CHARACTERIZATION AND MAPPING OF HISTIDINE GENES IN *SACCHAROMYCES LACTIS*[1]

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THERE is relatively little biochemical information on the gene control of biosynthetic pathways in eucaryotes. For this reason it is difficult to determine whether these organisms contain gene clusters which function as polarized units of integrated expression *i.e.* operons (JACOB and MONOD 1961). Comparative studies on the same biosynthetic pathway in eucaryotes and procaryotes would aid in determining the diversity of gene regulation.

The most intensively investigated pathway is that involving histidine biosynthesis. In *Salmonella typhimurium* the ten genes specifying the enzymes involved in histidine biosynthesis (see Figure 1) are clustered (the histidine operon) on the bacterial chromosome (for recent review see AMES *et al.* 1967). In two eucaryotes examined thus far, *Neurospora crassa* and *Saccharomyces cerevisiae*, the biochemical pathway is similar to that described in *Salmonella* (AHMED, CASE and GILES 1964; FINK 1964), although the genes controlling histidine biosynthesis are scattered in different linkage groups (AHMED, CASE and GILES 1964; MORTIMER and HAWTHORNE 1966). However, in both these fungi, a cluster of three genes remains which controls the third enzyme (PR-AMP 1, 6 cyclohydrase), the second enzyme (PR-AMP pyrophosphohydrolase) and the last enzyme (L-histidinol dehydrogenase), in the pathway (GILES 1963; FINK 1966).

The recent description of an independent mating system in a related but non-interbreeding yeast system, *Saccharomyces lactis* (*Kluyveromyces lactis*, VANDER WALT 1965) provides an opportunity for exploring homologies in regulatory behavior among the eucaryotes.

The results presented in this paper deal with the isolation, genetic analysis and biochemical characterization of histidine-requiring mutants in *S. lactis*. These studies were undertaken with independently derived histidine requiring strains to determine whether the biochemical pathway for histidine biosynthesis in *S. lactis* is similar to that in *S. cerevisiae*.

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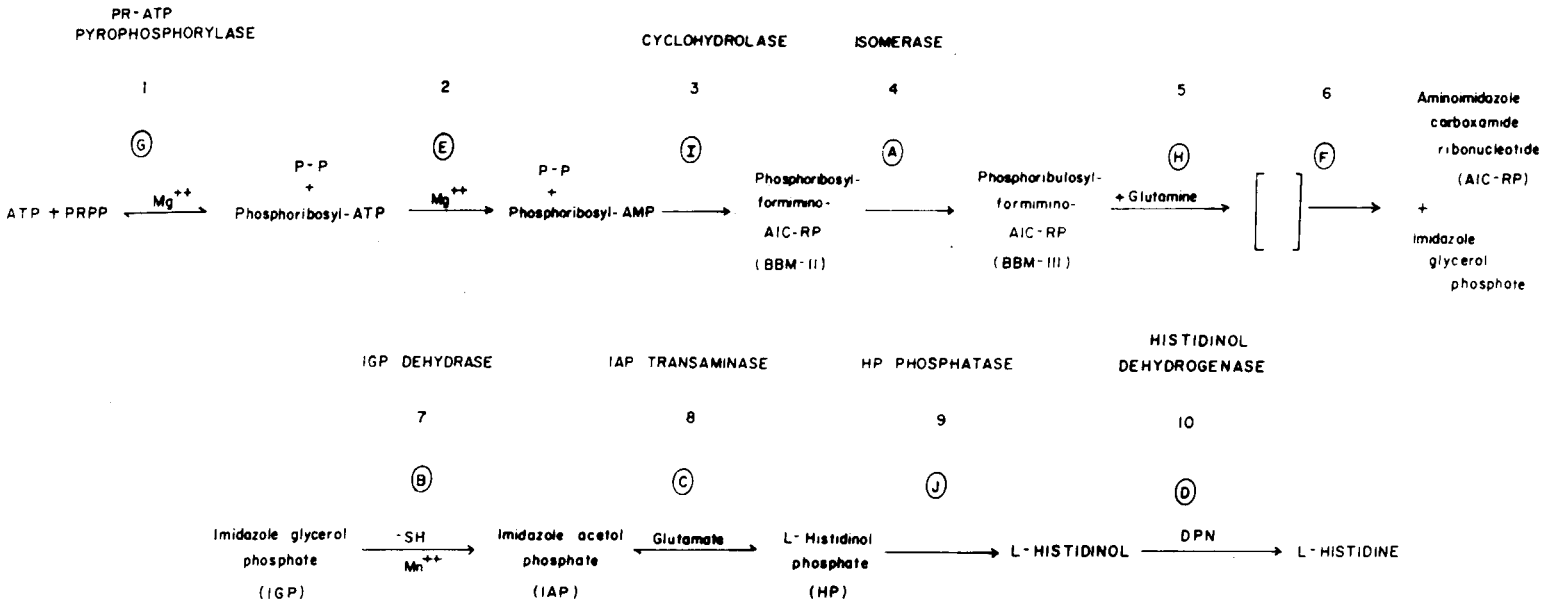

