INFECTION AND IMMUNITY, Aug. 1989, p. 2566–2568
0019-9567/89/082566-03$02.00/0

Vol. 57, No. 8

Evidence of Coordinate Regulation of Virulence in *Salmonella typhimurium* Involving the *rsk* Element of the 95-Kilobase Plasmid

JAMES L. VANDENBOSCH,[1]* DAVID R. KURLANDSKY,[2] ROBERT URDANGARAY,[2] AND GARTH W. JONES[2]

Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109,[2] and Department of Biology, Eastern Michigan University, Ypsilanti, Michigan 48197[1]

Received 5 December 1988/Accepted 24 April 1989

Integration of the *Salmonella typhimurium* virulence plasmid into the chromosome reduces mouse virulence, serum resistance, and HeLa cell adhesion-invasion while prolonging lag time in minimal medium. The proposed virulence plasmid regulatory element, *rsk*, partially restores virulence and fully restores the other three phenotypes to wild-type levels. Plasmid curing reduces virulence without affecting the other phenotypes. *rsk* has no apparent effect on the cured strain.

While the virulence of *Salmonella typhimurium* for the mouse clearly requires functions encoded on the 95-kilobase virulence plasmid (4, 11–13, 20), the identities and locations of genes associated with several traits have remained uncertain. Some studies suggest that all serum resistance structural genes of *S. typhimurium* are chromosomal (7, 11), although other evidence either indicates that some such genes are on the virulence plasmid (13) or implies that the plasmid is required for their expression (14, 24, 25). Similarly, although early studies suggested an association between the plasmid and adhesion-invasion of HeLa cells (16), apparently chromosomal adhesion-invasion genes (8) function in the absence of the plasmid (11).

Strains of *S. typhimurium* which carry a single copy of the 95-kilobase virulence plasmid integrated into the chromosome (24) are less serum resistant (24), less adhesive-invasive, and less virulent for mice (16), and they have an extended lag phase of growth in minimal media (J. L. VandenBosch, Ph.D. thesis, University of Michigan, Ann Arbor, 1988). *S. typhimurium* CR6260, with such an integrated virulence plasmid, is restored to normal levels of serum resistance by introducing plasmids bearing a translationally inactive 66-base-pair sequence of the virulence plasmid called *rsk* (25). The 21-nucleotide direct repeat motif of *rsk* suggests that *rsk* is a binding site for a regulatory element and may act as a titration site when isolated on high-copy-number plasmids (25).

Since these phenotypic changes follow plasmid integration, all may reflect regulatory disturbances associated with *rsk*. Accordingly, we have examined the modulation of these traits by *rsk*. The evidence presented here suggests that *rsk* may play a role in the regulation of *S. typhimurium* virulence.

Two pairs of *rsk*$^+$ and *rsk* mutant clones (pCF7212/pCF7218 and pCF7188/pCF7203, respectively) were compared in strain CR6260 (25) and with the parental strain CR6600. Each plasmid pair differed only by the deletion of the 66-base-pair *rsk* sequence in the *rsk* mutant constructs (25). Cultures and isolates were routinely examined (18) for plasmid content.

The lag times of cultures in minimal medium (19) were measured by incubating washed cells (5×10^6 bacteria per ml) from an overnight L-broth culture in M9 medium at 37°C with shaking (100 rpm). Lag times were calculated by regressing the slope of the growth curve. Strain CR6260 had a lag time significantly greater than that of the parental strain CR6600 ($P < 0.001$; Table 1). A normal lag time was restored in a CR6260 exconjugant (25) carrying autonomous copies of the virulence plasmid pCF810 (Table 1), showing that the defect in strain CR6260 is not chromosomal. The introduction of plasmid pBR322 into strain CR6260 extended the lag time, presumably because of the increased genetic load (data not shown). Comparison between strains with comparable *rsk*$^+$ and *rsk* mutant plasmids, however, showed significant reductions in lag time upon the introduction of *rsk*$^+$ clones pCF7188 and pCF7212 ($P < 0.001$; Table 1), equivalent to that caused by the virulence plasmid pCF810. *rsk* mutant clones pCF7203 and pCF7218 had no greater effect than plasmid pBR322. The ability of *rsk* to restore both lag time in M9 medium and serum resistance to strain CR6260 (25) suggests that these traits are associated.

