENBERG (1960). Cell extracts for enzyme assays were prepared by the following procedure. Derepressed cells were harvested and washed 3X in 0.1 M triethanol-amine-HCl (PO_4-free) buffer, pH 8.1 at 4°, followed by resuspension of cells in buffer containing 10% glycerol. Next, 5 mg of lysozyme (muramidase) was added to each cell suspension. The mixture was incubated 10 min at 34°. After this time, it was chilled in an ice bath and the partially-lysed cells were sonicated for 10 sec at 38 A with a Sonifier Cell Disruptor (Heat Systems—Ultrasonics, Incorporated, Plainview, New York). The resulting crude cell extract was then used in assays. The protein concentration of cell extracts was determined by the method of LOWRY *et al.* (1951).

Preparation of transforming DNA: The culture of donor strains for DNA isolation and the preparation of transforming DNA have been previously described (KLOOS 1969b).

Transformation procedures: Transformations were done using the procedures of KLOOS and ROSE (1970).

Two-point crosses: Representative *M. luteus* histidine auxotrophs of each biochemical class were crossed in pairwise combinations in transformation experiments to aid in determining the linkage relationships of histidine genes. Linkage was estimated by the use of recombination indices according to the method of CHAPMAN and NESTER (1969).

Cotransformation analysis: An additional test of linkage and an estimate of the relative distance of certain histidine genes from reference tryptophan markers (*trpE16* and *trpA6*) were accomplished using the ratio test (HARTMAN, LOPER and SERMAN 1960). Reciprocal transformation crosses were performed between different histidine auxotrophs and the reference tryptophan auxotrophs. Procedures for enumerating and verifying donor-type cotransformants have been previously described (KLOOS and ROSE 1970).

The rationale of the ratio test in estimating relative marker distances is that when one auxotrophic marker is in the donor DNA and another is in the recipient DNA, the frequency of prototrophic transformants will be directly related to the distance separating mutant sites;

whereas, the frequency of donor-type cotransformants will be inversely related to the distance.

Three-point crosses: Double mutants containing the reference marker *trpE16* together with the desired histidine marker were constructed by transforming the recipient strain *trpE16A6* with DNA from the histidine-requiring donor strain. Cells from crosses were plated onto defined agar media supplemented with 20 μg/ml anthranilic acid and 20 μg/ml L-histidine to permit growth of prototrophic, *hisX*−, *trpE16*, and *trpE16hisX*− recombinants. Recombinants were then tested for growth on unsupplemented defined agar media to identify the prototrophic recombinants and on defined agar media supplemented with either 20 μg/ml anthranilic acid or 20 μg/ml L-histidine to identify the *trpE16* and *hisX*− recombinants, respectively. Auxotrophs failing to grow on either of the above supplemented media were suspected of being *trpE16hisX*− double mutants and were verified by (1) their failure to accumulate tryptophan intermediates, (2) their failure to produce prototrophic recombinants when crossed with either mutant parent strain, but ability to produce prototrophic recombinants with DNA from the original prototrophic strain, and (3) their continuing requirement for both L-histidine and L-tryptophan for growth.

Three-point crosses were conducted according to the procedures of CARLTON (1967). Double histidine-tryptophan mutants were used as recipients in crosses with various histidine mutant donors. Cells from crosses were plated onto defined agar media supplemented with 20 μg/ml anthranilic acid to permit growth of prototrophic and *trpE16* recombinants. The recombinant colonies were then replica plated onto an unsupplemented defined agar medium to distinguish the prototrophs from *trpE16* auxotrophs.

The rationale for marker order assignment in three-point crosses is that if the single histidine mutant site of a donor is located between the reference marker *trpE16* and the histidine marker *hisX*− in the recipient, then it would require a quadruple crossover to yield a prototroph; however, if the donor mutant site is outside, then only a double crossover would be required. Prototrophs are calculated as the percentage of the recombinant classes *trpE+hisX+Y+* (*prototroph*) and *trpEhisX+Y+* (*trpE16*) (*X* and *Y* represent the two histidine markers in the cross).

RESULTS

Biochemical characterization of histidine auxotrophs: A total of 184 histidine auxotrophs (*his-1* through *his-63*; *his-64* through *his-184*) of *Micrococcus luteus* strain ATCC 27141 were isolated in two separate experiments using NTG as the inducing agent. Of these, 79 were characterized and are listed in Table 1. The remaining 105 mutants were omitted from further study due to excessive leakiness, poor growth, or high reversion rate. All auxotrophs selected were genetically recombinable and were, therefore, of independent origin.

