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Control of Bioluminescence in Phase Variants of *Photorhabdus luminescens* Hm and in Hyp, a Hyperpigmented Mutant Obtained from a Phase II Variant

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Abstract

Bioluminescence is a property that is common in marine bacteria and almost totally absent in terrestrial bacteria, the only known example being the entomopathogenic bacteria in the group *Photorhabdus luminescens*. Bioluminescence is also one of several traits that are modulated when secondary variants appear in *P. luminescens*; that is, the secondary variants are in general very dim, or dark. We present here some preliminary studies of the regulation of light emission in *P. luminescens* in the hope that they may elucidate some of the mechanistic details involved in the conversion between primary and secondary variants. Bioluminescence is a late function in *P. luminescens*, increasing dramatically after the culture reaches an optical density of 2.0 or more. Addition of rifampicin (RIF), an inhibitor of mRNA synthesis, results in early development of the luminous system; this enhancement is seen approximately two generations after RIF addition. Secondary form variants of strain Hm emit only about 1% as much light as the primary forms, and this low level of luminescence can be restored to the high level of the primary form by the addition of RIF, indicating that the *lux* genes are completely functional, and consistent with the hypothesis that the defect that leads to decreased luminescence in the secondary form is at least partially at the post-transcriptional level of regulation. The effect of RIF is specific for light emission, reversing none of the other properties associated with the primary/

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secondary conversion. In addition, a mutant called hyper was isolated as a hyperpigmented, hyperluminous strain. These two properties are apparently controlled together, as revertants to a wild type level of pigmentation also show a wild type (primary) level of luminescence. These data suggest that a variety of control points and mechanisms may be involved in the regulation of factors considered to be part of the primary/secondary form conversion.

Keywords: *Xenorhabdus, Photorhabdus,* phase variation, bioluminescence, rifampicin

1. Introduction

Akhurst (1980) first described form (phase) variation in *Xenorhabdus* and *Photorhabdus* as a type of variation that involved several phenotypic traits, but which could be consistently defined by changes in just two biochemical properties, namely the absorption of bromothymol blue, and the reduction of triphenyltetrazolium chloride (TTC). In subsequent studies (Akhurst, 1982; Akhurst and Boemare, 1990; Bleakley and Nealson, 1988; Boemare and Akhurst, 1988) several traits were often seen to vary simultaneously in the phase II forms. These included protease, lipase, intracellular protein crystal, antibiotic production, pigment production, and, for *Photorhabdus luminescens*, bioluminescence (Table 1). It was noted by Akhurst and Boemare (1990) however, that considerable variation can occur in these traits, even between phase I strains, and, furthermore, that for some species, the strains tested did not show variation in all characters.

Table 1. Phase-specific properties of *Photorhabdus luminescens* Hm

Property	HM1[c]	HM2[c]	HM2 + RIF[a]	HYP
Dye uptake	+++	–	–	nt
Bioluminescence	100	0.1	100	1,000[a]
Pigmentation	+++	–	–	++++[a]
Antibiotic production	+++	–	–	+++[b]
Lipase	+++	–	–	nt
Protease	+++	–	–	nt
Intracellular crystals	+++	–	nt	nt

[a]Data from Hosseini, 1994; [b]Unpublished results from Prof. A. Fodor; [c]Data from Bleakley et al., 1988; nt = not tested.

Bleakley and Nealson (1988) reported a phase II isolate of *P. luminescens* Hm (Hm2) that was deficient (but not totally negative) for many of the properties described above. Because the measurement of bioluminescence was fast, easy, and sensitive, it was used as a quantitative "handle" for the study of a phase-specific property. For instance, it was easy to see that the luminescence of phase II variants was only about 0.1% of that of phase I forms, although they often make substantially higher percentages (up to 10% of phase I levels) of the bacterial luciferase (Schmidt et al., 1989). Furthermore, growth conditions that favored high luminescence, favored the expression of other factors involved in the phase transition, suggesting that coordinate control might occur over these factors (Bleakley and Nealson, 1988).

