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Molecular genetic basis for complex flagellar antigen expression in a triphasic serovar of *Salmonella*

(flagellin/phase variation/plasmids/recombination/polymerase chain reaction)

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ABSTRACT **Strains of most *Salmonella* serovars produce either one (monophasic) or two (diphasic) antigenic forms of flagellin protein, but strains capable of expressing three or more serologically distinct flagellins ("complex" serovars) have occasionally been reported. A molecular genetic analysis of a triphasic strain of the normally diphasic serovar *Salmonella rubislaw* revealed that it has three flagellin genes, including the normal *fliC* (phase 1) and *fljB* (phase 2) chromosomal genes encoding type r and type e,n,x flagellins, respectively, and a third locus (herein designated as *flpA*) that is located on a large plasmid (pRKS01) and codes for a type d flagellin. The coding sequence of the plasmid-borne gene is similar to that of a phase 1 chromosomal gene, but the sequence of its promoter region is homologous to that of a phase 2 chromosomal gene. The irreversible loss of the ability to express a type d flagellin that occurs when the triphasic strain is grown in the presence of d antiserum is caused by deletion of part or all of the *flpA* gene. Thus, the molecular basis for the unusual serological reactions of the triphasic strain of *S. rubislaw* and, by inference, other complex serovars of *Salmonella* is explained. Plasmids of the type carried by the triphasic strain of *S. rubislaw* provide a mechanism for the generation of new serovars through the lateral transfer and recombination of flagellin genes.**

Strains of the diphasic serovars of *Salmonella* produce two serologically distinct types of flagellin, which is the major component of the bacterial flagellar filament. The alternate expression of different flagellin proteins is known as phase variation; cells in phase 1 express the *fliC* flagellin gene and those in phase 2 express the *fljB* flagellin gene and the associated repressor (*fljA*) of the phase 1 gene (1). Both flagellin genes are located on the chromosome (2), and phase transition is controlled by a site-specific recombination mechanism that periodically inverts the promoter region of the phase 2 flagellin operon. Antigenic variation of the phase 1 and phase 2 flagellins, in combination with that of the cell-wall lipopolysaccharide, is the basis of the Kauffmann–White serotyping scheme, which is universally employed to classify and identify strains of *Salmonella* (3, 4).

Isolates of the more than 2200 recognized *Salmonella* serovars are normally either diphasic or monophasic (producing only one type of flagellin), but strains that can express three or more flagellins ("triphasic" or other "complex" serovars) have occasionally been reported (4). For example, the serovar *Salmonella rubislaw* is normally diphasic, with the antigenic formula 11:r:e,n,x, which indicates a lipopolysaccharide of type 11, a phase 1 flagellin of type r, and a phase 2 flagellin exhibiting three antigenic factors, e, n, and x. However, a strain recovered from a human in 1963 was identified as a triphasic form of *S. rubislaw* by McWhorter *et al.* (5). The serological reactions of this strain, which in kind are typical of all triphasic serovars, are as follows: cells in phase 1 produce only antigenic type d flagellin, whereas those in phase 2 express flagellins with four antigenic factors, d, e, n, and x. Hence, the antigenic formula is 11:d:d,e,n,x, which involves a combination of phase 1 and phase 2 antigens not recognized in the Kauffmann–White scheme (4). When cells in either phase 1 or phase 2 are grown in the presence of d antiserum, colonies are recovered that have irreversibly lost the ability to produce type d flagellin and, instead, express a new phase 1 flagellin of an *r* antigenic type. The passaged cells are now serologically indistinguishable from normal diphasic strains of *S. rubislaw*.

Several hypotheses have been advanced to account for the occurrence of triphasic strains of *Salmonella*. On the arbitrary assumption that the long-term evolution of the salmonellae has involved progressive reduction in the number of flagellin genes (6), it has been suggested that the triphasic and other complex serovars are archetypal forms from which the diphasic and monophasic serovars evolved (7). Alternatively, Douglas and Edwards (8) concluded that some triphasic serovars are merely diphasic strains that happen to express flagellins of the same major antigen type in both phases. The occurrence of a third phase was interpreted as an example of "R phase" variation, in which serological challenge induces the expression of new, presumably mutant antigens (9).

