

Detoxification of nitric oxide by the flavorubredoxin of *Salmonella enterica* serovar Typhimurium

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Abstract

Salmonella possesses multiple enzymes that utilize NO as a substrate, and could therefore contribute to the organism's ability to resist nitrosative killing by macrophages. Flavorubredoxin is an oxygen-sensitive enzyme that reduces NO to nitrous oxide. The *Salmonella enterica* serovar Typhimurium *norV* gene encoding flavorubredoxin was disrupted and the NO sensitivity of the mutant was determined. The *norV* mutant showed a greater sensitivity to NO than wild-type *S. Typhimurium*, but did recover growth after a transient inhibition. The mutant phenotype suggests that multiple enzymes are employed by *S. Typhimurium* to detoxify NO under anaerobic conditions, one of which is flavorubredoxin.

Introduction

During infection, cells of pathogenic *Salmonella* species are typically internalized into free macrophages and the fixed macrophages of the liver and spleen. Here, bacterial cells are enclosed in the phagolysosome and are exposed to oxygen and nitrogen radicals that are included and derived from the superoxide and NO synthesized by the phagocyte oxidase and inducible nitric oxide synthase respectively [1]. Oxidative and nitrosative killing in the macrophage does not provide complete protection against infection, in part because of the ability of *Salmonella* to interfere with phagocyte oxidase and inducible nitric oxide synthase activities, to detoxify oxygen and nitrogen radicals and to reverse some of their harmful effects [1,2]. Several enzymes that contribute to resistance to oxidative and nitrosative killing have been identified in *Salmonella* serotype (ser.) Typhimurium (or *S. Typhimurium*) and related organisms [1]. The recently described flavorubredoxin of *Escherichia coli* is an A-type flavoprotein that reduces NO, probably to nitrous oxide, in the absence of oxygen or under microoxic conditions [3,4]. The *norVW* genes encoding the flavorubredoxin and its associated oxidoreductase are up-regulated in *S. Typhimurium* after macrophage internalization [5]. Here we report the construction and preliminary characterization of a *norV* mutant of *S. Typhimurium*, and show that the flavorubredoxin contributes to NO resistance *in vitro*.

Construction and characterization of a *norV* mutant of *S. enterica*

The *norV* gene of *S. Typhimurium* LT2 was replaced with a chloramphenicol resistance cassette using the method of

Datsenko and Wanner [6]. The flavorubredoxin is inactivated by oxygen, and is supposed to provide physiological protection against NO only at low or zero oxygen concentrations [3,7]. For this reason, the response of the *norV* mutant to exogenous NO was investigated in cultures growing under anoxic conditions. *S. Typhimurium* LT2 and the *norV::cat* mutant were cultured in sealed 3 ml cuvettes in a defined medium containing glucose as the carbon and energy source. The cuvettes were sparged with nitrogen before inoculation. In the early exponential phase (A_{600} 0.1), aqueous nitric oxide was added to a final concentration of 40 μ M, and growth was continued (Figure 1A).

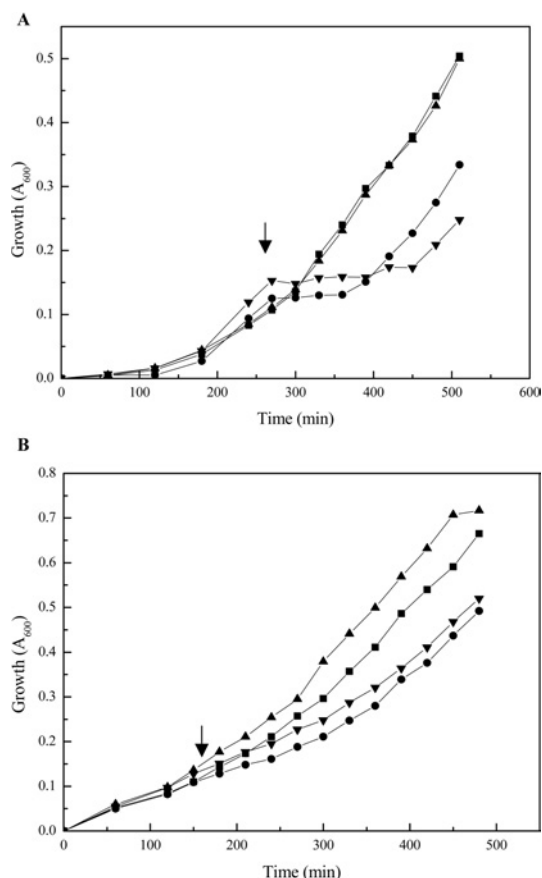
The *norV* mutation had no significant effect on anaerobic growth in glucose minimal medium, since similar growth kinetics were observed for the wild-type and mutant strains in the absence of NO (Figure 1A). After the addition of 40 μ M NO, the growth of both strains was immediately and transiently inhibited. The wild-type strain recovered approx. 100 min after NO exposure, and resumed growth at a rate similar to that seen in the control culture not exposed to NO. However, the *norV::cat* mutant reproducibly required an additional 80 min for recovery, before resuming growth at a rate similar to the control. The mutant phenotype demonstrates that the *norV* gene product contributes to NO resistance under these conditions, but is probably not the sole determinant of resistance. In *E. coli*, the growth of a *norV* mutant is indistinguishable from wild-type when cultured anaerobically in rich medium, and exposed to 240 ppm NO in an N₂ atmosphere [3]. As is shown here for *S. Typhimurium*, sensitivity to NO was observed when the *E. coli* mutant was grown in a glucose minimal medium, in which growth requires the activity of NO-sensitive enzymes containing Fe-S clusters [3]. *E. coli* *norV* mutants have also been reported to be sensitive to NO and to nitroprusside during anaerobic growth on rich media [8]. The preliminary