Accumulation patterns, growth responses, and imidazoleglycerol phosphate dehydrase (IGP dehydrase) activities separated histidine auxotrophs into classes that were consistent with those of *S. typhimurium* (HARTMAN, LOPER and SERMAN 1960; SMITH and AMES 1964). *his(IG)* mutants failed to accumulate Pauly-positive imidazoles, as tested by paper chromatography, or Bratton-Marshall positive diazotizable amines. Growth of these mutants was stimulated by exogenous L-histidinol and, as expected, they demonstrated IGP dehydrase (gene *B*) activity. In this study, we were unable to resolve *hisI* from *hisG* mutants and, hence, use the designation *his(IG)* to identify either type. Their resolution would require the additional enzyme assays for phosphoribosyl-adenosine monophosphate hydrolase and phosphoribosyl- adenosine triphosphate pyrophosphorylase, respectively.

his(EAHF) mutants accumulated 5-amino-1-ribosyl-4-imidazole carboxamide

TABLE 1

Biochemical characterization of Micrococcus luteus *histidine auxotrophs*

Mutant class	Representative strains	Growth response	Terminal accumulated intermediate	Imidazole glycerol phosphate dehydrase activity*
his(IG)	10,21,26,35,46,39,50,66, 68,74,75,80,88,96,103, 107,112,115,116,128	L-histidinol or L-histidine	none	+
his(EAHF)	2,14,17,19,33,36,43,45, 51,52,56,84,91,100,102, 109,110,118,120,121,130	L-histidinol or L-histidine	5-amino-1-ribosyl-4-imidazole carboxamide	+
hisB	7,8,9,25,31,34,37,38,40, 49,55,70,72,97,106,113, 123,124,129	L-histidinol or L-histidine	imidazole glycerol	—
hisC	22,77,86,95,126,127	L-histidinol or L-histidine	imidazole acetol	+
hisD	32,53,58,61,63,76,82,87, 90,92,98,114	L-histidine	histidinol	+
unknown	*his-94*	L-histidinol or L-histidine	imidazole glycerol	+

* Symbols: + = specific activity (μmoles substrate/min/mg protein) of 6.7–72 $\times$ 10^{-3}; — = specific activity of 0–2.5 $\times$ 10^{-3} (leaky).

(AIC-R), as detected by both paper chromatography (using Pauly's spray reagent) and the Bratton-Marshall test. These mutants were also stimulated by L-histidinol and had IGP dehydrase activity. In this study, we were unable to resolve *hisE*, *hisA*, *hisH*, or *hisF* mutants from each other and, hence, use the designation *his(EAHF)*. Their resolution would require the additional enzyme assays for phosphoribosyl-adenosine triphosphate pyrophosphohydrolase, isomerase, amidotransferase, and cyclase, respectively.

hisB mutants accumulated imidazoleglycerol and, in addition, some also accumulated AIC-R. They were stimulated by L-histidinol and, with the exception of mutant *hisB72*, failed to demonstrate detectable IGP dehydrase activity. Mutant *hisB72* had a low specific activity (2.5×10^{-3}) for this enzyme, which may be indicating an incomplete genetic block. One mutant, *his-94*, accumulated only imidazoleglycerol as did *hisB* mutants. But, unlike these mutants, it demonstrated a moderate specific activity (1.1×10^{-2}) for IGP dehydrase. It, therefore, remains unclassified.

hisC mutants accumulated imidazoleacetol and, in addition, some also accumulated imidazoleglycerol. They were stimulated by L-histidinol and had IGP dehydrase activity.

hisD mutants accumulated histidinol and, in addition, some also accumulated imidazoleacetol and/or imidazoleglycerol. They failed to grow with L-histidinol in place of L-histidine and had IGP dehydrase activity.

Linkage of histidine genes to the tryptophan gene cluster: Preferring the increased accuracy of map orientation conferred by cotransformation and three-point transformation analyses over two-point (best-fit) transformation, we selected *hisD* mutants and different nutritional mutants (*trpE16*, *trp(DF)2*, *purE1*, *purC4*, *aro-1*) to be used as donors in crosses with histidine auxotrophs of each biochemical class in a search for linkage. If linkage between certain histidine genes and another nutritionally-definable gene could be found, two of the necessary requirements for the above mapping techniques would be satisfied; i.e., the markers in the cross must be linked and two must be phenotypically distinguishable.

