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A Phosphopantetheinyl Transferase Homolog Is Essential for *Photorhabdus luminescens* To Support Growth and Reproduction of the Entomopathogenic Nematode *Heterorhabditis bacteriophora*

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The bacterium *Photorhabdus luminescens* is a symbiont of the entomopathogenic nematode *Heterorhabditis bacteriophora*. The nematode requires the bacterium for infection of insect larvae and as a substrate for growth and reproduction. The nematodes do not grow and reproduce in insect hosts or on artificial media in the absence of viable *P. luminescens* cells. In an effort to identify bacterial factors that are required for nematode growth and reproduction, transposon-induced mutants of *P. luminescens* were screened for the loss of the ability to support growth and reproduction of *H. bacteriophora* nematodes. One mutant, NGR209, consistently failed to support nematode growth and reproduction. This mutant was also defective in the production of siderophore and antibiotic activities. The transposon was inserted into an open reading frame homologous to *Escherichia coli* EntD, a 4′-phosphopantetheinyl (Ppant) transferase, which is required for the biosynthesis of the catechol siderophore enterobactin. Ppant transferases catalyze the transfer of the Ppant moiety from coenzyme A to a holo-acyl, -aryl, or -peptidyl carrier protein(s) required for the biosynthesis of fatty acids, polyketides, or nonribosomal peptides. Possible roles of a Ppant transferase in the ability of *P. luminescens* to support nematode growth and reproduction are discussed.

Photorhabdus luminescens (*Enterobacteriaceae*) bacteria are symbiotic with entomopathogenic rhabditid nematodes of the family *Heterorhabditidae,* with which they cooperate in infecting a wide variety of insect larvae (38, 45; for reviews, see references 25 and 26). The nematode requires *P. luminescens* for insect pathogenicity (34), while the bacteria depend on the nematodes for transmission between insect prey. The infective juvenile (IJ)-stage nematodes specifically retain symbiotic *P. luminescens* cells in their gut mucosa, and transmission of the bacteria is a requisite for insect pathogenicity (31, 32, 34). The nematodes require *P. luminescens* cells as a substrate for growth and reproduction (2, 21, 22, 30). It was suggested previously that symbiotic *P. luminescens* cells provide favorable nutritional conditions for *Heterorhabditis bacteriophora* nematodes to grow and reproduce (45).

During prolonged laboratory culture, *P. luminescens* strains show a tendency to undergo an apparent phase variation phenomenon (8, 9, 36). The native form of the bacteria, termed primary phase, is isolated from the IJ stage of the nematode. The secondary-phase variants appear at high frequency during prolonged culturing, while more rare is the generation of primary-phase cells from secondary phase (6). The secondary-phase cells differ from the primary-phase cells in colony morphology, cell size, and dye uptake characteristics (6, 7, 9, 52). Also, typical primary-phase characteristics such as bioluminescence, pigment synthesis, phospholipase and siderophore activities, and production of intracellular crystalline inclusion proteins are depressed or absent in secondary-phase cells. The mechanism and role of phase variation in *P. luminescens* are unknown. Particularly significant for the subject of this investigation is the inability of secondary-phase variant cells to support nematode growth and reproduction (22, 30).

Because *H. bacteriophora* nematodes have a strict requirement for *P. luminescens* for growth and reproduction, it seems likely that *P. luminescens* provides some nutrients and/or other factors to the nematode. Dead cells or culture supernatants of *P. luminescens* cells do not provide the nutrients and/or factors required for nematode growth and reproduction (34; T. A. Ciche, personal observation), suggesting that actively metabolizing *P. luminescens* cells are required. To better understand the contribution of *P. luminescens* to nematode growth and reproduction, we developed a genetic screen to identify *P. luminescens* genes necessary for nematode growth and reproduction.

Genetic studies of *P. luminescens* have been limited, probably because of a low frequency of transformation (7) and the current inability to introduce recombinant DNA into *P. luminescens* by conjugation (52). Success has been achieved in using allelic exchange to construct disruptions in the genes encoding intracellular inclusion proteins CipA and CipB (7) and insecticidal toxin genes *tca, tcb, tcc,* and *tcd* (10) of *P. luminescens*.

Here we describe the construction of a mini-Tn*5*-based transposon and a delivery vector for efficient mutagenesis and gene characterization in *P. luminescens.* We used this system to identify genes that are required for *P. luminescens* to support growth and reproduction of its nematode host, *H. bacteriophora.* We identified a transposon mutant that has lost the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Nematode strains		
H. bacteriophora	Strain NC1, symbiotic with NC1/1 bacteria	38
H. megidis	Strain Meg	H. Kaya (46)
Bacterial strains		
P. luminescens		
NC1/1	Primary phase, isolated from *H. bacteriophora* (identical to ATCC 29304, strain NC-19)	38
NC1/2	Secondary phase, isolated from NC1/1 cells	This study
Meg/1	Primary phase, isolated from *H. megidis*	46
NP394	NC1/1 with pUB394	This study
NGR209	NC1/1 *ngrA*::mini-Tn*5*	This study
NGR209A	NC1/1 with allelic-exchange vector p209A	This study
NGR209C	NC1/1 *ngrA*::mini-Tn*5* via allelic exchange	This study
NP*ngrA*::ΩKm	NC1/1 *ngrA*::ΩKm	This study
E. coli		
DH5α	Cloning strain	Gibco BRL
EC393	DH5α with pUB394	This study
EC209R	DH5α with p209, Km^r	This study
Plasmid vectors		
pGEM-3Z	Cloning vector	Promega
pGEM-7Zf(+)	Cloning vector	Promega
pBC SK(+)	Cloning vector	Stratagene
pUT-mTn*5*	Mini-Tn*5*; transposase	19, 35
pSU19	P15A replicon, Cm^r	5, 43
pSU39	P15A replicon, Km^r	5, 43
pSB101	pBC SK(+) with 2.6-kb *Pst*I fragment containing *sacR*/*sacB*	7
pHP45Ω	Source of interposon containing streptomycin resistance	24
pHP45Ω-Km	Source of interposon containing kanamycin resistance	24
pG325	pGEM-7 containing Sp^r Sm^r and *sacB*/*sacR*	This study
pUB394	Mini-Tn*5* delivery vector, Km^r Sm^r Sp^r $sucrose^s$	This study
p209	Retrieved plasmid with mini-Tn*5* and flanking *P. luminescens* DNA	This study
p209A	p209 with Sp^r Sm^r and *sacB*/*sacR Pst*I fragment into single *Nsi*I site of p209	This study
p*ngrA*::ΩKm	pBCSK containing *ngrA*, Sm^r Sp^r, and *sacB*/*sacR Pst*I fragment	This study
p*ngrA*	pBCSK containing *ngrA*	This study

ability to support nematode growth and reproduction, and we describe the analyses of the disrupted gene region.

