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Influence of Osmolarity on Phase Shift in *Photorhabdus luminescens*

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The influence of osmolarity and other environmental factors like low oxygen levels, light, extreme pH values, and temperatures on phase variation of *Photorhabdus luminescens*, the symbiotic bacterium of entomopathogenic nematodes of the genus *Heterorhabditis*, was investigated. Only subculturing in low-osmolarity medium triggered a phase shift to secondary phase reliably.

Photorhabdus luminescens (formerly *Xenorhabdus luminescens* [6]) cells are carried within the intestines of the infective dauerlarvae of *Heterorhabditis* spp., and when the nematode has penetrated a host insect, the bacteria are released. The insect is killed by the combined action of bacteria and nematodes, the bacteria multiply within the cadaver, and 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 economical production of the infective stages of the nematode. The symbiotic bacterium *P. luminescens* exhibits various phase variants. The primary phase supports nematode production, whereas the secondary phase mostly inhibits nematode propagation. Therefore, nematode mass production can be carried out successfully only when an unintended phase shift to secondary phase can be avoided. 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 changes in their environment (7–9, 12, 14, 15, 17, 19, 20). Phase variation is a common mechanism in pathogenic bacteria to adapt to different environments and to escape the host immune system (11). 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.

From each strain investigated (PSH1/2/3, Hb RS92-M1 [obtained from R. Gaugler, New Brunswick, N.J.], HP88 [I. Glazer, Bet Dagan, Israel], PE87.3 [obtained from L. J. M. Gerritsen, Wageningen, The Netherlands], HCH-W79 [obtained from J. Grunder, Wädenswill, Switzerland], HH [obtained from R. J. Akhurst, Canberra, Australia], and HF-85 [obtained from P. R. Westerman, Leeuwarden, The Netherlands]) primary-phase bacteria were selected according to phase designation (1) and, as a standard procedure, cultured in YS/1 broth (Table 1) (20 ml of broth in a 100-ml Erlenmeyer flask at 25°C and 180 rpm [New Brunswick G-53 shaker] for 24 h). Physical parameters such as high (35°C) or low (10°C) cultivation temperature, extreme pH values (adjustment of broth with HCl to pH 5 and with NaOH to pH 10), nonaeration (sealing of the culture flask with a 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 and Y broth (Table 1). Subcultures in either YS or Y broth were performed either every 24 h or in a parallel experiment every 96 h. The same medium components were used also for cultures on solid agar plates.

Physical parameters such as temperature, pH, low oxygen levels, and light were factors unable to trigger a significant phase shift from primary to secondary phase; results were highly variable, but certain forms of stress (extreme pH values, light, and lack of oxygen) destabilized the primary phase. However, when aliquots from such liquid cultures were streaked on Lab Lemco or MacConkey agar, they produced a majority of primary-phase colonies. In contrast to the above-mentioned environmental factors, low osmolarity significantly produced phase shift from primary to secondary phase. Table 2 shows the overall final average pH values, pigmentations, optical densities, dry weights, and cell and colony morphologies of all strains. After cultivation of the primary phase for 24 h in Y/1 broth, almost all cells could morphologically be characterized as secondary phase. No phase shift to secondary phase was observed in parallel subcultures in YS/1 broth. Phase shift to the secondary phase was reversible as soon as the influence of low osmolarity was removed. Subculturing from Y/1 broth to YS/1 broth resulted in the recovery of cells exhibiting primary-phase morphology. Prolonged subcultures in Y/1 broth (at least four times) with an interval of 96 h resulted in a vast majority of stable secondary-phase colonies (Table 2). Subculturing in a 24-h interval, without periods of starvation, did not have an equivalent effect triggering phase shift to secondary phase. Phase shift from primary to stable secondary phase was possible for all strains within four subcultures in Y/1 broth. The addition of proline, a known osmoprotector for many bacteria (9), had no protecting effect in either high or low osmolarity. On solid agar media a complete phase shift was never observed.

