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# Polyclonal Antisera To Distinguish Strains and Form Variants of *Photorhabdus* (*Xenorhabdus*) *luminescens*

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**In this study antisera against *Photorhabdus luminescens* strains were prepared for the first time. *P. luminescens* is a bacterial symbiont of entomopathogenic nematodes belonging to the genus *Heterorhabditis*. To characterize *P. luminescens* strains and form variants, we produced polyclonal antisera against *P. luminescens* PE (obtained from nematode strain NLH-E87.3) and against the primary and secondary forms of *P. luminescens* PSH (obtained from nematode strain DH-SH1). In double-diffusion tests all form variants of strain PE reacted with the antiserum against the primary form, but each variant produced a different diffusion pattern. The primary and secondary forms of strain PSH were also serologically different. Antiserum 9226 reacted with almost all *P. luminescens* strains tested, but it reacted differently with each strain in the double-diffusion test, showing that the strains were serologically different. The specificity of the antisera was increased by cross-absorption. After cross-absorption the antiserum against the strain PSH primary or secondary form was specific for that form and did not react with the other form. Using the cross-absorbed antisera in immunofluorescence cell-staining tests, we could distinguish primary and secondary form cells in a mixed strain PSH culture.**

*Photorhabdus luminescens*, a species which was recently removed from the genus *Xenorhabdus* (3, 8, 27), is an entomopathogenic bacterium that is symbiotically associated with nematodes belonging to the genus *Heterorhabditis* (22, 24, 27). The infective dauerlarvae of the nematodes carry the bacterial symbiont in their intestines. A nematode penetrates an insect host, moves into the hemocoel, and releases bacteria. Subsequently, the bacteria multiply and kill the host, assisted by excretion products of the nematode that repress the host immune system (17). *P. luminescens* also produces antibiotics which inhibit the growth of other microorganisms in the insect cadaver and provides nutrients utilized by the nematodes (2, 27).

The colony morphology and biochemical characteristics of *P. luminescens* isolates are highly variable. Two extreme colony forms have been designated phase 1 and phase 2 or primary form and secondary form, respectively (1, 4, 6, 16). The primary form, which can be isolated from infective dauerlarvae, often changes into the secondary form when the organism is cultured in vitro. The primary form has a unique colonial morphology, produces antibiotics, lipases, proteases, and a pigment, absorbs certain dyes from agar media, and is luminescent. The secondary form does not have or is diminished in these biochemical abilities, has a different colonial morphology, and does not support growth of nematodes as well as the primary form does (1, 4, 7, 12). Several colony forms that have intermediate properties have also been described (16, 19).

The mechanism and function of the phase shift phenomenon are not known, although this phenomenon has been studied for some time (1, 5–7, 9, 12, 15, 16, 19, 21, 22, 29, 30). The phase shift of *P. luminescens* is scientifically interesting and has practical importance for mass production of the nematode as a biocontrol agent, since the secondary form does not support the growth of the nematode very well (1, 4, 7, 12). One of the problems in phase shift research is the detection of primary and secondary cells in mixed populations. Although *P. luminescens* isolates can be separated into groups on the basis of DNA homology data (8, 13, 18, 25, 26), restriction fragment length polymorphism data (5, 16), and fatty acid analysis data (20), these data do not discriminate between form variants within strains (5, 16, 25). Frackman et al. (15) compared the *lux* genes of a luminescent primary form and a nonluminescent secondary form and found no differences. In addition, it is known that the genes coding for lipase activity are present in the secondary form and that the mRNA can be detected (30). Thus, we believe that it may be difficult to detect form variants at the DNA level. Form variants do differ in their protein contents, as shown by their sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein patterns (7, 14, 16). In this paper we describe serological techniques which can be used to discriminate between different strains and form variants of *P. luminescens.*