FIGURE 1.—Histidine biosynthetic pathway. (From AMES *et al.* 1967).

MATERIALS AND METHODS

Organisms and cultivation. Parental stocks of *Saccharomyces lactis*, Y-14 and Y-123, were used in these studies. The strains were originally obtained from L. J. WICKERHAM (NRRL Y1140 and NRRL Y1118 respectively). Histidine-requiring mutants were derived from each of these parents by ultraviolet irradiation. Maintenance of the stocks has been previously described (HERMAN and HALVORSON 1963). Yeast cells were cultured in yeast-malt extract broth (YM, WICKERHAM 1951) or in the following minimal medium: glucose, 20 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 8 g; trace elements, 1 ml, and water to 1000 ml. The carbohydrate, as a 20% aqueous solution, was autoclaved separately. After sterilization of the medium, 1 ml per liter of a vitamin solution (MACQUILLAN and HALVORSON 1962) was added. This medium was supplemented with 0.1 mg per ml of L-histidine for growth of histidine-requiring isolates. All media were solidified, as needed, with 20 g agar per liter. Malt extract agar (ME, WICKERHAM 1951) was used as both a mating and sporulation medium. Histidine-requiring mutants of *Salmonella typhimurium* deficient in the first six enzymes in the histidine pathway were obtained from DRS. G. FINK and B. AMES. The strains were cultivated, harvested and prepared for enzyme assay as described by SMITH and AMES (1964).

Genetic studies: The conditions for mating, the isolation of spores and determination of mating type have been previously described (HERMAN and HALVORSON 1963).

When complementary stocks of *S. lactis* are mated on ME and incubated under aerobic conditions, the diploid stage is transitory. Sporulation immediately follows formation of the zygote. To prevent sporulation and permit isolation of stable diploids the following procedure was employed: haploid cells of opposite mating type were mass-mated on ME and incubated at 30°C. After 8–12 hours, and prior to sporulation, individual zygotes (dumbbell-shaped cells with no spores visible) were isolated using a De Fonbrune micromanipulator and grown as isolated colonies on YM agar incubated under a nitrogen atmosphere Cells from these colonies were transferred to YM slants. After incubation for 2 to 3 days, at 30°C, the slants were stored at 5°C. Inocula from these slants were examined microscopically for the presence of characteristically shaped diploid-derived sporulated asci. The diploid nature of each of the isolates was verified by sporulating, dissecting and following the segregation of several independent markers.

The method of WINDISCH (1961), with the following modifications, was used for random spore analysis. Cells from a sporulated culture were incubated at 25°C for 10 hours in the gut juices of *Helix pomatia*. The reaction was stopped by dilution with twenty volumes of distilled water. The cells were then harvested by centrifugation at 10,000 $\times$ *g* for 10 minutes and washed twice with distilled water, placed in a tissue hand homogenizer, mixed with 3 volumes of liquid paraffin oil and treated for 10 minutes. Liberated spores were collected preferentially in the paraffin layer following centrifugation at 6,000 rpm for 10 min; vegetative cells remained in the aqueous phase.

Accumulation: The methods for accumulation and identification of Bound Bratton Marshall (BBM) compounds and imidozole intermediates in cultures of yeast histidine mutants have been described (FINK 1964).

