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O-Antigen Variation in *Salmonella* spp.: *rfb* Gene Clusters of Three Strains

NARESH K. VERMA,* NEIL B. QUIGLEY, AND PETER R. REEVES

Department of Microbiology, the University of Sydney, Sydney, New South Wales 2006, Australia

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The O antigens of *Salmonella* serogroups A, B, and D differ structurally in their side-chain sugar residue. The genes encoding O-antigen biosynthesis are clustered in the *rfb* operon. We report here the molecular cloning and analysis of the *rfb* operons of *Salmonella paratyphi A* (serogroup A) and *S. typhi* (serogroup D). The regions of DNA nonhomology between the *rfb* operons of these serogroup A, B, and D representatives are identified, and the evolutionary derivation of serogroup A from a serogroup D progenitor is discussed.

Lipopolysaccharides are integral components of the outer membranes of gram-negative bacteria and generally consist of three structural regions: the lipid A moiety, which is hydrophobic and is embedded in the outer membrane, an oligosaccharide core, and a polysaccharide chain commonly known as the O antigen. The O-antigen chains of *Salmonella typhi*, *S. paratyphi A*, and *S. typhimurium*, representative of *Salmonella* serogroups D, A, and B respectively, have identical trisaccharide subunit backbones (mannosyl-rhamnosyl-galactosyl). These chains can be distinguished, since each has a different 3,6-dideoxyhexose attached to the mannosyl residues as a side branch. The side branch is tyvelose in *S. typhi*, paratose in *S. paratyphi A*, and abequose in *S. typhimurium* (11, 13). Much of the antigenic variation among *Salmonella* species is the result of genetic variation in the *rfb* gene cluster, which maps at 42 min on the chromosome of *S. typhimurium* LT2 (17). We previously reported the cloning and restriction analysis of the *rfb* cluster from *S. typhimurium* LT2 (1, 2). We report here the cloning and analysis of the *rfb* loci from *S. typhi* and *S. paratyphi A* strains and compare them with the *S. typhimurium rfb* locus.

We have used the traditional taxonomy for *Salmonella* species, although as Brenner (3) points out, the statement in the 1974 (8th) edition of *Bergey's Manual* that "Scientifically none of the present methods of nomenclature of *Salmonella* is satisfactory" is as true today as more than a decade ago. It is clear that all *Salmonella* strains are in one species (3, 7), and Ewing (7) has used a single species name, although the International Subcommittee has still not made a recommendation. It is important to note that the strains of *S. typhimurium*, *S. paratyphi A*, and *S. typhi* used in this study are in reality not only members of the same species, but also of the same subspecies, and the homology and nonhomology we describe for different regions of the *rfb* gene cluster must be seen in this light.

MATERIALS AND METHODS

Bacteria and plasmids. Strains used are listed in Table 1. A series of plasmids which carry DNA spanning the entire *rfb* operon of *S. typhimurium* LT2 (2) were used in this study.

Enzymes and radiochemicals. All enzymes and radiochemicals used in this study were obtained as described previously (2).

DNA techniques. The methods used for DNA preparation, agarose gel electrophoresis, radioactive labeling of DNA, in situ DNA hybridization, autoradiography, ligation, and bacterial transformation were those described by Maniatis et al. (14). Molecular size standards were used as described in the companion paper (2).

Cosmid cloning. *Sau*3A partial digests of chromosomal DNA from *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 were prepared by the method of Maniatis et al. (14). The cosmid used in this experiment was pHC79 (10). It was digested with *Bam*HI and ligated with the partial *Sau*3A fragments in a final volume of 20 μl by mixing 2 μg of insert DNA with *Bam*HI-digested and phosphatase-treated vector DNA in a molar ratio of 1:3. A 3-μl portion of this ligation mixture was packaged in vitro by the method of Sternberg et al. (19), with a commercially available packaging kit (Promega Biotec, Madison, Wis.). DH1 was used as the host strain for infection and was plated out on ampicillin agar (containing 100 μg of ampicillin per ml). 2×10^4 recombinants per μg of ligated *S. typhi* DNA and 10^3 recombinants per μg of ligated *S. paratyphi* DNA were recovered.

Preparation of heteroduplex DNA and electron microscopy. Heteroduplex formation and DNA spreading were performed by the method of Davis and Parkinson (6) with minor modifications. Grids were prepared by the basic protein film technique (12) and stained with uranyl acetate or rotary shadowed with platinum-palladium.