## MATERIALS AND METHODS

**Bacterial isolates.** The sources of the nematodes from which bacterial strains were isolated are shown in Table 1. The bacteria were isolated directly from infective-stage nematodes (1, 16) or from the hemocoels of waxmoth (*Galleria mellonella*) larvae infected by nematodes. The bacteria were grown on nutrient agar (0.8% Lab Lemco broth [Oxoid] containing 1.5% agar) and were incubated in the dark at 25°C for 3 days. The following six form variants of strain PE were used: PE-red, PE-pink, PE-yellow, PE-white, PE-GB, and PE-P2. The first four of these variants were described by Gerritsen et al. (16). PE-red was the primary form of strain PE, and none of the other variants was a true secondary form. PE-pink and PE-yellow had some of the characteristics of a primary form and some of the characteristics of a secondary form. Most of the characteristics of PE-white were secondary-form characteristics, but PE-white formed small colonies and small cells and shifted back to PE-red constantly. PE-P2 and PE-GB were isolated as described by Gerritsen et al. (16). PE-P2 was a pink-pigmented variant that was a different shade of pink than PE-pink, and it had the same characteristics as PE-red (16). PE-GB had the same characteristics as PE-red,

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TABLE 1. Origins and double-diffusion patterns of *P. luminescens* strains used in this study

| Bacterial strain | Associated nematode strain or species | Original place of isolation | Double-diffusion pattern[a] |
|---|---|---|---|
| PE | NLH-E87.3 | Eindhoven, The Netherlands | A (1)[b] |
| PB | NLH-B87.1 | Bergeyk, The Netherlands | A (24) |
| PW | NLH-W79 | Wageningen, The Netherlands | A (25) |
| PFR | NLH-FR86 | Friesland, The Netherlands | B (35) |
| PNH | NLH-NH-87.1 | N-Holland, The Netherlands | B (34) |
| PL | NLH-L81 | Limburg, The Netherlands | C |
| PF | NLH-F85 | Flevoland, The Netherlands | D (33) |
| PNB | NLH-NB87 | N-Brabant, The Netherlands | E (32) |
| PJun | NLH-Jun91 | Papendal, The Netherlands | F (42) |
| PHi | NLH-Hi93 | Hilversum, The Netherlands | G (44) |
| PH1 | NLH-H92.1 | Eindhoven, The Netherlands | —[c] |
| PH2 | NLH-H92.2 | Eindhoven, The Netherlands | — |
| PSH | DH-SH-1 | Kiel, Germany | H (45, 52) |
| PDA | DH-Da1 | Darmstadt, Germany | I (54) |
| Pbac | *Heterorhabditis bacteriophora* | Brecon, Australia | I (55) |
| Pmol | CISH-Mol | Moldavia, Commonwealth of Independent States | J (53) |
| PK122 | IRH-K122 | Ireland | K (43) |

[a] Pattern in double-diffusion test performed with antiserum 9226. Strains with the same characters have similar precipitin patterns.
[b] The numbers in parentheses are the well numbers in Fig. 1.
[c] —, no reaction with the antiserum.

except that it was pale yellow and was more luminescent. Both variants were stable during subculturing. Most of the other *P. luminescens* strains had small-colony variants like PE-white (16), but only a few of these variants were used in double-diffusion tests. The PSH secondary form was isolated after induction of the primary form (20a).

**Antiserum production.** Antisera 9226, 9351, and 9352 were produced in rabbits against live, whole cells of PE-red, PSH primary form, and PSH secondary form, respectively. Bacterial cells were grown on nutrient agar plates for 4 days at 25°C, suspended in 0.85% NaCl, and washed three times by centrifugation and resuspension. The final concentration of each preparation was adjusted to $5 \times 10^8$ cells per ml. For the initial injection, 1 ml of a cell suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Sigma) and injected subcutaneously in the neck of a rabbit. After 3 weeks three intravenous injections consisting of 0.5, 1.0, and 1.5 ml of cell suspension without adjuvant were administered at 2- to 3-day intervals. Each rabbit was bled from a marginal ear vein 3 weeks after the final injection and at 1-week intervals thereafter; up to three blood samples were obtained from each rabbit.

