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

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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 aerobic 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* DH5a was used for subcloning of sodA, and Sm10pir was used as the host for the suicide vector derivatives of pGIP704 (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

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

DNA manipulation and cloning procedures. Plasmid DNA preparations were isolated with QiAprep kit (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 treatment, and polymerase were purchased from Pharmacia LKB (Freiburg, Germany). The digoxigenin-dUTP labeling and detection enzymatic digestion. A common 3.1-kb HindIII fragment was subcloned in PGPCAT, resulting in pTB3.K. The resulting plasmid, pTB3.K, was mobilized into E. coli K-12 thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu-Km(aphr).

The nucleotide sequence of the 3.1-kb sodA allele fragment was determined by the TaqDyeDideoxy terminator method with a 373A DNA Sequencer (Applied Biosystems GmbH, Darmstadt, Germany). In DH5α (lacZΔM15 Δ(lacZYA-argF)U169 thi-1 recA1 supE44 endA1 hsdR17 (rK- mK-) F− triPH dill) were infected with 4 × 108 bacteria. At the indicated days postinfection, the 3.120 bp of the sodA gene fragment was determined as a 3.1-kb HindIII fragment from pTB2.1 with a 3.1-kb HindIII insert encoding sodA from WA-C.

**RESULTS**

Cloning and sequencing of the Y. enterocolitica sodA gene. A cosmide 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 HindIII 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 assay that pTB2.1 encodes a gene product corresponding to SodA (Fig. 1, lanes 4 and 6). The 3.120 bp of the HindIII fragment encoded a protein of 153 amino acids with the calculated Mw of 16,626 Da. The GenBank database accession number X96852 has been assigned to the sodA gene fragment of Y. enterocolitica O8 strain WA-C.
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 *HindIII* 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 *HindIII* fragment encoding *sodA* was inserted into pSUP102, resulting in pTB4.1. After conjugation of pTB4.1 in WA-314, 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 × 10$^6$ ± 2.95 × 10$^6$ versus 3.46 × 10$^6$ ± 1.48 × 10$^6$). The differences between the CL curves of the isogenic pair were remarkably constant in parallel experiments (1.99 × 10$^6$ ± 1.46 × 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 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...
were infected with 5 days. For orogastric infection, groups of eight C57BL/6 mice numbers of surviving bacteria in the organs at the indicated elns. The progress of infection was determined by measuring the tested in the orogastric and intravenous mouse infection mod-

sodA

legend to Fig. 2.

the extracellular CL response was measured. Symbols are as explained in the

catalase, and the intracellular CL response was measured. (B) Intracellular

strains. (A) Extracellular oxygen metabolites were quenched with SOD and

Y. enterocolitica

S. typhimurium

MnSOD (three- to fivefold) (12). In contrast to

Y. enterocolitica

SodA

mutants to spleen and liver was observed. The low num-

TABLE 2. Killing of Y. enterocolitica strains by PMN

<table>
<thead>
<tr>
<th>Strain and culture</th>
<th>Mean % killing ± SD</th>
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<tbody>
<tr>
<td>WA-314</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>7 ± 13</td>
</tr>
<tr>
<td>NBD</td>
<td>4 ± 18</td>
</tr>
<tr>
<td>WA-314&lt;sub&gt;sodA&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>21 ± 15 (P &lt; 0.05)</td>
</tr>
<tr>
<td>NBD</td>
<td>17 ± 11 (P &lt; 0.05)</td>
</tr>
<tr>
<td>WA&lt;sub&gt;sodA&lt;/sub&gt;(pTB4.1)</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>13 ± 17 (NS)</td>
</tr>
<tr>
<td>NBD</td>
<td>6 ± 14 (NS)</td>
</tr>
</tbody>
</table>

a Bacteria were grown under the conditions described in Materials and Methods. PMN were incubated with different strains in the presence of 3% 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.

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<sub>sodA</sub> 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<sup>8</sup> bacteria of strain WA-314, WA-314<sub>sodA</sub>, or WA-314<sub>sodA</sub>(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 Pey-

er’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 num-

ber of reisolated sodA mutants at day 8 corresponded with a loss of mouse lethality of the mutant. The recomplementation of WA-314<sub>sodA</sub> with the sodA gene in trans [WA-314<sub>sodA</sub>(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<sub>sodA</sub> and WA-314<sub>sodA</sub>(pTB4.1)] showed kanamycin resistance, 2 of 20 tested WA-314<sub>sodA</sub>(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<sup>4</sup> bacteria of WA-314 and WA-314<sub>sodA</sub>, respectively. The wild-type strain colonized and multiplied in spleen and liver rapidly [(6.2 ± 3.2) × 10<sup>5</sup> CFU in the spleen and (1.8 ± 3.5) × 10<sup>5</sup> CFU in the liver on day 2; (8.5 ± 2.6) × 10<sup>5</sup> CFU in the spleen and (2.1 ± 4.1) × 10<sup>5</sup> CFU in the liver on day 4], whereas a mutation of the sodA gene in WA-

314</super><sub>sodA</sub> resulted in a marked decrease of reisolated bacteria [(9.6 ± 6.4) × 10<sup>3</sup> CFU in the spleen and (1.2 ± 4) × 10<sup>3</sup> CFU in the liver on day 2; (1.5 ± 4.5) × 10<sup>2</sup> CFU in the spleen and (8.8 ± 5.2) × 10<sup>2</sup> CFU in the liver on day 4].

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
Evidently, MnSOD is required for the survival and multiplication of yersiniae in spleen and liver tissue. Surprisingly, sodA mutants of Yersinia enterocolitica was intracellular and resistant to phagocytosis as the wild-type strain at 37°C. 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-

<table>
<thead>
<tr>
<th>Day and site</th>
<th>WA-314</th>
<th>WA-314 sodA</th>
<th>WA-314 sodA(pTB3.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>4.2 ± 0.71</td>
<td>3.42 ± 0.4 (NS)</td>
<td>4.11 ± 0.34 (NS)</td>
</tr>
<tr>
<td>PP</td>
<td>4.75 ± 0.56</td>
<td>4.31 ± 0.33 (NS)</td>
<td>4.57 ± 0.86 (NS)</td>
</tr>
<tr>
<td>MLN</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>S</td>
<td>&lt; 1.25</td>
<td>&lt; 1.25</td>
<td>&lt; 1.25</td>
</tr>
</tbody>
</table>

| 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                  |

* PP, Peyer’s patches; SI, small intestinal; MLN, mesenteric lymph nodes; S, spleen; L, liver.

* Results are means from eight animals ± standard deviations. P values were determined by the Student t test. NS, no significant difference; ND, not done.
ularly on day 4, could be due to different active macrophages in the organs. Reconcatenation of the sodA mutant with the sodA gene in trans restored virulence only in part. A similar observation was made for reconcatenation 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 oрогastric 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 mouse gut without generating a systemic infection makes the sodA mutant strain a suitable candidate for a live vaccine able to induce mucosal immunity.

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REFERENCES


