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EXPERIMENTAL ARTICLES

Pigmentation of *Serratia marcescens* and Spectral Properties of Prodigiosin

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Abstract—Pigmentation of *Serratia marcescens* depends on the composition of the cultivation medium. The cultures grown on glycerol–peptone medium and on the medium with acetate are red and yellow (yellowish orange), respectively, with the color depending on the ambient pH. *S. marcescens* cells growth on glycerol–peptone medium (visually of red color) contain two forms of prodigiosin: the "red" and "yellow" ones with absorption maxima at 535 and 460–470 nm, respectively. The absorption spectrum of prodigiosin in the native pigment–protein complex was different from the spectrum of the pigment dissolved in ethanol and resembled that of the cell suspension in the presence of an additional absorption maximum at 500 nm. Prodigiosin sensitivity to low-intensity visible light depended on the state of co-solubilized protein. The sensitivity of prodigiosin in the complex with a native protein is similar to the pigment photosensitivity in the composition of an intact bacterial cell. Prodigiosin extracted under denaturation conditions was less sensitive to illumination. Both the "red" and the "yellow" form of prodigiosin in solution fluoresced at 560–565 nm; the in vivo fluorescence of the red pigment form was more marked.

Keywords: *Serratia marcescens*, culture pigmentation, prodigiosin, absorption spectrum, fluorescence

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The ability to form the pigment prodigiosin is a species characteristic of *Serratia marcescens* [1–3]. The color of *S. marcescens* determined by prodigiosin is usually described in the literature as red, whereas prodigiosin in solution may be present in two forms, depending on the solvent and pH: with an absorption maxima at 535 and 460–470 nm [4–7] that have the red and yellow (yellow–orange) color, respectively. Despite the long history of investigation of the pigment formation of *S. marcescens*, the role of prodigiosin is still not clear. The photoprotective action of this pigment [6] and its involvement in respiration [5] were suggested. There is an opinion that prodigiosin biosynthesis only provides the cell with a sink for excessive reductive equivalents and energy [8, 9]. Antibiotic properties of prodigiosin have been noted most often [2, 10–15].

Structurally, prodigiosin (a linear tripyrrole) belongs to the class of compounds including such biologically important ones as heme, chlorophylls, and phycobilins [16, 17]. Therefore, the suggestions that prodigiosin is involved in cell energy metabolism are not unfounded.

Intense coloration indicates the potential significance of visible light for *S. marcescens*. Earlier, using the method of photomicrocalorimetry, we showed the possibility of storing light energy by a pigmented culture of *S. marcescens* [18], which arouses natural interest in the study of the spectral properties of prodigiosin and of the photoreactions occurring with the pigment under illumination with visible light. *S. marcescens* is a classical heterotrophic organism, and the revealed effect was quantitatively insignificant compared to photosynthesizing organisms (about 1% of energy storage by the culture of the microalga *Chlorella vulgaris*) [18].

Thus, it seems expedient to begin investigation of the physiological role of prodigiosin by revealing the photoreactions occurring in vitro with its involvement. The data on the properties of prodigiosin (including its spectral properties) available in the literature were obtained for the pure pigment. However, the involvement of prodigiosin in metabolic processes is only possible via interaction with cellular proteins. The spectral properties of the free pigment and of the protein-bound one may differ [16]. This presupposes extraction and investigation of prodigiosin together with the native protein preserving, if possible, the main characteristics of the pigment close to those in situ.

This work compares the spectral properties of prodigiosin in solution and as a part of the pigment–protein complex extracted from *S. marcescens* and their correlation with the spectral characteristic of the bacterial culture with a view to revealing the possible application of the pigment–protein complex to study the function of prodigiosin in the metabolism of the producer cell.

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MATERIALS AND METHODS

The pigmented strains *S. marcescens* ATCC 9986 and 33 obtained from the culture collection of the Department of Microbiology, Kazan University, were used in the work. Cultivation was carried out in glycerol–peptone medium (peptone, 10 g/L; glycerol, 5 g/L, pH 7–7.2) and in M9 mineral medium; modified nutrient media on their basis were also used. Their composition varied depending on substitution of the carbon source: the media contained glycerol (5 g/L), acetate (10 g/L), or glucose (5 g/L). The cells grown on agar medium and resuspended in saline were used as inoculum. The culture was grown at 28°C in 250-mL flasks with 50 mL of the medium under continuous stirring at 200 rpm.

