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Complete Physical Map of the *rfb* Gene Cluster Encoding Biosynthetic Enzymes for the O Antigen of *Salmonella typhimurium* LT2

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Biosynthesis of the *Salmonella typhimurium* LT2 O antigen is encoded by genes which map in the *rfb* cluster. The cloning and restriction enzyme analysis of part of this cluster have been described previously (H. N. Brahmbhatt, N. B. Quigley, and P. R. Reeves, Mol. Gen. Genet. 203:172–176, 1986). The entire *rfb* gene cluster has now been cloned, and a detailed restriction enzyme map has been constructed which has enabled us to map the approximate positions of individual *rfb* genes.

Lipopolysaccharides form a major component of the outer membranes of gram-negative bacteria. They are composed of three parts: the innermost lipid A, the middle oligosaccharide core, and the outermost linear polymer of repeating oligosaccharide units, the O antigen (9). The O-specific polysaccharide shows high structural variation within the genus *Salmonella* (7, 9, 10), whereas the core and lipid A remain constant. This variation in O-antigen structure in the genus *Salmonella* provides the basis for a major serological typing scheme, and its chemical nature has been studied in great detail (5, 11). The genetic basis of O-specific polysaccharide biosynthesis has been studied almost exclusively by using *Salmonella* strains, generally with *S. typhimurium* LT2 as a tool. The genes coding for the biosynthesis and assembly of O antigen are located mostly in the *rfb* gene cluster at map position 42 (21). A partial gene order for this cluster has been established by using a series of mutants with deletions extending from *his* into *rfb* (17). The basic repeating oligosaccharide unit of the *S. typhimurium* O antigen is a tetramer composed of abequose, mannose, rhamnose, and galactose. This unit is assembled from appropriate nucleoside diphosphate derivatives of the monosaccharides as an undecaprenol phosphate-linked intermediate before polymerization, possible modification, and translocation to the core. In a previous paper (1), we reported the cloning and physical mapping of a part of the *S. typhimurium* LT2 *rfb* gene cluster and determined the approximate location of the *rfbK* and *rfbM* genes. In this paper, we report the cloning of the complete *rfb* gene cluster and give a detailed physical map. We have also localized all deletion endpoints which are known to lie in the *rfb* region, thereby giving the approximate positions of most of the known genes on the map.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. *S. typhimurium* LT2 and *Escherichia coli* K-12 strains used are listed in Table 1. P9003 is wild type for *rfb* and is often referred to as wild type in the text. An *S. typhimurium* gene bank constructed in the vector λ1059 (6) was a kind gift from Russel Maurer (15).

Enzymes and radiochemicals. Restriction enzymes, DNA polymerase I, bacterial alkaline phosphatase, and T4 DNA ligase were purchased from Promega Biotec, Madison, Wis., Boehringer Mannheim Biochemicals, Indianapolis, Ind., New England BioLabs, Inc., Beverly, Mass., and BRESA, Adelaide, Australia. [α-^{32}P]dCTP was obtained from BRESA and Amersham Corp., Arlington Heights, Ill.

DNA techniques. The methods used to prepare plasmid and high-molecular-weight chromosomal DNA to digest with restriction enzymes and to ligate restriction fragments were those described previously (1). Recombinant λ1059 phage DNA was purified as described by Karn et al. (6). Competent cells were prepared and transformed with DNA by the method of Mandel and Higa (12). Agarose gel electrophoresis, radioactive labeling of DNA, in situ DNA hybridization, and autoradiography were performed as described by Maniatis et al. (13). *Eco*RI-digested bacteriophage SPP1 DNA (20), *Bgl*II-digested λ DNA, and *Msp*I-digested pUC9 DNA were used as molecular size standards.

