

## Simple Bacteriological Tests for Phenotypic Characterization of *Xenorhabdus* and *Photorhabdus* Phase Variants

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### Abstract

Simple bacteriological tests are described for characterizing phase variants of *Xenorhabdus* and *Photorhabdus* on Petri dishes. They are used for checking exoenzymatic activities as lipases, phospholipases and proteases, and for showing end-products secreted externally by variant colonies as pigments, extracellular material evidenced by dye adsorption, and agar diffusible antibiotics.

Keywords: Pigmentation, dye adsorption, antibiosis, proteases, lipases, phospholipases, hemolysis

### 1. Introduction

The present paper reports a standardization for characterization of *Xenorhabdus* and *Photorhabdus* phase variants on Petri dishes. They correspond to the commonly known techniques described in the usual bacteriological manuals, but are adapted to the special material that are the

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symbionts of Steinernematidae and Heterorhabditidae. They are the result of 15 years experience starting with the pioneer work of Akhurst (1980, 1982) describing differences of two colonial forms in terms of dye adsorption, production of antibiotics and phospholipase. With the characterization of other particular exoenzymatic properties linked with phase variants (Boemare and Akhurst, 1988), and a special annular hemolysis, formerly named *Xenorhabdus* hemolysis (Farmer et al., 1989), the results ended up at the concept of phase variation (Boemare and Akhurst, 1988). The phases of cultures are designated by the suffixes /1 and /2 to the strain designation (e.g. isolates F1/1 and F1/2 are phase I and phase II cultures, respectively, of strain F1), as previously described (Boemare and Akhurst, 1988).

## 2. Methods and Results

All tests have to be made in Petri dishes. Results have to be checked 2–5 days after inoculation and incubation at 28°C. To observe pigmentation and adsorption of dyes, each variant is streaked on one half of the Petri dish as shown on Fig. 1 in order to obtain a good separation of the colonies. Because both variants are streaked on each half part of the same plate, it is possible to observe the differential pigmentation and adsorption of dyes.

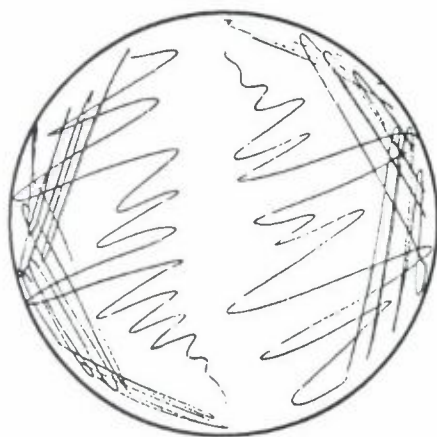


Figure 1. 1st, 2nd, 3rd and 4th: Steps of the streaking in order to test both variants on a same plate.

### *Pigmentation*

Pigmentation can be observed on nutrient agar (NA) or trypticase soya agar (TSA). Quantity of powder, as indicated by the manufacturer for 1 l, is dissolved in distilled water and agars are poured in Petri dishes after autoclave sterilization. Phase I of *Xenorhabdus* strains (except *X. nematophilus*) are pigmented and phase II are off-white. With the *Photorhabdus* strains, a differential pigmentation is observed in most of the cases (yellow/orange-red, or vice versa, pink/yellow). This character is very variable, depending on strains and species and the color cannot be established generally. Most of these pigments are pH-sensitive and consequently the color is related to the pH of the medium (Richardson et al., 1988). The only one general property is that no pigmentation or a differential pigmentation occurs for many strains. Consequently, pigmentation is only useful for descriptions of each strain and its phase variants.

### *Dye adsorption on NBTA*

Adsorption of dyes is quite universal for the phases I as the result of a dye binding on an external material produced around phase I colonies, as shown when dye solutions are spread on plates after incubation. But for routine test it is more convenient to use dyes incorporated in the media before pouring the plates.

The medium of Akhurst (1980), Nutrient Bromothymol Blue Agar (NBTA), is used to differentiate phase variants. To the quantity of nutritive agar powder, as indicated by the manufacturer for 1 l, and dissolved in distilled water, 25 mg/l of bromothymol blue (BTB) are added. This medium is sterilized in the autoclave. A solution of 4 g/l of Triphenyl 2,3,5 tetrazolium chloride (TTC) in distilled water is sterilized by filtration. 100 ml of the previous agar is allowed to cool to 45°C before adding 1 ml of the sterile solution of TTC (final concentration of TTC: 0.040 g/l).

