*EXTENDED DELETIONS IN THE HISTIDINE-ROUGH-B REGION OF THE SALMONELLA CHROMOSOME**

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The biosynthesis of O-antigenic lipopolysaccharides (LPS)[1] in *Salmonella* proceeds in two stages (for review see ref. 2). First, the R core, comprising the central portion of LPS, is synthesized. Then, the O side chains, which have been synthesized on a lipid carrier, are attached to the R core. The O side chains of group B *Salmonella* such as *Salmonella typhimurium* contain D-galactose, D-mannose, L-rhamnose, and abequose.

From smooth wild type, one can isolate rough mutants which synthesize LPS devoid of O side chains. Stocker and associates have separated rough mutants of *S. typhimurium* into two main genetic classes, which they called rough A (*rouA*) and rough B (*rouB*).[3, 4] Since it has become clear that *rouA* and *rouB* represent gene clusters rather than individual loci, they will hereafter be designated by the symbols *rfa* and *rfb* (followed by capital letters when individual loci in these clusters are specified).

Stocker's group suggested that the *rfa* gene cluster, located between *str* and *metA*, determines the synthesis of R core, whereas the synthesis of O side chains is determined by the genes of *rfb* cluster, located between *metG* and *his*.[3, 4] Intergroup crosses of *Salmonella* showed that the *rfb* cluster of group B strains contains the structural genes for at least eight out of the nine enzymes necessary for the synthesis of thymidine 5′-diphosphate (TDP)-rhamnose, cytidine 5′-diphosphate (CDP)-abequose, and guanosine 5′-diphosphate (GDP)-mannose.[5] Gene order could not be established because of extensive nonhomology of this chromosomal region between the stains used; that is, crossing-over appeared to take place only at a few sites within the *rfb* cluster.[6] A more standard approach, i.e., the genetic mapping of point mutations, is difficult because few of the *rfb* point mutants isolated had defects in the enzymes involved in nucleotide-sugar synthesis.[5, 7] In order to circumvent these difficulties, we undertook the biochemical-genetic analysis of mutants which are defective both in histidine biosynthesis and in biosynthesis of O side chains. These mutants were found to have deletions in the *his* operon which extended into the *rfb* gene cluster and caused the loss of one or more of the enzymes involved in nucleotide-sugar synthesis.

Materials and Methods.—Bacterial strains: Most of the strains were nonreverting histidine-requiring rough mutants isolated from wild-type *S. typhimurium* LT2 or its derivative, *ara-9*. Strains *his-96* and *his-101* were obtained from wild-type *S. typhimurium* LT7. Some of these were spontaneous mutants, some were isolated after treatment with X ray, fast neutrons, or nitrous acid. Other LT2 strains, used as controls, are described in footnotes to Table 1.

Genetic mapping of the his region: Completely blocked *rfb* mutants do not adsorb known transducing phages. Therefore, mutants were mapped through tests with a series of Hfr strains prepared from SU219 (*hisD23, gal-50*, HfrB2).[8] This strain, kindly supplied by Dr. K. E. Sanderson, Department of Biology, University of Calgary, Alberta, Canada, will not grow on histidinol

containing minimal medium due to the *hisD* mutation. Histidinol, however, supports the growth of *his* mutants with mutations in other genes of *his* operon. P22 phage lysates grown on these histidinol-utilizing *his* mutants were used to infect SU219, and selection of the donor *his* marker was performed on histidinol-containing minimal medium. A drop of an overnight culture of the *his* Hfr strains thus prepared was spotted on a lawn of the F^- *his-rfb* extended deletion mutants spread on a minimal agar plate. The presence or absence of prototrophic recombinants after 2 days' incubation at 37° indicated whether the *his* mutation of the donor was encompassed by, or was outside of, the extended deletion.

Cultivation of cells and preparation of extracts: These were performed as described previously.[5] The sonicates were centrifuged at 27,000 × *g* for 30 min. The pellets were washed once and were used for the assay of rhamnose-C^{14} incorporation.

