The PhlA Hemolysin from the Entomopathogenic Bacterium *Photorhabdus luminescens* Belongs to the Two-Partner Secretion Family of Hemolysins

Julien Brillard,^{1†} Eric Duchaud,² Noël Boemare,¹ Frank Kunst,² and Alain Givaudan^{1*}

Laboratoire EMIP, Université Montpellier II, IFR56, Institut National de la Recherche Agronomique (UMR 1133),
34095 Montpellier Cedex 5,¹ and Laboratoire de Génomique des Microorganismes Pathogènes,
Institut Pasteur, 75724 Paris Cedex 15,² France

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Photorhabdus is an entomopathogenic bacterium symbiotically associated with nematodes of the family Heterorhabditidae. Bacterial hemolysins found in numerous pathogenic bacteria are often virulence factors. We describe here the nucleotide sequence and the molecular characterization of the *Photorhabdus luminescens* *phlBA* operon, a locus encoding a hemolysin which shows similarities to the *Serratia* type of hemolysins. It belongs to the two-partner secretion (TPS) family of proteins. In low-iron conditions, a transcriptional induction of the *phlBA* operon was observed by using the chloramphenicol acetyltransferase reporter gene, causing an increase in PhlA hemolytic activity compared to iron-rich media. A spontaneous phase variant of *P. luminescens* was deregulated in *phlBA* transcription. The *phlA* mutant constructed by allelic exchange remained highly pathogenic after injection in the lepidopteran *Spodoptera littoralis*, indicating that PhlA hemolysin is not a major virulence determinant. Using the gene encoding green fluorescent protein as a reporter, *phlBA* transcription was observed in hemolymph before insect death. We therefore discuss the possible role of PhlA hemolytic activity in the bacterium-nematode-insect interactions.

Members of the family *Enterobacteriaceae* and the genus *Photorhabdus* (6) are associated with the entomopathogenic nematodes of the family Heterorhabditidae. These bacteria, which are highly pathogenic to insects, are transported by their nematode hosts into the hemocoel of the insect prey, which is killed probably via a combination of toxin action and septicemia. The bacterial symbionts contribute to the symbiotic relationship by establishing and maintaining suitable conditions for nematode reproduction. During the final stages of development, the bacteria and the nematode reassociate and subsequently leave the insect carcass in search of a new insect host (26). Recently, isolations of some nonsymbiotic *Photorhabdus* strains from infected humans in Australia and the United States were reported (21, 40).

The wild-type bacterium that is normally isolated from symbiotic infective-stage nematodes is referred to as phase I. Like many pathogenic bacteria, *Photorhabdus* strains spontaneously produce colonial variants *in vitro* which have been called phase II variants (5, 26). Both variants of the bacteria have generally been shown to be equally pathogenic for the larvae of the greater wax moth, *Galleria mellonella* (26).

The bacterial factors involved in killing the insect or in overcoming the insect's immune reactions are still under investigation. After invasion of the insect host by the nematodes, bacteria produce potential virulence factors, including lipase, protease, and lipopolysaccharides in the hemocoel (for a re-

view, see reference 26). It was shown that purified lipopolysaccharide or *Photorhabdus* protease fractions had no toxic effect after injection into insect hemocoel (7, 13). Recently, a novel toxin complex (Tc) with oral toxicity to a wide range of insects was identified in a supernatant of *Photorhabdus luminescens* W14 (8). Purified toxin complex a (Tca) has specific effects on the midgut epithelium of the insect (4). It has recently been shown that Tca, Tcd, and an RTX-like metalloprotease are expressed *in vivo* during insect infection (17).

Hemolysins are extracellular toxic proteins produced by many gram-negative (e.g., *Escherichia coli*, *Vibrio* spp.) and gram-positive (e.g., *Streptococcus* spp., *Listeria* spp.) bacteria and are known to function as virulence factors. Most of them are active against a wide range of nucleated cell types and thus are also called cytotoxins. Bacterial hemolysins are usually recognized on blood agar plates where a transparent zone appears around colonies. Hemolysis production in some *Photorhabdus* strains was first described by Farmer et al. (21). These bacteria have been shown to display an unusual reaction on sheep blood agar plate, designated annular hemolysis (1); this reaction was considered to be a marker in the identification of *Photorhabdus asymbiotica* isolated from clinical specimens (21, 25). Recently, we described the production of zones of hemolysis on sheep blood agar around several strains of *Photorhabdus* and *Xenorhabdus*, another symbiotic bacterium of nematodes (11). We also identified the presence of a cytotoxin active on insect hemocytes and on sheep erythrocytes in *Xenorhabdus* culture supernatants, whereas such activity could not be detected in *Photorhabdus* supernatants. In *Photorhabdus* spp., other factors might be responsible for the hemolysis observed on blood agar medium. Therefore, we investigated hemolysin production by *Photorhabdus*.