We here report the results of a molecular genetic analysis of the triphasic strain of *S. rubislaw* in which we have discovered that the type d flagellin is encoded by a third flagellin gene located on a plasmid and that the irreversible loss of this flagellin is caused by deletion of part or all of the plasmid-borne gene.†

MATERIALS AND METHODS

Bacterial Strains. The triphasic *S. rubislaw* strain (RKS 5310; CDC 2209-63) was obtained from A. C. McWhorter, of the Centers for Disease Control, Atlanta, GA (5). Strains of diphasic *S. rubislaw* (RKS 938), *Salmonella typhimurium* (RKS 284), *Salmonella muenchen* (RKS 3121), and *Salmonella typhi* (RKS 3333) were described by Smith *et al.* (10). Three derivative strains of RKS 5310 that cannot express a d flagellin antigen were selected by growth in semi-solid agar (cystine tryptic agar; Difco) containing d antiserum. Plates were inoculated with the triphasic *S. rubislaw* strain and, after overnight incubation, isolates were obtained from the leading edge of a 50-mm zone of growth extending from the point of inoculation.

Chromosomal DNA. Methods of preparation, restriction digestion, Southern transfer and hybridization, and polymerase chain reaction (PCR) amplification of chromosomal DNA

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Abbreviation: PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58145).

were as described (10, 11). Single-stranded DNA for sequencing was generated from gel-purified PCR products by the λ exonuclease method (12). Template DNA was sequenced on both strands, except as noted, as described elsewhere (10).

Plasmid DNA. The method of Kado and Liu (13) was used to prepare plasmid DNA for gel electrophoresis. DNA for sequencing was isolated by excision of ethidium bromide-stained fragments from an agarose gel and purified by extraction with phenol. To sequence the region in front of the *flpA* gene, a 3.5-kilobase (kb) *Kpn* I restriction fragment was identified by Southern hybridization with a *d* allele-specific probe. A *Kpn* I restriction digest of total DNA from the triphasic *S. rubislaw* strain was then size-separated on a NuSieve agarose gel, and fragments in the range 3.4–3.6 kb were purified with Geneclean (Bio 101, La Jolla, CA). The fragments were circularized with T4 ligase, and regions external to the *flpA* gene were PCR-amplified with primers 1 and 9. This inverse PCR amplification (14) generated a 2.2-kb product, and primer 8 was then used to sequence 200 base pairs (bp) adjacent to the start of the *flpA* gene.

Primers and Probes. Primers 1, 2, and 5 have already been described (10). The sequences of other primers used are as follows.

Primer 7: 5′-CCCACACCTAATGATGAAATTGAAGC-3′.
Primer 8: 5′-GCGGTGCCCAGAGCGGAC-3′.
Primer 9: 5′-CAGAACCGTTTCAACTCCG-3′.
Primer 10: 5′-CCTGTTTTATCATCCAGCCAG-3′.
Primer 11: 5′-TATGTAGCGGCATAGAAATCG-3′.

A universal probe for flagellin genes consisting of a 291-bp segment (from base pair 77 to base pair 368) of the *d* allele of the *fliC* gene reported by Wei and Joys (15) was generated by PCR amplification of chromosomal DNA extracts from *S. typhimurium* strain RKS 284. A 443-bp *Cla* I–*Pst* I restriction fragment cloned from a PCR product of the *fliC* gene of *S. typhi* (RKS 3333) was used as a *d* allele-specific probe (10). Probes specific for the *r* allele and the *hin* gene were previously described (10, 11).