MATERIALS AND METHODS

Microbiological methods. Sources of strains and plasmids are listed in Table 1. Dye reagents, Tween types, and antibiotics were purchased from Sigma Chemical Corp. (St. Louis, Mo.), and bacteriological growth media were purchased from Difco (Detroit, Mich.). Cells of *P. luminescens* were grown in 2% Proteose Peptone 3 (PP3), with 1.5% agar added when required, at 28°C in the dark. Kanamycin (15 μg/ml), streptomycin (25 μg/ml), spectinomycin (25 μg/ml), and sucrose (7.5% [wt/vol]) were added when required. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar (1.5% agar) at 37°C with ampicillin (100 μg/ml), kanamycin (50 μg/ml), streptomycin (25 μg/ml), spectinomycin (25 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml), chloramphenicol (35 μg/ml), and sucrose (5% [wt/vol]) added when required.

Secondary-phase (NC1/2) cells of *P. luminescens* were obtained and distinguished from the primary-phase (NC1/1) cells as described previously (7). Siderophore activity was determined using chrome azurol S (CAS) agar (49), and lipase activity was determined on spirit blue agar containing 0.5% (vol/vol) Tween 20, Tween 40, Tween 60, Tween 80, or Tween 85 (50). Antibiotic activity was determined by placing a 5-mm-diameter plug, taken 5 mm away from confluent growth of a 96-h culture of *P. luminescens* on PP3 agar, onto a plate of antibiotic medium 3 (Difco) that had been inoculated with *Micrococcus luteus* cells.

Nematode propagation. IJ nematodes were propagated by infecting greater wax moth larvae, *Galleria mellonella* (Ja-Da Bait Co., Antigo, Wis.), or by adding approximately 20 IJ nematodes to lawns of *P. luminescens* cells on lipid agar (LA) (53) (2.5% nutrient broth, 1.5% agar, 1% corn oil), nematode growth medium (12), or liquid culture medium (LCM) (22) as described previously (7).

The IJ nematodes were collected by flooding the LA plates or infected larvae at the time of IJ release and separated from adult nematodes by the water trap method of Wouts (53). Alternatively, IJ nematodes were grown on *P. luminescens* cells that were seeded onto LA or nematode growth medium contained on one side of a divided petri dish (Fisher Scientific, Pittsburgh, Pa.). After 14 days, newly formed IJ nematodes migrated into the sterile saline contained on the other side. IJ nematodes were surface sterilized as described previously (41).

Axenic nematodes. Axenic nematodes were obtained using a modification of the procedure of Han and Ehlers (33). *H. bacteriophora* nematodes were propagated on a *P. luminescens* strain, Meg/1, that was isolated from *Heterorhabditis megidis* nematodes. The *H. bacteriophora* nematodes grow and reproduce normally on Meg/1 bacteria, but the resulting surface-sterilized IJ nematodes do not retain Meg/1 bacteria and are therefore axenic (33).

Retention of bacteria by nematodes. The numbers of *P. luminescens* cells in the intestine of IJ nematodes were determined. For some experiments, 50 to 100 surface-sterilized nematodes were disrupted using an 0.1-ml microtissue grinder (Kontes, Vineland, N.J.). The homogenate was then serially diluted and plated on PP3 agar. Alternatively, a 10-μl sample of a water suspension containing 10 to 50 IJ nematodes was placed in the depression of a sterile hanging drop slide and dried in a laminar flow hood for 5 to 10 min, and then each nematode was disrupted with a sterile scalpel while being examined under a 40× dissecting microscope. The disrupted nematodes were suspended in 0.1 ml of PP3 broth, and the scalpel blade was rinsed in this suspension. The material was then transferred to a tube containing 0.9 ml of PP3 broth. The slide depression and scalpel were rinsed three times before plating serial dilutions of the tube onto PP3 agar. Colonies were counted following incubation at 28°C for 3 days.

DNA manipulations. Plasmid purification from *P. luminescens* and *E. coli* was carried out using Wizard Mini and Midi preps (Promega Corp., Madison, Wis.). Restriction enzymes and T4 ligase were used according to the manufacturer's instructions (Promega Corp.). When required, DNA fragments were extracted

from agarose gels using the Qiagen Gel extraction kit (Qiagen Inc., Valencia, Calif.). The bacterial DNA was purified using a modified cetyltrimethylammonium bromide (CTAB) method (14). Southern hybridization was performed under high-stringency conditions using the Genius kit (Boehringer Mannheim Corp., Indianapolis, Ind.). Transformation of *E. coli* and *P. luminescens* was done by electroporation using a Bio-Rad gene pulser according to the conditions suggested for *E. coli* by the supplier (Bio-Rad Laboratories, Hercules, Calif.).