The recently published partial genome sequence of *P. lumi-*

* Corresponding author. Mailing address: Laboratoire EMIP, CC101, INRA-Université Montpellier II (UMR 1133), Place E. Bataillon, 34095 Montpellier Cedex 5, France. Phone: 33-4-67144812. Fax: 33-4-67144679. E-mail: givaudan@crit.univ-montp2.fr.

† Present address: Unité de Biochimie Microbienne, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype ^a	Source or reference
Strains		
<i>P. luminescens</i> TT01	Wild type, phase I variant	CIP 105565
<i>P. luminescens</i> TT01/2	Phase II variant	Laboratory collection
<i>P. luminescens</i> TT01c	Col ^r natural mutant	Laboratory collection
<i>P. luminescens</i> TT01c- <i>phlA</i> ::Ω	TT01c <i>phlA</i> ::ΩCm	This work
<i>E. coli</i> XL1-Blue	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1 supE44</i>	Stratagene
MRF ^r	<i>thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI</i> ^q ΔM15 Tn5 (Km ^r)]	
<i>E. coli</i> S17-1	<i>pro</i> r ⁻ n ⁺ Tp ^r Sm ^r RP4-2-Tc::Mu::Tn7 <i>recA thi</i>	49
<i>E. coli</i> HB101	F ⁻ <i>mcrB mrr hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 (strR) supE44λ</i> ⁻	9
Plasmids		
pUC19	Ap ^r cloning vehicle	Biolabs
pJT02	1.2-kb <i>Hind</i> III internal fragment of <i>phlA</i> in the <i>Hinc</i> II site of pUC19	This work
pHP45-ΩCm	Ap ^r Cm ^r ΩCm	22
pJT02Ω	3.8-kb <i>Bam</i> HI fragment from pHP45-ΩCm in the <i>Nsi</i> I site of pJT02	This work
pJQ200KS	Gm ^r <i>sacRB mob oriV</i> (p15A replicon)	S. Forst
pJT421Ω	5-kb <i>Bam</i> HI- <i>Pst</i> I fragment of pJT02Ω in the <i>Bam</i> HI- <i>Pst</i> I sites of pJQ200KS	This work
pPLG-11B9	Bacterial artificial chromosome of <i>P. luminescens</i> genome (contains a 40-kb fragment overlapping <i>phlBA</i>)	This work
pRK404	Tc ^r low-copy mobilizable plasmid	19
pJT95	9.7-kb <i>Bam</i> HI fragment of pPLG-8C6 in the <i>Bam</i> HI site of pRK404 (contains <i>phlBA</i>)	This work
pRK2013	Km ^r (Tn903), ColE1 replicon, <i>tra</i> ⁺ (RK2); helper plasmid	24
pBBR1MAC2	Medium-copy mobilizable vector; Ap ^r ; <i>cat</i> reporter gene	38
pJT-PB-MAC	500-bp region overlapping <i>phlB</i> start codon cloned in <i>Bam</i> HI- <i>Kpn</i> I sites of pBBR1MAC2	This work
pBBR1KGFP	Medium-copy mobilizable vector; Km ^r ; <i>gfpmut3</i> reporter gene	33
pJT-PB-KGFP	500-bp region overlapping <i>phlB</i> start codon cloned in <i>Bam</i> HI- <i>Kpn</i> I sites of pBBR1KGFP	This work

^a Col^r, colicin resistant; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Tp^r, trimethoprim resistant; Cm^r, chloramphenicol resistant; Gm^r, gentamicin resistant; Tc^r, tetracycline resistant.

nescens W14 revealed a diverse array of genes that putatively encodes potential virulence factors (23). These factors include RTX-like toxins, hemolysin and cytotoxin homologs (23). Lately, the complete genome of *P. luminescens* subsp. *laumondii* TT01 was sequenced (E. Duchaud, unpublished data), confirming the presence of genes with similarities to various hemolysins.

In order to evaluate hemolysin contribution in *P. luminescens* TT01 to insect virulence, we characterized a hemolysin locus homologous to the loci of the pore-forming, calcium-independent hemolysins from *Serratia marcescens*, *Proteus mirabilis*, *Edwardsiella tarda*, and *Haemophilus ducreyi*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains and plasmids used in this study and their sources are listed in Table 1. *P. luminescens* was grown for 48 h at 28°C and strains of *Escherichia coli* for 24 h at 37°C, on Luria-Bertani (LB) broth or LB supplemented with 1.5% agar. When required, ampicillin, colistin, kanamycin, chloramphenicol (CHL), or tetracycline were added to the media at final concentrations of 100, 50, 20, 15, and 10 μg/ml, respectively. When necessary, growth media were supplemented with FeSO₄ to a final concentration of 100 μM or were modified to be iron deficient by addition of 0.3 mM 2,2'-dipyridyl. Colistin-resistant mutants arose at a frequency of ca. 10⁻⁹ (A. Givaudan, unpublished data). These strains behaved similarly to the parent strain in terms of growth rates, insect pathology, hemolysin production, and all other phenotypes that we tested. Phase variation was spontaneously obtained after long-term growth in LB broth as previously described (5). Wild type is referred to as phase I. Phase I colonies are blue when grown on NBTA (i.e., nutrient agar supplemented with 25 mg of bromothymol blue per liter and 40 mg of triphenyltetrazolium chloride per liter), are bioluminescent, and produce antimicrobial activity against *Micrococcus luteus* (from the culture collection of the Institut Pasteur, Paris, France), while phase II colonies are red, are not bioluminescent, and produce reduced or no antibacterial activity. Phase II of TT01 is indicated by addition of the suffix "2" to the strain designation.

