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A Homoserine Lactone Autoinducer Regulates Virulence of an Insect-Pathogenic Bacterium, *Xenorhabdus nematophilus* (*Enterobacteriaceae*)

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***N*-β-Hydroxybutanoyl homoserine lactone (HBHL), the autoinducer of the luminescent system of *Vibrio harveyi*, has been identified as the first small compound to restore virulence to avirulent mutants of *Xenorhabdus nematophilus*. HBHL stimulated the level of lipase activity excreted by avirulent *X. nematophilus* and lowered the phenoloxidase activity in the hemolymph of insects infected with *X. nematophilus*, parameters that are both associated with insect pathogenesis. Moreover, mortality of the insects infected with avirulent *X. nematophilus* was restored upon injection with HBHL. Chloroform extraction of medium conditioned with wild-type but not avirulent *X. nematophilus* led to the isolation of a compound with the same chromatographic mobility as HBHL as well as the ability to stimulate the luminescence of a dim autoinducer-dependent mutant of *V. harveyi*. Transfer of the *V. harveyi lux* operon into avirulent and wild-type *X. nematophilus* generated dim and bright luminescent strains, respectively, which responded to HBHL and an agonist and antagonist in a manner analogous to their effects on the luminescence of dim autoinducer-deficient and bright wild-type strains of *V. harveyi*, indicating that similar HBHL-dependent regulatory systems exist in these two bacterial species.**

Xenorhabdus nematophilus is a major insect pathogen that exists in the intestines of the parasitic nematode *Steinernema carpocapsae* (family *Steinernematidae*) (1, 9). The high susceptibility of many insect species to infection coupled with the strong reproductive capacity and mobility of the bacterium-nematode complex provides considerable potential for the use of parasitic nematodes for control of insects (11, 14). Invasion of the nematodes into the hemocoel of susceptible insect larvae results in the release of the bacteria into the hemolymph; growth of the bacteria and nematodes then results in the rapid death of the insect larvae.

Virulence of *X. nematophilus* and related insect-pathogenic bacteria is due to the expression of an interactive polygenic system comprising tolerance and evasion of the antimicrobial factors of the insect hemolymph (6, 15), changes in bacterial surfaces (6), and the excretion of toxic lipases and lecithinases (7, 19). Since insect mortality occurs at a critical concentration of *X. nematophilus* (6), a density-dependent global regulator may initiate the release of toxic factors (7). Identification of such a regulator could provide the foundation for not only understanding but also controlling virulence of *X. nematophilus*, which in turn may lead to the development of more effective insect pathogens (11). This paper reports the discovery that the β-hydroxybutanoyl homoserine lactone (HBHL) autoinducer, which causes induction of luminescence in the marine bacterium *Vibrio harveyi* (4), can restore virulence to avirulent mutants of *X. nematophilus*. The present results also demonstrate that HBHL or a closely related analog is excreted by wild-type and not by avirulent *X. nematophilus.*

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MATERIALS AND METHODS

Strains and growth conditions. *X. nematophilus* 19061 and avirulent mutants AV1, GF, and G (transpositional mutants of strain 19061 [7]), *V. harveyi* B392 and the autoinducer mutant D1 (4), and *Escherichia coli* were all grown at 27°C in Luria broth (LB) containing the appropriate antibiotics, if necessary.

Luminescent *X. nematophilus*. Plasmid pMGM221 containing the *lux* operon of *V. harveyi* was transferred into *X. nematophilus* from *E. coli* by triparental mating with the aid of the helper plasmid, pRK2013. Plasmid pMGM221 contains the genes for light emission (*luxCDABE*) plus the upstream promoter region. Luciferase (*luxAB*) and enzymes for synthesis of the fatty aldehyde substrate (coded by *luxCDE*), which reacts with host-derived $FMNH_2$ and O_2, will result in generation of light in *X. nematophilus* transconjugates grown in LB. Details of the construct and conjugation procedure will be described elsewhere (7a). Plasmid pMGM221 containing the *lux* operon was also transferred into *E. coli* MM294 by transformation.

