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# The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*

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## Summary

***Xenorhabdus nematophila*** **is a Gram-negative bacterium that leads both pathogenic and mutualistic lifestyles. In this study, we examine the role of Lrp, the leucine-responsive regulatory protein, in regulating both of these lifestyles.** ***lrp*** **mutants have attenuated virulence towards** ***Manduca sexta*** **insects and are defective in suppression of both cellular and humoral insect immunity. In addition, an** ***lrp*** **mutant is deficient in initiating colonization of and growth within mutualistic host nematodes. Furthermore, nematodes reared on** ***lrp*** **mutant lawns exhibit decreased overall numbers of nematode progeny. To our knowledge, this is the first demonstration of virulence attenuation associated with an** ***lrp*** **mutation in any bacterium, as well as the first report of a factor involved in both** ***X. nematophila*** **symbioses. Protein profiles of wild-type and mutant cells indicate that Lrp is a global regulator of expression in** ***X. nematophila*****, affecting ~65% of 290 proteins. We show that Lrp binds to the promoter regions of genes known to be involved in basic metabolism, mutualism and pathogenesis, demonstrating that the regulation of at least some host interaction factors is likely direct. Finally, we demonstrate that Lrp influences aspects of** ***X. nematophila*** **phenotypic variation, a spontaneous process that occurs during prolonged growth in stationary phase.**

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## Introduction

The ability of a microbe to establish a symbiotic relationship, whether beneficial or pathogenic, with a host depends on its successful adaptation to the conditions of the host environment, including fluctuating nutrient availability, host immunity and competing microbes. To cope with these factors and exploit the host niche, bacterial mutualists and pathogens have evolved strategies for nutrient scavenging and defence. For example, many pathogenic bacteria induce expression of iron-acquisition factors in response to iron-limiting conditions of the host (Crosa, 1997). Additionally, bacteria can modulate aspects of host immunity to ensure their own survival (Espinosa and Alfano, 2004; Kelly *et al*., 2005). Furthermore, commensal bacteria can utilize host cell-surface molecules as nutrient sources when in competition with other members of the microbiota (Bäckhed *et al*., 2005).

To date, the vast majority of research concerning microbe–host interactions has focused on beneficial or pathogenic relationships between one microbe and one host. Although many biologists view mutualism as 'mutual parasitism', the extent of similarities between mutualism and pathogenesis are not well established, especially with regard to their molecular foundations (Hentschel *et al*., 2000). *Xenorhabdus nematophila*, a member of the Enterobacteriaceae, provides the unusual opportunity to study both beneficial and pathogenic interactions engaged by a single microorganism. In doing so, it may be possible to understand the extent of overlap between factors involved in each interaction. *X. nematophila* mutualistically associates with a soil-dwelling nematode, *Steinernema carpocapsae*. A juvenile stage of the nematode carries a monoculture of *X. nematophila* within a specialized compartment of its intestinal tract as it waits in the soil for prey insect larvae (Forst and Nealson, 1996; Martens *et al*., 2003). In cooperation with the nematode, *X. nematophila* is able to infect and kill a variety of insect larvae. The bacteria and nematodes reproduce within the dead insect, reassociate, and exit the insect cadaver in search of a new insect host (Forst *et al*., 1997).

During prolonged growth in stationary phase, *X. nematophila* spontaneously switches between two cell types, primary and secondary, in a process termed

'phenotypic variation' (Forst and Clarke, 2002; Smits *et al*., 2006). Primary and secondary form cells display many phenotypic differences that vary depending on *Xenorhabdus* species and strain. Generally, primary form cultures produce a variety of extracellular factors, including haemolysins, proteases, antibiotics and lipases. Primary form cells also agglutinate sheep erythrocytes, bind bromothymol blue dye, and are motile in both swimming and swarming assays. Previously, *X. nematophila* ATCC 19061 and *X. nematophila* strain F1 secondary form variants were reported to produce reduced levels of haemolysin, antibiotics and intracellular crystalline inclusion proteins and were non-motile on swim plates (Givaudan *et al*., 1995; Volgyi *et al*., 1998). Despite these *in vitro* differences, both primary and secondary variants were virulent towards insects and colonized nematodes to the same level, making the selective advantage of this switch unclear. When *X. nematophila* F1 primary and secondary form cells are co-injected into insects in competition experiments, nematodes that emerged from the insect cadaver predominantly contained secondary form cells (Sicard *et al*., 2005), suggesting some competitive advantage for this variant during the life cycle.

The mechanism of phenotypic variation in *X. nematophila* is not understood at the molecular level. Phenotypic variation of lipase activity in *Photorhabdus luminescens*, another entomopathogenic bacterium, is post-translationally controlled (Wang and Dowds, 1993), and the putative transcription factor HexA represses variable phenotypes, including lipase, in secondary form *P. luminescens* (Joyce and Clarke, 2003). These findings indicate that in this bacterium, several regulators influence multiple stages of expression (e.g. transcription and protein stability) to control phenotypic variation. Global regulators and accumulation of GASP (growth advantage in stationary phase) mutations (Zambrano *et al*., 1993) are proposed mechanisms of phenotypic variation (O'Neill *et al*., 2002), but no data have been reported for *X. nematophila* to support this hypothesis. In *Escherichia coli*, early appearing GASP strains contain mutant alleles of the stationary phase sigma factor, RpoS (Zambrano *et al*., 1993), but an *X. nematophila rpoS* null mutant has no effects on phenotypic variation (Vivas and Goodrich-Blair, 2001). A mutant hunt for genes that convert *X. nematophila* from primary to secondary form revealed the involvement of *var1* (named for its involvement in variation) (Volgyi *et al*., 2000). The function of this gene, however, is not known, and thus, the regulatory mechanisms controlling *X. nematophila* phenotypic variation remain obscure. Furthermore, the role, if any, of such regulatory mechanisms in *X. nematophila* adaptation to each host environment has yet to be elucidated.

Whether in response to the transition between the external environment and the host or during different stages of colonization and infection, gene expression in many microbes is adjusted to produce the appropriate response (Mekalanos, 1992; Bader *et al*., 2005; Brencic and Winans, 2005; Rychlik and Barrow, 2005). Therefore, in *X. nematophila*, the cooperative relationship with the nematode, the pathogenic association with the insect, and the transition between hosts each likely require a distinct set of bacterial factors. *X. nematophila* genes and factors required for nematode colonization and virulence towards insects have been identified, but until this study, all factors required for colonization of the nematode are dispensable for virulence towards insects, and vice versa (Givaudan and Lanois, 2000; Vivas and Goodrich-Blair, 2001; Heungens *et al*., 2002; Cowles and Goodrich-Blair, 2004; 2005; Martens *et al*., 2005). Furthermore, to date, little is known about the regulation of these factors, including how host environmental stimuli are sensed and what regulatory cascades are required to induce gene expression appropriate for each host environment.

The study presented here focuses on a putative transcriptional regulator, Lrp (leucine-responsive regulatory protein). In *E. coli*, Lrp acts as a global regulator, controlling a large number of genes involved in amino acid biosynthesis and catabolism, transport, and production of pili (Calvo and Matthews, 1994; Tani *et al*., 2002). However, in other bacteria, Lrp has a more specific role in regulating branched-chain amino acid biosynthesis (Madhusudhan *et al*., 1993; Belitsky *et al*., 1997; Friedberg *et al*., 2001). Previous work from this lab identified the requirement for *lrp* in nematode colonization (Heungens *et al*., 2002) and presented two examples of Lrp-regulated colonization and virulence factors (Cowles and Goodrich-Blair, 2004; 2005), demonstrating functions for Lrp outside of a general metabolic role in *X. nematophila*. In this study, we provide a detailed analysis of Lrp involvement in virulence towards insects, as well as further characterization of its role in nematode colonization. Additionally, we illustrate a role for Lrp in *X. nematophila* phenotypic variation and define the scope of the Lrp regulon using two-dimensional (2D) gel electrophoresis. Our results indicate that Lrp is involved in both host interactions through the regulation of a large proportion of *X. nematophila* factors. To our knowledge, this study provides the first evidence that mutating *lrp* leads to virulence attenuation and identifies the first known bacterial factor to play a role in both *X. nematophila*–host interactions.