Preparation of cell extracts: One gram, wet weight, of yeast cells obtained from a culture growing logarithmically in YM broth, was suspended in 2.5 ml of 0.1 M triethanolamine buffer (TEA) at pH 7.5. The cell suspension was passed through a French pressure cell at 22,000 lb pressure per square inch. Following breakage, cell debris was removed by centrifugation at 10,000 $\times$ *g* for 30 min. Ribosomal particles were removed from the supernatant fluid by sedimentation for 1 hour at 144,000 $\times$ *g* in a Spinco Model L ultracentrifuge. The supernatant fraction was passed through a Sephadex (G-25, medium) column to reduce the salt concentration.

Enzyme assays: Phosphoribosyl-ATP (PR-ATP) pyrophosphorylase activity was assayed according to the procedure of AMES, MARTIN, and GARRY (1961). A unit of enzyme activity was defined as that amount of enzyme yielding a change of 0.001 absorbancy at 290 mμ units in 4 minutes. PR-ATP pyrophosphohydrase and PR-AMP cyclohydrase activities were measured indirectly by *in vitro* complementation tests as described by AHMED, CASE, and GILES (1964).

The activities of the enzymes involved in the conversion of BBMII to AICAR and IGP were

measured in an *in vitro* complementation system designed to test the ability of various yeast mutant extracts to supply a specific activity missing in Salmonella extracts. The system contained TEA buffer (pH 7.5); 0.02 to 0.05 ml yeast extract; BBMII or BBMIII (0.900 OD units) and glutamine, 8 μmoles. The reaction was started by the addition of extract from derepressed Salmonella cells defective in either A, H or F activity but supplying the other step in excess. The reaction was carried out at 30°C with a total volume of 0.03 ml in a cuvette with a 1 cm light path and followed spectrophotometrically by the loss in absorbance at 300 mμ as BBMII or BBMIII was converted to IGP or AICAR. The specific activity (rate of decrease in absorbance at 300 mμ per 4 min per mg protein in yeast mutant extract) was compared with the specific activity with extracts from wild-type yeast.

Imidazole glycerol phosphate dehydrase activity was assayed according to the procedure of AMES (1957). A unit is defined as the amount of enzyme which catalyzes the formation of 1 mμ mole of IAP per hr.

L-Histidinol dehydrogenase activity was assayed by the increase in absorbancy at 340 mμ due to the reduction of sodium nicotinamide adenine nucleotide (NAD). The reaction mixture contained: L-histidinol, 2 μmoles; NAD, 5 μmoles; a variable amount of enzyme preparation and 0.1 M TEA buffer (pH 8.5) to a final volume of 1.0 ml. To eliminate interfering activities, measurements were performed on fractionated extracts at the sacrifice of quantitative recovery. Qualitative results are therefore presented.

Enzymatic reactions were recorded automatically with a Gilford Multiple Sample Absorbance Recorder attached to a Beckman DU spectrophotometer. Protein concentration was determined as described by LOWRY *et al.* (1951) using Fraction V bovine serum albumin as a standard.

Reagents: The following chemicals were obtained from Pabst Laboratories: NAD, PRPP and ATP. L-Histidinol was purchased from the Cyclo Chemical Corp. AICAR was supplied by Calbiochem. BBMII, BBMIII and IGP were gifts from DR. BRUCE AMES.

RESULTS

Genetic Analysis: The hereditary nature of the factor(s) causing a histidine requirement was determined by dissection and tetrad analysis of ascospores. Each auxotroph was mated with a prototrophic stock and the progeny were tested for histidine dependence (his^-) or histidine independence (his^+). The results are summarized in Table 1. Fifteen to 30 tetrads from each mating were examined. In every tetrad a 2:2 segregation ratio for his^-:his^+ occurred. Since a 2:2 segregation ratio is characteristic for the segregation of a single genetic factor, the data

TABLE 1

Segregation of histidine requirements in crosses of his^- × his^+ parents

Cross his^- × his^+	Number of tetrads analyzed	Tetrad segregation ratios Mutant:Wild-type 2:2	3:1	4:0
WM20 × Y14	22	22	0	0
116 × Y14	15	15	0	0
145 × Y14	32	32	0	0
WM37 × Y123	38	38	0	0
43 × Y14	20	20	0	0
WM52 × Y123	17	17	0	0
WM12 × Y14	19	19	0	0
M17 × Y14	27	27	0	0
WM58 × Y123	16	16	0	0

indicated (1) that each UV induced histidine requirement was of genetic origin and (2) that each genetic defect of the histidine-requiring strains tested carried only a single *hi*$^-$ mutation; none was a double-mutant carrying two *hi*$^-$ mutations at two different loci.