Luminescence measurements. Luminescence was measured in light units for 1 ml of medium (LU), where 1 LU corresponds to 5×10^9 quanta/s, and plotted as LU/optical density at 660 nm (OD_{660}). A minimum of five independent experiments containing 10 samples each were used in luminescence measurements.

Generation and assay of autoinducer excreted by cells. Media conditioned by *X. nematophilus* grown to a low cell density (OD_{660} = 0.3 to preclude synthesis of antibiotics at higher cell densities) or by logarithmically growing wild-type *V. harveyi* cells were centrifuged, filter sterilized (0.22-μm-pore-size filter), and diluted to 20% (vol/vol) with fresh broth. Fresh D1 autoinducer-deficient cells, grown overnight, were inoculated into LB to an OD_{660} of 0.05, grown to an OD_{660} of 0.2, harvested by centrifugation, and mixed with 20% (vol/vol) conditioned medium to an OD_{660} of 0.05 to 0.10. Bacterial growth (OD_{660}) and light production for 1 ml of cells (LU) were then monitored.

Purification of the *X. nematophilus* autoinducer. *X. nematophilus* conditioned medium was extracted with chloroform (2:1, vol/vol). The extract was assayed for its ability to stimulate the luminescence of the dim *V. harveyi* D1 cells, and the activity was partially purified by separation on a 200/400-mesh silica gel column and on silica gel thin-layer chromatography (TLC) plates (4). The TLC plates were developed sequentially in 10:1 (vol/vol) chloroform-methanol and 10:1 (vol/vol) chloroform-acetic acid. The synthetic autoinducer from *V. harveyi*, HBHL, and chloroform extracts of *V. harveyi* were codeveloped on the plates as controls. Bands 1 cm wide were cut from the plates, eluted with 1.0 ml of methanol, and reextracted with 5 ml of chloroform, after which 2- to 10-μl aliquots were assayed with D1 cells.

Effect of HBHL and autoinducer analogs on luminescence of *X. nematophilus*. Both virulent and avirulent *X. nematophilus* transconjugants from overnight cultures were diluted in fresh LB to an OD_{660} of 0.05, shaken (150 rpm) to an OD_{660} of 0.2, and subsequently added to fresh LB containing added inducer or

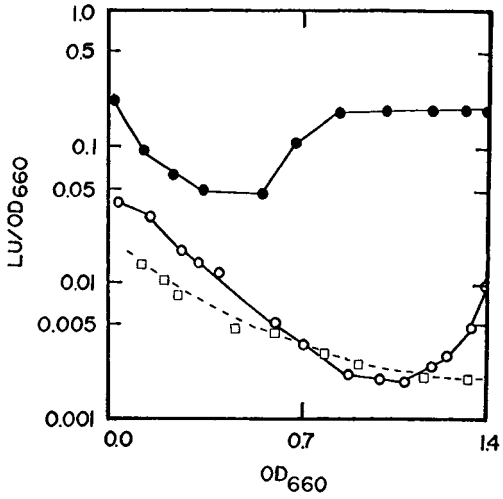

FIG. 1. Density-dependent production of luminescence by virulent (●) and avirulent (AV1) (○) *X. nematophilus* and *E. coli* (□) containing the *V. harveyi lux* operon. Overnight cultures were diluted in LB and incubated at 27°C in a reciprocating shaker (150 rpm), and light intensity was monitored as a function of cell density (OD_{660}).

analogs or no additions to an OD_{660} of 0.05. Both strains were sensitive to HBHL concentrations in excess of 1 μg ml^{-1}, with 0.1 μg ml^{-1} being optimal for light production used in this study. The agonist β-hydroxyvaleryl homoserine lactone (HVHL) at 1.0 μg ml^{-1} and the antagonist β-hydroxyhexanoyl homoserine lactone (HPHL) at 560 μg ml^{-1} did not inhibit bacterial growth and were thus used. AV1 logistically did not permit the direct analysis of the effect of HPHL on light production; thus, the effect was based on the ability of HPHL to inhibit the stimulatory effect of 0.1 μg of HBHL ml^{-1} on the bacteria. The effects of HVHL were larger with increasing concentrations (data not given).

