INFECTION AND IMMUNITY, Nov. 1997, p. 4705–4710 Vol. 65, No. 11
0019-9567/97/$04.00+0

Contribution of the Mn-Cofactored Superoxide Dismutase (SodA) to the Virulence of *Yersinia enterocolitica* Serotype O8

ANDREAS ROGGENKAMP,* THOMAS BITTNER, LORENZ LEITRITZ, ANDREAS SING, AND JÜRGEN HEESEMANN

Max von Pettenkofer Institute for Hygiene and Microbiology, Ludwig Maximilians University Munich, 80336 Munich, Germany

Received 18 June 1997/Returned for modification 19 August 1997/Accepted 1 September 1997

Enteric pathogens harbor a set of enzymes (e.g., superoxide dismutases [SOD]) for detoxification of endogenous and exogenous reactive oxygen species which are encountered during infection. To analyze the role of the Mn-cofactored SOD (SodA) in the pathogenicity of yersiniae, we cloned the *sodA* gene of *Yersinia enterocolitica* serotype O8 by complementation of an *Escherichia coli sodA sodB* mutant and subsequently constructed an isogenic mutant by allelic exchange. Sequence analysis revealed an open reading frame that enabled the deduction of a sequence of 207 amino acids with 85% identity to SodA of *E. coli*. In a mouse infection model, the *sodA* null mutant was strongly attenuated in comparison to its parental strain. After intravenous infection, the survival and multiplication of the mutant in the spleen and liver were markedly reduced. In contrast, inactivation of *sodA* had only minor effects on survival and multiplication in the gut and Peyer's patches, as could be demonstrated in the orogastric infection model. The reduction in virulence was accompanied by a low but significant increase of susceptibility of the *sodA* mutant to bacterial killing by polymorphonuclear leukocytes (PMN) and an alteration of the intracellular chemiluminescence response of PMN. These results suggest that the resistance of *Y. enterocolitica* to exogenous oxygen radicals produced by phagocytes involves the Mn-cofactored SOD. The important role of *sodA* for the pathogenicity of *Y. enterocolitica* could also be due to detoxification of endogenous, metabolically produced oxygen radicals which are encountered by extracellular enteric pathogens during the invasion of the host.

Yersinia enterocolitica is an enteric pathogen frequently causing food-borne disease in humans (7). The infection is initiated when ingested *Y. enterocolitica* cells penetrate Peyer's patches and proliferate within the lymphoid tissue of the small intestine (16). In contrast to *Shigella* and *Salmonella* spp., the evasion strategy of *Yersinia* results in extracellular survival and multiplication (20, 31). Pathogenicity is controlled by a 70-kb virulence plasmid which encodes for the cell adhesin YadA and by a series of yersinia outer proteins (Yops) which are secreted and subsequently translocated into the interacting host cells (6, 17, 23, 29, 33–35). Some of these Yops (YopH [protein-tyrosine-phosphatase] and YopE [cytotoxin]) are involved in inhibition of the oxidative burst and of phagocytosis by polymorphonuclear leukocytes (PMN) (4, 27, 28, 30). In PMN the generation of oxidative metabolites (reactive oxygen intermediates, such as superoxide anions and hydrogen peroxide) is thought to be one of the first lines of defense against extracellular and intracellular bacteria (10). For two intracellular pathogens, *Shigella flexneri* and *Salmonella typhimurium*, it could be shown that superoxide dismutases (SOD) are involved in detoxification of reactive oxygen species, mediating protection against killing by macrophages (13, 37). Deficiency of the iron-containing FeSOD (*sodB* mutant) resulted in severe attenuation of the virulence of *S. flexneri* in the rabbit ileal loop model, whereas the mutation of *sodA* (encoding Mn-cofactored SOD [MnSOD]) in *S. typhimurium* had only a minor effect for mouse virulence. The different contributions of SOD to pathogenicity could be explained by differences in the infectious process of these pathogens. For instance, under anaerobic growth conditions such as those in the gut lumen, *sodA* is suggested to be downregulated and most of the metabolically produced reaction oxygen would be detoxified by FeSOD (12, 36). Change to aerobic conditions and iron starvation, on the other hand, might be responsible for upregulation of *sodA* (12). These latter conditions would be met by extracellular pathogens invading host blood vessels or tissue such as liver and spleen. Besides superoxide produced by the microbe itself, the pathogen should suffer also from reactive oxygen generated by the defending professional phagocytes (21).

This assumption prompted us to construct a *sodA* mutant of *Y. enterocolitica* and to elucidate the contribution of MnSOD to pathogenicity in the mouse model. We isolated the *sodA* gene of *Y. enterocolitica* by complementation of a dismutase-negative strain of *Escherichia coli*. After an isogenic *sodA*-negative strain was created by allelic exchange, the function of *sodA* was tested in PMN assays (chemiluminescence [CL] and bacterial killing) and in the mouse infection model. Here we demonstrate that the *sodA* mutant is less resistant to the bactericidal activity of PMN and is severely attenuated in mouse virulence (by the intravenous route) in comparison to the parental strain. In contrast, functional *sodA* is of minor impact for survival in the gut and Peyer's patches, as is shown in orally challenged mice.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are summarized in Table 1. *E. coli* DH5α was used for subcloning of *sodA*, and Sm10λpir was used as the host for the suicide vector derivatives of pGP704 (22). Bacteria were cultured aerobically in Luria-Bertani medium (LB). For SOD activity assays, CL experiments, and kill assays, *Y. enterocolitica* overnight cultures at 27°C were diluted 1:40 in LB and grown at 37°C for 3 h with vigorous shaking. For the iron-deficiency condition, overnight cultures were grown in nutrition broth (NB) at 27°C, followed by 1:40 dilution in NBD medium (NB supplemented with 100 μM α,α′-dipyridyl) at 37°C for 3 h. Antibiotics were used at the