The situation in *P. luminescens* may be different from that seen in the *Xenorhabdus* species with regard to form variation, as to this time, no bona-fide revertants to phase I form have been seen in *P. luminescens*, while such revertants are apparently common in at least some of the *Xenorhabdus* species (Akhurst et al., 1992).

It is tempting to speculate, based on the above observations, that there is a master switch controlling phase transition in *P. luminescens*. This switch should have control over all properties associated with the primary/secondary forms, and operate at the same level (transcription, translation, etc.) for all functions. However, even in the first paper by Akhurst (1980), it was noted that some intermediate secondary-like cultures are seen, which appear to be altered in some of the properties, but not others. Further work by Akhurst and Boemare (1990) suggested that the situation is probably much more complex than a simple master switch mechanism controlling all factors. This is underscored by a recent paper by Gerritsen et al. (1992) in which a series of variants of *P. luminescens* were described with a range of properties in pigmentation, inclusion granules, bioluminescence, and antibiotic production, and all of which were deficient in dye uptake. Two of the variant types were unstable, reverting to the phase I after a few days in culture. While the authors concluded that these were not true phase II forms, it seems likely that the mechanism controlling the formation of these variants could somehow be connected with the more commonly studied phase conversion, and is therefore important to understand.

We report here luminous properties of phase variants of *P. luminescens* (strain Hm) and the fact that rifampicin (RIF), an inhibitor of mRNA synthesis can phenotypically reverse the luminous phenotype for the phase II dim form. We also report on the luminous properties of a chemically induced mutant strain called hyp (hyper), which is altered in pigmentation and luminescence, but is still sensitive to stimulation by RIF.

2. Materials and Methods

Bacterial strains

The bacteria used in these studies were phase I (Hm1) and phase II (Hm2) variants of *P. luminescens* (strain Hm) from the culture collection in our laboratory. Colonies were maintained and transferred weekly on Luria-Bertani (LB) agar (Miller, 1972). Colonies were routinely checked for light emission to verify that phase phenotypes were maintained. In addition, a mutant strain called hyp (hyper) was isolated after mutagenesis of a secondary variant with ICR-191, using standard methods of mutagenesis (Miller, 1972). Hyp was identified as a hyperpigmented colony, and later shown to be hyperluminous as well.

Media and growth conditions

LB liquid medium was used for routine growth and maintenance, and for most of the experiments described here. It consisted of 5 g yeast extract, 10 g tryptone, and 10 g NaCl in one liter of distilled water, with the pH adjusted to 7.4 (solid media contained 15 grams of agar). DMX (defined minimal) medium (Bleakley and Nealson, 1988) contained 1.7 mM $MgSO_4$, 9.4 mM NH_4Cl, 0.18 mM $CaCl_2$, 5 mM NaCl, 6.3 μM $FeCl_3$, 0.15 μM $MnCl_2$, 0.12 μM Na_2MoO_4, 30 mM sodium phosphate buffer (pH = 7.4), 30 mM DL-malic acid, and 10 mM proline.

Cells were grown in 250 ml culture flasks with shaking at approximately 150 rpm in a New Brunswick Psychrotherm temperature controlled shaker at 30°C unless otherwise indicated. After initial experiments with different volumes of culture medium, all experiments were begun with 50 ml of culture volume. With sampling, the volume decreased during the experiment so that 25 ml or less usually remained at the termination of the experiment. Under these conditions, no effects of oxygen limitation were noted.

Measurement of growth and luminescence

Growth was estimated by measuring absorbance (OD_{560}) in 1 cm cuvettes, using an LKB Ultraspec spectrophotometer at 560 nm. One ml samples were used, and were diluted when sample OD reached values greater than 0.2–0.3 units.

Luminescence was measured in scintillation vials containing a 1 ml sample of cells which were exposed to a photomultiplier tube in a light tight chamber fitted with a shutter. The samples were mixed vigorously and measured immediately after aeration. This was recorded as in vivo luminescence or

Light Units (LU)/ml. The samples were then removed, 100 µl of a solution of decanal (10 µl in 10 ml of dimethyl sulfoxide) (Schmidt et al., 1989) was added to each sample, and luminescence was again measured. This was recorded as aldehyde-stimulated in vivo luminescence. This has been shown to be a good measurement of luciferase content (Schmidt et al., 1989). Luminescence is expressed either as light per ml of culture (LU/ml), or as specific activity (LU/OD).