Phases I blue colonies mean an adsorption of BTB (reduction of TTC to formazan was hidden) and phase II red colonies only a reduction of TTC in formazan. In case of non pigmented strains, like *X. nematophilus*, the test is very obvious. But sometimes on this medium it is difficult to distinguish the colonies when they are highly pigmented. The test remains interesting when phases II are not pigmented, as most of the *Xenorhabdus* are, but not so easy with *Photorhabdus*.

*Dye adsorption on Mac Conkey agar*

The best medium to show the dye adsorption is the medium of Mac Conkey with the following composition:

Casein peptone	17 g	Neutral red	0.03
Meat peptone	3	Violet crystal	0.001
Lactose	10	Agar	12
Bile salts	1.5	H <sub>2</sub> O	1 l
NaCl	5		

Bile salts are not recommended. Normally this medium was devoted to the selection of *Salmonella-Shigella* and for the purpose of this test it is obvious that the bile salts are not necessary.

The phase I colonies are red, due to the adsorption of neutral red, while phases II remain off-white. This medium is very convenient, specially with *Photorhabdus* spp., because on other media responses are obscured by the pigmentation of the strain. On this medium a poor growth of *Xenorhabdus* or *Photorhabdus* is obtained due to the bile salts, and to be successful it is necessary to streak with a large amount of culture. So this test can be improved by discarding bile salts, and the commercial products containing them have to be avoided.

*Antibiosis with an overlay Micrococcus luteus culture for testing growth inhibition*

This test was initiated by Akhurst (1980) to control phase I variants producing diffusible antibiotics. Nutrient agar plates are spot inoculated with 24 h broth cultures of each variant (Fig. 2) and incubated 2 days. Then plates are exposed to chloroform (30 min) to kill spotted colonies. Plates are left 30 min in a laminar flow hood to allow evaporation of the chloroform. Sterile soft agar (100 ml containing 7 g/l of agar) is allowed to cool to 45°C before being inoculated with 1 ml of *Micrococcus luteus* 24 h old broth. When mixed, it is poured on the previous plates. An inhibition around a spot indicates a production of antibiotics. Phase I variants produce a very much higher quantity of agar diffusible antibiotics than phase II variants.

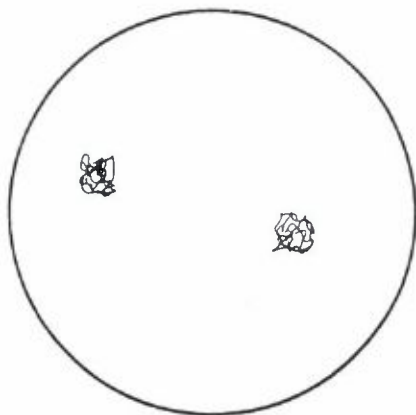


Figure 2. 1 and 2: Two spot inoculations for testing both variants on a same plate.

#### *Phospholipase (lecithinase) on yolk agar*

For this test yolk agar is used. An egg is surface disinfected with 70% ethanol. Under the laminar flow hood the yolk is collected aseptically and poured into an equal volume of 0.9% NaCl sterile saline solution. After homogenization, 10% vol./vol. of this yolk solution are added to nutrient agar at 45°C. Both variants are streaked as a line on the agar, in order to compare the response of each variant on the same plate (Fig. 3). An opaque halo around the inoculation line is a positive lecithinase reaction. Sometimes a positive reaction is indicated if an opacity is observed under the colonies (restricted positive reaction).

#### *Phospholipase on lecithin agar*

This method, recently adapted to characterize phase variants of *Xenorhabdus* and *Photorhabdus*, was adopted in routine in our laboratory. Isolates are spot inoculated (Fig. 2) on agar containing lecithin purified from egg yolk and delivered by manufacturers (e.g., Prolabo<sup>TM</sup>). Lecithin is first dissolved in 95% ethanol (10 g/l), and 1% (vol/vol) of this solution is incorporated into nutrient agar at 45°C. After 48h of incubation, an opaque zone surrounding the colonies reveals a phospholipase activity. Positive and negative responses are better expressed than the previous method, without any doubtful interpretation.

