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Phase II Variants of *Photorhabdus luminescens* are Induced by Growth in Low-Osmolarity Medium

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Abstract

Photorhabdus (*Xenorhabdus*) *luminescens* is a bacterium symbiotically associated with the entomopathogenic nematode of the genus *Heterorhabditis*. The influence of osmolarity and other environmental factors like oxygen level, light level, medium pH and temperature, were investigated for their potential to provoke a phase shift in *P. luminescens*. Only low osmolarity was able to trigger a shift from phase I to phase II variant reliably. Phase shift was reversible during the first 48 h of cultivation, but after several transfers and growth in low osmolarity medium (with periods of starvation), stable phase II variants were formed. The role of changing osmolarities in the symbiotic life cycle of *P. luminescens* is discussed.

Keywords: *Photorhabdus luminescens*, phase variants, environmental factors, osmolarity, symbiotic lifecycle

1. Introduction

Photorhabdus luminescens, formerly called *Xenorhabdus luminescens* (Boemare et al., 1993), isolates are associated obligatorily with its symbiotic nematode *Heterorhabditis*, whereas the bacteria *Xenorhabdus* spp. are facultative symbionts of nematodes in the genus *Steinernema*. The bacterial cells are carried within the intestine of the infective dauerlarvae and when the nematode has penetrated a host insect the bacteria are released. The insect

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is killed by the combined action of bacteria and nematodes, the bacteria multiply within the cadaver, the nematodes feed on them and propagate. The interactive nematode-bacterium complex has been applied for insect pest control with great success. Large scale application, however, requires an economical production of the infective stage of the nematodes. The symbiotic bacteria *P. luminescens* and *Xenorhabdus* spp. exhibit phase variation: the phase I types support nematode production, whereas the phase II variants tend to inhibit nematode propagation. Unintended transformation to phase II variants will therefore compromise nematode mass production. Reasons and mechanisms for phase variation must be understood in order to control phase shift.

Many bacteria – including those with pathogenic characteristics – have to face various forms of environmental stress such as high and low temperature, oxidative agents (H_2O_2), high and low osmolarity, alkaline and acid conditions and low oxygen supply. Therefore, most bacteria have developed mechanisms to adapt to these environmental changes (Gross, 1992). Phase variation is a common mechanism in pathogenic bacteria to adapt to different environments and to escape the host immune system. During their symbiotic and pathogenic life cycle *P. luminescens* and *Xenorhabdus* spp. also encounter different environmental conditions to which they have to adapt physiologically. Within the intestine of the infective dauerlarvae the cells rest dormant. When they are released into the hemocoel of the insect prey, the cells have favorable propagative conditions but have to resist the insect defense mechanisms. In this paper the influence of environmental factors such as temperature, pH, oxygen supply, light and different osmotic pressures on phase shift of *P. luminescens* is described.

2. Material and Methods

Bacterial strains and stock cultures

Bacterial strains and their associated nematodes used for experiments are listed in Table 1. Phase I bacteria were isolated from the hemocoel of *Galleria mellonella* 24 h after infection with nematodes and cultured in the dark at 25°C for 3 days on NBTA (8 g/l Lab Lemco broth [Oxoid], 15 g/l agar [Difco], 40 mg/l triphenyltetrazolium chloride and 250 mg/l bromothymol blue), Lab Lemco agar (8 g/l Lab Lemco broth [Oxoid] containing 15 g/l agar [Difco]) or modified MacConkey agar (MacConkey broth [Merck] supplemented with 15 g/l agar). After several subcultures on NBTA, Lab Lemco agar or MacConkey agar phase II variants appeared and were purified by subculturing phase II colonies (Akhurst, 1980). Phase variants were identified according to characteristics

listed in Table 3. From each strain a single phase I colony was picked and cultured in YS broth (0.5 g/l NH_4H_2POv, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$*7H20, 5.0 g/l NaCl, 5.0 g/l yeast extract [all chemicals from Merck], 1000 ml distilled water), 20 ml broth in a 100 ml Erlenmeyer flask at 25°C and 180 rpm (New Brunswick G-53 shaker) for 24 h. Glycerol was added to the bacterial suspension to a concentration of 15% (v/v) and 2 ml portions were stored as stock cultures at –30°C.

