

# Anaerobic metabolism of primary and secondary forms of *Photorhabdus luminescens*

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## Abstract

An oxygen electrode inserted into a dead *Manduca sexta* larva infected with *Heterorhabditis* nematodes carrying the bacterium *Photorhabdus luminescens* showed barely detectable levels of oxygen in a 1 to 2 mm zone below the cuticle, and virtual anaerobiosis deeper in the carcass. This observation indicates that the bacteria in this habitat, where they are actively growing, are probably carrying out a fermentative metabolism. Therefore, the anaerobic metabolism of the primary and secondary form variants of *P. luminescens* Hm and NC1 was compared. Amino acids were not fermented by either strain, either singly or in mixtures. Glucose was fermented by both forms of both organisms, forming products typical of mixed acid fermentation by Enterobacteriaceae. The fermentation patterns were the same in the primary and secondary forms. Growth rates of the secondary form cells were higher in defined medium with glucose as energy and carbon source. Growth yields of the primary and secondary forms of strain Hm were nearly identical, whereas the growth yield of secondary form cells of strain NC1 was slightly higher than that of the primary form. The results of this study indicate that the observed predominance of primary form cells in infected insect larvae cannot be explained by an advantage over the secondary form cells related to the efficiency of anaerobic growth or fermentative metabolism.

**Keywords:** *Photorhabdus luminescens*; *Heterorhabditis bacteriophora*; Phase variant; Mixed acid fermentation; Oxygen limitation; Anaerobic metabolism

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## 1. Introduction

The facultatively anaerobic bacterium *Photorhabdus luminescens*, formerly *Xenorhabdus luminescens* [1], lives in mutualistic association with entomopathogenic nematodes of the genus *Heterorhabditis* [2]. The bacteria are carried within the intestinal tract of the infective stage juvenile nematodes. The nematodes locate a susceptible insect larva, penetrate into

the hemocoel and release the *P. luminescens* cells into the hemolymph. The bacteria proliferate, killing the insect host, and provide permissive conditions for reproduction of the nematodes. The insect carcass is red in color and emits light due to bioluminescence of the bacteria. After 1–2 weeks, several hundred juvenile stage nematodes, each carrying *P. luminescens* in their intestine, leave the insect and search for a new host [3].

*P. luminescens* characteristically forms two form variants, designated primary and secondary form [3,4]. The two forms differ in that the primary cells

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produce larger amounts of antibiotics, pigments and extracellular proteases and lipases and are more bioluminescent than the secondary cells. Also the primary cells but not secondary cells form intracellular crystalline inclusion proteins. The two forms also differ in absorption and reduction of dyes from growth media [3,5,6]. The nematodes grow preferentially in the presence of the primary form cells. This is the form of the bacteria isolated from nematode intestines and is the predominant form found in infected insects. Secondary form cells appear in cultures after prolonged cultivation *in vitro* [4] and usually not in insect hosts [3]. The primary form has been suggested to be better adapted to conditions in the nematodes and in the insect while the secondary form may be better adapted as free-living organism in soil [7]. The physiological or genetic basis and regulation of the phase variation is not known. The shift from primary to secondary form was reported to occur more frequently under anoxic or microaerophilic conditions [8,9]. The anaerobic metabolism of the primary and secondary form has not been studied in detail to date. Because an infected insect cadaver is most probably an oxygen-limited environment, we compared fermentation balances, growth rates and growth yields of primary and secondary form cells of *P. luminescens* to ascertain if one form is better adapted to anoxic growth conditions than the other.

## 2. Materials and methods

### 2.1. Organisms and growth conditions

*Photorhabdus luminescens* strain NC1 was obtained from Dr. W. Brooks, University of North Carolina, USA, and strain Hm from Dr. G. Poinar, University of California, Berkeley, USA. *Manduca sexta* larvae were a gift from Dr. S. Dierkes, Köln, Germany, and *Heterorhabditis bacteriophora* nematodes carrying *P. luminescens* monoxenically were kindly provided by Dr. R.-U. Ehlers, Kiel, Germany.

*P. luminescens* was cultivated under anoxic conditions in bicarbonate-buffered mineral salts medium [10] containing 0.05% (w/v) yeast extract, with 2 mM cysteine as reducing agent. The pH of the medium was adjusted to 7.3 with 0.5 M Na<sub>2</sub>CO<sub>3</sub>. Substrates were added from sterile stock solutions.