Rifampicin (RIF) and chloramphenicol (CM) were obtained from Sigma Chemical Co. (St. Louis). Production of protease, lipase, pigments and antibiotics were qualitatively determined as previously described (Bleakley and Nealson, 1988).

3. Results

The bioluminescence of Hm1, and Hm hyp are shown in Fig. 1, in which the specific activity of luminescence (LU/OD) is plotted as a function of time (1A) and as a function of growth as measured by optical density (1B). A time lag in synthesis of 5–6 hours is seen for both Hm1 and hyp, followed by a steady increase in light and luciferase content (aldehyde stimulated luminescence) for about 10 hours (1A). For both Hm1 and hyp, the luminescence begins to increase only after the cultures have reached an OD of approximately 2.0, equal to 10^9 cells per ml (1B).

In Fig. 2, it is shown that while different levels of rifampicin (RIF) inhibit growth as expected (2A), low concentrations actually stimulate luminescence (2B) of strain Hm1. Low levels (2 µg/ml) of RIF have little effect on growth, but have a major stimulatory effect on the development of luminescence. Higher levels stimulate luminescence and inhibit growth, while very high levels stop growth, and inhibit luminescence. Because it had almost no effect on growth, but strongly stimulated luminescence, a concentration of 2 µg/ml RIF was chosen for subsequent experiments.

Fig. 3 shows that 2 µg/ml RIF increases both the unstimulated and the aldehyde-stimulated luminescence for Hm1 (3A) and Hm2 (3B). The aldehyde stimulation of the RIF-treated cultures is still apparent, but considerably less than is seen in the absence of RIF. That is, for the primary form (panel A), the aldehyde stimulation is a factor of about 20 fold in the control, but only about a factor of 1.5–2 in the RIF-enhanced culture. For the secondary form the aldehyde stimulation is almost 100 fold in the control, but decreases to about 1.5–2 fold in the RIF-stimulated cultures.

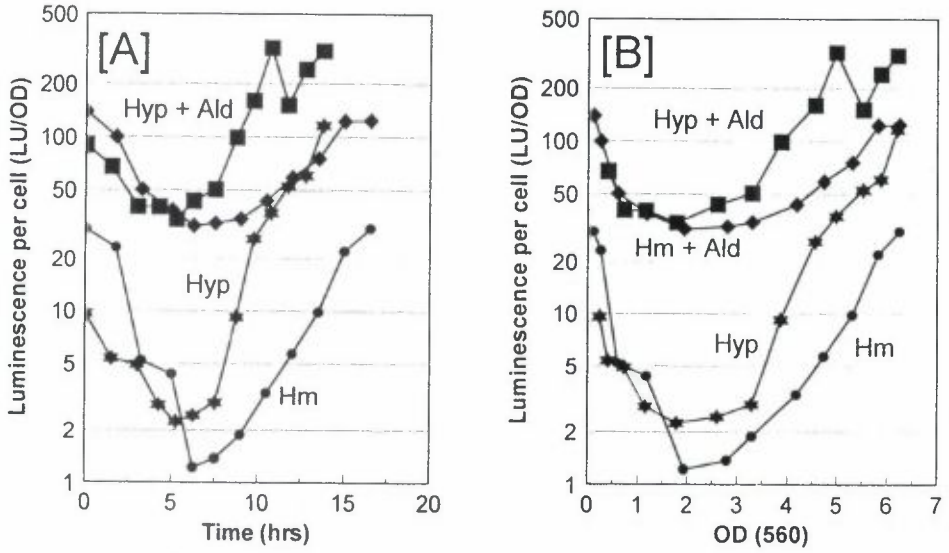

Figure 1. Development of the luminous system in strain Hm1 and hyp. Luminescence is presented as LU/OD (relative luminescence activity per cell), and plotted versus time (panel A) and versus OD_{560} (panel B) to demonstrate the kinetics of the development, and the relationship to cell density when the increase in luminescence begins, respectively. The solid circles represent luminous activity of Hm1 cells with no additions, while the solid diamonds show the aldehyde stimulated luminescence of Hm1 (proxy measurement for luciferase content). The unstimulated and aldehyde-stimulated activity of hyp are represented by the solid stars and solid squares respectively.