Detailed restriction enzyme mapping. A detailed restriction enzyme map was made for the *rfb* region. The strategy used in mapping each clone was as follows. The plasmid DNA

TABLE 1. Bacterial strains

Strain	Genotype
S. typhimurium LT2	
P9003 (CL4419)	*hsdL6 trpC2 nml H1b fla-66 H2*-enx *rpsL120 xyl-1404ilv-452 metE551 metA22 hsdSA29*
P9073	Δ(*hisI-rfbK*)642[a]
P9077	Δ(*hisD-rfbK*)1462[a]
P9078	Δ(*hisI-rfbK*)1463[a]
P9033	Δ(*hisD-rfbM*)660[a]
P9031	Δ(*flaA-rfbH*)515[a]
P9034	Δ(*hisO-rfbF*)801[a]
P9030	Δ(*hisO-rfba*)399[a]
P9074	Δ(*hisB-rfbA*)695[a]
P9035	Δ(*hisO-galF*)809[a,b]
P9051	Δ(*hisA-rfbL*)101[a]
P9029	Δ(*hisO-metG*)388[c]
E. coli K-12	
P334 (C600)	*thr1 leu6 tonA1 lacY1 supE44 thi-1*

[a] Endpoint within *rfb* is shown in Fig. 1.
[b] Note that *galF* is within the *rfb* cluster deletion.
[c] Note that this includes all of the *rfb* cluster. P9003 was obtained from I. Beacham, and other LT2 strains were obtained from H. Nikaido (17). C600 was from our collection.

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was cleaved in single digests with each of 28 enzymes to determine the frequency of cleavage. Unique sites could be mapped in relation to vector polylinker sites, and for enzymes with two or three sites the two terminal locations could be mapped with respect to polylinker and cloning sites. The third site could then be mapped in relation to other defined sites. This rough map was then refined by doing a series of double digests to accurately measure the sizes of the small fragments between adjacent sites and to determine the order where it was ambiguous. Sufficient double digests were then done to enable us to see each fragment, defined by sites adjacent on the map, and to estimate its size. All such estimates were the average of at least two replicates with the most appropriate gels. The sizes of the larger fragments were also accurately measured to confirm the map as derived.

Deletion endpoint mapping. The deletion endpoints in *rfb* were mapped by analysis of the autoradiographs produced by Southern hybridization of various cloned *rfb* DNA probe fragments, against restriction enzyme digests of chromosomal DNA from the deletion strains. The restriction enzymes used were those for which sites were known to exist adjacent to or within the region spanned by the probe. Adjacent to every deletion DNA digest track, a track containing wild-type DNA digested with the same restriction enzyme was included as a positive control. Comparison of band sizes in the test and control tracks on the autoradiographs defined specific restriction enzyme sites as lost or retained in the deletion strain DNA. The deletions run from within or to the left of *his* and into *rfb* (17), and hence the restriction enzyme sites to the right (distal to the *his* operon) of a deletion endpoint were expected to be present, and those to the left (proximal to the *his* operon) of this endpoint were expected to be absent.

Cosmid cloning. High-molecular-weight chromosomal DNA from strain P9003 was partially digested with *Sau*3A1 by the method of Maniatis et al. (13). Cosmid vector pcos2EMBL was used as described by Poustka et al. (18) for cosmid libraries, but without the use of phosphatase on the chromosomal DNA.

RESULTS

Cloning of the *rfb* gene cluster. The *Mlu*I-*Hin*dIII insert fragment (from approximately position 4.52 to position 5.76 [note that the *Mlu*I site is just outside the *rfb* region cloned]) of plasmid pPR259 (Fig. 1) (2) was used as a probe to screen

