COVER SHEET

TITLE: Function of a Nematode Intestine Localization Protein in Nematode Colonizing Xenorhabdus nematophila

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ABSTRACT

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nematode *Steinernema carpocapsae*. This association is species specific; *S. carpocapsae* can
only be colonized by *X. nematophila*, and not by other *Xenorhabdus* species. A protein called
NilB (nematode intestine localization protein B) is essential for *X. nematophila* to colonize
nematode epithelial cells. NilB function is as yet unknown, but several experiments have raised
the possibility that it functions as a metal transporter, with an increase in its expression being
observed upon addition of manganese. Furthermore, our data suggest NilB confers resistance
to manganese toxicity. Identification of nematode factors that interact with NilB using cross-
linking and co-precipitation is also in progress.

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*Senior Honors Thesis*
*College of Agriculture & Life Sciences Honors Program*
*Honors in the Major Microbiology*
Abstract

The bacterium *Xenorhabdus nematophila* forms a mutualistic relationship with a nematode *Steinernema carpocapsae*. This association is species specific; *S. carpocapsae* can only be colonized by *X. nematophila*, and not by other *Xenorhabdus* species. A protein called NilB (nematode intestine localization protein B) is essential for *X. nematophila* to colonize nematode epithelial cells. NilB function is as yet unknown, but several experiments have raised the possibility that it functions as a metal transporter, with an increase in its expression being observed upon addition of manganese. Furthermore, our data suggest NilB confers resistance to manganese toxicity. Identification of nematode factors that interact with NilB using cross-linking and co-precipitation is also in progress.
Introduction

*Xenorhabdus* is a Gram-negative bacterium that forms a symbiotic relationship with a nematode *Steinernema* (3). *Xenorhabdus* not only forms mutualistic symbiosis with the nematode but together with the nematode they form parasitic symbiosis with the larvae of the insect (typically Lepidoptera, lab insect model: *Manduca sexta*) (3). The bacterium colonizes the intestine of the nematode (Figure 1) and together they infect and kill the insect larvae for reproduction (Figure 2). The mutualistic benefits include the nematode providing the bacterium with entry to the insect host while the bacterium kills the insect and yields increased reproductive fitness to the nematode (3).

![Image of nematodes and bacteria](image-url)

**Figure 1** – Fluorescence microscopy image showing GFP-expressing *Xenorhabdus bovienii* colonized within *Steinernema puntavense* (courtesy K. Murfin). In the nematode intestine, the bacteria colonize the anterior portion of the intestine.
Mutualism (with nematode) | Pathogenesis (with insect)
---|---
IJs emerge from insect cadaver | IJ infects insect host

IJ development and colonization | Bacteria released into insect haemolymph

Nematode growth and development using bacterial biomass as food | Bacterial infection kills the insect host

Figure 2 – Bacterium *Xenorhabdus* life cycle in association with nematode *Steinernema*. The bacteria colonize and grow in the infective juvenile (IJ) stage of the nematode and the nematodes reproduce within the insect cadaver (1).

In the association between *Steinernema carpocapsae* and *Xenorhabdus nematophila*, NilB is one of the nematode intestine localization proteins (NilA, NilB, and NilC), which determine the host specificity and are essential for the bacterium to colonize within the nematode’s intestine (2). *nilA and nilB* are transcribed together from a promoter upstream of *nilA*, while *nilC* is transcribed separately in the opposite direction (Figure 3). However, the exact functions of nematode intestine localization proteins are unknown (2).
NilB is an outer trans-membrane protein with exposed surface loops, and has its N-terminal region in the periplasm (Figure 3) (4). Such orientation is consistent with the model that it may be a metabolite transporter. A preliminary experiment had shown that NilB is more highly expressed when manganese is added, but not when other metals are added (Figure 4) (J.M. Chaston and H. Goodrich-Blair, unpublished). Although all cells require manganese for essential cell functions, sensitivity to manganese is not a rare phenotype among bacterial species. Toxicity of manganese is thought to be due to its ability to cause protein oxidation or to replace functional metal ions in essential enzymes (11). Bacillus stearothermophilus cells grew slower as manganese concentration was increased (7) and Escherichia coli cells regulate intracellular manganese levels as excess manganese is toxic to them (8). Moreover, a recent study has revealed a manganese exporting protein (or bacterial manganese resistance factor, MntX) that contributes to manganese tolerance in the human pathogen Neisseria meningitidis (6). The potential link between NilB and manganese raised the possibility that X. nematophila NilB may confer resistance to manganese.