Fig. 4 demonstrates that the stimulation of luminescence by RIF can occur at any time during the growth cycle, with luminescence stimulation occurring a few hours after the addition of RIF. This is true for both the cellular luminescence (as shown), and the aldehyde-stimulated luminescence (data not shown; Hosseini, 1994). Hosseini (1994) showed that under a variety of different conditions used to control growth rate, this effect occurs only after 1–2 cell divisions following the addition of RIF and that the effect is reversible. That is, if RIF stimulated cells are diluted into media with no RIF, the stimulatory effect is lost in a few hours. (Hosseini, 1994). Panel B shows the data plotted as a function of cell density – after the cells have reached a density of $OD_{560} = 3$ or more, the response to added RIF is markedly damped, probably due to the slow growth rate of the cultures.

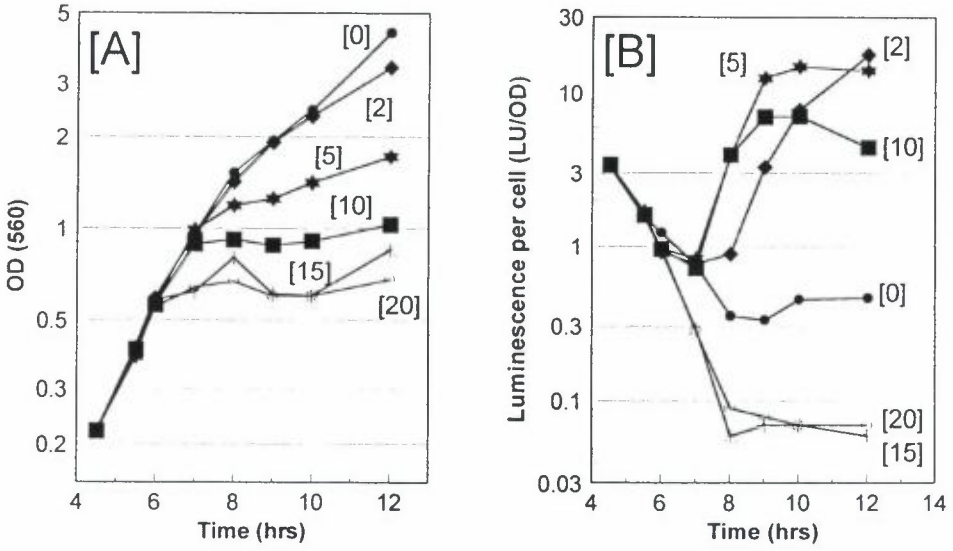

Figure 2. Effect of different levels of the mRNA synthesis inhibitor rifampicin (RIF) on the growth (panel A) and luminescence of strain Hm1. At 12 hours, the control culture has still not begun the increase in luminescence, while those cultures with 2, 5, and 10 μg/ml RIF have all increased markedly. Concentrations that totally inhibit growth lead to a decrease in bioluminescence. Concentrations of RIF are indicated on the figure.

The luminescence intensity of all three strains (Hm1, Hm2 and hyp) is enhanced by 2 μg/ml rifampicin (RIF) (Fig. 5). It is difficult to distinguish between the three strains in the RIF-enhanced cultures, suggesting that they are all maximally expressed.

Table 1 shows a summary of what is known about several traits commonly thought to be involved with the phase conversion. As previously reported by Bleakley and Nealson (1988) all of these properties are positive for Hm1 and negative for Hm2. Treatment of Hm2 with RIF resulted in highly luminous cells, but did not reverse any of the other traits. The hyper mutant was not examined with regard to all traits, but both luminescence and pigment are strongly expressed, and according to Dr. A. Fodor (personal communication), hyp also produces high levels of antibiotic activity.