The reduced adhesiveness of strain CR6260 (16) to HeLa cells, measured at 30 min (16, 17), was reversed by the *rsk*$^+$ but not the *rsk* mutant clones (Table 2). Invasion was measured after a 3-h period of attachment and internalization by killing extracellular bacteria with gentamicin (21) and releasing intracellular bacteria with 0.1% *N*-lauroyl sarcosine (Sigma Chemical Co., St. Louis, Mo.). The numbers of intracellular bacteria were normalized to the inoculum (10^8 bacteria) and then expressed as percentages of the normalized value of strain CR6600. The low numbers of intracellular bacteria of strain CR6260 were significantly increased ($P < 0.001$; Table 2) following the introduction of *rsk*$^+$ clone pCF7212 to levels statistically indistinguishable from that of parental strain CR6600. Increased invasion appeared to result from the increased adhesiveness (Table 2). As with invasion, the *rsk* mutant clone pCF7218 had no effect on adhesion.

Mouse virulence was examined in 8-week-old male BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine) injected by the intraperitoneal route with 0.1-ml volumes of washed bacterial suspensions. Log LD_{50} values were calculated by probit analysis (9).

Strain CR6260 is as avirulent for the mouse intraperitoneally ($P < 0.001$; Table 3) as it is per os (16). The LD_{50} is restored to normal levels, however, following the introduction of the autonomous virulence plasmid pCF810 ($P < 0.001$; Table 3). The decrease in the virulence of strain CR6260, therefore, is similar to that of cured strains (11; see below) and an interruption of a chromosomal locus is not

* Corresponding author.

TABLE 1. Lag time in M9 minimal media

Strain	Virulence plasmid		Clone		Mean ± SEM M9 lag time (h) (*n*)
	Name	Status[a]	Name	*rsk*[b]	
CR6600	pCF601	Autonomous	None	NA	6.3 ± 0.1 (4)
CR6260	pCF610	Integrated	None	NA	8.6 ± 0.2 (4)
CR7179	pCF610	Integrated	None	NA	6.6 ± 0.1 (3)
	pCF810	Autonomous			
CR7188	pCF610	Integrated	pCF7188	+	6.9 ± 0.1 (3)
CR7203	pCF610	Integrated	pCF7203	−	9.1 ± 0.1 (3)
CR7212	pCF610	Integrated	pCF7212	+	7.3 ± 0.2 (3)
CR7218	pCF610	Integrated	pCF7218	−	9.3 ± 0.1 (3)

[a] Physical state of the virulence plasmid (24).
[b] Presence (+) or absence (−) of the *rsk* sequence in clones (25). NA, Not applicable.

responsible for avirulence. Virulence is increased about 10-fold by the *rsk*$^+$ clones ($P < 0.005$; Table 3). In contrast, the *rsk* mutant construct, strain CR7203(pCF7203), is statistically indistinguishable from strain CR6260.

Two smooth, independently isolated, virulence plasmid-free strains (CR6605 and CR6625) were obtained from strain CR6600 following novobiocin curing of a miniTn*10*-tagged virulence plasmid, pCF601 (16). No plasmid sequences were detected in colony blots with ^{32}P-labelled virulence plasmid probes generated with random primers (Amersham Corp., Arlington Heights, Ill.) from cloned sequences of virulence plasmid pCF801 (24); strains CR6260 and CR6600 gave approximately equal signals. Serum resistance, adhesion-invasion, and lag time in minimal medium of both cured strains are statistically indistinguishable from that of parental strain CR6600. Plasmids pCF810 and pCF7212 have no significant effect on any of these three phenotypes of cured strains (data not shown). Curing the virulence plasmid, however, greatly reduces virulence, giving an LD_{50} near that of CR6260, but the introduction of virulence plasmid pCF810 restores virulence to wild-type levels ($LD_{50} \leq 100$). The *rsk*$^+$ plasmid pCF7212 had no greater apparent effect than *rsk* mutant plasmid pCF7218, giving an LD_{50} similar to that of strain CR6260 (data not shown).

