Xenorhabdus nematophila Requires an Intact iscRSUA-hscBA-fdx Operon To Colonize Steinernema carpocapsae Nematodes

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An insertion between iscA and hscB of the Xenorhabdus nematophila iscRSUA-hscBA-fdx locus, predicted to encode Fe-S assembly machinery, prevented colonization of Steinernema carpocapsae nematodes. The insertion disrupted cotranscription of iscA and hscB, but did not reduce hscBA expression, suggesting that X. nematophila requires coordinated expression of the isc-hsc-fdx locus for colonization.

The intestines of Steinernema carpocapsae infective juvenile-stage (IJ) nematodes are mutualistically colonized by Xenorhabdus nematophila bacteria (4). Germfree S. carpocapsae nematode eggs applied to lawns of X. nematophila will develop through juvenile and reproductive stages (32) until high nematode population density and low nutrient concentrations result in formation of progeny IJ nematodes colonized by X. nematophila (13, 17). Our lab is investigating molecular mechanisms mediating X. nematophila-S. carpocapsae interactions by identifying X. nematophila genes required for IJ nematode colonization.

Identification of a colonization-defective X. nematophila mutant. X. nematophila HGB081 (Table 1) was mutagenized with mini-Tn10, using plasmid pKV124 (31) transferred by conjugation from S17-1 (Apri) (7). Exconjugants selected on rifampin (100 μg/ml) and chloramphenicol (30 μg/ml) were individually cultivated with S. carpocapsae (Strain All) nematodes. Progeny IJ nematodes were harvested from each coculture and microscopically examined for the presence or absence of X. nematophila colonizers (32). One of 692 bacterial mutants screened was deficient in colonization and was designated HGB166. This frequency (0.16%) is within the range found in an independent Tn5 screen (8) and suggests that colonization genes comprise a small mutagenesis target.

In a quantitative colonization assay (8), HGB166 exhibited a severe colonization defect (Table 2) but was indistinguishable from its parent in exponential growth rate in Luria-Bertani medium, survival for 8 days on solid medium, swimming motility, attachment to polyvinyl chloride, dye binding, or lipase and protease activities (5, 14, 16, 22, 32; data not shown). HGB166 was fully virulent toward Manduca sexta larvae (W. Goodman, University of Wisconsin—Madison) in three separate experiments, at injection levels of 4 × 107 to 8 × 105 CFU, both the wild type and HGB166 were able to kill 90 to 95% of insects (32; data not shown).

Southern hybridization (18) with a pKV124 probe (ECF random prime kit; Amersham Pharmacia, Piscataway, N.J.) performed on EcoRI- or BglII (Promega, Madison, Wis.)-digested HGB166 DNA revealed one hybridizing band for each digestion (data not shown), indicating a single Tn10 insertion in HGB166. The transposon and flanking DNA were cloned as a BglII insert in BamHI-digested pBluescript II KS+ (pMP1) or as a self-ligated EcoRI fragment (pMP2E) (Fig. 1 and Table 1). Plasmid isolation, sequencing, and sequence analysis were carried out as previously described (8).

The HGB166 colonization defect is caused by Tn10 insertion in an isc-hsc-fdx locus. The transposon insertion of HGB166 is in a conserved locus with the gene order iscRSUA-hscBA-fdx, 3 nucleotides downstream of the predicted iscA stop codon and 56 nucleotides upstream of the putative hscB start codon (19, 34) (Fig. 1). In Escherichia coli, this locus encodes iron-sulfur center assembly machinery (12, 19, 26, 28, 29). Iron-sulfur centers are components of many cellular proteins with redox, regulatory, or catalytic function (3), and the mechanism of their assembly by isc-hsc-fdx-encoded proteins has begun to be elucidated. IscS, a cytochrome desulfurase, donates sulfur to a nascent cluster (6, 29, 34) forming on the scaffolding protein IscU (1). Plasmid isolation, sequencing, and sequence analysis were carried out as previously described (8).