Cultures were incubated in 50-ml screw top glass bottles at 30°C in the dark. Cultivation under air was performed in defined mineral salts medium containing per litre of distilled water: MOPS, 5.23 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.66 g; NaCl, 1.00 g; MgCl<sub>2</sub> · 6 H<sub>2</sub>O, 0.40 g; KH<sub>2</sub>PO<sub>4</sub>, 0.20 g; NH<sub>4</sub>Cl, 0.25 g; KCl, 0.50 g; CaCl<sub>2</sub> · 2 H<sub>2</sub>O, 0.15 g. The pH was adjusted to 7.0 with 5 M NaOH. After autoclaving, 1 ml of trace element solution SL10 [10] and 1 ml of vitamin solution [11] were added. Cultures were incubated on a rotary shaker at 230 rpm at 30°C in the dark. Agar plates contained nutrient broth (8 g/l) and 1.5% agar. Dye-containing agar plates (nitroblue tetrazolium chloride, NBTA) were prepared as described [3].

Primary and secondary forms were differentiated by colony pigmentation and morphology on nutrient agar or on NBTA plates.

Growth in liquid media was followed via optical density at 578 nm wavelength. After samples were withdrawn from anaerobically grown cultures, the removed volume was replaced by sterile glass rods. All growth experiments were run at least in triplicate.

Cell dry mass was determined gravimetrically after filtration and drying at 80°C.

### 2.2. Analytical methods

Glucose, trehalose, succinate, lactate, formate, acetate, and ethanol were analyzed by HPLC using a HDX-87H ion exclusion column for organic acids analysis (300 × 7.8 mm; Biorad, Hercules, CA, USA) at 40°C and an ERC-7512 refractive index detector (Erma, Tokyo, Japan) at 206 nm. A quantity of 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml × min<sup>-1</sup> was used as eluent. Concentrations were calculated via peak areas (Hitachi D-2500 chromatointegrator, Hitachi, Tokyo, Japan) compared with external standards.

The oxygen concentration in dead *M. sexta* larvae that had been infected with *H. bacteriophora* nematodes carrying *P. luminescens* was measured with a Clark-type oxygen microelectrode which was kindly provided by Dr. A. Brune, Konstanz, Germany, and was calibrated with nitrogen- or air-saturated water. For measurements, the larva was embedded in 0.5% (w/v) agarose in Ringer solution inside a small glass

cuvette to allow optical control. The microelectrode was moved with a micromanipulator from the agarose surface downwards in 0.5 mm steps towards and into the larva.

### 3. Results

#### 3.1. Oxygen gradient in a *Manduca sexta* larva killed by nematodes

A polarographic oxygen microelectrode inserted slowly into the agar matrix surrounding a dead larva and into the cadaver produced the oxygen gradient profile shown in Fig. 1. The oxygen content of the agarose gel decreased rapidly from the agar-air interface to the outer cuticle layer of the cadaver. The oxygen concentration below the cuticle layer was very low and the cadaver was virtually anoxic at a depth greater than 2 mm. The anoxic zone may actually be closer to the insect surface than is indicated in Fig. 1. The surface of the cadaver was elastic and inserting the electrode caused an indentation of the surface before breaking through the cuti-

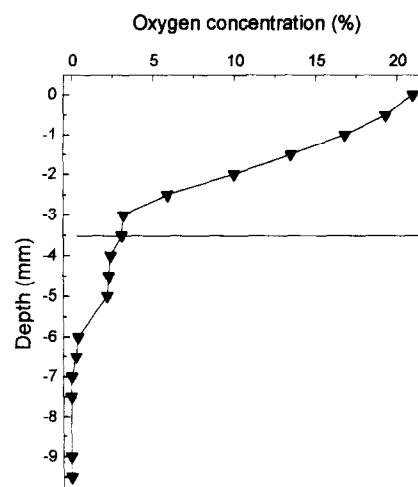


Fig. 1. Oxygen concentration in a dead *Manduca sexta* larva infected with *Heterorhabditis bacteriophora* nematodes carrying *Photorhabdus luminescens*. Four days after infection the larva glowed in the dark, indicating the presence of *P. luminescens*. The dead larva was embedded in agarose. Oxygen concentration was measured with a Clark-type oxygen microelectrode that was moved with a micromanipulator from the agarose surface (0 mm) towards and into the larva. The surface of the larva (-3.5 mm) is indicated by the horizontal line.