Dominance studies were conducted on diploids constructed by mating each his$^-$ mutant with a his$^+$ clone. The procedure used in the isolation of the diploids is described under MATERIALS AND METHODS. For each diploid heterozygosity for his$^-$:his$^+$ was verified by tetrad analysis. A minimum of 10 tetrads from each diploid were examined. In all cases a 2:2 segregation ratio for his$^-$:his$^+$ prevailed. When these diploids were tested for their histidine requirement, each proved to be phenotypically wild type *i.e.*, to grow on minimal media lacking histidine. From this result it follows that the mutations causing a histidine requirement are recessive to histidine independence.

Complementation studies were initiated to establish whether any of the mutants were blocked at the same step in the biosynthesis of histidine. Diploids arising from the matings in all combinations of the various histidine mutants were isolated and scored for growth in the absence of histidine. The growth responses are summarized in Table 2 and indicate that the mutants fell into four complementation groups designated Locus 1 through Locus 4.

Mutants within each locus did not complement. To determine whether these auxotrophs were alleles, stocks from within complementation groups were intercrossed, sporulated, and the segregants examined for histidine independence. The results appear in Table 3. In crosses between mutants 116 and WM20 and 145 and WM 20, between 43 and WM 37, and between M 17 and WM 58 and WM 12 and WM 58, no his$^+$ recombinants were observed. Parental ditype (PD) tetrads only were recovered. When random spore analyses were carried out on these same crosses (see Table 3) less than 1 percent of the spores isolated from each mating were his$^+$. Since extensive genetic analysis failed to reveal his$^+$

TABLE 2

*Complementation responses between various histidine mutants**

		Locus 1	Locus 2			Locus 3		Locus 4		
		WM52	WM20	116	145	WM37	43	M17	WM58	WM12
Locus 1	WM52	—	+	+	+	+	+	+	..	+
Locus 2	WM20	+	—	—	—	+	+	+	+	+
	116	+	—	—	—	+	+	+	+	..
	145	+	—	—	—	+	+	+	+	..
Locus 3	WM37	+	+	+	+	—	—	+	..	+
	43	+	+	+	+	—	—	+	+	..
Locus 4	M17	+	+	+	+	+	..	—	—	..
	WM58	..	+	+	+	..	+	—	—	—
	WM12	+	+	+	+	+	..	..	—	—

* Up to 6 independently derived diploids were tested at each locus. + and — refer to complementation as indicated by growth on minimal media.

TABLE 3

Segregation ratios from his⁻ × his⁻ *crosses originating from within complementation groups*

Cross*	Number of tetrads analyzed	Tetrad distribution patterns† PD	NPD	TT	Random spore analysis: Number of spores examined	Random spore analysis: Number of *his*⁻ spores recovered
Locus 2						
116 × WM20	15	15	0	0	363	363
145 × WM20	12	12	0	0	337	337
Locus 3						
43 × WM37	24	24	0	0	784	781
Locus 4						
M17 × WM58	16	16	0	0	619	619
WM12 × WM58	16	16	0	0	. . .	. . .

* Locus 1–4 indicates independent complementation groups.
† PD = parental ditype; NPD = non-parental ditype; TT = tetratype.

recombinants from matings between these non-complementing stocks, we assume that mutants 116 and 145 are alleles of WM 20; 43 to WM 37, and M 17 and WM 12 to WM 58.

