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Photobactin: a Catechol Siderophore Produced by *Photorhabdus luminescens*, an Entomopathogen Mutually Associated with *Heterorhabditis bacteriophora* NC1 Nematodes

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The nematode *Heterorhabditis bacteriophora* transmits a monoculture of *Photorhabdus luminescens* bacteria to insect hosts, where it requires the bacteria for efficient insect pathogenicity and as a substrate for growth and reproduction. Siderophore production was implicated as being involved in the symbiosis because an *ngrA* mutant inadequate for supporting nematode growth and reproduction was also deficient in producing siderophore activity and *ngrA* is homologous to a siderophore biosynthetic gene, *entD*. The role of the siderophore in the symbiosis with the nematode was determined by isolating and characterizing a mini-Tn*5*-induced mutant, NS414, producing no detectable siderophore activity. This mutant, being defective for growth in iron-depleted medium, was normal in supporting nematode growth and reproduction, in transmission by the dauer juvenile nematode, and in insect pathogenicity. The mini-Tn*5* transposon was inserted into *phbH*; whose protein product is a putative peptidyl carrier protein homologous to the nonribosomal peptide synthetase VibF of *Vibrio cholerae*. Other putative siderophore biosynthetic and transport genes flanking *phbH* were characterized. The catecholate siderophore was purified, its structure was determined to be 2-(2,3-dihydroxyphenyl)-5-methyl-4,5-dihydro-oxazole-4-carboxylic acid [4-(2,3-dihydroxybenzoylamino)-butyl]-amide, and it was given the generic name photobactin. Antibiotic activity was detected with purified photobactin, indicating that the siderophore may contribute to antibiosis of the insect cadaver. These results eliminate the lack of siderophore activity as the cause for the inadequacy of the *ngrA* mutant in supporting nematode growth and reproduction.

Photorhabdus luminescens (*Enterobacteriaceae*) is an insect pathogen mutually associated with and transmitted by the entomopathogenic nematode *Heterorhabditis bacteriophora* (29, 42; for reviews, see references 21 and 22). The specialized dauer juvenile (DJ) stage of the nematode contains a pure culture of *P. luminescens* cells in the anterior region of the gut mucosa (14, 19). The DJ nematodes seek insect larvae and enter the hemocoel through natural openings or by penetrating the cuticle and then regurgitate their charge of *P. luminescens* (14, 35, 42). The bacteria alone are highly lethal to insect larvae when injected into the hemocoel, with less than 30 *P. luminescens* cells causing 50% mortality to insect larvae (LD_{50}), but are not pathogenic when ingested by insect larvae (29, 35). The nematode requires *P. luminescens* for growth and reproduction in insect larvae and on artificial medium (2, 18). The bacteria produce antibiotics and bacteriocins that appear to inhibit other saprophytic microorganisms in the infected insect cadaver (1, 40, 44). Before nutrients in the insect are depleted, the nematodes again differentiate to the DJ stage, each colonized by *P. luminescens* in the intestine and disperse from the cadaver in search of another insect victim.

Iron is essential to most bacteria and is often found at limiting concentrations in soil and water habitats and in eukaryotic hosts. Larval stages of the tobacco hornworm, *Manduca sexta*, contain a ferritin-type iron binding protein in the hemolymph (26, 38). One mechanism that bacteria use to acquire iron from eukaryotic hosts is to produce siderophore molecules that have high affinity for iron and form soluble iron complexes to sequester and transfer ferric iron into the bacterial cells (for reviews, see references 16, 37, and 43). Members of the family *Enterobacteriaceae* typically produce catechol and hydroxamate siderophores (16, 17, 43), some of which are considered to be virulence factors that capture iron from its bound form, usually as ferritin, in eukaryotic hosts (43). Siderophores can also function in antibiosis; i.e., siderophores produced by rhizobacteria can inhibit the growth of pathogenic organisms in the rhizosphere and enhance plant growth (30, 54). Siderophore activity was detected in cultures of *P. luminescens* (4) but its role in nematode symbiosis or insect virulence was not studied.

We recently reported a mini-Tn*5* transposon mutant of *P. luminescens* that was inadequate for nematode growth and reproduction and unable to express siderophore and antibiotic activities (13). The transposon-disrupted gene, *ngrA*, encodes a protein that is homologous to *entD*, a 4′-phosphopantetheinyl transferase (PPTase) that is required for the biosynthesis of the catechol siderophore enterobactin (15, 31). Members of the PPTase superfamily are required for the acyl or peptidyl carrier protein (ACP or PCP) activity (9, 31) involved in the biosynthesis of a great diversity of fatty acid, polyketide, and nonribosomally synthesized peptide molecules. It is plausible that the defect of the *ngrA* mutant in supporting nematode growth and reproduc-

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TABLE 1. Strains and plasmids used in this study

Organism or plasmid	Relevant characteristics	Source or reference
Nematodes		
Heterorhabditis bacteriophora	Strain NC1	29
Heterorhabditis megidis	Meg	41; H. Kaya
Bacteria		
Photorhabdus luminescens		
NP1/1	Primary phase, isolated from *H. bacteriophora* (identical to ATCC 29304, strain NC-19)	29
NC1/2	Secondary phase, isolated from NP100	13
Meg/1	Primary phase, isolated from *H. megidis*	41
NP394	NP1/1 with pUB394	13
NGR209	NP1/1 *ngrA*::mini-Tn*5*	13
NS414	NP1/1 *phbH*::mini-Tn*5*	This study
E. coli DH5α	Cloning strain	Gibco BRL
Plasmid vectors		
pGEM11Z(+)	Cloning vector	Promega
pBC SK(+)	Cloning vector	Stratagene
pUB394	Mini-Tn*5* delivery vector; Km^r, Sm^r Sp^r sucrose	13
p414	Retrieved plasmid with mini-Tn*5* and flanking *P. luminescens* DNA from NS414	This study

tion is directly linked to siderophore biosynthesis since *P. luminescens* may produce a siderophore that requires a holo-PCP that is covalently modified by a PPTase enzyme for siderophore biosynthesis.