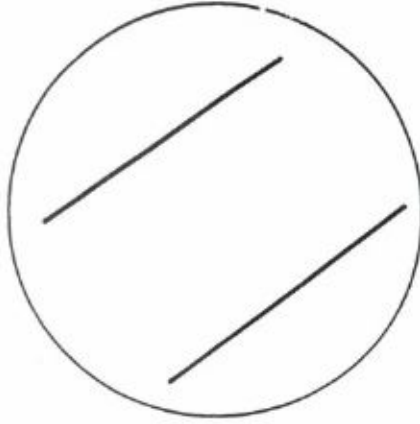


Figure 3. 1 and 2: Two lines of inoculation for testing both variants on a same plate.

*Proteolysis on gelatine agar according to Frazier's method (1926)*

To the quantity of nutrient agar powder indicated by the manufacturer, 12 g/l of gelatine are added and the plates streaked as shown Fig. 3. After the prerequisite incubation, plates are flooded with the following mixture:

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HgCl <sub>2</sub>	12 g
12N HCl	16 ml
H <sub>2</sub> O	80 ml

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A clear halo due to the proteolysis is evidenced without any difficulty. Most of the *Xenorhabdus* strains are positive in phase 1, some *Photorhabdus* are positive for both phases.

*Lipolysis on "calcium" agars according to Sierra's method (1957)*

CaCl<sub>2</sub> · 2 H<sub>2</sub>O (0.1 g) is dissolved in 1 l of distilled water and the quantity of nutrient agar powder indicated by the manufacturer is added. Tweens are used as lipid substrates and sterilised by moderated autoclaving (110°C, 30 min) and mixed at 1% vol./vol. into "calcium" agar at 45°C. Tweens are fatty acids

combined with polyoxyethylenesorbitan (tween 20 = polyoxyethylenesorbitan monolaurate; tween 40 = polyoxyethylenesorbitan monopalmitate; tween 60 = polyoxyethylenesorbitan monostearate; tween 80 = polyoxyethylenesorbitan monooleate; tween 85 = polyoxyethylenesorbitan trioleate).

Both variants are streaked as a line on the agar, in order to compare the response of each variant on the same plate (Fig. 3). Fatty acids precipitate around the colonies indicating a lipolysis. The responses of the phases are strictly depending on the combined fatty acid with the polyoxyethylene-sorbitan. For instance, *Photorhabdus* strain Hm/1 is lipolytic for the tweens 20, 40, 60, 80, 85, and Hm/2 negative for all. With most other *Photorhabdus*, both phases produce a positive reaction in this test. Sometimes *Xenorhabdus* phases II are more lipolytic than phases I. This characteristic is relevant to an accurate physiological study comparing phases and strains of each species.

#### *Hemolysis on "Blood" agars*

TSA or the following "blood" agar are used:

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Composition:	Proteose-peptone	15 g/l
	Liver hydrolysate	2.5
	Yeast extract	5
	NaCl	5
	Agar	12

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TSA or "blood" agar are allowed to cool to 45°C before adding 5 to 10% of defibrinated blood from sheep or horse. Both variants are streaked as a line on the agar, in order to compare the response of each variant on the same plate (Fig. 3), or spot inoculated (Fig. 2). A green halo is interpreted as an hemolysis  $\alpha$  (partial) and a clear halo as an hemolysis  $\beta$  (total)<sup>1</sup>. These tests clearly give a differential response depending on the strains: phases I of some strains have a partial hemolysis and phase II nothing, or a total hemolysis and a partial, respectively. The special "*Xenorhabdus* hemolysis" described by Farmer et al. (1989), as a clear ring at short range surrounding the colonies, can be also seen.

<sup>1</sup> The term of "hemolysis" is improperly used by bacteriologists. In physiology, hemolysis is the result of a disruption of erythrocyte membrane releasing the hemoglobin (e.g. by osmotic pressure). In these bacteriological tests, there are also, either a partial digestion of hemoglobin in biliverdin (green), or a total digestion of the hemoglobin which clears the medium around the colonies. So these tests indicate two steps of the hemoglobin hydrolysis.

Because this phenotypic trait is described for *Photorhabdus* strains (formerly *Xenorhabdus luminescens*) it is suggested to name it as an "annular hemolysis" (Akhurst et al., 1996).

### 3. Discussion

These methods as described above should allow the standardization of methods for a universal characterization of phase variation in *Xenorhabdus* and *Photorhabdus* spp. Other properties have been shown for some *Xenorhabdus* phase I variants, like for example presence of fimbriae (Brehélin et al., 1993; Binnington and Brooks, 1994; Moureaux et al., 1995) or flagella (Givaudan et al., 1995). These latter are not yet popularized and consequently not included in this report.

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