Salmonella typhimurium and its host : host-pathogen cross-talk, immune evasion, and persistence

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Isolation and characterization of *Salmonella enterica* serovar Typhimurium mutants with increased or decreased susceptibility to superoxide

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appendix one

Abstract

One of the early host defense mechanisms against microorganisms like *Salmonella enterica* serovar Typhimurium is the production of superoxide radicals and derivatives (4, 8). *Salmonella*, however, has developed mechanisms to resist this killing mechanism by preventing the production of these toxic compounds or by neutralizing the oxidants. A range of genes necessary for bacterial survival during oxidative stress has been identified but the exact role of many of these genes in survival of *Salmonella* within macrophages is still not completely understood.

We have generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion and selected for those strains with increased susceptibility to redox cycling agents that release superoxide intracellularly and next tested these strains for their ability to replicate intracellularly within RAW264.7 macrophages. By inverse PCR we determined the position in the genome where the MudJ transposon had inserted. Combining the data from several mutants might yield insight into the survival mechanisms of *S. enterica* serovar Typhimurium during superoxide stress encountered outside and within macrophages.

We have isolated 24 mutants that displayed hypersusceptibility to menadione. Of these 13 mutants retained full virulence, whereas 11 mutants were attenuated in vitro in macrophages. We have also isolated 6 mutants that were less susceptible to menadione than the wild-type strain and 4 were attenuated in vitro. We concluded that the fate of replication within macrophages cannot be predicted on the basis of the in vitro susceptibility to the redox cycling agent releasing intracellular superoxide, as some of the superoxide-susceptible mutants appeared to be just as or even more virulent than the wild-type while in the group of superoxide-resistant strains four out of six mutants displayed an attenuated in vitro phenotype. Still, mutational analysis should be helpful to predict the in vitro and in vivo phenotype.

Introduction

Research on mutant strains of *Salmonella* has led to the identification of a number of genes that are necessary for bacterial survival during oxidative stress but the exact contribution of many of these genes for survival of the bacteria within macrophages is still poorly understood (reviewed in (3)). To gain insight into strategies that are used by *Salmonella* to resist superoxide stress and to survive and replicate within macrophages, we generated *S. enterica* serovar Typhimurium mutants by random MudJ transposon insertion and selected for strains with increased susceptibility or resistance to superoxide. These strains were tested for their ability to replicate intracellularly within RAW264.7 macrophages and by inverse PCR we determined the position in the genome where the MudJ transposon had been inserted.

Materials and Methods

Cells and culture conditions. RAW264.7 were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 µg/ml) at 37ºC and 5% CO_2.

Bacterial strains. The bacterial strain used in this study is *S. enterica* serovar Typhimurium 14028s (ATCC). Single colonies of the different strains were grown in Luria-Bertani (LB) medium (10 mg of tryptone, 5 mg of yeast extract, and 10 mg of NaCl/ml) at 37°C while being shaken (225 rpm).

Generation and isolation of *S. enterica* serovar Typhimurium mutants. Mutants were generated by random P22 MudJ transposon insertion in 14028s and selected for superoxide sensitivity by streaking single colonies onto series of M9 agar plates containing 0.5 mg/ml, 0.25 mg/ml, or no menadione (Sigma).

Disk diffusion assay. To determine susceptibility to superoxide, disk diffusion assays were performed as described by Bauer et al. (2). Briefly, overnight cultures of *Salmonellae* were spread on M9 plates. A cotton disk containing 30 mmol menadione was placed in the center. After overnight incubation at 37°C the diameter of the bacterium-free zone was determined (mm) as a measure for susceptibility.

Inverse PCR. To identify the gene in which the MudJ transposon had inserted, the DNA flanking the left end of the transposon was amplified and sequenced by inverse PCR using primers: 5'-CCGGGAGGACATTGGATTAT-3' (sense) and 5'-CGTACTTCAAGTGAATCAATAC-3' (antisense). The PCR product was purified using the QIAquick PCR purification kit (Qiagen) and was sequenced.