RESULTS

A 2.25-kilobase (kb) *Kpn*I fragment, which lies approximately in the middle of the *S. typhimurium rfb* operon (positions 9.95 to 12.20), was isolated from pPR300 (2) and used as a radioactive probe to screen cosmid libraries for the presence of homologous DNA. Eight clones in the *S. typhi* Ty21a library and two clones in the *S. paratyphi A* IMVS1316 library were identified with this probe. Cosmid DNAs from these 10 clones were digested with *Eco*RI and run on agarose gels; they were found to have a number of common bands, many of which also occur in *S. typhimurium* (2). These fragments were assumed to be homologous to those of *S. typhimurium*, and on this basis partial maps of the cosmids were deduced (Fig. 1).

Hybridization of the same 2.25-kb radioactive probe with blotted *Eco*RI digests of all cosmids and with chromosomal DNA from *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 identified a single fragment in each case which, with the exception of pPR429, had the same mobility as the 11.02-kb

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Characteristics	Source or reference
E. coli K-12 DH1	*supE44 gyrA96 recA endA1 thi-1 hsdR17 relA1*	9
S. typhimurium LT2 SL1654	*hsdL6 trpC2 nml H1b fla-66 H2*-enx *rpsL120 xyl-404 ilv-452 metE551 metA22 hsdSA29*	I. Beacham
S. paratyphi A IMVS1316		S. Dixon
S. typhi Ty2 Ty21a	*galE*	8

*Eco*RI fragment of *S. typhimurium* DNA (positions 9.57 to 20.59) from which the probe was derived (Fig. 2).

All *Eco*RI fragments from the *S. typhi* and *S. paratyphi A* cosmid clones were subcloned into plasmid vectors pBR325 and pUC9 (Fig. 1). However, only the relevant subclones are discussed here. The 11.0-kb fragments corresponding to the *S. typhimurium* fragment from positions 9.57 to 20.59, which covers most of the *rfb* gene cluster, were examined in detail. Restriction enzyme digests with *Hin*dIII, *Kpn*I, *Cla*I, *Hpa*I, *Pst*I, *Xba*I, and *Bgl*II gave identical patterns to those given with the corresponding *S. typhimurium* fragment. A *Sac*I site which was absent in *S. typhimurium* was found in *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 and mapped at a position corresponding to coordinate 12.6 of *S. typhimurium*, and a *Sac*I site at coordinate 9.97 in *S. typhimurium* LT2 was absent in both *S. typhi* Ty21a and *S. paratyphi A* IMVS1316. We conclude that DNA between the *Eco*RI sites at positions 9.57 and 20.59 in *S. typhimurium* LT2 is essentially homologous with the corresponding regions in *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 DNA. This conclusion was supported by hybridizing radioactive *S. typhimurium* DNA from this region with blotted DNA fragments from double enzyme digests of *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 DNA from this region.

Restriction site mapping was carried out on the 6.8-, 2.3-, and 1.8-kb *Eco*RI fragments from *S. typhi* Ty21a and the 6.8-, 2.8-, 2.3-, and 1.8-kb *Eco*RI fragments from *S. paratyphi A* IMVS1316. We determined the order of these fragments by using them separately as probes to hybridize with double enzyme digests of chromosomal DNA; we then constructed a restriction map of this region, from positions 0 to 10.9 of *S. typhi* Ty21a and positions 0 to 16.5 of *S. paratyphi A* IMVS1316 (Fig. 3). All restriction sites on the 6.8-, 2.3-, and 1.8-kb fragments from *S. paratyphi A* IMVS1316 were found to map at the same positions on the corresponding fragments of *S. typhi* Ty21a. The 2.8-kb *Eco*RI fragment, which stains with an intensity approximately twice that expected by comparison with adjacent bands, appears from its restriction map to duplicate the adjacent ends of the 6.8- and 1.8-kb *S. typhi* Ty21a *Eco*RI fragments (Fig. 3). We conclude that a 2.8-kb region is triplicated (Fig. 4). *Nru*I, *Hpa*I, *Eco*RI, and *Cla*I digests all gave a 2.8-kb fragment, which was shown by densitometry of gel photographs to be present at twice the molarity of other fragments. This confirmed the 2.8-kb triplication and showed that one end lies between positions 4.87 and 5.50. Further confirmation was given by the existence of the expected 12.1-kb *Kpn*I fragment in the chromosome (pPR615) which extends from position 2.47 to position 14.55 (Fig. 3).