Chemicals: Sources of most chemicals have been described.[5] Phosphomannoisomerase was partially purified from extracts of wild-type *S. typhimurium* (prepared from frozen cells kindly given by Dr. B. N. Ames of the National Institutes of Health). Glucose-1,6-diP was obtained from Calbiochem; and phosphoglucoisomerase from Boehringer. DEAE-cellulose was "Cellex D, standard capacity" (control no. 2885) from Bio-Rad Laboratories.

Results.—Extent of deletion in the his region: A series of histidine-requiring, P22-resistant mutants were newly isolated, or culled by screening stable mutants in Dr. Philip E. Hartman's culture collection. These mutants did not revert to histidine independence and showed the phage-sensitivity pattern[3] typical of *rfb* mutants. The genetic lesions in the *his* region of these mutants were investigated by crossing them with various *his* deletion and point mutants. The majority were found to have multisite mutations, most probably deletions in the *his* operon (Fig. 1). It is significant that in all mutants of this majority class, the deletion extended up to (and presumably beyond) the "left" (i.e., the farthest from the operator) end of *his* operon. A few mutants (not shown in Fig. 1) had point mutations or short deletions in the *his* region and independent mutations in the *rfb* cluster.

Extent of deletion in the rfb region. We screened various strains for their content of enzymes of nucleotide-sugar synthesis, many of which are known to be determined at the *rfb* gene cluster.[5] The results are summarized in Table 1.

(1) Smooth strains contained all of the nucleotide-sugar biosynthetic enzymes assayed. The enzyme levels in these strains were similar to those found in the wild-type strain, LT2 (Table 1). In addition, some strains exhibiting the *rfb* phenotype (e.g., the double mutant *his-1327 rfb-811*) contained normal enzyme levels; this indicates that activities other than those examined here are coded for by the *rfb* gene cluster, and that the probable accumulation of nucleotide-sugars that occurs with the rough phenotype[15] did not affect the enzyme levels.

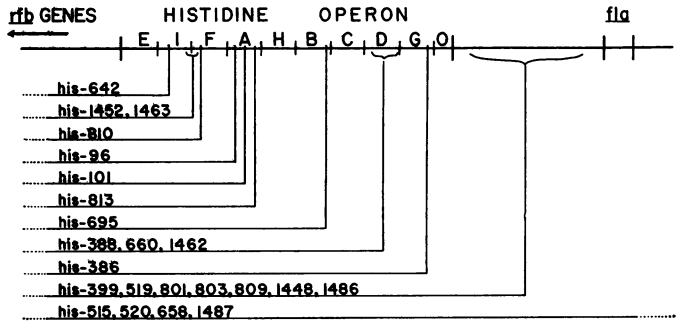

FIG. 1.—Extent of deletion in histidine operon in various mutants. For the detailed genetic map of the *his* region see ref. 11.

TABLE 1

ACTIVITIES OF ENZYMES INVOLVED IN NUCLEOTIDE-SUGAR SYNTHESIS

	Specific Activity of Enzymes									
	UDPG pyro-phosphorylase	RHA-2	RHA-1	RHA-3	ABE-1	ABE-2	ABE-3	MAN-3	MAN-2	Incorp. of C^{14}-rhamnose
LT2	100	100	100	100	100	100	100	100	100	100
hisO1202	95	91	72	109	86	111	103	97	109	
hisR1200		85	86	95	84	102	112	103	81	
his-1327 rfb-811	113	105	113	105	101	106	119	103	106	
his-612		92	96	97	106	87	80	104	85	
his-96	108	126	115	113	64	115	119	129	4	*
his-642	98	99	107	98	54	108	126	84	7	*
his-1462	88	90	96	93	55	78	103	71	6	
his-1463	93	96	101	91	54	81	121	71	4	
his-660	99	98	132	86	60	108	103	*	6	*
his-1486	91	101	83	82	53	95	129	*	4	
his-1487	102	100	82	86	53	88	98	*	4	
his-515	89	109	101	100	54	52	*	*	4	*
his-801	102	101	105	75	*	*	*	*	5	*
his-399	84	82	2	*	*	*	*	*	22	
his-695	91	119	3	*	*	*	*	*	20	
his-809	163	4	18	*	*	*	*	*	25	
his-810	172	25	22	*	*	*	*	*	12	*
his-101	160	2	28	*	*	*	*	*	*	*
his-386	190	30	34	*	*	*	*	*	*	*
his-388	198	14	25	*	*	*	*	*	*	
his-519	149	19	23	*	*	*	*	*	*	
his-520	171	22	28	*	*	*	*	*	*	
his-658	171	36	28	*	*	*	*	*	*	
his-803	183	18	21	*	*	*	*	*	*	
his-813	176	16	21	*	*	*	*	*	*	
his-1448	155	38	40	*	*	*	*	*	*	