Replication within macrophages. RAW264.7 cells (2×10^5) were challenged with *S. enterica* serovar Typhimurium at a 10:1 multiplicity of infection (MOI) as described previously (7). At 0 and 24 hours the cells were lysed in 1 ml milliQ and serial dilutions were made to determine the number of intracellular CFU.

Results and Discussion

By multiple random MudJ transposon insertions in wild-type *S. enterica* serovar Typhimurium 14028s a total of 306 mutants were selected from the series of M9 plates supplemented with 0, 0.25, or 0.5 mg/ml menadione. These mutants were assayed for superoxide sensitivity using disk diffusion assays with the redox cycling agent menadione. Diameters of bacteria-free zones showed that from the mutants tested, 24 showed an increased and six showed a decreased susceptibility to intracellular superoxide induced by menadione when compared to the wild-type strain 14028s (Table 1). These mutants were selected for further research and were next tested for the ability to survive within RAW264.7 macrophages (Fig. 1).

On the basis of the in vitro susceptibility to intracellular superoxide and in vitro replication potential within macrophages the mutants can be divided into four groups: susceptible/attenuated (group 1), susceptible/virulent (group 2), more resistant/attenuated (group 3), and more resistant/virulent (group 4). For some of the mutants in each group, we identified the gene in which the MudJ transposon had inserted by amplifying and sequencing the left flanking DNA by inverse PCR. For reasons at present unknown, we so far succeeded in getting inverse PCR products for 9 mutants (Table 1).

Group 1 consists of 11 mutants of which four (AVD101, MM4, DvD32, and DvD71) are extremely attenuated. For mutant MM4 the MudJ transposon had inserted into *hycE*, the gene encoding a protein that is similar to the *E. coli* large subunit of hydrogenase 3 (part of FHL complex) and for mutant DvD71 in *ybiT*, encoding a putative ATPase component of ABC transporter with duplicated ATPase domain. The other 7 mutants (MM11, MM18, MKS6.2, DvD46, and DvD66) were moderately attenuated compared to the wild-type strain. The position of the MudJ transposon is only known for mutant ND6, MM18 and MM11. The phenotype of mutant ND6 is caused by MudJ transposon insertion in *pabC*, the gene encoding PabC, a 4-amino-4-deoxychorismate lyase (ADC lyase) that converts ADC into p-aminobenzoate (PABA) which is a precursor of folate and component of folic acid (1). Folate cannot be taken up from the environment so endogenous synthesis is essential for growth and cell processes. For MM18 the transposon had inserted into *nupC*, the gene that encodes a protein similar to *E.coli* NUP family nucleoside transport protein. Mutant MM11 contains a MudJ transposon insertion in *sitC* that encodes a *fur* regulated *Salmonella* iron transporter. *SitC* is is part of the *sit* operon encoding an ABC transporter that is involved in the transport of a siderophore-iron complex across the inner membrane and that is regulated by *fur* (5). Fur is the regulator of genes involved in the uptake of iron from the iron-limiting environment such as within a host. Uptake of iron needs to be tightly

regulated since free Fe(II) is toxic to the bacteria as it reacts with hydrogen peroxide in the Fenton reaction. Chelation of intracellular iron is a method to protect the bacteria from killing by hydrogen peroxide (reviewed in (3)).

