Detection of *Pseudomonas* Colonies That Accumulate Poly-β-Hydroxybutyrate on Nile Blue Medium

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ABSTRACT

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A simple procedure was developed to detect colonies of bacteria that accumulate poly-β-hydroxybutyrate (PHB), a storage product characteristic of many nonfluorescent pseudomonads. Colonies of PHB-positive bacteria fluoresced bright orange to yellow under long-wave ultraviolet light when grown on a medium containing PHB and Nile blue dye. Bright orange fluorescent granules were visible within the cells when examined microscopically under ultraviolet light. Colonies of fluorescent pseudomonads did not fluoresce on this medium, nor were fluorescent granules visible microscopically. This technique allows detection of PHB-positive pseudomonads without microscopic examination. Nile blue medium also was useful as a differential isolation medium for *Pseudomonas solanacearum* and may be useful for other nonfluorescent pseudomonads.

The pseudomonads are usually divided into two groups based on their fluorescence under ultraviolet light when grown on King's medium B (5). The nonfluorescent pseudomonads are so called because they do not fluoresce under these conditions. In this paper, when we refer to the fluorescent or nonfluorescent pseudomonad groups, we are referring only to their reaction on King's medium B under ultraviolet light.

Poly-β-hydroxybutyrate (PHB) is a polymeric ester that serves as an energy reserve. It is of diagnostic value in the identification of many nonfluorescent pseudomonads, including *Pseudomonas andropogonis, P. gladioli*, and *P. solanacearum*. None of the fluorescent pseudomonads accumulate PHB.

The standard method to detect PHB is to stain bacteria with Sudan black and observe the cells microscopically for presence of blue-black granules (3,5). The granules often are difficult to visualize because of staining difficulties such as the presence of Sudan black dye particles on the slide. An improved method was developed using Nile blue to stain the PHB granules. Nile blue fluoresces orange under ultraviolet light and appears to have greater affinity for PHB than does Sudan black (4). The orange fluorescent granules are easy to detect with an ultraviolet microscope. This paper describes an improvement of the Nile blue technique that is much less tedious than previous methods that require staining and microscopy. Nile blue was incorporated into a high carbon: nitrogen medium (NB). Colonies of bacteria that contain PHB fluoresced bright orange or yellow when viewed with a long-wave ultraviolet lamp.

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MATERIALS AND METHODS

A medium with a high carbon:nitrogen ratio is most favorable for production of PHB granules (3). The following NB medium resulted in ample production of PHB granules: 900 ml distilled water, 1.0 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 5.0 g DL-β-hydroxybutyric acid sodium salt, 20.0 g Difco proteose peptone No. 3, 1 ml 1% Nile blue solution, 4.5 ml 1 N NaOH, and 17.5 g agar. The pH was about 7.0. After autoclaving, a filter-sterilized or autoclaved solution of 20.0 g dextrose in 100 ml H_2O was added. Care should be taken not to autoclave the dextrose solution for more than 15 min, as it will affect growth of some nonfluorescent pseudomonads. Proteose peptone and dextrose were added to improve growth.

Bacteria tested on NB included 25 strains of nonfluorescent pseudomonads, seven strains of fluorescent pseudomonads, five strains of *Xanthomonas campestris*, and six strains of unknown bacteria isolated from various diseased plants that fluoresced orange on NB (Table 1). Strains were streaked on NB and incubated at 28 C until good growth appeared (2–4 days). Colonies then were illuminated with a long-wave (366-nm) ultraviolet lamp and given a subjective rating for brightness.

Strains also were checked microscopically under ultraviolet light (Nikon G-1B filter, 546/10 nm) for the presence of fluorescent granules. A loopful of cells grown on NB was mixed with a drop of water on a slide, covered with a coverslip, and then examined at high power under ultraviolet light. A subjective rating was made of the brightness of fluorescent granules.

To further confirm that PHB granules caused the colonies to fluoresce, all strains were streaked on basal NB (NB without Nile blue). Bacterial cells then were stained by the Nile blue (4) or Sudan black method (5) and examined microscopically under ultraviolet light or full-spectrum light, respectively, and rated as described above.