* Corresponding author. Mailing address: Max von Pettenkofer-Institut for Hygiene and Microbiology, Ludwig Maximilians University Munich, Pettenkoferstr. 9a, 80336 Munich, Germany. Phone: 49-89-51605200. Fax: 49-89-5380584.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
Strains		
WA-314	*Y. enterocolitica* serotype O8; clinical isolate; pYVO8+	18
WA-C	Plasmidless derivative of WA-314	18
WA-314$_{sodA}$	*sodA* mutant of WA-314; Km	This study
TG1 *sodA sodB*	*E. coli sodA sodB* mutant	C. Spiegelhalder (Freiburg, Germany)
DH5α	*E. coli endA1 supE44 hsdR17*(r_K^- m_K^+) *thi-1 recA1 gyrA relA1* α(*lacZYA-argF)U169* (ϕ80 *lacZ*αM15)	15
Sm10λpir	*E. coli* K-12 *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu-Km(λpir)	22
Plasmids		
pBluescriptKS	ColE1 replicon; *bla*	Stratagene
pGPCAT	R6K replicon; suicide vector; Cm	26
pSUP102	p15A replicon; *mob*; Cm	14
pTB1	pLAFRII with a 25-kb insert encoding *sodA* from WA-C	This study
pTB2.1	pBluescriptKS with a 3.1-kb *Hin*dIII insert encoding *sodA* from WA-C	This study
pTB2.K	pTB2.1 with *sodA* inactivated by a Km-GenBlock in the *Bcl*I site of *sodA* (*sodA*::Km)	This study
pTB3.K	pGPCAT; *sodA*::Km	This study
pTB4.1	pSUP102 with a 3.1-kb *Hin*dIII insert encoding *sodA* from WA-C	This study

following concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 25; nalidixic acid, 60; chloramphenicol, 20; tetracycline, 20.

DNA manipulation and cloning procedures. Plasmid DNA preparations were isolated with Qiagen kits (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Conjugations between bacterial strains were performed overnight on blood agar plates as described previously (19). Restriction enzyme digestions, recovery of DNA fragments from agarose gels by DEAE membrane (Schleicher & Schuell, Inc.), ligations, transformations, and Southern blot hybridization were performed as described by Ausubel et al. (1). Enzymes, deoxynucleoside triphosphates, and *Taq* polymerase were purchased from Pharmacia LKB (Freiburg, Germany). The digoxigenin-dUTP labeling and detection kit from Boehringer (Mannheim, Germany) was used for DNA probes in hybridization experiments. Oligonucleotides were synthesized by Roth (Karlsruhe, Germany) in TRITYL-OFF mode.

The construction of the gene bank from *Y. enterocolitica* WA-C in the cosmid vector pLAFRII has been described elsewhere (24). For isolation of *Y. enterocolitica* dismutase genes, the *E. coli* strain TG1 *sodA sodB* was conjugated with *E. coli* Sm10λpir carrying the *Y. enterocolitica* gene bank and subcultured on LB agar supplemented with chloramphenicol, tetracycline, and paraquat. Plasmids from 12 exconjugants were isolated and characterized by restriction enzyme digestion. A common 3.1-kb *Hin*dIII fragment was subcloned in pKS, resulting in pTB2.1. This fragment restored the growth defect of TG1 *sodA sodB* on paraquat agar. The nucleotide sequence of the 3.1-kb *Hin*dIII fragment was determined by the TaqDyeDideoxy terminator method with a 373A DNA Sequencer (Applied Biosystems GmbH, Darmstadt, Germany).

For construction of a *sodA* mutant in *Y. enterocolitica*, a *Bam*HI-digested 1.2-kb Km-GenBlock derived from pUC-4K (Pharmacia LKB) was introduced in the *Bcl*I site of pTB2.1, resulting in pTB2.K. The *Bcl*I site is located inside the *sodA* gene, 166 bp downstream of the start codon. In DH5α the *Bcl*I site was methylated. Therefore, pTB2.1 was passed through a *dam E. coli* (ATCC 47045). The *sodA*::Km gene fragment was transferred to the suicide vector pGPCAT (26) by using the restriction sites *Sac*I and *Kpn*I. The resulting plasmid, pTB3.K, was mobilized into *Y. enterocolitica* WA-314. Exconjugants resistant to nalidixic acid and kanamycin but sensitive to chloramphenicol were further characterized. The allelic exchange (disruption of *sodA* by insertion of the Km cassette) resulting in the WA-314 *sodA* mutant (WA-314$_{sodA}$) was confirmed by Southern hybridization and a SOD activity assay.

To restore the *sodA* mutant in *trans*, the *sodA* gene was cloned as a 3.1-kb *Hin*dIII fragment from pTB2.1 into the low-copy-number vector pSUP102, resulting in pTB4.1.