The influence of illumination was studied using the white light from a Hitachi white fluorescent lamp with a light flow intensity of 22.8 μM photons/(m^2 s); the light filters ZS-10 (500–600 nm) and SS-5 (340–470 nm) were used to identify the spectral regions where prodigiosin absorption occurred.

The growth of bacterial cultures was assessed by optical density (E670) spectrophotometrically. For the quantitative determination of prodigiosin, it was extracted with HCl-acidified ethanol (1 : 9); the prodigiosin concentration was calculated by absorbance at 535 nm using the extinction coefficient 51.3 × 10^3 L/(g cm) [19].

The pigment–protein complex was obtained by treating the biomass with 0.1% sodium dodecyl sulfate or Triton X-100 with subsequent centrifugation to remove the biomass residue. The pigment–protein complex was purified by salting out with ammonium sulfate (0.2 of the saturation concentration); the floating sediment obtained was dialyzed and resuspended in water or 0.1% detergent solution. The preparation thus obtained contained the purified pigment–protein complex with only one band in the electrophoregram in PAAG [20].

The suspension of nongrowing cells in 50 mM Tris-HCl buffer (pH 7.0) served as the control for assessment of the photosensitivity of prodigiosin within the pigment–protein complex.

Absorption spectra were recorded using a Lambda-25 spectrophotometer (Perkin Elmer); fluorescence spectra were determined using a Fluorat-02 Panorama fluorimeter (Lumex).

RESULTS AND DISCUSSION

The pigmented cultures of *S. marcescens* ATSS 9986 and 33 grew in peptone media containing glycerol, glucose, or acetate as a source of carbon and energy. The greatest biomass accumulation (10–12 g/L) and maximum pigment formation (0.3–0.4 mg/L) occurred in the medium with glycerol; a slightly lesser amount of prodigiosin was accumulated in the medium with acetate (0.08–0.15 mg/L). The nutrient medium containing glucose as a source of carbon and energy did not provide conditions for prodigiosin synthesis. Cultivation on glycerol– and acetate–peptone was accompanied by alkalization of the medium (pH up to 7.8–8.5 on glycerol and up to 9–9.5 on acetate after 6–7 days of cultivation), whereas strong acidification (pH up to 4–4.5, sometimes up to 3.5) was observed on glucose.

Pigment formation of the *S. marcescens* cultures studied depended also on the nitrogen source. Both cultures grew well on media with both organic (peptone) and mineral (nitrate) source of nitrogen; however, the pigment was accumulated only on media with peptone. Addition of proline (up to 3–5 mg/mL), a precursor of prodigiosin biosynthesis [21], to the mineral medium did not stimulate pigment formation.

Upon growth on liquid or solid glycerol–peptone medium, both *S. marcescens* cultures were red-colored in various tints (young cultures were bright red; with age they acquired a cherry tint). The culture of strain 9986 on agar medium had a well-defined metallic luster, which was slightly pronounced or absent in the case of strain 33. When the cultures grew on acetate medium, they acquired the yellow (yellow-orange) color. The yellow pigmentation of *S. marcescens* due to prodigiosin has been described in the literature [5]. The yellow coloration of bacteria of this species may also be caused by the formation of muconic acid derivatives [22] or carotene [23]. Pigment extraction from the biomass of these strains grown on acetate–peptone medium under the standard conditions (with acidified ethanol) resulted in a red-colored solution with a spectrum characteristic of the "red" form of prodigiosin (absorption maximum at 535 nm). Extraction with neutral ethanol resulted in the yellow solution with an absorption maximum at 460–470 nm; acidification caused a shifting of the absorption maximum to the 535-nm range with the resultant change in the solution coloration. Such a pH-dependent color change is characteristic of prodigiosin [4, 7]. Thus, the pigmentation of the *S. marcescens* cultures growing in acetate medium is determined by the "yellow" form of prodigiosin. On the whole, the coloration of both the growing culture of *S. marcescens* and the suspension of nongrowing cells depended on pH of the medium.