Bacterial cell morphology within the dauerlarvae (only for strain PSH1) was studied in vivo (interference-contrast microscope; magnification, ×1,600) with dauerlarvae that were kept in tissue culture flasks in Ringer's solution (Merck) at 4°C for at least 3 months. During storage lipid reserves of dauerlarvae were reduced and the morphology of the bacterial cells resting within the intestines was visible: cells were rather long and never contained inclusion bodies. They resembled secondary-phase cells. After being released from the dauerlarvae and multiplying within the insect cadaver, the bacteria possessed inclusion bodies and obtained primary-phase characteristics.

Several factors responsible for phase variation were discussed in the past: prolonged culture time, lack of oxygen, and environmental factors (2–4, 10, 13, 16). The addition of insect

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TABLE 1. Compositions of media used for experiments to trigger phase shift from primary to secondary phase in *P. luminescens* strains

Component	Concn (g/liter) in:			
	YS/1[a]	Y/1[b]	YS/2[c,d]	Y/2[c,e]
$NH_4H_2PO_4$	0.5	0.5	0.5	0.5
K_2HPO_4	0.5	0.5	0.5	0.5
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2
Yeast extract	5.0	5.0	5.0	5.0
NaCl	5.0		20.0	
Sucrose				120.0

[a] Osmolarity, 265 mosmol/liter.
[b] Osmolarity, 60 mosmol/liter.
[c] Also contained 1 mM proline.
[d] Osmolarity, 720 mosmol/liter.
[e] Osmolarity, 350 mosmol/liter.

hemolymph and extracts of reproductive larvae and dauerlarvae and of monoxenic nematode-bacterium cultures to various media failed to produce phase shift to secondary phase (5). The results presented here show that low osmolarity is a factor that reproducibly triggers phase variation in *P. luminescens*. Phase shift was shown to be reversible within the first subculture; after prolonged subculture under low-osmolarity conditions, phase shift becomes irreversible. A stable secondary phase might be created only under in vitro conditions, while in vivo only a reversible secondary phase would have a function. During its symbiotic and pathogenic life cycle, *P. luminescens* encounters different environmental conditions to which it has to adapt physiologically: high osmolarity and abundant nutrients within the insect prey during the reproduction phase and low osmolarity and starvation during dormant phases within the intestines of the infective dauerlarvae or outside in the soil environment (18, 21). Phase variation can be assumed to enable *P. luminescens* to adapt to changing environmental conditions during its symbiotic life cycle.

TABLE 2. Characteristics of *P. luminescens* primary-phase variants subcultured one and four times in YS and Y medium[a]

Parameter	Result for			
	YS		Y	
	1 subculture	4 subcultures	1 subculture	4 subcultures
pH	6.05	8.45	6.59	8.52
OD_{725}[b]	11.95	3.2	12.35	2.35
DW (g/liter)[c]	3.75	1.17	4.29	1.09
Pigmentation	White	Orange	Red	Red
Degree of bioluminescence[d]	Strong	Reduced	None	None
Cell length (μm)	3–5	<3	>5	3–5
Presence of inclusion bodies	Abundant	Frequent	None	None
Characteristics on McM agar[e]	Primary[f]	Primary	Primary	Secondary[g]
Characteristics on LL agar[h]	Primary	Primary	Primary	Secondary

[a] Average values for pH, optical density, dry weight, pigmentation, bioluminescence, and cell length in the culture broth, as well as the colony characteristics from streakings on Lab Lemco and MacConkey agar plates, are shown. Samples were taken at 96 h.
[b] OD_{725}, optical density at 725 nm.
[c] DW, dry weight.
[d] As judged with dark-adapted eyesight.
[e] McM agar, MacConkey agar.
[f] Primary, primary-phase colonies.
[g] Secondary, secondary-phase colonies.
[h] LL agar, Lab Lemco agar.

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