RESULTS

The triphasic *S. rubislaw* strain (RKS 5310) and a diphasic strain of *S. rubislaw* (RKS 938) were analyzed by multilocus enzyme electrophoresis (16, 17) to estimate their overall genetic relatedness. They proved to be very similar in chromosomal genotype, differing in alleles at only 1 of the 21 chromosomal enzyme loci assayed.

Triphasic *S. rubislaw* Has Three Flagellin Genes. The hypothesis that the triphasic serovars of *Salmonella* are merely diphasic strains producing phase 1 and phase 2 flagellins that share a major antigen (8) implies that only two flagellin genes are present. To test this hypothesis, total DNA was prepared from the triphasic and diphasic *S. rubislaw* strains, restriction digested with *Eco*RI, and tested with the universal flagellin gene probe to identify fragments containing flagellin gene sequences. Three hybridizing fragments, of 3.6, 15.5, and 17.5 kb, were identified in the DNA from the triphasic *S. rubislaw* strain (Fig. 1, lane 2), two of which—the 3.6-kb and 15.5-kb fragments—were shared with the diphasic strain (Fig. 1, lane 1). The flagellin allele associated with each of the three restriction fragments was identified by hybridization with probes for the *d* allele (17.5-kb), the *r* allele (3.6-kb), and the *hin* gene (15.5-kb) (Fig. 1, lanes 3–5). By analogy with the condition in *S. typhimurium* (2), we assume that the *hin* gene is adjacent to the *fljB* gene (phase 2 flagellin) in these strains. We conclude that the triphasic *S. rubislaw* strain has three flagellin genes: *fliC* (*r* allele) on the 3.6-kb *Eco*RI fragment, *fljB* (*e,n,x* allele) on the 15.5-kb fragment, and a third gene of the *d*-allele type on the 17.5-kb fragment. In contrast, the diphasic *S. rubislaw* strain has only two flagellin genes, *fliC* and *fljB*.

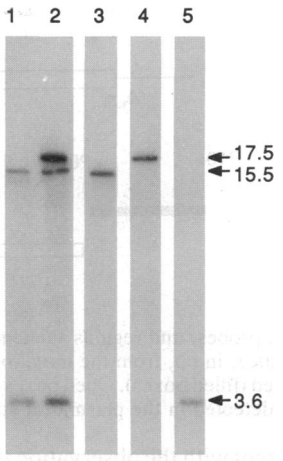

FIG. 1. Southern hybridization of *Eco*RI restriction-digested DNA from the diphasic *S. rubislaw* (lane 1) and triphasic *S. rubislaw* strains (lanes 2–5) with four specific probes. Lanes 1 and 2, universal flagellin gene; lane 3, *hin*; lane 4, *d* flagellin allele; and lane 5, *r* flagellin allele. The size (in kb) of each hybridizing fragment, estimated by comparison with molecular weight markers of known size, is indicated.

The Third Flagellin Gene Is Plasmid-Borne. The irreversibility of the loss of a major flagellin antigen that is characteristic of triphasic serovars of *Salmonella* when grown on selective medium suggested that the third gene is located on an accessory DNA element. To explore this possibility, we analyzed plasmid DNA from the triphasic *S. rubislaw* strain by agarose gel electrophoresis (Fig. 2, lane 6). This strain carries two plasmids, the largest of which (herein designated pRKS01) is approximately the same size as the 214-kb standard plasmid of *S. flexneri* strain 2457O (Fig. 2, lane 5) (20). The plasmid DNA was tested with the universal flagellin gene probe and, subsequently, with the *d* allele-specific probe. Both probes hybridized strongly to pRKS01, but a probe for the *hin* gene did not anneal with any of the extrachromosomal DNA. The absence of a *hin* gene on