4. Discussion

These studies began with the notion that luminescence might be valuable to study the mechanism of phase conversion, being easily and quickly measured,

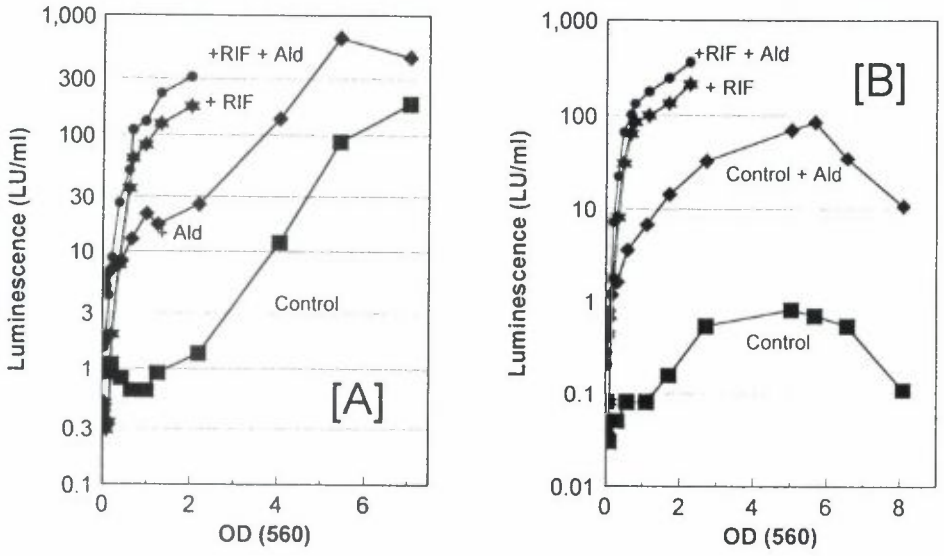

Figure 3. Effect of 2 μg/ml RIF on the development of the luminous system in Hm1 (panel A) and in Hm2 (panel B). Symbols: solid circles = unstimulated luminescence of control cultures; open circles = aldehyde stimulated luminescence of control cultures; solid diamonds = unstimulated luminescence of RIF-treated cultures; solid stars = aldehyde-stimulated luminescence of RIF-treated cultures.

without the need for breaking open the cells. The level of aldehyde-stimulated luminescence can be taken as a measure of luciferase content. Since substrate (aldehyde) limitation usually, but not always occurs, it is prudent to make both unstimulated and stimulated measurements.

In other species of luminous bacteria, a control mechanism called auto-induction operates at the level of transcription (Nealson, 1977). This mechanism involves a small molecule called autoinducer (homoserine lactone or HSL derivative) that is produced by the bacteria (Nealson and Hastings, 1991). When it accumulates in the growth medium it acts as the specific transcriptional inducer of the bioluminescence system. Autoinduction is blocked by both inhibitors of protein and mRNA synthesis (Nealson, 1977), and is now known to occur by the interaction of autoinducer with a specific receptor protein called LuxR (Fuqua et al., 1994). Hosseini (1994) showed that any concentration of chloramphenicol (CM) that inhibits growth also inhibited luminescence. Surprisingly, the addition of low levels of RIF which partially inhibited growth caused a dramatic increase in bioluminescence. This increase was seen in both in vivo light production, and aldehyde stimulated luminescence, indicating that luciferase synthesis was stimulated by RIF