## Results

### *An* lrp *mutant has attenuated virulence towards* Manduca sexta *larvae*

To assess the contribution of Lrp to *X. nematophila* virulence, we constructed an *lrp-2*::Km mutant. When injected

 

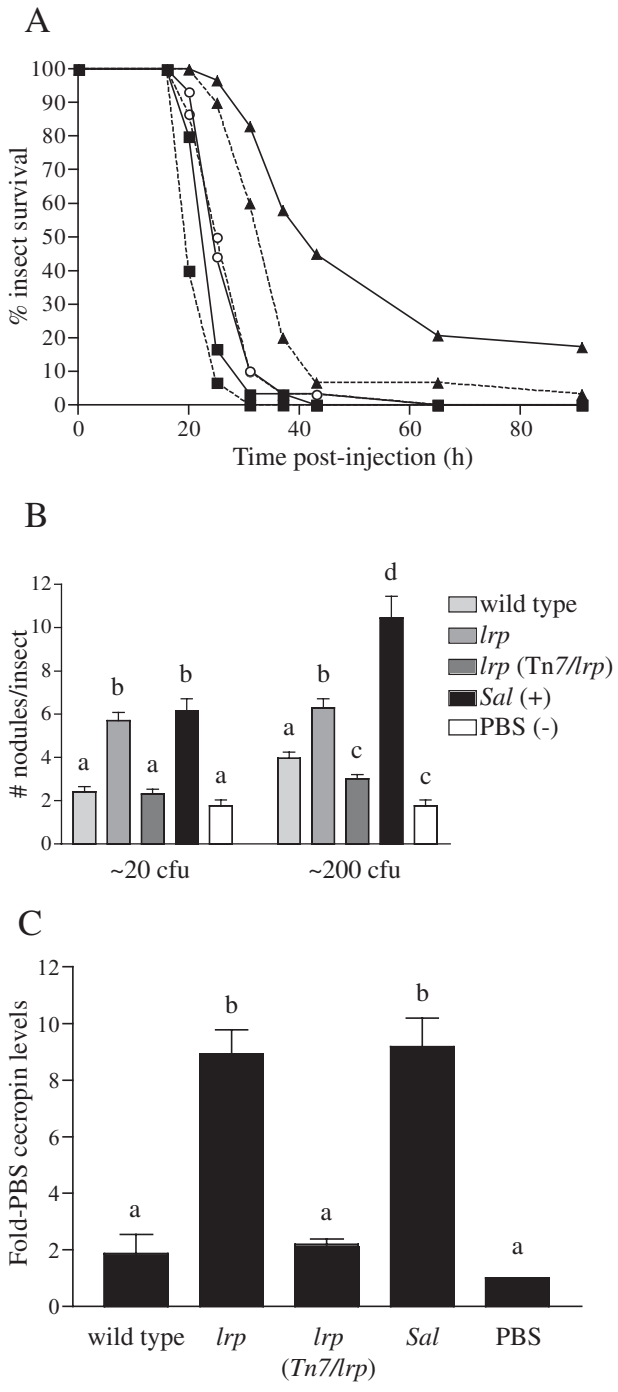


**Fig. 1.** *lrp* is necessary for virulence and immune modulation in *M. sexta* insects.
A. Insect survival was monitored over time after injection with wild-type *X. nematophila* (squares), *lrp* mutant (triangles) and *lrp* (Tn*7*/*lrp*) (open circles) at 20 cfu (solid lines) and 200 cfu (dashed lines) per insect. The survival curves shown are from a representative experiment ($n = 5$).
B. The number of nodules formed per insect was examined after injection with ~20 or ~200 cfu of each strain listed.
C. Transcript levels of the antimicrobial peptide cecropin A were measured after injection of each strain (200 cfu/insect). Resulting amounts of cecropin transcript were compared with the response to a PBS injection and expressed as fold-PBS. Error bars indicate standard error ($n = 20$ insects for nodulation assays and 10 insects for cecropin assays). The same letter indicates no significant difference between treatments at the same dosage [$P < 0.001$ for 20 cfu (B), $P < 0.05$ for 200 cfu (B), and $P < 0.001$ for (C)]. Injections with *S. enterica* serovar Typhimurium are denoted by *Sal*.

at a level of ~20 colony-forming units (cfu)/insect, the *lrp* mutant had a significantly higher $LT_{50}$ (50 h as compared with 23 h in wild type, $P < 0.001$) and killed fewer overall insects by 72 h than did wild type ($P < 0.001$) (Fig. 1A). A higher injection dose (~200 cfu/insect) resulted in more similar virulence patterns for wild-type and *lrp* mutant strains ($LT_{50}$ of 19 h versus 32 h respectively, $P < 0.01$) (Fig. 1A). The *lrp* mutant virulence deficiencies at both injection levels were complemented by providing the *lrp* locus *in trans* at the *att*Tn*7* site [denoted as *lrp* (Tn*7*/*lrp*)] on the *X. nematophila* chromosome (Fig. 1A). To determine if the virulence attenuation of the *lrp* mutant was due to a growth defect, we examined the growth of wild type and the *lrp* mutant in Luria–Bertani (LB) broth and insect haemolymph (blood), and found no significant difference in growth rate, final concentration of cells, or overall growth curve appearance between strains under either growth condition (data not shown). As yet, there is no minimal medium available that supports the growth of *X. nematophila*, precluding an analysis of the *lrp* mutant growth under that particular condition. However, *lrp* mutant colony size and number were indistinguishable from wild type on defined medium agar plates (data not shown).

### lrp *is required for* X. nematophila *suppression of insect immunity*

To better understand the *lrp* virulence defect, we examined aspects of the *Manduca sexta* immune response towards an *X. nematophila lrp* mutant. First, we tested the cytolytic activity of *lrp* mutant cell-free supernatants against *M. sexta* immune cells (haemocytes). Supernatants from the *lrp* mutant lysed fewer haemocytes compared with wild-type and *lrp* (Tn*7*/*lrp*) cell-free supernatants ($P < 0.001$) regardless of the bacterial growth phase examined (Table 1).

To further evaluate the cellular immune response to the *lrp* mutant, we injected insects with bacteria and measured nodule formation at 16 h post injection. Nodules are melanized haemocyte aggregates that form around bacteria. Previous studies have shown that non-pathogens are effectively sequestered into nodules while *X. nematophila* cells are not (Park *et al*., 2003). Regardless of dose, nodule formation in insects injected with the *lrp* mutant was comparable with the response towards *Salmonella enterica* serovar Typhimurium LT2 (denoted

**Table 1.** Cytolytic activity of *X. nematophila* supernatants.

| Strain | % cytolysis[a] | | |
|---|---|---|---|
| | Log[b] | Early stationary[b] | Late stationary[b] |
| Wild type | 53.4 (± 2.1) | 90.9 (± 1.3) | 97.7 (± 0.5) |
| *lrp* mutant | 17.3 (± 1.8)[d] | 18.9 (± 1.9)[d] | 25.1 (± 2.2)[d] |
| lrp (Tn*7*/*lrp*) | 50.1 (± 4.6) | 98.9 (± 0.3) | 99.4 (± 0.2) |
| (–) control[c] | 16.1 (± 0.9)[d] | 9.3 (± 1.1)[d] | 6.4 (± 0.7)[d] |

**a.** % cytolysis indicates the average percentage dead haemocytes per field; standard error ($n = 30$ fields) is given in parentheses.
**b.** Log, early stationary, and late stationary phase time points were taken at $OD_{600} = 1.0$, 4.0 and 8.0 respectively.
**c.** Grace's Insect Medium was used as a negative control for measuring spontaneous lysis of haemocytes.
**d.** Indicates statistically significant differences from wild type ($P < 0.001$).

as *Salmonella*), a bacterium that is not pathogenic towards *M. sexta* insects, whereas wild-type and *lrp* (Tn*7*/*lrp*) cells elicited significantly fewer nodules ($P < 0.001$ for 20 cfu/insect and $P < 0.05$ for 200 cfu/insect), similar to PBS-injected controls (Fig. 1B).

