Avirulence of LT2 Strains of Salmonella typhimurium Results from a Defective rpoS Gene

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In order to identify the genetic basis for the attenuation of Salmonella typhimurium LT2 strains, experiments were performed to identify a gene(s) which restores virulence to an avirulent LT2 strain. These and further experiments confirmed that an rpoS mutation is the sole determinant of the attenuation of S. typhimurium LT2.

Since its original isolation in the 1940s by Lilleengen, Salmonella typhimurium LT2 has been widely used in genetic studies (15, 21, 26). The descendants of the original LT2 isolate are most accurately described as a set of clades, most of which are highly attenuated for virulence in susceptible mice (2, 21). It has previously been shown that virulence can be restored to avirulent LT2 strains (hereafter collectively referred to as the LT2A clade) by mutations in a single gene, designated mouse virulence A, or mviA (2). The mutation found in virulent LT2 (LT2V) is a single nucleotide change in mviA that causes a valine-to-glycine change at position 102 (mviA

V102G) (4). More recent reports have shown that mviA encodes a protein with homology to the response regulator family (4) and that it is a determinant of the stability of the stationary-phase sigma factor σ7 (2, 16, 19).

Examination of a panel of virulent S. typhimurium isolates has revealed that all have a functional mviA+ gene (3). Therefore, it has been speculated that the LT2A clade harbors an attenuating mutation ( provisionally designated mviB) which is functionally reverted by the inactivation of mviA+ (mviA:: Kan). We now report the identification of the mviB mutation as rpoS.

The mviA genotype of a low-passage LT2 strain (SG5C1412, hereafter referred to as LT2L) was examined by targeted gene transfer into LT2 strain WB4188, which is virulent as a result of an mvi4:: Kan insertion but is otherwise an avirulent LT2 strain, as described previously (3). The desired recombination was selected for as the gain of the mviA+ linked marker (Cam+) from the donor and screened for by replica patching for the loss of the marker within the mviA gene of the recipient (Kan+). The resulting recombinants have the mviA genotype of the donor strain (LT2L) in the LT2A genetic background. Although the donor (LT2L) and the recipient (WB4188) are avirulent, all LT2L-derived mviA recombinants examined were avirulent (Fig. 1). These results show that LT2L has mviA+ and is virulent and is thus indistinguishable from other virulent S. typhimurium strains.

Isolation of mutations which restore virulence to LT2A. Because LT2L presumably has mviB function, we used LT2L as a donor strain in random genetic exchanges to recombine the mviB+ gene into LT2A strain WB435 (3). A library of random MuJ transpositions in strain LT2L was prepared as described previously (5, 13) and recombined via P22 transduction (22) into LT2A, and the entire pool of recombinants was used to infect genetically susceptible (BALB/c ByJ) mice to select for virulent recombinants. Bacteria were isolated from the spleens and livers of infected mice and used to infect other groups of mice at lower doses. Following three rounds of passage in mice, transformants which had fully restored virulence (50% lethal dose < 103) were isolated. Because mviA:: MuJ mutations would restore virulence, transformants were screened for cotransductional linkage to an mvi4:: 4093::Cam insertion. Several mviA-linked insertions but only one MuJ insertion, not linked to mviA+, were found. The unlinked insertion was chosen for further study. When this mutation, zgd4388:: MuJ, was recombined via transduction into an LT2A strain, the virulence was increased to a level comparable to the virulence of a wild-type strain (Fig. 2).

Chromosomal DNA from mutants carrying the insertion was prepared by a rapid method which has been described in detail (7, 9). Junction fragments were cloned from mutants carrying the insertion and sequenced with primers directed to the MuL attL element, as described previously (7, 10). The nucleotide sequence was compared to sequences in existing databases by BLAST computer searches (1). The attR junction fragment clone contained a 1.5-kbp insertion. The sequence from the vector end of the clone was homologous with the sequence from 63 bp 5′ of S. typhimurium rpoS, with 100% homology through 200 bp of the rpoS gene. The restriction map of the insert was consistent with the remainder of rpoS being contained on this fragment. When we sequenced from the attR junction site we were surprised to find homology to Escherichia coli hycI region encoding 50 of 58 amino acids [aa] starting at aa 64), with the hycI gene and the rpoS gene convergent. In E. coli (hyc has not been mapped or sequenced for S. typhimurium), hycI and rpoS are tandem and about 31 kbp apart. Unexpectedly, when the sequence from the attL junction was obtained we found the region coding for the C-terminal 105 aa (plus 23 bp) of rpoS. At this point, we had already found that rpoS was responsible for the activity that we had found, we did not pursue this obviously complex rearrangement further.

