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Mutation of *waaN* Reduces *Salmonella enterica* Serovar Typhimurium-Induced Enteritis and Net Secretion of Type III Secretion System 1-Dependent Proteins

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Mutation of *waaN*, a gene involved in lipid A biosynthesis, reduced enteropathogenic responses induced by *Salmonella enterica* serovar Typhimurium in bovine ligated ileal loops. However, the secretion of key virulence determinants was also reduced, and therefore the reduction in enteropathogenicity cannot be solely attributed to a reduction in biological activity of lipid A.

Lipopolysaccharide (LPS) is the major component of the outer leaflet of the outer membrane of gram-negative bacteria. LPS has been described as having three structural and functional domains: the lipid A, core, and O antigen domains. The biological and toxic activities associated with LPS lie within the lipid A domain. The genetic basis for lipid A biosynthesis has largely been determined (15), allowing the construction of defined mutations that result in bacteria synthesizing altered lipid A structures. Previously, mutations in lipid A biosynthesis genes resulted in conditional lethality, but recently mutations in *msbB* (renamed *waaN*) in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium which do not affect bacterial growth have been described (11, 12, 16). *htrB* (renamed *waaM*) mutants have also been generated which are conditional for growth at less than 32°C for survival (17). The *waaM* and *waaN* genes are responsible for the late acylation reactions that complete lipid A biosynthesis. Loss of these acyl chains from lipid A reduces the ability of the molecule to induce release of cytokines and other mediators of the immune response (7, 9, 12, 13, 16).

In mice, mutation of *waaM* in serovar Typhimurium resulted in reduced virulence and reduced growth in vivo, probably in large part due to the temperature sensitivity of these mutants (9). Mutation of *waaN* led to a very different phenotype. These mutants were able to grow at the same rate as the wild-type bacteria in murine livers and spleens following intravenous inoculation but reached higher numbers (approximately 10^9 CFU per organ) than those typically associated with death during infection with wild-type serovar Typhimurium. Only a small proportion of the mice died, and the surviving mice eventually cleared the infection (12). The levels of proinflammatory cytokines and nitric oxide production were considerably lower during the course of infection with the *waaN* mutant than with the wild-type bacteria. This suggests that death in the mouse typhoid model of infection is dependent on high levels of cytokine release in response to lipid A.

In order to assess the role of lipid A in other *Salmonella* infection systems, Everest et al. (3) tested a *waaN* mutant in a rabbit ligated ileal loop model for enteropathogenesis and found that it showed no difference compared with wild-type serovar Typhimurium. This result was surprising in that the mutant is reduced in its ability to induce cytokines, which have been implicated in the induction of enteropathogenic responses (1, 2, 14). We have therefore reevaluated the role of lipid A in *Salmonella* enteropathogenesis by testing the *waaN* mutant in the bovine ligated ileal loop model. The results from this model using defined isogenic bacterial mutants correlate well with the severity of enteritis in orally inoculated calves (18, 20, 21).

Effect of mutation of *waaN* on induction of enteropathogenic responses by serovar Typhimurium. Bacteria were incubated in bovine midileal loops for 12 h, during which time polymorphonuclear leukocytes (PMNs) from each calf were isolated, labeled with ^{111}In, and reinjected. The surgical procedure is described in detail elsewhere (18). After 12 h, the secretory response (volume of fluid within a loop/length of loop in milliliters per centimeter) and the γ emission of PMNs in the test loops compared with the negative control loops (PMN influx ratio) were recorded. The bacterial strains used, serovar Typhimurium C5 and its derivative *waaN* mutant and *S. enterica* serovar Dublin SD2229 and its derivative *sipB* mutant, have been described previously (12, 22). The inocula were incubated overnight in Luria-Bertani (LB) broth at 25°C and 100 rpm, subcultured approximately 1:3 into fresh LB broth, and incubated for 2 h at 37°C and 130 rpm. The optical density of the subcultures was adjusted by the addition of LB broth as required. The mean inoculum ± standard error of the mean (SEM) was 9.4 ± 0.08 $\log_{10}$ CFU per loop, and the mean secretory response ± SEM in the negative control loops (inoculated with sterile LB broth) was 0.02 ± 0.02 ml cm^{-1}.

The *waaN* mutant induced significantly lower secretory and inflammatory (PMN influx) responses than wild-type serovar Typhimurium C5 ($P < 0.01$) in each of three calves (Fig. 1). The reduction associated with mutation of *waaN* was less than that associated with mutation of serovar Dublin *sipB*, which has previously been shown to consistently abolish both responses (4, 10). The effect of the *waaN* mutation was partially complemented by introducing an intact copy of *waaN*, cloned into plasmid pUC18, into the *waaN* mutant. Differences between the results presented here and those of Everest et al. (3) may be attributed to the relative sensitivity of the assays, since inoculation of rabbit ligated ileal loops with stationary-phase

