

NOTES

Relevance of DNA Alkylation Damage Repair Systems in *Salmonella enterica* Virulence[∇]

Gerard Álvarez, Susana Campoy, Denis A. Spricigo, Laura Teixidó, Pilar Cortés, and Jordi Barbé*

Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

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Systematic inactivation of pathways involved in DNA alkylation damage repair demonstrated that inactivation of the *ada*, *ogt*, *tag*, *uvrA*, and *mfd* genes is required to detect a *Salmonella enterica* virulence decrease. Furthermore, the fitness of *S. enterica*, defective in these genes, is lowered only when the bacterium is orally, but not intraperitoneally, inoculated.

Bacteria are exposed to a wide variety of DNA-injuring agents, including alkylating agents, and their effects have been well studied (12, 17, 18). The repair of alkylated DNA in bacterial cells has been described mainly in *Escherichia coli* (12, 18), which presents the following two different mechanisms to eliminate alkyl radicals from its DNA: (i) the alkyl-induced expression of genes encoding the necessary repair enzymes (12, 17) and (ii) the constitutive synthesis of such proteins (9).

Many bacterial species have a genetic network, known as the adaptive response, which functions to repair alkyl lesions in the bacterial DNA (18, 19) and is regulated by the Ada protein, which also possesses methyltransferase activity (18). Transfer of a methyl from DNA-methylated phosphates to the Cys-37 residue of Ada (17) triggers a conformational change in this protein that converts it into a positive transcriptional regulator. Once activated, Ada stimulates the expression of its own transcriptional unit, including the *alkB* gene, which encodes an *N*¹-meA-DNA dioxygenase, and the *alkA* and *aidB* genes, which encode a *N*³-meA-DNA-glycosylase and a flavin-containing DNA binding protein, respectively (9).

Bacteria also possess the following two additional enzymes involved in the repair of alkylated DNA: Ogt (*O*⁶-meG-DNA methyltransferase) and Tag (*N*³-meA-DNA glycosylase) (9). Expression of the *ogt* and *tag* genes is constitutive and does not depend on the presence of DNA alkylation (9). Despite the importance of alkylated DNA repair, there is very little information concerning the relevance of this process in pathogenic bacteria.

To determine the implications of DNA alkylation damage repair in *Salmonella enterica* virulence, mutants defective in the *ada* gene, or genes under its control (*alkA*, *alkB* and *aidB*), or mutants defective in the *ogt* and *tag* genes were constructed using the one-step PCR-based gene replacement method (6).

As expected, survival assays showed that each of these single mutants was more sensitive than the wild-type strain to several alkylating agents, including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl methanesulfonate (MMS), and diethyl sulfate (DES) (data not shown). Furthermore, competition assays between each of these mutants and the wild-type strain, carried out as reported previously (3, 4), demonstrated that the inactivation of neither *ada*, *ogt*, nor *tag* had any effect on the virulence of *S. enterica* cells, regardless of whether the bacteria were inoculated orally or intraperitoneally (i.p.) in BALB/c mice (data not shown).

In light of these results, *S. enterica* strains carrying all possible double combinations of the *ada*, *tag*, and *ogt* mutations were constructed. These double mutants were much more sensitive to MMS, MNNG, or DES than either the wild-type or the single-mutant strains (data not shown). Nevertheless, their virulence was not diminished when inoculated either i.p. or orally, as shown by the competitive index (CI) determined with competition assays (data not shown). An *S. enterica ada ogt tag* triple mutant was subsequently constructed, and although it was extremely sensitive to the alkylating agents (Fig. 1), its virulence, when inoculated either orally (Fig. 2) or i.p. (data not shown), was not diminished compared with that of the wild-type strain. Likewise, the virulence of an *S. enterica ada ogt tag alkA alkB aidB* mutant, i.e., defective in all genes specifically involved in DNA alkylation damage repair (9, 17), was not affected (Fig. 2). These results show that the combined inactivation of the *ada* network and the *ogt* and *tag* genes does not decrease the fitness of *S. enterica* cells during the infective process.

The nucleotide excision repair system (associated with the UvrABC excinuclease) is also involved in the repair of alkylated DNA (25). Likewise, the *mfd* pathway has been shown to participate in the repair of DNA lesions that block bacterial transcription and prevent RNA polymerase elongation, both of which being induced by alkylating agents and by other injurious compounds (16, 20). Accordingly, a mutation in the *uvrA* gene, the *mfd* gene, or both was introduced into the *S. enterica ada ogt tag* mutant. Survival assays showed that only the presence of both mutations (*uvrA* and *mfd*) increased the sensitivity of the

* Corresponding author. Mailing address: Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Phone: 34-93-581-1837. Fax: 34-93-581-2387. E-mail: jordi.barbe@uab.cat.

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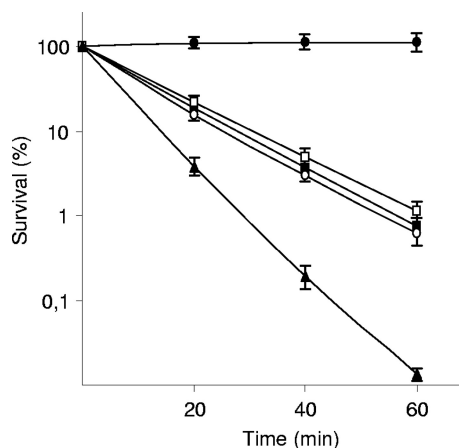


FIG. 1. Sensitivity of *S. enterica* wild-type (●), *ada tag ogt* (■), *ada tag ogt mfd* (□), *ada tag ogt uvrA* (○), and *ada tag ogt uvrA mfd* (▲) strains to 30 mM DES. Sensitivity is expressed as the survival rate of exponential-phase cells treated with the corresponding alkylating agent. The data are the average results from at least three independent experiments.

