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Sequence Analysis of *rsk*, a Portion of the 95-Kilobase Plasmid of *Salmonella typhimurium* Associated with Resistance to the Bactericidal Activity of Serum

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Increased sensitivity to killing by human serum complement occurs in *Salmonella typhimurium* strains in which the 95-kilobase virulence plasmid is integrated into the chromosome. This phenotypic change appears to be due to alterations in plasmid gene expression and is reversed by the presence of an autonomous plasmid bearing a cloned region of the virulence plasmid. Accordingly, this region has been termed *rsk* for reduced serum killing. Sequence analysis of the region reveals that *rsk* is composed of a series of direct 10-base-pair (bp) repeats with a 21-nucleotide periodicity. Two adjacent repeats are identical, but increasing loss of conservation is apparent with increased distance both 5′ and 3′ of these highly conserved 10-mers. The smallest isolated sequence which restores the serum-resistant phenotype is only 66 bp long and contains the two identical 10-mers and one degenerate 10-mer (8 of 10 bp conserved) 3′ of these. The minimal *rsk* region of 66 bp does not appear to contain a coding sequence, or a promoter, for a structural gene. It is proposed that the minimal *rsk* is an isolated regulatory site involved in the regulation of the serum resistance of *S. typhimurium*. Integration of the 95-kilobase plasmid disrupts the normal regulation of virulence plasmid genes, resulting in an increase in the killing of the bacteria by complement activated by the classical pathway. The introduction of the minimal *rsk* on a multiple-copy plasmid restores resistance to serum killing, possibly through the titration of a *trans*-acting regulatory factor.

Integration of the presumed low-copy-number *Salmonella typhimurium* 95-kilobase (kb) virulence plasmid (33) into the chromosome elevates the susceptibility of smooth strains of *S. typhimurium* to complement-mediated killing while affecting neither growth in decomplemented serum nor the quantity or the composition of the lipopolysaccharide (36). This shift from a serum-resistant to a more serum-sensitive state cannot be ascribed to plasmid-mediated insertional mutations in chromosomal genes, since the serum resistance phenotype is restored by the introduction of an autonomous copy of the virulence plasmid into these strains, which continue to maintain the integrated plasmid copy (20, 36). The chromosomal sites involved in plasmid integration, moreover, differ in both of the strains examined (D. K. Rabert, Ph.D. thesis, University of Michigan, Ann Arbor, 1987). Furthermore, the serum sensitivity phenotype is not caused by the interruption of a plasmid locus at the site of Tn*10* insertion or at the site of plasmid integration. Both strains carrying the Tn*10*-marked virulence plasmids, pCF610 and pCF810, exhibit wild-type levels of serum resistance and have Tn*10* inserted at the same site; in this site which is interrupted upon their integration into the chromosome (Rabert, Ph.D. thesis) to create the serum sensitivity phenotype (36).

Other studies have also correlated the 95-kb plasmid of *S. typhimurium* with resistance to the bactericidal activity of serum complement (14, 15) and with mouse virulence (3, 12, 13, 20, 30). Contrary to these findings, Gulig and Curtiss (12) concluded that the 95-kb plasmid did not increase resistance to serum killing. Interpretation of these results is complicated by the use in the latter study of human serum which had been absorbed with salmonellae to specifically eliminate antibody-dependent activation of complement by the classical pathway. VandenBosch et al. (36), however, demonstrated that differential serum killing of smooth strains of *S. typhimurium* results primarily from the action of human serum complement activated by the antibody-dependent classical pathway and that smooth strains resist killing by the antibody-independent alternative pathway (36).

Resistance to complement-mediated killing may be multifactorial. Studies on rough variants of *S. typhimurium* have demonstrated the quantity and composition of the lipopolysaccharide to be the main determinant of serum resistance of such strains (2, 8). Hackett et al. (14) have described an 11-kilodalton (kDa) protein, encoded by a gene of the 95-kb plasmid, which confers serum resistance on rough *S. typhimurium* and *Escherichia coli* strains (14). The *S. typhimurium* 95-kb plasmid-associated serum resistance described by VandenBosch et al. (36), however, is apparent for *S. typhimurium* cells of a smooth phenotype but was not detected for rough isolates tested (unpublished data).