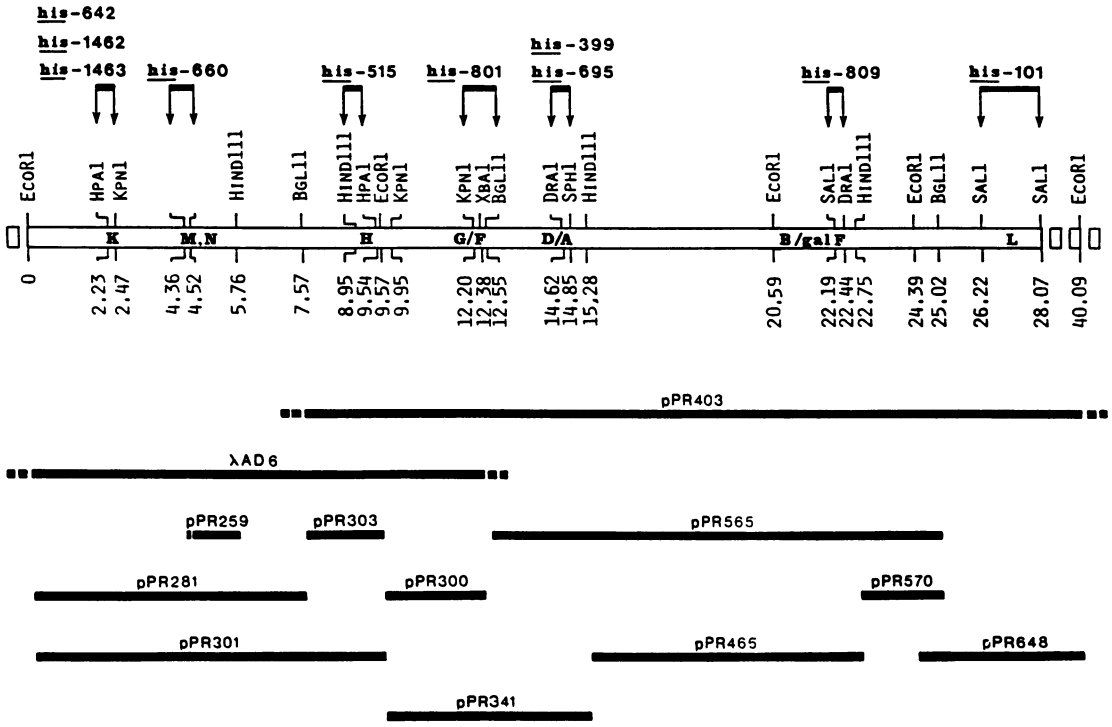

FIG. 1. Location of *his-rfb* deletion endpoints within the *rfb* cluster of *S. typhimurium* LT2. The *his* and *gnd* genes are located to the left of the region shown, and the deletions all extend from within or to the left of *his* through *gnd*, into *rfb*. The names of specific deletions (see also Table 1) are positioned above short intervals of *rfb* DNA. These intervals are defined by specific restriction sites indicated by paired arrows and contain the *rfb* endpoints of the deletions indicated. Other restriction sites shown were used for subcloning *rfb* DNA segments and are discussed in the text. The *rfb* endpoint of the *his-660* deletion was mapped previously (1). The *rfb* genes known to be interrupted by each deletion (17) are in boldface type on the thick open bar representing the chromosome. Genes separated by a slash are those for which the relative order has not been determined. Heavy lines represent specific *rfb* DNA segments which have been subcloned into lambda or plasmid vectors. Plasmids pPR259 and pPR281 have been described previously (1); other fragments were subcloned as follows. λAD6 was selected from a λ1059 bank by using the *Mlu*I-*Hin*dIII (Fig. 1) (2) fragment of pPR259 as a probe; pPR300 has an *Eco*RI-*Xba*I fragment from λAD6 in pJRD158b (2, 4); pPR301 has an *Eco*RI fragment from λAD6 in pEMBL8 (3); pPR340 (not shown) has the 11.02 *Eco*RI fragment (positions 9.57 to 20.59) of pPR403 in pEMBL8; pPR341 has an *Eco*RI-*Hin*dIII fragment (positions 9.57 to 20.59) of pPR340 in pPR328 (19); pPR403 is a cosmid clone selected from the pcos2EMBL bank; pPR465 has a *Hin*dIII fragment from pPR403 in pUC9 (23); pPR565 has a *Bgl*II fragment from pPR403 in pJRD158b; pPR570 has the *Hin*dIII-*Bgl*II fragment from pPR565 in pUC9; and pPR648 has the *Eco*RI fragment (15.7 kb) from pPR403 in pKO1 (16). The units are in kilobases, and position 0 is arbitrarily chosen to signify the *his*-proximal end of *rfb*. The exact location of the *rfb* cluster terminus has not been defined, but is thought to lie close to position 0 (1).