SOD activity assay. Bacteria were washed with phosphate-buffered saline and lysed by sonication on ice. After centrifugation at 15,000 × *g* for 20 min, the protein concentration of the crude lysate was determined by bicinchoninic acid protein assay (Pierce, Freiburg, Germany). Equivalent amounts of total protein (40 μg) were loaded onto a polyacrylamide gel run under nondenaturing conditions. Five micrograms of purified FeSOD (Sigma, Deisenhofen, Germany) served as a control in one lane. SOD bands were visualized in gels by the activity staining method of Beauchamp and Fridovich (3).

CL and killing assays with PMN. PMN were isolated from peripheral blood obtained from healthy volunteers by a one-step separation method using Mono-Poly Resolving Medium (Flow Laboratories, Irvine, United Kingdom) (11). The activation of the neutrophil oxidative burst was measured as luminol-enhanced CL and monitored with a microplate-chemiluminometer (Hamamatsu Photonics, Herrsching, Germany) as described previously (30). Bacteria were tested preopsonized with 5% normal human serum (NHS) and mixed with PMN at a multiplicity of infection (MOI) of 40:1. To discriminate between intra- and extracellular CL, SOD and catalase or horseradish peroxidase and azide were added to the wells to quench intra- or extracellular oxygen metabolites, respectively (5, 8). For restimulation of PMN, opsonized zymosan (250 μg/ml) was added and the secondary CL signal was measured. Each assay was performed in duplicate and repeated at least three times. Only representative and reproducible CL graphs are shown.

For killing experiments, bacteria were grown under different conditions and incubated with PMN at an MOI of 40:1 in the presence of 5% NHS. For evaluation of the ratio of killed yersiniae, PMN were lysed with ice-cold distilled water containing 0.5% tergitol after incubation with bacteria for 90 min at 37°C. Serial dilutions were plated on Mueller-Hinton agar, and CFU were counted after 48 h of incubation at 26°C. Control samples, containing no PMN, were treated in parallel under the same conditions. The killing rate was quantified as the mean percent (± standard deviation) killed bacteria in comparison to the total number of bacteria from at least four independent experiments.

Mouse virulence test. Virulence was tested in the intravenous and orogastric mouse infection models as described previously (25). For the intravenous infection route, groups of four BALB/c mice (6- to 8-week-old females; Charles River WIGA, Braunschweig, Germany) were infected with 4×10^4 bacteria (40 times the minimal lethal dose [38]). For the oral infection route, groups of eight C57BL/6 mice (female, 6 to 8 weeks old; Charles River WIGA, Sulzfeld, Germany) were infected with 5×10^8 bacteria. At the indicated days postinfection, the numbers of bacteria in the organs were determined by plating serial dilutions of homogenized tissue (animal licensing committee permission no. 621-2531.01-52/95).

Statistics. The significance of the differences among the control and experimental groups was determined by the Student *t* test. *P* values of <0.05 were considered statistically significant.

Nucleotide sequence accession number. The GenBank database accession number X96852 has been assigned to the *sodA* gene fragment of *Y. enterocolitica* O8 strain WA-314.

RESULTS

Cloning and sequencing of the *Y. enterocolitica sodA* gene. A cosmid library of *Y. enterocolitica* serotype O8 strain WA-C constructed in pLAFRII was conjugated into *E. coli* TG1 *sodA sodB*. Exconjugants able to grow on minimal agar supplemented with paraquat and the appropriate antibiotics were further studied. Restriction enzyme analysis of cosmids of 12 strains showed a common 3.1-kb *Hin*dIII fragment. This fragment was subcloned into pBluescriptKS, resulting in pTB2.1. Transformation of pTB2.1 in TG1 *sodA sodB* restored the growth defect of the *E. coli* mutant and showed in a SOD activity assay that pTB2.1 encodes a gene product corresponding to SodA (Fig. 1, lanes 4 and 6). The 3,120 bp of the *Hin*dIII

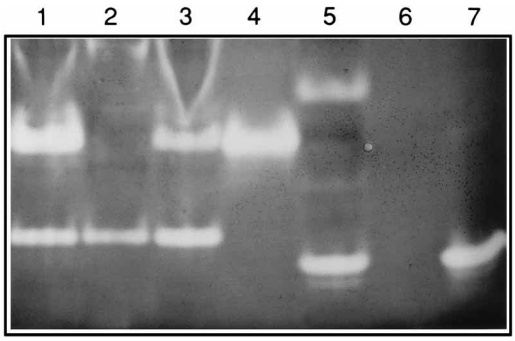


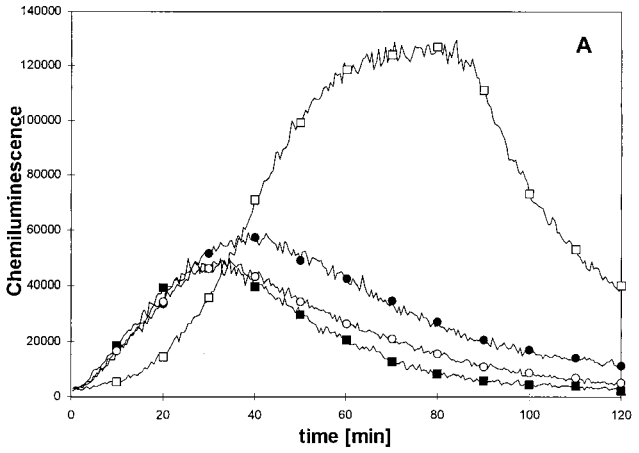
FIG. 1. SOD activity gel. Crude cell extracts (40 μg of protein) were loaded on a nondenaturing 10% polyacrylamide gel and stained for SOD activity. Lane 1, WA-314$_{sodA}$(pTB4.1); lane 2, WA-314$_{sodA}$; lane 3, WA-314; lane 4, TG1 *sodA sodB*(pTB2.1); lane 5, DH5α; lane 6, TG1 *sodA sodB*; lane 7, *E. coli* SodB (5 μg).