As a final measure of the role of *lrp* in modulating insect immunity, we injected insects with bacteria (~200 cfu/insect) and monitored the resulting levels of an antimicrobial peptide, cecropin A, which is normally induced in response to bacterial infection (Gillespie *et al*., 1997). As observed in previous studies (Ji and Kim, 2004; Park *et al*., 2007), insects injected with wild-type *X. nematophila* had significantly reduced levels of cecropin expression compared to injections with a non-pathogen, such as *Salmonella* ($P < 0.001$, Fig. 1C). When the *lrp* mutant was injected into insects, we observed cecropin levels equivalent to those seen with *Salmonella* ($P > 0.05$, Fig. 1C). The ability to suppress cecropin transcription was restored in the *lrp* (Tn*7*/*lrp*) strain (Fig. 1C). We were unable to evaluate the cecropin response to an injection of ~20 cfu/insect due to a lack of induction towards *Salmonella* over that of a PBS control at that dose and time point (data not shown).

### *Effects of* lrp *on recovery of bacteria from nematodes, colonization initiation, and nematode yield*

We previously reported that a *X. nematophila lrp-1*::Tn*5*Km mutant (HGB321) has reduced levels of nematode colonization, as calculated by nematode sonication and plating to determine the average cfu/nematode (Heungens *et al*., 2002). However, preliminary analyses of a GFP-expressing *lrp* mutant during colonization revealed the presence of higher numbers of cells within nematodes than would be expected for that colonization level (data not shown). To resolve these contrary results, we first considered the possibility that the *lrp* mutant is more sensitive to the sonication treatment used to liberate bacterial cells from surface-sterilized nematodes. Treated in parallel, the *lrp* mutant was no more sensitive to sonication than wild type (20% ± 7% survival versus 9.5% ± 3.4% survival respectively). We next examined whether the *lrp* mutant is defective in growth after recovery from nematodes. In previous colonization assays, colonies obtained from nematode sonicates were observed at 16 h post plating and *lrp* mutant colonization levels were reported to range from 0.05 to 2.95 cfu/nematode, relative to 44.6 ± 11.7 in wild type (Heungens *et al*., 2002). When the incubation time was extended to 40 h, we observed two populations of colonies: an early emerging population of colonies (0.1 ± 0.04 cfu/nematode), visible at 16 h and similar in number to the previously reported low colonization level of HGB321, and, at 40 h, more numerous, late emerging colonies (7.0 ± 2.5 cfu/nematode). Combining the number of early and late emerging colonies, the total cfu/nematode for the *lrp* mutant is, nonetheless, significantly lower than that of wild-type cells (30.3 ± 4.3 cfu/nematode, $P < 0.001$). To determine if the mutant-derived early and late emerging colonies are genetically distinct, we retested their separate abilities to colonize nematodes and found that they each gave rise to both early and late emerging colonies at similar levels (data not shown). We re-examined nematode colonization by the *lrp-1*::Tn*5*Km mutant HGB321 (Heungens *et al*., 2002) and observed the same early and late colony effects as with the *lrp-2*::Km mutant described here (HGB1059) (data not shown).

To test the hypothesis that the delayed recovery phenotype of *lrp* mutants results from a defect in adjusting to the transition between different growth conditions, we pregrew wild-type and *lrp* strains in LB broth, in insect haemolymph, on LB plates, or on defined medium plates and then plated these cultures onto LB plates. No delay in recovery was seen when the *lrp* mutant was grown in rich LB medium or insect haemolymph. However, when pregrown on defined medium plates, the *lrp* mutant exhibited the same delayed growth phenotype observed when cells were recovered from *S. carpocapsae* nematodes (data not shown).

To further quantify the nematode colonization defect of the *lrp* mutant, we examined the distribution of GFP-expressing bacteria within populations of nematodes using fluorescence microscopy. Early in colonization, *lrp* mutant cells were observed within 84% of nematodes, whereas wild-type and *lrp* (Tn*7*/*lrp*) cells were observed within 97% of nematodes ($P < 0.001$). Combining these data with the average cfu/nematode from this experiment, we calculated the average number of bacterial cells per colonized nematode and found that the *lrp* mutant colonized at ~12 cfu/colonized nematode, significantly lower

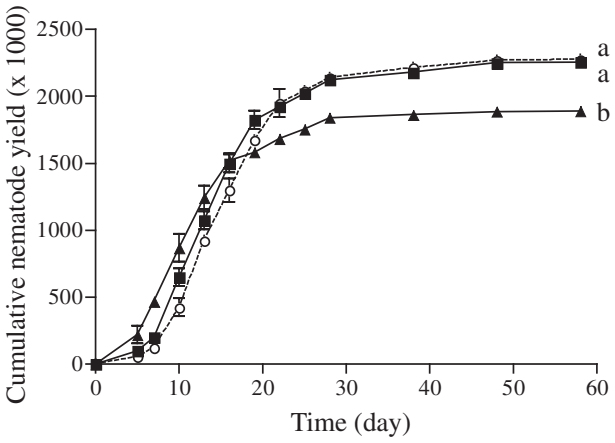


**Fig. 2.** *lrp* is required for normal nematode fecundity. The production of offspring nematodes reared on lawns of wild-type *X. nematophila* (squares), *lrp* mutant (triangles) and *lrp* (Tn*7*/*lrp*) (open circles) was monitored at the indicated time points. Replicate populations of nematodes ($n = 5$) were tested for each strain. Error bars indicate standard deviation from the mean at each time point. Different letters indicate a significant ($P < 0.001$) difference in the levels of cumulative nematode production.

than the averages of ~39 and 44 cfu/colonized nematode for wild-type and *lrp* (Tn*7*/*lrp*) strains respectively ($P < 0.001$).

During the course of the experiments described above, we noted that nematodes reared on lawns of the *lrp* mutant appeared to yield fewer offspring nematodes than those reared on lawns of wild type. To further characterize this observation, we collected nematodes from lawns of wild-type, *lrp* mutant, and *lrp* (Tn*7*/*lrp*) cells and quantified nematode yield over time. Nematodes reared on lawns of the *lrp* mutant reproduced to lower overall levels than those reared on wild-type and *lrp* (Tn*7*/*lrp*) lawns (Fig. 2).

### *Lrp regulates a large proportion of* X. nematophila *factors*

To investigate the mechanisms underlying the *in vivo* defects of the *lrp* mutant, we examined several *in vitro* phenotypes that are predicted to be important for *X. nematophila*–host interactions. Unlike wild-type cells, *X. nematophila lrp* mutants were negative for haemolysis of mammalian erythrocytes (sheep, rabbit and horse), Tween 20 lipase activity, sheep erythrocyte haemagglutination, swimming motility, swarming motility, antibiotic activity towards *Bacillus subtilis* and *Micrococcus luteus* indicator strains, intracellular crystalline protein production, and dye binding activity (Table 2).