the λ1059 bank of *S. typhimurium* DNA by plaque hybridization. Phages from several positive plaques were purified, and their DNA was isolated, nick translated, and used in dot blot hybridization against chromosomal DNA from all deletion strains (Table 1) to identify which cloned DNA extended farthest into the *rfb* region. The isolate selected for further analysis, λAD6, hybridized to DNA from the wild-type strain P9003 and to DNA from strains carrying deletions which extend to the *rfbF* and *rfbG* genes (Fig. 1). The insert in λAD6 presumably carries *rfbK*, *rfbM*, *rfbN*, *rfbH*, and possibly *rfbG* and *rfbF* also. However, we do not have a functional test for the expression of these *Salmonella* genes by this lambda clone.

Plasmids pPR300 and pPR301 (Fig. 1) were obtained from λAD6, and pPR403 was selected from the cosmid bank by using a *Kpn*I fragment (9.95 to 12.20) of pPR300 as probe. Several cosmid clones hybridized with this probe, and preliminary analysis showed that pPR403 extended farthest to the right: plasmids pPR341, pPR465, pPR565, pPR570, and pPR648 were obtained from it in a series of steps (Fig. 1).

Sufficient restriction map analysis was done as the subcloning proceeded to facilitate the subcloning itself and to start the mapping of deletion endpoints (see below) so that progress along the *rfb* gene cluster could be monitored. The deletion endpoint data also indicated that *rfb* did not extend beyond the region covered by pPR648.

Southern blot analysis of pPR403 and wild-type chromosome DNA, with pPR341, pPR465, and pPR570 as probes, was carried out to confirm that the general pattern of *Eco*RI sites was the same on both clone and chromosome and hence that there had been no major structural rearrangement during cloning. This was further confirmed for a variety of sites during the analysis of deletion endpoints (see below), which involved extensive Southern blot analysis of chromosomal DNA, with all results being consistent with the absence of structural rearrangements.

Detailed restriction enzyme analysis of the *rfb* gene cluster. We have already published a detailed restriction enzyme map of *rfb* DNA between positions 0 and 7.57 (1). The complete physical map presented in Fig. 2 was established up to position 25.02 by performing detailed restriction enzyme analysis on plasmids pPR341, pPR465, and pPR570 (see Materials and Methods for details of the mapping procedure). Plasmids pPR303 and pPR648 were analyzed only on a preliminary basis, since they carry DNA which is either being sequenced in our laboratory (pPR303) or lies predominantly beyond the last deletion endpoint (*his* operon distal), that of Δ(*his-rfb*)*101*, known to lie in or beyond *rfbL*.