The *rsk* sequence (25), therefore, has clear pleiotropic effects on *S. typhimurium* gene expression when tested in a strain with an integrated virulence plasmid, but not when examined in strains from which the plasmid has been cured. The results suggest that *rsk* is involved in the coordinated regulation of these traits. It is unlikely that *rsk* complements a defect in a structural gene common to each of the phenotypes, since the smallest *rsk*$^+$ sequence is translationally inactive and only 66-base pairs in size. The role of *rsk* in regulation is consistent both with a recent proposal (8) that aspects of *S. typhimurium* virulence are regulated and with the recognized similarity of the DNA sequence of *rsk* (25) to known binding sites of regulatory elements (1, 5, 6, 15, 23).

TABLE 2. HeLa cell adhesion-invasion

Strain	Virulence plasmid		Clone		% Invasion[a]	Adhesion[b]
	Name	Status[c]	Name	*rsk*[d]		
CR6600	pCF601	Autonomous	None	NA	100.0	13.1 ± 4.2
CR6260	pCF610	Integrated	None	NA	5.0	0.4 ± 0.7
CR7212	pCF610	Integrated	pCF7212	+	98.4	13.0 ± 2.7
CR7218	pCF610	Integrated	pCF7218	−	5.6	0.8 ± 1.2

[a] Intracellular bacteria after 3 h of exposure as a percentage of that in parental strain CF6600. Standard errors were approximately 10%.
[b] Number of bacteria attached per HeLa cell (± standard deviation) after 30 min (16, 17).
[c] Physical state of the virulence plasmid (24).
[d] Presence (+) or absence (−) of the *rsk* sequence in clones (25). NA, Not applicable.

TABLE 3. Virulence for BALB/c ByJ mice challenged by the intraperitoneal route

Strain	Virulence plasmid		Clone		Mean log LD_{50}[a] ± SEM
	Name	Status[b]	Name	*rsk*[c]	
CR6600	pCF601	Autonomous	None	NA	2.0 ± 0.1
CR6260	pCF610	Integrated	None	NA	5.8 ± 0.3
CR7179	pCF610	Integrated	None	NA	2.3 ± 0.3
	pCF810	Autonomous			
CR7188	pCF610	Integrated	pCF7188	+	4.6 ± 0.2
CR7212	pCF610	Integrated	pCF7212	+	4.1 ± 0.3
CR7203	pCF610	Integrated	pCF7203	−	5.6 ± 0.3

[a] Calculated by probit analysis (9).
[b] Physical state of the virulence plasmid (24).
[c] Presence (+) or absence (−) of the *rsk* sequence in clones (25). NA, Not applicable.

A possible lesion(s) incurred by plasmid integration and reasons for the restoration of serum resistance by *rsk* have been discussed elsewhere (25). We have proposed (25) that *rsk* normally functions on the autonomous virulence plasmid to control the production of a plasmid gene-encoded regulatory molecule. We speculate that this molecule could be a repressor, since with the exception of mouse virulence (11), curing the virulence plasmid does not change these phenotypes, whereas plasmid integration apparently decreases their expression. The negative control of chromosomal genes by plasmid loci is similar to that of the calcium-limited growth of *Yersinia pestis* (27). It suggests that cured strains appear to be wild type for these traits either because gene expression is at or near maximal expression in broth culture or because strain CR6600 is capable of a more rapid genetic response. It is perhaps only when the regulatory system is perturbed, such as by plasmid integration, that the regulatory role of the plasmid becomes apparent.

Both serum resistance and adhesion-invasion appear to be involved in mouse virulence (7). The contribution of a shorter period of adaptation to minimal medium may reflect the adjustment of the pathogen to nutrient-limited conditions within an animal cell vacuole. Virulence is undoubtedly multifactorial, involving processes in addition to those provided by chromosomal genes. At least one aspect of mouse virulence is encoded by the plasmid (11), and it could be the expression of this latter plasmid gene(s) that *rsk* does not restore.

The coordinate regulation of virulence and virulence traits is known in *Y. pestis* (27), *Bordetella pertussis* (26), *Vibrio cholerae* (22), and *S. typhimurium* (3, 8), and *S. typhimurium* has regulons similar to those of *Escherichia coli* (2, 3, 10). Our studies indicate that the coordinate regulation of the virulence of *S. typhimurium* involves the 95-kilobase virulence plasmid.

This work was supported by Public Health Service grant AI19647 from the National Institutes of Health to G.W.J.

We gratefully acknowledge the assistance of Kristy Paquette and Susan Miller.