Evidence for linkage of *trpE* to *his(IG)* and *his(EAHF)* genes was obtained by the appearance of donor-type cotransformants in crosses between mutant *trpE16* and *his(IG)* and certain *his(EAHF)* mutants. Donor-type cotransformants were not observed in crosses between *trpE16* and other *his* mutants or in crosses using *hisD* or other nutritional mutants as donors. Also, some *his(EAHF)* mutants used as recipients failed to produce donor-type cotransformants with DNA from *trpE16* and are presumably unlinked to those that produced cotransformants. Those failing to produce cotransformants include *his(EAHF)19*, *-45*, *-33*, *-100*, *-118*, and *-120*. The relative distances of other *his(EAHF)* mutant sites and *his(IG)* mutant sites from the tryptophan gene (*trp*) cluster are demonstrated by the probability of independent integration (p) as shown in Table 2. Data are the average of five separate crosses. The total number of recombinant colonies obtained in each cross to estimate p was in a range of 2,032–6,224.

TABLE 2

The probability of independent integration of different histidine markers into trpE *and* trpA *mutants of* Micrococcus luteus*

Donor strain	Recipient strain† *trpE16*	*trpA6*
his(IG)116	0.23	0.36
-75	0.20	0.32
-74	0.20	0.39
-26	0.19	0.44
his(EAHF)14	0.18	0.31
-56	0.17	0.50
-91	0.16	0.32
-109	0.15	0.34
-43	0.14	0.31
-102	0.14	0.33
-36	0.12	0.27
-52	0.11	0.34

* Figures represent the probability of independent integration (p), which is equal to the number of prototrophic transformants divided by the number of prototrophic + donor-type cotransformants.

† *trpE16* and *trpA6* mutant sites are at opposite ends of the tryptophan gene cluster (Kloos and Rose 1970).

Reciprocal crosses were made using the histidine mutants as recipients and *trp* mutants as donors; however, since many of the histidine mutants had rather poor recipient competency, data from these crosses have been omitted. As can be seen from the data in Table 2, histidine mutant sites appear to be located near the *trpE* end of the *trp* cluster. Based on the *p* values obtained with *trpE16*, the presumed closer and more useful tryptophan marker, *his(IG)* mutant sites appear to be distal and *his(EAHF)* mutant sites appear to be proximal, possibly even contiguous, to the *trp* cluster. Some ambiguity was observed in the relative distances between mutant sites if comparisons were made with both *trp* markers.

Three-point transformation crosses were used to gain further information on the arrangement of histidine markers linked to the *trp* cluster. The results of these crosses are shown in Table 3.

CARLTON (1966) found that in mapping tryptophan mutant sites in *Bacillus subtilis* such crosses fell into two classes. Those assumed to require a quadruple crossover to produce prototrophic recombinants had less than 25% prototrophs, and those assumed to require a double crossover had percentages greater than 40. KLOOS and ROSE (1970) obtained rather similar results mapping tryptophan mutant sites in *M. luteus*. In this study, the percentage of prototrophic recombinants in crosses where the donor mutant site was assumed to be between the two recipient mutant sites was usually less than 40; whereas, in crosses where the donor mutant site was assumed to be outside these sites, it was usually 60 or greater. Only a few crosses produced ambiguous results with percentages of prototrophic recombinants between 40 and 60. The total number of recombinants scored in each cross was in a range of 100–1,123.

The sequence of histidine mutant sites, determined by three-point transforma-

TABLE 3

Three-point transformation mapping of histidine mutant sites linked to the tryptophan gene cluster of Micrococcus luteus*

Donor strain(Y⁻)	Recipient strain (*trpE16hisX*⁻)											
	his(EAHF)14	*-36*	*-52*	*-109*	*-56*	*-102*	*-43*	*-91*	*his(IG)74*	*-26*	*-75*	*-116*
his(EAHF)14	0	15	18	17	13	13	11	15	12	6	7	9
-36	73	0	34	49	20	18	30	20	12	9	11	14
-52	79	43	0	42	27	16	18	12	16	13	15	12
-109	53	87	74	0	52	32	53	24	18	14	19	16
-56	77	69	67	59	0	31	25	25	11	19	15	15
-102	73	81	78	76	64	0	18	29	18	11	13	10
-43	79	68	78	78	68	69	0	29	18	16	19	10
-91	76	76	77	80	70	66	74	0	14	7	8	7
his(IG)74	86	79	78	77	74	70	72	76	0	30	7	18
-26	82	81	82	73	69	77	77	78	60	0	19	20
-75	87	81	76	82	67	73	72	80	58	60	0	24
-116	83	80	82	81	72	69	77	73	72	74	65	0