NB was also tested as an isolation medium for *P. solanacearum* from tomato plants. When tested for sensitivity to several common antibiotics using standard techniques, *P. solanacearum* was found to be resistant to polymyxin B. Therefore, 5 ml of 1% polymyxin B and 5 ml of 1% cycloheximide (to reduce fungal contamination) were added to NB after autoclaving. Stem sections from symptomatic, inoculated tomato plants were ground in a mortar with 100 ml of sterile water.

Dilution plates of this suspension were made on NB and Nile blue amended with antibiotics (NBA). Counts of orange fluorescent colonies on the respective media were made. Five plates per dilution were counted. The recovery efficiency of NBA was tested by plating dilutions of a heavy suspension of *P. solanacearum* on NBA and King's medium B. Ten plates per dilution were counted.

RESULTS AND DISCUSSION

All nonfluorescent pseudomonad strains with the exception of *P. cissicola* and *P. corrugata* that ostensively accumulated PHB produced a fluorescent orange to pink or yellow color on NB when illuminated with long-wave ultraviolet light (Table 1). Strains of *P. solanacearum* and *P. rubrisubalbicans* were very bright orange. Only strains of *P. andropogonis* fluoresced bright yellow. Other strains exhibited an orange to pink color.

Under full-spectrum light, colonies of nonfluorescent pseudomonads appeared white or slightly pinkish white. Most turned blue after 2–4 days of culture and no longer fluoresced under ultraviolet light. This loss of fluorescence is most likely a reaction of the dye to a pH change. In aqueous solutions, Nile blue is a mixture of oxazine, a blue dye that stains acidic lipids, and oxazone, a red dye that fluoresces yellow under long-wave ultraviolet light. Oxazone stains nonacidic lipid tissue, as does Sudan

black. The blue color of oxazine is intense enough to mask the red color of oxazone (2,6). It is likely that colonies become more acidic as they age, causing the oxazine to turn blue and mask the fluorescent oxazone dye. The addition of a drop of dilute acid to a white fluorescent colony caused it to turn blue and cease fluorescing. A drop of dilute base caused an old blue colony to become pinkish white and fluoresce under ultraviolet light (*data not shown*). For these reasons, colonies should be examined before they age and turn blue.

Table 1. Fluorescence of colonies on Nile blue medium and microscopic fluorescence of poly-β-hydroxybutyrate (PHB) granules within bacterial cells[a]

Species	Strain no.	Colony fluorescence on Nile blue medium[b]	Microscopic fluorescence of PHB granules		
			No stain[c]	NB stain[d]	Sudan black[e]
Nonfluorescent pseudomonads					
Pseudomonas andropogonis	061	+++	+++	+++	+++
	914	+++	+++	+++	+++
P. avenae	860	++	++	+	+
P. caryophylli	437	++	+++	+++	++
P. cattleyae	232	++	+++	+++	+++
	364	++	++	+++	+++
P. cepacia	454	+	+	++	++
	455	+	+	+	+
P. cissicola	616	−	−	−	−
P. corrugata	736	−	−	−	−
P. gladioli	556	++	+++	+++	+++
	557	++	++	+	++
	558	++	+++	++	++
	560	++	+++	++	+++
P. pseudoalcaligenes subsp. *citrulli*	625	++	+	+	−
subsp. *konjaci*	996	++	++	+	−
P. rubrilineans	644	+	+	nf	−
	645	++	+	nf	+
	646	+	++	+++	++
P. rubrisubalbicans	647	++	+++	+++	+++
	648	+++	+++	+++	+++
P. solanacearum	573	+++	+++	+++	+++
	574	++	+++	+++	+++
	575	+++	+++	+++	+++
	581	+++	+++	+++	+++
Fluorescent pseudomonads					
P. aeruginosa	001	−	nf	nf	−
P. cichorii	648	−	−	nf	−
P. fluorescens	495	−	−	nf	−
P. marginalis	496	−	−	nf	−
P. putida	679	−	−	nf	−
P. syringae	937	−	−	nf	−
P. viridiflava	586	−	−	nf	−
Xanthomonads					
Xanthomonas campestris	745	−	−	nf	−
X. c. begoniae	139	−	−	nf	−
X. c. dieffenbachiae	720	−	−	nf	−
X. c. juglandis	167	−	−	nf	−
X. c. vesicatoria	165	−	−	nf	−
Unknown[f]					
Cherry	108	++	++	+++	+
Dracaena	049	++	+++	++	+++
Peach	980	++	++	++	+
Pear	988	++	++	++	++
Soil	795	++	++	++	+
Tomato	899	++	+++	++	++