Assays of virulence-related enzymes. Lipase activity of conditioned media with (0.1 μg ml^{-1}) and without HBHL was based on calcium-induced precipitation of fatty acids hydrolyzed in Tween 80 agar (12) and reported as units/milligram of protein. Endotoxin of virulent and avirulent (AV1) *X. nematophilus* inhibits the activation of the opsonin phenoloxidase in the prophenoloxidase cascade in insect hemolymph (7). However, AV1 has a leaky outer membrane that releases periplasmic enzymes activating phenoloxidase (7). To determine if HBHL influenced AV1 whole bacterial activation of phenoloxidase, hemolymph (10 μl) from insects with and without HBHL and AV1 was incubated with laminarin (1 mg ml^{-1}) to activate residual phenoloxidase not inhibited by the bacterial endotoxins. Phenoloxidase activity was spectrophotometrically determined (A_{490}) by the formation of dopachrome from the substrate L-dihydroxyphenylalanine (7).

Insect mortality and the effect of HBHL. Ten microliters of LB containing 10^3 early-exponential-phase wild-type and avirulent bacteria (diluted in fresh LB from cultures with OD_{660} of 0.05) was injected in the base of the prothoracic legs of sixth instar *Galleria mellonella* larvae (7). Insects were then injected with 10 μl of phosphate-buffered saline (7) or with HBHL (0.1 μg μl^{-1}). Larvae were incubated at 29°C with continuous lighting and 70% relative humidity and checked for mortality at regular intervals. Because of the linearity in insect mortality, traditional probit mortality transformation of data was not required. A minimum of five independent experiments, each done with 10 larvae, were performed.

RESULTS

To monitor the fate of *X. nematophilus* as an infectious agent of insects, the mobilizable plasmid pMGM221, containing the *V. harveyi lux* genes (*luxCDABE*) (10), was introduced into *X. nematophilus* bacteria by conjugation. Light emission by the conjugated *X. nematophilus* was very high, the luminescence of the cells being over 50 times brighter than that for *E. coli* cells containing the same *lux* plasmid (Fig. 1). Moreover, the luminescence intensity of *X. nematophilus* (given as LU/OD_{660}) was growth dependent and exhibited a lag characteristic of light emission in the native *V. harveyi* strain (16). The lag in luminescence in *V. harveyi* is primarily due to excretion of the *lux* autoinducer, HBHL, which accumulates in the media and triggers the induction of luminescence at higher cell densities.