* Figures represent the percentage of *trpE*+ recombinants in the X+Y+ recombinant classes.

tion analysis, is *his(IG)116 –75 –26 –74 – his(EAHF)91 –43 –102 –56 –109 –52 –36 –14 – trpE16*. This result is consistent with the general separation of *his(IG)* and *his(EAHF)* mutant sites by cotransformation analysis.

Several results of these studies would suggest that the histidine mutant sites linked to the *trp* cluster extend out from the *trpE* end rather than extend between *trpE* and *trpA* and into the cluster. For example, the more distal position of *his(IG)* compared to *his(EAHF)* mutant sites from *trpE* does not appear to be associated with a closer positioning of these sites to *trpA*, as determined by cotransformation analysis. Furthermore, the very close and continuous *p* values obtained in transformation crosses between different tryptophan mutants (KLOOS and ROSE 1970) would suggest that additional genes are not dispersed between those recognized in the *trp* cluster.

Other evidence for excluding histidine mutant sites from within the *trp* cluster comes from three-point transformation crosses between selected histidine and double tryptophan mutants. These crosses were conducted and analyzed by two different procedures: by CARLTON's (1966) procedure, as discussed above, and by reciprocal transformation. The two hypothetical gene arrangements being considered are diagrammed in Figure 3 and the results of both types of three-point transformation crosses are shown in Table 4.

In crosses between *hisY⁻* and *hisX⁻trpE16* mutants (Table 3) or between *trpY⁻* and *trpX⁻trpE16* mutants (KLOOS and ROSE 1970) analyzed by the CARL-

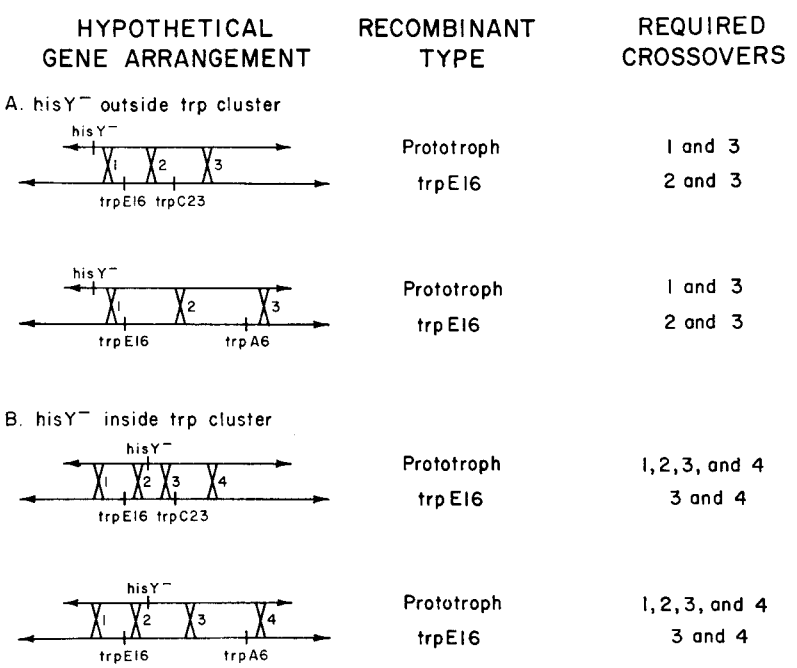

FIGURE 3.—Hypothetical three-point cross gene arrangements. The donor DNA fragment is indicated by the upper line and the recipient genome is indicated by the lower line in each configuration. Crossover regions are marked by cross lines and numbered.