To determine if siderophore activity is necessary for *P. luminescens* to support the growth and reproduction of its nematode host, we used mini-Tn*5* mutagenesis to produce mutants producing no detectable activity. One such mutant was tested for its effect on nematode symbiosis and for virulence to insect larvae. Purification and structure determination of the siderophore molecule and analysis of the genes involved in its synthesis and transport are also described.

MATERIALS AND METHODS

Microbial methods. The sources of the strains and plasmids used in this study are shown in Table 1. Dyes, antibiotics, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), and microbiological media were obtained from Difco (Detroit, Mich.). Cells of *P. luminescens* were grown in 2% Proteose Peptone no. 3, with 1.5% agar added when required, at 29°C in the dark. Kanamycin (10 μg/ml), streptomycin (20 μg/ml), spectinomycin (20 μg/ml), and sucrose (7.5%, wt/vol) were added when required. For siderophore production, cells were grown in acid-washed glassware with iron-depleted A-2 minimal medium [20 mM glucose, 10 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0), 5 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.5 mM NaCl] or morpholinepropanesulfonic acid (MOPS) defined medium (36) supplemented with 3% (vol/vol) glycerol and 1× minimal essential medium-vitamin solution (Gibco Invitrogen Corp., Carlsbad, Calif.) that was prepared with Milli-Q purified water (Millipore Corp., Bedford, Mass.) and batch treated with Chelex resin in accordance with the manufacturer's (Bio-Rad Laboratories, Hercules, Calif.) instructions. *Escherichia coli* was propagated at 37°C in Luria-Bertani medium with 1.5% agar; ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (10 μg/ml), streptomycin (20 μg/ml), spectinomycin (20 μg/ml), and sucrose (7.5%, wt/vol) were added when required.

Transposon mutagenesis and screen for siderophore mutants. Transposon-induced mutants were generated from NP394, which contains a transposon delivery vector, as described previously (13). Siderophore activity was assayed on chrome azurol S (CAS) medium (45). Bacteria producing siderophore activity formed a clear halo around the colony on the blue CAS medium because of the removal of Fe^{3+}. One mutant, named NS414, not producing a halo on CAS medium was isolated and characterized.

Phenotypic characterization. Expression of phase-dependent characteristics by *P. luminescens* cells was determined as described previously (4, 13) and repeated three times. The ability of *P. luminescens* mutants to support nematode growth and reproduction was determined by adding 30 DJ nematodes to lipid agar containing a lawn of *P. luminescens* cells as described previously (13). Nematode growth and reproduction were characterized by the recovery of DJ nematodes, development to hermaphrodites 7 days after the addition of DJ nematodes, and total DJ nematode yields 20 days after DJ nematode addition. The ability of *P. luminescens* cells to be retained in the DJ nematode gut mucosa was also determined as described previously by counting the CFU from homogenized, surface-sterilized, freshly harvested (<3 days old) DJ nematodes and DJ nematodes that had been incubated in saline for 30 days (14).

Insect pathogenicity was tested by injecting third-instar larvae of *Manduca sexta* (Carolina Biological Supply, Burlington, NC) or *Galleria mellonella* larvae (Ja-Da Bait Co., Antigo, Wis.) with serial dilutions of *P. luminescens* cells as described previously (13). Oral insecticidal activity (5) was assayed with 15×-concentrated cell-free supernatants obtained from 72-h Proteose Peptone no. 3 cultures. A 0.05-ml portion of the concentrate was applied to the surface of a 1-cm^3 disk of insect diet, and a single first- or second-instar larva of *M. sexta* was added.

Molecular biological techniques. Plasmid preparations were performed with Wizard minipreps in accordance with the manufacture's (Promega Corp., Madison, Wis.) instructions. Restriction enzymes were used in accordance with the manufacturer's (Promega Corp.) instructions, as were *Nsi*I and T4 ligase (New England Biolabs Inc., Beverly, Mass.). The bacterial DNA was purified with a modified cetyltrimethylammonium bromide method (10). Transformation of *E. coli* and *P. luminescens* was done by electroporation with a Bio-Rad Gene Pulser under the conditions suggested for *E. coli* by the supplier (Bio-Rad).