To identify the region of nonhomology between these three *Salmonella* species, we purified a 9.57-kb *Eco*RI *S. typhimurium* DNA fragment from pPR301 (2) for use as a radioactive probe against *Eco*RI digests of chromosomal DNA from *S. typhi* Ty21a and *S. paratyphi A* IMVS1316. The probe, which contained *S. typhimurium* DNA between map positions 0 and 9.57, hybridized strongly with the 6.8-kb and weakly with the 2.3-kb *Eco*RI fragments from these strains. In addition to these fragments, the probe hybridized weakly with the 2.8-kb *Eco*RI fragment unique to the *S. paratyphi A* IMVS1316 digests (Fig. 5). The common 1.8-kb fragment did not exhibit any detectable homology with the 9.57-kb *S. typhimurium* DNA probe.

To determine whether the homology extended beyond *rfb* toward the *his* operon, we used plasmid pPR294 (1), which covers 3.35 kb directly to the left of the *Eco*RI site at

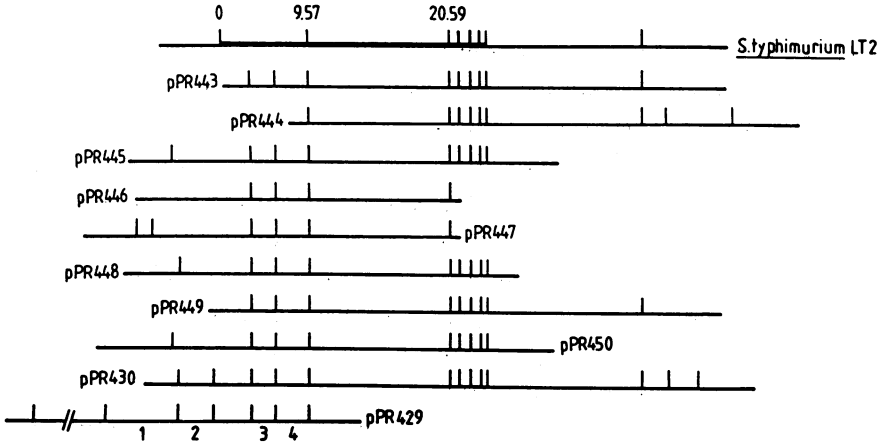

FIG. 1. *Eco*RI restriction maps of cosmids carrying part of the *rfb* operons from *S. typhi* Ty21a (pPR443, pPR444, pPR445, pPR446, pPR447, pPR448, pPR449, and pPR450) and *S. paratyphi A* (pPR430 and pPR429). The approximate extent of the *rfb* region of *S. typhimurium* LT2 is indicated by a heavy line. The other cosmids are aligned at the conserved *Eco*RI site at coordinate 9.57 of *S. typhimurium* LT2. The numbers 1, 2, 3, and 4 indicate the *rfb* DNA fragments that were subcloned to form plasmids pPR505, pPR434, pPR436, and pPR437, respectively.

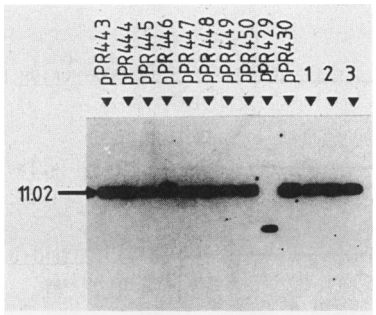

FIG. 2. *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 cosmid clones digested with *Eco*RI and probed with the 2.25-kb *Kpn*I radioactive fragment of *S. typhimurium* LT2 *rfb* DNA (positions 9.95 to 12.20) from pPR300. Lanes 1, 2, and 3 contain *S. typhimurium* LT2, *S. typhi* Ty21a, and *S. paratyphi A* IMVS1316 chromosomal *Eco*RI digests, respectively.

position 0, as a molecular probe to hybridize with several restriction enzyme digests of chromosomal DNA from *S. typhi* Ty21a and *S. paratyphi A* IMVS1316. The restriction enzyme sites toward the *his* operon for *Eco*RI, *Hpa*I, *Bgl*II, and *Pst*I were mapped previously in *S. typhimurium* (1, 4) and found to be conserved in *S. typhi* Ty21a and *S. paratyphi A* IMVS1316.

