The *Mycobacterium tuberculosis* ESAT-6 Homologue in *Listeria monocytogenes* Is Dispensable for Growth In Vitro and In Vivo

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ESAT-6 is a virulence determinant in *Mycobacterium tuberculosis* and a member of a conserved group of proteins in a variety of other bacteria. A targeted deletion of the homologous gene in *Listeria* was generated, and in contrast to that observed for mycobacteria, this locus was not required for *Listeria* virulence.

Infection with *Mycobacterium tuberculosis* is a major global public health problem. Over one-third of the world's population is infected, and an estimated 2 million deaths occur each year as a result. Comparative genome analysis between the nonpathogenic mycobacterium bacille Calmette-Guerin and the pathogenic mycobacterium *M. tuberculosis* or *Mycobacterium bovis* has revealed a 9.5-kb region (RD1) to be important for virulence (1, 2, 9). Spontaneous deletion of this region is thought to be the primary mutation that occurred in *M. bovis* during serial passage by Calmette and Guérin between 1908 and 1921, resulting in the current widely used vaccine strain BCG. This hypothesis is supported by the severe in vivo attenuation of *M. tuberculosis* after targeted deletion of this region and restoration of virulence by complementation of this region in BCG (7, 8, 13).

In *M. tuberculosis*, 11 open reading frames (ORFs) are present in the 9.5-kb RD1 region. Three of these ORFs encode critical components of a secretion system that exports two additional protein products encoded within RD1, ESAT-6 and CFP-10 (14). Both ESAT-6 and CFP-10 contain immunodominant epitopes in the T-cell response to *M. tuberculosis* infection (12). Although the mechanism of action of ESAT-6 is unknown, this specific locus is essential for mycobacterial virulence because targeted deletion of this gene in either *M. tuberculosis* or *M. bovis* results in profound attenuation following in vivo infection (14, 15).

Homologues of the *M. tuberculosis* ESAT-6 protein have been identified in a variety of other bacterial species. While initial studies identified homologues in high-G+C bacterial species such as actinobacteria and other mycobacteria (6), further analyses have identified more distant homologues in various species of gram-positive bacteria in the low-G+C group, including *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp., and *Listeria* spp. (10). Although the level of sequence similarity of these proteins compared with ESAT-6 is relatively low (~30% similarity and ~15% identity), conservation of the internal tryptophan-X-glycine (W-X-G) and ~100-residue

length supports the inclusion of these proteins in an ESAT-6 superfamily of "WXG100" proteins. Recently, the homologue of *M. tuberculosis* ESAT6 in *Staphylococcus aureus* has been identified and found to play an essential role in *S. aureus* virulence following intravenous infection (3).

Listeria monocytogenes is a gram-positive bacterium that causes human clinical disease ranging from self-resolving gastroenteritis to more serious illnesses, including abortion, sepsis, and central nervous system infection. Neonates and other immunocompromised hosts are especially susceptible to more serious infection. The pathogenesis and virulence determinants of *L. monocytogenes* have been well characterized with in vitro and in vivo infection models (4). During infection, *L. monocytogenes* coordinates the expression of an array of bacterial gene products to gain access and reside within the intracytoplasmic compartment of infected cells, thereby evading humoral immunity, and as a result protective immunity to this infection is mediated predominantly by cellular mediators (11). *L. monocytogenes* infection is currently widely used as a model to study and identify host mediators of innate and adaptive immunity to intracellular bacterial pathogens.

The *L. monocytogenes* ESAT-6 homologue is a 99-residue protein and is encoded by the *lm00056* locus (*Lmesat6*) identified by genome sequencing (National Center for Biotechnology Information accession no. AL591973). At the protein level, it is 14% identical and 30% similar to *M. tuberculosis* ESAT-6 and contains the conserved W-X-G motif (Fig. 1A). During infection, transcription of this locus can be detected in the spleens of infected mice by reverse transcription-PCR (Fig. 1B). The importance of this protein in the virulence of other bacterial and mycobacterial pathogens and the conserved nature of homologous proteins in diverse bacterial species suggested they may play a conserved role in bacterial replication and/or pathogenesis. Thus, we sought to examine the role of this locus in *L. monocytogenes* infections by comparing the in vitro and in vivo virulence properties of an *Lmesat6* deletion mutant with wild-type (WT) *L. monocytogenes*. A targeted internal 170-bp deletion within the *Lmesat6* open reading frame was generated by homologous recombination after cloning upstream and downstream fragments into the temperature-sensitive construct pKSV7 (Fig. 1C and D) and electroporation

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FIG. 1. (A) Alignment of *M. tuberculosis* ESAT-6TB and the homologous protein in *L. monocytogenes*, ESAT-6LM. Identical residues are in bold, and similar residues are shaded. (B) Reverse transcription-PCR demonstrating expression of the *L. monocytogenes* ESAT-6 locus (lmo0056; National Center for Biotechnology Information accession no. AL591973) by bacteria recovered ex vivo from the spleens of mice infected for 72 h with 10^5 CFU of *L. monocytogenes* strain 10403s. RT, reverse transcriptase. (C) *L. monocytogenes* genomic map containing the *L. monocytogenes* ESAT-6 locus (lmo0056) and the construct used for deletion of bases 57 to 227 in the 294-base coding region. Five hundred base pairs of the genomic upstream sequence flanking the deletion sites was cloned into the HindIII/KpnI sites of the temperature-sensitive plasmid pKSV7 using the following primers: forward primer 5'-aagcttcagtggaactccgcg-3' and reverse primer 5'-ggatccgtaagttttcgcgatc-3'. The following primers were used for the downstream flanking region: forward primer 5'-ggatccttgagaagacgaaacg-3' and reverse primer 5'-ggtaccactttctgcaactccgcg-3'. Underlined regions indicate introduced restriction sites. (D) PCR of genomic DNA isolated from *L. monocytogenes* strains 10403s (WT) and 10403s Δ esat6 demonstrating the 170-bp deletion.

into WT *L. monocytogenes* strain 10403s using previously described methods (5).