Mutants from each of the four complementation groups were intercrossed in various combinations and the linkage relationships expressed in the progeny were studied. The results of these tetrad analyses are summarized in Table 4. Most of the tetrads recovered from intercrosses between mutants at the first three loci were parental ditypes (PD). Since a parental ditype (PD) to non-parental ditype (NPD) segregation ratio greater than 1:1 indicates linkage between two factors (PERKINS 1953), it appears that Loci 1, 2 and 3 occupy independent sites in a single linkage group. Tetratype (TT) and NPD frequencies reflect the intensity with which two factors are linked (PERKINS 1953). NPD and TT frequencies in the Locus 1 × Locus 3 cross were higher than in either the Locus 2 × Locus 3 or Locus 2 × Locus 1 matings (Table 4). We assume that these differences in linkage intensity reflect differences in distances between the three loci and suggest that the linked sites occur in the order Locus 1, Locus 2, Locus 3.

TABLE 4

Tetrad distributions from his⁻ × his⁻ *crosses originating between complementation groups*

Cross	Loci involved*	Number of tetrads analyzed	Tetrad distribution patterns† PD	NPD	TT
WM 20 × WM 43	2 × 3	42	30	1	11
WM 20 × WM 52	2 × 1	29	15	1	13
WM 52 × WM 43	1 × 3	29	10	4	15
WM 12 × WM 20	4 × 2	12	2	3	7
WM 12 × WM 43	4 × 3	55	8	9	38
WM 12 × WM 52	4 × 1	36	9	10	17

* Locus 1–4 indicate independent complementation groups.
† PD = parental ditype; NPD = non-parental ditype; TT = tetratype.

Mutants at Locus 4 were not linked to mutations mapping in the other three regions but mapped at a site in an independent linkage group close to that occupied by the mating locus (tetrad distributions for his⁻: mating type were PD 23; NPD 0; TT 3) and adenine-1 (HERMAN, unpublished results).

A PD:NPD:TT ratio of 1:1: less than 4 indicates that the two factors undergoing segregation occupy centromere-linked positions (PAPAZIAN 1952). Examination of ratios (Table 4) obtained from the cross between his⁻ mutants at Locus 1 and those from Locus 4 (PD:NPD:TT = 9:10:17) suggest that these two sites are each centromere linked. In summary we conclude that the relative order of the three complementation groups showing linkage is centromere, Locus 1, Locus 2, Locus 3. Locus 4 occupies a centromere-linked site in an independent linkage group.

Biochemical analysis: The enzymatic defect associated with mutants from each of the four complementation groups was identified by accumulation studies and direct enzyme assays.

Mutants blocked in the early steps of histidine biosynthesis do not accumulate BBM or imidazole intermediates. Therefore, the activity of each of the first three enzymes in the pathway was measured in extracts prepared from wild type and mutant cells. The results are summarized in Table 5. PR-ATP pyrophosphorylase activity was detected spectrophotometrically in all extracts examined. The other activities were assayed as discussed in MATERIALS AND METHODS. These results indicate that all yeast strains tested exhibit activity associated with each of the first three steps in histidine biosynthesis.

Accumulation studies were undertaken to establish whether the mutants were blocked prior to or following formation of the imidazole ring. When extracts from

TABLE 5

Activity of histidine biosynthetic enzymes in various yeast mutants

Strain	PR-ATP pyrophosphorylase units/mg protein	*In vitro* complementation with Salmonella mutants G	E	I	Imidazole glycerol phosphate dehydrase units/mg protein	L-Histidinol dehydrogenase
Wild type						
Y-14	.052	+	+	+	30.6	+
Y-123	.055	+	+	+	33.3	+
Mutant Locus						
1. WM52	.039	+	+	+	63.0	+
2. WM20	.048	+	+	+	34.6	+
116	.039	+	+	+	30.6	+
145						+
3. WM37	.043	+	+	+	0	+
43					0	+
4. WM12						0
M17	.021	+	+	+	28.6	0
WM58						0

mutant cultures were examined, the imidazole intermediates, imidazole glycerol and L-histidinol were identified in extracts from mutants of Locus 3 and Locus 4 respectively. When extracts prepared from wild type as well as mutants from each of the four loci were examined, imidazole glycerol phosphate dehydrase activity was demonstrated in all preparations except those obtained from mutants at Locus 3 (WM-37 and 43) (see Table 5). Also in agreement with the accumulation data, L-histidinol dehydrogenase activity was not detected in extracts from mutants at Locus 4 (WM-12, M-17 and WM-58). Since mutants of Loci 1 and 2 failed to accumulate imidazole compounds, the blocks in these mutants appear to be prior to formation of the imidazole ring.