**Absorption of antiserum.** Portions of the second antiserum 9226 blood sample were absorbed with bacterial suspensions containing either PE-white or Pbac; the resulting preparations were designated antisera 9226-W and 9226-B, respectively. Portions of the first antiserum 9351 and 9352 blood samples were absorbed with bacterial suspensions containing PSH secondary form and PSH primary form, respectively; the resulting preparations were designated antisera 9351-S and 9352-P, respectively. Bacterial cells were grown on nutrient agar plates for 3 days at 25°C and harvested in 2 ml of antiserum, mixed thoroughly, and incubated at 37°C for 30 min. The agglutinates were removed by centrifugation. This procedure was repeated until agglutination was not observed (three to seven times). The levels of absorption were determined by performing double-diffusion tests.

**Immunodiffusion.** The agar plates used for Ouchterlony double-diffusion tests (23) were prepared with 15-ml portions of a preparation containing 1% Difco purified agar, 0.85% NaCl, and 0.02% sodium azide; the medium was dispensed into plastic petri dishes (diameter, 9 cm). Sets of six peripheral wells that were 3 mm in diameter and 4 mm apart were cut around a center well; five sets were cut in each plate. Bacterial cells were grown on a nutrient agar plate for 4 days at 25°C and harvested in 0.5 ml of distilled water (cell concentration, approximately $10^9$ to $10^{10}$ cells per ml). Each center well was filled with antiserum 9226, 9351, or 9352 that was diluted with an equal volume of glycerol or with undiluted antiserum after cross-absorption. The surrounding wells were filled with 10-μl portions of bacterial suspensions. Preimmune serum and a heterologous bacterium, an *Enterobacter* sp., were used as negative controls. The plates were incubated at 25°C for 2 to 4 days and examined with a stereomicroscope with dark-field illumination for precipitin bands. All strains were tested with antiserum 9226. Only strains PE and PSH were tested with antisera 9351 and 9352.

**Indirect IF cell staining.** An indirect immunofluorescence (IF) test was performed as described by van Vuurde et al. (28). Microscope slides with 24 4-mm wells (type 10-342-A; Nutacon) were used. Each well was filled with 5 μl of a bacterial suspension containing $10^5$ to $10^7$ cells per ml. The bacterial suspension was air dried and fixed for 10 min in 96% ethanol. After the slides were washed with demineralized water, they were dried, and each well was filled with 5 μl of antibacterial antiserum. Threefold serial dilutions of each antiserum in phosphate-buffered saline (PBS) (0.8% NaCl, 0.27% $Na_2HPO_4 \cdot 12H_2O$, 0.04% $H_2NaPO_4 \cdot 2H_2O$ [pH 7.2]) were tested to determine the titer of the antiserum. The slides were incubated for 30 min under humid conditions in the dark. After the slides were washed with demineralized water, they were dried, 5 μl of anti-rabbit fluorescein isothiocyanate-conjugated antibodies (Sigma) diluted 1:200 in PBS was added to each well, and the slides were incubated for 30 min. After the slides were washed with demineralized water, each slide was dried and covered with 60 μl of mounting buffer (2.1% $Na_2HPO_4 \cdot 12H_2O$, 0.1% $H_2NaPO_4 \cdot 2H_2O$, 33.3% glycerol [catalog no. 4095; Merck] [pH 7.6]) and a coverslip. The slides were examined with a UV microscope by using a type 50W/N.A.100 objective and incident blue (490-nm) fluorescent light. Each antiserum was tested with the homologous bacterium and form variants of the homologous bacterium, but not with other strains.