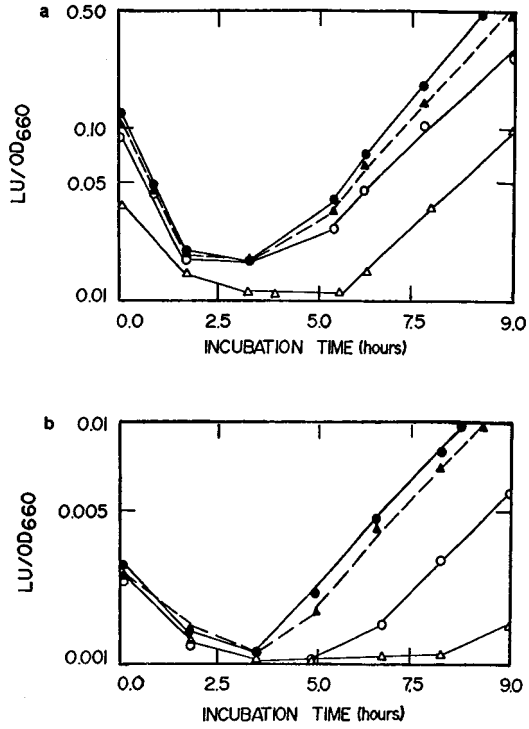

FIG. 2. Synthetic autoinducer of *V. harveyi* and its analogs influence luminescence of *X. nematophilus*(pMGM221) strains. (a) Effects of autoinducer (HBHL; ●), agonist (HVHL; ▲), and inhibitor (HPHL; △) on the luminescence of wild-type *X. nematophilus*(pMGM221). (b) HBHL (●) and HVHL (▲) enhanced luminescence in the avirulent transconjugant AV1, and HPHL inhibited light induction by HBHL (△). O in both panels, no additions. Additional details are given in Materials and Methods.

Transfer of the *lux* operon into an avirulent *X. nematophilus* strain (AV1) generated, in contrast, a dim phenotype whose light intensity remains at a low level and starts to increase only at a much higher cell density (Fig. 1). Indeed, the luminescence of the avirulent *X. nematophilus*(pMGM221) is as low as that seen for *E. coli*(pMGM221). The difference in expression of luminescence in avirulent and virulent strains of *X. nematophilus* suggests that regulators of virulence in *X. nematophilus* may control and be related in structure to the regulators of the *V. harveyi lux* operon (i.e., the HBHL *lux* autoinducer).

Addition of synthetic HBHL to the transconjugated wild-type and avirulent strains of *X. nematophilus*(pMGM221) caused a shift in induction of light to an earlier stage of growth and a large increase in the luminescence of the avirulent strain, while a small increase occurred in the luminescence of the wild-type *X. nematophilus* strain (Fig. 2). In contrast, HBHL has no effect on the luminescence of *E. coli*(pMGM221) or on dim autoinducer-deficient mutants of the luminescent bacterium *Vibrio fischeri*, which has a closely related homoserine lactone autoinducer (8). These results indicate that the stimulatory factor in *X. nematophilus* is HBHL or a very closely related molecule. The effects on light emission of *X. nematophilus* transconjugants by the agonist (HVHL) and the antagonist (HPHL) of HBHL were similar to that observed for the *lux* system of *V. harveyi* (5). The HVHL agonist caused the luminescence of the avirulent *X. nematophilus*(pMGM221) to increase at lower cell density, while addition of the HPHL

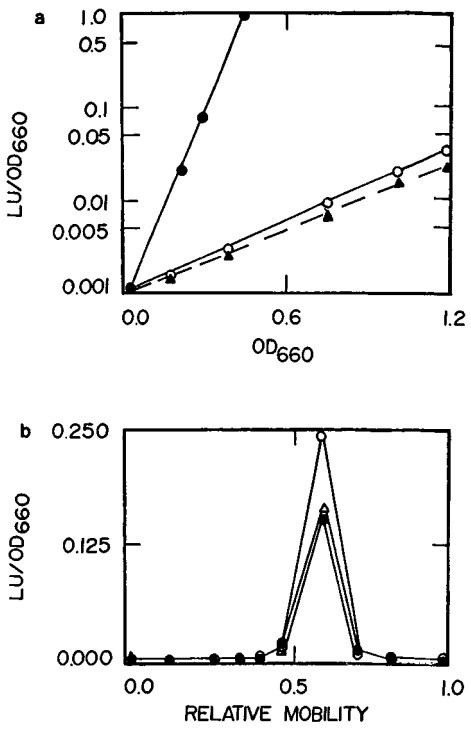

FIG. 3. Autoinducer produced by wild-type *X. nematophilus* strain 19061 induces bioluminescence in the *V. harveyi* autoinducer-deficient dim mutant D1. (a) Both *X. nematophilus* conditioned medium (○) and its chloroform extract (▲) enhanced light production by D1 cells but less effectively than did *V. harveyi* conditioned broth (●). (b) Partially purified autoinducer isolated from *X. nematophilus* conditioned medium (○), which stimulates light production of D1 cells, has a mobility on TLC identical to that of *V. harveyi* autoinducer extracted from conditioned medium (△) and synthetic HBHL (●).