fragment were sequenced. An open reading frame of 621 bp enabled the deduction of an amino acid with 85% identity to SodA of *E. coli* or *S. typhimurium* SodA. The putative promoter region of this open reading frame was highly homologous to that of *E. coli sodA*, including a potential Fur box. Forty-one base pairs downstream of the stop codon, a strong terminator follows the open reading frame (data not shown). These data demonstrated that the cloned *Hin*dIII fragment encodes the *sodA* gene of *Y. enterocolitica* O8.

Construction of a *Y. enterocolitica sodA* mutant. The *sodA* gene of *Y. enterocolitica* in pTB2.1 was interrupted by insertion of a Km-GenBlock. After transfer of the *sodA*::Km fragment into a suicide vector, the resulting plasmid, pTB3.K, was mobilized into WA-314. The inactivated gene was introduced into the chromosome by allelic exchange. The resulting clones (WA-314$_{sodA}$) were analyzed by Southern hybridization for correct replacement of the wild-type *sodA* by *sodA*::Km. The SOD activity assay confirmed the inactivation of *sodA* in WA-314 (Fig. 1, lanes 2 and 3).

For complementation of the *sodA* mutation in WA-314$_{sodA}$, the 3.1-kb *Hin*dIII fragment encoding *sodA* was inserted into pSUP102, resulting in pTB4.1. After conjugation of pTB4.1 in WA-314$_{sodA}$, the complementation of the gene defect was confirmed by SOD activity assay (Fig. 1).

Interaction of *sodA* mutant strains with PMN. To analyze the role of MnSOD in the defense of *Y. enterocolitica* against oxygen-dependent microbicidal mechanisms, we studied the interaction of parental strain WA-314 and *sodA* mutant strains with PMN and monitored the oxidative burst and the bacterial killing. As has been shown previously, the wild-type strain WA-314 resisted the bactericidal attack of the PMN and suppressed the oxidative burst, whereas the interaction of PMN with plasmidless WA-C resulted in a high CL response accompanied by bacterial killing (30). The *sodA* mutant WA-314$_{sodA}$ was still able to suppress the CL response of PMN (Fig. 2), but the primary CL curve differed significantly from that of WA-314: (i) the maximum value (t_{max}) was markedly delayed for WA-314$_{sodA}$ (14.5 ± 2.5 min) and (ii) the integral of the CL curve was higher for WA-314$_{sodA}$ ($5.46 \times 10^6 \pm 2.95 \times 10^6$ versus $3.46 \times 10^6 \pm 1.48 \times 10^6$). The differences between the CL curves of the isogenic pair were remarkably constant in parallel experiments ($1.99 \times 10^6 \pm 1.46 \times 10^6$): the integral of the WA-314$_{sodA}$ CL curve was about 1.48 ± 0.15 times higher than that of the WA-314 CL curve. The WA-314$_{sodA}$ strain complemented in *trans* with *sodA* [WA-314$_{sodA}$(pTB4.1)] showed behavior identical to that of the wild-type strain in terms of configuration of the CL curves. All three strains were



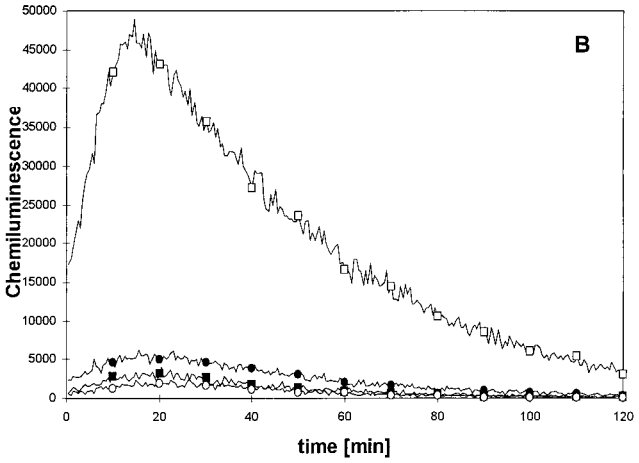


FIG. 2. Different WA strains were incubated with PMN at a ratio of 40:1, and the primary or secondary CL response was measured as described in Materials and Methods. (A) The bacteria were opsonized with 5% NHS and incubated with PMN. The primary CL response was measured. (B) After 120 min of incubation, the PMN were restimulated with opsonized zymosan and the secondary CL response was measured. Symbols: ○, WA-314; □, WA-C (plasmidless); ●, WA-314$_{sodA}$; ■, WA-314$_{sodA}$(pTB4.1).

able to inhibit a secondary CL response after zymosan treatment (Fig. 2B), indicating efficient translocation of YopH and YopE.

The CL response of PMN can be divided into an early intracellular and a late extracellular response (5). Wild-type *Yersinia* strains like WA-314 were able to suppress the extracellular CL response completely, probably due to Yop translocation (30). As expected, mutation in *sodA* did not affect the ability of WA-314 to suppress the extracellular CL response (Fig. 3B). The intracellular CL curves showed the same delayed maximum values for the *sodA*-mutated strain as did the curves of the total CL response (Fig. 2A and 3A).