Targeted exchange of rpoS alleles alters virulence. The exchanges of rpoS alleles between LT2A and virulent strains were achieved with an rpoS1005::Amp selectable marker (8) in the recipient and a zgd5181:: Tn10 (dtc) transposition insertion, which is 50% linked to rpoS (14). The resulting strains had the rpoS alleles of the donors (rpoS7TM and rpoS7T2) in the genetic backgrounds of the recipients (LT2A and virulent non-LT2 S. typhimurium TML, respectively). The virulence of the resulting recombinants for mice was then examined. The mutants fell into two classes: those with the rpoS gene of a virulent strain in
an LT2A genetic background and those with LT2A rpoS in a virulent-strain background. As shown in Fig. 2, the virulence of these recombinants corresponded with the source of the rpoS gene. The replacement of LT2A rpoS with the rpoS gene of a virulent strain was sufficient to restore full virulence. Conversely, the rpoS gene from an LT2A strain attenuated virulent strains.

**s levels are decreased in LT2A compared to those in virulent strains.** The s protein levels in different S. typhimurium strain lineages were examined by immunoblotting with a monoclonal antibody against the s protein (17). Figure 3 shows the amounts of s protein in fractions of whole-cell lysates of LT2A, LT2V, LT2L, and TML. The amount of s protein found in LT2A is much less than that seen in the virulent clades (Fig. 3).

It has previously been difficult to reconcile the conflicting virulence phenotypes conferred by mviA on different S. typhimurium strains. Because LT2 is the only strain in which mviA causes avirulence, it was proposed that LT2A strains have some mutation which affects the relationship of mviA to vir-
ulence. The data presented here identify the mutant gene (previously designated mviB) as rpoS. The presence of an rpoS mutation in strain LT2 has been inferred by the demonstration of alterations in expression of the genes of the spv operon, which are under the control of σ^+ (6, 18), and by an unusual start codon described for one LT2 strain which affects expression of the genes necessary for the acid tolerance response (14). It is therefore not surprising that the in vivo experiments reported here have shown that a mutation in rpoS is the key attenuating factor in LT2 strains. The present study demonstrates that the nonfunctional allele of rpoS found in LT2A strains is apparently the only key attenuator, as virulence is restored by the introduction of a wild-type rpoS allele.

Data obtained with S. typhimurium (2) and E. coli (16, 19) have shown that mviA^+ (also designated rssB or sprE) facilitates the degradation of σ^+ probably through interaction with the CtpXP protease (23). When these data are considered in combination with the present study, it seems clear that the mechanism for the restored virulence of LT2A mviA mutants is the restoration of normal intracellular levels of σ^+, probably as a result of decreased protein turnover. We have also found that increased levels of RpoS, possibly present at inappropriate times, in virulent S. typhimurium also result in avirulence (24).

The only virulence-related attribute of S. typhimurium which has been shown to be regulated by σ^+ is the expression of genes of the spv regulon, which determine the ability of salmonellae to grow in deep tissues in a manner which is not yet clear (11, 12). What role σ^+ plays in human typhoid is not known, since the spv regulon is not found in Salmonella typhi. The attenuated vaccine strain S. typhi Ty21a has been shown to have a mutant rpoS allele, which is likely to contribute to its attenuation (20). An improved understanding of the contributions of rpoS and mviA to mouse typhoid may help in the design of a new attenuated typhoid vaccine.

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ADDENDUM

Following the initial submission of the manuscript, we became aware of a related study by Mary Wilmes-Riesenberg et al. in which virulence was restored to an LT2A strain by the introduction of the rpoS gene from S. typhimurium UK1 (25). These results serve to further validate our findings.

REFERENCES


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