TABLE 4

Three-point transformation crosses between histidine and double tryptophan mutants of Micrococcus luteus

Y^-	Recipient strain* (*trpE16X*$^-$) *trpC23*	*trpA6*	Ratio of frequency of prototrophic recombinants in reciprocal cross of Y^- × *trpE16X*$^-$† *trpC23*	*trpA6*
hisY$^-$:				
his(EAHF)14	33	25	—‡	—
-36	45	28	—	—
-52	37	30	—	—
-109	34	30	1.8	2.4
-56	37	27	—	—
-102	34	32	1.2	1.9
-43	45	28	0.7	—
-91	46	33	—	—
his(IG)74	45	36	—	—
-26	52	40	0.7	1.0
-75	48	39	1.2	2.0
-116	41	41	—	—
trpY$^-$:				
trpA6 (outside marker)	75	0	1.3	0
trpC23 (inside marker)	0	19	0	0.15

* Figures represent the percentage of *trpE*+ (prototroph) recombinants in the X^+Y^+ recombinant classes in crosses between single histidine or tryptophan mutants (Y^-) as donors and double tryptophan mutants (*trpE16X*$^-$) as recipients.

† Figures represent the ratio of prototrophic recombinants in reciprocal crosses between single histidine or tryptophan mutants (Y^-) and double tryptophan mutants (*trpE16X*$^-$). This ratio is equal to the frequency of prototrophs produced when a double *trpE16X*$^-$ mutant is a recipient/frequency of prototrophs produced when it is a donor.

‡ Symbol: — = crosses not tested.

TON procedure, the percentages of prototrophic recombinants were clearly separated into a high and low class, depending on whether the *hisY*$^-$ or *trpY*$^-$ donor mutant sites were located outside or between the two mutant sites in the recipient, respectively. As can be seen from the data in Table 4, crosses between *hisY*$^-$ and *trpE16X*$^-$ mutants produced intermediate percentages of prototrophic recombinants, lower than would be expected if the *hisY*$^-$ mutant sites were located outside of the *trp* cluster, as compared to the data from the above crosses. The reason for this is not clear. It may be related to the fact that the unselected site (*trpE*) is the middle marker in these crosses while it was a terminal marker in the crosses of Table 3. Although the percentages of prototrophs are somewhat ambiguous, we believe this experiment supports the contention that *hisY*$^-$ is located outside of the *trp* cluster. If it were, one would expect to find a higher percentage of prototrophic recombinants in crosses with *trpE16C23* mutants than with *trpE16A6* mutants due to the closer position of *trpE16* and *trpC23* mutant sites, as supported by earlier cotransformation studies with tryptophan mutants (KLOOS and ROSE 1970). The data in Table 4 are consistent with this expectation.

Results of the reciprocal transformation crosses of competent mutants (Table 4) further supported the contention that *hisY*$^-$ is located outside of the *trp* cluster. It follows that if *hisY*$^-$ were located outside, the frequency of prototrophs would be nearly equal in reciprocal crosses as shown in the data. If *hisY*$^-$ were located within the *trp* cluster, we would have expected the relative frequency of prototrophs to be very small in the cross where the double *trpE16X*$^-$ mutant was used as a recipient.

Evidence for a histidine cluster unlinked to the tryptophan genes: Representative histidine mutants of each biochemical class that failed to produce cotransformants with tryptophan mutants were crossed among themselves and to a *his(IG)* and *his(EAHF)* mutant with sites linked to the *trp* cluster. The results of certain key two-point transformation crosses are given in Table 5. The unlinked markers *trpC23* and *purE(ISU)* were used in crosses for comparisons and in calculating recombination indices. The total number of prototrophs scored in crosses was in a range of 31–2,846, which is presumably influenced by the distances separating mutant sites. The low recombination indices (<1.0) obtained in crosses between *hisB*, *hisC*, and those *his(EAHF)* mutants with sites unlinked to the *trp* cluster provide evidence for a second histidine gene (*his*) cluster in *M. luteus*. The recombination data from these crosses suggest a tentative sequence of histidine genes of *his(EAHF) – hisB – hisC – his(EAHF)* for this cluster.

Non-linkage of hisD: The high recombination indices (≥ 1.0) shown in Table 5 and the lack of cotransformants obtained in crosses between *hisD* and other histidine mutants would indicate that the *hisD* gene is not linked to any of the histidine genes examined in this study. Two minor exceptions were noted where the recombination indices were 0.89 and 0.80. *hisD* mutants also had high recombination indices of 1.0 or greater and failed to produce cotransformants in crosses with various tryptophan auxotrophs.