The simplest explanation of the serum sensitivity phenotype caused by plasmid integration is that alterations in the levels of expression of plasmid genes follow integration. Accordingly, a study was undertaken to clone and analyze plasmid sequences that reverse this unique serum sensitivity phenotype of smooth strains. This strategy should allow both structural and regulatory genes involved in serum resistance to be recognized. In this study, a cloned fragment of the virulence plasmid that restores serum resistance to a serum-sensitive strain with an integrated plasmid copy is described. The region found to confer serum resistance, termed *rsk* for reduced serum killing, has the characteristics of a regulatory site.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The serum-sensitive test strain CR6260 (20, 36) carries only a single integrated copy of the virulence plasmid pCF610 (Rabert, Ph.D. thesis). Strain CR6260 was isolated as a tetracycline-sensitive derivative of the wild-type virulent strain CR6600 (20, 36) by introducing a Tn*10* into the wild-type virulence plasmid pCF601 to give plasmid pCF610 and then selecting for loss of the tetracycline marker (20). Integration has been shown to involve Tn*10* sequences and to have occurred by inverse transposition with the concomitant loss of the resistance to tetracycline (Rabert, Ph.D. thesis). Plasmid pCF810 is a Tn*10*-tagged derivative of virulence plasmid pCF801, found originally in strain CR8500 (20, 36); it was constructed with the same bacteriophage lysate used to make plasmid pCF610 (20). Southern hybridization and restriction enzyme analyses demonstrated that the Tn*10* insertions are in similar, if not identical, locations in plasmids pCF801 and pCF601 and that these plasmids are indistinguishable (Rabert, Ph.D. thesis). Plasmid pCF801 provided the source DNA for clone construction.

Culture conditions. Unless otherwise stated, all cultures were incubated at 37°C for 18 h in L broth with gentle shaking. Viable cell counts were done on tryptic soy agar plates (Difco Laboratories, Detroit, Mich.). Tetracycline (25 μg/ml) or ampicillin (50 μg/ml) was used when appropriate for the selection of transconjugants and transformants.

Genetic methods. Restriction enzyme digestion and DNA ligation were performed by standard procedures (27). Plasmids to be digested with *Bcl*I were prepared from cultures of *E. coli* GM119, which is deficient in adenine methylase. Exonuclease III deletions were performed by using the Erase-a-base system (Promega Biotec, Madison, Wis.) according to the instructions of the manufacturer.

Initial clones of the virulence plasmid pCF801 were constructed by digesting the 95-kb plasmid with *Kpn*I and ligating the fragments into *Kpn*I-digested pKB111. This vector was derived by the insertion of a pCR1 fragment, which carries a unique *Kpn*I site, between the *Eco*RI and the *Hin*dIII sites of pBR322 (5).

Transformations were performed essentially as described by Davis et al. (7) for the transfection of bacteriophage lambda DNA. Plasmid constructs were transformed initially into either *E. coli* HB101 or *S. typhimurium* SGSC452 (obtained from K. E. Sanderson, University of Calgary, Calgary, Canada), both of which are rough and deficient in their respective restriction systems. Plasmids from *E. coli* hosts were modified in *S. typhimurium* SGSC452 before being transformed into other salmonellae.

Tn*10*-tagged virulence plasmids were mobilized to *S. typhimurium* with an F′ *lac* plasmid. Exconjugants were then cured of the F′ *lac* plasmid at 40°C and identified on MacConkey-lactose agar plates containing tetracycline (36).

All cultures were screened routinely for plasmids by the rapid lysis method of Kado and Liu (21). Large quantities of plasmid DNA were purified by cesium chloride-density gradient centrifugation (27) after alkaline lysis of the cells (19).

Protein product analysis. Proteins encoded by the cloned fragments were detected by a DNA-directed in vitro transcription-translation system (Amersham Corp., Arlington Heights, Ill.) with [^{3}H]leucine (Dupont, NEN Research Products, Boston, Mass.) to radiolabel the proteins. To avoid protein aggregation, which occurs at 100°C (data not shown), samples were routinely heated at 40°C for 30 min in 1% sodium dodecyl sulfate. The denatured proteins were resolved in 15% polyacrylamide gels by using a 5% stacking gel and the Laemmli buffer system (25). Protein bands were identified by fluorography by using Amplify (Amersham). Molecular weight markers were supplied by Amersham (CFA.645) and Sigma Chemical Co., St. Louis, Mo. (SDS-7B).

Serum resistance assays. Cloned sequences of plasmid pCF801 were transformed into the serum-sensitive strain CR6260, and transformants were examined for serum resistance levels in comparative tests with the progenitor strain CR6600.

Cultures were screened for serum resistance by a modification of the colorimetric assay of Moll et al. (28). Overnight broth cultures were diluted to 5×10^5 bacteria per ml in Veronal-buffered saline (BR16; Oxoid U.S.A., Inc., Columbia, Md.) containing 1% gelatin and were inoculated into four times the volume of pooled normal human serum (PNHS) in a microdilution plate. Plates were incubated in an atmosphere of 10% CO_2 to maintain the correct pH (36). After 2 h of incubation at 37°C, an equal volume of peptone-glucose broth (1% peptone, 1% glucose, 0.01% bromothymol blue) was added to inactivate the remaining complement and to facilitate bacterial growth, and the plates were reincubated overnight. Cells not damaged by complement are able to grow normally after treatment and effect a color change in the indicator after the fermentation of glucose. In contrast, complement-damaged cells exhibit delayed growth in rich medium and no color change is apparent after the same incubation period.