[a] All cultures were from the University of California plant pathology collection.
[b] Appearance of colonies under ultraviolet light on Nile blue medium; colonies fluoresce orange or yellow. +++ = Bright, ++ = moderate, + = low, and − = no fluorescence.
[c] Appearance of PHB granules when viewed microscopically under ultraviolet light. Cultures were grown on Nile blue medium (NB). +++ = Fluorescent granules visible in almost all cells, ++ = fluorescent granules visible in about 20–60% of cells, + = fluorescent granules visible in less than 10% of cells, − = no fluorescent granules visible but some dull cell wall fluorescence, and nf = no granule or cell wall fluorescence.
[d] Same as footnote c except culture grown on basal NB (no Nile blue) and stained after growth by the Nile blue method.
[e] Cultures grown on basal NB, stained by the Sudan black method, and viewed with full-spectrum light. +++ = Black staining granules visible in almost all cells, ++ = black granules visible in 20–60% of cells, + = black granules visible in less than 10% of cells, and − = no black granules visible.
[f] All cultures isolated from plant hosts were nonfluorescent on King's medium B, negative for Gram stain and Hugh Leifson (anerobic fermentation of glucose), and positive for oxidase.

Microscopic examination under ultraviolet light of bacterial cells grown on NB revealed orange fluorescent granules. The cell walls did not stain. Cells from older blue colonies also contained fluorescent granules. Apparently, the oxazine form of the dye did not interfere with fluorescence when viewed microscopically with transmitted ultraviolet light. There was a positive correlation between the number of fluorescent granules visible and the brightness of the fluorescence of the colony (Table 1). Ostle and Holt (4) confirmed that strains containing orange fluorescent granules also contained PHB. Comparable results were obtained when cells of colonies grown on basal NB media were stained with Nile blue or Sudan black.

Fluorescent granules were not seen in cells of *P. cissicola* and *P. corrugata*. This is not surprising, since colonies of these species did not fluoresce on NB under ultraviolet light. Also, PHB granules were not detected with the Sudan black stain. Although these species are listed in Bergey's manual (1) as accumulating PHB, this did not occur under these cultural conditions.

Colonies of fluorescent pseudomonads tested on NB did not fluoresce orange under ultraviolet light but appeared dark blue under full-spectrum light. Microscopic examination of cells revealed no fluorescent granules, nor were black granules seen when stained with Sudan black.

Colonies of all xanthomonads tested appeared yellow on NB under full-spectrum light. Under long-wave ultraviolet light they appeared slightly orange, which is probably due to the intense yellow color of the colony. Microscopic examination of the cells revealed no fluorescent granules or black-staining granules with Sudan black. Xanthomonad colonies should not be confused with a PHB-positive orange fluorescent colony because nonfluorescent pseudomonad colonies are never yellow under full-spectrum light.

When cells from colonies of *X. campestris* pathovars or fluorescent pseudomonads grown on NB were examined microscopically, the cell walls appeared orange. Thus, it is important when viewing cells to differentiate between the cell walls that are orange and granules that fluoresce orange. When colonies were grown on basal NB and then stained with Nile blue, the cell walls did not appear as orange. Since Nile blue stains lipids, it is expected that lipid components in cells grown on the medium would take up more dye. Among the nonfluorescent *Pseudomonas* strains, only granules, and not the cell walls, took up the stain. It is possible that PHB granules may have

a greater affinity for the dye than cell walls.

NBA worked well as a differential isolation medium for detecting *P. solanacearum* from inoculated tomato plants. It was very easy to differentiate *P. solanacearum* from contaminants that grew on the semiselective medium. An average of 86% of the colonies were fluorescent orange on NBA, whereas 7% of the colonies were fluorescent orange on NB (average of three trials). The addition of antibiotics greatly reduced the number of colonies that did not fluoresce orange. The recovery efficiency of NBA as compared to King's medium B was 83%. All fluorescent orange colonies cannot be assumed to be nonfluorescent pseudomonads, since other bacteria may accumulate PHB. Further tests would be required to confirm identification. NB is a useful isolation medium for detecting PHB-containing colonies. The addition of antibiotics could be tailored to enhance the detection of other desired nonfluorescent pseudomonads.

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