Thus, the restriction analyses presented above showed that most of the sites from positions 0 to 5.5 and from position 9.57 rightward on the *S. typhimurium rfb* map were conserved, with the 9.57-kb *Eco*RI site of *S. typhimurium* corresponding to the *Eco*RI sites at position 10.9 of *S. typhi* Ty21a and position 16.5 of *S. paratyphi A* IMVS1316. The junction between the homologous and nonhomologous regions was localized by heteroduplex analysis as follows.

Plasmid pPR505, which consists of a 6.8-kb *Eco*RI DNA fragment from *S. paratyphi A* IMVS1316 (positions 0 to 6.8) cloned in p*cos*2EMBL (16) was digested with *Eco*RI. Plasmid pPR508, which is pGB2 (5) carrying the 9.57-kb *Eco*RI fragment (positions 0 to 9.57) from *S. typhimurium*, was linearized with *Sal*I. Vectors pGB2 and p*cos*2EMBL were used because they exhibit no homology with each other (data not shown). The digestion products were allowed to hybridize in a heteroduplex reaction. Denaturation and renaturation of these plasmid DNA fragments gave rise to double-stranded linear molecules which branched into two single strands at one end (Fig. 6). By measuring the lengths of double-stranded segments of these molecules, we found that the length of homologous DNA was about 5.8 ± 0.06 kb (average of 10 measurements), showing that the region of homology extends from positions 0 to 5.8 of this strain.

To localize the other end of the nonhomologous region, plasmid pPR617, which contains the 2.3-kb *Eco*RI fragment of *S. paratyphi A* IMVS1316 cloned in pUC9, and plasmid pPR507, which is pGB2 carrying the 9.57-kb fragment of *S. typhimurium* in the opposite orientation to that in pPR508, were used in a similar heteroduplex reaction, since pUC9 also has no homology with pGB2. Plasmids pPR617 and pPR507 were linearized with *Bam*HI and *Sal*I, respectively before being mixed in a heteroduplex reaction. The double-stranded region was measured and found to be about 1.4 ± 0.05 kb (average of eight measurements), showing that the region of homology extends from position 9.57 to about 8.17 on the *S. typhimurium* map (Fig. 7).

DISCUSSION

Southern hybridization and heteroduplex analysis demonstrated that the *rfb* gene clusters of *S. typhimurium* LT2, *S. typhi* Ty21a, and *S. paratyphi A* IMVS1316 share substantial homology. The LT2 regions from positions 0 to 5.8 and from 8.17 to 9.57 were shown to be homologous by heteroduplex analysis, and with the exception of one *Sac*I site, all restriction sites examined in the region from 9.57 (the first *Eco*RI