As wild-type and *lrp* strains of *X. nematophila* proved to be different in each of the measured phenotypes, we used 2D gel electrophoresis to compare the profiles of soluble *X. nematophila* proteins from stationary-phase wild-type and *lrp* mutant cells and evaluate the number of proteins whose expression is directly or indirectly regulated by Lrp. Counting only spots that were clearly different between strains, we found that of 290 spots, the *lrp* mutant had decreased levels of ~31% of spots and increased levels of ~34% of spots, as compared with wild type. A representative section of each gel is shown in Fig. 3; other regions of gels showed similar numbers of visible changes (Fig. S1).

### *Lrp controls aspects of* X. nematophila *phenotypic variation*

Because *lrp* mutant characteristics resemble those of secondary form strains (Table 2), we hypothesized that Lrp is a key transcriptional regulator of phenotypic variation in *X. nematophila*. To determine if *lrp* effects on secondary form phenotypes are at the transcriptional level, we measured RNA levels of several genes known to encode

**Table 2.** *In vitro* phenotypes of *X. nematophila* strains.

| | Strain[a] | | | |
|---|---|---|---|---|
| *In vitro* phenotype[b] | Primary | *lrp* mutant | *lrp* (Tn*7*/*lrp*) | Secondary |
| Haemolysis of RBCs[c] | + | – | + | – |
| Haemagglutination of RBCs | + | – | + | – |
| Antibiotic production | + | – | + | – |
| Crystal proteins | + | – | + | – |
| Dye binding | + | – | + | – |
| Lipase production | + | – | + | ++ |
| Swimming motility | + | – | + | + |
| Swarming motility | + | – | + | + |

**a.** Primary form (HGB800), *lrp* mutant (HGB1059), *lrp* (Tn*7*/*lrp*) complementation (HGB1060), and secondary form (HGB1061) strains were tested for the above phenotypes according to the assays described in *Experimental procedures*.
**b.** Phenotype results are described as '+' when the activity is present at primary form levels, '–' when the activity is absent, and '++' when the activity is increased compared with primary form.
**c.** RBCs denote mammalian red blood cells. Haemolytic activity was tested against sheep, rabbit and horse erythrocytes. The same pattern of results was seen with each blood type. Haemagglutination was performed for sheep erythrocytes only.

 

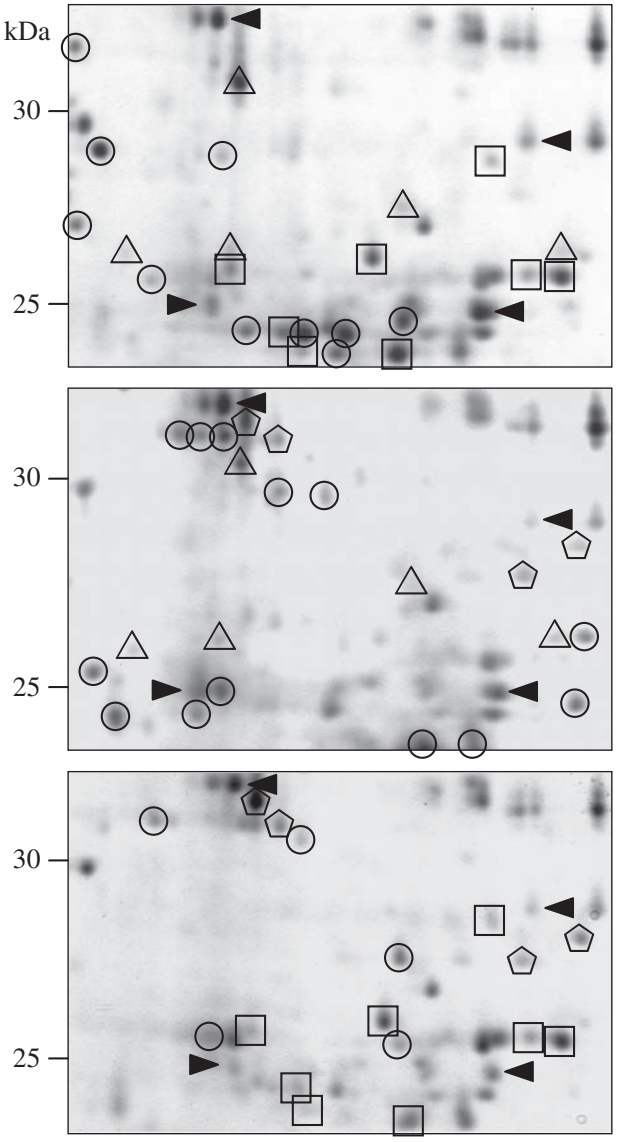


**Fig. 3.** *lrp* regulates expression of a large proportion of *X. nematophila* proteins. 2D gels for wild-type primary *X. nematophila* (top gel), the *lrp* mutant (middle gel) and secondary form *X. nematophila* (bottom gel) are shown. Circles denote spots that were unique to one gel (either in intensity or presence) (10 in wild type, 13 in *lrp* and 5 in secondary). Arrows designate reference spots that are similar in intensity between all three gels. Pentagons (4), squares (8) and triangles (5) indicate spots absent from wild type, *lrp* or secondary respectively, but present at similar intensities in the other two gels.

activities subject to phenotypic variation. Using quantitative PCR (qPCR), we determined that the putative haemolysin locus *xaxAB* (Brillard *et al*., 2001; A. Givaudan, pers. comm.) and the crystalline inclusion protein gene *pixA* (Goetsch *et al*., 2006) are expressed at significantly lower levels in both the *lrp* mutant and secondary form cells (Fig. 4), which matches the phenotypic patterns observed (Table 2). Relative to primary form, secondary form variants exhibit increased lipase activity while the *lrp* mutant produces no detectable activity against Tween 20. In primary form cells, this activity depends on the presence of *xlpA* (Park and Forst, 2006; G.R. Richards and H. Goodrich-Blair, unpubl. data), and we therefore anticipated that *xlpA* transcript levels would be unaffected or elevated in secondary form cells and reduced in the *lrp* mutant. As predicted, *xlpA* transcript levels are significantly lower in the *lrp* mutant (Fig. 4). Unexpectedly, *xlpA* transcript in secondary form cells is also significantly lower than in wild type (Fig. 4). In addition, we found that levels of *var1*, a gene previously shown to be involved in phenotypic variation (Volgyi *et al*., 2000), are unaffected in the *lrp* mutant and secondary strains (Fig. 4).

As the genes associated with the remaining variable phenotypes are, as yet, uncharacterized, we compared the overall protein profiles of the *lrp* mutant and secondary form variant. Of approximately 130 spots clearly visible by 2D gel electrophoresis, secondary form had 25 spots that were absent or at lower intensities than primary form and 27 spots that were present at higher intensities compared with primary form (Fig. 3 and Fig. S1). Of the 52 spots that differed between primary and secondary form variants, 36 were affected similarly in the *lrp* mutant (Fig. 3 and Fig. S1).