Sequence analysis. The genome of the strain TT01 of *P. luminescens* has been sequenced by using the whole-genome shotgun strategy (Duchaud, unpublished).

Cloning, sequencing, and assembly were done as described by Frangeul et al. (27). Annotation was performed during the finishing phase by using GMP-Tool-Box (L. Frangeul and E. Duchaud, unpublished data). Coding sequences (CDS) were defined by combining Genemark predictions (30) with visual inspection of each open reading frame for the presence of a start codon preceded by a ribosome-binding site and BLASTP similarity searches on the Nprrot database (2).

Molecular genetic techniques. Genomic DNA from *P. luminescens* TT01 was prepared according to a method described previously (44). Endonucleases (Roche Molecular Biochemicals), DNA polymerase, calf intestine phosphatase, and T4 DNA ligase (Q-Biogene) were used according to the recommendations of the manufacturers. In order to control the *phlA* mutant, Southern blotting experiments were performed as previously described (44).

Construction of a *phlA*-null mutant. PCR was performed with the primers 1F (5'-CCACTCGTTAATCAGAGCA-3') and 1R (5'-CCCTTTATGCTAACCTG TGA-3') (Fig. 1A) to amplify the *phlBA* locus by using the *Tfu* polymerase, a high-fidelity polymerase. The 7-kb amplified fragment was subsequently digested with *Hind*III, and the fragments were separated on a 0.8% agarose gel. Fragments of 1.2 kb corresponding to the internal *phlA* region were collected by using Nucleotrap (Macherey-Nagel), blunt ended by using Klenow polymerase, ligated to *Hinc*II-digested and dephosphorylated pUC19 to yield to pJT02 (Table 1), and cloned in *E. coli* XL1MRF^r. The CHL-resistant Ω interposon, which has been described previously (22), was used for *phlA* disruption. Construction of pJT02Ω was performed by insertion of the *Bam*HI-digested and blunt-ended CHL-resistant Ω cassette ligated to blunt-ended, dephosphorylated *Nsi*I-digested pJT02. The *Nsi*I site is located approximately in the middle of the 1.2-kb *phlA* fragment of pJT02. Thereafter, the Ω cassette-interrupted *phlA* fragment was isolated by *Bam*HI-*Pst*I digestion and ligated to corresponding sites of the mobilizable plasmid pJQ200KS to create pJT421Ω (Table 1), which was transformed in *E. coli* S17-1. Conjugations between S17-1(pJT421Ω) and TT01c, followed by allelic exchange, were performed as described previously (28). Briefly, mating between bacterial cells was performed overnight on a nitrocellulose filter. After we made sure that transconjugants harbored pJT421Ω, we selected clones with allelic exchange on medium containing saccharose (4% [wt/vol]) and CHL. Insertion of the Ω cassette in the *phlA* gene of the resulting mutant (Fig. 1A) was confirmed by PCR amplification and Southern blot analysis. This mutant was termed TT01c *phlA*::Ω.

Complementation of *phlA* mutant. In the framework of the genome project, we have end sequenced 1,200 BAC clones, constructed in pBeloBAC11 (32). One

FIG. 1. Map of the *P. luminescens* *phlBA* hemolysin locus. (A) Transcription orientation of *phlB* and *phlA* (open arrows) are represented with the positions of the restriction endonucleases recognition sites used. The mutation obtained by Ω interposon insertion is indicated. Primers used and their orientation are represented by solid arrows. (B) Possible Fur-binding sites in the promoter region of the *phlBA* operon. The identities with the 19-nucleotide sequence of the Fur-binding consensus sequence reported previously (18) are indicated below the sequence, with matches represented by “+” and mismatches represented by “-”; the total number of identities is indicated at the end. The putative -10 box is overlined, and start codon is underlined.

BAC spanning the *phlBA* locus has been selected. This BAC (59 kb), called pPLG-11B9, was digested by *Bam*HI in order to isolate a 9.7-kb fragment containing *phlBA* and 1,000 bp upstream of *phlB* and then cloned in the *Bam*HI dephosphorylated site of the mobilizable plasmid pRK404, yielding pJT95 (Table 1). Because pJT95 lacks the *tra* genes, mating with TT01c *phlA::Ω* was performed with *E. coli* S17-1 harboring pJT95 and *E. coli* HB101 harboring pRK2013, a helper plasmid.