Table 1

Growth rates, growth yields, and fermentation patterns of primary and secondary forms of *Photorhabdus luminescens* strains Hm and NCI in mineral salts medium with 5 mM glucose and 0.05% yeast extract

	Hm		NCI	
	Primary	Secondary	Primary	Secondary
$\mu$ ( $h^{-1}$ )	0.064	0.078	0.063	0.081
Doubling time $t_d$ (h)	10.9	8.8	11.0	8.5
Growth yield	33.9	30.7	16.3	23.6
(g dry mass mol glucose $^{-1}$ )				
Substrate used (mM)	3.46	4.55	4.75	5.00
Substrate assimilated <sup>a</sup> (mM)	0.81	0.92	0.53	0.81
Products formed (mM)				
Succinate	0.24	0.36	0.37	0.40
Lactate	< 0.2	1.20	0.74	0.67
Formate	5.52	6.46	4.55	4.07
Acetate	2.14	2.90	3.68	2.44
Ethanol	4.11	4.64	6.07	5.59
pH (final)	6.5	6.4	6.4	6.5
Carbon recovery (%) <sup>b</sup>	119/91	122/97	109/97	94/79
Electron recovery (%) <sup>b</sup>	127/97	128/102	124/110	114/96

Data are means of at least three independent growth experiments in every case, which differed by 10% at most.

<sup>a</sup> Substrate assimilation was calculated after the following equation with  $\langle C_4H_7O_3 \rangle$  representing cell matter [18]:  $17 C_6H_{12}O_6 \rightarrow 24 \langle C_4H_7O_3 \rangle + 6 CO_2 + 18 H_2O$ .

<sup>b</sup> Carbon and electron recovery were calculated considering that glucose was assimilated (first number) or that cell mass was exclusively formed from yeast extract (second number).

cle and penetrating further. It is apparent from this experiment that the bacteria and nematodes inside the cadaver are in an anoxic environment.

### 3.2. Anaerobic growth of primary and secondary forms of *P. luminescens*

Several organic components of insect hemolymph, including amino acids and trehalose [12], were tested as substrates for anaerobic growth of *P. luminescens*. No growth of either the primary or secondary form cells of strains Hm and NC1 occurred with the following substrates tested at 10 mM concentration in the presence of 0.05% yeast extract: triacetin, glycerol, aspartate, valine, glycine, alanine, leucine, lysine, valine + proline, alanine + proline, alanine + glycine, leucine + proline, leucine + glycine, aspartate + glycine, glutamate + proline, glutamate + glycine and 0.1% (w/v) casamino acids. Both primary and secondary form cells of strain Hm grew anaerobically with 5 mM glucose or 2.5 mM trehalose. Growth of the Hm primary form cells with trehalose occurred only after a lag phase of three weeks. The primary and secondary form cells of NC1 fermented glucose but did not ferment or grow anaerobically using trehalose. The primary and secondary form cells of both strains Hm and NC1 grew aerobically with glucose or trehalose in the mineral salts medium.

### 3.3. Fermentation of glucose by primary and secondary form cells

Primary and secondary form cells of *P. luminescens* strains Hm and NC1 fermented glucose in the presence of 0.05% yeast extract, forming formate, acetate, ethanol, succinate and lactate (Table 1). These are typical products of mixed acid fermentations by Enterobacteriaceae. Growth rates of the secondary form cells of both strains were higher than those of the primary form cells, and higher optical densities were reached (Fig. 2). Growth yields of the primary and the secondary form cells of strain Hm did not differ significantly whereas the growth yield of the primary form cells of strain NC1 was lower than that of the secondary form cells.

Comparisons of the fermentation patterns of the primary and the secondary form cells of both strains