Retrieval of DNA adjacent to the transposon insertion. The DNA from NS414 was purified, restriction enzyme digested with *Nsi*I (the mini-Tn*5* transposon contains no *Nsi*I sites), intramolecularly ligated (ligation reaction carried out in a 0.25-ml volume), ethanol precipitated, and transformed by electroporation into *E. coli* DH5α cells. Transformed cells containing the transposon were selected by their resistance to kanamycin. The plasmid was purified and restriction enzyme digested with *Nsi*I and *Sfi*I to verify that it contained a single restriction fragment and the mini-Tn*5* transposon (determined by the presence of a 2.9-kb *Sfi*I restriction fragment). A 26-kb plasmid containing the mini-Tn*5* transposon and DNA flanking the insertion was retrieved from mutant NS414 and named p414.

Sequence analysis of p414. The sequence of DNA flanking the transposon insertion of p414 was obtained by using M13 forward and reverse primers located 60 or 40 bp from the I-end or O-end inverted repeats of the transposon and by primer walking. The oligonucleotide primer 5′TAAGCGCCTTCCTGCATGG CTT3′ was used to sequence DNA flanking the alternative O end (13). An *Eco*RI restriction fragment from p414 was cloned into pGEM11Zf(+) (Promega Corp.) and sequenced with M13 forward and reverse sequencing primers and by primer walking. Dye terminator cycle sequencing with an ABI terminator mixture was performed under the conditions suggested by the supplier (Perkin-Elmer Corp., Foster City, Calif.), and the products were analyzed on an ABI 377 automated sequencer. The similarity of the DNA sequences to known sequences was determined by BLAST analysis (3).

Siderophore purification. The siderophore was initially purified from 1.5 liters of culture broth obtained from *P. luminescens* cells grown while shaken at 29°C to stationary phase (72 h) in iron-depleted A-2 minimal medium (250 ml in each of eight 2-liter flasks). The cells were sedimented from the culture by centrifugation at 6,000 × *g* for 15 min. The supernatant fluid was extracted twice, for 15 min each time, with equal volumes of ethyl acetate, and the combined extracts were evaporated to dryness under vacuum at 50°C. The residue was dissolved in 1.0 ml of ethyl acetate to which 4.0 ml of methanol-water-acetic acid (90:9:1) was added. The resulting solution was diluted to 100 ml with water, and an insoluble residue was removed by centrifugation. The supernatant was applied to a C_{18} cartridge (Burdick & Jackson, Inc., Muskegon, Mich.) equilibrated with water. The cartridge was washed with 10 ml of water and then eluted sequentially with 10 ml each of 20, 40, 60, 80, and 100% acetonitrile. Siderophore activity was confined to the 40% acetonitrile fraction. Siderophore purification was performed on a Hewlett Packard 1100 high-performance liquid chromatograph equipped with a diode array detector. Aliquots from the 40% acetonitrile fractions were diluted fourfold with 0.1% trifluoroacetic acid (TFA), applied to a C_4 column (4.66 by 250 mm; Vydac; Hesperia, Calif.), and eluted with a 1% min^{-1} gradient of acetonitrile in 0.1% TFA at 0.5 ml min^{-1}.

Siderophore purification was improved by modifying the above-described procedure as follows. Siderophore was produced in 4.75 liters of MOPS defined medium and extracted twice overnight with 0.5 volume of ethyl acetate. The

TABLE 2. Comparison of the phenotypes of NS414 with those of NC1/1, NC1/2, and NGR209

Phenotype assayed	Reaction of strain:			
	NC1/1 (primary)	NC1/2 (secondary)	NGR209 *ngrA*	NS414
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	+	−	+	+
Support of nematode growth and reproduction[c]				
Development of DJ nematodes[a]	19 (6)	0	0	18 (3)
Final yields of DJ nematodes[b]	>10,000	0	0	>10,000
Colonization of DJ nematodes				
3 days postcollection	88 (42)	ND[d]	72 (27)[e]	94 (37)
30 days postcollection	122 (60)	ND	102 (39)	110 (25)
G. melonella infectivity	+	ND	+	+
Insect pathogenicity				
Injected cells[f]	+	+	+	+
Oral cell-free activity[g]	+	ND	+	+

[a] Mean numbers of adult hermaphrodites present from 30 DJ nematodes added; standard deviations are in parentheses.
[b] Nematode growth and reproduction on LA and LCM (13) and in *G. mellonella* hosts from an inoculum of 30 DJ nematodes.
[c] Numbers of CFU per DJ 3 or 30 days after collection of DJ nematodes. *G. melonella* infectivity is positive if the LD_{50} is <30 DJ nematodes 72 h after addition.
[d] ND, Not determined.
[e] When cocultured with Meg/1 to support nematode growth and reproduction.
[f] LD_{50} of <10 CFU 72 h postinjection of third-instar *M. sexta* larvae.
[g] No weight gain of third-instar *M. sexta* larvae when 0.05 ml of concentrated 20× cell-free supernatant from a 72-h culture was added to a 1-cm piece of diet.

combined extract was evaporated to dryness under vacuum at 50°C and resuspended in 4.8 ml of 100% methanol, and then 115.2 ml of Milli-Q water was added. Ten-milliliter aliquots were applied to C_{18} SepPak cartridges (Alltech Associates, Deerfield, Ill.) equilibrated with water, and 20, 40, 60, 80, or 100% methanol was used instead of acetonitrile where the siderophore eluted in the 60% methanol fraction. Active fractions were pooled; methanol evaporated under a gentle stream of nitrogen gas; extracted with ethyl acetate, which was then evaporated under nitrogen gas; dissolved with 0.4 ml of 100% methanol, where 3.6 ml of Milli-Q water was added; applied to a Nucleosil C_{18} 5-μm column (4.6 by 250 mm Supelco, Bellefonte, Pa.); and eluted with a 0.5% min^{-1} 30 to 80% methanol gradient at 0.5 ml min^{-1} on an Isco 2350 high-performance liquid chromatograph equipped with a 2360 gradient programmer and a V^4 absorbance detector set at 320 nm. A single peak containing siderophore activity was collected (92- to 94-min eluates); evaporated; dissolved in 0.05 ml of 100% methanol, where 0.45 ml of Milli-Q water was added; and applied to and eluted from the C_{18} column as described above. This procedure yielded 10.5 mg of pure siderophore that was used for subsequent structure determination.