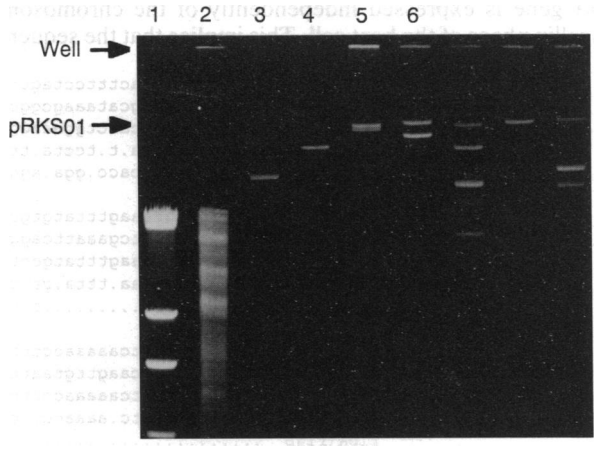

FIG. 2. Agarose gel electrophoresis of extrachromosomal DNA prepared from *Escherichia coli* strain V517, containing a 55-kb plasmid (lane 3) (18); *S. typhimurium* strain LT2, containing a 90-kb plasmid (lane 4) (19); *Shigella flexneri* strain 2457O, containing two plasmids of 160 kb and 214 kb (lane 5) (20); and triphasic *S. rubislaw* strain RKS 5310 (lane 6), containing two plasmids, one of which is pRKS01, as indicated. Lane 1 is the *Hin*dIII fragments of λ DNA and lane 2 is an *Eco*RI restriction digestion of chromosomal DNA from *S. typhimurium* laboratory strain LT2. The three unmarked lanes are other triphasic strains of *Salmonella* (see text).

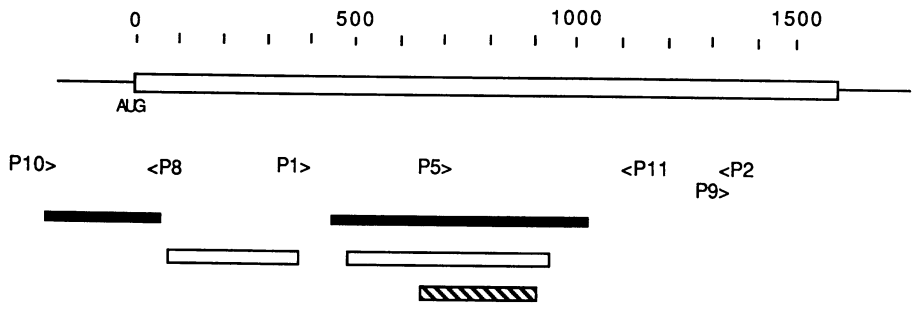

FIG. 3. Primers, probes, and regions sequenced. Schematic representation of the plasmid-borne *flpA* gene and flanking regions; the scale represents the distance, in bp, from the initiation codon (AUG) of the gene. The positions and directions of PCR primers are marked, as are the regions sequenced (filled boxes). Open boxes represent the position of the universal flagellin probe (left) and the *d* flagellin allele probe (right). The small deletion detected in the plasmid of strain RKS 5313 is indicated by the hatched box.

pRKS01 is consistent with the observation that only one copy of this gene is present in the triphasic *S. rubislaw* strain (Fig. 1, lane 3). To confirm that the third flagellin gene is plasmid-borne, pRKS01 DNA was excised from an agarose gel, purified, and subjected to PCR amplification with primers 1 and 2 (Fig. 3), which have been used to amplify phase 1 and phase 2 flagellin genes from strains of various *Salmonella* serovars (10, 11). A single fragment was amplified, and digestion with *Pst* I revealed a restriction pattern identical to that of the chromosomal *d* allele of *S. typhi* (strain RKS 3333) (10). The extracted plasmid DNA was also subjected to PCR amplification with primers 2 and 7, a combination that we have used to specifically amplify the *fliC* gene in strains of several other *Salmonella* serovars (unpublished data). [Primer 7 was derived from the sequence of the *fliD* gene, which is adjacent to *fliC* in *S. typhimurium* (21).] But under various reaction conditions with pRKS01 DNA as template, we were unable to amplify a flagellin sequence with this primer.