## RESULTS

**Reaction of bacteria in double-diffusion tests.** Figure 1 shows the results of the double-diffusion test performed with antiserum 9226. PE-red produced four precipitin bands (Fig. 1, well 1), one thick band close to the well containing bacteria, two bands close together in the middle, and one weak band near the center well. The weak band near the center well was broad and vague, running out to the center well. The two bands in the middle were not always separated distinctly.

The different form variants of PE produced different precipitin patterns (Fig. 1, wells 1 through 5, 23, and 24). All six variants produced the first bright band, and all except PE-white (well 3) produced the last broad band. The PE-white and PE-yellow (well 4) patterns lacked the two middle bands. One or both of the middle bands produced by PE-pink (well 2), PE-P2 (well 22), and PE-GB (well 23) differed from the bands produced by PE-red and from each other, but all of the bands fused with the PE-red bands without spur formation.

Most of the *P. luminescens* strains tested reacted with antiserum 9226 (Fig. 1). Only two strains, PH1 and PH2, did not react with this antiserum (data not shown). PHi (well 44) gave only a very weak reaction. The precipitin patterns of PB (well 24) and PW (well 25) were very similar to the pattern of PE-red. The small-colony variants of PW and PB produced the same precipitin pattern as PE-white (data not shown). The small-colony variants of PSH1, PFR, and PK122 did not react with antiserum 9226 (data not shown). PFR and PNH (wells 34 and 35) produced similar patterns, which differed from the