Hemolytic assays. Based on the description of an hemolytic assay to reveal the presence of ShlA activity in *Serratia marcescens* (10), we developed a liquid hemolytic assay with horse erythrocytes. It was performed with horse defibrinated blood (BioMérieux, Marcy l'Etoile, France), and hemolysis was expressed as the percentage of the total erythrocytes lysed. Phosphate-buffered saline (PBS)-washed erythrocytes were diluted to a final concentration of 25% (vol/vol). Aliquots of 1 ml of exponentially growing *Phototribadus* cells were mixed with 50 μ l of an erythrocyte suspension and then incubated for 3 h at 28°C. After centrifugation (3,000 $\times$ g for 15 min), the A_{540} of the resulting supernatant was measured to evaluate the hemoglobin release. Hemolytic activity on blood agar plates was determined as previously described (11).

Detection of hemolysin mRNA by reverse transcription-PCR (RT-PCR). Total RNA from exponential-phase bacteria growing in low-iron conditions was extracted by using the High Pure RNA Isolation Kit (Roche). cDNA synthesis from 2 µg of total RNA was performed by using AMV-RT polymerase according to the instructions given by the Access RT-PCR System kit (Promega). Specific amplifications were performed with the primers BF (5'-GCCATGAAGTACTCATCTT-3') and BR (5'-ACTACCAACCCACTGAATG-3') for the *phlB* region, AF (5'-GGAAATCGCAGCTTAATGC-3') and AR (5'-CCGATATTGAGTGCCCTGAT-3') for the *phlA* region, and 2F (5'-CTCGCCAACAGCA CATTATC-3') and 2R (5'-GTTATGTGACAGCCCGAAG-3') for a region overlapping *phlB* and *phlA*. This step was coupled with 30 cycles of PCR amplification with *Tfl* polymerase (Promega) as follows: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C.

Construction of *cat* transcriptional fusion. Transcriptional fusion between *phlB* and *cat* genes were constructed by using plasmid pBBR1MAC2 provided by S. Köhler (38). This plasmid was derived from pBBR1-MCS (34): T3 and T7 promoters were deleted, and the antibiotic resistance gene was replaced by a β -lactamase gene *bla* encoding ampicillin resistance and a promoterless reporter gene encoding chloramphenicol acetyltransferase (CAT). PCR amplification of a 500-bp fragment corresponding to the 350 bp upstream of *phlB* ATG and the 150 bp of the 5' end of *phlB* was performed with primers 3F (5'-GTCTTGGTACC TCTTCTAGTACAACCCAT-3') and 3R (5'-GATGTGGATCTTAGCTG

CTGCTTTAGTT-3') containing *Bam*HI and *Kpn*I sites, respectively. *Bam*HI-*Kpn*I digestion of the PCR product was performed for adequate orientation when introduced into the corresponding sites of pBBR1MAC2 to yield pJT-PB-MAC. Because *cat* expression confers CHL resistance to the bacteria, the absence of such expression in TT01c harboring pBBR1MAC2 was controlled by determination of the CHL MIC compared to TT01c without plasmid. Values were comparable in both strains (<7.5 and <2 µg of CHL/ml, respectively); thus, pBBR1MAC2 without an insert was used as a negative control for transcriptional analysis of *phlB*.

Determination of CAT expression in *P. luminescens*. Intracellular fractions of exponentially growing *P. luminescens* harboring pBBR1MAC2 or pJT-PB-MAC were collected in order to determine their CAT concentrations. When a bacterial culture reached an optical density of ca. 1.0, a growth phase for which the presence of hemolytic activity could be observed, the bacterial pellet of 500 μ l of culture was resuspended in PBS and immediately frozen at -80°C . Lysis of bacterial cells was performed by repeated brief freezing-thawing cycles (at -80°C and 37°C), and the lysate was centrifuged for 15 min at $10,000 \times g$ to eliminate bacterial debris. The concentration of total proteins in extract was determined by Pierce dosage by using bicinchoninic acid assay (Interchim, Montluçon, France). In parallel, the CAT concentration was determined by an enzyme-linked immunosorbent assay (ELISA) technique with anti-CAT digoxigenin-marked antibodies, followed by a colorimetric assay (CAT-ELISA Kit; Roche), as previously described (12). Each measurement was performed in triplicate. For each sample, CAT concentration was expressed relatively to the total protein concentration. These values were analyzed by the Student *t* test in order to determine *P* values for differences in expected versus actual values.