In sum, the triphasic *S. rubislaw* strain has, in addition to the two normal chromosomal flagellin genes, a third, plasmid-borne, type *d* flagellin gene. Because the plasmid-borne gene is not associated with either a *fliD* gene or a *hin* gene, it cannot be identified as either a *fliC* or a *fljB* gene. Accordingly, we designated it as *flpA* (flagellin-plasmid located).

Promoter Sequence of the *flpA* Gene. The serological reactions of the triphasic serovars of *Salmonella* indicate that the *flpA* gene is expressed independently of the chromosomal flagellin phase of the host cell. This implies that the sequence of the promoter region of the *flpA* gene differs from the corresponding sequences of the *fliC* and *fljB* genes. Support for this conclusion comes from the absence of a *hin* gene on the plasmid and our failure to amplify the *flpA* gene with primers derived from the *fliD* gene. To obtain the sequence of the promoter region of the *flpA* gene, we used an inverse PCR (14), and from this sequence we designed a PCR primer located 181 bp from the initiation codon of the *flpA* gene (primer 10, Fig. 3). This primer was used, in combination with *d* allele-specific primers, to PCR amplify and sequence the promoter region of the *flpA* gene; the region sequenced extends from 164 bp in front of the initiation codon to 60 bp inside the coding region (Fig. 3).

To assess the extent of sequence variation in the promoter region of *d* alleles from various strains, we used primers 2 and 7 to PCR amplify and sequence 244 bp directly in front of the *fliC(d)* gene of strains of *S. muenchen* (RKS 3121) and *S. typhi* (RKS 3333) (10). The segment extends from the coding region of the *fliC* gene to within 10 bp of the coding region of the *fliD* gene and was in both cases identical in sequence to the published sequence of this region of the *S. typhimurium* chromosome (22, 23).

In Fig. 4, the promoter sequence and promoter-proximal coding sequence of the *flpA* gene may be compared with the corresponding sequences of the *fliC* gene of *S. typhimurium* (22, 23) and the *fljB* gene of *Salmonella adelaide* (21). At five of the six sites that are polymorphic in the first 20 codons of the these genes, *flpA* and *fljB* share nucleotides, and this

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flpA        gggggtacttcactttcctagttagtcccgtggagccttaacgaatgtgatcgaagccaatcc 63
fliC        ggaataatgatgcataaagcggctatttcgccgcctaagaaaaagatcgggggaagtgaaaaa 63
fljB        gacacagtattcacctggaaaggctttttaatcaaaatgttagatgtaagcaattacggacag 63
flpA\fliC   ..gggt.cttca.t.tccta.ttag.cc..tg.agcctt..cg.atgt.atc....cc..tcc
flpA\fljB   .acacagta.tcacc.gga.aggct.tttaatc.aaa.gtta..tgtaagcaatta.gg.cag

flpA        aaaaattagtaaagtttatgtgctaaatgtcgataacctggatgacacaggtaagcctggcat 126
fliC        ttttctaaagttcgaaattcaggtgccgatacaagggttacggtgagaaaccgtgggcaacag 126
fljB        aaaaaatagtaaagtttatgcctcaactgtcgataacctggatgacacaggtaagcctggcat 126
flpA\fliC   aaaaa.t.gtaaa.ttta.gt.c.aaatg.cg.taacc.ggatgacac.ggtaa.cctgg..t
flpA\fljB   .....a.............cctc..c.....................................

flpA        aacattggttatcaaaaaccttccaaaaggaaaatttt ATG GCA CAA GTA ATC AAC  182
fliC        cccaataacatcaagttgtaattgataaggaaaagatc ATG GCA CAA GTC ATT AAT  182
fljB        aacattggttatcaaaaaccttccaaaaggaaaatttt ATG GCA CAA GTA ATC AAC  182
flpA\fliC   aa..t.ggttatc.aaaacct.cc.a.....tt.t ... ... ... ..A ..C ..C
flpA\fljB   ..................................... ... ... ... ... ... ...