In *E. coli*, prolonged growth in nutrient-limiting conditions leads to accumulation of mutations that increase the fitness of the cell (Zambrano *et al*., 1993). These GASP mutations reproducibly occur first in the gene encoding the stationary phase sigma factor, RpoS, but *E. coli rpoS* mutants are out-competed by further GASP mutations in *lrp* (Zinser and Kolter, 2000). To determine if spontaneous mutations in *lrp* are involved in *X. nematophila* phenotypic variation, we sequenced the *lrp* region from primary and

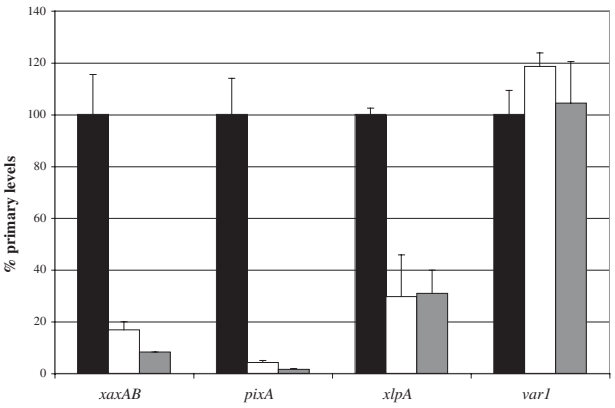


**Fig. 4.** Effects of *lrp* mutant and secondary form on *X. nematophila* transcriptional regulation. Expression levels of *xaxAB*, *pixA*, *xlpA* and *var1* were measured in primary form (HGB800) (black bars), *lrp* mutant (HGB1059) (white bars), and secondary form (HGB1061) (grey bars) strains. Data are presented as per cent primary form levels. Error bars represent standard deviation ($n = 3$).

 

secondary form variants and found no changes in the 2920 bp containing *lrp* and flanking sequences (data not shown). We also detected no changes in *var1* and regions flanking that gene (2950 bp) (data not shown). Attempts to monitor *lrp* transcription using qPCR failed due to *lrp* levels below the limit of detection for this assay (data not shown). Complementation of *lrp* expression in the *lrp* mutant restored primary form characteristics (Table 2), but increased levels of *lrp* in wild-type primary or secondary form strains had no effect on phenotypic variation, as monitored by dye binding plate assays (data not shown).

### *Lrp directly interacts with basic metabolism, insect virulence, and nematode colonization gene promoters*

Previously, we showed that *lrp* is required for activation of *xhlA* haemolysin expression and repression of *nilC* outer-membrane lipoprotein expression (Cowles and Goodrich-Blair, 2004; 2005). In this study, we demonstrate that Lrp also controls a number of other genes involved in measurable *in vitro* phenotypes (Fig. 4). In *E. coli*, studies of the interactions between Lrp and the promoter regions of genes involved in the synthesis of branched-chain amino acids, such as *ilvIH*, have provided a great deal of insight into the biochemistry of Lrp–DNA interactions (Calvo and Matthews, 1994). To examine the *in vitro* binding of Lrp to representative *X. nematophila* promoter regions, we purified and titrated Lrp in promoter DNA gel-shift assays. Lrp bound *xhlBA*, *nilC* and *ilvIH* promoters with a similar affinity (approximately 1 μM, data not shown) under these conditions. Furthermore, the addition of a 10-fold excess of unlabelled promoter DNAs decreased the proportion of bound DNA in each reaction, whereas the addition of a 10-fold excess of unlabelled, non-specific DNA had no effect on the proportion of bound DNA in reactions (Fig. 5).

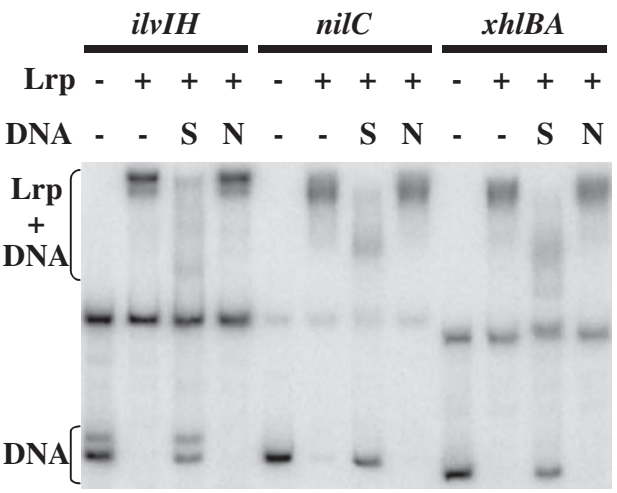


**Fig. 5.** Characterization of Lrp binding to *ilvIH*, *nilC* and *xhlBA* promoters. To examine Lrp binding specificity, reactions were performed with no Lrp (first lane) or 1 μM Lrp (remaining lanes) and a 10-fold excess of unlabelled specific DNA fragment (S, third lane) or non-specific fragment (N, last lane). Note that the positions of contaminating bands do not change under any condition tested.

## Discussion

In this study, we show that Lrp regulates, directly or indirectly, important aspects of *X. nematophila* pathogenesis and mutualism. *lrp* is the first gene identified to play a role in both of these lifestyles, as it is necessary for normal virulence and immune modulation, as well as for normal nematode colonization and development. We further demonstrate that Lrp is a global regulator in *X. nematophila*, affecting the expression of approximately 65% of the proteins observed and numerous phenotypes associated with wild-type strains of this bacterium. Given the dramatic role of *lrp* in gene expression, general and non-specific defects in metabolism or growth might reasonably explain the pathogenesis and mutualism defects of the *lrp* mutant. However, the *lrp* mutant does not have growth defects in LB, insect haemolymph, or on defined medium agar. Furthermore, the *lrp* mutant has altered expression of known virulence (*xhlA*) and mutualism (*nilC*) factors (Cowles and Goodrich-Blair, 2004; 2005), and we have shown here that Lrp binds to the regions upstream of these genes. Therefore, in addition to its influence on basic metabolism, Lrp appears to contribute to pathogenesis and mutualism by regulating specific factors necessary for these processes. Considering the large proportion of proteins that are significantly affected in expression in an *X. nematophila lrp* mutant, the measured defects in pathogenesis, mutualism and basic metabolism are surprisingly subtle. Such subtle defects could have a profound impact on competitive fitness that may be revealed by monitoring the performance of the *lrp* mutant in competition with wild type.

The *X. nematophila lrp* mutant is attenuated in virulence as well as modulation of cellular and humoral immunity in *M. sexta* larvae, suggesting that Lrp regulates many aspects of the infection process. Lrp homologues are found extensively among bacteria, including several pathogens (Brinkman *et al*., 2003), but until this study none has been directly linked to virulence. Furthermore, previous research has established that *X. nematophila* fails to elicit or actively suppresses several aspects of insect immunity, including nodulation and antimicrobial peptide induction (Park *et al*., 2003; 2007; Ji and Kim, 2004), but *lrp* is the first *X. nematophila* gene known to be necessary for these activities. Due to the scope of factors affected by Lrp, we hypothesize that this global regulator controls one or more regulatory cascades that ultimately lead to expression of virulence and immune modulation factors. We previously identified one Lrp-dependent

 

virulence determinant, the haemolysin encoded by *xhlA* (Cowles and Goodrich-Blair, 2005). Heterologously expressed XhlA lyses insect haemocytes but an *X. nematophila xhlA* mutant retains wild-type levels of cytolytic activity (Cowles and Goodrich-Blair, 2005). The profound defect of the *lrp* mutant in secreted cytolytic activities suggests that Lrp controls other haemolysins/cytolysins besides XhlA. Indeed, our data demonstrate *lrp* mutants also have reduced expression of *xaxAB*, encoding the *flhDC*-dependent C1 haemolysin (Brillard *et al*., 2001; A. Givaudan, pers. comm.). If, as in mammals (Trinchieri, 2003), *M. sexta* haemocytes induce downstream immune responses such as the induction of antimicrobial peptides and nodule formation, then the immunosuppressive defects of the *lrp* mutant could be explained simply by loss of cytolytic activity in this mutant.