Serum resistance was measured quantitatively as previously described (36). Briefly, overnight L broth cultures were diluted in Veronal-buffered saline containing 1% gelatin and inoculated into PNHS at a final concentration of approximately 1×10^5 bacteria per ml and 90% PNHS. Incubation was continued at 37°C in 10% CO_2 for 2 h, and viable counts were determined in triplicate. Serum sensitivity was expressed as the percent survival after 2 h of incubation (36) and is defined here as a loss in viability relative to strain CR6600 during this time. Increased serum resistance is defined as significantly higher viable counts by 2 h, compared with strain CR6600.

DNA sequencing. The Sanger dideoxy method was used for DNA sequencing. Sequencing was performed on templates of single-stranded M13 clones grown in *E. coli* JM101 or double-stranded pBR322 clones grown in *E. coli* HB101 by using commerical kits (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and Promega Biotec, respectively) and Klenow DNA polymerase. Primers for the pBR322 clones were purchased from New England BioLabs, Inc. (Beverly, Mass.). The 17-base-pair primer for M13 sequencing was obtained from Bethesda Research Laboratories and [^{32}P]dATP was purchased from Amersham.

Details of the construction of the pBR322 clones are described in Results. M13mp18 clones were generated from plasmid pCF7096 (Fig. 1) by ligating the *Sma*I-*Bam*HI and *Bgl*II-*Eco*RI fragments into the *Bam*HI and *Kpn*I (blunt-ended with Klenow) and the *Eco*RI and *Bam*HI sites of M13mp18, respectively. An M13mp19 clone was generated by cloning the *Bgl*II-*Eco*RI fragment of pCF7096 into *Eco*RI-*Bam*HI-digested M13mp19. Both strands of the cloned DNA were sequenced by using overlapping clones.

The sequences generated were analyzed by two computerized data management systems: Microgenie Version 4.0 (Beckman Instruments, Inc., Palo Alto, Calif.) and Pustell sequence analysis program version 4.0 (International Bio-

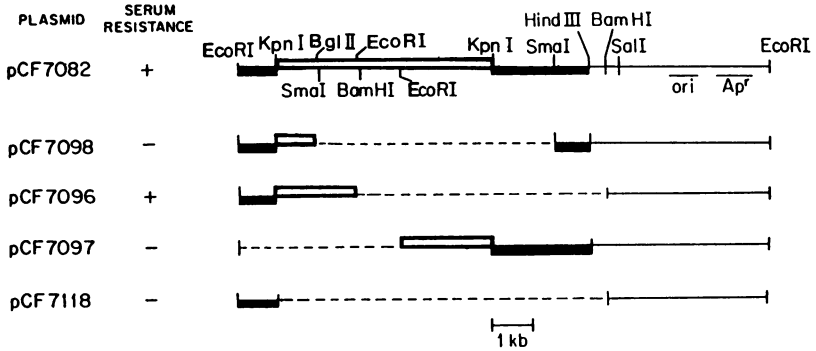

FIG. 1. Deletions of the 5.3-kb insert of plasmid pCF7082 generated by religating single restriction enzyme digests of the plasmid. Plasmids were tested for their ability to confer serum resistance in the screening assay. Symbols: ■, DNA from pCR1; □, DNA from pCF801; ——, DNA from pBR322; - - - -, areas which have been deleted.

technologies, Inc.). Searches against known sequence data were done by using the Genetic Sequence Data Bank (GenBank release 48) furnished by the National Institutes of Health.

RESULTS

Cloning of the *rsk* site of pCF801. We initiated studies designed to identify the region of the 95-kb virulence plasmid responsible for restoring serum resistance to strain CR6260 (36) by cloning sequences of plasmid pCF801 (20) into the *Kpn*I site of plasmid pKB111 (5). The total cloned DNA represents over 60% of plasmid pCF801. Of the six different clones tested for serum resistance in the colorimetric assay, only plasmid pCF7082, which contains a 5.3-kb insert (representing about 5.5% of the total plasmid DNA), conferred resistance to serum killing (Fig. 1).