Table 1. Susceptibility of *S. enterica* serovar Typimurium mutants to menadione

Mutant	bacterial-free zone (mm)	inactivated gene	in vitro phenotype
14028s	26 ± 1		V[b]
ND102	36 ± 1	nd[a]	V
ND103	30 ± 1	nd	V
MM3	37 ± 1	*yhgE*	V
MKS2	34 ± 1	*kup*	V
MKS6.2	36 ± 1	nd	V
DvD1	29 ± 2	nd	V
DvD50	30 ± 2	*ybdM*	V
DvD51	31 ± 3	nd	V
DvD54	31 ± 1	nd	V
DvD69	31 ± 1	nd	V
DvD70	32 ± 1	nd	V
M30	34 ± 2	nd	V
MdR2	37 ± 2	nd	V
ND6	32 ± 2	*pabC*	A[c]
AVD101	31 ± 1	*pnp*	A
ND104	34 ± 2	nd	A
MM11	31 ± 1	*sitC*	A
MM18	32 ± 1	*nupC*	A
MM4	40 ± 1	*hycE*	A
MKS6.1	40 ± 2	nd	A
DvD32	29 ± 1	nd	A
DvD46	29 ± 2	nd	A
DvD66	29 ± 1	nd	A
DvD71	30 ± 1	*ybiT*	A
ND23	21 ± 1	nd	V
MdR23	19 ± 1	nd	V
ND32	20 ± 1	nd	A
ND34	20 ± 1	nd	A
ND37	20 ± 1	nd	A
ND38	21 ± 1	nd	A