The data presented here show that the *lrp* mutant is defective in two stages of mutualism with the nematode host, initiation of colonization and outgrowth of colonizing bacteria [a fully colonized nematode results from the outgrowth of one to two founding bacterial cells (Martens *et al*., 2003)], and thus it is likely that Lrp regulates factors involved in these processes. Previously, we showed that Lrp represses the colonization factor *nilC* (Cowles and Goodrich-Blair, 2004). Therefore, we hypothesized that the *lrp* mutant colonization defects may result from inappropriate expression of *nilC*. However, a mutation in *nilR* (a second repressor of *nilC*) has the same effects on *nilC* expression but does not cause a colonization defect (Cowles and Goodrich-Blair, 2006), indicating that Lrp must regulate additional factors involved in nematode colonization. Two additional nematode colonization genes, *nilA* and *nilB*, also require *nilR* and *lrp* for repression (Cowles and Goodrich-Blair, 2006). By extension of the same logic, the overexpression of these additional nematode colonization genes is likely not the cause of the nematode colonization defects observed in the *lrp* mutant.

The *lrp* mutant is defective in outgrowth within nematodes as evidenced by the observation that the average number of *lrp* mutant cells within colonized nematodes is 33% of wild type. As *lrp* regulates metabolism in other bacteria, this defect could result from inappropriate co-ordination of metabolic functions during growth within the nematode. In addition, the *lrp* mutant displays a recovery phenotype, which may reflect a defect of the *lrp* mutant in making the transition from the nematode environment to that of an LB agar plate. Furthermore, the delayed colony emergence phenotype appears in both the nematode-to-LB and the defined medium-to-LB transitions, indicating that the nematode growth environment may be nutrient limiting. Taken together, these results suggest that, as with other *lrp* homologues (Yokoyama *et al*., 2006), *X. nematophila* requires *lrp* for 'feast or famine' adaptation to nutrient availability during interactions with the host. Interestingly, as the *lrp* mutant presents no recovery phenotype during the transition from growth in LB broth to insect haemolymph, this adaptation does not appear to be necessary for early stages of insect infection and does not explain the delayed virulence of this strain. However, it is possible that Lrp is necessary for *X. nematophila* adaptation to alternate nutrient sources (e.g. upon haemolymph depletion or within sequestered tissues).

We have further extended the analysis of the role of *lrp* in mutualism by demonstrating that the *lrp* mutant supports the production of fewer nematode progeny than wild type. This phenomenon cannot be explained by growth or survival defects of *lrp* mutant during the assay because *lrp* mutant survival is indistinguishable from wild type under the nematode cultivation conditions (Heungens *et al*., 2002). One possible explanation for our findings is that Lrp may control bacterial contributions to nematode sexual reproduction. In the absence of *X. nematophila*, *S. carpocapsae* reproduction fails to occur and nematode reproductive organs fail to form normally (Poinar and Thomas, 1966). Heat-killed or fractionated *X. nematophila* do not support nematode development (Volgyi *et al*., 1998; C.E. Cowles and H. Goodrich-Blair, unpubl. obs.), suggesting that *X. nematophila* actively provides functions necessary for this process. If sexually reproductive adult nematodes develop in a synchronized fashion within reproducing nematode populations, then failure of the *lrp* mutant to properly direct sexual reproduction could result in reduced fecundity. Alternatively, because nematodes feed on *X. nematophila* during development, lower nematode yield may indicate that the *lrp* mutant is a poor nutrient source compared with wild type.

In this work, we present evidence that the regulation of *X. nematophila* phenotypic variation is controlled, at least in part, by the global regulator Lrp. Multiple phenotypes of secondary form variants resemble those of the *lrp* mutant, and ~70% of differences between primary and secondary form protein profiles are also found in the *lrp* mutant. Using qPCR, we provide the first example of a transcriptional mechanism for phenotypic variation in this bacterium and demonstrate that Lrp acts as an activator of primary form traits in *X. nematophila*. These results contrast with regulation of phenotypic variation in another entomopathogenic bacterium, *P. luminescens*, which is controlled by HexA-dependent transcriptional repression of primary form traits, as well as an uncharacterized post-translational mechanism (Wang and Dowds, 1993; Joyce and Clarke, 2003).

The fact that secondary form variants are virulent towards insects and colonize nematodes (Volgyi *et al*., 1998; K.N. Cowles and H. Goodrich-Blair, unpubl. data) suggests that activities subject to phenotypic variation are not required for these processes. However, phenotypic variation is reversible (K.N. Cowles, C.E. Cowles

and H. Goodrich-Blair, unpubl. data) and therefore functions essential for host interactions may be expressed appropriately in reverted cells. This topic is difficult to address until genes encoding variable activities have been identified and characterized for their role in host interactions. A recent study identified the gene, *pixA*, that encodes one of two variant crystal proteins (Goetsch *et al*., 2006), and we show here that Lrp and phenotypic variation transcriptionally regulate *pixA*. Goetsch *et al*. (2006) demonstrated that *pixA* is not required for virulence or nematode colonization. However, a *pixA* mutant still produces one of the two crystal proteins absent in secondary form variants and this protein may provide redundant functions. Future experiments examining the effect of a strain completely deficient in crystal proteins will help to resolve this matter.

Lrp is involved in many, but not all, aspects of *X. nematophila* phenotypic variation. *X. nematophila* ATCC 19061 secondary form variants overproduce lipase activity while the *lrp* mutant has no activity in this assay. Lipase activity is encoded by *xlpA* and depends on the flagellar export apparatus for secretion (Park and Forst, 2006; G.R. Richards and H. Goodrich-Blair, unpubl. data). Intriguingly, despite its hyper-lipolytic phenotype, the secondary variant, like the *lrp* mutant, has reduced *xlpA* transcript levels relative to wild type. At least two scenarios may explain these results: *X. nematophila* may encode a second lipase activity that is Lrp-dependent (and therefore absent in an *lrp* mutant) and only expressed in secondary form. Alternatively, secondary variants may have a mechanism to increase translation, stability, or secretion of the XlpA lipase, thus compensating for reduced *xlpA* transcript. Preliminary data suggest the latter scenario is more likely: a secondary form variant of the *xlpA* mutant does not express lipolytic activity *in vitro* indicating *X. nematophila* does not express a secondary specific, Lrp-dependent lipase (G.R. Richards and H. Goodrich-Blair, unpubl. data).

The *lrp* mutant and the secondary variant are further distinguished by the fact that 16 of the 52 spots detected that differ between primary and secondary protein profiles are unchanged in the *lrp* mutant. Interestingly, 12 of those 16 spots are more intense in secondary form relative to the other two strains and may be connected to overproduction of lipase activity in this variant. Lrp-independent control of secondary phenotypes may occur through the previously identified *var1* gene (Volgyi *et al*., 2000). Var1 is a 121-amino-acid protein of unknown function, but an *X. nematophila var1* mutant loses the same activities as secondary form (although lipase production was not reported), and expression of these activities is complemented by expression of *var1 in trans* (Volgyi *et al*., 2000). We demonstrate here that transcriptional levels of *var1* are unaffected by phenotypic variation and an *lrp* mutation, indicating that *var1* is not a member of the Lrp regulon. This result does not preclude the possibility that Var1 interacts with Lrp to regulate phenotypic variation. For example, Volgyi *et al*. (2000) suggested that Var1 might serve as a chaperone promoting folding or secretion of primary-form expressed factors. However, it is also plausible that Var1 is part of a distinct regulatory pathway.