Photobactin characterization. Accurate mass measurements were performed on an Applied Biosystems Mariner time-of-flight spectrometer configured with a turbo-ion spray source operated in positive-ion mode (spray tip potential, 4,500 V; spray chamber temperature, 400°C; nozzle potential, 110 V). ^1H (400 MHz) and ^{13}C (100 MHz) nuclear magnetic resonance (NMR) spectra were recorded as a CD_3OD solution at 300 K with a Bruker DRX 400 spectrometer equipped with a Nalorac 3-mm MdG-400B probe head. Chemical shifts were referenced to δ 3.30 and 49.0 for ^1H and ^{13}C spectra, respectively. A series of two-dimensional (2D) NMR experiments were used to secure the structure of photobactin. One-bond proton-carbon couplings were determined with a multiplicity-edited heteronuclear single quantum coherence experiment. Constant-time heteronuclear proton-carbon multiple-bond coherence data were used to establish long-range proton-carbon couplings. Homonuclear proton couplings were measured with a pure-absorption total correlated spectroscopy experiment.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence flanking the mini-Tn*5* transposon of p414 is AY042783.

RESULTS

Isolation and characterization of a siderophore-deficient mutant. A mutant producing no detectable siderophore activity with the CAS plate assay (45), NS414, was obtained by screening 650 mini-Tn*5*-induced mutants.

NS414 resembles the wild type in most characteristics except in siderophore production and growth in iron-deficient medium. The NS414 cells grew poorly in iron-depleted A-2 minimal medium, reaching a maximal A_{600} of 0.05. Cells of NC1/1 reached an A_{600} of 0.3 in this medium. The addition of 10 μM (final concentration) ferric chloride or ferric sulfate to the A-2 medium allowed the NS414 cells to grow to an A_{600} of 0.3. These data indicate that siderophore production is involved in sequestering Fe^{3+} and is required for *P. luminescens* to grow efficiently under iron-limiting conditions.

NS414 and NC1/1 (wild type) were indistinguishable with respect to the symbiotic interactions with the nematode under the same experimental conditions in which an *ngrA* mutant

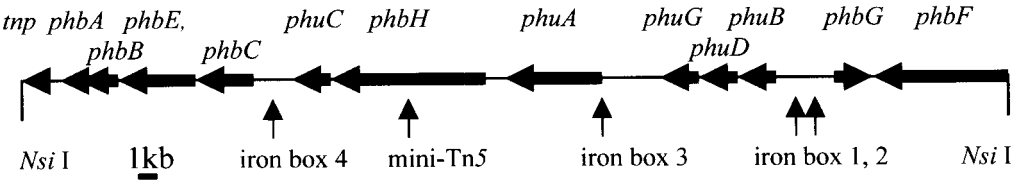

FIG. 1. Structure of the DNA flanking the mini-Tn*5* transposon insertion of NS414. Shown are the locations of the site of the mini-Tn*5* transposon insertion and the locations of putative iron boxes.

(also unable to produce detectable siderophore activity) was unable to support nematode growth and reproduction (Table 2). Nematodes grew equally well on LA medium (13) in the presence of NS414 or NC1/1; inoculated DJ nematodes developed to hermaphrodites to the same extent, and final DJ nematode yields were similar. The DJ nematodes retained the NS414 and NC1/1 cells equally well, and these bacteria persisted equally well in DJ nematodes incubated in saline for 30 days (Table 2). Furthermore, DJ nematodes retaining NS414 and NC1/1 cells were equally pathogenic to insects (LD_{50} at 72 h, <30 DJ nematodes). NS414 and NC1/1 were similar in insect virulence when injected into *G. mellonella* or *M. sexta* larvae (LD_{50} at 72 h, <30 cells). The levels of insecticidal activity of NS414 and NC1/1 given orally were also similar. In summary, NS414 supported nematode growth and reproduction, colonized and persisted in DJ nematodes, and had insect virulence indistinguishable from that wild-type NC1/1 *P. luminescens*.

NS414 cells were identical to NC1/1 cells in all of the characteristics observed, including the production of antibiotics, pigments, exopolysaccharides, and crystalline inclusion proteins; bioluminescence; and dye uptake (Table 2). The only detectable difference between NS414 and NC1/1 cells was the loss of siderophore production by NS414. Furthermore, no phase instability greater than that of NC1/1 was noticed when NS414 was subcultured for more than 6 months. It is evident that siderophore activity does not affect the expression of many primary-phase phenotypes or alter the stability of the primary phase.