Table 1. *P. luminescens* strains and associated nematodes used in this study

Bacterial strains	Associated nematodes	Sources
PSH1, PSH2, PSH3	*Heterorhabditis megidis*	Ehlers, Germany
HB RS92-M1	*Heterorhabditis bacteriophora*	Gaugler, USA
HP88	*Heterorhabditis bacteriophora*	Glazer, Israel
PE87.3	*Heterorhabditis megidis*	Gerritsen, The Netherlands
HCH-W79	*Heterorhabditis megidis*	Grunder, Switzerland
HH	*Heterorhabditis heliothidis*	Akhurst, Australia
HF-85	*Heterorhabditis megidis*	Westerman, The Netherlands

Factors influencing phase shift

The standard procedure for the cultivation of *P. luminescens* strains in liquid culture was realized in YS broth at 25°C and 180 rpm. Physical parameters like high (35°C) or low cultivation temperature (10°C), extreme pH values (adjustment of broth with HCl to 5 and NaOH to 10), non-aereation (sealing culture flask with rubber stopper) and exposure to visible light (8 W fluorescent lamp) were used for liquid cultures to investigate their influence on phase shift. Different osmotic pressures in liquid cultures were realized by varying the components of YS broth. Table 2 describes media with different osmolarities used in the experiments to investigate effects on phase shift. Proline (1 mM), a known osmoprotector for many bacteria (Cairney et al., 1984), was added to the media with low and high sodium chloride concentrations. Phase I stock cultures (2 ml) were inoculated in 20 ml of each medium in a 100 ml Erlenmeyer flask and cultured at 25°C and 180 rpm. Subcultures in either YS or Y broth were performed with 5% inoculum either every 24 h or in a parallel experiment every 96 h. Before subculturing, samples (3 ml) were removed from the flask and used for measurements of pH value, cell density (optical density at 725 nm [OD_{725}] in a Kontron spectrophotometer) and biomass [dry weight in g/l].

Table 2. Composition of media used for experiments to trigger phase shift from phase I to phase II in *P. luminescens* strains mentioned in Table 1

	YS	Y	YSS	YSCC
$NH_4H_2PO_4$ [g/l]	0.5	0.5	0.5	0.5
K_2HPO_4 [g/l]	0.5	0.5	0.5	0.5
$MgSO_4x7H_2O$ [g/l]	0.2	0.2	0.2	0.2
Yeastextract [g/l]	5.0	5.0	5.0	5.0
NaCl [g/l]	5.0		20.0	
Sucrose [g/l]				120.0
Proline [mMol/l]			1	1
Osmolarity [mOsmol/l]	265	60	720	350

The influence of environmental factors on phase shift was investigated by evaluation of cell morphology, pigmentation, bioluminescence and colony morphology on Lab Lemco agar, NBTA and MacConkey agar. Previously purified phase I and II variants served as standards. Bacterial cell morphology within the dauerlarvae (only strain PSH1) was studied (interference microscope/Zeiss Axioskop, magnification * 1600) of dauerlarvae that were kept in tissue culture flasks in Ringer's solution [Merck] at 4°C for at least 3 months. Samples of *Galleria mellonella* hemolymph were taken 24 h after infection with nematodes.

3. Results

Characterization of isolated phases

When phase I cells of *P. luminescens*, isolated from infective dauerlarvae of *Heterorhabditis*, were repeatedly subcultured, phase II colonies were observed. Phase I and II variants were obtained by picking the corresponding colonies and subculturing on NBTA, Lab Lemco agar or MacConkey agar over a period of several months. The phase I and II variants differed significantly from each other in cell and colony morphology and in physiological properties (Table 3). In addition, *P. luminescens* was able to exhibit a great variety of characteristics either by intermediate phase variants or the occurrence of phase I and II variants in mixed populations. Figs. 1a and 1b show the cell morphology of the phase I variant (PSH1/1), and phase II variant (PSH1/2), respectively.

Table 3. Cell- and colony-morphology and bioluminescence of phase I and phase II variants of *P. luminescens* in YS broth and on Lab Lemco-, MacConkey- and NBT-agar

	Phase I variant	Phase II variant
Pigmentation in/on:		
YS broth	white-yellow	orange-red
Lab Lemco agar	orange-red	yellow
MacConkey agar	blue-lilac	yellow
NBTA	blue-green	red
Bioluminescence	visible	not visible
Colony morphology	convex, rough, dense	flat, smooth, translucent
Colony texture	hydrophobic, "sticky"	hydrophilic, not "sticky"
Cell morphology	cocco-bacilli	bacilli
Inclusion bodies	visible	not visible

Characterization of bacterial cell morphology during in vivo conditions

With a storage time of three months lipid reserves of dauerlarvae were reduced, and morphology of the bacterial cells resting within the intestine was visible: cells were rather long and never contained inclusion bodies, thus resembling phase II cells (Fig. 1c). After being released from the dauerlarvae and multiplying within the insect cadaver the bacteria possessed inclusion bodies (Fig. 1d) and obtained phase I characteristics.

