ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 5, pp. 585–589. © Pleiades Publishing, Ltd., 2007.
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EXPERIMENTAL ARTICLES

Application of the Complex of DNA with the Congo Red Anionic Diazo Dye for Detection of Nuclease-Producing Colonies of Marine Bacteria

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Received July 25, 2006

Abstract—This study was aimed at the development of a method for detection of colonies of nuclease-secreting marine bacteria. The BAL nuclease-producing marine bacterium *Pseudoalteromonas espejiana* BAL-31 was used as the test object. A new method was developed involving the congo red (CR) anionic dye. The *P. espejiana* culture was plated on nutrient agar with CR and denatured DNA. In such media, CR was found to form complexes with DNA. After two days of incubation at 30°C, halos were found around the *P. espejiana* colonies. No halos appeared when DNA was not introduced, when BAL nuclease was inactivated, or when the plates were inoculated with *Escherichia coli.* It was concluded that the halos around the colonies indicated nuclease secretion. The halos were shown to result from the coagulation of CR released after digestion of the CR–DNA complex by the nuclease. This method for detection of nuclease-producing colonies can probably be used for all marine bacteria and possibly for halophilic bacteria as well.

Key words: Pseudoalteromonas espejiana, BAL nuclease, congo red, secretion.

DOI: 10.1134/S0026261707050116

Microbial environments often contain free nucleic acids originating from dead cells. A number of bacteria secrete nucleases and phosphatases. They can therefore utilize exogenous nucleic acids as a source of carbon and energy. Detection and identification of the colonies of nuclease-secreting microorganisms can be useful for analysis of complex microbial ecosystems and for species and strain identification; it is essential in screening for mutants in nuclease secretion.

The goal of the present work was to find the method for detection of nuclease-producing colonies of marine bacteria. The marine γ-proteobacterium *Pseudoalteromonas espejiana* BAL-31 [1] (formerly *Pseudomonas* BAL-31 [2], *Alteromonas espejiana* BAL-31 [3]) was the object of our study. *P. espejiana* secretes a number of hydrolytic enzymes, including gelatinase, amylase, lipase [2], alkaline phosphatase [4], and BAL nuclease [5]; the latter is a commercial product. *P. espejiana* supports growth of a lipid-containing bacteriophage PM2 [6].

The procedure used to differentiate between the colonies with or without nuclease secretion consists of the following stages: denatured DNA is introduced into the nutrient agar; the medium is inoculated with the analyzed mixture of microorganisms; the plates are incubated; and the DNA-free zones resulting from nuclease activity are revealed. To reveal these zones (halos), several methods are widely used. In the first and the oldest one [7], acid (1 N HCl) is poured over the agar; the areas containing DNA then become turbid, while DNA-free zones remain transparent (halos are formed) [8]. The cells in the colonies die after the application of acid; replicas are therefore required to preserve the variants. In the second method, the cationic triphenylmethane dye methyl green is added into the medium or poured over the plates; the dye binds to DNA. After DNA digestion with a nuclease, at pH above 5.0, the liberated dye molecules react with hydroxyl ions and form a colorless carbinol [9]; the halos are formed [10]. In the third method, the cationic thiazine dye toluidine blue is added into the medium or poured over the plates; the dye binds to DNA. After DNA digestion with a nuclease, the liberated dye binds to agar and changes color from blue to pink (metachromasia, [11]); the halos are formed [12]. A number of companies manufacture DNase Test Agar for the three above-mentioned methods (without dyes, with methyl green, or with toluidine blue). Cationic dyes, e.g., acridine orange, are sometimes used for this purpose; their fluorescence changes color and/or increases sharply on binding with DNA [13].

We have tried the methods with methyl green and toluidine blue for detection of BAL nuclease around

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espejiana colonies (the dyes were added to the agar). None of these methods was acceptable for this bacterium. The goal of the present work was to develop a new method for detection of nuclease-producing colonies of marine and halophilic bacteria involving the *anionic* dye congo red.

MATERIALS AND METHODS

The following materials were used in the present work: EGTA, ethylene glycol-*bis*-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Serva, 11290); agar, Bacto-Agar Typ USA (Ferak, Berlin, Germany); peptone, Veta (Moscow, Russia); DNA, high-polymeric DNA from chicken erythrocytes, sodium salt (Reanal); methyl green, pure for analysis (Soyuzglavreaktiv, Russia); toluidine blue, pure (Minkhimprom USSR); and congo red, pure for analysis (DiaM, Germany). In a 1-cm cuvette, the aqueous solution of this batch of congo red (20 μg/ml) had an optical density of 0.84 at 505 nm (at the absorption maximum). The apparent molar extinction coefficient was therefore 2.9×10^4 M^{-1} cm^{-1}, lower then the reported values ($4.2–5.4 \times 10^4$ M^{-1} cm^{-1}). All the dyes were used without additional purification.

Bacteria. *Pseudoalteromonas espejiana* Pae4 [14] sensitive to the phage PM2 is a descendant of *P. espejiana* BAL-31 obtained in 1976 from the All-Russian Institute for Genetics and Selection of Industrial Microorganisms. *Escherichia coli* K12, strain AB 1157 (F^- thrA1 leu-6 thi-1 proA2 his-4 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 str-31 supE44) was obtained from S.V. Vasil'eva, Institute of Biochemical Physics, Russian Academy of Sciences.

Nutrient media The BAL 50-10 broth (BAL broth [15]) contained the following: NaCl, 450 mM; KCl, 9.4 mM; $MgSO_4$, 50 mM; $CaCl_2$, 10 mM; and peptone, 8 g/l. LCM (low-calcium medium) [14] was the BAL 50-10 medium without $CaCl_2$ and with 1.5 mM EGTA.