For killing-rate experiments, the bacteria were grown in LB or in iron limitation medium (NBD) at 37°C. Wild-type strains of *Y. enterocolitica* grown at 37°C were shown to be resistant to phagocytosis and thus were localized extracellularly attached to PMN (30). Mutation in *sodA* did not affect resistance to phagocytosis (data not shown). As demonstrated in Table 2, the mutation of *sodA* in *Y. enterocolitica* resulted in increased susceptibility to PMN-mediated killing. For *E. coli* it has been shown that iron starvation resulted in increased expression of

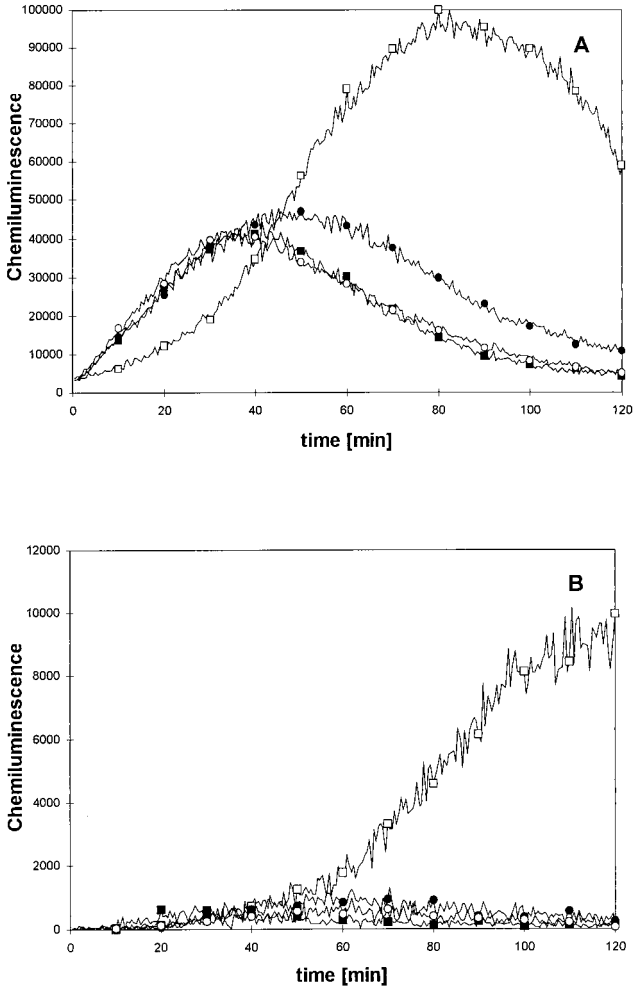


FIG. 3. Intra- and extracellular CL responses of PMN to different WA strains. (A) Extracellular oxygen metabolites were quenched with SOD and catalase, and the intracellular CL response was measured. (B) Intracellular oxygen metabolites were quenched with horseradish peroxidase and azide, and the extracellular CL response was measured. Symbols are as explained in the legend to Fig. 2.

MnSOD (three- to fivefold) (12). In contrast to *S. typhimurium* (37), preincubation of *Y. enterocolitica* WA-314 in NBD medium had no effect on resistance against killing by PMN (Table 2).

Virulence of WA-314$_{sodA}$ for mice. The effect of insertional inactivation of *sodA* on the virulence of *Y. enterocolitica* O8 was tested in the orogastric and intravenous mouse infection models. The progress of infection was determined by measuring the numbers of surviving bacteria in the organs at the indicated days. For orogastric infection, groups of eight C57BL/6 mice were infected with 5×10^8 bacteria of strain WA-314, WA-314$_{sodA}$, or WA-314$_{sodA}$(pTB4.1). The results are summarized in Table 3. The course of infection of WA-314 was progressive, with dissemination of the bacteria in lymphatic organs at day 5 and a high bacterial load at day 8. At the beginning of the infection (day 1), comparable amounts of bacteria of both the *sodA* mutant strain and the wild-type strain colonized the Peyer's patches. However, at day 5 the number of reisolated *sodA* mutant bacteria in Peyer's patches was 10 times less than that of the parent strain bacteria. Moreover, no dissemination of *sodA* mutants to spleen and liver was observed. The low number of reisolated *sodA* mutants at day 8 corresponded with a loss of mouse lethality of the mutant. The recomplementation of WA-314$_{sodA}$ with the *sodA* gene in *trans* [WA-314$_{sodA}$(pTB4.1)] did not restore mouse virulence, as indicated by a reduced number of reisolated bacteria from the Peyer's patches and the lack of dissemination to spleen and liver. *Yersinia* colonies isolated from Peyer's patches 5 days postinfection were screened on antibiotic-containing agar plates for resistance to kanamycin and chloramphenicol. While all tested *sodA* mutant strains [WA-314$_{sodA}$ and WA-314$_{sodA}$(pTB4.1)] showed kanamycin resistance, 2 of 20 tested WA-314$_{sodA}$(pTB4.1) colonies were found to be chloramphenicol sensitive, indicating loss of plasmid pTB4.1. The results of the intravenous infection route were in good correlation with those of the orogastric infection model. We determined the bacterial loads in spleen and liver 2 and 4 days after intravenous infection with 4×10^4 bacteria of WA-314 and WA-314$_{sodA}$, respectively. The wild-type strain colonized and multiplied in spleen and liver rapidly [$(6.2 \pm 3.2) \times 10^5$ CFU in the spleen and $(1.8 \pm 3.5) \times 10^5$ CFU in the liver on day 2; $(8.5 \pm 2.8) \times 10^7$ CFU in the spleen and $(2.1 \pm 4.1) \times 10^7$ CFU in the liver on day 4], whereas a mutation of the *sodA* gene in WA-314$_{sodA}$ resulted in a marked decrease of reisolated bacteria [$(9.6 \pm 6.4) \times 10^2$ CFU in the spleen and $(1.2 \pm 4) \times 10^3$ CFU in the liver on day 2; $(1.5 \pm 4.5) \times 10^2$ CFU in the spleen and $(8.8 \pm 5.2) \times 10^4$ CFU in the liver on day 4].