To determine if the HGB166 colonization defect is caused by the transposon, we transformed (33) this strain with plasmids carrying portions of the isc-hsc-fdx locus (Fig. 1, Tables 1 and 3) PCR amplified with ExTaq polymerase (Takara Shuzo, Shiga, Japan) and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer's directions. Plasmids were sequenced, and the insert genes are congruent with the plasmid's lac promoter. HGB081 wild-type or HGB166 transformants (selected with 50 μg of kanamycin/ml) were tested for their colonization proficiency (Table 2). As previously observed in other mutants (8), no plasmid fully rescued HGB166 colonization. However, HGB166 carrying multicopy isc-hsc-fdx colonized 4,000-fold higher than the minimum detection level and only 50-fold lower than the wild type, demonstrating that the transposon insertion is responsible for the colonization defect. Consistent with the idea that the iscR

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gene product might be a negative regulator of isc operon transcription, as it is in E. coli (20), slightly higher levels of colonization were obtained for HGB166 carrying pJGS19 (lacking iscR) than for HGB166 carrying pJGS5 (with iscR).

Complementation was also observed when the isc-hsc-fdx locus was present in single copy in the chromosome. Tn7 constructs with and without the isc-hsc-fdx locus were transposed to the attTn7 site (13) of HGB081 and HGB166 after conjugation from E. coli S17-1 (pir) by triparental mating using the helper plasmid pUX-BF13 (2). HGB166 Tn7 isc-hsc-fdx was able to colonize nematodes at ~50-fold higher levels than HGB166 carrying the Tn7 construct alone and at ~10-fold lower levels than the wild-type control (Table 2). The failure of the isc-hsc-fdx locus to fully complement the mutant to wild-type colonization levels may be due to an additional detrimental effect of the insertion mutation that cannot be rescued by a second intact copy, or it may be due to an additional independent defect in the strain background.

In all of our initial experiments, the colonization levels of HGB166 were reproducibly below the level of detection of our assays (i.e., below 0.0001 CFU/IJ nematode). However, in subsequent experiments we began to observe a very low frequency of colonization in nematodes derived from HGB166 lawns (see, for example, HGB166 Tn7 in Table 2). One colony derived from this colonization assay was isolated, designated HGB609, and characterized. Although this strain still carries the transposon insertion (data not shown and Fig. 2), it is able to colonize nematodes at wild-type levels (data not shown), suggesting it has acquired a second-site suppressor(s) of the colonization defect.

The HGB166 transposon uncouples iscA-hscB transcription but does not eliminate expression of iron-sulfur cluster assembly genes. To test whether the intergenic transposon in HGB166 affects transcription of the isc-hsc-fdx locus (19, 27,
we carried out real-time quantitative PCR (q-PCR) on a Bio-Rad iCycler. cDNA was synthesized with random primers (Amersham Pharmacia, Piscataway, N.J.) and reverse transcriptase on RNA templates derived from three independently grown cultures. Reactions were performed in duplicate in 25 μl with the iCycler SYBR Green PCR master mix (Bio-Rad, Hercules, Calif.) and a two-step cycling protocol, in accordance with the manufacturer’s protocol. Wild-type samples yielded a product spanning the iscA and hscB coding regions, suggesting that these two genes are cotranscribed in wild-type cells (Fig. 2). Although products indicative of hscB and hscA transcription were detected in RNA samples derived from HGB166, no product was observed representing cotranscription of iscA and hscB. Furthermore, a small but reproducible increase in hscB transcription was observed in HGB166 compared to HGB081, perhaps due to an additional promoter in the transposon (11). These data suggest that the transposon insertion of HGB166 does not prevent expression of genes encoding the iron-sulfur-

FIG. 1. Organization of the X. nematophila isc-hsc-fdx locus. Open reading frames are indicated by open arrows, with the name of the gene indicated above. The location of the transposon (at top) insertion of HGB166 is represented by a vertical line between iscA and hscB. The transposon is not shown to scale, inverted repeats (IR) are represented by black boxes, and the origin of replication (oriR6K) is indicated by an open circle. Relevant restriction sites are shown: B, BgII; E, EcoRI; H, HindIII; S, SacI. Horizontal lines below indicate the approximate locations of fragments that were subcloned (see Table 1). To the right, the names of the plasmids containing the cloned fragment are indicated. Plasmids pMP1 and pMP2E have the transposon insertion within the cloned fragment, indicated by the solid arrowhead. pJGS5, pJGS19, pJGS6, and pJGS18 have wild-type fragments cloned.