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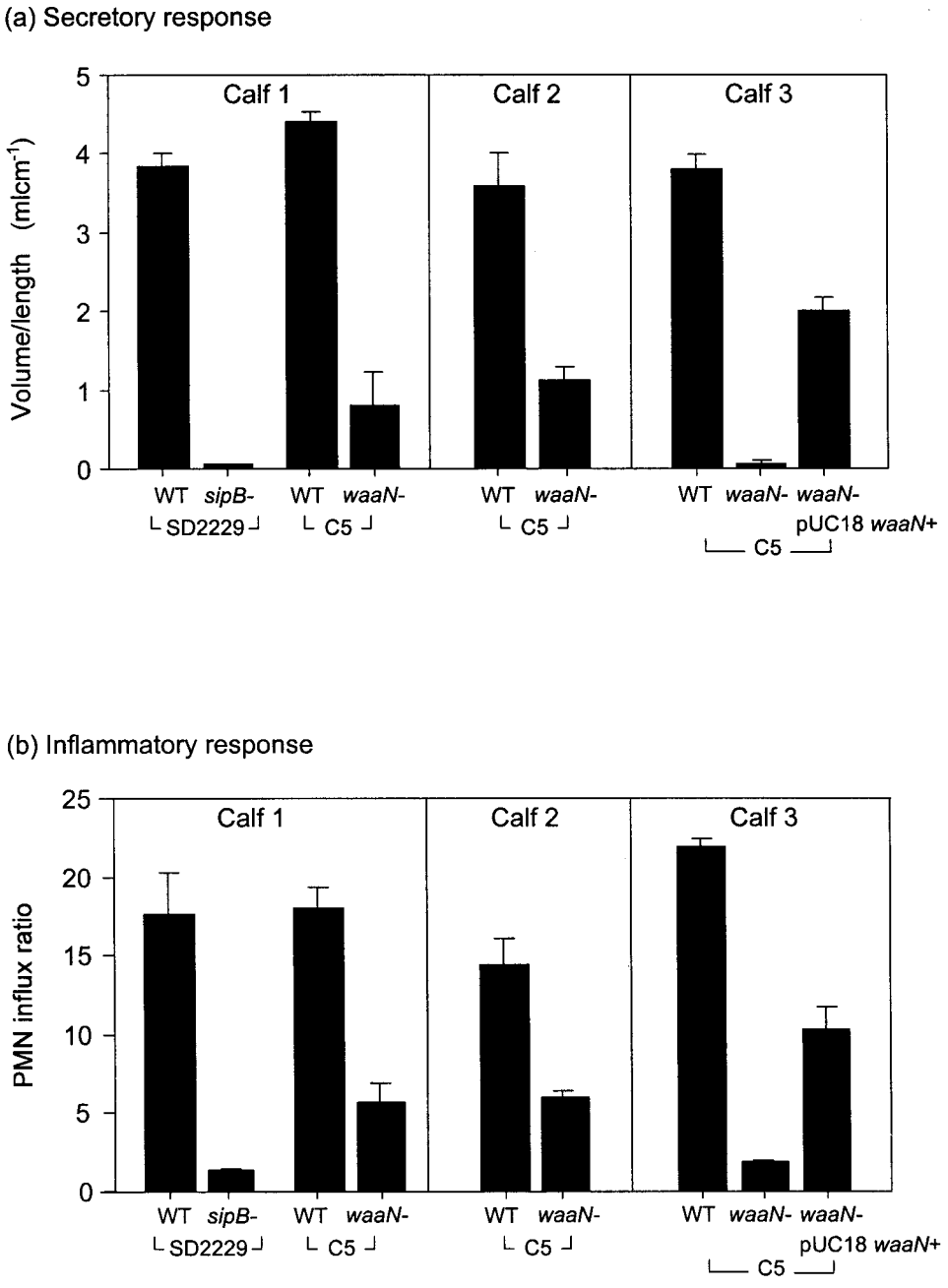

FIG. 1. Induction of enteropathogenic responses by serovar Dublin SD2229, serovar Typhimurium C5, and derivative mutants in bovine ligated ileal loops. (a) Secretory response. (b) Inflammatory response. Each bar represents the mean from three ligated loops and is presented with the SEM. WT, wild type.

Salmonella cultures, as done by Everest et al., results in relatively low enteropathogenic responses (19).

The *waaN* mutant is impaired in secretion of proteins required for invasion and enteropathogenesis. Induction of enteropathogenic responses by serovars Typhimurium and Dublin requires the appropriate secretion and translocation of type III secretion system-1 (TTSS-1) *Salmonella* invasion proteins (Sips) and *Salmonella* outer proteins (Sops) (4, 10, 21). The effect of the *waaN* mutation on the secretion of proteins by serovar Typhimurium was assessed. Bacterial cultures were prepared by incubation overnight in LB broth at 25°C and 100 rpm, subculture 1:10 into fresh LB broth, and incubation for 4 h at 37°C and 130 rpm. There were no differences in the optical densities of the cultures (1.106 and 1.109 at 600 nm) or the number of viable bacteria (2.5×10^9 and 4.0×10^9 CFU ml^{-1}) between the wild-type and *waaN* mutant, respectively, in a representative experiment. The culture supernatant was obtained by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C and filtration with 0.45-μm-pore-size disposable filters. Proteins present in the supernatant were precipitated by the addition of trichloroacetic acid, separated on a sodium dodecyl sulfate–12% polyacrylamide gel, and stained with Coomassie brilliant blue as described previously (22). Several proteins were present in larger amounts in wild-type serovar Typhimurium