Localization of deletion endpoints. Plasmid pPR648 was

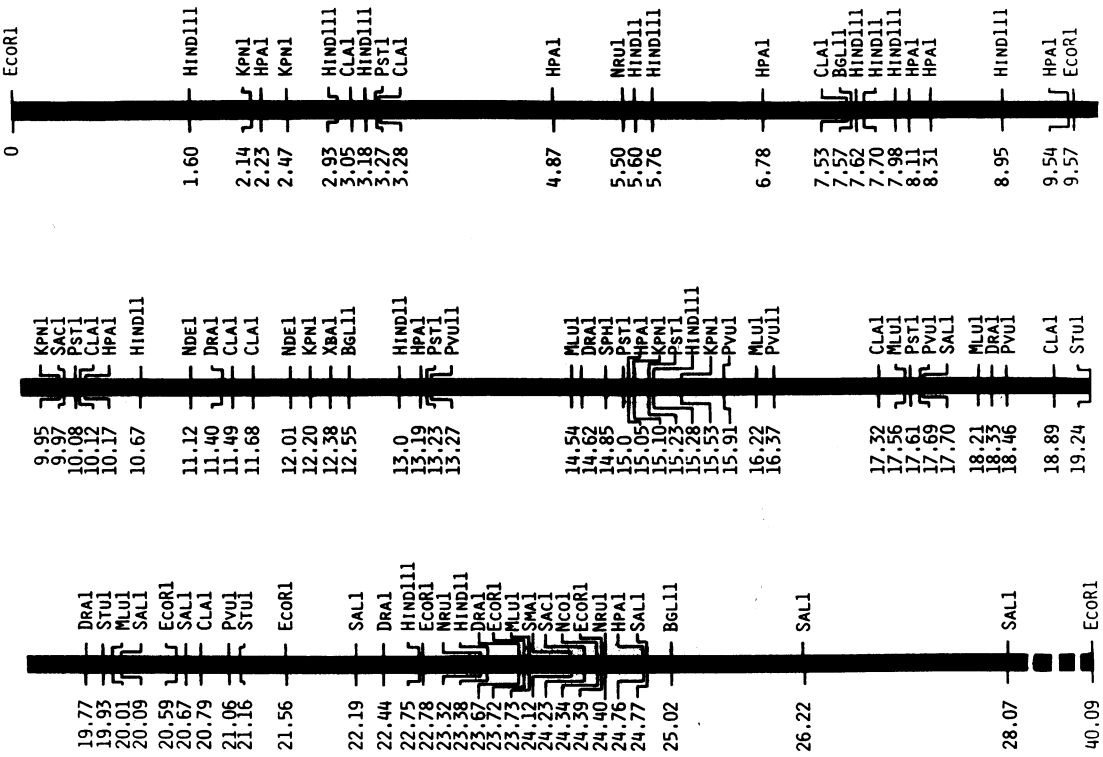

FIG. 2. Detailed restriction enzyme map of the *rfb* gene cluster of *S. typhimurium* LT2 from positions 0 to 25.02 (units in kilobases). The map from positions 0 to 7.57 has been previously published (1). The *Hin*dIII site at position 2.93 was previously mapped erroneously at position 1.35 (1). Plasmids pPR303, pPR341, pPR465, and pPR570 (Fig. 1) were digested with a set of 28 restriction enzymes which have 6-base-pair recognition sites. These enzymes were *Apa*I, *Ava*I, *Bam*HI, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Nae*I, *Nco*I, *Nde*I, *Nru*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sca*I, *Sma*I, *Sph*I, *Stu*I, *Xba*I, and *Xho*I. Digested DNAs were electrophoresed in 0.8%, 1.2%, or 2.0% agarose gels to resolve fragments of 2.73 to 8.0 kb, 0.36 to 2.73 kb, and 0.08 to 0.36 kb, respectively. Single digests were used to position unique sites relative to known vector sites and to identify enzymes with more than three sites in the mapping plasmids. Recognition sites for these enzymes (*Hin*cII and *Nru*I in the interval 15.28 to 22.75) were not mapped. Double digests were used to map sites which appeared two or three times in a cloned fragment and to determine the order of ambiguous preliminary position assignments or to measure accurately the sizes of small fragments. There are no recognition sites for the enzymes *Ava*I, *Bam*HI, *Nae*I, *Sac*II, and *Sca*I between positions 9.57 and 25.02. Only the *Sal*I and *Eco*RI sites between positions 25.02 and 40.09 have been mapped.

used as a probe for Southern hybridization analysis against *Bgl*II-cleaved chromosomal DNA from all of the *his-rfb* deletion strains listed in Table 1, with P9003 included as a positive control. The probe showed hybridization to DNA from P9003 and all of the deletion strains other than that carrying Δ(*his-metG*)*388*. The deletions known to end within *rfb* thus all terminate before the end of pPR648 at position 40.09, whereas the deletion in P9029, known to extend from within *his* through *rfb* into *metG*, terminates beyond position 40.09 (Fig. 1). Precise localization of the deletion endpoints was done by Southern hybridization analysis as described in Materials and Methods. A series of probes and enzymes were used to define the endpoints, and in Fig. 3 we present the autoradiographs for some of these experiments which localized seven of the endpoints to short intervals between adjacent restriction sites. The location of each deletion endpoint is marked in Fig. 1, together with the two determined previously (1). The probe DNA used in localizing each of the deletion endpoints is specified in Fig. 3. All the deletion endpoints are shown as lying between two restriction enzyme sites, of which only the right-hand one is conserved. In each case, several sites to the right of the deletion endpoint were also shown to be present and several sites to the left were shown to be absent.