NilR, a transcription factor, synergistically acts with the global regulator Lrp to repress nilAB, and nilC transcription (Figure 3) (5). A study on NilR had demonstrated that deleting nilR significantly increases the expression of NilB by ~16-fold compared to the wild type (5). Therefore, nilR mutant strain (ΔnilR, HGB1102) was used along with ΔnilABC (HGB777) and ΔnilABC ΔnilR (HGB1251) strains to study NilB’s role in response to manganese.

Since manganese toxicity may be due to manganese binding to iron binding sites in essential enzymes to cause malfunctions, the manganese to iron ratios may be the factor that decides the degree of toxicity of manganese. Dipyridyl is an organic compound that chelates iron, and thus can be used to create iron-limiting conditions (10). A study had shown that an
iron-dependent protein expression (*X. nematophila* haemolysin proteins, XhI BA) changes significantly when 2,2-dipyridyl is added, and supplying with additional iron restores the expression to the wild type level (9). Therefore, dipyridyl can be a useful compound to control iron levels, which will enable further studies on manganese toxicity.

**Figure 3** – Illustration of predicted localization of NilA, B, and C. NilB, as an outer-surface localized protein, may have a function of transporting out manganese. Lrp and NilR synergistically repress *nilAB* and *nilC* transcription (3). Abbreviations: nt – nucleotide, aa – amino acid.
Figure 4 – β-galactosidase assay results showing nilB-lacZ expression in X. nematophila ΔnilR grown in minimal medium supplemented with different metals at approximately 30 μM (J.M. Chaston, and H. Goodrich-Blair, unpublished). **nilAB500-lacZ** fusion strain (lacZ gene fused 500 base pairs downstream of nilAB promoter to allow use of β-galactosidase assay in measuring NilB expression) in ΔnilR background was used. Manganese dramatically increases NilB expression in contrast to other metals. n=3 replicates. Error bars represent the standard deviation of the replicates.
Methods and Materials

Strains

Four *X. nematophila* strains were used for manganese study including wild type (HGB800), *nilR* mutant (HGB1102), Δ*nilABC* (HGB777), and a double mutant (Δ*nilR* Δ*nilABC*, HGB1251) (Table 1). The mutant strains were created previously in our lab for use in related studies (2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Control, has wild type <em>nilR</em> and <em>nilABC</em> genes</td>
</tr>
<tr>
<td>Δ<em>nilR</em></td>
<td>NilR represses NiIIB expression. This strain, with the deletion of <em>nilR</em> gene, has higher NiIIB expression than wild type.</td>
</tr>
<tr>
<td>Δ<em>nilABC</em></td>
<td>Deletion of <em>nilABC</em> genes in this strain means NiIIB protein will not be present. Such absence of NiIIB in this strain allows determining the importance of NiIIB on manganese resistance</td>
</tr>
<tr>
<td>Δ<em>nilR</em> Δ<em>nilABC</em></td>
<td>NilR may also be repressing genes other than <em>nilABC</em> in the bacteria. This double mutant strain makes sure that it is really the NiIIB protein that is responsible for manganese resistance.</td>
</tr>
</tbody>
</table>

Table 1 – List of strains used in the present study and the purposes of using each strain.

Procedures and Materials

Each strain was grown for 22 hours at 30°C in 2 ml dark LB (Lysogeny Broth), and then was washed with minimal medium. A 2 μl sample of the washed cells was inoculated in total volume of 200 μl of minimal medium (with/without 50μM MnCl₂) in 96-well plates. Over the next 96 hours, optical density (O.D.) at the wavelength of 600 nm was taken every 30 minutes, creating a growth curve. Then maximum O.D. was determined and maximum velocity (growth rate) was calculated from the growth curve. The maximum O.D. data were used to calculate percent decrease from growth in the no manganese condition relative to 50 μM manganese.
condition. These data were statistically analyzed using ANOVA multiple comparison with Tukey's post-test (SAS statistical package, SAS Institute Inc., Cary, NC). Random effects based on variability of dates of each experiment were incorporated into the analysis.