An additional study using the rationale of GOODGAL (1961) was done to further clarify the linkage relationship of *hisD* to the *trp* cluster and the associated histidine genes. It is apparent that if two markers are closely linked they may be cotransformed by a single transforming DNA fragment; however, if two markers are unlinked, they may be cotransformed only by two transforming DNA fragments in two separate, single transformation events. GOODGAL observed that as the concentration of DNA added to the transformation system was reduced, the number of single transformation events was also reduced. Therefore, a double mutant whose two markers were unlinked would have a more rapid reduction in the rate of cotransformation of the two markers than would be observed in a double mutant whose two markers were linked. In this study, we compared the rate of cotransformation of the double mutant *trpE16his(IG)26*, carrying linked markers, with the double mutant *trpE16hisD61*. Donor DNA for crosses was obtained from the prototrophic parent strain. It can be seen in Figure 4 that the rate of cotransformation (prototrophs) fell more rapidly when *trpE16hisD61* was the recipient than when the double mutant composed of two linked markers, *trpE16(IG)26*, was the recipient. These results provide more evidence that the

TABLE 5

Mean recombination indices of reciprocal two-point transformation crosses between histidine auxotrophs of Micrococcus luteus*

Mutant sites	*his(IG)51*	*his(EAHF)14*	*hisD61*	*hisD76*	*his-94*	*his(EAHF)45*	*–118*	*–120*	*hisB25*	*hisB113*	*hisC77*	*hisC22*	*his(EAHF)100*
his(IG)51	0.00	...	...	...	...	...	...	...	...	...	...	...	...
his(EAHF)14	—†	0.00	...	...	...	...	...	...	...	...	...	...	...
hisD61	2.57	1.10	0.00	...	...	...	...	...	...	...	...	...	...
hisD76	—	1.07	0.01	0.00	...	...	...	...	...	...	...	...	...
his–94	—	1.80	1.10	—	0.00	...	...	...	...	...	...	...	...
his(EAHF)45	—	—	—	—	—	0.00	...	...	...	...	...	...	...
–118	—	—	—	—	—	0.12	0.00	...	...	...	...	...	...
–120	—	—	—	—	—	0.13	0.03	0.00	...	...	...	...	...
hisB25	1.30	1.20	1.20	0.80	1.10	0.12	0.13	0.04	0.00	...	...	...	...
hisB113	1.70	1.00	—	—	—	—	—	—	<0.01	0.00	...	...	...
hisC77	—	—	1.07	—	—	0.17	0.23	0.17	0.02	—	0.00	...	...
hisC22	1.60	2.00	1.11	—	—	—	—	—	0.06	0.06	0.01	0.00	...
his(EAHF)100	—	—	—	—	—	0.78	0.66	0.31	0.20	—	0.12	—	0.00

* Low (<1.00) recombination indices from crosses involving presumed linked histidine mutant sites are in bold face type. The recombination index (RI) was calculated from the equation RI = (*loc m/ref m*) / (*loc w/ref w*), where *loc* is the number of transformants to the *hisX* marker, *ref* is the number of transformants to the unlinked marker *purE*, *m* is mutant *hisY* DNA, and *w* is prototrophic DNA (CHAPMAN and NESTER 1969). Mutant sites are arranged in an order showing the best fit, i.e., that producing the best uninterrupted sequence of increasing recombination indices from the closest to the most distant mutant site (HARTMAN, LOPER and SERMAN 1960).

† Symbol: — = crosses not tested.

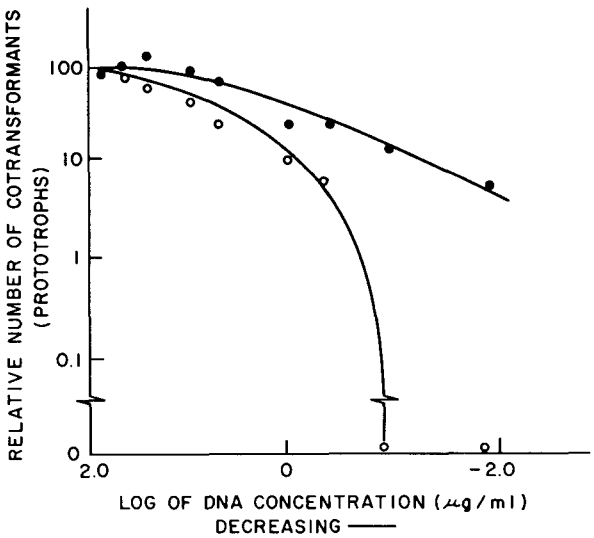

FIGURE 4.—The effect of DNA concentration on the relative number of cotransformants (prototrophs) produced in crosses between a prototrophic parent donor and double histidine-tryptophan mutant recipients. Symbols: ●, cotransformants in crosses with double mutant containing linked markers *trpE16his(IG)26*; ○, cotransformants in crosses with double mutant *trpE16-hisD61*.

hisD gene is not linked to the *trp* cluster nor to the histidine genes associated with that cluster.