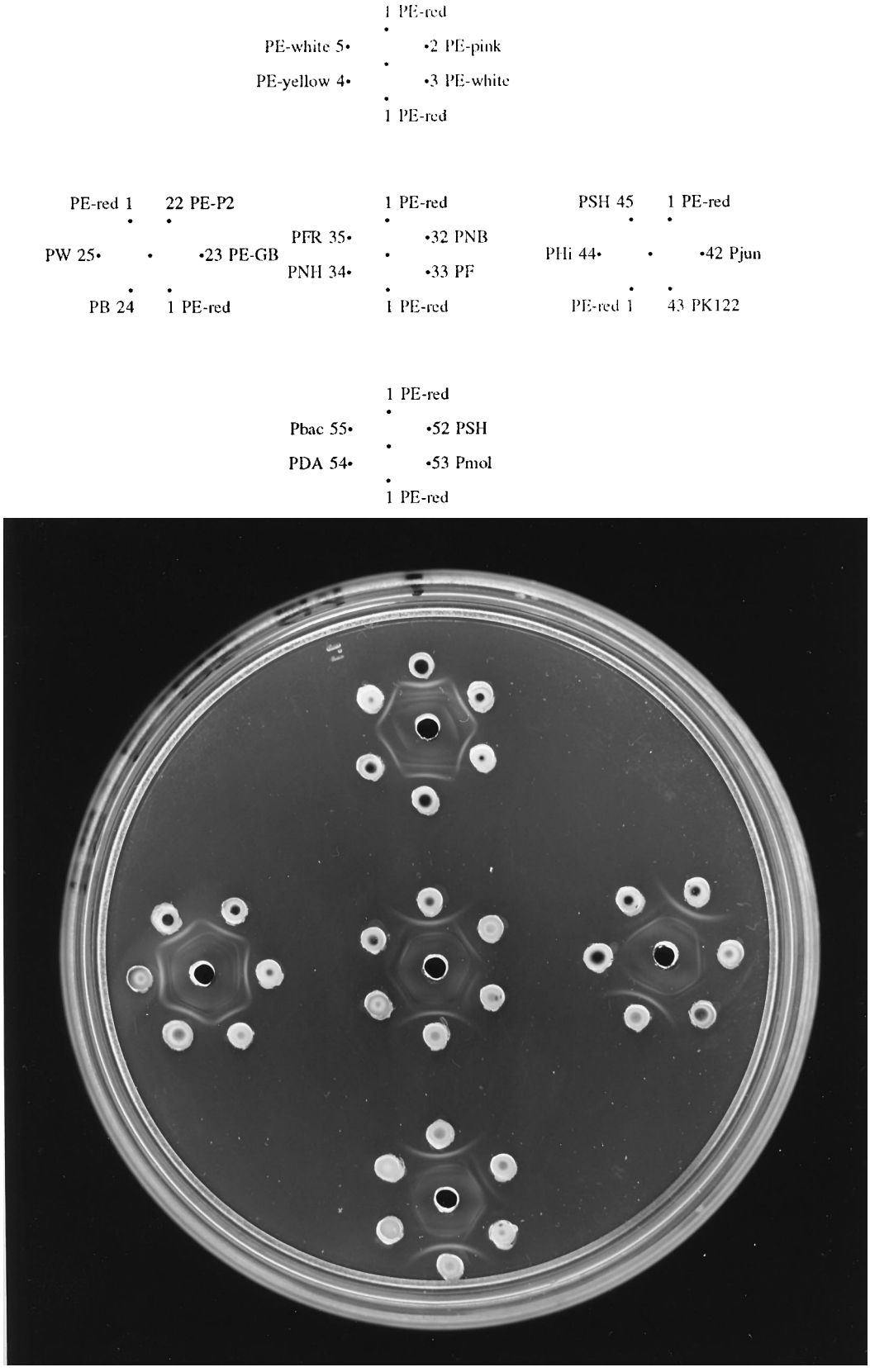


FIG. 1. Ouchterlony double-diffusion patterns showing the reactions of antiserum 9226 with different *P. luminescens* strains and form variants.

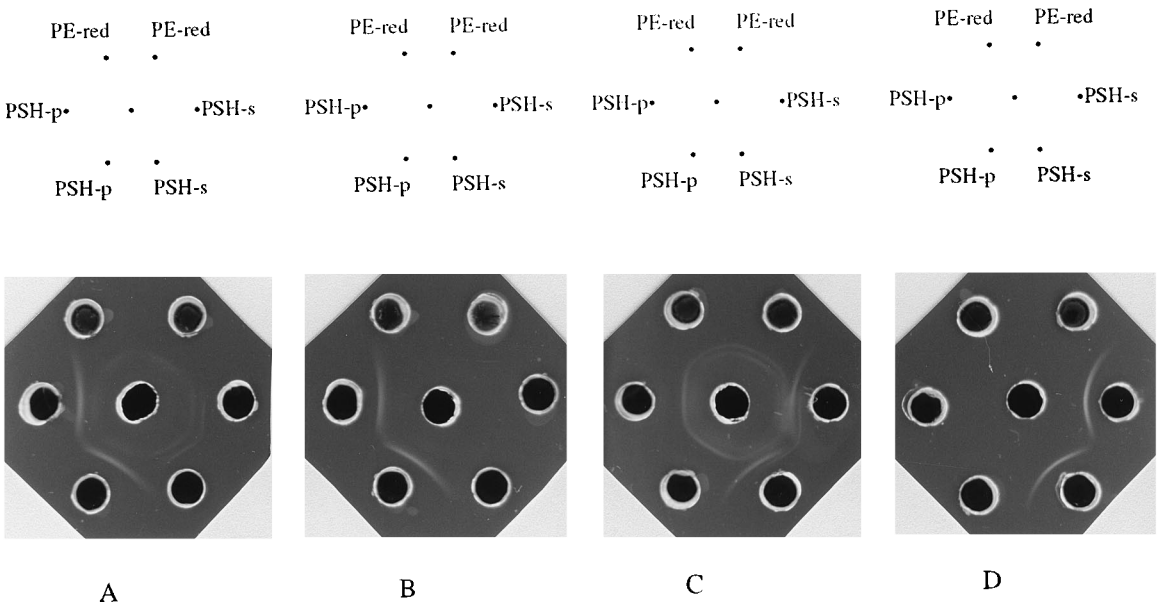


FIG. 2. Ouchterlony double-diffusion patterns showing reactions of *P. luminescens* PE-red, PSH primary form (PSH-p), and PSH secondary form (PSH-s) with antiserum 9351 (A), antiserum 9351-S (cross-absorbed with PSH secondary form) (B), antiserum 9352 (C), and antiserum 9352-P (cross-absorbed with PSH primary form) (D).

pattern of PE-red. All other strains produced different precipitin patterns (Fig. 1 and Table 1).