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Figure 1 | Anaerobic growth of *S. Typhimurium* and a *norV::cat* mutant in (A) minimal glucose medium and (B) minimal glycerol nitrate medium

Duplicate cultures of *S. Typhimurium* LT2 (■, ●) and the *norV::cat* mutant (▲, ▼) were grown in the absence (■, ▲) and presence (●, ▼) of NO. NO was added to a final concentration of 40 μ M at the time indicated by the arrow. The data shown are representative of three separate experiments.



characterization of the *S. Typhimurium norV* mutant implies that the flavorubredoxins of *S. Typhimurium* and *E. coli* have similar physiological roles.

Other enzymes which could detoxify NO in *Salmonella*

The fact that the *norV* mutant eventually recovers from NO exposure implies that there may be other enzymes capable of removing NO during anaerobic growth. Indeed, *S. Typhimurium* possesses at least two additional enzymes that utilize NO as a substrate and may have roles in NO

resistance. The periplasmic cytochrome *c* nitrite reductase, NrfA, catalyses the six-electron reduction of nitrite to ammonia. It has recently been shown that the *E. coli* NrfA may also be able to reduce NO, and thereby protect the cell against exogenous NO [9]. The *S. Typhimurium* genome includes *nrf* genes that potentially encode NrfA activity, and we have confirmed the presence of this activity in cultures grown anaerobically on glycerol nitrate medium. Intriguingly, under these growth conditions there is minimal inhibition of growth of wild-type or the *norV* mutant strains by NO (Figure 1B).

The bacterial flavohaemoglobin oxidizes NO to nitrate in the presence of oxygen and reduces NO to nitrous oxide in the absence of oxygen [10]. It has been suggested that in *E. coli* the flavohaemoglobin only provides physiological protection against NO in aerobic cultures [11]. Crawford and Goldberg [12] showed increased sensitivity of an *bmp* mutant of *S. Typhimurium* to acidified nitrite and nitrosothiols under both aerobic and anaerobic conditions. The flavohaemoglobin of *S. Typhimurium* has been shown to have a role in protecting against macrophage killing [13].

Salmonella serovars contain multiple enzymes that are capable of detoxifying NO under both oxic and anoxic conditions. It will be of interest to evaluate and compare the individual roles of these enzymes in providing resistance against NO under different growth conditions, both in pure culture and during macrophage infection.

References

- Vazquez-Torres, A. and Fang, F.C. (2001) Trends Microbiol. **9**, 29–33
- Chakravorty, D., Hansen-Wester, I. and Hensel, M. (2002) J. Exp. Med. **195**, 1155–1166
- Gardner, A.M., Helmick, R.A. and Gardner, P.R. (2002) J. Biol. Chem. **277**, 8172–8177
- Gomes, C.M., Giuffre, A., Forte, E., Vicente, J.B., Saraiva, L.M., Brunori, M. and Teixeira, M. (2002) J. Biol. Chem. **277**, 25273–25276
- Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. and Hinton, J.C.D. (2003) Mol. Microbiol. **47**, 103–118
- Datsenko, K.A. and Wanner, B.L. (2000) Proc. Natl. Acad. Sci. U.S.A. **97**, 6640–6645
- Silaghi-Dumitrescu, R., Coulter, E.D., Das, A., Ljungdahl, L.G., Jameson, G.N., Huynh, B.H. and Kurtz, Jr, D.M. (2003) Biochemistry **42**, 2806–2815
- Hutchings, M.I., Mandhana, N. and Spiro, S. (2002) J. Bacteriol. **184**, 4640–4643
- Poock, S.R., Leach, E.R., Moir, J.W.B., Cole, J.A. and Richardson, D.J. (2002) J. Biol. Chem. **277**, 23664–23669
- Wu, G., Wainwright, L.M. and Poole, R.K. (2003) Adv. Microb. Physiol. **47**, 255–310
- Gardner, A.M. and Gardner, P.R. (2002) J. Biol. Chem. **277**, 8166–8171
- Crawford, M.J. and Goldberg, D.E. (1998) J. Biol. Chem. **273**, 12543–12547
- Stevanin, T.M., Poole, R.K., Demoncheaux, E.A. and Read, R.C. (2002) Infect. Immun. **70**, 4399–4405

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