Construction of pUB394. The structure of the transposon delivery vector, pUB394, is shown in Fig. 1A. A pSU39 plasmid (5, 43) was inserted between the I and O ends that define the termini of the mini-Tn*5* by removing the chloramphenicol resistance gene from the mini-Tn*5* of pUT-mTnCm (24) by digesting it with *Sma*I and replacing it with a *Hin*cII and *Sma*I fragment of pSU39. The resulting plasmid is named pUS39. The correct orientation of the insert was determined. Because *P. luminescens* organisms are resistant to ampicillin and conjugation techniques have not been established with these bacteria, the ampicillin resistance and Mob RP4 genes were removed by *Bam*HI and *Sfi*I digestion of pUS39 followed by self-ligation, resulting in pUSF39. pGTn, pGHP, and pGHS were constructed to isolate the transposase, streptomycin and spectinomycin resistance, and levansucrase genes, respectively. Plasmid pGTn was constructed by removing a 1.5-kb *Sal*I fragment containing the transposase gene from pUT-mTn*5*Cm (19, 35) and inserting it into pGEM-7Zf(+) (Promega). The orientation of the insert was verified. The transposase-encoding gene was inserted into the *Xba*I site located outside the I-end inverted repeat of the mini-Tn*5* of pUB39 as a *Xba*I fragment from plasmid pGTn. The resulting plasmid is named pUGS394. The orientation of the insert was verified. pUGS394 was digested with *Bam*HI to remove the ampicillin resistance and Mob RP4 genes and self-ligated. The resulting plasmid is named pUS394. The streptomycin and spectinomycin resistance gene was removed as a 2.0-kb *Hin*dIII fragment from pHP45Ω (24) and inserted into pGEM-7Zf(+). The resulting plasmid is named pGHP. Plasmid pGHS was constructed by inserting a 2.6-kb *Xba*I fragment from pBS101 (7) containing *sacB*/*sacR* (27, 47) into *Xba*I-digested pGHP. The orientation of the insert was verified. The streptomycin and spectinomycin resistance and the *sacB*/*sacR* sucrose sensitivity genes were removed as a 4.6-kb *Bam*HI fragment from pGHS and inserted into a *Bam*HI site of pUS394, yielding pUB394. A second, alternative O end was located 1.7 kb 5′ to the O end of the mini-Tn*5*. Between the two O ends are the R6K *ori* and DNA encoding the N-terminal (amino acids 1 to 202) region of the Tn*5* transposase-encoding genes. No difference was observed in the stability of these and normal O-end mini-Tn*5* insertions.

Transposon mutagenesis. The use of pUB394 for transposon mutagenesis and retrieval of DNA containing mini-Tn*5* insertions is shown in Fig. 1B. Low transformation efficiency and the inability to conjugate or transduce DNA in *P. luminescens* make the standard transposon mutagenesis techniques utilizing suicide plasmids inefficient. The *sacB* gene, conferring sucrose sensitivity, allows selection against cells containing pUB394 and, when used with selection for the transposon (resistance to kanamycin), allows cells containing insertions, but not pUB394, to be selected. Cells of *P. luminescens* NC1/1 were transformed with the transposon delivery vector pUB394 to create strain NP394. As a consequence of pUB394 replication in NP394, mini-Tn*5* insertions may accumulate. NP394 containing a mini-Tn*5* insertion and pUB394 will cause, at high frequency (10^{-3}), kanamycin- and sucrose-resistant cells by loss of pUB394. To select mini-Tn*5* mutants that have lost pUB394, cells of NP394 (checked for the absence of a mini-Tn*5* insertion prior to mutant generation) were grown overnight in PP3 containing kanamycin, and 10^{-1} and 10^{-2} dilutions were plated on PP3 agar containing kanamycin and sucrose to select for cells containing the mini-Tn*5* but not pUB394. The mutants were transferred to PP3 agar containing kanamycin to verify the resistance to kanamycin, PP3 agar containing streptomycin and spectinomycin to verify the absence of pUB394, M9 minimal medium to determine auxotrophy, and eosin-methylene blue to determine the phase state of the mutants. Mutant cells unable to grow on M9 medium were assumed to be auxotrophs, and those not accumulating dye on eosin-methylene blue were assumed to be secondary-phase cells. Secondary-phase cells do not support nematode growth and reproduction and were not characterized, because they are likely to be spontaneous phase variants of NP394 and not transposon-induced secondary-phase cells.

Screening for the ability of mutants to support growth and reproduction of *H. bacteriophora* nematodes. The screen for the ability of bacteria to support nematode growth and reproduction is shown in Fig. 2A. Individual colonies of transposon-induced mutants of *P. luminescens* were inoculated into 0.25 ml of PP3 with 10 μg of kanamycin/ml and were incubated statically overnight at 28°C. Samples of 0.05 ml were added to individual wells of 24-well tissue culture plates (Falcon 1143; Becton Dickinson Labware, Lincoln Park, N.J.) with 1.5 ml of LA-containing kanamycin. Following incubation overnight at 28°C, an average of 12 axenic IJ nematodes was added to each well. Bacteria able to support nematode growth and reproduction were detected by the appearance of a white mass of nematodes 21 days later. NP394 and secondary-phase (NC1/2) *P. luminescens* containing pUB394 were included in the assay as positive and negative controls, respectively. Putative nematode growth and reproduction mutants were verified by repeating the nematode growth and reproduction experiments twice, each with 12 replicates. Mutants were named NGR for nematode growth and reproduction mutants.

Retrieval of DNA flanking the mini-Tn*5* of NGR209. DNA from mutant NGR209 was purified, restriction enzyme digested with *Nsi*I (the mini-Tn*5* contains no *Nsi*I site), intramolecularly and ethanol precipitated, and transformed by electroporation into *E. coli* DH5α (Fig. 1B). Transformants containing the mini-Tn*5* were selected by being resistant to kanamycin. The retrieved plasmid, p209, was purified and restriction enzyme digested with *Nsi*I and *Sfi*I to verify that the plasmid contained a single *Nsi*I restriction fragment and the mini-Tn*5* (determined by the presence of a 2.9-kb *Sfi*I restriction fragment), respectively.

Causality determination of the mini-Tn*5* insertions. A 4.6-kb *Pst*I fragment from pG325 (Table 1) containing the *sacB*/*sacR* genes and a gene conferring resistance to streptomycin and spectinomycin was ligated into the *Nsi*I site of the retrieved mini-Tn*5* plasmids to create the allelic-exchange plasmid p209C. The plasmid was transformed into wild-type NC1/1 cells. Allelic exchange was selected for by growing NC1/1 cells containing the allelic-exchange plasmid overnight in PP3 containing kanamycin and then plating the cells on PP3 containing kanamycin and sucrose. The mutants were tested for sensitivity to streptomycin and spectinomycin to verify the loss of the allelic-exchange plasmid. The phenotype of the allelic-exchange mutant, NGR209A, was compared to that of the original mini-Tn*5* mutant, NGR209.