The precipitin patterns of the bacterial strains changed when we used different antisera. The reactions of PE-red, PSH primary form, and PSH secondary form were stronger with the homologous antisera (antisera 9226, 9351, and 9352, respectively) than with the other antisera (Fig. 1 and 2A and C). The precipitin patterns of the PSH primary form and the PSH secondary form obtained with antisera 9351 and 9352 were different (Fig. 2A and C).

Figure 2B and D show the results of the double-diffusion test obtained with antisera 9351 and 9352 after cross-absorption. Absorbed antisera 9351-S and 9352-P exhibited no reactions with PSH secondary form and PSH primary form, respectively (Fig. 2B and D). After cross-absorption the reaction of an antiserum with the homologous bacteria was less intense than the reaction observed previously (Fig. 2). Pbac and PE-white did not produce precipitin bands in reactions with antiserum 9226 after cross-absorption with Pbac (9226-B) and PE-white (9226-W), respectively (data not shown). PE-red did not produce most of the first bright band in a reaction with 9226-W.

**IF cell staining.** The maximum dilution of antisera that still detected bacterial cells in IF tests was 1:3,000 (titer = 3,000). After cross-absorption the titer decreased to 1,000 in reactions with the homologous bacteria. In IF tests performed with PE-red and PE-white cells (separate or mixed) and antiserum 9226-W, only the large PE-red cells were fluorescent green, while the small PE-white cells were not fluorescent, even when undiluted antiserum was used. Furthermore, PSH primary form and PSH secondary form were not fluorescent in reactions with antisera 9352-P and 9351-S, respectively (Fig. 3).

## DISCUSSION

In this paper we report that we produced polyclonal antisera which could distinguish between *P. luminescens* strains, as well as between form variants. We produced antisera against the primary forms of strains PE and PSH (antisera 9226 and 9351, respectively) and against the secondary form of strain PSH (antiserum 9352). These antisera were polyclonal and therefore contained antibodies against different antigens. In the double-diffusion test performed with antiserum 9226, the four PE-red bands each represented a different antibody-antigen complex. The antigens that diffused in the medium could have been lipopolysaccharide vesicles, unmodified outer membrane vesicles, exoenzymes, or other small molecules (10). All form variants of PE reacted with the antiserum, but each variant produced a different double-diffusion pattern. Some bands were identical, which means that the antigens were identical (reaction of identity). The bands that differed from the PE-red bands showed that the epitopes (antigenic sites on molecules) were located on antigens (molecules) that had different diffu-

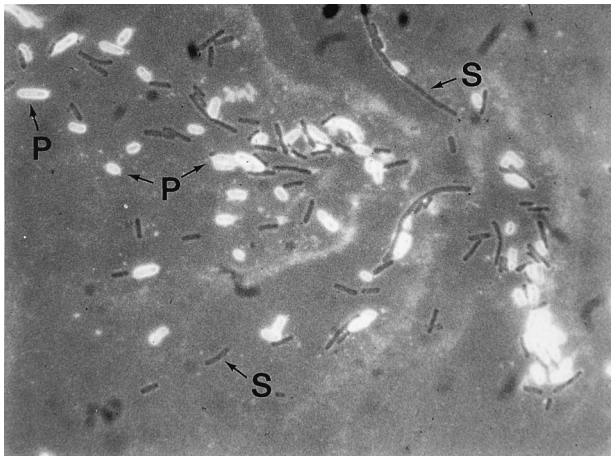


FIG. 3. IF cell staining of *P. luminescens* PSH primary and secondary form cells with antiserum 9351-S (specific for PSH primary form). The primary form cells (P) are coated with fluorescent labelled antibodies, while the secondary form cells (S) are dark. The film was exposed first to UV light and then to visible light.

sion speeds (reaction of partial identity). These differences made it possible to discriminate between form variants. A truly secondary form, like the PSH secondary form, was not isolated from PE cultures (16). The results obtained with antisera 9351 and 9352 showed that the primary and secondary forms of PSH, like the form variants of PE, possess different antigens.