Deletion derivatives of plasmid pCF7082 were generated by digestion with either *Sma*I, *Bam*HI, or *Eco*RI and by subsequent religation. Only plasmid pCF7096, which contains a 2.1-kb insert bordered by *Kpn*I and *Bam*HI sites, conferred serum resistance to strain CR6260 (Fig. 1), while other deletion derivatives did not. Plasmid pCF7118, from which the entire *Kpn*I and *Bam*HI insert of plasmid pCF7096 has been deleted, did not confer serum resistance. The 1.1-kb *Bgl*II-*Eco*RI fragment of the insert in plasmid pCF7096 was subsequently cloned between the *Eco*RI and *Bam*HI sites of pBR322 to give plasmid pCF7123, and this plasmid (Fig. 2) was shown to confer serum resistance to strain CR6260.

A set of nested deletion derivatives of plasmid pCF7123 was created by exonuclease III digestion from the internal *Mlu*I site of the cloned DNA after blockage of the *Eco*RI site of pBR322 with alpha-phosphorothiolate deoxynucleoside triphosphates (Promega Biotec). The smallest of these clones, which conferred serum resistance, plasmid pCF7188 (Fig. 2), measures 540 bp from the *Bgl*II-*Bam*HI site to the terminus created by exonuclease III digestion (designated X in Fig. 2).

Further deletions of plasmid pCF7123 were made by restriction enzyme digestion to reveal that only the portion of plasmid pCF7188 between site X and the *Bcl*I site is required for the serum resistance phenotype. This region of the plasmid associated with serum resistance is referred to as

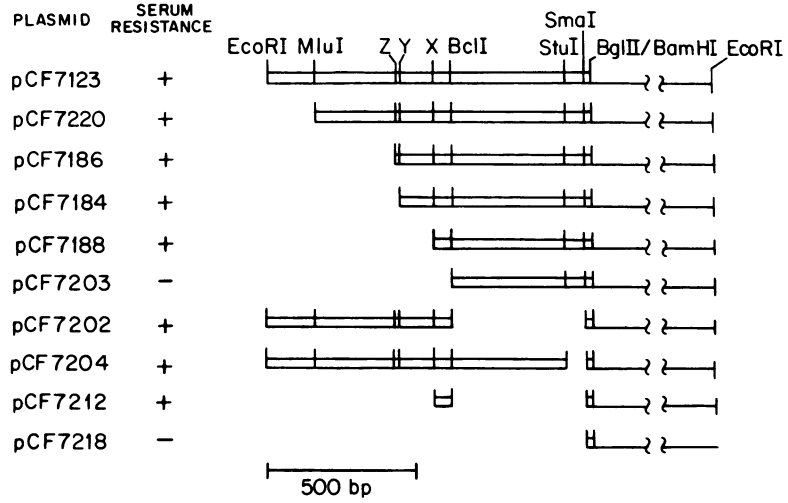

FIG. 2. Development of *rsk*$^+$ and *rsk* mutant plasmids from plasmid pCF7123 containing the 1.1-kb *Eco*RI-*Bgl*II fragment of plasmid pCF7096. Single lines represent pBR322 DNA; double lines represent cloned DNA of plasmid pCF801. Plasmids pCF7203, pCF7202, and pCF7204 were produced, respectively, from *Eco*RI-*Bcl*I, *Bcl*I-*Sma*I, and *Stu*I-*Sma*I double digestions of pCF7123. Plasmid pCF7212 was produced from a *Bcl*I-*Sma*I digestion of pCF7188. All other plasmids arose from exonuclease III digestion of pCF7123 from the *Mlu*I site; X, Y, and Z designate termini generated by exonuclease III digestion from this site. The *rsk* region is situated between the X and *Bcl*I sites.

TABLE 1. Percent survival of *S. typhimurium* after 2 h of incubation in PNHS

Strain	Virulence Plasmid		Clone		% Survival (*n*)[a]
	No.	Status[b]	No.	*rsk*[c]	
CR6600	pCF601	Autonomous	None	NA	112 ± 7 (4)
CR6260	pCF610	Integrated	None	NA	41 ± 7 (4)
CR7179	pCF610	Integrated	None	NA	278 ± 36 (3)
	pCF810	Autonomous			
CR7172	pCF610	Integrated	pCF7096	+	142 ± 5 (3)
CR7171	pCF610	Integrated	pCF7123	+	166 ± 18 (3)
CR7188	pCF610	Integrated	pCF7188	+	140 ± 14 (3)
CR7202	pCF610	Integrated	pCF7202	+	170 ± 20 (3)
CR7212	pCF610	Integrated	pCF7212	+	121 ± 2 (3)
CR7203	pCF610	Integrated	pCF7203	−	44 ± 6 (3)
CR7218	pCF610	Integrated	pCF7218	−	28 ± 8 (3)

[a] Mean percentage of inoculum ± standard error of the mean surviving after 2 h of incubation in 90% PNHS; *n*, number of assays (36).
[b] Physical state of the virulence plasmid determined by gel electrophoresis for autonomous plasmids and by Southern blot for integrated copies (Rabert, Ph.D. thesis).
[c] Presence (+) or absence (−) of the *rsk* sequence (Fig. 1 and 2; also see text). NA, Not applicable.

rsk in subsequent sections of this paper. Subsequently, the sequence common to all clones that conferred serum resistance (i.e., the *rsk* sequence) was subcloned by deleting the *Bcl*I-*Sma*I fragment of pCR7188 (Fig. 2, plasmid pCF7212). The control plasmid for this construct was made by deleting the *Eco*RI-*Sma*I fragment from plasmid pCF7123 (Fig. 2, pCF7218); serum resistance was not associated with the latter clone.