Influence of temperature, pH, low oxygen and light on phase shift

Physical parameters like temperature, pH, low oxygen and light did not stimulate the formation of phase II variants. Although the results were highly variable, certain forms of stress (extreme pH values, light, lack of oxygen) resulted in reduced bioluminescence of the cultures, accompanied by a higher portion of cells with phase II characteristics. However, when aliquots from such liquid cultures were streaked on Lab Lemco agar, NBTA or MacConkey agar they produced a majority of phase I colonies.

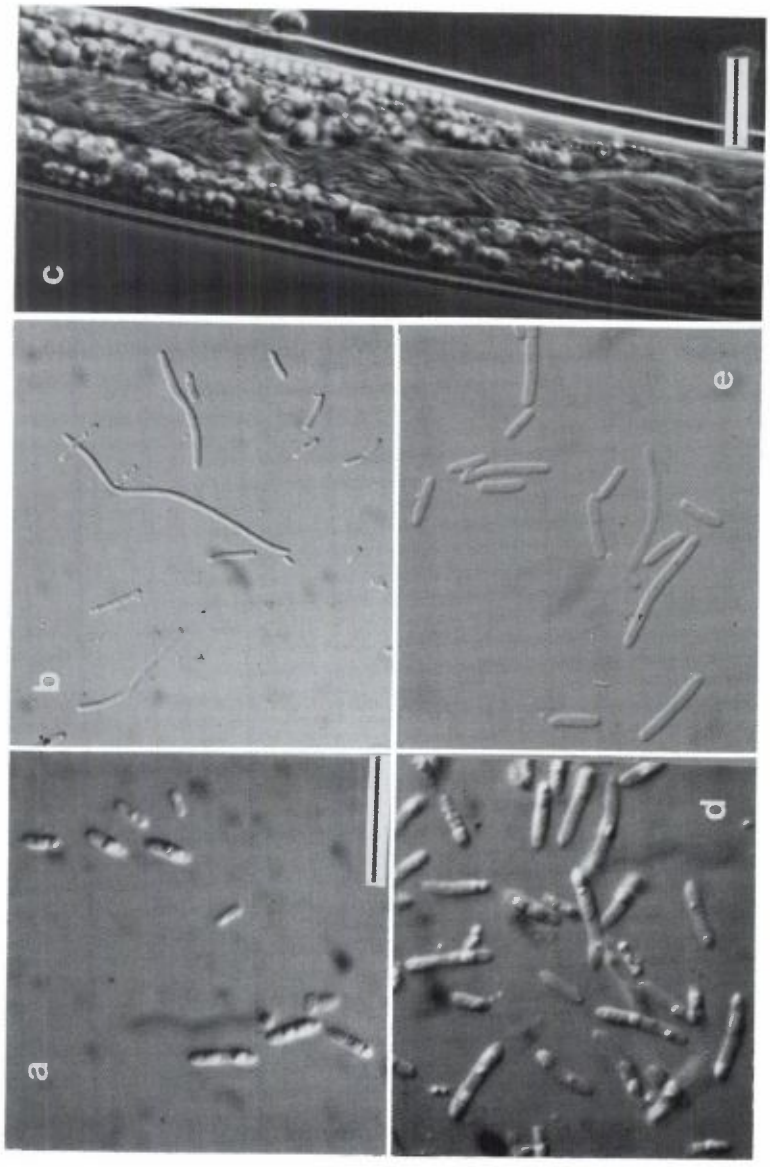

Figure 1. Phase variants of the *Photorhabdus luminescens* strain PSH1. Bar = 10 μm. (a) Phase I cells of the *P. luminescens* strain PSH1/1 cultured on Lab Lemco agar in the dark at 25°C for 72 h. (b) Phase II cells of the *P. luminescens* strain PSH1/2 cultured on Lab Lemco agar in the dark at 25°C for 72 h. (c) Cells within the intestine of infective dauerlarvae nematodes (*Heterorhabditis megidis*, strain HSH1). (d) Cells of the *P. luminescens* strain PSH1 isolated from an insect cadaver 48 h after penetration of infective dauerlarvae (*Heterorhabditis megidis*, strain HSH1). (e) Phase I cells of the *P. luminescens* strain PSH1 inoculated into Y broth after 24 h: phase II cell morphology.