DISCUSSION

Salmonella O antigens constitute one of the best-documented examples of polymorphism involving a surface polysaccharide. The structural analysis of the lipopolysaccharides of different groups (9) and the demonstration that nearly all the genes determining O-antigen biosynthesis were essentially in the one *rfb* cluster (17) showed that this variation could be analyzed in detail at the genetic level. However, owing largely to the instability of strains with point mutations and the absence of good selection for the *rfb* region, further elucidation of the genetic basis of this variation has been limited. Recently, we reported the cloning and physical mapping of part of the *rfb* region of *S. typhimurium* (1). We have now cloned the complete *rfb* gene cluster and established a detailed restriction enzyme map, thus laying the foundation for an analysis of the organization of the cluster and determination of the genetic basis for the variation in the O antigen of *Salmonella* spp. We have also located all of the available deletion endpoints known to lie in the *rfb* locus, thereby providing an approximate position for the genes located near each endpoint (17). The deletions all lack a group of contiguous genes (17), and the last gene deleted (i.e., distal to the *his* operon) is indicated in Fig. 1. It

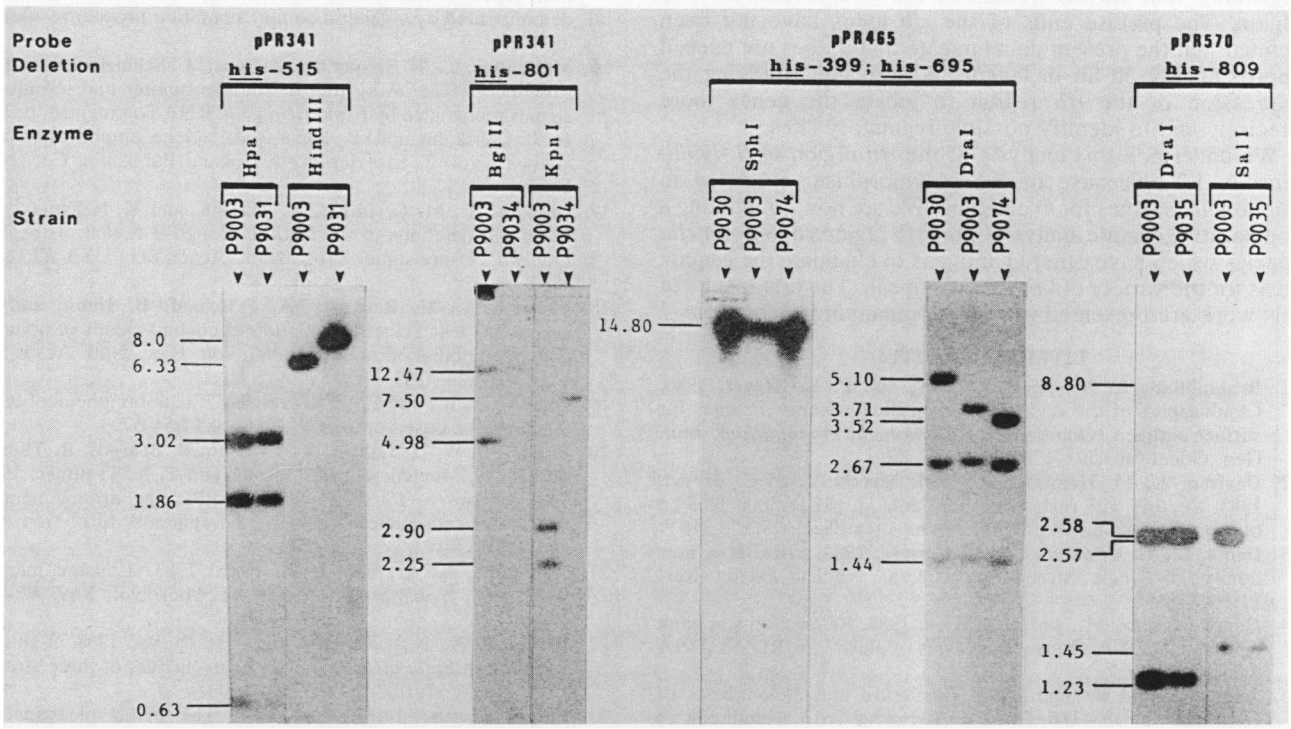

FIG. 3. Southern hybridization analysis of deletion endpoints. Data are shown only for the two restriction enzymes which cleave nearest to, and on either side of, each deletion endpoint. A control track containing wild-type DNA digested with the same enzyme is also shown for each case. The probe DNA used is indicated in the top line, and the sizes of the bands are in kilobases. Note that the 8.8-kb band in the *Sal*I digest of P9035 is very faint owing to the short region of homology with probe DNA. In several cases we can interpret the data only if we assume that fragments with only a short region of homology with probe DNA are not detected. For example, in the *his-515* deletion endpoint, the expected 9.84-kb *Hpa*I fragment (between positions 15.05 and 24.75) is not seen, but the detection of the 0.63-kb *Hpa*I fragment (between positions 9.54 and 10.17) from P9031 DNA shows that the *Hpa*I site at 9.54 is conserved. At the same time, the presence of an 8.0-kb *Hin*dIII fragment from P9031 instead of the 6.33-kb fragment (positions 8.95 to 15.28) seen in the P9003 *Hin*dIII digest shows that the *Hin*dIII site at position 8.95 is absent in P9031 DNA. Therefore, the *his-515* deletion endpoint lies between the *Hin*dIII (position 8.95) and *Hpa*I (position 9.54) sites. Other fragments having limited overlap with the probe DNA which are not detected include a 7.48-kb *Kpn*I fragment (positions 2.47 to 9.95) in the P9003-*Kpn*I track for the *his-801* deletion endpoint; a 0.25-kb *Kpn*I fragment (positions 15.23 to 15.53) in the P9003 and P9034 *Kpn*I digests for the *his-801* deletion endpoint; and a 1.23-kb *Dra*I fragment (positions 22.44 to 23.67) in the P9003, P9030, and P9074 *Dra*I digests for the *his-399* and *his-695* deletion endpoints.