Sequence analysis of p209. The sequence of DNA flanking the transposon insertion of p209 was obtained by using M13 forward and reverse primers located 60 or 40 bp from the inverted repeat termini of the transposon and by primer walking. If the sequence obtained from the M13 forward primer revealed the alternative O-end insertion, the DNA sequence flanking the alternative O end was obtained by using the oligonucleotide primer (5′ TAAGCGCCTTCCTGC ATGGCTT 3′). Dye terminator cycle sequencing using ABI terminator mix was performed using the conditions suggested by the supplier (Perkin-Elmer Corp., Foster City, Calif.), and then the reaction products were analyzed on an ABI 377 automated sequencer (Perkin-Elmer Corp.) at the University of Wisconsin Biotechnology Center. Comparison of the DNA sequence to database sequences was done using BLAST programs using nonredundant databases (4).

Complementation of NGR209 with pNgrA. The disrupted allele of NGR209 was designated *ngrA*. Intact *ngrA* was obtained by PCR amplification using the oligonucleotide primers Edf (5′ ATTAAGTATAGACTGTAGGATA 3′) and Edr (5′ TGATCAGGGACGGTATCAGCT 3′) and *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, Calif.). The Edf primer was designed to include the intergenic region between *ngrA* and the *phfB* gene that might contain a promoter element (see Fig. 4). An 0.8-kb band was extracted from an agarose gel and blunt end ligated into pBC SK− (Stratagene) that had been treated with *Hin*cII and shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, Ohio). Clones containing intact *ngrA* were obtained by screening transformants on LB medium containing chloramphenicol and X-Gal. Plasmid preparations were made on white colonies, resulting in a clone containing an 0.8-kb insert with a sequence identical to *ngrA* of p209 and in the same orientation as the *lacZ* gene to allow the *lac* promoter to be utilized for transcription of *ngrA*. The resulting plasmid, pNgrA, was transformed into NGR209, and the phenotype of the resulting clone was determined.

Phenotypic characterization. Analyses of phase-dependent characteristics and the ability of the cells to support nematode growth and reproduction or to be retained by IJ nematodes were performed as described above. The pathogenicity of bacteria for insects was determined as described previously (11). Positive insect pathogenicity was defined 72 h postinjection as 50% mortality of insect larvae resulting from a dose of less than 30 cells. To test for oral insecticidal activity (11), growth liquors from 72-h PP3 cultures were filter sterilized and concentrated 15 times using a Microcon 40 (Amicon, Inc., Beverly, Mass.) microconcentrator. An 0.05-ml sample of retentate was added to a 1.0-g portion of gypsy moth diet (ICN Pharmaceuticals Inc., Costa Mesa, Calif.), and a single first- or second-instar larva of *Manduca sexta* was then added. Larvae were observed for weight gain and/or death following 72 h of incubation. Experiments were performed twice with 12 replicates each.

Nucleotide sequence accession number. The GenBank accession number for DNA sequence flanking the mini-Tn*5* insertion of mutant NGR209 is AF288077.