The results are expressed as percentage of the specific activity found in strain LT2. Some of the values are the mean of two to four independent determinations. *, Undetectable (usually less than 1–2%). Mixed extract experiments in representative strains showed no indication of the presence of inhibitor.

Abbreviations for the names of enzymes:[5] RHA-1, TDP-glucose pyrophosphorylase; RHA-2, TDP-glucose oxidoreductase; RHA-3, "TDP-rhamnose synthetase"; ABE-1, CDP-glucose pyrophosphorylase; ABE-2, CDP-glucose oxidoreductase; ABE-3, "CDP-abequose synthetase"; MAN-1, phosphomannoisomerase; MAN-2, phosphomannomutase; MAN-3, GDP-mannose pyrophosphorylase.

Enzymes were assayed essentially as described previously,[5] with the following changes. (1) The UDPG pyrophosphorylase and ABE-1 reactions were measured spectrophotometrically by following the pyrophosphate-dependent production of glucose-1-P from UDPG or CDP-glucose. (2) For the assay of MAN-2, the reaction mixture contained glucose-1,6-diP, 5 mμmoles; phosphoglucoisomerase, 0.8 unit;[9] and phosphomannoisomerase, 0.1 unit, in addition to the components described previously.[5] (3) Incorporation of C^{14}-rhamnose into the acid-insoluble fraction was tested in the presence of nonradioactive UDP-galactose, GDP-mannose, and CDP-abequose.[10]

ABE-1 activity appears to be about one-half of the wild type in mutants *his-96* through *his-515*. Most probably this is due to the absence of 6-phosphogluconate dehydrogenase in these mutants; a gene located between the *his* operon and *rfb* region determines this enzyme (Dan Fraenkel and Grace Peyru, personal communication), which generates extra NADPH from glucose-6-P during assay and increases the *apparent* activity of ABE-1 in extracts of wild-type and control strains. Similarly, MAN-2 activity in wild type is overestimated approximately by the factor of two. Thus, the actual activity of MAN-2 in mutants *his-96* through *his-801* is presumably around 10% of the wild type, and that in mutants *his-399* through *his-810* is about 40% of the wild type.

Strains *hisO1202* and *hisR1200* are constitutive mutants of *his* operon, both smooth;[12] *his-612* has a deletion involving the left half of the *his* operon and a few genes beyond it but presumably not reaching the *rfb* cluster (as it is smooth);[13] *his-1327 rfb-811* has a mutation in the *rfb* region and a deletion linking the operator end of *his* operon to another unknown operon, so that *his* genes are no longer under the control of histidine repressor.[14]

(2) The levels of the nucleotide-sugar biosynthetic enzymes were not affected by derepression of the *his* operon in the constitutive strains *hisO1202* and *hisR1200* Thus, although closely located on the chromosome, the rate of functioning of one group of genes appears to be independent of the other.