<table>
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<th>Oligonucleotide</th>
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<td>qPCR for control 16s RNA</td>
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is relieved by exposure to H$_2$O$_2$, the to be coregulated with upstream genes: when IscR repression mutants (Table 2). In

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...r nicotine in HGB166 have not yet been elucidated, it is clear that one phenotypic consequence is a severe defect in colonization. X. nematophila are metabolically active within young IJ nematodes (13), and the data presented here suggest that this metabolism requires an intact locus encoding the iron-sulfur center assembly machinery.

**Nucleotide sequence accession number.** The X. nematophila *isc-hsc-fdx* sequence was submitted to GenBank under the accession number AY138456.

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**REFERENCES**


assembly machinery. Consistent with this, we found that activities of succinate dehydrogenase (a Fe-S enzyme whose activity is ~85% lower in *iscS*, *iscU*, *hscB* or *hscA* mutants of *E. coli* and *Salmonella enterica* serovar Typhimurium than in the wild types [19, 25, 28]) in *X. nematophila* HGB081 and HGB166 were not significantly different (data not shown). We conclude that the transposon insertion in HGB166 does not eliminate expression of the *hscBA* genes but does affect their normal transcriptional regulation, uncoupling them from cotranscription with upstream genes. As expected, placement of the *isc-hsc-fdx* locus in single copy at the *Hn7* att site of HGB166 restores *iscA-hscB* cotranscription. The suppressor strain, HGB609, has retained the transposon insertion and lacks transcription between *iscA* and *hscB* (Fig. 2).

It is possible that uncoupling of *hscBA-fdx* transcription from control by IscR in *X. nematophila* leads to a defect in some aspect of Fe(II) metabolism. Consistent with this hypothesis is the fact that the entire *isc-hsc-fdx* locus was required to complement the colonization defect of HGB166; neither *iscRSUA* nor *hscBA-fdx* fragments restored colonization to the mutant (Table 2). In *E. coli*, the *hscBA-fdx* genes do not appear to be coregulated with upstream genes: when IscR repression is relieved by exposure to H$_2$O$_2$, the *iscRSUA* genes are in-

FIG. 2. q-PCR on transcripts expressed from the *isc-hsc-fdx* locus. q-PCR was carried out on cDNA derived from RNA isolated and DNase treated as previously described (8) from HGB081 (wild-type) (solid bars), HGB166 (*iscA::Tn7::iscB*) (diagonal hatched bars), HGB571 (HGB166 Tn7) (shaded bars), HGB573 (HGB166 Tn7-*isc-hsc-fdx*) (open bars), or HGB609 (HGB166 sup-1) (crosshatched bars) cells grown in Luria-Bertani medium and harvested at an optical density (*A$_{600}$*) of 0.7. Reactions were carried out with primers specific for internal portions of *iscS* (primers JGP12 and JGP13) (expected product size, 403 bp; annealing temperature [*T$_{m}$*], 58.1°C), *iscA* (primers JGP5 and JGP6) (expected product size, 135 bp; *T$_{m}$*, 50.8°C), *hscB* (primers JGP1 and JGP2) (expected product size, 308 bp; *T$_{m}$*, 50.8°C), and *hscA* (primers JGP7 and JGP8) (expected product size, 629 bp; *T$_{m}$*, 58.1°C), as well as intergenic transcripts spanning *iscA* and *hscB* (*iscA-hscB*; primers JGP1 and JGP4) (expected product size, 327 bp; *T$_{m}$*, 50.8°C) or *hscB* and *hscA* (*hscA-hscB*; primers JGP10 and JGP11) (expected product size, 248 bp; *T$_{m}$*, 58.1°C). See Table 3 for primer sequences. Control reactions lacking either reverse transcriptase or RNA did not yield products (data not shown). Arbitrary units of RNA levels were determined by subtracting the threshold cycle (determined by maximum curvature approach as set by machine parameters) of each reaction from the threshold cycle obtained in the no-DNA control for the relevant primer set. Each reaction was then normalized using the threshold cycles obtained using 16S rRNA primers (RT16srRNAfor and RT16srRNArev) (expected product size, 272 bp; *T$_{m}$*, 50.8°C). The transcript being detected is indicated below each series.