Culture media from wild type and mutant cells were examined for the accumulation of BBM compounds. The results are summarized in Figure 2. A higher concentration of BBM compounds relative to that found in wild-type cultures was observed in culture media of mutants at Loci 1 and 2. These results indicate that the enzymatic lesions in these two groups of mutants must be involved with the conversion of BBMII to IGP since activity associated with earlier steps had been previously demonstrated in these extracts (see Table 5).

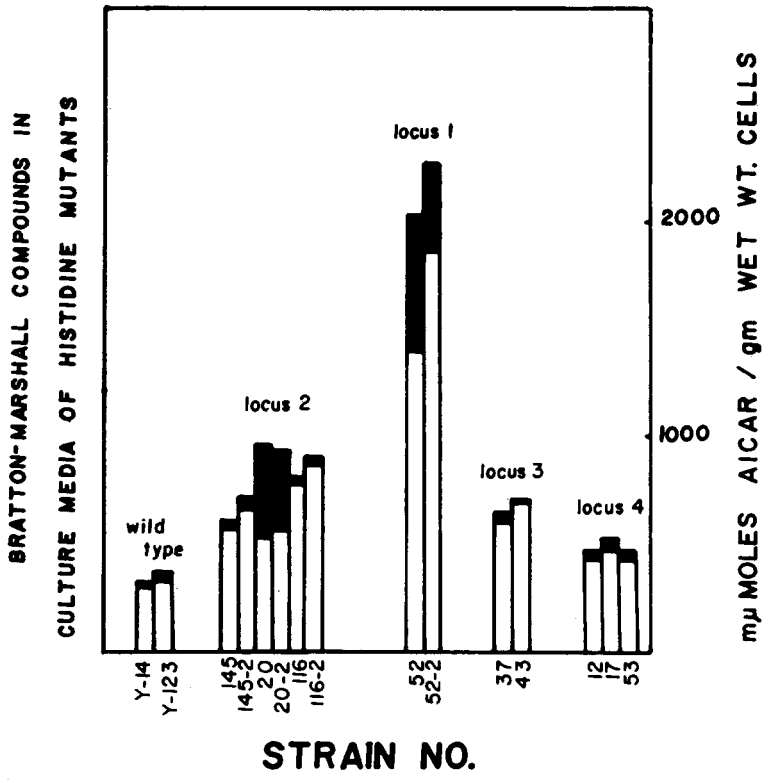

FIGURE 2.—Accumulation of Bound Bratton Marshall compounds in culture media of histidine mutants. Shaded areas show increase in diazotizable amines following acid hydrolysis. Some of the mutants were cultured in duplicate.

TABLE 6

Activity of yeast histidine mutants in A, H and F steps

Mutant	Substrate	Salmonella extract added	Enzymes supplied	Percent specific activity of wild-type yeast
WM20	BBMII	A	HF	0
WM20	BBMIII	H	F	20
WM20	BBMIII	F	H	73
WM52	BBMII	A	HF	22
WM52	BBMIII	H	F	0
WM52	BBMIII	F	H	115

The ability of the various yeast mutants to catalyze the conversion of BBMII to IGP was therefore measured in an *in vitro* complementation system (see MATERIALS AND METHODS). The results are summarized in Table 6. When tested for isomerase activity (BBMII to BBMIII), no activity was observed in extracts of mutant WM 20 (Locus 2). No H activity (BBMIII to IGP) could be demonstrated in extracts from mutant WM 52 (Locus 1). Extracts prepared from mutants WM 20 and WM 52 contained F activity. It is concluded that Locus 1 is the structural gene for amidotransferase activity and Locus 2 is concerned with isomerase activity.