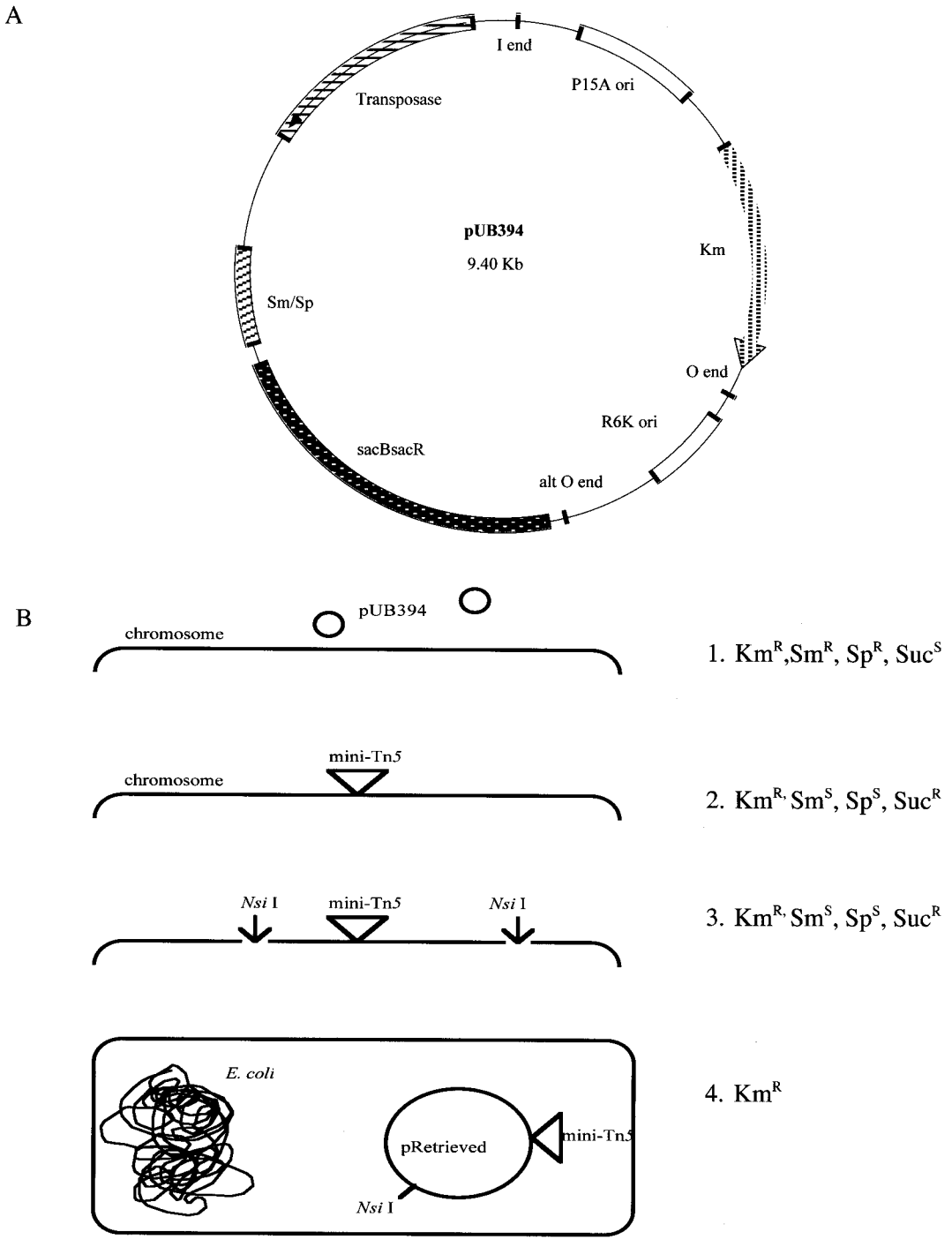

FIG. 1. (A) The structure of pUB394. Genes conferring resistance to kanamycin (Km) and streptomycin and spectinomycin (Sm/Sp) and the *sacB*/*sacR* gene conferring sensitivity to sucrose are shown. The mini-Tn*5* is located between the I and O ends. (B) Use of pUB394 for transposon mutagenesis of *P. luminescens*. (Line 1) Transformants containing pUB394 were selected for by resistance to streptomycin. (Line 2) Transposon-induced mutants containing the mini-Tn*5* mutants that have lost pUB394 were selected for by resistance to kanamycin and sucrose. (Line 3) The mini-Tn*5* was retrieved from the mutants by *Nsi*I digestion of mutant DNA, self-ligation, and transformation into *E. coli*. (Line 4) Transformants containing the mini-Tn*5* were selected for by their resistance to kanamycin. The DNA sequence flanking the insertion was obtained using M13 forward and reverse priming sites present in the mini-Tn*5*. Causality of the mini-Tn*5* insertion was determined by inserting a *Pst*I causality cassette (containing the *sacB*/*sacR* sucrose sensitivity genes and a gene conferring resistance to streptomycin and spectinomycin) into the *Nsi*I site present in the retrieved plasmids. Allelic exchange was then performed with this plasmid and the wild-type allele as described previously (7).

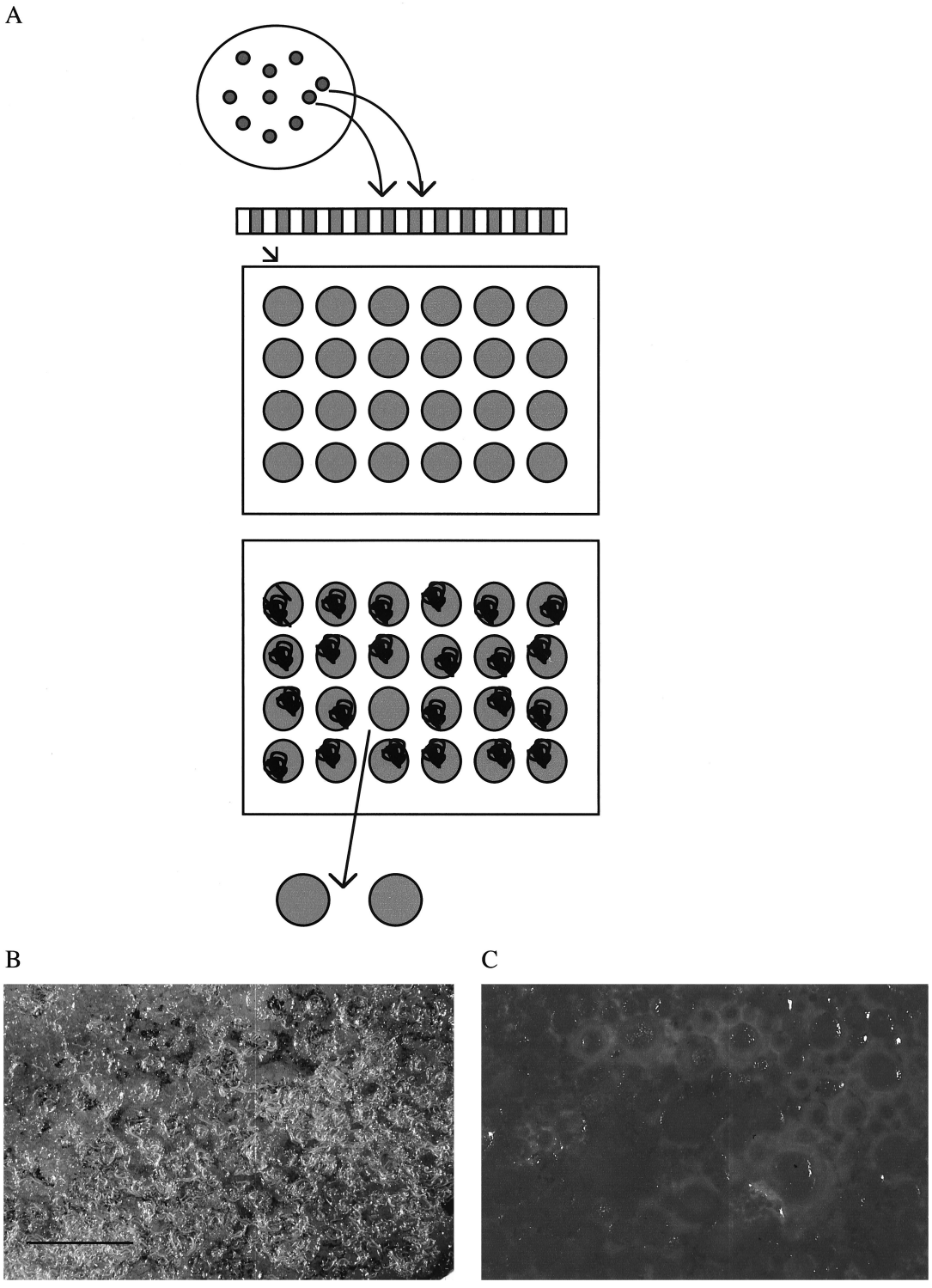

FIG. 2. Illustration of the in vitro screen for the ability of mutants to support nematode growth and reproduction. (A) Transposon-induced mutants were grown overnight in liquid culture, and a sample was placed onto LA in titer dishes and incubated overnight. An average of 12 IJ nematodes was added to the wells. Following incubation for 20 days, the wells were then observed for the presence of nematode growth. Mutant cells in wells not showing nematode growth were further characterized. (B and C) Photographs of the surface of LA wells showing growth of nematodes on NC1/1 cells (B) and no growth on NGR209 cells (C). Bar, 1 cm.