Non-linkage of his-94: The high recombination indices (≥ 1.0) shown in Table 5, obtained in crosses between mutant *his-94* and other histidine mutants, indicate a lack of linkage of this unclassified histidine mutant site to (other) histidine genes examined in this study. *his-94* also had high recombination indices of 1.0 or greater and failed to produce cotransformants in crosses with various tryptophan auxotrophs. The above results provide suggestive evidence of a fourth separate region on the *M. luteus* genome containing genes that are associated with histidine biosynthesis.

DISCUSSION

The biochemical and genetic analysis of histidine auxotrophs of *M. luteus* appears to have resolved at least eight of the nine to ten genes for histidine biosynthesis found in other microorganisms. *his(IG)* mutants require enzyme analyses to determine whether they represent *hisI* or *hisG* genes, or both. *his(EAHF)* mutants also require enzyme analyses for the identification of *hisE*, *hisA*, *hisH*, or *hisF* genes. However, transformation mapping data would suggest that at least three of the four genes have been resolved genetically. The mutant *his-94* can be separated from other histidine mutants, genetically, but lacks biochemical clarification. It could conceivably have a mutation in an L-histidinol phosphate phosphatase gene as demonstrated by its ability to accumulate imidazoleglycerol

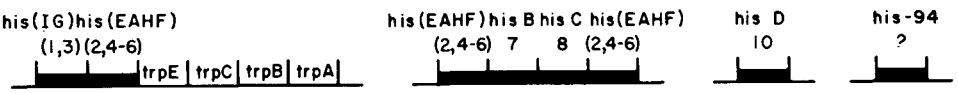

FIGURE 5.—Linkage groups of histidine genes in *Micrococcus luteus*. Genes are identified by the corresponding Salmonella letter designation and number of the step they control in the biosynthetic pathway. Parentheses indicate that the exact designation of the gene is not known.

while possessing IGP dehydrase activity. However, additional enzyme analyses would be required for complete identification.

Cotransformation and two-point transformation analyses were useful as tests for linkage of histidine genes. These procedures were also useful in intergenic mapping but were not designed for accurate intragenic mapping. The best fine structure mapping in this study was accomplished by three-point transformation and, together with earlier studies of tryptophan mutants, has unfolded a sequence of 21 mutant sites extending through at least two histidine and four tryptophan genes. A summary of the histidine linkage groups found in *M. luteus* is shown in Figure 5. These groups are different from those found in other bacteria.

Comparisons of the linkage relationships of genes in bacteria suggest the possibility that there exists a conservation of linkage between histidine and tryptophan genes. As shown in this study with *M. luteus*, at least two histidine genes are closely linked to a *trp* cluster. *Bacillus subtilis* also has a number of histidine genes closely linked to a *trp* cluster (NESTER, SCHAFER and LEDERBERG 1963; CHAPMAN and NESTER 1969). In both species, the histidine and tryptophan genes appear to be contiguous, but differ in their arrangement. In *M. luteus*, an 'early' reaction histidine gene is adjacent to the anthranilate synthetase gene, whereas in *B. subtilis* the imidazoleacetol phosphate transaminase gene is adjacent to the tryptophan synthetase A gene. *Streptomyces coelicolor* has a *his* cluster located between two closely linked *trp* clusters (ENGEL 1969). The related species *S. typhimurium* (SANDERSON 1970) and *E. coli* (TAYLOR 1970) have histidine and tryptophan operons within 10% and 19% (minute length) of the total chromosome map, respectively. In *Pseudomonas aeruginosa*, histidine and tryptophan genes are somewhat dispersed; however, one tryptophan gene is within a region of 5–10% of the linkage group II map from two histidine genes, and four histidine genes are concentrated within a region covering about one-third of this map (LOUTIT 1969). It will be interesting to see if this linkage pattern will continue to be observed as additional bacterial species are studied. It is hopeful that patterns of linkage relationships may eventually lend insight into the evolution of gene clusters and regulation mechanisms.

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