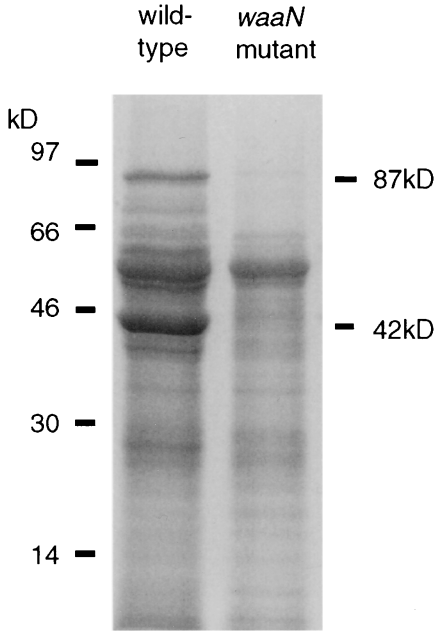

FIG. 2. Secretion of proteins by serovar Typhimurium C5 wild-type and its isogenic *waaN* mutant analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The sizes of molecular weight markers are indicated on the left. The gel was scanned with a Kodak DCS420 digital camera, and the contrast of the image was adjusted by using Adobe Photoshop 3.0.

C5 than in the *waaN* mutant (Fig. 2) in each of three separate experiments. Two of the most prominent of these proteins had molecular sizes similar to those reported for SipA (87 kDa) and SipC (42 kDa) (8, 21). We have previously demonstrated that a 42-kDa secreted protein from serovar Typhimurium is recognized by an anti-SipC monoclonal antibody (21).

In addition to enteropathogenesis, Sips are also required for bacterial invasion, and it was confirmed that mutation of *waaN* caused a reduction in bacterial invasion in a standard gentamicin protection assay with cultured human epithelial (Int 407) cells as described previously (20). Briefly, bacterial cultures were prepared by incubation overnight in LB broth at 25°C and 100 rpm, subculture 1:100 into fresh LB broth, and incubation for 4 h at 37°C and 130 rpm. Monolayers of Int 407 cells were prepared by seeding 5×10^5 cells per ml in RPMI medium containing 5% fetal calf serum into 24-well tissue culture plates and incubating overnight. The monolayers were infected at a ratio of approximately 5 bacteria per eukaryotic cell for 1 h, and then extracellular bacteria were removed by washing followed by incubation with medium containing gentamicin (100 μg ml^{-1}) for 1 h. The monolayers were washed twice, Int 407 cells were lysed with 0.1% deoxycholate, and the numbers of bacteria were estimated by counting viable cells. The invasiveness of strain C5 was significantly reduced ($P < 0.001$), from 4.94 ± 0.03 to 4.48 ± 0.01 $\log_{10}$ CFU ml^{-1} by mutation of *waaN* in an experiment representative of a total of three, each performed in triplicate. This reduction was complemented (4.95 ± 0.02 $\log_{10}$ CFU ml^{-1}) by introducing an intact copy of *waaN* in *trans*. The number of viable bacteria in the inoculum for the wild-type, *waaN* mutant, and *waaN*-complemented strains were 5.97, 6.16, and 6.18 $\log_{10}$ CFU ml^{-1}, respectively. Taking the protein secretion and invasion results together, *waaN* is likely to reduce enteropathogenesis by reducing the net secretion of TTSS-1-dependent proteins, and any direct effect of reduced lipid A toxicity is difficult to evaluate.

The reduction in *Salmonella* virulence associated with mutation of *waaN* cannot be attributed solely to a reduction in cytokine induction, as was concluded in previous studies (12, 13), because of the pleiotropic effects associated with the mutation. Even the reduction in cytokine expression following infection of mice with the *waaN* mutant compared to infection with wild-type serovar Typhimurium may be the result of a nonspecific effect, because mutation of genes associated with TTSS-1 reduces the synthesis or activation of proinflammatory cytokines (5, 6). Despite this postulated nonspecific effect on cytokine induction, the reduced cytokine induction by viable *waaN* mutants can still be attributed, at least in part, to altered lipid A, because experiments with heat-killed bacteria and purified LPS clearly demonstrate that LPS from *waaN* mutants induces less cytokine release than wild-type LPS (12, 13).

Perhaps the most important relevance of this study is the use of serovar Typhimurium *waaN* mutants as a cancer therapeutic, in which the tumor targeting and antitumor activities of wild-type serovar Typhimurium are retained but with reduced toxicity (13). For such a strain to be used in humans, the mechanism of attenuation must be clearly defined. The results of this study, demonstrating the pleiotropic effects associated with mutation of *waaN*, contribute significantly to our understanding of this attenuation.

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