The coloration of prodigiosin solution depended on the ratio of the red (absorption maximum 535 nm) and yellow (460–470 nm) pigment forms, which, in turn, depended on the final pH of the solution. The neutral and slightly alkaline solutions were characterized by the presence of both absorption peaks (Fig. 1); a change in the final pH resulted in a corresponding change in the absorption spectrum. This circumstance makes it impossible to determine the ratio of different pigment forms in the native culture by extraction.

Figure 2 shows the absorption profiles of the pigmented cultures of *S. marcescens* strains ATCC 9986 and 33 used in the work. The absorption regions of the cultures grown in glycerol– and acetate–peptone

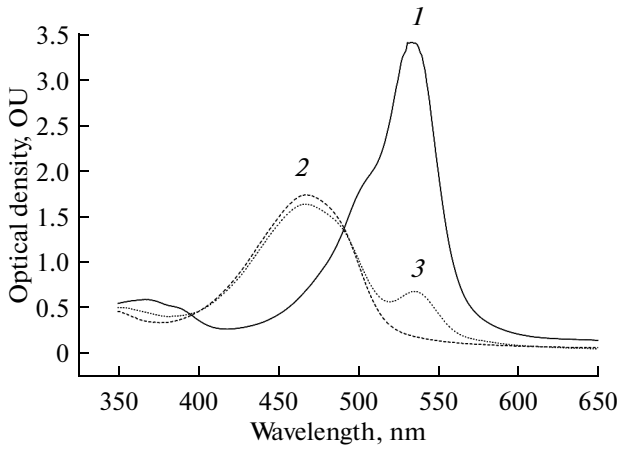

Fig. 1. Influence of pH on the absorption spectrum of the alcohol solution of prodigiosin:, pH 4 (*1*), pH 10 (*2*), and pH 7.5 (*3*).

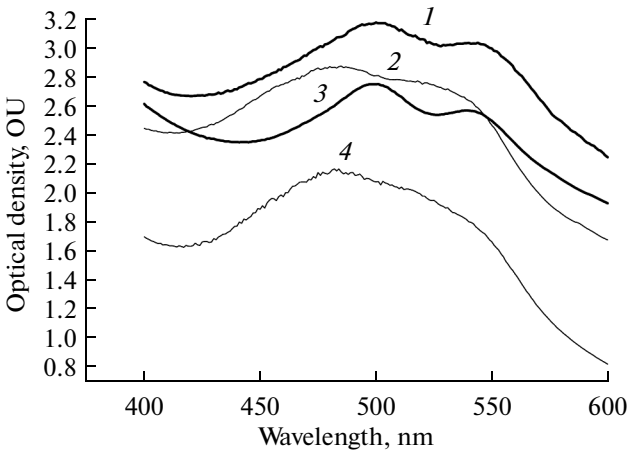

Fig. 2. Influence of the medium composition on absorption of *S. marcescens* in the visible range: strain 9986 (*1, 2*) and strain 33 (*3, 4*); in the glycerol–peptone medium (*1, 3*) and in the acetate–peptone medium (*2, 4*).

media were slightly different but, on the whole, overlapped the absorption spectra of both forms of prodigiosin. On visual inspection, the coloration of the cultures grown on glycerol was determined by the red pigment form, and slightly marked maxima in the 500- and 530–540-nm regions can be noted on absorption curves. The absorption curves of the cultures growing on acetate and colored yellow (yellow–orange) were slightly shifted to the short-wave region and had no marked maxima.

Prodigiosin is sensitive to the visible light. Illumination of the pigmented *S. marcescens* cultures with low-intensity white light [6–7 kJ/(m^2 s)] caused a decrease in color intensity without influencing cell growth.

Figure 3 shows the data on the effect of light in different spectral regions on the pigmentation of *S. marcescens* ATCC 9986 growing in the standard glycerol–peptone medium and having the red color characteristic of this species. Illumination with green light (the absorption region of the prodigiosin "red" form, 535 mn) with an intensity of 40–45 J/(m^2 s) decreased the pigment content (by approximately 50% at 6–7 days). A similar effect for the blue light (the absorption region of the prodigiosin "yellow" form, 460–470 mn) was observed at a light intensity of 160 J/(m^2 s). With a greater intensity of blue light [250–300 J/(m^2 s)], the amount of prodigiosin decreased by more than 80%. The coloration of the culture of *S. marcescens* 33 was also sensitive to both the green and the blue light. A 50% reduction in the prodigiosin content in the culture at the end of the observation period occurred at a light intensity of 40–45 J/(m^2 s) for the green light and 250–300 J/(m^2 s) for the blue light. Thus, both pigment forms were present in the growing culture appearing visually as red.