flpA        ACT AAC AGT CTG TCG CTG TTG ACC CAG AAT AAC CTG AAC AAA         224
fliC        ACA AAC AGC CTG TCG CTG TTG ACC CAG AAT AAC CTG AAC AAA         224
fljB        ACT AAC AGT CTG TCG CTG CTG ACC CAG AAT AAC CTG AAC AAA         224
flpA\fliC   ..T ... ..T ... ... ... ... ... ... ... ... ... ... ... ...
flpA\fljB   ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ...
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FIG. 4. Sequence comparison of the promoter and promoter-proximal coding region of the *flpA* gene (this study), the *fliC* gene (22, 23), and the *fljB* gene (21). The sequence shown extends from 164 bp in front of the coding region of each gene (lowercase letters) to 60 bp within the coding region (uppercase letters). Also shown are the nucleotide differences between the *flpA* and *fliC* (flpA\fliC) and between *flpA* and *fljB* (flpA\fljB).

sequence homology continues for 101 bp in front of the genes. However, the 63-bp region farthest from the coding region of *flpA* shows little similarity with the corresponding region of *fljB* (Fig. 4); the promoter region of *flpA* has less homology with that of *fliC*.

In sum, the promoter region and the promoter-proximal coding sequence of the plasmid-borne flagellin gene (*flpA*) has greater homology with a phase 2 gene (*fljB*) than with a phase 1 gene (*fliC*).

Molecular Basis for Loss of the d Antigen. Three independent derivatives of the triphasic *S. rubislaw* strain that migrated through semi-solid agar containing d-specific flagellin antiserum were designated as strains RKS 5313, RKS 5314, and RKS 5315. When extrachromosomal DNA prepared from these strains and the parental triphasic strain was visualized by agarose gel electrophoresis, no variation in either plasmid content or size was apparent. The plasmid DNA was then tested with the universal flagellin gene probe, to which the *flpA*-bearing plasmid (pRKS01) hybridized strongly, as did the plasmid of similar size from RKS 5313; but the plasmid DNA from RKS 5314 and RKS 5315 did not anneal with this probe. The extrachromosomal DNA was then used as a template for PCR amplification with primers specific for *d* flagellin alleles. Primers 5 and 11 (Fig. 3) amplified a 378-bp segment from the central region of the *flpA* gene of pRKS01 but failed to amplify sequences from the three derivative strains. Primers 10 and 11 (Fig. 3) amplified a 1217-bp segment that included the promoter region and much of the *flpA* gene from pRKS01 but did not amplify sequences from the plasmid DNA of strains RKS 5314 and RKS 5315. However, a 950-bp sequence was amplified from the plasmid of RKS 5313. These findings indicate that most of the *flpA* gene was deleted from the plasmids of RKS 5314 and RKS 5315 and that a 261-bp segment was deleted from the plasmid of RKS 5313.

It has been reported that a type *j fliC* allele can be generated by deletion of a 261-bp segment from the antigen-coding region of the normal *fliC* (*d*) allele of *S. typhi* (24). Because d antiserum does not cross-react with type j flagella, cells expressing type j flagellin are unaffected by d antiserum in motility experiments. To confirm that the plasmid of derivative strain RKS 5313 carries a *j* allele, we sequenced the appropriate region of the *flpA* gene and compared it with the sequence of the same region of pRKS01 from the triphasic strain. Single-stranded DNA was derived from segments amplified by PCR primers 10 and 11, and the region from base pair 457 to base pair 1027 (15) was sequenced (Fig. 3). This segment of pRKS01 was only slightly divergent in sequence from that of the *fliC*(*d*) allele of *S. muenchen* reported by Wei and Joys (15); we observed only two changes in 570 bp. (The full sequence of the *flpA* gene will be reported elsewhere.) When the same region of the plasmid from strain RKS 5313 was sequenced on one strand, it was found to have a deletion of 261 bp (Fig. 3) and was identical in sequence to the *j* allele derivative of the *fliC* (*d*) gene of *S. typhi* (24).