TABLE 2. Killing of *Y. enterocolitica* strains by PMN[a]

Strain and culture	Mean % killing[b] ± SD
WA-314	
LB	7 ± 13
NBD	4 ± 18
WA-314$_{sodA}$	
LB	21 ± 15 ($P < 0.05$)
NBD	17 ± 11 ($P < 0.05$)
WA$_{sodA}$(pTB4.1)	
LB	13 ± 17 (NS)
NBD	6 ± 14 (NS)

[a] Bacteria were grown under the conditions described in Materials and Methods. PMN were incubated with different strains in the presence of 5% NHS at 37°C at an MOI of 40:1. After 90 min of incubation, PMN were lysed and the numbers of viable bacteria were determined by plating. The experiments were repeated at least four times.

[b] Mean percent killed bacteria with respect to the total number of bacteria (control sample without PMN). *P* values were determined by the Student *t* test. NS, no significant difference.

DISCUSSION

Y. enterocolitica strains of biotype IB (serotypes O8, O13, O20, and O21) and *S. typhimurium* belong to the family of *Enterobacteriaceae* and are highly pathogenic for mice after oral infection. These enteric pathogens use the Peyer's patches as the port of entry and then disseminate to the spleen and liver. However, on a cellular level yersiniae can be considered extracellularly multiplying pathogens, whereas salmonellae have developed efficient strategies for intracellular survival and multiplication. Therefore, we expected that the contribution of MnSOD to pathogenicity would differ between these two pathogens. Indeed, we could demonstrate that the *sodA* null mutant of *Y. enterocolitica* was strongly attenuated in mouse virulence when applied by the intravenous or the orogastric route. In contrast, *sodA* mutation of *S. typhimurium* had been reported to play a minor role in mouse virulence (37). These

TABLE 3. Number of bacteria per organ in C57BL/6 mice 1, 5, and 8 days after orogastric infection with 5×10^8 bacteria

Day and site[a]	Mean $\log_{10}$ CFU ± SD[b]		
	WA-314	WA-314$_{sodA}$	WA-314$_{sodA}$(pTB3.3)
1			
SI	4.2 ± 0.71	3.42 ± 0.4 (NS)	4.11 ± 0.34 (NS)
PP	4.75 ± 0.56	4.31 ± 0.53 (NS)	4.57 ± 0.86 (NS)
MLN	< 0.5	< 0.5	< 0.5
S	< 1.25	< 1.25	< 1.25
5			
SI	6.02 ± 0.72	4.89 ± 0.56 (NS)	5.25 ± 0.44 (NS)
PP	5.91 ± 0.33	4.62 ± 0.41 ($P < 0.05$)	5.15 ± 0.64 (NS)
MLN	3.85 ± 0.82	< 0.5	< 0.5
S	4.01 ± 0.43	< 1.25	< 1.25
L	3.66 ± 0.43	< 1.25	< 1.25
8			
SI	6.42 ± 0.81	2.1 ± 0.33 ($P < 0.001$)	ND
PP	6.81 ± 0.61	3.66 ± 0.69 ($P < 0.01$)	ND
MLN	5.34 ± 0.82	< 0.5	ND
S	6.26 ± 0.78	< 1.25	ND
L	5.53 ± 0.73	< 1.25	ND

[a] PP, Peyer's patches; SI, small intestine; MLN, mesenteric lymph nodes; S, spleen; L, liver.
[b] Results are means from eight animals ± standard deviations. *P* values were determined by the Student *t* test. NS, no significant difference; ND, not done.

authors argued that salmonellae probably encounter few bactericidal superoxides within the phagosomal environment, so that the detoxifying MnSOD is not strictly required.

Evidently, MnSOD is required for the survival and multiplication of yersiniae in spleen and liver tissue. Surprisingly, *sodA* mutation did not play a crucial role for invasion, survival, and multiplication of yersiniae in Peyer's patches. One possible explanation could be that during the entry process (e.g., translocation through M cells) yersiniae are located intracellularly. However, it has been clearly demonstrated that yersiniae multiply extracellularly in Peyer's patches and form abscesses which break through the mucosal wall (2, 16). Thus, we can assume an environment similar to the anaerobic or microaerophilic one of the gut lumen, which favors downregulation of *sodA* and upregulation of *sodB* (12). This assumption is supported by the results obtained with *sodB* mutants of *S. flexneri*, which were found to be strongly attenuated compared with the parental strain in the rabbit ileal loop model (13).