Prodigiosin is localized in the surface structures of *S. marcescens* cells [24]. Prodigiosin has two spectral forms with absorption maxima at 535 and 460–470 nm, depending on the solvent and pH of the medium [4–7], and their mutual conversion is considered to depend on the protonation–deprotonation process [6]. On the one hand, simultaneous presence of both pigment forms in the cells of *S. marcescens* can be explained by their simultaneous existence at the neutral (or near-neutral) pH values. On the other hand, the complex and heterogeneous structure of biological membranes, including that of the prokaryotic cytoplasmic membrane [15, 25], the transmem-

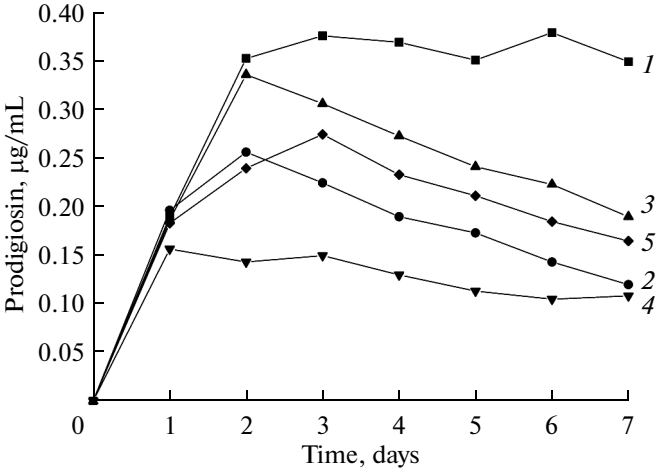

Fig. 3. Influence of illumination on the prodigiosin content in the growing culture of *S. marcescens* 9986: dark control (*1*); illumination with white light at 6 kJ/(m^2 s) (*2*); illumination with green light at 40 J/(m^2 s) (*3*); illumination with blue light at 160 J/(m^2 s) (*4*); and illumination with blue light at 250 J/(m^2 s) (*5*).

Photosensitivity of prodigiosin in different samples

Time, h	Cell suspension (prodigiosin, μg/mL)		Triton X-100 (prodigiosin, μg/mL)		SDS (prodigiosin, μg/mL)	
	light	darkness	light	darkness	light	darkness
0	0.194 ± 0.003		0.193 ± 0.001		0.195 ± 0.001	
24	0.073 ± 0.005	0.194 ± 0.002	0.081 ± 0.003	0.195 ± 0.002	0.157 ± 0.005	0.195 ± 0.001
48	0.049 ± 0.004	0.193 ± 0.001	0.046 ± 0.003	0.194 ± 0.001	0.144 ± 0.004	0.195 ± 0.001

brane proton gradient, lateral proton transfer [26–28], and nonuniform distribution (concentration) of negatively charged groups in the protein molecules that contribute to protonation of some of their regions [29] can create different local conditions for the protonation–deprotonation of prodigiosin molecules in a bacterial cell.

Prodigiosin is a water-insoluble pigment bound to the cytoplasmic membrane and probably associated with a structure of lipoprotein nature [24]. Prodigiosin can be extracted from the biomass with both polar organic solvents (acetone, ethanol) and some detergents. The biomass treatment with the sodium dodecyl sulfate or Triton X-100, which solubilize cellular proteins [30], resulted in transition of the water-insoluble pigment into the water phase as a part of the pigment–protein complex; in the case of Triton X-100, this occurred under non-denaturing conditions [30]. Prodigiosin was solubilized in the complex with a 105-kDa protein; the hydrodynamic diameter of the native pigment–protein complex was 7.5–8 nm [20]. The prodigiosin-bound protein was extracted from the biomass only under the action of a detergent and therefore belonged to the group of integral proteins [25].