antagonist delayed the appearance of luminescence for both wild-type cells and avirulent cells stimulated with HBHL (Fig. 2); the effects with both HVHL and HPHL were almost identical to that observed on *V. harveyi* luminescence and moreover varied with antagonist and agonist concentration in a similar manner (data not shown). The results provide good evidence that the terrestrial insect pathogen *X. nematophilus* has a regulatory system containing an autoinducer similar to that controlling light production in the marine bacterium *V. harveyi* (2, 17, 20, 21).

Direct support for the production of HBHL by *X. nematophilus* was provided by the stimulation of light emission of a dim autoinducer-deficient mutant, D1, of *V. harveyi* (5) by medium (20%, vol/vol) conditioned by wild-type *X. nematophilus* cells (Fig. 3a). Although the level of light stimulation was 200-fold lower for medium conditioned by *X. nematophilus* than for medium conditioned by *V. harveyi*, it should be noted that the *X. nematophilus* cells are grown to only a low cell density (see Materials and Methods) to preclude the production of antibiotics which occurs at high cell density. Indeed, media conditioned by *X. nematophilus* grown to high cell density will kill *V. harveyi* cells, as reported by Paul et al. (18) for some *V. harveyi* strains. Recently, conditioned medium from a second species of *Xenorhabdus* has been shown to stimulate luminescence of the dim mutant to an even higher level than medium conditioned by *X. nematophilus* (7b). In contrast, media conditioned by three phase 1 avirulent mutants, AV1, GF, and G (7, 11, 22), had no effect on the luminescence of the D1 mutant, confirming that an autoinducer was not being produced by avirulent *X. nematophilus* (data not shown). The avirulent mutants had been produced by insertion of the Tn*5* transposon at different sites in the genome and differed in a number of other biochemical characteristics (11, 22). However, none of the three mutants at doses of up to 10^5 bacteria could kill test insects, in contrast with wild-type *X. nematophilus*, where 1 to 10 bacteria are lethal.

TABLE 1. Effect of the autoinducer HBHL (0.1 μg/ml) on bacterial lipase and insect phenoloxidase activities associated with the virulence of *X. nematophilus*

Strain	Activity (U mg^{-1})[a]	
	Lipase	Phenoloxidase
AV1		
Alone	2.1 ± 0.2	8.3 ± 0.2
+HBHL	5.8 ± 0.2	1.8 ± 0.3
Wild type	12.7 ± 0.2	1.2 ± 0.3

[a] Mean ± standard error of five independent experiments, each performed with 10 insects.

As agents in the spent medium (9) of *X. nematophilus* might decrease the light response of the D1 mutant, the stimulatory factor (autoinducer) excreted by virulent *X. nematophilus* was extracted with chloroform and partially purified by silica gel chromatography followed by TLC on silica gel plates. The stimulatory factor had chromatographic properties identical to those of HBHL through all stages of purification, including an R_f of 0.6 on thin-layer silica gel plates (Fig. 3b). Autoinducer was not detected in chloroform extracts of media conditioned by any of the three avirulent mutants of *X. nematophilus* at the optical density tested.

As HBHL is not produced by the avirulent strains of *X. nematophilus*, suggesting that it is connected to virulence, the effects of HBHL on enzymes associated with the virulence of the bacterium-nematode complex (6, 11) were tested. The HBHL autoinducer increased lipase activity of avirulent AV1 by 175% and reduced the activation of the phenoloxidase in the hemolymph infected with larvae containing avirulent *X. nematophilus* by 80%, to levels more closely associated with those containing the wild-type strain (Table 1).