We show here that Lrp is a global regulator in *X. nematophila*, while in some other bacteria it has a narrow influence on gene expression (Madhusudhan *et al*., 1993; Belitsky *et al*., 1997; Friedberg *et al*., 2001). Furthermore, we demonstrate that Lrp regulates both virulence and mutualism functions. How are these regulatory functions co-ordinated, and what dictates Lrp function as a global versus specific regulator? Lrp regulation of promoters can be modulated through direct interactions with other DNA-binding proteins. For example in *E. coli*, Lrp, CpxR and PapI interact during binding at the *papI-papX* promoter region (Hernday *et al*., 2003; 2004). These types of interactions can expand the range of stimuli sensed by Lrp and alter its specificity for subsets of promoters. Similarly, *X. nematophila* Lrp activity at distinct classes of promoters may be modulated by transcription factors that, in turn, respond to specific nematode or insect environmental stimuli. Indeed, NilR and Lrp synergistically repress expression of the nematode colonization factor *nilC*, but a *nilR* mutant does not show the pleiotropic phenotypes of the *lrp* mutant examined herein (Cowles and Goodrich-Blair, 2006). Analysis of Lrp-interactive transcription factors necessary for mutualism or pathogenesis should shed light on the co-ordination of these two processes.

## Experimental procedures

### *Bacterial strains and culture conditions*

*X. nematophila* ATCC 19061 (HGB800) was used as wild-type for all experiments except where specifically indicated. *S. enterica* serovar Typhimurium LT2 (D. Downs, University of Wisconsin-Madison) was used as a positive control for insect immunity experiments. Secondary form variant HGB1061 was isolated by repeated isolation of red colonies from NBTA plates (Boemare and Akhurst, 1988). Primary variants grow as blue colonies on NBTA plates while secondary form colonies are red (Boemare and Akhurst, 1988). Liquid bacterial cultures were grown in LB broth (Miller, 1972) at 30°C. Liquid media used with *X. nematophila* strains were stored in the dark and solid media were solidified with 2% agar and supplemented with 0.1% sodium pyruvate (Xu and Hurlbert, 1990). Defined agar plates were made as previously described (Orchard and Goodrich-Blair, 2004). *S. carpocapsae* nematode strain All (H. Kaya, UC-Davis) stocks were maintained in *Galleria mellonella* larvae and were reared on lawns of bacteria grown on lipid agar plates for colonization assays (Vivas and Goodrich-Blair, 2001).

### *Molecular biological methods*

Standard molecular biological methods were used for this study (Sambrook *et al*., 1989). Oligonucleotides (Integrated DNA Tech-

 

**Table 3.** Primers used in this study.

| Primer | Sequence (5′ to 3′)[a] | Use |
|---|---|---|
| CecropinForGC | CAGCGCATTCGCCATGGC | Cecropin assay |
| CecropinRev | ACGGTCGCGACTGCAGCC | Cecropin assay |
| MsActMiniFor | GGAAATCGTTCGTGACATCA | Cecropin assay |
| MsActMiniRev | CGGAACCTCTCGTTACCGAT | Cecropin assay |
| Mg primer | CGGGCAGTGAGCGCAACGTTTTTTTTTTTT | cDNA development |
| LrpcompFwd | GGGCCTAGGCTAATCAATTGTTTCTATAGAATAG | *lrp* complementation |
| LrpcompRev | GCGGGTACCGTGCCAGCTTGGATCGGATGG | *lrp* complementation |
| XaxAquant1 | CTGATAAGCAGCTGGCTG | qPCR (*xaxA*) |
| XaxAquant2 | CCATCTGATAACTCACCGCC | qPCR (*xaxA*) |
| XaxBquant1 | GGATGCGGATCGGGAGAAG | qPCR (*xaxB*) |
| XaxBquant2 | CTGGCTATCTCCGCACCTTG | qPCR (*xaxB*) |
| PixAquant1 | TATAGGTGACATGATCCG | qPCR (*pixA*) |
| PixAquant2 | CAACAGATCTCACAACGC | qPCR (*pixA*) |
| XlpAquant1 | CGCTGCATTGGCAACAGGAAA | qPCR (*xlpA*) |
| XlpAquant2 | GCCAATCGTGCTGAACGGTAT | qPCR (*xlpA*) |
| Var1quant1 | AGTCAGTTCTGGTCAGCAGGG | qPCR (*var1*) |
| Var1quant2 | ACCGATAAGATTGCCAAAGGG | qPCR (*var1*) |
| RecAminFor | TGTCCGTTTGGATATCCGCC | qPCR (*recA*) |
| RecAminRev | CCCAGAGTATTAATACCTTCCCCAT | qPCR (*recA*) |
| NcoLrpFor | NNNCCATGGTTGATAATAAGAAGC | Lrp purification |
| SalLrpRev | NNNGTCGACACGAGTCTTAATCACC | Lrp purification |
| IlvI-200 | ATTGAATCTATGTATCGG | Gel shift assay |
| IlvIrev | GTTTGCAAGTTAATTGTCG | Gel shift assay |
| NilC-200 | AAGGGCCCGATATTTTGCTGGCAAGA | Gel shift assay |
| NilCLacZRev | GCGAATTCTTGTATTCATATAATGATT | Gel shift assay |
| XhlBAgs1 | CCAAAATGAGTTATTATAAACCAC | Gel shift assay |
| XhlBApeATG | GCCGTCCTTTTAATCATGGG | Gel shift assay |

**a.** Engineered restriction enzyme sites are underlined.

nologies, Coralville, IA) are described in Table 3. All DNA constructs were sequenced to ensure that inserts did not contain errors.

### *Construction of* lrp *mutant and complementation strains*

The HGB800 *lrp-2*::Km mutant (HGB1059) was constructed by inserting a kanamycin (Km)-resistance cassette into a unique BglII site at bp 32 of the *X. nematophila lrp* gene using allelic exchange. To complement this mutation, the *lrp* locus, including the predicted promoter region, was PCR-amplified from *X. nematophila* genomic DNA with primers LrpcompFwd and LrpcompRev (Table 3) and cloned into the miniTn*7*–delivery vector pEVS107. The resulting plasmid was introduced into HGB1059 to create *lrp* (Tn*7*/*lrp*) (HGB1060) and confirmed as described (Cowles and Goodrich-Blair, 2004).

### *Insect immunity and virulence assays*

Tobacco hornworm *M. sexta* larvae were raised from eggs (NCSU Insectary, Raleigh, NC) as described (Cowles and Goodrich-Blair, 2005). For virulence assays, *X. nematophila* strains were grown overnight in LB broth, subcultured 1:100 into fresh LB broth for 24 h, subcultured 1:500, and grown to $OD_{600} = 0.8$ (log phase cells). [Log phase cells exhibit higher virulence at low doses than do stationary phase cells such as those used in protocols described in previously published reports (Cowles and Goodrich-Blair, 2005).] Cultures were diluted to the desired concentration in sterile PBS and injected into fourth-instar insect larvae as described (Cowles and Goodrich-Blair, 2005). Insects were monitored for 90 h post injection. Insects still alive by this time point showed no disease symptoms and were considered clear of bacterial infection.

Cytolytic assays were performed as described previously (Cowles and Goodrich-Blair, 2005). Briefly, haemolymph from fifth-instar larvae was drained into cold anticoagulant buffer (Mead *et al*., 1986) at a ratio of approximately 5:1 (v/v). Haemocytes were pelleted, resuspended in the same volume of Grace's Insect Medium and allowed to bind to sterile glass coverslips for 15 min at room temperature (RT). The medium was then replaced with 40 µl of cell-free supernatants from *X. nematophila* strains grown in LB broth to $OD_{600} = 1.0$, 4.0 or 8.0. Samples were incubated for 1 h at RT and dead cells were identified using propidium iodide staining.