LITERATURE CITED

1. **Abeles, A. L., K. M. Snyder, and D. K. Chattoraj.** 1984. P1 plasmid replication: replicon structure. J. Mol. Biol. **173**:307–324.

2. **Aliabadi, Z., F. Warren, S. Mya, and J. W. Foster.** 1986. Oxygen-regulated stimulons of *Salmonella typhimurium* identified by Mu d(Ap *lac*) operon fusions. J. Bacteriol. **165:**780–786.
3. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51:**509–518.
4. **Baird, G. D., E. J. Manning, and P. W. Jones.** 1985. Evidence for related virulence sequences in plasmids of *Salmonella dublin* and *Salmonella typhimurium*. J. Gen. Microbiol. **131:**1815–1823.
5. **Baskhaus, H., and J. B. Petri.** 1984. Sequence analysis of a region from the early right operon in phage P22 including the replication genes *18* and *12*. Gene **32:**289–303.
6. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35:**79–87.
7. **Fields, P. I., R. V. Swanson, C. G. Haiddaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
8. **Finlay, B. R., F. Heffron, and S. Falkow.** 1989. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science **243:**940–943.
9. **Finney, D. J.** 1978. Statistical method in biological assay. Charles Griffin & Co., Ltd., London.
10. **Foster, J. W., and M. P. Spector.** 1986. Phosphate starvation regulon of *Salmonella typhimurium*. J. Bacteriol. **166:**666–669.
11. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55:**2891–2901.
12. **Hackett, J., I. Kotlarski, V. Mathan, K. Francki, and D. Rowley.** 1986. The colonization of Peyer's patches by a strain of *Salmonella typhimurium* cured of the cryptic plasmid. J. Infect. Dis. **153:**1119–1125.
13. **Hackett, J., P. Wyk, P. Reeves, and V. Mathan.** 1987. Mediation of serum resistance in *Salmonella typhimurium* by an 11-kilodalton polypeptide encoded by the cryptic plasmid. J. Infect. Dis. **155:**540–549.
14. **Helmuth, R., R. Stephan, C. Bunge, B. Hoog, A. Steinbeck, and E. Bulling.** 1985. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. Infect. Immun. **48:**175–182.
15. **Ho, Y.-S., D. L. Wulff, and M. Rosenberg.** 1983. Bacteriophage lambda protein *c*II binds promoters on the opposite face of the DNA helix from RNA polymerase. Nature (London) **304:**703–708.
16. **Jones, G. W., D. K. Rabert, D. M. Svinarich, and H. J. Whitfield.** 1982. Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. Infect. Immun. **38:**476–486.
17. **Jones, G. W., L. A. Richardson, and D. L. Uhlman.** 1981. The invasion of HeLa cells by *Salmonella typhimurium*: reversible and irreversible bacterial attachment and the role of bacterial motility. J. Gen. Microbiol. **127:**352–360.
18. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Pardon, P., M. Y. Popoff, C. Coynault, J. Marly, and I. Miras.** 1986. Virulence-associated plasmids of *Salmonella* serotype typhimurium in experimental murine infection. Ann. Inst. Pasteur Microbiol. **137B:**47–60.
21. **Small, P. L. C., R. R. Isberg, and S. Falkow.** 1987. Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. **55:**1674–1679.
22. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84:**2833–2837.
23. **Tsutsui, H., A. Fujiyama, T. Morotsu, and K. Matsubara.** 1983. Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bacteriol. **155:**337–344.
24. **VandenBosch, J. L., D. K. Rabert, and G. W. Jones.** 1987. Plasmid-associated resistance of *Salmonella typhimurium* to complement activated by the classical pathway. Infect. Immun. **55:**2645–2652.
25. **VandenBosch, J. L., D. K. Rabert, D. R. Kurlandsky, and G. W. Jones.** 1989. Sequence analysis of *rsk*, a portion of the 95-kilobase plasmid of *Salmonella typhimurium* associated with resistance to the bactericidal activity of serum. Infect. Immun. **57:**850–857.
26. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. Infect. Immun. **43:**263–269.
27. **Yother, J., T. W. Chamness, and J. D. Goguen.** 1986. Temperature-controlled plasmid regulon associated with low calcium response in *Yersinia pestis*. J. Bacteriol. **165:**443–447.