(3) Most of the stable histidine-requiring mutants that concomitantly exhibit the *rfb* phenotype showed significant changes in activities of enzymes of nucleotide-sugar synthesis (Table 1). The pattern of these alterations showed that *rfb* genes can be arranged in a definite, linear order. For example, MAN-2 activity was drastically lowered in all the mutants of this class while at the other extreme, RHA-2 activity was altered only in about half of the mutants. We conclude that these changes are the result of deletions of the respective structural genes and infer that

the order of the genes in the *rfb* region is as shown in Figure 2. The genetic studies mentioned above support the conclusion that the enzyme alterations are the result of extended deletions encompassing the terminal portions of the histidine operon and variously sized segments of the *rfb* gene cluster.

In all the extended deletion mutants studied, phosphoglucomutase, MAN-1, and UDP-N-acetylglucosamine pyrophosphorylase showed close to normal activity and UDP-galactose 4-epimerase was always present (not shown in Table 1). This suggests that structural genes for these enzymes are probably located outside the *rfb* cluster; the genes for UDP-galactose 4-epimerase (*galD*) and MAN-1 (*pmi*) are indeed known to have such locations.[4, 8] These results are not surprising, as UDP-N-acetylglucosamine pyrophosphorylase is not involved in the synthesis of O side chains in this organism, and as the other three enzymes are amphibolic enzymes with important catabolic functions.

Patterns of alteration in enzyme activity: Some of the enzymes assayed, e.g., RHA-3, ABE-2, and ABE-3, had either close to 100 per cent activity or no detectable activity at all. In contrast, other enzymes frequently showed intermediate activity, or activities higher than the wild-type level. Our results, summarized below, indicate that *each of the activities RHA-2, MAN-2, RHA-1, and UDPG pyrophosphorylase is determined by more than one structural gene.*

(1) *RHA-2:* This enzyme was present in normal levels in mutants *his-96* through *his-695*, was present at a very low level in mutants *his-809* and *his-101*, and was present at "intermediate" levels in mutants *his-386* through *his-1448* (Table 1).[16] Chromatography of crude extracts on DEAE-cellulose showed two distinct peaks (I and II) in the case of the "normal-level group" such as LT2 or *his-695*, whereas

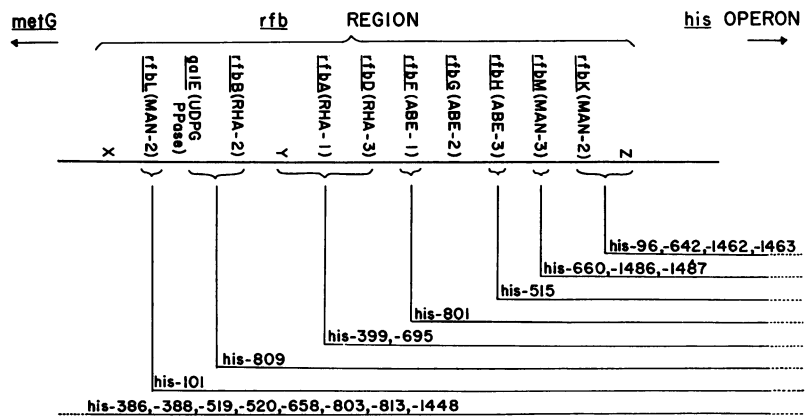

FIG. 2.—Proposed partial map of *rfb* region. The function determined by each locus is shown in parentheses. Although the locus for UDPG pyrophosphorylase is present in the "*rfb*" region," it is called *galE* because the loss of this enzyme will result in the inability to ferment galactose. Distance between loci is arbitrary, and other genetic loci might be present between any two genes which are shown as adjacent to each other. Thus the extent of deletion shown is the *minimal* estimate.

The order of genes in parentheses has not been established. The locus *rfbF* was placed provisionally to the left of *rfbG* because we felt that the lowering of ABE-2 activity in *his-515* may be due to the "bipolarity effect." [18]

For the functions of *X* and *Y* region, see text. *Z* refers to structural gene(s) the product of which is essential for the incorporation of rhamnose-C^{14} from TDP-rhamnose into the acid-insoluble fraction.

only a single peak (III) was seen in the case of "intermediate" strains, such as *his-386* or *his-519* (Fig. 3). Peak III consistently was eluted slightly earlier than peak II.