For nodulation and cecropin suppression assays, fourth-instar insect larvae were injected with cultures prepared as described above for virulence assays. Insects were frozen at −80°C at 9 h and 16 h post injection for cecropin and nodulation experiments respectively. For nodulation assays, insects were individually thawed and dissected (Park *et al*., 2003) with 20 insects per treatment. For cecropin experiments, total RNA was extracted from three insects per treatment per experiment using TRIzol® (Invitrogen, Carlsbad, CA), treated with DNase I (Boerhinger, Mannheim, Germany), and used to make cDNA with primer Mg (Table 3) and AMV Reverse Transcriptase (Promega, Madison, WI). Reactions for qPCR were performed as described (Cowles and Goodrich-Blair, 2005). Cecropin transcript levels were measured using CecropinForGC and CecropinRev primers (Table 3). Cecropin cycle threshold (Ct) results for each sample were normalized to actin Ct values (amplified with MsActMiniFor and MsActMiniRev, Table 3), compared with cecropin levels in response to PBS injection, and expressed as 'fold PBS'.

 

### *Nematode colonization assays and nematode yield*

Nematode colonization assays were performed by cultivating *S. carpocapsae* nematodes on lipid agar plates (Vivas and Goodrich-Blair, 2001) seeded with bacterial lawns and sterile *S. carpocapsae* nematode eggs. Nematode colonization was assayed as described (Cowles and Goodrich-Blair, 2004), except dilution-plated sonicates were monitored at 16 h and 40 h. GFP-expressing *X. nematophila* cells were observed inside nematodes using fluorescence microscopy as described (Martens *et al*., 2003). To determine the proportion of nematodes in a population that were colonized, 100 nematodes from five separately reared populations were observed by fluorescence microscopy. Nematode yield was assayed by collecting nematodes at the times indicated in Fig. 2. The total number of nematodes present was determined by comparison with a standard of nematodes at known concentrations.

### *Phenotypic assays*

Haemolysin activity (Rowe and Welch, 1994) towards sheep, rabbit and horse erythrocytes (Colorado Serum Company, Denver, CO), Tween 20 lipase activity (Sierra, 1956), haemagglutination (Moureaux *et al*., 1995), swimming (Vivas and Goodrich-Blair, 2001) and swarming (Givaudan *et al*., 1995) motility, antibiotic production (Maxwell *et al*., 1994), crystal protein production (Vivas and Goodrich-Blair, 2001), and dye binding assays (Boemare and Akhurst, 1988) were performed as described.

### *Two-dimensional gel electrophoresis*

Cultures were grown to stationary phase, pelleted, and washed three times in 50 mM NaCl, 10 mM Tris, pH = 7.5, followed by a single wash in deionized water. Cells were resuspended into 5 ml of deionized water and lysed by French Pressure lysis. The lysates were sonicated using a probe sonicator to shear genomic DNA then cleared by centrifugation and passage through a 0.2 μm syringe filter. The cleared lysate was then subjected to ultracentrifugation (30 min at 100K rpm in a Beckman TLA100.2 rotor) to remove cell membranes and insoluble proteins. Twenty or 50 μg of the soluble fraction was focused on a 13 cm Amersham pH4-7L IPG strip using an Ettan IPGphor IEF system (15 h, 50 V; 1 h, 500 V; 1 h, 1000 V; 5 h, 8000 V), using buffers recommended by the manufacturer. The focused strips were then equilibrated and proteins were separated on a 14% SDS/PAGE gel. Gels were silver stained and digitized on a flatbed scanner. Triplicate experiments were run to confirm the reproducibility of results.

### *Quantitative measure of transcript levels*

Total RNA from wild-type primary form (HGB800), secondary form (HGB1061), and *lrp* mutant (HGB1059) strains was isolated at $OD_{600}$ = 8.0 (stationary phase cells) using TRIzol® extraction protocol (Invitrogen, Carlsbad, CA), treated with DNase I (Boerhinger, Mannheim, Germany), and used to make cDNA with random hexamer primers (Integrated DNA Technologies, Coralville, IA) and AMV Reverse Transcriptase (Promega, Madison, WI). Reactions for qPCR were performed as described (Cowles and Goodrich-Blair, 2005). Transcript levels of *xaxAB*, *pixA*, *xlpA* and *var1* were measured with primers listed in Table 3. Results for *xaxA* and *xaxB* expression levels were the same (data not shown) and consequently all transcription data are shown as *xaxAB* transcript levels. As expected, no product was detected using water and DNase-treated RNA from each sample as template. Ct results for each sample were normalized according to *recA* levels (amplified with RecAminFor and RecAminRev), converted to arbitrary units factoring in a twofold change in product amounts per cycle, compared with wild-type primary form levels, and expressed as fold wild-type.

### *Purification of Lrp*

The Lrp-$His_6$ overexpression plasmid was constructed by PCR amplifying *lrp* from *X. nematophila* genomic DNA using NcoLrpFor and SalLrpRev primers (Table 3) and cloning the digested, amplification product into pET28a (Novagen, Madison, WI). Lrp was purified from *E. coli* BL21 (λDE3) harbouring pET28a/CtagLrp. Two litres of culture were grown for 24 h at 30°C with vigorous shaking in LB broth + Km (50 μg $ml^{-1}$). Cells were pelleted, washed twice in 300 mM NaCl, 50 mM $NaPO_4$, pH = 8.0 and resuspended in the same buffer + 20 mM imidazole, followed by French Pressure lysis. Protein was batch-purified from cleared lysates using 2 ml of Ni-NTA resin (Qiagen, Valencia, CA), according to the manufacturer's instructions. Imidazole was removed by two rounds of dialysis against 250 mM NaCl, 50 mM $NaPO_4$, pH = 8.0. Purified proteins were estimated to be > 99% pure, as judged by Coomassie staining of an SDS/PAGE gel of the purified protein. Both N-terminal- (not described) and C-terminal-$His_6$-tagged variants of the protein were purified, but the N-terminal-$His_6$-tagged variant showed a low affinity for promoter DNAs in gel-shift assays and was not studied further.

### *Gel-shift assays*

Fragments from the *ilvIH*, *nilC* and *xhlBA* promoter regions (231 bp, 223 bp and 207 bp upstream of the predicted start codon respectively) were generated using ExTaq polymerase, *X. nematophila* genomic DNA and the primers listed for this purpose in Table 3. DNA fragments were end-labelled using γ-$^{32}$P-ATP and T4 polynucleotide kinase. Unincorporated radioactivity was removed using the Qiagen Nucleotide Removal Kit. To determine the binding constant for Lrp and promoter DNAs, gel shift assays were performed using 100 ng of DNA fragment, Lrp-$His_6$ (0, 0.33, 1 or 3 μM), 10 μg of salmon sperm DNA, 100 mM NaCl, 20 mM Tris, pH = 7.5 in a total volume of 20 μl. The specificity of Lrp-$His_6$ for each fragment was addressed by performing the same binding assay with 0 or 1 μM Lrp-$His_6$, and a 10-fold excess of either unlabelled promoter DNA fragment (1 μg) or salmon sperm DNA (100 μg). Samples were incubated at RT for 15 min and were separated on 5% polyacrylamide 0.5× TBE gels.

### *Statistics*

*P*-values were calculated using one-way ANOVA with Tukey's post-test at a 95% confidence interval.

### *GenBank accession numbers*

The GenBank accession numbers of *lrp*, *xhlBA*, *nilC*, *xaxAB*, *pixA*, *recA*, *ilvIH* and *xlpA* are AY077463, AY640584, AY077465, DQ249320, AY563156, AF127333, EF123200 and EF123201 respectively.

 

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Lrp regulates expression of a large proportion of *X. nematophila* proteins, many that are also regulated by phenotypic variation. Two-dimensional gels for wild-type primary form *X. nematophila* (left gel), the *lrp* mutant (middle gel), and secondary form *X. nematophila* (right gel) are shown.

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