The mini-Tn*5* transposon of NS414 was inserted into an ORF homologous to nonribosomal peptide synthetases. A 26-kb plasmid, p414, containing 22 kb of DNA flanking the mini-Tn*5* transposon insertion was retrieved from NS414 cells. The physical map of this DNA region is shown in Fig. 1. The mini-Tn*5* transposon was inserted into an ORF that is homologous to PCP modules of nonribosomal peptide synthetases (34, 46). The protein product of this gene, named *phbH* (for photobactin synthetase protein), is 34% identical and 50% similar to amino acid residues 1423 to 2155 of VibF (accession no. AAF02102), which are required for production of the siderophore vibriobactin by *Vibrio cholerae* (6, 27) (Table 3). This region corresponds to the thiolation and condensation domains of PCP modules (6, 34, 46). A serine residue present in the thiolation domain is indicative for covalent binding of phosphopantetheinate by a PPTase (31).

Two other genes, *phbF* and *phbG*, which are also homologous to nonribosomal peptide synthetases, were found in the vicinity of *phbH*. The protein product of *phbF*, like PhbH, is also similar to VibF, but to amino acid residues 187 to 391 and 490 to 1392, which correspond to aminoadenylation domains A1 to A10 (34). The adenylation domain determines the substrate specificity of the module, and "codons" have been suggested in 10 important amino acid residues that are indicative of the molecule to be adenylated (47). The codon of PhbF is 80% identical to the VibF codon (6, 28), which adenylates threonine, forming each of the two oxazoline rings found in the vibriobactin molecule (25, 27). The third putative nonribosomal peptide synthetase gene is *phbG*, whose protein product is similar to VibH of *V. cholerae* and EntF of *E. coli* (Table 2). Only the condensation-elongation C3 (His) domain characteristic of nonribosomal peptide synthetases is present in PhbG (34).

TABLE 3. Characteristics of genes flanking the mini-Tn*5* transposon in mutant NS414[a]

Gene	% Similarity/ % identity	Significance (E value)	Accession no.	Protein	Function(s)	Organism
phbF	49/65	0	AAF02102	VibF	Nonribosomal peptide synthetase	*V. cholerae*
phbG	32/49	4e − 54	AAD48879	VibH	Nonribosomal peptide synthetase	*V. cholerae*
phuB	56/73	2e − 44	AAD48880	ViuP	Periplasmic binding protein	*V. cholerae*
phuG	46/61	5e − 78	AAD48882	ViuG	Cytoplasmic membrane permease	*V. cholerae*
phuD	52/65	7e − 75	AAD48881	ViuD	Cytoplasmic membrane permease	*V. cholerae*
phuA	46/64	1e − 163	AAF28471	VuuA	Vulnibactin outer membrane receptor	*V. vulnificus*
phbH	34/50	1e − 106	AAF02102	VibF	Nonribosomal peptide synthetase	*V. cholerae*
phuC	64/77	1e − 92	AAD29087	FepC	Siderophore transport ATP binding	*Y. enterocolitica*
phbC	56/68	8e − 89	AAA16100	EntC	Isochorismate synthase	*E. coli*
phbE	66/79	0	AAC73695	EntE	2,3-Dihydroxybenzoate-AMP ligase	*E. coli*
phbB	55/70	1e − 85	AAB40795	EntB	2,3-Dihydro-2,3-dihydroxy, benzoate synthase	*E. coli*
phbA	63/79	2e − 88	AAA76836	EntA	2,3-Dihydro-2,3-dihydroxy, benzoate dehydrogenase	*E. coli*
tnpA	56/76	2e − 89	AAA58209	YhgA	Hypothetical transposase	*E. coli*

[a] Similarity values are for the most similar proteins, determined by BLASTp analysis (3), whose accession numbers, names, functions, and source organisms are also shown.

Iron box	location	sequence	identity
		--------> <--------	
Consensus		GATAATGATAATCATTATC	
Iron Box 1	16026	A**ATAATGAT**T**ATCATA**T**AC**	14/19, 74%
Iron Box 2	15965	C**AT**TCA**GATAATC**CAA**A**GT	10/19, 53%
Iron Box 3	12379	A**ATA**GT**AATAATTATCATA**	13/19, 68%
Iron Box 4	5829	A**ATAAT**A**ATAATTATC**A**AT**	13/19, 68%

FIG. 2. Alignment of putative iron boxes in p414. Bold and underlined residues are identical to the consensus sequence. Arrows above the sequence represent the inverted repeat of the iron box. The locations and percent identities of the four putative iron boxes are also shown.

The *phbA*, *phbB*, *phbC*, and *phbE* genes are homologous to *entA*, *entB*, *entC*, and *entE*, which encode 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, 2,3-dihydro-2,3-dihydroxybenzoate synthase, isochorismate synthase, and 2,3-dihydroxybenzoate-AMP ligase, respectively (Table 3) (32, 33, 55). These proteins are involved in the synthesis of the 2,3-dihydroxybenzoic acid moiety that is characteristic of the catechol class of siderophores. Homologous genes are also required for the biosynthesis of vibriobactin, which contains three 2,3-dihydroxybenzoic acid moieties (25, 52; see Fig. 4B). EntB is a multifunctional protein that has a C-terminal aryl carrier protein containing a thiolation domain that requires covalent modification by a PPTase (31). The predicted protein encoded by *phbB* also contains the thiolation domain (34). The protein encoded by *phbE* contains an adenylation domain with a codon that is similar to domains that adenylate 2,3-dihydroxybenzoate like EntE and VibE (28).