In vivo pathogenicity assays. The pathogenicity assays were performed on the common cutworm *Spodoptera littoralis* as previously described (28). Briefly, 20 μ l of exponentially growing bacteria diluted in PBS were injected into the hemolymph of 20 fifth-instar larvae of *S. littoralis* reared on an artificial diet. Insect larvae were then individually incubated at 23°C for up to 96 h, and the CFU of bacteria were determined by plating dilutions on LB agar. Insect death was monitored every 5 h. Three independent experiments for each treatment (bacterial doses or strains) were performed. Statistical analysis with SPSS version 11.0.1 (SPSS, Inc., Chicago, Ill.) was performed to construct a life table. Survival curves were compared by using Gehan's generalized Wilcoxon test. This test compares each survival time in each group with every survival time in other group.

A second construction was performed with *gfpmut3* as a reporter gene by using pBBR1-KGFP (33). Cloning of the same region upstream of *phlB* as in pJT-PB-

MAC was performed into the *Bam*HI and *Kpn*I sites of pBBR1-KGFP. About 3,000 CFU of the bacterial cells harboring these plasmids were injected in *S. littoralis* larvae as described above or, as a control, cultured in LB medium. Bacteria collected from LB medium or from insect hemolymph at 24 h postinoculation were examined for fluorescence under a Leica microscope.

Nucleotide sequence accession number. The nucleotide sequence of *P. luminescens* hemolysin genes *phlB* and *phlA* were deposited in EMBL under accession no. AL662784.

RESULTS

***phlBA* sequence analysis.** The deduced amino acid sequence of two large open reading frames of 4,440 and 1,665 bp found in the *P. luminescens* TT01 genome showed 62 and 76% similarity, respectively, with the products responsible for hemolytic activity in *Serratia marcescens*, ShlA and ShlB (accession no. M22618). By analogy, we designated these two *P. luminescens* genes *phlA* and *phlB*. The deduced amino acid sequence of *phlA* and *phlB* also showed similarities (44 to 72% similarity to the all length of the protein sequence) with products of hemolysin genes from *E. tarda* (accession no. D89876), *Proteus mirabilis* (accession no. M30186), and *H. ducreyi* (accession no. U32175) and with putative products of hemolysin genes identified in the *Yersinia pestis* genome (accession no. AJ414158). All of these hemolysins were genetically organized as two adjacent genes, in the same orientation, as were *phlB* and *phlA* (Fig. 1A). In *Serratia marcescens*, Schiebel et al. showed that ShlB is essential for activation and secretion of ShlA (47).

A novel family of protein secreted by the two-partner secretion (TPS) pathway was recently proposed to include HpmaA (52), EthA (29), and HhdA (39) hemolysins from *Proteus mirabilis*, *E. tarda*, and *H. ducreyi*, respectively (31). However, this TpsA family not only includes hemolysins but also potential virulence proteins with other functions such as adhesins. A multiple alignment between PhlA, these four hemolysins, and a putative hemolysin from *Y. pestis* was performed by using Multalin program (Fig. 2) (15). This alignment revealed three groups of at least four amino acids each that were conserved between all of these hemolysins as follows: ILNEV (at positions 111 to 115), NPNG (positions 140 to 143), and LVVGNP (positions 159 to 164). Two cysteine residues found in PhlA showed a conserved position in the five other hemolysins of this family (Fig. 2). Multiple alignments between PhlB and the five other secretion or activation proteins showed that the positions of two cysteine residues and the C-terminal phenylalanine, which is characteristic of outer membrane proteins (50), were conserved (data not shown).

As shown on Fig. 1B, a putative -10 promoter region and a putative ribosome-binding site were found upstream of the start codon of *phlB*. In *E. coli*, iron regulation is achieved via the ferric uptake regulator (Fur) repressor protein, which binds to a 19-nucleotide consensus sequence (GATAATGAT AATCATTATC) (16, 18). Overlapping Fur boxes are frequently found in the promoter regions of iron-regulated genes in *E. coli* (37). Four putative overlapping Fur boxes with homology with the *E. coli* consensus ranging from 9 to 12 of 19 were found in the 5' upstream region of *phlB* (Fig. 1B). A putative rho-independent terminator was found 18 bp downstream of the stop codon of the *phlA* gene (data not shown).

Identification of the *phlA*-encoded function. In *Serratia marcescens*, studies of C-terminally truncated ShlA polypep-

tides have indicated that this region is involved in the hemolytic activity (42). Because of the strong homologies between PhlA and ShlA, we decided to truncate 62% of the C-terminal region of PhlA. This mutation was performed after allelic exchange by insertion of an Ω interposon in *phlA*, leading to a truncated PhlA polypeptide lacking its 910 C-terminal amino acid residues. The resulting mutant, TT01c-*phlA*:: Ω , was assayed for hemolytic activity. Comparison of TT01c and TT01c-*phlA*:: Ω on a sheep or horse blood agar plate did not show any differences, since both strains displayed a perceptible halo of hemolysis with a size of <5 mm around colonies (data not shown). These data indicated that an additional hemolysin was produced on blood agar medium and that *phlA* was not involved in the hemolytic phenotype observed on such media.