DISCUSSION

It has been previously established by means of accumulation studies, direct enzyme assays and mixed *in vitro* complementation tests that the pathways for histidine biosynthesis are similar in *Salmonella typhimurium*, *Saccharomyces cerevisiae* and *Neurospora crassa* (AMES 1967; FINK 1964 and 1965 and AHMED, CASE and GILES 1964). The use of similar methods of analysis has led to identical conclusions for histidine biosynthesis in *S. lactis* which we summarize in Figure 3. Mutants which map at two loosely linked loci accumulate BBM compounds. Determination of enzyme activities in mixed extracts (Salmonella and yeast) showed that mutant WM-52 did not convert BBMIII to AICAR and IGP. Extracts from mutant WM 20 were unable to convert BBMII to AICAR and IGP. Thus, if the biochemical synthesis of histidine is identical in these organisms then Locus 1 controls amidotransferase activity and mutants at this locus are analogous to H mutants (Figure 1) in Salmonella. Similarly, Locus 2 governs isomerase activity and mutants mapping at this locus are comparable to A mutants in Salmonella.

Locus 3 controls IGP dehydrase activity. Mutants WM 35 and 43, which map at this locus, accumulate imidazole glycerol. Imidazole glycerol phosphate dehydrase activity could not be demonstrated in extracts from these strains. Locus 4 which maps on another linkage groups controls L-histidinol dehydrogenase activity. Mutants which map at this locus (WM 12, 17, and WM 58) accumulate histidinol. L-histidinol dehydrogenase activity was not detectable in extracts from these strains.

A comparison of the chromosomal distribution of genes controlling histidine

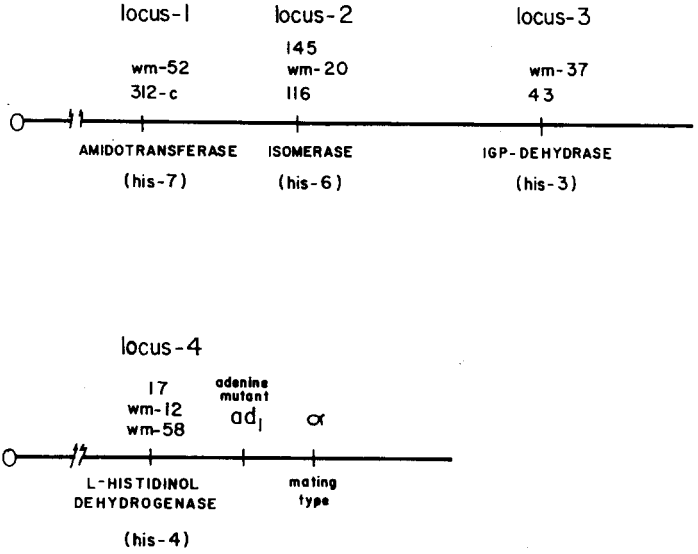

FIGURE 3.—A summary of the gene-enzyme relationships in *S. lactis*. The stock numbers of specific histidine mutants of independent origin are listed below the locus in which they map. The gene designations and corresponding enzyme defects or physiological properties are indicated below the linkage map. The map distances, corrected for 2, 3 and 4 strand crossovers (PERKINS 1953), are proportional to their recombination frequencies. Locus 4 and Locus 1 both show weak linkage with their respective centromeres. Locus designations in parentheses e.g. (*his*-7) or (*his*-6), refer to the histidine mutants in *S. cerevisiae* (FINK 1964).

biosynthesis in *S. cerevisiae* and *S. lactis* indicates the absence of genetic homology between these two non-interbreeding yeasts. In *S. lactis* the genes controlling amidotransferase, isomerase and dehydrase activities are loosely linked on one chromosome. In *S. cerevisiae* the genes controlling these enzymes are scattered on linkage group 9, fragment 1, and linkage group 2, respectively. If, as suggested by the distribution of histidine genes in *S. cerevisiae* and *N. crassa*, gene scattering increases with the number of chromosomes, then the clustering of genes in *S. lactis* may reflect the presence of fewer chromosomes in this organism.

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SUMMARY

Nine histidine-requiring mutants of *Saccharomyces lactis* were subjected to genetic and biochemical analysis. The mutants mapped at four distinct loci. Three of the genes are loosely linked while the fourth gene is located in a second linkage group close to the centromere-linked gene determining mating type. The gene-enzyme relationships for these four loci were established. The pathway of histidine biosynthesis appears to be similar to that described in other organisms.

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