These results may be explained as follows (Fig. 2). The deletion of the structural gene *rfbB* results in a loss of both peaks I and II. Peak III may be the product of a structural gene, presumably not located in the *rfb* region, which becomes derepressed after the deletion of chromosomal region covered by the *his-386* deletion but not by the *his-101* deletion (*X* in Fig. 2).

(2) *MAN-2:* The activity of this enzyme was low in all those extended deletion mutants which retained most of the other *rfb* enzymes (*his-96* through *his-801* in Table 1). When an additional portion of the *rfb* gene cluster (*Y* in Fig. 2) was lost by deletion, a higher, "intermediate" activity was found (*his-399* through *his-810* in Table 1). In cases where the deletion extended still further into the *rfb* cluster, as in mutants *his-101* through *his-1448*, the residual MAN-2 activity was absent (Table 1). DEAE-cellulose chromatography of extracts from wild-type LT2 revealed two peaks with MAN-2 activity; with an extract from *his-642*, a mutant belonging to the "low-level group," only one peak was seen (Fig. 4*a*, *b*).

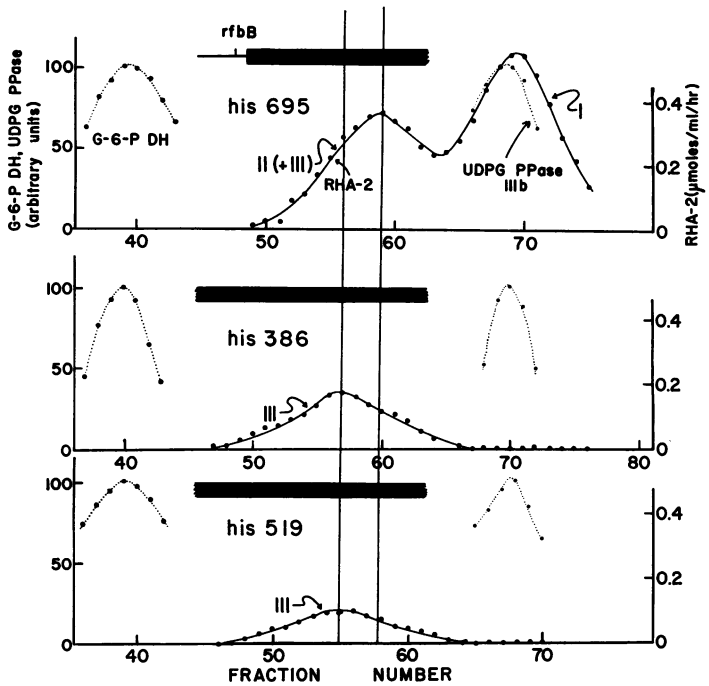

FIG. 3.—DEAE-cellulose chromatography of RHA-2. Crude extract (containing *ca.* 60 mg protein) was applied to a column (1.9 cm^2 × 18 cm), and the elution was made by a linear gradient of NaCl. To the mixing flask was added 200 ml of Tris succinate-Mg acetate (16 mM–8 mM, pH 7.6) containing 0.08 *M* NaCl, and to the reservoir 200 ml of the same buffer containing 0.24 *M* NaCl. Each fraction contained 4.1 ml. In order to correct for minor differences in fraction volume, etc., the graphs are drawn so that the peaks of two "marker" enzymes (glucose-6-P dehydrogenase and UDPG pyrophosphorylase IIIb) come out at the same points. The insets show the inferred genetic constitution of *rfb* region in each strain; areas covered with thick black bars are believed to be deleted.