Using a liquid hemolytic assay, we found that exponentially growing TT01c bacteria produced hemolytic activity on horse erythrocytes. In contrast, no hemolysis production by TT01c-*phlA*:: Ω strain could be detected under the same conditions (Fig. 3). After complementation of the *phlA* mutant with pJT95, hemolytic activity was partially restored (Fig. 3). Thus, this assay allowed us to identify the presence of PhlA hemolytic activity. Interestingly, this liquid hemolytic assay did not reveal any hemolytic activity in filter-sterilized bacterial supernatants of the strains tested (data not shown).

All attempts to clone the entire *phlBA* locus in *E. coli* failed with the medium-copy plasmid pBBR1MCS as a vector, but it was possible with the low-copy vector, pRK404. This may indicate a toxicity of the *phlBA* locus in *E. coli*, as was also observed for the *ethBA* locus, which could only be cloned on a low-copy number vector (29). Surprisingly, pJT95 containing the *phlBA* locus did not confer a hemolytic phenotype when cloned in *E. coli* either on a blood agar plate nor in a liquid hemolytic assay.

Increase of hemolytic activity in low-iron conditions. The effects of iron deprivation were assayed on *P. luminescens* hemolytic activity. After addition of dipyriddy, a nonutilizable iron chelator, the hemolytic activity of *P. luminescens* was increased compared to that of iron-supplemented cells (Fig. 3). As expected, TT01c-*phlA*:: Ω displayed no hemolytic activity in either iron-rich or iron-depleted media (Fig. 3). Surprisingly, the phase II variant TT01/2, which usually has many reduced exoenzymatic activities or is deficient in these activities compared to the wild-type phase I (5), displayed a hemolytic activity at least twice that of the wild type in iron-rich medium. In iron-depleted medium, TT01/2 hemolytic activity was not increased (Fig. 3).

Transcriptional analysis of *phlBA*. RT-PCR analysis revealed the amplification of fragments of expected sizes corresponding to mRNA of *phlB* (Fig. 4, lane 1) and *phlA* (Fig. 4, lane 3) when *P. luminescens* phase I cells were grown in low-iron conditions, whereas no such transcript could be observed in cells growing in standard medium (data not shown). In contrast, an intensive band was observed on an agarose gel after RT-PCR amplification of TT01/2 total RNA cells grown in standard medium (data not shown). These data indicate that the level of transcription of *phlB* and *phlA* is higher in TT01/2 than in phase I cells under these growth conditions.

When a couple of primers for which the amplified fragment overlapped *phlB* and *phlA* were used, a fragment of the expected size was observed after RT-PCR amplification (Fig.

FIG. 2. Multiple alignments between the N-terminal regions of the hemolysins of *Serratia marcescens* (ShlA), *P. luminescens* (PhlA), *E. tarda* (EthA), *Proteus mirabilis* (HpmA), and *H. ducreyi* (HhdA) and the putative hemolysin of *Y. pestis* (YhlA) as determined by using Multalin version 5.4.1 (15). The total numbers of residues are as follows: 1,608 for ShlA, 1,481 for PhlA, 1,594 for EthA, 1,577 for HpmA, 1,175 for HhdA, and 1,635 for YhlA. Cystein residues are marked by an asterisk. Consensus levels: high, 90% (dark gray shading); low, 50% (light gray shading). Consensus symbols: \$, any of LM; !, any of IV; %, any of FY; #, any of NDQEBZ.

4, lane 2), indicating that *phlB* and *phlA* were cotranscribed. Therefore, transcriptional analysis with the CAT reporter gene was only performed on the 5'-upstream region of *phlA*.

Studies with *cat* transcriptional fusions indicated transcription of *phlA* compared to the negative control (Table 2). In low-iron conditions, when hemolytic activity is induced, transcription of *phlA* was significantly induced (Table 2). Inter-

estingly, the CAT production by a phase II variant containing *phlA* transcriptional fusions was at least twofold increased compared to those measured in TT01c in the same conditions. We confirmed that the phase II variant harboring pBBR1MAC2 (negative control) showed no significant transcription of the reporter gene (Table 2). These results were correlated to the measurements of the hemolytic activity previously described (Fig. 3).

FIG. 3. PhlA hemolytic activity as represented by the percentage of hemoglobin release from horse erythrocytes. Bacterial strains were grown in LB broth supplemented with 100 μ M FeSO₄ (■) or iron depleted by the addition of 300 μ M 2,2'-dipyridyl (▨) to an optical density at 540 nm (OD₅₄₀) of 1.0 prior to the hemolytic assay (see Materials and Methods). The data represent the mean values $\pm$ the standard error of the mean for triplicate determinations from one of four similar experiments.