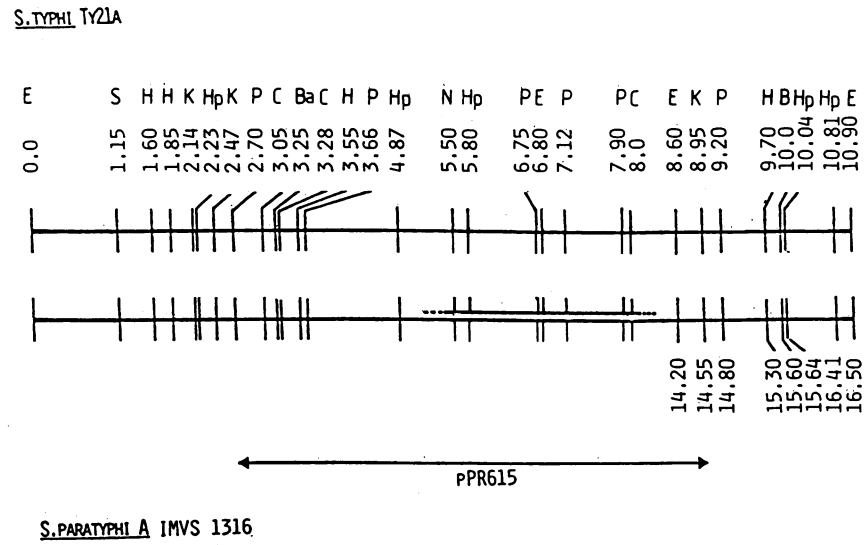

FIG. 3. Restriction map of *rfb* DNA of *S. typhi* Ty21a from positions 0 to 10.9 and *S. paratyphi A* IMVS1316 from positions 0 to 16.5. *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 have substantial similarity in their restriction maps. The *S. paratyphi A* IMVS1316 map is drawn such that sites present between positions 0 and 4.87 and positions 14.2 and 16.5 correspond to sites present between positions 0 and 4.87 and positions 8.6 and 10.9 on the *S. typhi* Ty21a map, respectively. The sites found to be triplicated in *S. paratyphi A* IMVS1316 (see text) are within a 2.8-kb region which includes the 2.5-kb segment from coordinates 5.5 to 8.0, indicated by double lines in the *S. paratyphi A* IMVS1316 map. Restriction enzyme sites: E, *Eco*RI; S, *Sac*I; H, *Hin*dIII; K, *Kpn*I; Hp, *Hpa*I; P, *Pst*I; C, *Cla*I; Ba, *Bam*HI; N, *Nru*I; B, *Bgl*II. Plasmid pPR615 is pUC19 carrying a 12.1-kb *Kpn*I fragment which extends from positions 2.47 to 14.55 on the *S. paratyphi A* IMVS1316 map.

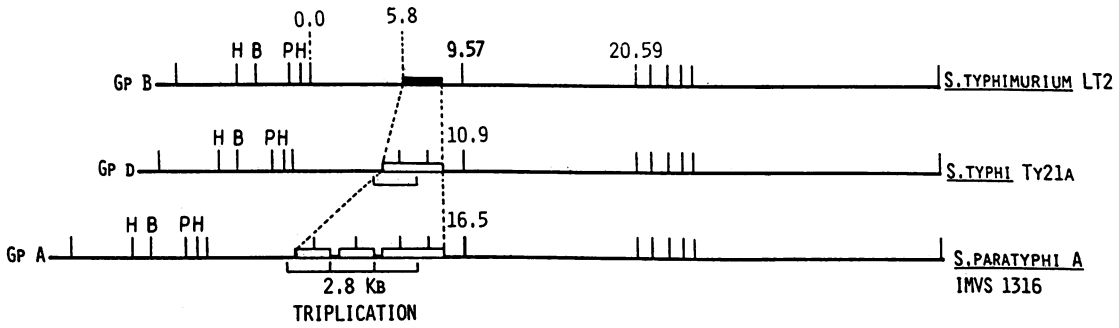

FIG. 4. Comparative map of *rfb* operons from *Salmonella* groups B, D, and A. *Eco*RI sites are indicated by vertical bars. H, B, and P are *Hpa*I, *Bgl*II, and *Pst*I recognition sites, respectively. Regions shown by restriction site homology and heteroduplex analysis to be homologous or unique are indicated as follows: —, common to all; ■, *S. typhimurium* LT2 specific; □, common to *S. typhi* Ty21a and *S. paratyphi A* IMVS1316. Note that digestion at any site present only once in a triplicated interval produces a fragment of the same length as this interval and that the fragment is present at twice the molarity of other fragments generated by this enzyme.

site known after position 8.17) to 20.59 of *S. typhimurium* were conserved in *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 DNA. However, it should be noted that although heteroduplex analysis has indicated homology in the region from positions 8.17 to 9.57, some restriction site nonhomology was detected. The remaining region of homology rightward of position 20.59 has not been examined in the same detail, but presumably the same high level of homology exists throughout this conserved region.

The DNA between positions 5.8 and 8.17 in *S. typhimurium* is replaced by a nonhomologous segment in *S. typhi* Ty21a and *S. paratyphi A* IMVS1316 (Fig. 4). The segment in *S. paratyphi A* IMSV1316 differs from that of *S. typhi* Ty21a only in having a 2.8-kb fragment triplicated, giving rise to 5.6 kb of additional DNA. The extent of the triplication was established by the presence of a 2.8-kb fragment at twice the molarity of other fragments in separate digests with four different enzymes. One end of the triplicated region must lie between the *Cla*I and *Nru*I sites at positions 8.0 and 8.6 in *S. paratyphi A* IMVS1316, and hence the other end must lie between positions 4.87 and 5.5. Thus, the 2.8-kb repeated region includes a short piece of DNA homologous with *S. typhimurium* LT2, accounting for the homology of the 2.8-kb *Eco*RI fragment with pPR301. Abequose is replaced by paratose and tyvelose in the O antigens of *S. paratyphi* and *S. typhi*, respectively. Other studies in our laboratory on *S. typhimurium* LT2 have shown that the genes for abequose synthesis (*rfbF*, *rfbG*, and *rfbH*) map in the general region of the structural difference between the three strains studied (2), and we are continuing our study of this variable region to precisely locate the genes involved in the synthesis of these three sugars.