Influence of osmolarity

In contrast to the above mentioned factors, growth in media of low osmolarity reproducibly resulted in the appearance of colonies with one or more phase II properties. After cultivation of phase I variants for 24 h in Y broth almost all cells could morphologically be characterized as phase II variants: they were elongate with no inclusion bodies (Fig. 1e). In addition, the culture was orange-red pigmented and only weakly luminescent. No phase shift to phase II was observed in parallel subcultures in YS broth. In contrast to other phase variants of *P. luminescens*, these phase II variants reverted to phase I forms upon growth in high osmolarity medium. Subculturing from Y broth to YS broth resulted in yellow pigmentation, recovery of bioluminescence and the majority of cells exhibiting phase I morphology. Prolonged subcultures in Y (at least four times) with an interval of 96 h resulted in a vast majority of stable phase II colonies when samples were streaked on Lab Lemco agar or MacConkey agar (Table 4), media which lack osmotic stress. Subculturing in a 24 h interval, without periods of starvation, did not have an equivalent effect favoring the phase II variants.

Table 4. Characteristics of *P. luminescens* phase I variants (strains listed in Table 1), subcultured 1 and 4 times in YS and Y medium; samples taken at 96 h. Table shows average pH, optical density, dry weight, pigmentation, bioluminescence and cell length in the culture broth, and the colony characteristics from streaking on LL and McC agar plates.

	YS 1. Subc.	YS 4. Subc.	Y 1. Subc.	Y 4. Subc.
pH	6.05	8.45	6.59	8.52
OD_{725}	11.95	3.2	12.35	2.35
DW [g/l]	3.75	1.17	4.29	1.09
Pigmentation	white	orange	red	red
Bioluminescence	+++	+	–	–
Cell length	middle	short	long	middle
Inclusion bodies	+++	++	–	–
McC agar	1°	1°	1°	2°
LL agar	1°	1°	1°	2°

1° = phase I colonies; 2° = phase II colonies.

Physiological reactions during subculturing in media with different osmolarity

The pH value increased during subculturing being slightly more alkaline in Y than in YS. Although pigmentation is pH dependent (Nealson et al., 1990), the minor pH differences between YS and Y cultures (Table 4) could not be responsible for the strong differences in pigmentation: YS cultures were yellow-white whereas Y cultures were orange-red pigmented. Optical density and dry weight in Y initially were slightly higher but decreased during subculturing. All strains investigated had similar physiological reactions. Table 4 shows the final average pH value, optical density and dry weight of all strains. Shifting from phase I to stable phase II was possible for all strains within four subcultures in Y broth. In media with high osmolarity (YSS and YSCC) no phase shift from phase I to phase II was observed. Shifting from phase I to phase II was not prevented in low osmolarity by the addition of proline.

4. Discussion

Phase variation is a general characteristic of *Xenorhabdus* and *Photorhabdus*, although its expression can vary significantly between species (Akhurst, 1994). While the process is reasonably well described, the mechanism of phase variation remains unknown. Appearance of phase II variants frequently occurs during stationary phase and is unpredictable in its timing and extent (Boemare and Akhurst, 1990). When phase shift was first described, it was assumed that phase II was stable (Akhurst 1980). Only few reports mention reverse phase shift; *X. nematophilus*, *X. bovienii* and *X. poinarii* show phase shift from phase II to phase I (Akhurst and Boemare, 1990) and in *P. luminescens* reversible phase shift between selected variants was frequently observed (Gerritsen et al., 1992; Hurlbert et al., 1989). The impression that phase II is stable might be caused by prolonged subculturing. In the literature it is described that in contrast to *Xenorhabdus* strains a reverse phase shift was impossible after long subculturing for *Photorhabdus* strains. In the results presented here, a reversible phase shift was observed for all tested *Photorhabdus* strains. But prolonged subculturing under conditions that favor the phase II bacteria (low osmolarity and starvation) is necessary to obtain stable phase II variants which will not shift back. It can be assumed that there is an early period of subculturing in which a reversible phase shift is possible and after which there is a point of no return and phase shift from phase II to phase I is rarely observed.