DISCUSSION

We have shown that a triphasic strain of *S. rubislaw* has a plasmid-borne flagellin gene (*flpA*) in addition to the normal phase 1 and phase 2 chromosomal flagellin genes (*fliC* and *fljB*). The *flpA* gene hybridizes with a probe specific for the *d* flagellin allele and has a central antigen-coding region that is virtually identical in sequence to the *d* allele of the *fliC* gene of *S. muenchen* (15). Although *flpA* encodes a type d flagellin and, therefore, represents an allele that is almost invariably associated with phase 1 flagellins (4), the promoter and promoter-proximal coding sequences show greater homology with a phase 2 flagellin gene. We consider this homology significant because the four *fliC* alleles that have been sequenced (refs. 21 and 22; this study) show little or no nucleotide variation in this region. The sequence homology between the promoter regions of *flpA* and *fljB* does not extend to the *hin* region; indeed, a *hin* gene could not be detected on these plasmids by Southern hybridization. Consequently, *flpA* has a mosaic structure and appears to consist of a phase 2 flagellin promoter coupled to a central, antigen-determining region derived from a phase 1 gene.

Our findings explain at the molecular level the unusual, and hitherto puzzling, serological reactions of the triphasic *S. rubislaw* strain and, by inference, complex *Salmonella* serovars in general. Because the sequence of the promoter of *flpA* is similar to that of a phase 2 gene, *flpA* is unlikely to be sensitive to repression by the product of the *fljA* gene, which is cotranscribed with the phase 2 flagellin gene (22). For the triphasic strain of *S. rubislaw*, we suggest that cells in phase 2 express the phase 2 chromosomal flagellin gene (*fljB*) and the plasmid-borne gene (*flpA*). Simultaneous expression of these genes results in flagellar filaments composed of both flagellin proteins (see ref. 25), and, thus, the antigenic reactions of these strains are d, e, n, and x. In contrast, when *fljB* and the repressor of *fliC* are not being expressed, chromosomally encoded phase 1 flagellin is absent and only a type d flagellin encoded by the plasmid-borne gene is produced. This observation suggests the presence of a repressor of the *fliC* gene that is cotranscribed with the *flpA* gene in a manner analogous to the *fljA* gene of the phase 2 flagellin operon. Finally, the irreversible loss of the type d flagellin is explained by the selection of cells with plasmids from which part or all of the *flpA* gene has been deleted. When the gene is absent, cells express the normal diphasic *S. rubislaw* phase 1 and 2 flagellins; but when the deletion is partial and merely converts *flpA* from a *d* to a *j* allele, the cells remain triphasic and fail to express the chromosomal type r phase 1 flagellin. The observation that growth in d antiserum selects for deletions rather than mutations that silence the *flpA* gene suggests that expression of *flpA* is not controlled by a site-specific inversion system (1).

In related work, we have interpreted the occurrence of the same *fliC* alleles in very distantly related serovars of *Salmonella* as evidence that the lateral transfer and chromosomal integration of flagellin gene sequences is a major evolutionary process generating new serovars (10, 16). We now suggest that plasmids of the type we have identified are vehicles for the lateral transfer of flagellin genes, and, significantly, we have recently discovered that strains of several other, chromosomally unrelated triphasic *Salmonella* serovars contain plasmids that are similar in size to pRKS01 (see Fig. 2) and also carry *flpA* (*d*) genes (unpublished data). Transfer of the pRKS01 plasmid, either by conjugation or as a phage, would alter the array of antigenic flagellin types produced by the recipient cell and, thus, would generate a novel complex serovar. Moreover, because their flagellin genes are available for homologous recombination with chromosomal flagellin genes, plasmids of this type also provide a mechanism for the evolution of new monophasic and diphasic serovars.

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