The *phuA*, *phuG*, *phuB*, and *phuC* (photobactin uptake) genes are homologous to genes whose products are involved in TonB-dependent high-affinity transport systems (Fep) (17, 50). (Table 3). The *phuA* gene encodes a protein that is homologous to the outer membrane receptor for vulnibactin, VuuA, in *V. vulnificus* (51) and ViuA for vibriobactin in *V. cholerae* (7). The *phuG* gene encodes a protein that is homologous to ViuG, FepG, and other cytoplasmic membrane permease proteins (11, 12, 53). The *phuB* gene likely encodes a periplasmic ferric-siderophore binding protein analogous to the lipoprotein ViuP (53). The *phuC* gene encodes a protein that is homologous to ViuC and FepC, which are homologous to ATPase proteins of ABC transporters (11, 12, 53).

The *tnpA* gene is located at one 5′ end of the photobactin region, and its protein product is homologous to a group of hypothetical transposases and is most similar to YhgA from *E. coli*.

The photobactin gene region also contains four potential iron boxes (Fig. 1). Iron boxes 1 and 2, upstream of *phuB*, are 74 and 53% identical to the consensus iron box (8, 20) (Fig. 2). Iron box 3, located 5′ of *phuA*, is 68% identical to the consensus iron box. Iron box 4, located 5′ of *phbC*, is also 68% identical to the consensus iron box.

Photobactin is a novel catechol-type siderophore. Two peaks with siderophore activity were detected from the C_4 column eluted with a gradient of acetonitrile in 0.1% TFA. Most of the activity was observed in a peak eluting at 37 min, with lesser activity eluting at 29 min (Fig. 3A). However, on standing in the presence of 0.1% TFA, material from the 37-min peak also eluted at 29 min when reanalyzed under identical conditions, indicating alteration of the siderophore under acidic conditions (Fig. 3B). The UV spectrum of the 37-min peak obtained with the diode array detector was characteristic of catechol siderophores such as vibriobactin (25) and agrobactin (39), with absorbance maxima at 257 and 316 nm (Fig. 3C). The UV spectrum of the 29-min peak exhibited UV maxima at 250 and 316 nm. The shift of the absorbance maximum from 257 to 250 nm and the loss of absorbance in the 280-nm range were similar to those observed for the siderophore agrobactin under acidic conditions and indicate the presence of an oxazoline ring (39).

No alteration of the siderophore was observed when a methanol rather than an acetonitrile–0.1% TFA gradient was used to elute the siderophore from the C_{18} columns. This preparation of photobactin was used for structure determination. A molecular formula of $C_{22}H_{25}N_3O_7$ for photobactin was determined from electrospray ionization time-of-flight mass spectrometry (*m/z* 444.1765 [M^+ + H calculated 444.1765]) and ^{13}C NMR data. The structure of photobactin (Fig. 4A) was readily determined from a series of 2D NMR experiments. The full chemical shift assignments and key 2D NMR correlations appear in Table 4. The International Union of Pure and Applied Chemistry name for this molecule is 2-(2,3-dihydroxyphenyl)-5-methyl-4,5-dihydro-oxazole-4-carboxylic acid [4-(2,3-dihydroxybenzoylamino)-butyl]-amide. The structure of photobactin (Fig. 4A) is related to that of vibriobactin (Fig. 4B).

A limited amount of pure photobactin was assayed for antibiotic activity. Approximately 100 μg was dissolved in 0.1 ml of 100% methanol, and 10 μg was applied to a 6-mm-diameter filter disk, which was placed on an overnight culture of indicator bacteria. These experiments were repeated twice. Antibiotic activity was detected with *Micrococcus luteus* (3-mm zones of inhibition), *Bacillus cereus* (4-mm zones of inhibition), and *Staphylococcus aureus* (1.5-mm zones of inhibition) but not with *E. coli* or *Bacillus subtilis*.

DISCUSSION

Here we report the purification, the proposed structure, and most of the genes involved in the biosynthesis and transport of a novel catecholate siderophore, photobactin, produced by *P. luminescens*. This study was initiated to determine if siderophore production is required for *P. luminescens* to support the growth and reproduction of its nematode host. The rationale for this hypothesis was that *ngrA*, encoding a putative PPTase often involved in siderophore biosynthesis, is necessary for the bacteria to support the growth and reproduction of its