To compare these results with experiments presented in the literature it must be stressed that the evaluation of phase variants in the cultures described in this article is fundamentally different from the methods reported by other

workers (Akhurst and Boemare, 1990). Usually phase variants are identified by their properties growing on solid agar plates. In the experiments presented here, cells were grown in liquid media and morphologically examined while they were still in liquid. Morphological changes of phase I variants in liquid media with low osmolarity to phase II characteristics (elonged cell size and shape, no inclusion bodies, no bioluminescence) were not detected when cultured on solid media. Cell morphology might have altered as a function of starvation during prolonged cultures in liquid media (Givskov et al., 1995).

Several factors responsible for phase variation were discussed in the past. Prolonged culture time and lack of oxygen were proposed to favor the appearance of phase II colonies (Bleakley and Nealson, 1988). However, our results indicate that lack of oxygen is only a weak factor for phase shift. Environmental factors were proposed to be involved in phase variation (Landfald and Strøm, 1986). The addition of insect hemolymph, extracts of reproductive and dauerlarvae and of monoxenic nematode/bacterium cultures to various media failed to produce phase shift to phase II variants (Boemare and Akhurst, 1990). The results presented here show that low osmolarity is a factor that reproducibly triggers phase variation in *P. luminescens*, being confirmed for several *P. luminescens* strains.

The influence of changing osmotic values, especially on enteric bacteria, has been investigated intensively (Bukau et al., 1986; Cairney et al., 1985). Changes of osmolarity in the environment are known to produce the expression of different phase variants in pathogenic bacteria (Gross, 1992). Mechanisms of osmoregulation cause changes in cell surface properties (van Alphen and Lutgenberg, 1977) and the intracellular accumulation of compatible solutes (Patchett et al., 1992). The adaptation to changing environmental conditions with different osmolarities involves a great number of sensor proteins, transport proteins and enzyme systems (Landfald and Strøm, 1986). DNA coiling has been described as a transcriptional sensor for environmental changes like cold and osmotic shock (Wang and Syvannen, 1992). Environmental conditions like anaerobiosis and changing osmotic pressure can alter DNA supercoiling, thus influencing promotor activity and regulation of transcription (McClellan et al., 1990). Proline stimulates growth of enteric bacteria in otherwise inhibitory osmotic strength (Cairney et al., 1984). In our case, proline did not prevent phase shift from phase I to phase II in liquid cultures with low osmolarity.

Within their lifecycle the *Xenorhabdus* and *Photorhabdus* bacteria must face different environments: high osmolarity (400 mOsmol) and rich nutrients within the insect cadaver during reproduction phase of bacteria and nematodes and low osmolarity (60 mOsmol) and starvation during dormant phases within the intestine of the infective dauerlarvae or outside in soil environment. The

internal osmotic pressure in nematodes is approx. 60 mOsmol (Wright and Newall, 1980). This corresponds to the osmolarity of Y broth that favors phase II bacteria. Cells within the intestine of the dauerlarvae morphologically resemble to the cells cultured in vitro in low osmolarity and to isolated phase II cells: they are longer than phase I cells and lack inclusion bodies. Starvation seems to be a factor promoting phase shift when osmotic stress is applied. Physiological and metabolic changes of phase variants after starvation were described for *P. luminescens* and *Xenorhabdus nematophilus* with the postulation that phase II cells may be better adapted to conditions in soil as free-living organisms (Smigielski et al., 1994). The release of the bacteria into the host hemolymph implies a switch to rich nutrients and a change of osmotic pressure to approx. 400 mOsmol, a value that stabilizes phase I variants. In the presented experiments phase shift was shown to be reversible within the first subculture, whereas after prolonged subculture under low osmotic conditions phase shift became irreversible. A stable phase II variant might be created only under in vitro conditions, in vivo only a reversible phase II variant would have a function. Further investigations on bacterial cells cultured under in vivo conditions must clarify if this hypothesis is suitable. Additional experiments can verify, if the switch from phase I to phase II variants under low osmolarity and starvation conditions is a conversion or a selection for the occasionally appearing phase II bacteria. A general, renewed discussion on the definition of phase variants of *Xenorhabdus* and *Photorhabdus* also seems to be necessary.

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