We also observed several differences between strains. Most strains produced a specific precipitin pattern. Only strains PW and PB were serologically similar to PE. All strains except PH1 and PH2 reacted with antiserum 9226; this was true even for strains that were isolated from different nematode species and were genetically different, such as Pbac, PDA, Pmol, and PK122 (26). Although antiserum 9226 reacted with almost all *P. luminescens* strains, it reacted differently with each strain in the double-diffusion test. Even bacteria isolated from nematodes that originated from the same area (PE, PH1, and PH2) reacted differently. This suggests that within a nematode species each nematode isolate carries its own specific symbiont. Whether the nematodes actually carry different symbiont species or whether the symbionts differ in their antigenic regions in only minor ways is not clear. The differences might even be caused by subculturing (PE was subcultured for 3 years, while PH1 and PH2 were isolated recently). The differences in serological reactions which we observed are reproducible, and therefore it is possible to produce strain- or variant-specific antisera which can be used to identify organisms and to perform research on form variation in *P. luminescens.*

One way to produce a more specific antiserum is by cross-absorption. PE-red has epitopes that are not present in other bacteria. For example, during cross-absorption with PE-white all antibodies that react with PE-white are removed, leaving only antibodies that react with epitopes specific for PE-red. Thus, it is possible to make an antiserum more specific for PE-red.

Alternatively, monoclonal antibodies (antibodies that react to only one epitope) could be used to distinguish strains or form variants. The differences observed in double-diffusion patterns show that it might be possible to make specific monoclonal antibodies.

Specific antisera that distinguish between the primary and secondary forms of a bacterial strain, like 9351-S and 9352-P, are important for mass rearing of nematodes and for research on form variation. In mass rearing of nematodes a mixed culture of primary and secondary cells must be avoided, because the secondary cells do not support nematode growth as well as the primary cells do (12). By testing a bacterial culture with the IF test a shift to secondary cells can be detected, and only truly primary cultures can be used for mass production of nematodes. Other serological methods, like the enzyme-linked immunosorbent assay, might be even more suitable for this purpose.

In research on form variation in *P. luminescens* the possibility of detecting primary and secondary forms in cells instead of in colonies opens new perspectives. Primary and secondary form cells can be detected in insects and nematodes without subculturing them. During subculturing a shift to a different form often occurs; therefore, direct detection with antiserum is a major improvement. A whole range of serological techniques can be used to study the mechanism and function of phase variation.

In IF tests the antiserum only reacts with the cell surface. Since cross-absorbed antisera react with homologous bacteria but not with their variants, the surfaces of the variants must be different. The outer membrane contains lipopolysaccharides and membrane proteins as major antigens. Some of the proteins are used to transport products into and out of the cell. If the serological differences are located on the membrane proteins, this might be the key to the mechanism of form variation. Most of the characteristics of the primary form that are not observed in the secondary form involve products which need to be transported into or out of cells; uptake of dye, antibiotic production, lipase and lecithinase activities, and even luminescence can be regulated by an autoinducer which diffuses through the cell membrane (11). It is known that the genes coding for lipase activity and luminescence are present in the secondary form and also that the mRNAs can be detected (15, 29, 30). A possible explanation for the lack of lipase activity and luminescence in the secondary form, which can be supported by our results, is that the products are not excreted or that the products are modified during excretion. In further research this hypothesis will be tested.

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