Quantitative measurement of serum resistance. As described previously (36), strain CR6260 is more serum sensitive than its parental strain, CR6600 ($P < 0.001$), and acquisition of an autonomous copy of the 95-kb virulence plasmid pCF810 (strain CR7179) (Table 1) not only restores serum resistance ($P < 0.001$) (Table 1) but also enhances it above that of strain CR6600. The latter feature may be due to increased copy number of some structural gene involved in resistance to the bactericidal activity of PNHS. The net increase in viable counts of strain CR6600 during short-term killing tests varied with the batch of serum and was not always evident. After 6 to 8 h of incubation, however, the viable count of strain CR6600 always increased substantially. In contrast, strain CR6260 exhibited little or no increase in viable counts during this extended incubation time because of continued killing (data not shown).

All derivatives of strain CR6260, harboring plasmids with the *rsk* sequence described above, also exhibited a statistically higher survival in PNHS after 2 h than did transformants carrying plasmids lacking this sequence ($P < 0.001$) (Table 1). Small differences in the serum resistance of strains with rsk^+ plasmids are apparent. Sequence analysis (see below) has not revealed any defects in their construction, and differences may therefore be due to the influences of DNA sequences contiguous to the *rsk* region. It is noteworthy that transformants containing the cloned rsk^+ sequences are more serum resistant than the progenitor strain CR6600, which carries only an autonomous copy of the wild-type virulence plasmid, but are less resistant than strain CR7179, which carries both an autonomous and an integrated copy of the virulence plasmid.

Sequence determination. The region of the virulence plasmid which restored serum resistance was localized by biological assays to the DNA sequence between the exonuclease III terminus (site X) and the *Bcl*I site of plasmid pCF7123 (Fig. 2). The sequence of plasmid pCF7123 DNA was determined to obtain the sequence of the *rsk* region and evidence of adjacent structural genes. Sequence determinations were done with the M13 system and by the double-stranded DNA method with the same constructs as were used in the biological assays (see above). The complete sequence of the DNA cloned into plasmid pCF7123, including the *rsk* region, is given in Fig. 3. The *rsk* region between site X and the *Bcl*I site measures 66 bp (nucleotides 521 to 586 in Fig. 3). The insert cloned in plasmid pCF7123 is 1,065 bp.

Computer-assisted analysis of the 66-bp *rsk* region revealed a series of three asymmetric direct 10-mer repeats (Fig. 3). The two 10-mers positioned 5′ within the cloned fragment were identical, whereas the third sequence, 3′ to these, had 8 of 10 bases conserved. Further analysis of sequences immediately outside this 66-bp section revealed a fourth 10-mer, 5′ of the 66-bp region, in which again 8 of 10 bases were conserved. One of the nonconserved bases in this sequence, however, differed from the nonconserved bases of the degenerate 3′ 10-mer sequence. Most significantly, the start of each of these four 10-mer repeats was located precisely 21 bases from the start of the previous 10-mer. Further searches 3′ and 5′ to the central group of four 10-mers revealed the existence of additional possible 10-mer sequences, the starts of which are spaced at 21-bp intervals. These distal sequences show increasing loss of conservation as the distance from the two highly conserved central 10-mers increases (Fig. 4). Some of the bases composing the 11-bp spaces between 10-mers also appear to be conserved, particularly at positions 3, 4, and 7 of the spacer sequences (Fig. 4).

Examination of *rsk* clones for potential ORF and protein products. Although the functional 66-bp *rsk* site is too small to encode a protein, *rsk* could be a regulatory signal influencing the expression of a structural gene on plasmid pCF801 (see below). Since the identification of such a gene would be informative of the role of *rsk* in wild-type strains, the area surrounding the *rsk* site was examined for evidence of a structural gene.