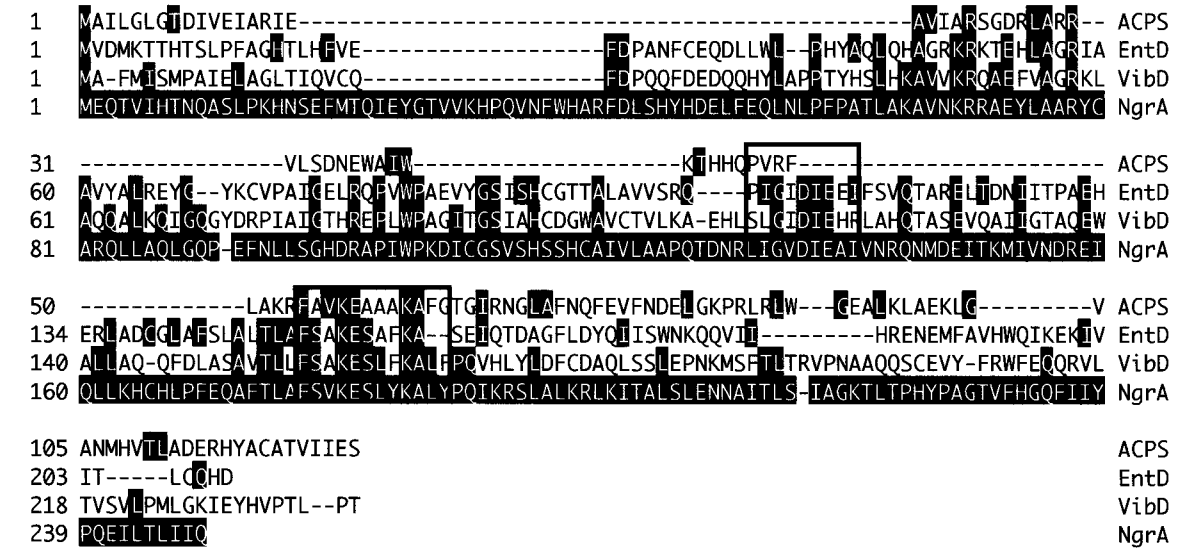

FIG. 3. Alignment of NgrA with Ppant transferase proteins. Shaded residues indicate identity to NgrA. Boxed are the two Ppant transferase motifs (39). Proteins were aligned using Clustal W (DNAStar, Madison, Wis.): ACPS (accession no. sp P24224); EntD, enterobactin synthetase component D (accession no. P19925), *E. coli*; VibD, phosphopantetheinyl transferase (accession no. AAD48884), *V. cholerae*.

RESULTS

Transposon mutagenesis. The frequency of transposition and loss of the pUB394 delivery vector, defined as the ratio of cells resistant to kanamycin and sucrose to total cells present, was 1.7×10^{-7}. A large number of strains with putative mini-Tn*5* insertions were obtained. Southern analysis of 20 randomly picked mutants showed that more than 90% of the putative transposon mutants contained single insertions on different locations of DNA (data not shown). Less than 2% of the strains with putative insertions were resistant to streptomycin and spectinomycin. This suggests that resistance to sucrose in these rare strains occurred by a mechanism other than loss of the delivery vector pUB394. Approximately 1 to 3% of the mutants were auxotrophic. This frequency would be expected if the mini-Tn*5* was inserted randomly into the genome of *P. luminescens*, assuming the genome to be approximately the same size as that of *E. coli.*

Mutant screening. Of 2,800 transposon-induced mutants screened, a mutant (NGR209) was obtained that consistently failed to support nematode growth and reproduction. A white mass of nematodes is evident when nematodes are grown on lawns of NC1/1 (Fig. 2B), while no adult nematodes are seen on lawns of NGR209 (Fig. 2C).

Cloning and analyses of DNA flanking the mini-Tn*5* insertion of mutant NGR209. A 10.6-kb plasmid containing the mini-Tn*5* was retrieved from NGR209. The *ngrA* gene, disrupted by the mini-Tn*5* in mutant NGR209, appears to encode a 4′-phosphopantetheinyl (Ppant) transferase enzyme. The deduced protein product shows a significant degree of similarity to the two Ppant transferase motifs (boxed residues) characteristic of these proteins (39) (Fig. 3). NgrA is most similar to the Ppant transferases EntD from *Salmonella enterica* serovar Typhimurium and *E. coli* and VibD from *Vibrio cholerae*, which are required for the biosynthesis of the catechol siderophores enterobactin (16, 39) and vibriobactin (54), respectively (residues identical to NgrA are shaded). Ppant transferases transfer the Ppant moiety from coenzyme A to acyl carrier proteins (ACP), aryl carrier proteins, and peptidyl carrier proteins (39). The Ppant-modified carrier proteins are required for the biosynthesis of lipids, lipoproteins, polyketide, and nonribosomal peptides, many with siderophore, antibiotic, or pharmacological activities (37, 42).

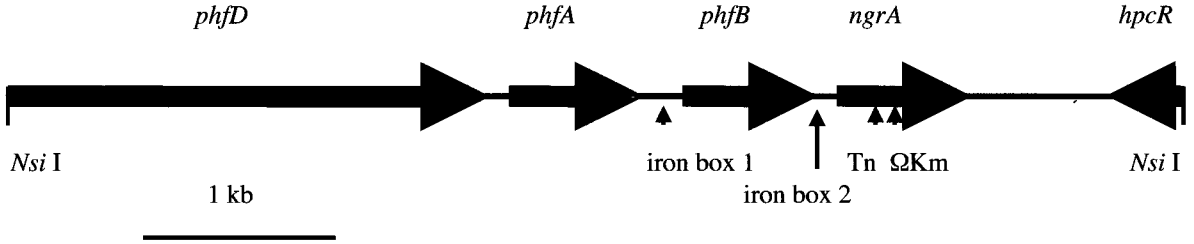

FIG. 4. Physical structure of DNA flanking the mini-Tn*5* insertion of mutant NGR209. Shown are the insertion site of the mini-Tn*5* transposon, the location of two potential iron boxes, the site of an ΩKm insertion, and *Nsi*I sites at the termini of the retrieved DNA.