[a]nd, not determined
[b]V, virulent
[c]A, attenuated

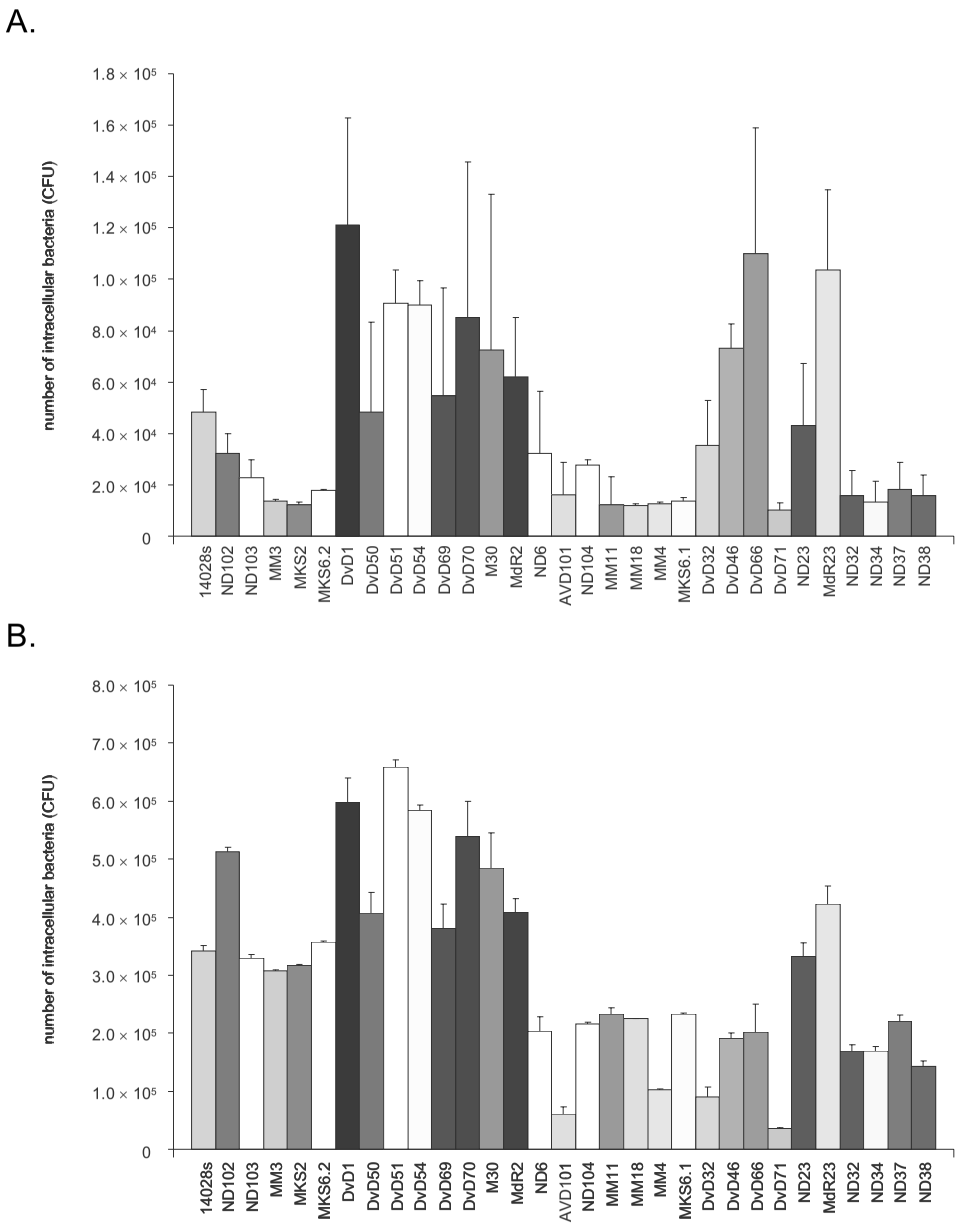

Figure 1. Number of intracellular bacteria in RAW264.7 murine macrophage-like cells infected with wild-type *S. enterica* serovar typhimurium 14028s and several random MudJ transposon insertion mutants with increased or decreased susceptibility to intracellular superoxide induced by menadione. At 0 (A) and 24 h (B) after infection of the cells the numbers of intracellular bacteria were determined bacteriologically. Average data ± SEM from 2-6 in vitro infection experiments is shown.

Seven strains in group 2 were as virulent as the wild-type (ND103, ND104, MKS2, MKS6.2, DvD50, DvD69, MdR2) and six mutants even seemed to be more virulent (ND102, DvD1, DvD51, DvD54, DvD70, M30). Of these mutants the MudJ transposon position is only known for MKS2 in which *kup*, encoding a protein similar to *E. coli* low affinity potassium transport system is inactivated and for DvD50 in which the putative transcriptional regulator of *ybdN* (= putative 3'-phosphoadenosine 5'-phosphosulphate sulfotransferase (PAPS reductase)/FAD synthetase encoded by *ybdM* is inactivated.

Group 3 contains four mutants (ND32, ND34, ND37, and ND38) that are moderately attenuated in RAW264.7 macrophages. For neither of these mutants we were able to get an inverse PCR product, so the position of the MudJ transposon in the genome is unknown.

Group 4 only consists of two mutants (ND23 and MdR23) that display an in vitro phenotype that is comparable to the wild-type strain. As for the mutants of group three no inverse PCR products were obtained.

The main conclusion that can be drawn from the data presented here is that the in vitro phenotype cannot be predicted from the in vitro susceptibility to intracellular superoxide generated intracellularly by the redox cycling agent menadione. The in vitro phenotypes of the selected mutants were all completely different and this strongly suggests that factors other than susceptibility to superoxide play a role in in vitro virulence. With respect to prediction of virulence in mice, the situation becomes even more complex since strain AVD101, as described in Chapter 5, is extremely susceptible to intracellular superoxide, is attenuated in macrophages, but is more virulent than the wild-type strain upon subcutaneous infection of C3H/HeN mice (6). The genetic defects caused by the MudJ transposon insertion leading to increased susceptibility to superoxide also appeared to be very diverse and did not directly explain the observed in vitro susceptibility to superoxide nor the in vitro intracellular phenotype.

To be able to predict the in vitro and in vivo phenotype on the basis of the in vitro susceptibility to superoxide in the future, more research is needed. Many more mutants need to be tested in in vitro infection experiments and for all mutants the inactivated gene should be identified. In addition, these mutants need to be tested in vivo as well, since even the in vivo phenotype cannot always be predicted from the intracellular behavior.

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