Analysis of rsk^+ clones for protein products showed that a 40-kDa protein and a 13-kDa protein are produced by plasmid pCF7096 (2.1 kb) and by plasmid pCF7123 (1.1 kb), respectively (Fig. 5). These proteins were of interest, since either of them could represent gene products initiated from promoters associated with *rsk*. However, plasmid pCF7188, bearing the 540-bp rsk^+ insert, produced no proteins other than those produced by a *rsk* mutant control plasmid, plasmid pCF7118 (Fig. 5). Therefore, while either of these

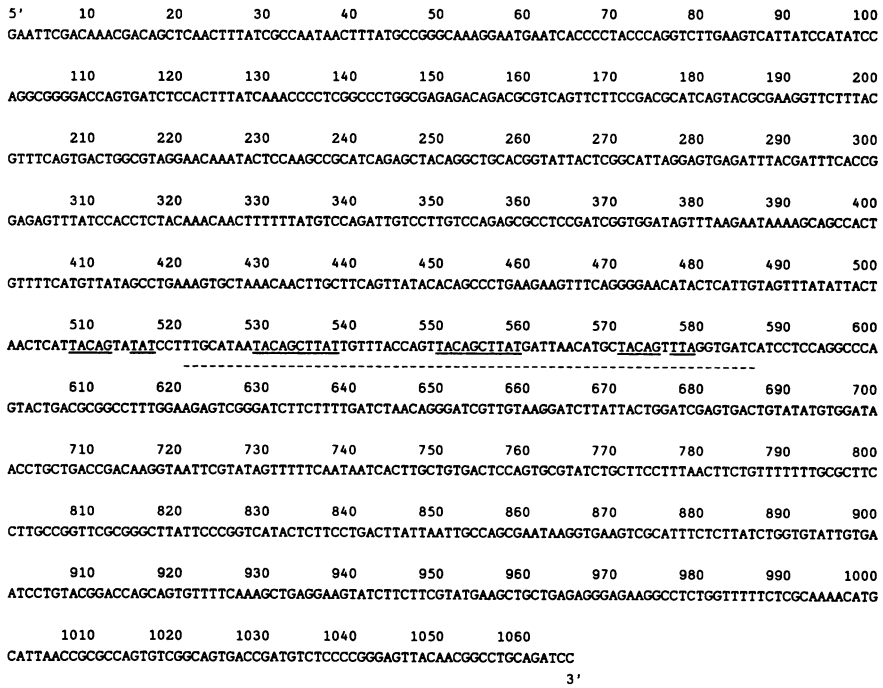

FIG. 3. DNA sequence of pCF7123 from the *Eco*RI site to the fused *Bam*HI-*Bgl*II site. The entire sequence of the upper strand of the inserted pCF801 DNA is shown with numbering beginning from the *Eco*RI site. The 10-mer repeats are underlined. The dashed line from nucleotide 521 to 586 represents the 66-bp insert of the *rsk*$^+$ plasmid, plasmid pCF7212.

proteins may be produced by a structural gene controlled by *rsk* on the native plasmid, pCF801, it is clear that inclusion of these structural genes on the *rsk* clones is not required in order to generate the Rsk phenotype in strain CR6260.

Repeats: 21mer / 10mer	Conservation:
5'	
ctTgcttcagtTAtAcacagc	3/10
ccTgaagaagtTtCAGgggAa	5/10
caTactCattgTAgtttaTAT	5/10
tacTaaCtcatTACAGtaTAT	8/10
ccTTTgCataaTACAGCTTAT	10/10
tgTTTaCcagtTACAGCTTAT	10/10
gaTTaaCatgcTACAGtTTAg	8/10
gtgaTcatcctccagGCccAg	3/10
tacTgaCgcggcctttagaAg	1/10
agTcgggatctTcttttgatc	1/10
taacagggatcgttgtaagga 3'	0/10

FIG. 4. The distribution of conserved sequences and nucleotides aligned as 21-mers around the central 10-mers (Fig. 3), positions 529 to 559. Conserved bases are capitalized and in bold type; the two central 10-mers are underlined. Conservation of the central 10-mer sequence (TACAGCTTAT) is shown on the right.

The sequenced insert of plasmid pCF7123 contains eight possible open reading frames (ORF) following ATG start codons that are 40 bp or larger. None of these, however, is associated with classical procaryotic consensus promoter sequences, none extends through the *rsk* region, and none reads out of *rsk* from an internal start codon. Moreover, none of the ORF are of sufficient size to accommodate the 13-kDa protein which this plasmid produces. Production of this protein requires sequences 5′ of the exonuclease III site X and is probably initiated from a translation start site (ATG) located in pBR322, 53 bp 5′ of the vector-insert junction at coordinate 4309. The coding sequence for the 40-kDa protein has not been located but must be associated with sequences distal to the sequenced 1,065-bp region around the *rsk* site. Three ORF, with translation start sites at positions 96, 952, and 1031 (Fig. 3), read out of the insert and terminate in the vector, and therefore they may represent the 5′ ends of structural genes which have the *rsk* site situated upstream of the coding region. The start sites of all three, however, are more than 350 bp 3′ from *rsk*, and, should there be an undetected promoter, it is unlikey that the promoter would coincide with the *rsk* region to form a classical operator site.