TABLE 2. Phenotypic characterization of NC1/1, NC1/2, NGR209, and NGR209 reconstituted with pNgrA

Phenotype assayed	Reaction of strain:			
	NC1/1 (primary)	NC1/2 (secondary)	NGR209	NGR209 + pNgrA
Dye absorption				
Eosin *Y*-methylene blue	+	−	+	+
Neutral red	+	−	+	+
Bromthymol blue	+	−	+	+
Bioluminescence	+	−	+	+
Extracellular products				
Lipase activity	+	−	+	+
Hemolytic activity	+	−	+	+
Protease activity	+	−	+	+
Antibiotic activity	+	−	−	+
Siderophore activity	+	−	−	+
Colony morphology	Convex, mucoid	Flat, nonmucoid	Convex, mucoid	Convex, mucoid
Pigmentation	+	−	+	+
CipA and CipB production	+	−	+	+
Insect pathogenicity				
Injected cells	+	+	+	+
Oral cell-free activity	+	ND[b]	+	+
Support of nematode growth and reproduction[a]	+	−	−	+

[a] Nematode growth and reproduction on LA and LCM and in *G. mellonella* hosts.
[b] ND, not determined.

The sequence of the entire 5.9 kb of p209 flanking the mini-Tn*5* was analyzed. The physical map of this DNA (Fig. 4) shows that three open reading frames similar to those for fimbrial proteins, *phfB, phfA,* and *phfD,* are located 5′ and on the same strand as *ngrA.* The *phfB* gene is located 54 bp from *ngrA* and is 1,014 bp in length, with residues 215 to 337 of the predicted protein product being 37% identical and 53% similar to the type I fimbrial subunit (FimA/PapA family) (spP12903) (29). The next gene 5′ of *ngrA*, *phfA,* is 966 bp in length with residues 115 to 320 of the predicted protein product being 28% identical and 42% similar to the fimbrial adhesin MrkD from *Klebsiella pneumoniae* (spP21648) (3). The *phfD* gene is at least 2,397 bp in length (the start codon was not retrieved). Its protein product is 38% identical and 58% similar to the S-fimbrial usher SfaF (prf1713397E) (48). Two possible iron boxes (ferric iron uptake [Fur] regulator-binding sites) (13, 18) were identified. Iron box 1 is located between *phfA* and *phfB* and contains 15 bases identical, with a 3-bp insertion, to the palindromic 19-bp consensus iron box (13, 17, 23). Iron box 2 is located between *phfB* and *ngrA* and contains 10 bases identical to the 19-bp consensus iron box.

Phenotypic characterization of mutant NGR209. The phenotype of NGR209 was compared to those of the primary (NC1/1)- and secondary (NC1/2)-phase cells and to NGR209 reconstituted with pNgrA (complementation of NGR209 with intact *ngrA*) (Table 2). NGR209 is identical to NC1/1 in all properties except in not supporting nematode growth and reproduction or producing siderophore and antibiotic activities. Complementation of NGR209 with pNgrA restored these properties. The restoration of these properties was not due to gene replacement of the mini-Tn*5*-disrupted *ngrA* with intact *ngrA,* because NGR209 cured of pNgrA reverted to the NGR209 phenotype.

The causality of the transposon for the phenotype of NGR209 was demonstrated by performing allelic exchange with the mini-Tn*5*-disrupted *ngrA* gene into the wild-type *ngrA* gene of *P. luminescens* cells. The resulting mutant, NGR209C, had all the phenotypic characteristics of NGR209 (data not shown). An omega cassette disruption of *ngrA*, NP*ngrA*::ΩKm, also had a phenotype identical to that of NGR209 (data not shown).

The results of the analyses of growth, reproduction, and mortality of nematodes when grown on cells of NC1/1 and NGR209 and retention of NC1/1 and NGR209 by nematodes are shown in Table 3. Nematode development from the IJ to the J4 stage was reduced significantly when nematodes were grown on LA medium seeded with cells of NGR209 compared to the equivalent nematodes propagated on NC1/1 cells. The inability of nematodes to reproduce on NGR209 cells was shown by the observation that no IJ nematodes were present after 20 days of growth on the NGR209 cells. In contrast, large numbers of nematodes were produced by nematodes propagated on NC1/1 cells. The NGR209 cells are not toxic to the nematodes, as indicated by a similar percent mortality of IJ nematodes after incubation for 3 days with NGR209 or NC1/1 cells. Also, the numbers of IJ nematodes were essentially the same following 10 to 14 days of growth on 1:1 mixtures of NGR209 and NC1/1 or NGR209 and Meg/1 cells, again indicating that NGR209 cells are not inhibitory for nematode growth and reproduction.

The addition of large numbers (250) of IJ nematodes did not overcome the inability of NGR209 cells to support nematode

TABLE 3. Ability of NC1/1 and NGR209 to associate with *H. bacteriophora*

Characteristic	Value for strain[g]:	
	NC1/1	NGR209
Nematode development[a]	5.1 (2.5)	1 (1.1)
Nematode yields[b]	2,730 (1,750)	0 (0)
% Nematode mortality[c]	13.7 (12.0)	7.7 (7.0)
Retention by IJ nematodes[d]		
Grown on NC1/1	106.6 (102)	—[e]
Grown on NGR209 and Meg/1	—[f]	78 (35)
Grown on NGR209 and NC1/1	83.5 (70)	0

[a] Average numbers of J4 or adult nematodes observed on LA 4 days following the addition of an average of 12 IJ nematodes ($n = 12$).
[b] Average numbers of IJ nematodes observed on LA at day 20 (same cultures as for nematode development; $n = 12$).
[c] Percent nonviable IJ nematodes per milliliter of LCM 5 days after addition of 150 IJ nematodes per ml, determined by observing no movement 1 min after the addition of 1.0% commercial bleach ($n = 3$).
[d] IJ nematodes were propagated on LA seeded with bacteria on one half of divided petri dishes as described in Materials and Methods. Results show the average number of bacteria per disrupted IJ nematode ($n = 3$).
[e] NGR209 was not present in the experiment.
[f] NC1/1 was not present in the experiment.
[g] Values in parentheses are standard deviations.