To test the effect of HBHL on virulence directly, *G. mellonella* insects were injected with *X. nematophilus* and their survival was measured with time of injection of HBHL into the hemolymph (Fig. 4). The avirulent strain AV1 killed the insects 30 to 40 h after injection of HBHL. In contrast, insects containing the avirulent strain without HBHL or simply injected with HBHL did not die over the next 2 weeks. The time for the death of 50% of the insects, LT_{50}, decreased in the presence of HBHL after infection with either avirulent or virulent strains (AV1 plus HBHL, LT_{50} = 35.2 ± 0.5 h; AV1 minus HBHL, LT_{50} = nil; wild type plus HBHL, LT_{50} = 8.6 ± 0.1 h; wild type minus HBHL, LT_{50} = 9.6 ± 0.2 h [$P < 0.01$]).

DISCUSSION

The ability of HBHL to restore virulence to the avirulent mutants provides strong proof that HBHL or a closely related compound plays a key role in the development of virulence by *X. nematophilus* and would explain the need for a critical concentration of bacteria in the hemolymph before the onset of insect mortality (7). The dependence of the virulence of *X. nematophilus* on HBHL provides the first documented example of an autoinducer-dependent virulence mechanism by insect-pathogenic bacteria. *N*-Acyl homoserine lactone-depen-

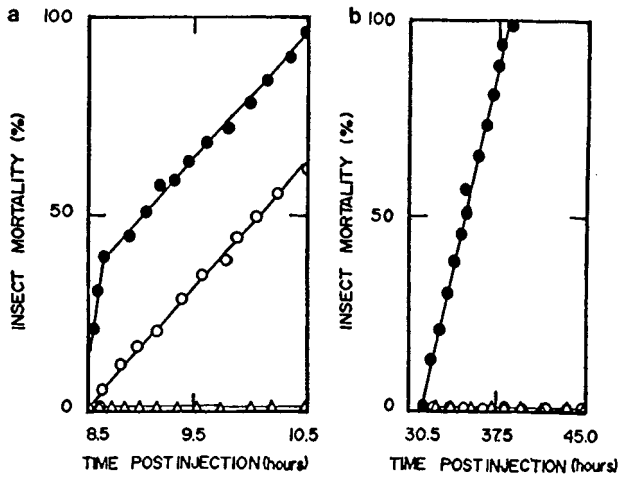

FIG. 4. The synthetic autoinducer of *V. harveyi*, HBHL, enhances the virulence of strains of *X. nematophilus* for larvae of the insect *G. mellonella*. (a) Larval mortality caused by the wild-type bacteria. (b) Mortality caused by the avirulent strain AV1. ●, with HBHL; ○, without HBHL; △, injection of HBHL alone. Additional details are given in Materials and Methods.

dent systems have been implicated directly in virulence for human and plant pathogens; homoserine lactone autoinducers trigger the production of multiple toxic exoproducts associated with the invasion of host tissues by the human pathogen *Pseudomonas aeruginosa* (3) and are involved in the global control of exoenzymes facilitating tissue necrosis and bacterial growth in plants produced by the pathogen *Erwinia caratovora* (13). However, these systems are more closely related to the *N*-β-oxohexanoyl homoserine lactone-dependent system of *V. fischeri* and contain proteins homologous to those involved in autoinducer synthesis (LuxI) and transcriptional activation (LuxR) in *V. fischeri*. In contrast, LuxR from *V. harveyi* is unrelated in sequence to *V. fischeri* LuxR (20, 21), and the protein implicated in autoinducer synthesis in *V. harveyi* (LuxM) is unrelated to LuxI (2), although LuxM is related in sequence to another protein (AinS) implicated in synthesis of a second homoserine lactone autoinducer in *V. fischeri* (10). The establishment of a second system with a dependence on HBHL similar to that for the *lux* system of *V. harveyi* suggests that such a homoserine lactone may be involved in different regulatory mechanisms, and thus HBHL may play a role in the development of processes at high cell density more universal than previously thought.

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