Virulence of *phlA* mutant for *S. littoralis*. In order to evaluate whether *phlA* is involved in the pathogenicity of *P. luminescens* for susceptible insect larvae, two doses (10 to 100 and 250 to 500 CFU) of the wild-type strain and TT01c-*phlA*:: Ω were injected into *S. littoralis* larvae. We monitored insect mortality for 3 days postinjection (data not shown). After injection of fewer than 30 CFU into the *S. littoralis* hemolymph, all larvae were dead within 48 h, indicating that both strains are highly virulent. When the survival curves for two doses of injected bacteria were significantly different ($P < 0.001$), survival analysis with grouped injection levels of bacteria indicated no sig-

TABLE 2. Transcriptional activity of 5' upstream region of *phlBA* operon as measured by expression of CAT reporter gene

Phase: strain	Mean CAT concn (pg/ μ g of protein) ^a	
	With iron	Without iron
Phase I (negative control): TT01c(pBBR1MAC2)	8.7 $\pm$ 3.4 ^b	5.9 $\pm$ 0.1 ^b
Phase I: TT01c(pJT-PB-MAC)	289.9 $\pm$ 15.8 ^c	566.2 $\pm$ 26 ^c
Phase II (negative control): TT01/2(pBBR1MAC2)	21.3 $\pm$ 1.2 ^d	ND
Phase II: TT01/2(pJT-PB-MAC)	661.8 $\pm$ 2.4 ^d	ND

^a Means $\pm$ standard deviations of three measurements are presented. Bacteria were grown in LB medium either supplemented with FeSO₄ (100 μ M) or iron depleted with 2,2'-dipyridyl (300 μ M) until reaching an OD₅₄₀ of 1.0. ND, not determined.

^b Differences between data are not significant ($P > 0.05$).

^c Differences between data are significant ($P < 0.01$).

^d Differences between data are highly significant ($P < 0.001$).

nificant difference ($P > 0.05$) between the wild type and the *phlA* mutant. For instance, 50% of larvae were dead after 31.5 and 32 h with injected doses per insect of 430 CFU of TT01c and 475 CFU of TT01c-*phlA*:: Ω , respectively. Thus, it can be concluded that TT01c-*phlA*:: Ω displays behavior similar to that of the wild type during pathology in *S. littoralis*.

Septicemia was observed 24 h after injection of both strains in insect larvae. Transcriptional fusions with *gfpmut3* reporter gene revealed a positive signal in bacteria collected from *S. littoralis* hemolymph at 24 h after the injections (ca. 6 h before insect death), whereas no induction was observed in the negative control (data not shown). Similar signals were also observed for bacteria grown in LB medium (data not shown).

DISCUSSION

Characterization of *phlBA* hemolysin locus from *P. luminescens*. The *phlBA* locus of *P. luminescens* was identified on the basis of its sequence similarities with the *Serratia marcescens* hemolysin locus. In *Serratia marcescens*, ShlB that belongs to the TpsB protein family has been shown to be essential for secretion and activation of the ShlA hemolysin (47). HpmB and HpmA in *Proteus mirabilis* (52), EthB and EthA in *E. tarda* (29), and HhdB and HhdA of *H. ducreyi* (39), which all belong to the TpsB and TpsA protein families, have the same functions (31). Furthermore, putative hemolysin genes were also identified in the *Y. pestis* genome. In view of the strong similarities with the hemolysins (Fig. 2) and the secretion or activation proteins of these bacteria, the conserved positions of the cysteine residues, and an identical genetic organization, it might therefore be concluded that PhlB and PhlA most probably have the same functions in *P. luminescens*.

Several groups of at least four amino acids were conserved between all of these hemolysins (Fig. 2). In particular, the NPNG and the ANPN consensus found in PhlA were reported to be important for activation and secretion of ShlA (48). These conserved regions are located in the first 200 residues, the region involved in ShlA secretion, whereas the C-terminal region is responsible for hemolytic activity. This N-terminal region also showed similarity with nonhemolytic members of the TpsA family, such as FHA hemagglutinin of *Bordetella*

FIG. 4. RT-PCR detection of *phlB*, *phlA*, and a region overlapping the *phlB* and *phlA* open reading frames with *P. luminescens* TT01c RNA grown in iron-depleted LB broth. The following primers were used: lane 1, BF and BR (nucleotide positions in *phlBA* sequences 380 and 915, respectively); lane 2, 2F and 2R (positions 1471 and 1967); and lane 3, AF and AR (positions 3027 and 4685, see Materials and Methods).

pertussis (36), and the haem:haemopexin-binding protein of *Haemophilus influenzae* (14).