The minimal medium was made with appropriate salts and concentrations for *X. nematophila* at pH 7.0 (Table 2). 1 M MnCl$_2$ liquid stock was diluted to 10 mM first in deionized distilled water (ddw) before being added to minimal medium as higher MnCl$_2$ concentration (>10 mM) caused precipitation upon addition into the medium.

For the experiment with iron limiting conditions, 2,2-bipyridyl (dipyridyl) was dissolved in ethanol in 100 mM. The 100 mM dipyridyl stock was then added to minimal medium, with final concentration of 10 µM. For making iron rich conditions, freshly made 10 mM FeSO$_4$ solution was added to minimal medium to the final concentration of 500 µM.

<table>
<thead>
<tr>
<th>Salts:</th>
<th><strong>SL-6 stock:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41 mM nicotinic acid (<em>X. nematophila</em> is an auxotroph for this vitamin)</td>
<td>500 mg/L MnCl$_2$•4H$_2$O</td>
</tr>
<tr>
<td>22 mM KH$_2$PO$_4$</td>
<td>300 mg/L H$_3$BO$_3$</td>
</tr>
<tr>
<td>40.2 mM K$_2$HPO$_4$</td>
<td>200 mg/L CoCl$_2$•6H$_2$O</td>
</tr>
<tr>
<td>15.1 mM (NH$_4$)$_2$SO$_4$</td>
<td>100 mg/L ZnSO$_4$•7H$_2$O</td>
</tr>
<tr>
<td>1-2 pellets of NaOH (final pH = 7.0)</td>
<td>30 mg/L Na$_2$MoO$_4$•2H$_2$O</td>
</tr>
<tr>
<td><strong>Supplements:</strong></td>
<td>20 mg/L NiCl$_2$•6H$_2$O</td>
</tr>
<tr>
<td>10 ml/L of SL-4 salts*</td>
<td>10 mg/L CuCl$_2$•2H$_2$O</td>
</tr>
<tr>
<td>0.91% glucose</td>
<td><strong>SL-4 trace elements stock:</strong></td>
</tr>
<tr>
<td>0.25mM MgCl$_2$•(H$_2$O)$_6$</td>
<td>0.5 g/L EDTA</td>
</tr>
</tbody>
</table>

Table 2 – List of salt ingredients and their concentrations in *X. nematophila* minimal medium. *SL-6 stock* is used to make *SL-4 trace elements stock*, which is then used to make the supplements that are added to the main salts. The final pH of the medium should be 7.0. *SL-4 salts reference: Atlas, R.M. 1997. Handbook of microbiological media, 2nd ed. CRC Press, Boca Raton, FL.*
Results

Manganese sensitivity was calculated based on the percent decrease in maximum growth (maximum O.D.) from each strain's growth in minimal medium without any treatment to growth in minimal medium with 50 μM MnCl₂ (Figure 5). The average percent decrease of maximum growth for ΔnilR strain was 13%, 30% for ΔnilABC strain, 20% for wild type, and 43% for ΔnilR ΔnilABC strain (Figure 5). A significant difference was observed between the ΔnilR strain and the ΔnilR ΔnilABC strain (p < 0.01). In addition, nearly significant differences (p=0.08) were observed between wild type and the double mutant ΔnilR ΔnilABC and between ΔnilR and ΔnilABC.

The average growth rate (maximum velocity) of no manganese treatment vs. 50 μM MnCl₂ treatment for ΔnilR strain was 0.78 vs. 0.70, with 0.53 vs. 0.39 for ΔnilABC strain, 0.67 vs. 0.64 for wild type, and 0.64 vs. 0.41 for ΔnilR ΔnilABC strain (Figure 6).

Among dipyriddylic (DP)/iron/manganese treatments on wild type X. nematophila, treatment of only 500 μM FeSO₄ without (curve E) and with (curve D) 10 μM DP resulted in the highest growths (Figure 7). Addition of 50 μM MnCl₂ with 10μM DP (curve B) gave lowest growth, but adding 500 μM FeSO₄ with those treatments (curve C) was able to recover growth to the level of the control (Figure 7). DP concentration of 10 μM (curve A) did not cause less growth than the control (Figure 7).
Figure 5 – Relative sensitivity of each X. nematophila mutant strains to manganese in minimal media. The data represents percent decrease in maximum optical density from samples with no manganese added to samples with 50 μM manganese added. Higher ‘percent decrease’ values indicate higher manganese sensitivity and lower manganese resistance. n=4, error bars represent the standard error of the mean.