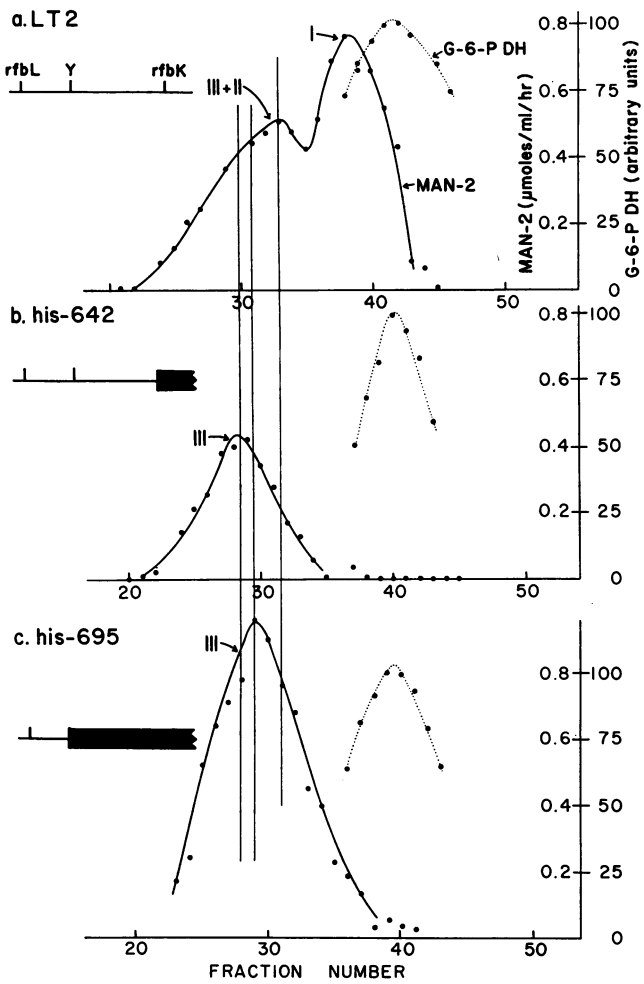

FIG. 4.—DEAE-cellulose chromatography of MAN-2. Conditions are similar to those for the experiments of Fig. 3. The graphs are drawn so that the peak of "marker" enzyme, glucose-6-P dehydrogenase, comes out at the same point. The fractions were assayed by determining the decrease in acid-labile P after incubation with mannose-1-P and glucose-1,6-diP. The insets show the inferred genetic constitution of *rfb* region in each strain; areas covered with thick black bars are believed to be deleted.

Only one peak was also obtained with an extract of *his-695* (a representative of the "intermediate-level group"), but the peak was larger than with *his-642* (Fig. 4*c*). No significant MAN-2 activity was found when an extract of *his-519* was chromatographed (not shown in Fig. 4).

The MAN-2 peaks of *his-642* and *his-695* appeared to be eluted slightly earlier than the early peak of the wild type. It should also be noted that the elution profile of the early peak of wild type shows a marked asymmetry; this suggests that this peak actually contains two components (II and III), one of which presumably corresponds to the peak (III) seen in the mutant *his-642*.[17]

In analogy with RHA-2, it may be that both of the two major wild-type peaks (I and II) are lost by the deletion of a structural gene (*rfbK* of Fig. 2), leaving the

third peak (III) coded for by the structural gene *rfbL*, which becomes derepressed in "intermediate" mutants upon deletion of the Y region (Fig. 2). In contrast to RHA-2, however, MAN-2 is completely lost in those mutants with more extensive deletions. Therefore, the second structural gene *rfbL* is inferred to be located within the *rfb* gene cluster (Fig. 2).