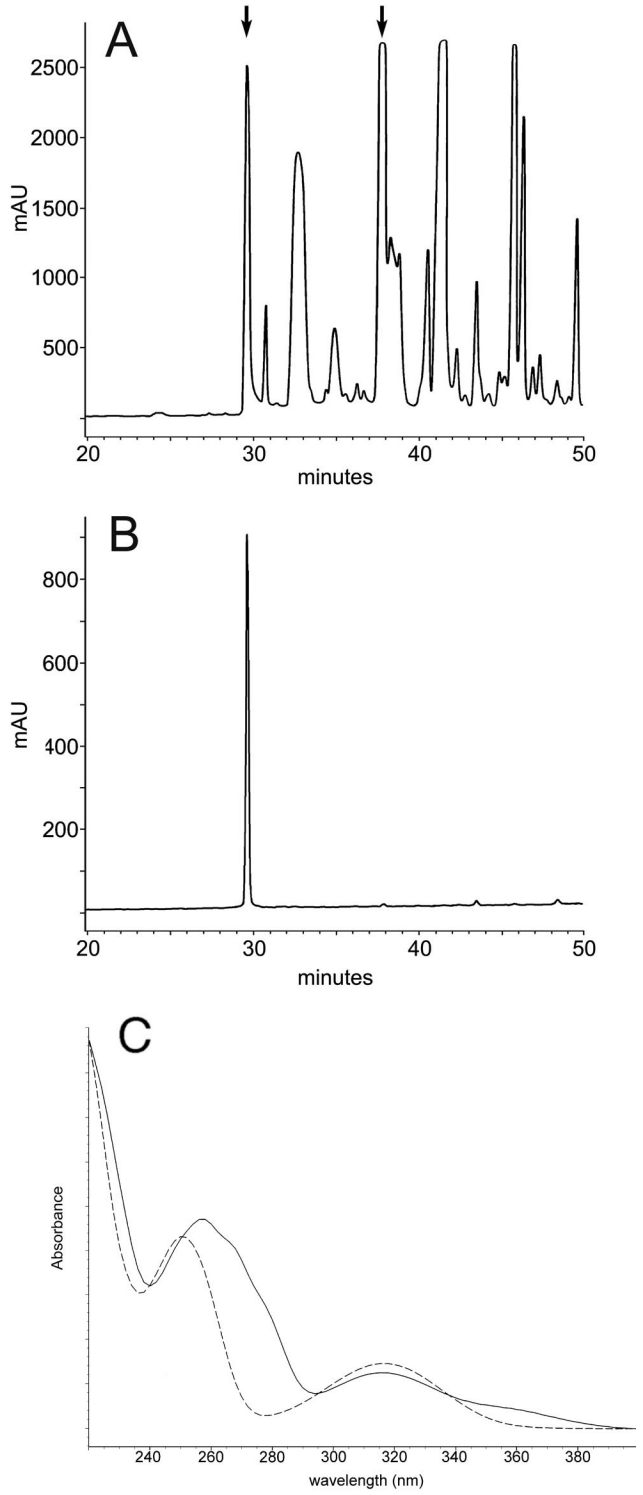

FIG. 3. (A) C_4 reverse-phase chromatograph of the siderophore-containing fraction obtained from the C_{18} cartridge. The arrows denote the peaks containing siderophore activity (29 to 30 min and 38 min). (B) When stored in 0.1% TFA, the 38-min peak shifted to 29 to 30 min. (C) UV spectra of the 38-min (solid line) and 29- to 30-min (dashed line) siderophore peaks. The shift of the absorbance maximum from 257 to 250 nm and the loss of absorbance in the 280-nm range indicate the opening of an oxazoline ring.

nematode host and to produce siderophore activity (13). Indeed, *phbH*, encoding a putative PCP likely requiring a PPTase for its activity, is required for siderophore activity but not for symbiosis with the nematode. Thus, the siderophore is not the nematode growth- and reproduction-promoting factor that was absent in the *ngrA* mutant.

The proposed structure of photobactin (Fig. 4A) is related to the siderophore molecules vibriobactin (25; Fig. 4B) and agrobactin (39) produced by *V. cholerae* and *Agrobacterium tumefaciens*, respectively. Photobactin contains a putrescine backbone versus the spermidine and norspermidine backbones of agrobactin and vibriobactin, respectively, and as a consequence of one less amide group of putrescine, photobactin contains one less oxazoline-2,3-dihydroxybenzoicacid or 2,3-dihydroxybenzoic acid moiety.

Since photobactin and vibriobactin are structurally related, it is not surprising that the corresponding biosynthetic and transport genes are also similar, with some important differences. Genes involved in photobactin biosynthesis and transport are clustered in a single gene region, in contrast to the vibriobactin biosynthesis uptake and transport genes that are present in two regions of the *V. cholerae* chromosome, and many of these genes are arranged differently. One terminus of the photobactin gene cluster contains a transposase-like gene that may indicate that this region is present in or originated from a mobile genetic element like a virulence plasmid or pathogenicity island. The thiolation and adenylation domains of the unusual nonribosomal peptide synthetase VibF (27) are present as two subunits in *P. luminescens*, with PhbH containing the thiolation domain and PhbF containing the adenylation and cyclization domains. Because putrescine, versus norspermidine, is the amine backbone of photobactin, biochemical characterization of PhbF may reveal different specificities for acylation of primary amines than that of VibF (27). These unique characteristics of PhbH and PhbF may be useful in engineering polyketide or nonribosomal peptide synthetases for the biosynthesis of novel natural products. Finally, it is interesting that a PPTase seems to be absent from the photobactin gene cluster while it is present in the vibriobactin gene cluster, suggesting that NgrA (13) may perform this function.