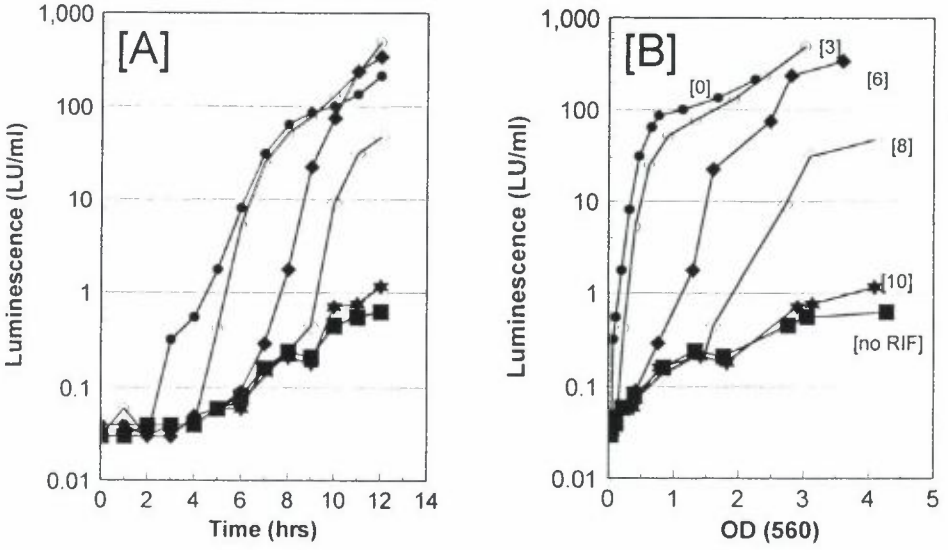

Figure 4. Effect of 2 μg/ml RIF added at various times on the luminescence of Hm2. For these experiment, the secondary variant was used in order to reduce the level of background luminescence. The times of addition are indicated on the figure in panel B. The luminescence of the culture is presented as a function of time (panel A) and as a function of cell growth (panel B). Symbols: solid squares = control (no RIF addition); solid circles = RIF at T=0; open circles = RIF at 3 h; solid diamonds = RIF at 6 h; open diamonds = RIF at 8 h; solid stars = RIF at 10 h.

addition (Figs. 2–5). The stimulation could be initiated at any time in the growth cycle of the cells until late stationary phase, as shown in Fig. 4, and the resulting increase in luminescence occurred few hours after addition of the RIF (i.e. after approximately 2 cell divisions). The fact that intermediate levels of RIF, which inhibit growth strongly, also stimulated luminescence, suggests that the mRNA for the lux system is already in place, or that its synthesis is unaffected by the RIF. Wang and Dowds (1991) have reported high levels of *lux* mRNA in the primary and secondary forms of *P. luminescens*, and Frackman and Nealson (unpublished observations) have seen high levels of *lux* mRNA at all stages of growth, for both the primary and secondary forms. These observations are consistent with some regulation of the *lux* system at a post-transcriptional level.

The RIF effect is apparently specific for the *lux* system: none of the other phase-specific properties tested (pigment, antibiotic, lipase, protease) was enhanced by low levels of RIF (Table 1). This suggests that the effect of RIF is on a luminescence-specific regulatory factor that can operate over and above