growth and reproduction. The addition of 110 IJ nematodes to NGR209 cells caused the development of IJ to J4 nematodes to increase from 1 to 5.7 (standard deviation, 2.1; $n = 3$). Although this is approximately equal to the number of J4 nematodes seen on NC/1 cells (Table 3), the J4 nematodes growing on NGR209 cells did not develop to hermaphrodites. This suggests that the decreased development from IJ to J4 observed on NGR209 cells was not the only cause for the inability of NGR209 to support nematode growth and reproduction. Furthermore, a mixture of developmental stages (J1 to adult) added to NGR209 cells did not grow and reproduce. Some nematode development and reproduction were initially seen but quickly ceased. This again indicates that the defect of NGR209 for nematode growth and reproduction does not involve only the development of IJ nematodes to reproductive hermaphrodites; other stages of nematodes were also unable to develop and reproduce.

Because of the close proximity of fimbrial genes to *ngrA,* it is conceivable that the defect in NGR209 for growth and reproduction might also affect colonization by NGR209 of the IJ nematode intestine. Therefore, the ability of NGR209 to be retained by the IJ nematodes was determined. To do this, the *H. bacteriophora* nematodes were first propagated on Meg/1 bacteria. This resulted in IJ nematodes that were nearly axenic (approximately one Meg/1 cell per 200 IJ nematodes). The nematodes were incubated for 10 to 14 days on LA inoculated with NC1/1 cells, a 1:1 (vol/vol) mixture of NGR209 and Meg/1 cells, and a 1:1 mixture of NGR209 and NC1/1 cells. The IJ nematodes cultured on NC1/1 cells retained an average of 106 NC1/1 cells (Table 3). The IJ nematodes retained an average of 78 NGR209 cells and no Meg/1 cells when cultured on a mixture of NGR209 and Meg/1. This is essentially the same as the amount of NC1/1 cells retained by IJ nematodes. However, IJ nematodes retained an average of 83 NC1/1 and no NGR209 cells when propagated on a mixture of NC1/1 and NGR209. At day 20, the proportions of bacteria on the LA were about the same as those added initially. Thus, competition of the bacterial strains on the LA prior to IJ retention is not the cause for the differential retention in the IJ nematodes. It is evident that the mini-Tn*5* insertion in *ngrA* causes NGR209 not to be retained in the presence of NC1/1 cells but to be retained in the absence of NC1/1 and the presence of Meg/1 cells.

DISCUSSION

The entomopathogenic nematode *H. bacteriophora* will grow and reproduce only when feeding on living cells of its symbiotic bacterium, *P. luminescens.* Spontaneous phase variants of the bacterium that have lost expression of multiple characteristics will not support nematode growth. A screen of 2,800 transposon mutants of *P. luminescens* yielded only one mutant, NGR209, which lost the ability to support nematode growth and reproduction while retaining most primary-phase characteristics. The transposon is inserted into a gene, *ngrA,* which database analyses show to be most similar to the *entD* gene that encodes the enzyme Ppant transferase. The enzyme transfers the Ppant moiety from coenzyme A to EntB and EntF, which are required for the biosynthesis of the siderophore enterobactin (16, 28, 39).

The nematode growth and reproduction mutation also caused loss of detectable antibiotic and siderophore production. It is unlikely that loss of these properties is involved in the nematode growth phenotype, because adding growth liquor from a *P. luminescens* culture, which contained both activities, to the nematode growth medium did not overcome the growth defect. In addition, we isolated a transposon mutant of *P. luminescens* producing no detectable siderophore activity, and this mutant supports nematode growth and reproduction (15).

The *ngrA* gene is more likely to be involved in biosynthesis of a hormone or signal regulator of nematode development than in that of a nutritional factor. The gene is probably not involved in the biosynthesis of fatty acids or lipids because the Ppant transferase of *E. coli* that activates ACP required for fatty acid biosynthesis is essential (40, 51). Other possible functions of NgrA are the biosynthesis of polyketide or nonribosomally synthesized peptide molecules (39) that are good candidates for hormonal or signal molecules. One possible candidate is the quorum-sensing homoserine lactone molecules that require ACP and ACP synthase (ACPS) for biosynthesis (44). It is unlikely that NgrA is involved in homoserine lactone biosynthesis because NgrA is not equivalent to ACPS based on amino acid similarity.

The NgrA product might be very unstable or active at a critical threshold level, since adding growth liquor of exponential- or stationary-phase cultures of *P. luminescens* to LA does not restore nematode growth. It is also possible that the putative signal molecule is produced by the bacteria only when they are grown in the presence of the nematode.

The *ngrA* mutant cells retain most of the characteristics of the parent and have clearly not been converted to the secondary-phase variant. The parent and *ngrA* mutant cells produce two crystalline inclusion proteins, CipA and CipB (7). The secondary-phase cells do not produce them. Inactivation of

either *cipA* or *cipB* by omega cassettes resulted in cells exhibiting secondary-phase characteristics (7). These mutants did not support nematode growth and reproduction. It thus seems clear that the *ngrA* mutant is specifically related to nematode growth and is not involved in the secondary-phase variation phenomenon.

The genes located near *ngrA,* having putative functions involving fimbrial biogenesis and adhesion and the iron boxes (Fig. 4), might be relevant to the nematode-bacterium symbiosis. Fur is a global regulator in *E. coli* and regulates some virulence genes (17). Fimbriae are often responsible for specific binding of bacterial cells to eukaryotic cells (20), which can signal changes in gene expression in both bacteria and host cells (1). Knowing the nucleotide sequence of these genes will allow us to specifically disrupt the genes to determine their possible roles in the symbiotic association. Our isolation of the mini-Tn*5* insertion in the *ngrA* gene provides a starting point for genetic and physiological analysis of this symbiotic relationship.

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