DISCUSSION

Strain CR6260, unlike strains cured of the 95-kb virulence plasmid, has the entire complement of plasmid DNA (Rabert, Ph.D. thesis) but appears to express the genes associated with serum resistance less fully as a consequence of plasmid integration. The reason for the reduced expression of the putative serum resistance gene(s) is unknown, but the reduced expression does not seem to be due to insertional mutagenesis caused either by the Tn*10* inserted in the plasmid or by the insertional inactivation of chromosomal serum resistance genes resulting from plasmid integration. Since Tn*10*-tagged plasmids restore the serum resistance

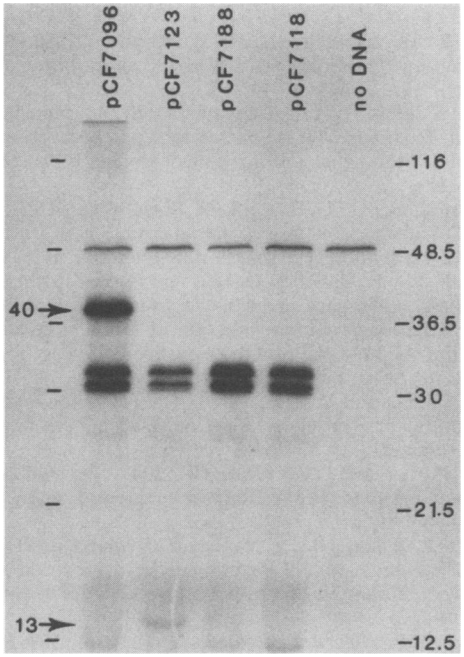

FIG. 5. Polyacrylamide gel electrophoresis of polypeptides produced by *rsk*$^+$ and *rsk* mutant clones (Fig. 1 and 2) in an in vitro transcription-translation system in the presence of [^{3}H]leucine. Samples were heated with sodium dodecyl sulfate at 40°C to avoid aggregation and resolved on 15% gels, and the protein bands were identified by fluorography; the molecular sizes of 116 to 12.5 kDa, shown on the left, were obtained from measurements made on kits of commercial markers (Sigma and Amersham). All plasmids are *rsk*$^+$ with the exception of the control plasmid pCF7118. The 40-kDa protein was produced by plasmid pCF7096, and the very weak 13-kDa protein band was produced by plasmid pCF7123. The *rsk*$^+$ plasmid pCF7188 yielded the same proteins as the control *rsk* mutant plasmid pCF7118.

phenotype to strains maintaining an integrated plasmid (36; Rabert, Ph.D. thesis), our cloning strategy had the potential to detect either serum resistance structural genes or *trans*-acting regulatory elements of the plasmid which modify the expression of the putative serum resistance gene(s), irrespective of these being chromosomal (12), or on the (integrated) plasmid (14). Deletion mutagenesis of the original 5.3-kb cloned DNA revealed that the minimum virulence plasmid sequence which restores serum resistance to strain CR6260 does not produce any detectable protein and is 66 bp long (i.e., the *rsk* region). Sequence analysis demonstrates a motif reminiscent of a regulatory protein-binding site, suggesting that *rsk* may act by binding a regulatory factor.

The minimum *rsk* element is a series of direct repeats with a well-defined periodicity characteristic of a protein-binding site. The presence of equally spaced direct repeats is not likely to be a random event. In a chromosome of 3×10^6 bp, three copies of single 10-mers may occur randomly, and indeed, searches conducted on the Genetic Sequence Data Bank revealed the existence of two other identical 10-mers in over 1.5×10^6 bp of bacterial and bacteriophage sequences searched. These 10-mers occur as single isolated sequences in the coding regions of the *tar* (24) and the *umuD* (22) genes of *E. coli* but have no recognized regulatory significance. The presence of solitary 10-mers at the frequency expected of a random event suggests that for *rsk* to constitute a functional unit, additional bases are required, and a minimal *rsk* sequence, therefore, may be composed of at least two 10-mers spaced on the DNA helix with a precise 21-bp periodicity. This and the existence of highly conserved bases within the spacer regions between 10-mer repeats suggest that the *rsk* regions can be usefully considered a series of 21-mer direct repeats, analogous to the direct repeats seen in the replication regions of the plasmids mini-F (35) and mini-P (1), the binding sites of the bacteriophage transcriptional activators C1 in P22 (4) and *c*II in lambda (17), and the binding site of the eucaryotic activator Sp1 (11). The organization of these direct repeats allows regions of highly conserved bases to be aligned on the same surface of the DNA helix, which in the case of Sp1 provides for the cooperative binding of the regulatory factor subunits essential for transcriptional activation (34). Likewise, alignment of the subunits on the same face of the helix would seem to be required for the *c*II stabilization of the polymerase complex (17) and for the DNA looping interaction seen at the lambdoid P1 RepA-binding sites (29).