Illumination of suspensions of nongrowing *S. marcescens* cells decreased the coloration intensity by 60 and 75% after 24 and 48 h, respectively. The photosensitivity of prodigiosin extracted with Triton X-100 was close to the photosensitivity of the pigment in the composition of the integral cell. Prodigiosin extracted with dodecyl sulfate was considerably less sensitive to the low-intensity white light: during the experiment, its color intensity decreased by only 20–25% (table). It may be concluded that the prodigiosin-containing complex solubilized by Triton X-100 is close to the native structure present in the intact cell.

The involvement of prodigiosin in cell metabolism is only possible via its interaction with cellular proteins.

The data on the spectral properties of prodigiosin extracted with organic solvents and devoid of its natural proteinaceous environment are available in the literature. However, it is known that proteins may influence the spectral properties of the chromatophores bound to them [16]. Figure 4 shows the absorption spectra of prodigiosin in alcohol solution and in the composition of the pigment–protein complex (in acidified solutions). The absorption spectrum of prodigiosin solubilized by dodecyl sulfate was similar to the pigment spectrum in ethanol: they had a narrow absorption maximum at 535 nm and a shoulder at 500 nm, whereas prodigiosin in the composition of the native pigment–protein complex revealed two absorption maxima: 535 nm characteristic of prodigiosin and a new one, at 500 nm, corresponding to the shoulder in the spectrum of the alcohol solution of the pigment. It is interesting to note that this makes the spectrum of the native pigment–protein complex similar to the absorption profile of the culture growing on glycerol. Acidification of an aqueous solution of the pigment–protein complex caused the spectral change characteristic of prodigiosin—emergence of absorption at 460–470 nm and disappearance of the 535-nm peak. In the case of the native pigment–protein complex, both maxima in this region (500 and 535 nm) disappeared.

Prodigiosin as a part of the native pigment–protein complex is similar in photosensitivity and spectral properties to prodigiosin in its natural environment (in the composition of the bacterial cell) and may be

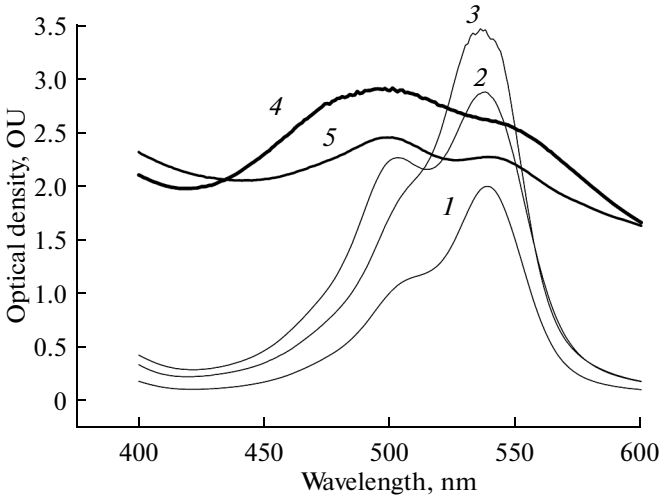

Fig. 4. Prodigiosin absorption in different samples: in 0.1 DDS (pH 5.5) (*1*); in 0.1 Triton X-100 (pH 5.5) (*2*); in ethanol (pH 5.5) (*3*); 3-day *S. marcescens* culture (pH 7.5) (*4*); and cell suspension in 50 mM Tris-HCl (pH 5.5) (*5*).

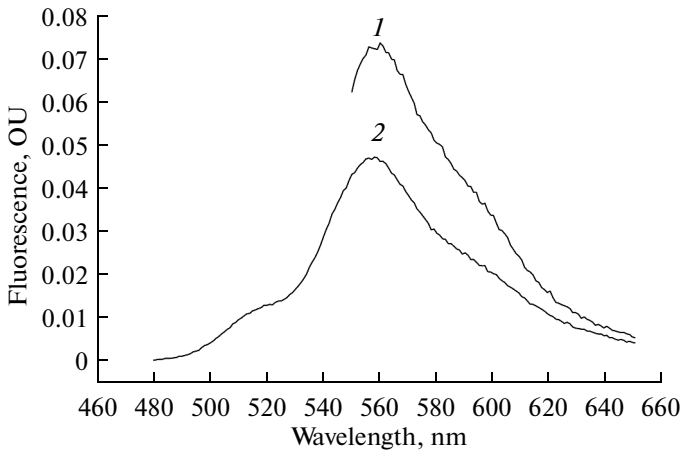

Fig. 5. Prodigiosin fluorescence (ethanol solution): the "red" form (pH 4), excitation at 535 nm (*1*) and the "yellow" form (pH 10), excitation at 465 nm (*2*).