(3) *UDPG pyrophosphorylase and RHA-1:* The activity of UDPG pyrophosphorylase was normal unless the deletion extended far into the *rfb* region. The activity was *elevated* in strains *his-809* through *his-1448* (Table 1). In contrast, RHA-1 activity was almost completely absent in mutants *his-399* and *his-695*, and exhibited an intermediate level in strains *his-809* through *his-1448*, concomitantly with the increase in UDPG pyrophosphorylase. The peculiar behavior of these enzyme activities was clarified by the results of DEAE-cellulose chromatography of extracts from various strains (Table 2). In wild type four peaks with UDPG pyrophosphorylase activity were found. All of these peaks also had activity toward TDP-glucose. Peak I enzyme had a much higher affinity for TDP-glucose than for UDPG, and thus is considered TDP-glucose pyrophosphorylase (RHA-1). Enzymes in peaks II, IIIa, and IIIb, however, had lower K_M for UDPG than for TDP-glucose, and thus are considered UDPG pyrophosphorylases. In *his-695*, where RHA-1 activity was extremely low, peak I was absent. The traces of RHA-1 activity in *his-695* and *his-399* extracts are apparently due to the broad substrate specificities of the remaining UDPG pyrophosphorylases. When the deletion extended further to the left, as in *his-519*, peak II and peak IIIa were absent, and peak IIIb activity was strongly derepressed (about 50-fold increase). The net result of the loss of II and IIIa and the derepression of IIIb was the gain of only 50–90 per cent in *total* UDPG pyrophosphorylase activity. The residual RHA-1 activity, however, was increased appreciably (Table 1, *his-809* and below) because the derepressed enzyme in peak IIIb had a higher affinity toward TDP-glucose than the enzymes in peaks II and IIIa.

These results show that the gene for II and IIIa must be located within the *rfb* gene cluster (*galE* of Fig. 2). It is also clear that there must be another UDPG pyrophosphorylase structural gene coding for enzyme IIIb; the location of this gene is now under investigation.

Discussion.—A partial map of the *rfb* region of *S. typhimurium* has been constructed on the basis of the levels of a number of enzymes assayed in extracts of mutants with extended deletions (Fig. 2). The present results confirm our previous conclusion that many enzymes of nucleotide-sugar synthesis are determined at the *rfb* gene cluster.[5] Furthermore, genes concerned with related functions appear to be clustered within the *rfb* region, i.e., those determining TDP-rhamnose synthesis, those determining CDP-abequose synthesis, and those determining GDP-mannose synthesis.

There are several cases where more than one enzyme catalyzes a single reaction.

TABLE 2

UDPG (TDP-GLUCOSE) PYROPHOSPHORYLASES IN VARIOUS STRAINS

	Peaks on DEAE-Cellulose Chromatography			
	I	II	IIIa	IIIb
LT2	++	++	++	+
his-695	−	++	++	+
his-519	−	−	−	+++

In some of these cases, a low degree of substrate specificity seems to be responsible. For example, one of the four "UDPG pyrophosphorylase" peaks found on DEAE-cellulose chromatography is the result of loose specificity of TDP-glucose pyrophosphorylase.

Other cases appear to be more complex. Interestingly, three sets of enzymes, RHA-2, MAN-2, and UDPG pyrophosphorylase, show a very similar pattern. DEAE-cellulose chromatography of extracts of wild type shows two major peaks and one minor peak of activity catalyzing the same reaction; in deletion mutants both of these major peaks are absent, and the remaining peak frequently becomes derepressed. We assumed the presence of duplicate structural genes in each case, but this hypothesis does not explain the presence of *two* major peaks in the wild type, or the *simultaneous* loss of these two peaks in deletion mutants. One possibility might be the random association of two nonidentical subunits, whose structure is determined by the two structural genes mentioned above. This will produce three kinds of molecules (i.e., AA, AB, and BB), *two* of which will be lost upon deletion of either one of the structural genes. Other hypotheses, however, can explain our results equally well, and further studies are in progress to clarify these points.

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[1] Abbreviations used: LPS, lipopolysaccharide; genetic loci are symbolized with lower-case, italicized designations: *rfb*, rough B; *str*, streptomycin resistance; *met*, *his*, and *try*, requirements for methionine, histidine, and tryptophan, respectively; *ara*, inability to ferment arabinose; *fla*, inability to synthesize flagella. Enzyme activities are abbreviated with nonitalicized capital-letter designations; these are given in the footnote to Table 1.

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[16] It is not clear why *his-810* has an intermediate, instead of low, level of RHA-2. One possibility would be an independent mutation at the X region (Fig. 2).

[17] In a more recent experiment using a column of DEAE-polyacrylamide gel, the "early peak" of the wild type has been resolved into two distinct peaks.

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