Because of the strong amino acid sequence conservation of these hemolysins, including both secretion/activation protein (protein B) and the hemolysin (protein A), PhlB and PhlA should belong to the TpsB and TpsA protein families, respectively. However, with regard to the relatively low conservation in the corresponding nucleotide sequences of these family members, the hypothesis of a recent horizontal transfer may be excluded. Alternatively, we can predict the existence of a common ancestral gene that might have evolved only slowly because of strong selective pressures, indicating an important role of this hemolysin family for the maintenance of the bacteria in their respective ecological niches.

In an investigation on the distribution of *Serratia* hemolysin genes among hemolytic bacteria, all *Serratia marcescens* strains tested, including two strains isolated from insects, have been found to contain sequences homologous to *shlB* and *shlA* (43). In *P. luminescens* W14, the genomic sample sequence presented similarities to *shlA* and *shlB* (23). We also detected some homologous segments between *phlBA* and several clones from this genomic sample sequence by using tblastX in the genome survey sequences database of GenBank (23). The present study shows the functionality of PhlA in *P. luminescens* subsp. *laumondii* TT01c. Moreover, homologous genes were recently found in the genome of *Y. pestis*, which persists in the flea gut for long periods. These aspects of life cycle of *Yersinia* in insects bears a striking resemblance to one stage of the interaction between *Photorhabdus* and its nematode hosts when the bacterial symbionts are within the midgut of infective juvenile larvae. Because of the occurrence of these genes in insect-parasite combinations, insect pathogenic bacteria, or nematode symbionts, we may therefore hypothesize that TpsA hemolysins could be involved in mutualistic or detrimental relationships between the invertebrate hosts and bacteria.

The transcription of *phlBA* operon is iron regulated. For many pathogenic bacteria, iron is often a rate-limiting growth factor in their host. Production of cytolysins might be a mean to release iron present in eucaryotic cells (35). In the insect hemocoel, the free-iron concentration is probably low, as indicated by the isolation of transferrins in the hemolymph of several orders of insects, including *Lepidopteran* (3). Measurements of *P. luminescens* hemolysis in iron-supplemented or iron-depleted media showed an induction of PhlA activity in low-iron conditions (Fig. 3). These results have been confirmed by transcriptional analysis of 5' upstream region of *phlBA* (Table 2). We also found four putative overlapping Fur boxes upstream of *phlB*, with identities of 9 to 12 nucleotides with the *E. coli* Fur box (Fig. 1B). Apparently, the *E. coli* Fur protein tolerates considerable variability in operator sequences (41), as illustrated by the Fur-regulated *E. coli* genes *exbD* and *fhuE*, which have a conservation of only 9 and 10 nucleotides with the Fur box consensus sequence (20, 45, 46). Furthermore, the presence of predicted sequences homologous to the *E. coli* Fur protein have been identified in *P. luminescens* TT01 genome (Duchaud E., unpublished data), and in *P. luminescens* W14 genomic sample sequence (23). The iron regulation of PhlA is in accordance with the previously described iron regulation of ShlA and EthA hemolysins from *Serratia* and *Edwardsiella* (29, 41).

The hemolytic activity of the phase II variant was not affected by iron rates in growth media (Fig. 3), and it was at least twofold higher than that of the wild type (phase I). RT-PCR analysis (data not shown) and studies of *phlB* fusions with *cat* reporter gene (Table 2) indicated a deregulation of *phlBA* transcription in this phase variant. The expression of other iron-regulated genes in this strain compared to the wild type is currently being studied.

In vivo role of PhlA hemolysin. In a mouse challenge, intravenous injection of *Proteus mirabilis* or its corresponding *hpmA* mutant was performed. The results show a sixfold increase in the 50% lethal dose in the *hpmA* mutant compared to the wild type (51). Our insect pathology experiments revealed that the *phlA* mutant is still highly virulent. These data showed that PhlA is not a major virulence factor when *P. luminescens* is injected into *S. littoralis* hemolymph.

However, transcriptional studies with *gfpmut3* as a reporter gene showed a positive signal for bacteria grown in vivo in insect hemolymph. In a previous work, we assayed insect hemocyte cytotoxicity in bacterial supernatants only and concluded that no cytolysin could be detected in *P. luminescens* supernatant (11). Although TPS hemolysins are secreted by their specific secretion/activation protein, the hemolytic activity detected in natural bacterial producer supernatant is usually absent or very low. Furthermore, pulse-labeling experiments have determined that ShlA has a half-life of only 3 min at 37°C (47). Our experiments indicate that bacteria and erythrocytes need to be coincubated to detect the hemolytic activity caused by PhlA. With regard to the present results, we can hypothesize a possible cytolytic activity of PhlA against insect hemocytes when bacteria are present in the hemolymph.

The transcriptional expression of *phlBA* promoter in hemolymph before insect death might indicate a unexpected role of PhlA hemolysin in this ecological niche. Once the septicemia is well established, the insect cadaver tissues are bioconverted into a source of nutrients for growth of both nematode and bacteria. PhlA hemolysin probably causes some insect cell disruption, which might play a role in nematode and bacteria multiplication.

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