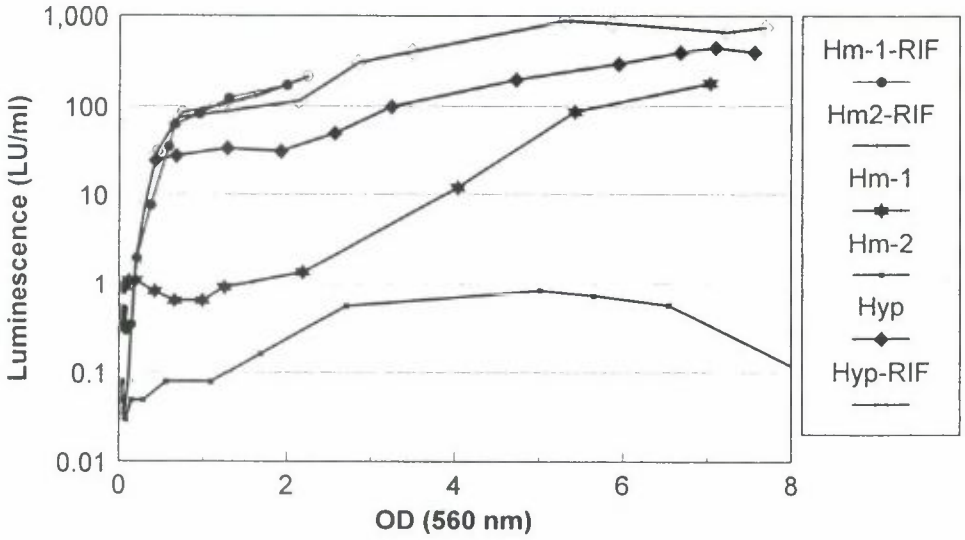

Figure 5. Effect of 2 μg/ml RIF added to primary (Hm1), secondary (Hm2), and hyp mutant. In all cases, the addition of RIF enhances the luminescence, leading to cultures that are nearly equal with regard to luminescence activity. Symbols: solid stars = Hm1 control; solid squares = Hm2 control; solid diamonds = hyp control; solid circles = Hm1 + RIF; open circles = Hm2 + RIF; open diamonds = hyp + RIF.

the factor that normally leads to low levels of luminescence in the secondary form.

Hyp is a mutant isolated after treatment of Hm2 with the chemical mutagen ICR-191 (Miller, 1972) in an attempt to isolate revertants to the primary form. The mutant was picked from a plate as a pigmented colony in a background of non-pigmented secondary types and checked for several other properties involved with the primary/secondary transition. Luminescence was notably increased, as shown in Figs. 1–5, but the mechanism by which this occurs remains unclear. Fig. 1 shows two important properties of the luminescence of hyp. First, the mutant is still aldehyde (substrate) limited, and second, the time of increase in luminescence is roughly the same for Hm1 and hyp. The major difference would appear to be that the rate of increase in luminescence activity is higher in hyp than in Hm1 (expressed as luminescence per cell), as shown in Fig. 1. The hyp mutant thus reveals that while there may be a master switch controlling many factors in the primary/secondary transition, it is nevertheless possible to obtain mutants that are apparently reversed for only some of the many traits. It is also clear that the lesion that leads to increased

brightness in hyp is at a site distinct from the site at which RIF exerts its stimulatory effect, since RIF causes enhanced luminescence of hyp (Fig. 5).

The data presented here, when considered along with the work of Akhurst (1980), Akhurst and Boemare (1990), Boemare and Akhurst (1989), and the more recent observations of Gerritsen et al. (1992) suggest that there are a variety of levels at which regulation of the primary/secondary transformation can occur. Krasomil-Osterfeld has reported the formation of transitional secondary forms that can be induced by growth of *P. luminescens* in low salt (Krasomil-Osterfeld, pp. 155–166 in this volume). These variants may be intermediates in the formation of more stable phase 2 types.

Occasionally, stable secondary forms are found that appear to be negative for most, if not all, of the traits involved in the phase I/II conversions. Treatment of such a secondary (Hm2) with ICR-191 resulted in a mutant called hyp, which expresses at least two properties at levels above that of the parent strain (Hm1), suggesting that a level of control exists over selected traits associated with phase shifts. Furthermore, as shown here, even in these stable forms phenotypic reversion of luminescence occurs in response to RIF addition: even in phase II variants, another level of control exists that can make the cells bioluminescent. To our knowledge, this is the first such indication of the phenotypic reversal of any of the traits that are involved with the primary/secondary transformation. Understanding the mechanism of RIF stimulation of luminescence may thus provide a clue to understanding the variability seen in secondary forms as reported by many workers.

Acknowledgments

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REFERENCES

Akhurst, R.J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* **121**: 303–309.

Akhurst, R.J. and Boemare, N.E. 1990. Biology and taxonomy of *Xenorhabdus*. In: *Entomopathogenic Nematodes in Biological Control*, R. Gaugler and H. Kaya, eds., CRC Press, Boca Raton, pp. 75–87.

Akhurst, R.J., Smigielski, A.J., Mari, J., Boemare, N.E., and Mourant, R.G. 1992. Restriction analysis of phase variation in *Xenorhabdus* spp. (Enterobacteriaceae),

entomopathogenic bacteria associated with nematodes. *Systematic and Applied Microbiology* **15**: 469–473.

Bleakley, B. and Nealson, K.H. 1988. Characterization of primary and secondary forms of *Xenorhabdus luminescens* strain Hm. *FEMS Microbial Ecology* **53**: 241–250.