It seems plausible that the role of microbial SOD in pathogenicity should be closely associated with defense against reactive oxygen produced by neutrophils (21). *Y. enterocolitica* has developed a strategy to inhibit production of reactive oxygen by professional phagocytes by translocating the protein-tyrosine-phosphatase YopH and the cytoskeleton-destroying YopE (30). In spite of this, the contact of yersiniae with PMN initially elicited a short oxidative burst which could be harmful for *sodA* mutants. Measuring the oxidative burst of PMN as luminol-enhanced CL, we found that a mutation in the *sodA* gene of *Y. enterocolitica* impaired the suppression of the oxidative burst to some degree. In comparison to the response to the wild-type strain, the primary CL response of the PMN induced by the *sodA* mutant was markedly delayed (14.5 min) and the integral of the CL curve, a marker for the extent of the oxidative burst, was significantly higher. However, the zymosan-induced secondary CL response, measured after 2 h of incubation with the yersinia strains, showed no differences between the *sodA* mutant and the wild-type strain, indicating effective suppression of the oxidative burst by YopH/YopE translocation of the isogenic pair. The change in the primary CL curve was accompanied by a low but significant increase in the susceptibility of the *sodA* mutant to bacterial killing by PMN. The differences between the wild type and the *sodA* mutant could be compensated by restoration of *sodA* expression in WA-314$_{sodA}$(pTB4.1).

The oxidative burst can be divided into an early intracellular and a late extracellular component (5). Quenching experiments indicated that the differences in the CL curve were due to early intracellular changes. The *sodA* mutant strain was as resistant to phagocytosis as the wild-type strain at 37°C. The intracellular difference in the CL response might be due to diffusion of oxygen radicals across cell membranes into the pathogen (21). The delay of the CL maximum generated by the *sodA* mutant could be explained by the fact that dismutases react with a substrate (OH^-) of myeloperoxidase (MPO), the most bactericidal component of the oxidative burst (9). Since luminol-enhanced CL first of all signals the activity of MPO, a shift of the CL curve is not surprising.

Differences in susceptibility to bacterial killing of wild-type *S. typhimurium* and a *sodA* mutant might be detected only after maximal induction of *sodA*, for example, in iron limitation medium. We tested this possibility by preincubation of the WA strains in NBD medium. Growth under iron limitation did not cause further resistance (or even multiplication) of the tested *Y. enterocolitica* strains. Different localizations of the bacteria (*Salmonella* was intracellular and *Yersinia* was extracellular at 37°C) could explain these findings.

In the orogastric mouse infection model, the mutation of the *sodA* gene resulted in an attenuation of virulence. While the colonization of the Peyer's patches and the initiation of an infection were comparable between the wild-type and the *sodA* mutant strains, the further course of infection differed. The *sodA* mutant strain was not able to generate a systemic infection with dissemination in lymphatic organs such as mesenteric lymph nodes or spleen. The attenuation of the virulence could also be demonstrated in the intravenous infection model. The amount of WA-314$_{sodA}$ reisolated from the spleen was 100 to 1,000 times lower than the amount of WA-314. The different bacterial loads of WA-314$_{sodA}$ in the spleen and liver, partic-

ularly on day 4, could be due to different active macrophages in the organs. Recomplementation of the *sodA* mutant with the *sodA* gene in *trans* restored virulence only in part. A similar observation was made for recomplementation of other *Yersinia* mutants as well (32). A possible explanation might address the stability of the plasmid in *trans.*

In summary, MnSOD plays an important role in the pathogenicity of *Y. enterocolitica*. This could be demonstrated in the orogastric and intravenous mouse infection models. Whether the attenuation of virulence resulting from mutation of the *sodA* gene is due to increased susceptibility to killing by PMN and change in the primary CL response or to reduced detoxification of metabolically produced bacterial superoxide remains to be elucidated. The ability of WA-314$_{sodA}$ to colonize the Peyer's patches without generating a systemic infection makes the *sodA* mutant strain a suitable candidate for a live vaccine able to induce mucosal immunity.

ACKNOWLEDGMENTS

We thank C. Spiegelhalder for providing the *sodA sodB*-negative strain TG1 and K. Baus for excellent technical assistance.