cells to DES (Fig. 1). Similarly, results obtained from competitive experiments indicated that the presence of either the *uvrA* or the *mfd* mutation in the *S. enterica ada ogt tag* mutant did not lower its CI compared to that of the wild-type strain when inoculated either i.p. (data not shown) or orally (Fig. 2). However, the addition of both mutations (*uvrA* and *mfd*) to the triple (*ada ogt tag*) mutant, generating strain UA1869, decreased the CI by about 200-fold (Fig. 2). However, this decrease in virulence was apparent only when the bacteria were inoculated orally; the fitness of the i.p.-inoculated UA1869 mutant was the same as that of the wild type (data not shown). Moreover, there was no alteration in the virulence of either the

S. enterica uvrA or the *S. enterica mfd* single mutant or in that of the *S. enterica uvrA mfd* double mutant when the bacteria were administered orally or i.p. (data not shown).

Several *in vitro* tests were carried out to understand the UA1869 strain fitness reduction. The growth kinetics of this strain under oxic and anoxic conditions were found to be the same as those of the wild-type strain (data not shown). Therefore, the competitive defect of orally inoculated UA1869 cells was not due to any putative DNA-damaging effect of endogenous alkylating agents generated in bacterial cells during anaerobic respiration or metabolically (23). In addition, UA1869 displayed the same sensitivity to pH and bile salts as the wild-type strain (data not shown).

Taken together, these data suggest that the effect of DNA alkylation damage on the *S. enterica* infective process is not particularly significant, since despite the high sensitivity of *ada ogt tag alkA alkB aidB* cells to alkylating agents, there is no decrease in their virulence (Fig. 2). However, our results suggest that DNA alkylation damage in *S. enterica* must occur before the orally ingested bacteria are disseminated in the bloodstream, since the virulence of i.p.-inoculated strain UA1869 was not affected. Indeed, the production of alkylating compounds in the mammalian gut has been reported (15, 24).

Furthermore, it could be argued that spontaneous mutation rates are higher in *S. enterica* cells harboring several inactivated DNA repair systems, such that their fitness during the infective process is altered. This possibility is unlikely, since it has been widely described that there is no decrease in the virulence of *S. enterica* hypermutable phenotypes (5, 26).

Although *Salmonella* lives primarily in the intestinal tracts of animals, dissemination of this pathogen also requires that it be able to survive outside the host. In fact, these bacteria are readily isolated from environmental sources, e.g., water and soil contaminated with *Salmonella*-containing feces (7), due to

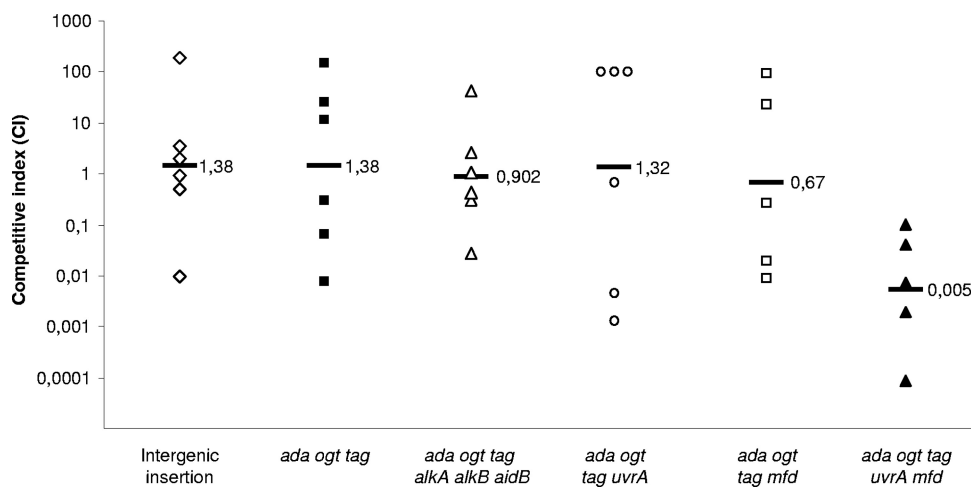


FIG. 2. *In vivo* competition infection experiments. The *S. enterica* wild-type strain was mixed with each of the mutant derivatives at a ratio of 1:1 before being used in the oral infection of BALB/c mice. The competitive index (CI) was calculated as the ratio of the mutant to the wild-type strain in the output (bacteria recovered from the host after infection) divided by that ratio in the input (initial inoculum). Each symbol indicates the CI value for one mouse. Bars represent the geometric mean results, with their values written alongside them. A CI of <1 indicates a competitive disadvantage of the mutant compared to that of the wild-type strain. The CI for a wild-type *S. enterica* derivative strain containing only a chloramphenicol resistance cassette inserted in the chromosomal coordinate 16088, corresponding to an intergenic region and with no effect on virulence (data not shown), was used as the control. Statistical analysis was carried out using a two-tailed *t* test, which unequivocally demonstrated that the virulence of *S. enterica ada ogt tag uvrA mfd* was significantly lower than that of the wild type ($P = 0.00000096$).

the application of manure or sewage sludge as fertilizers on agricultural fields (8, 10, 13, 19, 22). Moreover, the survival of these contaminating pathogens, such as *S. enterica*, may range from several days to a few months, depending on the environmental conditions (13, 14, 21). Besides its protective role against DNA alkylation damage generated during the intestinal steps of oral infection (Fig. 2), the extensive alkylation repair system of *Salmonella* may be involved in the long-term selective pressure to increase the survival of these bacteria outside the infected animal and, thus, in enabling them to overcome the potentially massive DNA injuries caused by alkylating agents present in the environment (1, 2, 11).

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