The growth and virulence properties of this mutant, *L. monocytogenes* Δ esat6, were compared with the parental WT *L. monocytogenes* strain. The growth rates of this mutant in cell-free medium under both aerobic (300 rpm on an orbital shaker) and microaerophilic (soft agar) conditions were identical to those of the parental strain. Similarly, the size and frequency of bacterial plaques formed after in vitro infection of HeLa cell monolayers were identical between the mutant and parental strains (data not shown).

We further evaluated the importance of Lmesat6 during in vivo infection. Following intravenous infection with either WT *L. monocytogenes* or *L. monocytogenes* Δ esat6, bacterial replication uniformly occurred in the spleens and liver at day 3 compared with the initial inocula, and bacterial clearance uniformly occurred by day 7 (Fig. 2). However, at each of these time points, no significant differences in bacterial burden were detected in either organ between mice infected with *L. monocytogenes* Δ esat6 and WT *L. monocytogenes*. In the time course

FIG. 2. Numbers of *L. monocytogenes* bacteria recovered from the spleens (top panel) and livers (bottom panel) of mice (C57BL/6 [*H-2^b*] $\times$ B10.D2 [*H-2^d*]) inoculated intravenously with 2.0×10^4 CFU of either 10403s (squares) or 10403s Δ esat6 (triangles) on day 3 (closed symbols) and day 7 (open symbols) postinfection. Each datum point indicates the $\log_{10}$ CFU from an individual mouse. These data are pooled from two independent experiments that yielded similar results. Bar, geometric mean. Liver day 7 number of CFU of 10403s compared with the number of 10403s Δ esat6 CFU ($P = 0.59$) (Mann-Whitney test; Graph Pad, San Diego, CA).

of primary *L. monocytogenes* infection, innate immune cells such as macrophages, neutrophils, and natural killer cells are responsible for control of bacterial replication in the first 5 days following infection, while immunity thereafter is mediated by antigen-specific CD8 and CD4 T cells. Thus, the normal bacterial clearance from day 3 to day 7 following infection by *L. monocytogenes* Δ esat6-infected mice suggests that the *L. monocytogenes* homologue of ESAT6 is not required for generation of *L. monocytogenes* antigen-specific CD8 and CD4 T cells. This was tested by examining the percentage and total numbers of *L. monocytogenes*-specific CD8 and CD4 T cells after infection with either WT *L. monocytogenes* or *L. monocytogenes* Δ esat6. At the peak of the *L. monocytogenes*-specific T-cell response (day 7), splenocytes from infected mice were stimulated with either the major histocompatibility complex (MHC) class I antigenic peptide LLO 91-99 (CD8 T cells) or the class II peptide LLO 189-201 (CD4 T cells) and specific cells were quantified by cell surface and intracellular cytokine staining as previously described (16) (Fig. 3). While infection with both strains elicited a robust CD8 and CD4 T-cell response, there was no difference in either the percentage or the total number of *L. monocytogenes*-specific CD8 or CD4 T cells after infection with *L. monocytogenes* Δ esat6 or WT *L. monocytogenes*.

These data demonstrate that the *L. monocytogenes* homologue to *M. tuberculosis* ESAT6 does not play a detectable role in *L. monocytogenes* virulence or in the triggering of *L. mono-*

FIG. 3. (A) Percentage of gamma interferon (IFN- γ)-positive splenic CD8 and CD4 T cells on day 7 after infection with 10403s (solid bars) or 10403s Δ esat6 (shaded bars) after in vitro stimulation with MHC class I-restricted *L. monocytogenes* peptide LLO 91-99 or class II-restricted *L. monocytogenes* peptide LLO 189-201 with corresponding controls. (B) Calculated total numbers of gamma interferon-producing CD8 and CD4 T cells per spleen in mice infected with 10403s (solid bars) or 10403s Δ esat6 (shaded bars) after stimulation with the indicated MHC class I- or class II-restricted *L. monocytogenes*-specific peptides. These data represent six mice per group combined from two independent experiments. Bar, standard error. Percent CD8 10403s cells compared with 10403s Δ esat6 cells, $P = 0.48$; percent CD4 10403s cells compared with 10403s Δ esat6 cells, $P = 0.59$; total number of CD8 10403s cells compared with 10403s Δ esat6 cells, $P = 0.24$; total number of CD4 10403s cells versus 10403s Δ esat6 cells, $P = 1.0$ (Mann-Whitney test; Graph Pad, San Diego, CA).

cytogenes-specific adaptive T-cell-mediated immune responses. The lack of an obvious role for *L. monocytogenes* ESAT-6 could be due to the intracytoplasmic residence of *L. monocytogenes* compared with the intravacuolar residence of *Mycobacterium* spp. and *Staphylococcus* spp. or other intrinsic differences between the virulence properties of these bacterial pathogens. The data presented in this report extend our knowledge regarding the function of the WXG100 protein family in bacterial virulence by showing that it is not required in all contexts. Thus, further evaluation of this protein family's role in the virulence of other bacterial pathogens is warranted.

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