NgrA is most similar to *E. coli* EntD, which is a PPTase required for the biosynthesis of the catechol siderophore enterobactin (15, 23, 31) and to the putative *V. cholerae* VibD PPTase found in the vicinity of vibriobactin biosynthesis and uptake genes (51). The disrupted *phbH* gene of NS414 encodes a PCP-containing thiolation domain. Covalent modification of PCPs with a 4′-phosphopantetheinyl moiety by a PPTase is required for PCP activity (24, 48, 49). Another gene, *phbB*, located in the vicinity of *phbH*, likely encodes an aryl carrier protein that also requires a PPTase for its activity (23). NgrA may covalently modify the thiolation domains of PhbH or PhbB, since a PPTase was not found in this siderophore region. However, the ability of the *phbH* mutant NS414 to support nematode growth and reproduction suggests that the phosphopantethenylation of another PCP or ACP, involved in the biosynthesis of an unknown metabolite, is the cause for the inadequacy of the *ngrA* mutant of *P. luminescens* in supporting nematode growth and reproduction. It is possible that NgrA regulates the activity of multiple PCPs or ACPs through post-translational phosphopantethenylation.

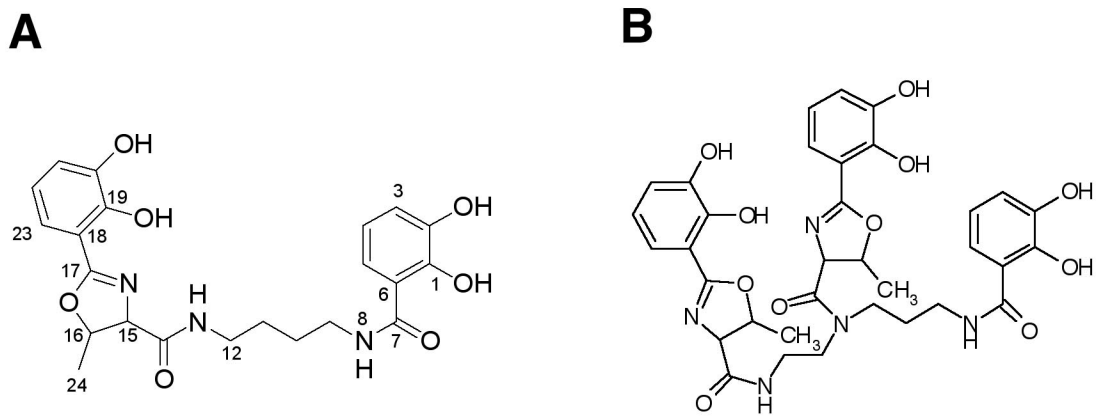

FIG. 4. Structures of photobactin (A) and vibriobactin (B). The atom numbering scheme used to describe data from the NMR experiments is shown for photobactin.

Mutant NS414 resembled the wild-type bacteria in all characteristics other than siderophore production. It is significant that the characteristics of nematode symbiosis, insect virulence, and phase variation were unchanged. Therefore, the mini-Tn*5* transposon-disrupted siderophore gene *phbH* is not essential for nematode symbiosis or insect virulence. However, mutant NS414 grows poorly in iron-deficient medium, indicating that *phbH* is required for growth in low-iron environments. Insects, like humans, produce high-affinity iron binding proteins, possibly to maintain a low concentration of iron in the hemolymph (26, 38). Since NS414 grows poorly in iron-deficient medium, it appears that the insect cadaver is not iron limited or that *P. luminescens* may have other mechanisms by which to obtain iron from insects, such as the action of secreted hemolysins, lipases, or proteases. Although NS414 does produce antibiotic activity, probably through the production of other broad-spectrum antibiotics (1, 40, 44), siderophore production may contribute to antibiosis in the insect host since purified siderophore has antibiotic activity. When growing in insect larvae, *P. luminescens* produces antibiotics that are thought to inhibit competing saprophytic bacteria (1, 40, 44). Photobactin may perform a similar role by sequestering iron in the insect cadaver.

The data presented herein clearly show that the inability of the *ngrA* mutant to support nematode growth and reproduction is not due to loss of siderophore activity. The symbiotic defect of the *ngrA* mutant must therefore be due to the inability to produce another nonribosomally synthesized peptide, polyketide, or lipopeptide molecules that require PPTase for its biosynthesis. The search for the NgrA target required for nematode symbiosis is thus narrowed.

TABLE 4. NMR data for photobactin

Position[a]	δ_C	δ_H, multiplicity, J (H_z)	HMBC[b]
1	150.3		
2	147.3		
3	119.56 or 119.52	6.91, dd, 8.0, 1.5	C-1, C-5
4	119.56 or 119.52	6.69, t, 8.0	C-2, C-6
5	118.6	7.18, dd, 8.0, 1.5	C-1, C-3, C-7
6	116.7		
7	171.5		
9	40.13 or 40.06	3.38, br t, 6.5	C-7, C-10 or C-11
10	27.79 or 27.75	1.61, m	C-11
11	27.79 or 27.75	1.61, m	C-10
12	40.13 or 40.06	3.28, br t, 6.5	C-10 or C-110, C-14
14	172.9		
15	75.8	4.41, d, 7.5	C-14, C-16, C-17, C-24
16	80.7	4.86, dd, 7.5, 6.5	C-14, C-17
17	168.4		
18	111.8		
19	149.5		
20	146.7		
21	120.3	6.94, dd, 8.0, 1.5	C-19, C-24
22	119.92 or 119.87	6.72, t, 8.0	C-18, C-20
23	119.92 or 119.87	7.15, dd, 8.0, 1.5	C-17, C-19, C-21
24	21.3	1.51, t, 6.5	C-15, C-16

[a] Position as indicated in Fig. 4A.
[b] HMBC, heteronuclear multibond conductivity.

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