is interesting that the three deletions ending in or just beyond *rfbK* all end within the same 0.24-kilobase (kb) region, although they are known to include at least two different endpoints at the other end (Table 1). A similar result is seen with the two endpoints terminating in or just beyond *rfbD* or *rfbA*, both endpoints lying in the same 0.66-kb region. It remains to be seen whether the endpoints in each cluster are at specific sites within these two regions. The deletion endpoints given here provide only an approximate estimate for the locations of the genes. Each deletion must end in or beyond the gene indicated as the deletion endpoint in Fig. 1: each gene could therefore lie anywhere between that point and the deletion endpoint to the left. Several other unmapped enzyme functions are expected to lie in the *rfb* locus; for example, only one of the four transferase genes (rhamnosyltransferase, *rfbN*) has been definitively located (8), and although Yuasa et al. (24) have suggested that the galactosyltransferase gene is at the left-hand end of the *rfb* cluster closest to *his*, where several point mutations have been identified, the other two are unmapped. Furthermore, *rfbH* is the genetic symbol for CDP-abequose synthetase, which by analogy with *Yersinia pseudotuberculosis* types II, III, and V (14), may well comprise three or more enzymes and hence genes. Two large gaps have been found between the *rfb* endpoints of deletions *his-695* and *his-809* and that of *his-101*, but only a few known genes are located here, indicating that further functions may be located in this region. The precise ends of the *rfb* locus have not been defined, but the present data indicate that it does not exceed approximately 30 kb in length. We are now studying the expression of the *rfb* region to locate the genes more precisely and to identify possible regulatory sites.

We undertook this analysis of the *rfb* region of *S. typhimurium* LT2 because of the polymorphism exhibited in *Salmonella* species for O antigens. We are now conducting a comparative genetic analysis of the *rfb* regions of *Salmonella* species which have other O antigens to elucidate the genetic basis for the variety of known O antigens. The first results of this work are presented in the accompanying paper (22).

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