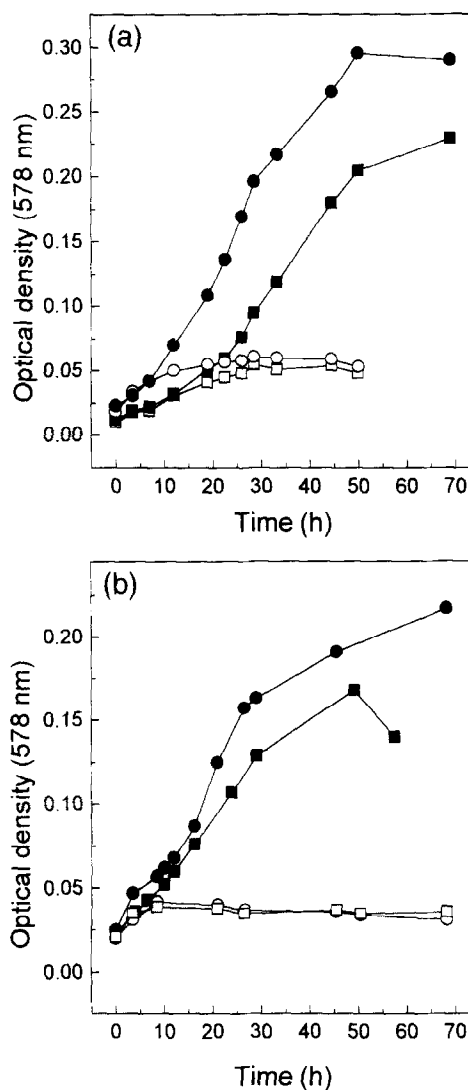


Fig. 2. Growth of the primary ( $\square$ ,  $\blacksquare$ ) and the secondary form ( $\circ$ ,  $\bullet$ ) of *P. luminescens* under anoxic conditions in mineral salts medium containing 5 mM glucose and 0.05% yeast extract (filled symbols) or 0.05% yeast extract alone (open symbols). (a) strain Hm; (b) strain NC1. Data are means of at least three independent growth experiments in every case, which differed by 10% at most.

showed only minor differences in the mineral salts medium used (Table 1). Cells of both forms of strain NC1 produced more ethanol and less formate than strain Hm. Calculations of carbon and electron recovery indicated that cell mass was formed mainly from yeast extract, and glucose was used nearly exclusively as energy source.

#### 4. Discussion

Microelectrode measurements proved that virtually no oxygen was present in the interior of *Manduca sexta* larvae cadavers that had been killed by the bacteria. The steep oxygen gradient obtained as the electrode neared the larva indicated that oxygen was being actively consumed, probably by respiratory metabolism within the peripheral region of the cadaver. This respiratory activity was probably due to both the nematodes and bacteria inside the larva. The insect cadaver visibly emits light due to bioluminescence of the *P. luminescens* pathogen. Bioluminescence is an oxygen consuming reaction which might also contribute to the level of anoxia inside the cadaver. Once anoxic conditions were established inside the cadaver, the facultatively anaerobic *P. luminescens* would be expected to switch to fermentative metabolism. Such metabolic differentiation phenomena are known for other Enterobacteria, even in apparently homogeneous colonies growing on agar media [13].

No shift from the primary to secondary form cells or vice versa was observed when *P. luminescens* strain Hm or NC1 were cultivated anaerobically. Conversion of primary to secondary form cells was reported previously [8,9]. Two recent reports suggested that growth in liquid media of low osmolarity, and not anaerobiosis, is involved in the primary to secondary phase shift [14,15].

The concentration of amino acids in insect hemolymph is relatively high [12] and so it is interesting that *P. luminescens* strains Hm and NC1 did not ferment amino acids. Glycerol and triacetin, which could represent model compounds for fats, were not fermented either. Both forms fermented glucose to products typical of enterobacterial fermentations. Glucose is a component of *M. sexta* hemolymph [16] and could be a substrate for the bacteria growing in the cadavers. The fermentation patterns of the primary and secondary form cells did not differ significantly, so their mode of energy conservation under anaerobiosis appears not to vary. This is interesting since secondary cells have decreased amounts or loss of many physiological properties. Growth rates of the secondary form cells were higher than primary form cells in both strains. Higher growth rates for the secondary form cells in mineral

medium under oxic conditions were reported earlier [6,8]. Growth yields of the primary and secondary cells of strain Hm did not differ significantly. For strain NC1, the growth yield of the primary cells was lower than that of the secondary cells.

Trehalose is the most abundant sugar in insect hemolymph [16,17]. Both forms of strain Hm fermented trehalose. However, growth of the primary cells was slower than that of secondary cells (data not shown). Neither form of strain NC1 fermented trehalose. It is surprising that the organism does not take advantage of this energy source in the larvae.

The original purpose of this investigation was to determine if fermentation metabolism of *P. luminescens* is involved in the reported predominance of primary over secondary stage cells in the infected insect cadaver environment. The fermentation data obtained, showing no significant differences between the two forms, do not provide an answer to the question why nearly exclusively the primary form is found in the insect cadaver. The secondary form cells appear at high frequency in laboratory culture. Smigielski et al. [7] reported a significantly higher level of respiratory enzyme activities in stationary cultures of secondary stage cells as compared to primary stage cells. From this they suggested that the secondary stage (phase II) cells are better adapted to the low nutrient soil environment in contrast to the rich insect hemolymph which might favor the primary (phase I) cells. There have been no authenticated reports of isolation of *P. luminescens* from environments other than from infected insects and nematodes, or of reversion of secondary to the primary stage. The physiological basis and ecological significance of phase variation in *Photorhabdus* remains a mystery.

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