The failure to identify any significant ORF or consensus promoter sequence within 1,000 bp surrounding the *rsk* site may indicate that *rsk* is not directly associated with any structural gene. However, although regulatory sequences are usually located at the promoter, the palindromic binding sites for the *deo* (6), *gal* (18, 26), *araBAD* (10), *glnALG* (32), and *lac* (23) operons are considerably distant from their promoters, and yet they allow for cooperative interaction with proteins bound to sites adjacent to the promoter (23, 31). Likewise, RepA dimers bound to the direct repeats of the P1 *incA* gene can interact with RepA dimers bound to the *repA* promoter, although the former are separated from the latter by approximately 1,000 bp. Similarly, the direct repeats of the *rsk* site on pCF801 may also act at a distance from the gene(s) it putatively regulates, thereby explaining the lack of an adjacent ORF. Significantly, *repA* protein-mediated binding between the *incA* and *repA* direct repeats appears to occur in *trans* when these sites are on separate replicons (29).

In the cloned state, the 66-bp *rsk* region does not appear to be translationally active and is far too small to encode the 11-kDa protein reported to confer serum resistance on rough *S. typhimurium* (14). This does not exclude the possibility that this 11-kDa protein is required for the serum resistance of the progenitor strain CR6600. Indeed, the ability of the low-copy-number plasmid pCF810 to confer greater serum resistance on strain CR6260 than any of the high-copy-number *rsk*-bearing plasmids implies that ancillary genes, quite possibly including the serum resistance structural gene(s), are located on the virulence plasmid. Throughout our studies we have not found serum resistance to be restored to rough strains of *S. typhimurium* or *E. coli* either by the entire 95-kb plasmid or by clones carrying the *rsk* region (data not shown). The use of *Kpn*I for the construction of the original clones, however, may have precluded our isolation of this structural gene together with its putative upstream regulatory element (14). Isolation of both of these components on a high-copy-number plasmid may confer higher levels of serum resistance than does our system. The evidence presented by Gulig and Curtiss (12) conflicts with that of Hackett et al. (14) and suggests that any genes associated with serum resistance are chromosomal. Accordingly, the effects mediated by *rsk* may occur not only at loci distal on the plasmid but also at chromosomal sites.

On the basis of these observations and the similarity of *rsk* to known regulatory elements, we propose that *rsk* represents the binding site of a regulatory molecule and that the *rsk* regulatory unit is involved in the control of serum

resistance genes located on the plasmid or on the chromosome. Integration of the virulence plasmid into the chromosome presumably alters the expression of the gene(s) associated with *rsk*, perhaps by increasing production of a *rsk*-binding factor or by increasing the affinity of such a factor for *rsk*. (Disruption of *rsk* by plasmid integration, however, cannot explain this phenomenon, since *rsk* maps to a location at least 1.5 kb from the site of plasmid integration [Rabert, Ph.D. thesis].) These changes could be due to topological alterations in the plasmid DNA consequent upon insertion; precedents for such a mechanism influencing the expression of genes exist (9, 16). Consistent with the recognized ability of bound regulatory proteins to act at a distance (31), the structural gene(s) affected by *rsk* may be at a considerable distance from *rsk* on plasmid pCF801. The structural gene(s) immediately affected by the formation of the *rsk-rsk*-binding factor complex may encode either the mediator of serum resistance or another regulatory molecule, either protein or RNA. *rsk*, therefore, is presumably a *cis*-acting element on the virulence plasmid and functions in *trans* in strain CR6260 either as a simple, multiple-copy titration site which sequesters the *rsk*-binding molecule or acts in a more complex manner involving a protein-mediated DNA-DNA interaction as proposed for *incA* (29). In either case, binding of this factor to *rsk* leads to increased expression of a serum resistance gene. The small differences seen in the serum resistance of strain CR6260 carrying the *rsk* clones are, if real, probably reflective of the influence of contiguous pBR322 or pCF801 sequences on the formation or stability of this complex.

In conclusion, strain CR6260, which harbors a single integrated copy of the virulence plasmid, behaves very differently in serum killing tests (36) than do cured strains (12; J. L. VandenBosch, D. R. Kurlandsky, R. Urdangaray, and G. W. Jones, submitted for publication). The difference can be ascribed to an alteration in the regulatory mechanism involving the serum-resistance phenotype after plasmid integration. *rsk*, although only 66 bp long, is able to restore the wild-type serum-resistant state, perhaps by removing effector molecules which normally act to repress serum resistance.

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