Figure 6 – Growth rate of each strain in both no manganese and 50 μM manganese condition represented by maximum velocity (Vmax) values. These rates were calculated from growth curves plotted using O.D.600 measurements made every 30 minutes for 96 hours in minimal media containing given manganese conditions. n=3, error bars represent the standard error of the mean.
Figure 7 – Growth curves of wild type *X. nematophila* strain treated with dipyridyl (DP)/iron/manganese in minimal media. The legend lists the concentration of DP/iron/manganese added for each curve. Control curve was grown without any treatment in the minimal medium. Time points were taken at 30 minute intervals. n=3 replicates, error bars represent standard error of the mean.
Discussion

Based on the results from manganese sensitivity experiment, the presence of *nilABC* determined the level of *X. nematophila*'s resistance to manganese. Both mutant strains lacking *nilABC* showed greater percent decrease in maximum growth when treated with 50 μM MnCl₂ (30% for ΔnilABC and 43% for ΔnilR ΔnilABC) than strains with wild type *nilABC* loci (13% for ΔnilR and 20% for wild type) (Figure 5). Moreover, the growth rates (maximum velocity) of ΔnilABC containing strains dropped in greater portions, consistent with the trends shown in the percent decrease in maximum growth (Figure 6). ΔnilABC strain's growth rate dropped by 0.14 and ΔnilR ΔnilABC strain's dropped by 0.23 while both ΔnilR strain and the wild type had less than 0.08 decline in their growth rates (Figure 6). Hence, these results imply that NilABC is necessary for manganese resistance. Since NilB is the outer membrane protein as diagramed in Figure 3, NilB is likely to be the crucial protein among NilABC to confer resistance to manganese potentially by transporting excess manganese out of the cell.

The ΔnilR strain played a major role in demonstrating the necessity of NilB in manganese resistance. Although the difference of percent decrease in maximum growth between ΔnilR strain and the wild type are not significantly different according to the statistical analysis, minor difference (~7%) was still observed between those strains (13% for ΔnilR strain to 20% for wild type) (Figure 5). This minor difference is also consistent with the trend that more NilB provides more resistance to manganese. As ΔnilR strain has highest level of NilB among other strains (5), the significant differences of observed between ΔnilR strain and strains lacking ΔnilABC loci gave a strong indication that NilB plays a crucial role in *X. nematophila*'s manganese resistance.
The wild type strain was extremely sensitive to 50 μM MnCl₂ in dipyridyl-induced iron-limiting condition, but adding 500 μM FeSO₄ in that condition was able to rescue growth to the normal level (Figure 7). This implies that the manganese to iron ratio determines manganese sensitivity, and it supports the idea that excess manganese can be toxic by replacing functional metal ions in essential enzymes (11).

Overall, the experimental design of this study was successful in achieving valid and consistent data. Using O.D. measurements was most suitable for studying manganese sensitivity to growth. Other possible methods such as measuring zone of inhibition after placing a manganese soaked disk or serial dilution plating on manganese containing plates did not work as well. In minimal medium, growth time of less than 72 hours was not enough to represent a solid growth trend as there were longer lags than in LB. Growth time of 96 hours was enough to obtain a valid growth curve. As there was possible biological variability in experiments done on different dates (i.e. factors affecting growth of every strain), percent decrease instead of raw values of maximum O.D. was used to represent the data in order to reduce effects from such variability. Taking this into consideration, random effects of dates were incorporated in the statistical analysis.

In future studies, nematode factors such as receptors that interact with NilB could be possibly identified using cross-linking and co-immunoprecipitation. Several experiments regarding these have already been attempted but yet have encountered some technical difficulties in obtaining pure and enough samples from crude nematode extracts. Nevertheless, successful identification of NilB-interacting nematode factors will provide details in understanding the NilB function.
Acknowledgements

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References