At this stage, it seems reasonable to conclude that *S. paratyphi A* IMVS1316, which is expected to differ from *S. typhi* Ty21a in lacking CDP paratose-2-epimerase (15, 20), nonetheless carries all of the DNA present in *S. typhi* Ty21a, but that the gene for tyvelose synthesis is presumably inactive in *S. paratyphi A* IMVS1316. Sasaki and Uchida (18) showed that a group D strain could mutate to lose the epimerase and then resemble a group A strain by producing paratose. Our data show that in at least one instance, a naturally occurring group A strain arose in this way.

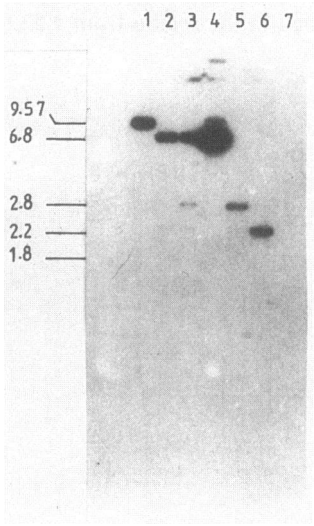

FIG. 5. Southern hybridization analysis of *Eco*RI digests of *S. typhimurium* LT2, *S. typhi* Ty21a, and *S. paratyphi A* IMVS1316 chromosomal DNA and of various plasmids by using the *Eco*RI fragment of *S. typhimurium* LT2 *rfb* DNA from positions 0 to 9.57 as a radioactive probe. Lanes: 1, *S. typhimurium* LT2 chromosomal DNA; 2, *S. typhi* Ty21a chromosomal DNA; 3, *S. paratyphi A* IMVS1316 chromosomal DNA; 4, pPR505; 5, pPR434; 6, pPR436; 7, pPR437. The *Eco*RI subclones in lanes 4 to 7 carry the 6.8-, 2.8-, 2.3-, and 1.8-kb *rfb* DNA fragments, respectively, from *S. paratyphi A* IMVS1316. The two bands of least intensity visible in lane 4 correspond to partial digestion products of pPR505.

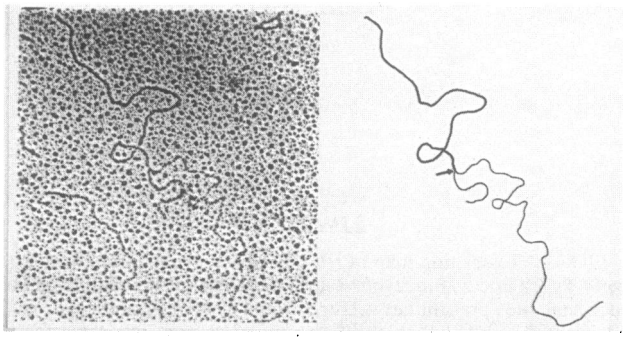

FIG. 6. Heteroduplex formed between a 6.8-kb *Eco*RI fragment from pPR505 (*S. paratyphi A* IMVS1316) and plasmid pPR508 (*S. typhimurium* LT2) linearized with *Sal*I. The arrow indicates the end of the region of homology.

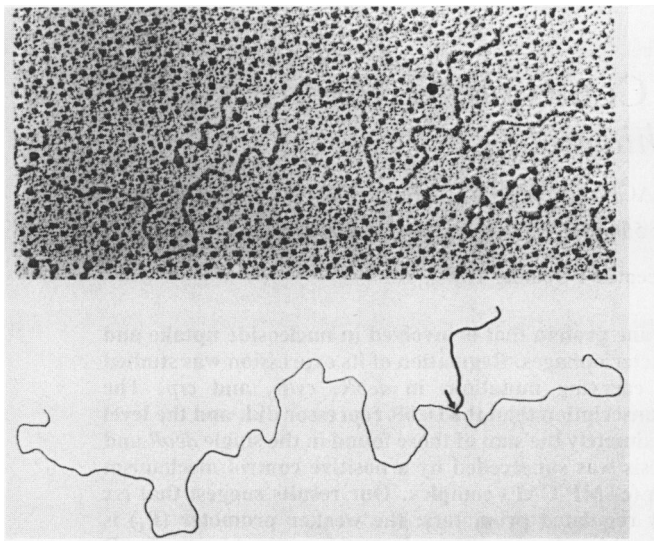

FIG. 7. Heteroduplex formed between plasmids pPR617 and pPR507 linearized with *Bam*HI and *Sal*I, respectively. The arrow indicates the end of the region of homology.

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