Boemare, N.E. and Akhurst, R.J 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Journal of General Microbiology* **134**: 751–761.

Ehlers, R.-U., Wyss, U., and Stackebrandt, E. 1988. 16S rRNA cataloguing and the phylogenetic position of the genus *Xenorhabdus*. *Systematic and Applied Microbiology* **10**: 121–129.

Frackman, S. and Nealson, K.H. 1990. The molecular genetics of *Xenorhabdus*. In: *Entomopathogenic Nematodes in Biological Control*, R. Gaugler and H. Kaya, eds., CRC Press, Boca Raton, pp. 285–300.

Frackman, S., Anhalt, M., and Nealson, K.H. 1990. Cloning, organization, and expression of the bioluminescence genes of *Xenorhabdus luminescens*. *Journal of Bacteriology* **172**: 5767–5773.

Fuqua, W.C., Winans, S., and Greenberg, E.P. 1994. Quorum sensing in bacteria, the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* **176**: 269–275.

Gerritsen, L.J.M., deRaay, G., and Smits, P.H. 1992. Characterization of form variants of *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* **58**: 1975–1979.

Hosseini, P. 1994. Regulation of Bioluminescence in *Photorhabdus luminescens* Hm, Primary and Secondary Forms. Masters Thesis. Univ. of Wisconsin-Milwaukee. 160 pp.

Meighen, E.A. 1991. Molecular biology of bacterial bioluminescence. *Microbiological Reviews* **55**: 123–142.

Meighen, E.A. and Szittner, R.B. 1992. Multiple repetitive elements and organization of the lux operons of luminescent terrestrial bacteria. *Journal of Bacteriology* **174**: 5271–5381.

Miller, J.H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

Nealson, K.H. 1977. Autoinduction of bacterial luciferase: occurrence, mechanism and significance. *Archives of Microbiology* **112**: 73–79.

Nealson, K.H. 1991. Luminous bacteria symbiotic with entomopathogenic nematodes. In: *Symbiosis as a Source of Evolutionary Innovation*, L. Margulis and R. Fester, eds., MIT Press, Boston, MA, pp. 205–218.

Nealson, K.H. and Hastings, J.W. 1991. The Luminous bacteria. In: *The Prokaryotes*. Second Edition. Vol. I. A. Balows, H.G. Trueper, M. Dworkin, W. Harder, and K.-H. Schleifer, eds., Springer Verlag, New York, NY, pp. 625–639.

Nealson, K.H., Schmidt, T.M., and Bleakley, B. 1990. Physiology and biochemistry of *Xenorhabdus*. In: *Entomopathogenic Nematodes in Biological Control*, R. Gaugler and H. Kaya, eds., CRC Press, Boca Raton, pp. 271–284.

Nearhos, S.P. and Fuerst, J.A. 1987. Reanalysis of 5S rRNA sequence data for the vibrionaceae with the clustan program suite. *Current Microbiology* **15**: 329–335.

Poinar, G.O., Thomas, G., Haygood, M.G., and Nealson, K.H. 1980. Growth and luminescence of the symbiotic bacteria associated with the terrestrial nematode, *Heterorhabditis bacteriophora*. *Soil Biology and Biochemistry* **12**: 5–10.

Schmidt, T.M., Kopecky, K., and Nealson, K.H. 1989. Bioluminescence of the insect pathogen *Xenorhabdus luminescens*. *Applied Environmental Microbiology* **55**: 2607–2612

Smigielski, A., Akhurst, R.J., and Boemare, N.E. 1994. Phase variation in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: differences in respiratory activity and membrane energization. *Applied Environmental Microbiology* **60**: 120–125.

Wang, H. and Dowds. B.C. 1991. Molecular cloning and characterization of the lux genes from the secondary form of *Xenorhabdus luminescens*. *Biochemical Society Transactions* **20**: K122.

Wang, H. and Dowds, B.C. 1993. Phase variation in *Xenorhabdus luminescens*: cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. *Journal of Bacteriology* **175**: 1665–1673.