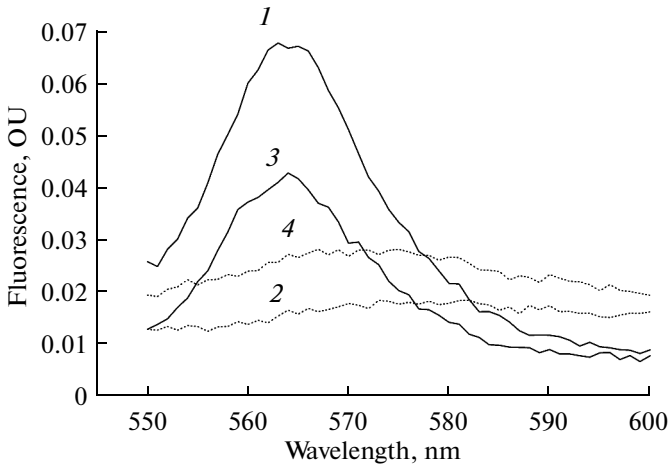

Fig. 6. Fluorescence of the *S. marcescens* 9986 culture grown in different media: in the glycerol–peptone medium (*1, 2*) and in the peptone medium with acetate (*3, 4*); the excitatory wavelength 535 nm (*1, 3*) and 465 nm (*2, 4*).

regarded as a model for studying the light-induced reactions proceeding in a bacterial cell with the involvement of prodigiosin.

The pigment molecules can transfer the absorbed light energy to other molecules or to a photochemical reaction (as it occurs in photosynthesis). Moreover, the excitation state energy liberated when the pigment is transferred to its usual state can either be emitted as radiation (fluorescence) or diffused in the form of heat (heat dissipation). The chlorophyll fluorescence intensity in vitro is at a relatively high level, whereas in operating plant photosystems the processes, in which the energy is lost, are not very essential [31].

In Figure 5, the prodigiosin fluorescence spectra are shown. Both the "red" and "yellow" prodigiosin forms in ethanol had a fluorescence maximum of 560–565 nm. Prodigiosin as a part of the pigment–protein complex fluoresced in the same region.

When the suspension of *S. marcescens* intact cells was excited by light at a wavelength of 535 nm, it also fluoresced in the region characteristic of prodigiosin (Fig. 6); excitation of intact cells by the light at a wavelength of 465 nm caused weak fluorescence but did not yield a well-defined peak, although it could be detected when sensitivity was increased. Interestingly, on excitatory irradiation (465 nm), the picture was similar to both the culture grown in the glycerol–peptone medium (red color) and on acetate (yellow color); only the total level of fluorescence was different. Considering the photosensitivity of pigmentation of *S. marcescens* culture at both 500–600 and 380–470 nm, it may be suggested that the fluorescence of the "yellow" form of prodigiosin (in contrast to the "red" form) is partly quenched by the pigment microenvironment, which may indicate a different role of both pigment forms in the metabolism of *S. marcescens*.

Thus, the coloration of the growing culture of *S. marcescens* depends on the cultivation conditions, primarily on pH of the culture. In the cells of the *S. marcescens* cultures grown on the standard glycerol–peptone medium and having the red pigmentation characteristic of this species, both forms of prodigiosin are present simultaneously: the "red" (with an absorption maximum of 535 nm) and the "yellow" one (460–470 nm). Prodigiosin possesses its own fluorescence, with the maximum in the alcohol solution within the 560–565 nm range. Prodigiosin fluorescence was also revealed in the culture of *S. marcescens*. While fluorescence of the "red" form of prodigiosin was observed in intact cells, the fluorescence of the "yellow" form was scarcely pronounced.

Prodigiosin extracted with detergent solutions was present in the micelles containing the protein and detergent molecules; such pseudohomogeneous colloid systems are successfully used in enzymology for studying the membrane proteins [25]. The native pigment–protein complex solubilized with Triton X-100 is similar in photosensitivity and spectral properties to prodigiosin in natural environment (as a part of a bacterial cell) and can also be used for studying the primary photoreactions occurring in the bacterial cell with the involvement of prodigiosin.

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