This study was supported by Bayerischer Forschungsverbund, FORGEN.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc, New York, N.Y.
2. **Autenrieth, I. B., and R. Firsching.** 1996. Penetration of M cells and destruction of Peyer's patches by *Yersinia enterocolitica*: an ultrastructural and histological study. J. Med. Microbiol. **44:**285–294.
3. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
4. **Bliska, J. B., K. L. Guan, J. E. Dixon, and S. Falkow.** 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. Proc. Natl. Acad. Sci. USA **88:**1187–1191.
5. **Briheim, G., O. Stendahl, and C. Dahlgren.** 1984. Intra- and extracellular events in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. Infect. Immun. **45:**1–5.
6. **Cornelis, G. R.** 1994. Yersinia pathogenicity factors. Curr. Top. Microbiol. Immunol. **192:**243–263.
7. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
8. **Dahlgren, C.** 1987. Polymorphonuclear leukocyte chemiluminescence induced by formylmethionyl-leucyl-phenylalanine and phorbol myristate acetate: effects of catalase and superoxide dismutase. Agents Actions **21:**104–112.
9. **DeChatelet, L. R., G. D. Long, P. S. Shirley, D. A. Bass, M. J. Thomas, F. W. Henderson, and M. S. Cohen.** 1982. Mechanism of the luminol-dependent chemiluminescence of human neutrophils. J. Immunol. **129:**1589–1593.
10. **Elsbach, P., and J. Weiss.** 1992. Inflammation: basic principles and clinical correlates, 2nd ed. Raven Press, Ltd., New York, N.Y.
11. **Ewald, J. H., J. Heesemann, H. Rüdiger, and I. B. Autenrieth.** 1994. Interaction of polymorphonuclear leukocytes with *Yersinia enterocolitica*: role of the *Yersinia* virulence plasmid and modulation by the iron-chelator desferrioxamine B. J. Infect. Dis. **170:**140–150.
12. **Fee, J. A.** 1991. Regulation of sod genes in *Escherichia coli*: relevance to superoxide dismutase function. Mol. Microbiol. **5:**2599–2610.
13. **Franzon, V. L., J. Arondel, and P. J. Sansonetti.** 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. **58:**529–535.
14. **Guiney, D. G., and D. R. Helinski.** 1979. The DNA-protein relaxation complex of the plasmid RK2: location of the site-specific nick in the region of the proposed origin of transfer. Mol. Gen. Genet. **176:**183–189.
15. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
16. **Hanski, C., U. Kutschka, H. P. Schmoranzer, M. Naumann, A. Stallmach, H. Hahn, H. Menge, and E. O. Riecken.** 1989. Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O8 with intestinal mucosa during experimental enteritis. Infect. Immun. **57:**673–678.
17. **Heesemann, J., and L. Grüter.** 1987. Genetic evidence that the outer membrane protein Yop1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. FEMS Microbiol. Lett. **40:**37–41.
18. **Heesemann, J., C. Keller, R. Morawa, N. Schmidt, H. J. Siemens, and R. Laufs.** 1983. Plasmids of human strains of *Yersinia enterocolitica*: molecular relatedness and possible importance for pathogenesis. J. Infect. Dis. **147:**107–115.
19. **Heesemann, J., and R. Laufs.** 1983. Construction of a mobilizable *Yersinia enterocolitica* virulence plasmid. J. Bacteriol. **155:**761–767.
20. **Lian, C.-J., W. S. Hwang, and C. H. Pai.** 1987. Plasmid-mediated resistance to phagocytosis in *Yersinia enterocolitica*. Infect. Immun. **55:**1176–1183.
21. **Miller, R. A., and B. E. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1–18.
22. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
23. **Persson, C., R. Nordfelth, A. Holmström, S. Hakansson, R. Rosqvist, and H. Wolf-Watz.** 1995. Cell-surface-bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. Mol. Microbiol. **18:**135–150.
24. **Rakin, A., E. Saken, D. Harmsen, and J. Heesemann.** 1994. The pesticin receptor of *Yersinia enterocolitica*: a novel virulence factor with dual function. Mol. Microbiol. **13:**253–263.
25. **Roggenkamp, A., H.-R. Neuberger, A. Flügel, T. Schmoll, and J. Heesemann.** 1995. Substitution of two histidine residues in YadA protein of *Yersinia enterocolitica* abrogates collagen binding, cell adherence and mouse virulence. Mol. Microbiol. **16:**1207–1219.
26. **Roggenkamp, A., S. Schubert, C. A. Jacobi, and J. Heesemann.** 1995. Dissection of the *Yersinia enterocolitica* virulence plasmid pYVO8 into an operating unit and virulence gene modules. FEMS Microbiol. Lett. **134:**69–73.
27. **Rosqvist, R., I. Bolin, and H. Wolf-Watz.** 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. **56:**2139–2143.
28. **Rosqvist, R., A. Forsberg, M. Rimpilainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic protein YopE of *Yersinia* obstructs the primary host defence. Mol. Microbiol. **4:**657–667.
29. **Rosqvist, R., K. E. Magnusson, and H. Wolf-Watz.** 1994. Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. **13:**964–972.
30. **Ruckdeschel, K., A. Roggenkamp, S. Schubert, and J. Heesemann.** 1996. Differential contribution of *Yersinia enterocolitica* virulence factors to evasion of microbicidal action of neutrophils. Infect. Immun. **64:**724–733.
31. **Simonet, M., S. Richard, and P. Berche.** 1990. Electron microscopic evidence for in vivo extracellular localization of *Yersinia pseudotuberculosis* harboring the pYV plasmid. Infect. Immun. **58:**841–845.
32. **Skrzypek, E., and S. C. Straley.** 1995. Differential effects of deletions in *lcrV* on secretion of V antigen, regulation of the low-Ca^{2+} response, and virulence of *Yersinia pestis*. J. Bacteriol. **177:**2530–2542.
33. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia spp*. Mol. Microbiol. **3:**517–529.
34. **Sory, M. P., and G. R. Cornelis.** 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. **14:**583–594.
35. **Straley, S. C., E. Skrzypek, G. V. Plano, and J. B. Bliska.** 1993. Yops of *Yersinia* spp. pathogenic for humans. Infect. Immun. **61:**3105–3110.
36. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:**2305–2314.
37. **Tsolis, R. M., A. J. Bäumler, and F. Heffron.** 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Infect. Immun. **63:**1739–1744.
38. **Vogel, U., I. B. Autenrieth, R. Berner, and J. Heesemann.** 1993. Role of plasmid-encoded antigens of *Yersinia enterocolitica* in humoral immunity against secondary *Y. enterocolitica* infection in mice. Microb. Pathog. **15:**23–36.

Editor: J. T. Barbieri