Broth cultures of *P. espejiana* were grown in BAL 50-10 medium at 29–30°C without forced aeration [14].

Preparation of denatured DNA: DNA (200 mg) was mixed with water (10 ml) and stored for a day in a refrigerator; the resulting gel was sonicated with a UZDN-2T disintegrator at 44 kHz. The viscous solution was then heated for 20 min in a boiling water bath and cooled quickly.

Preparation of the nutrient agar with DNA and congo red or other dyes. Stock solutions: (A) agarized (2%) BAL 50-10 broth; (B) denatured DNA in water (20 mg/ml); (C) congo red solution in 0.5 M NaCl (1 mg/ml). The solutions were heated in a boiling water bath and mixed in the ratio 5 : 1: 4; solution (C) was added at the last stage. The mixture was poured into 9-cm petri dishes (10 ml per plate). Agars with DNA and other dyes were prepared similarly; freshly prepared aqueous solutions of the dyes were used.

Absorption spectra were determined with a Specord UV VIS spectrophotometer.

Photographs of the plates with bacterial colonies were made with a Camedia C-765 Ultra Zoom digital camera (Olympus).

RESULTS AND DISCUSSION

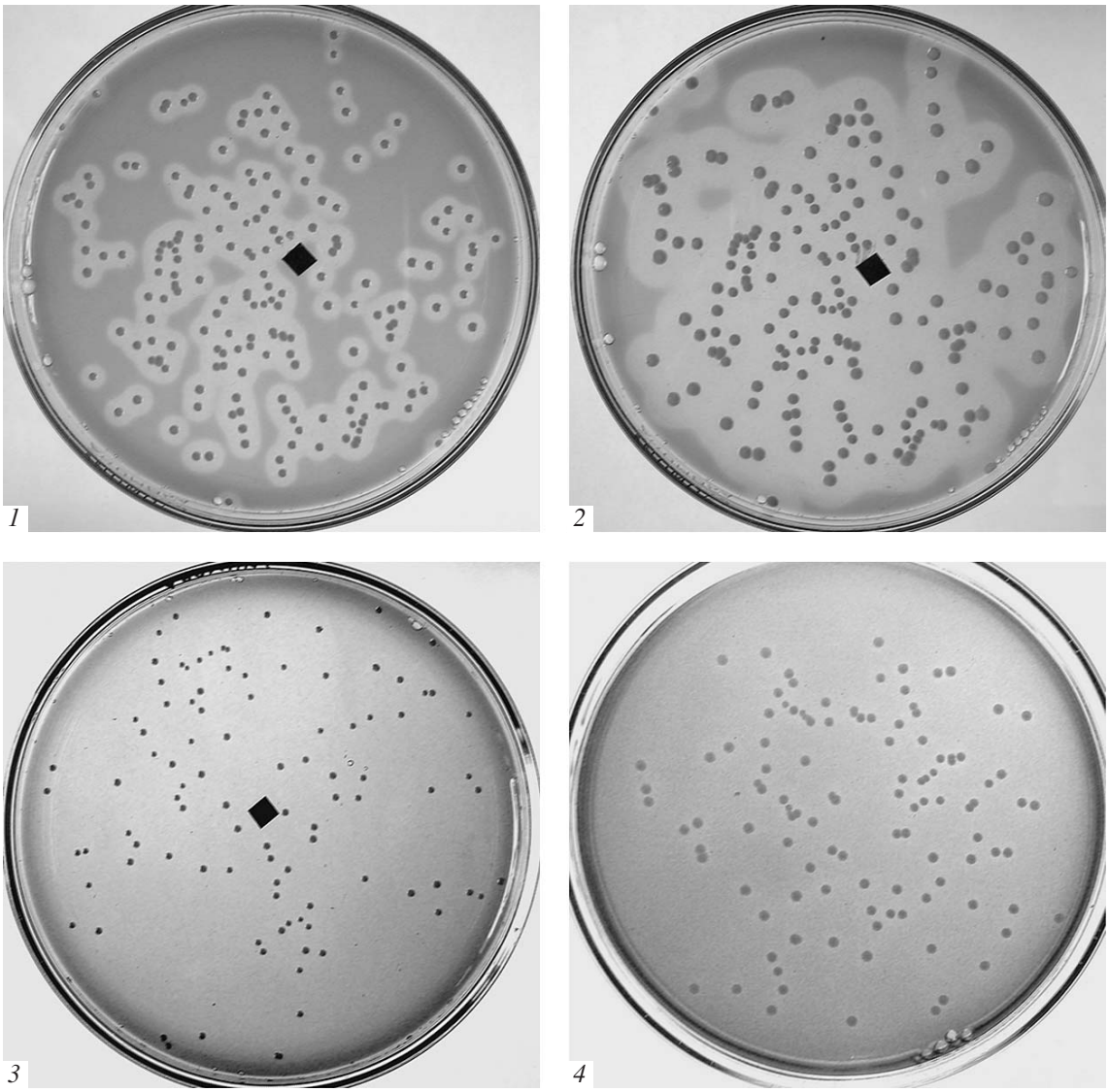
The dye used to reveal BAL nuclease-secreting *P. espejiana* colonies should not be toxic to *P. espejiana* when added to BAL 50-10 nutrient medium. It should also bind to DNA; its coloration in bound and unbound states should be visually different. Methyl green (MG) and toluidine blue (TB), the dyes usually applied for this purpose, were tested.

P. espejiana was plated on the medium with DNA and MG (approx. 10^2 CFU per plate). At MG concentration of 0.05 mg/ml, no inhibition of colony growth occurred; agar coloration, however, was weak. Poorly discernable halos appeared around the colonies after two days of incubation. At MG concentration of 0.1 mg/ml, inhibition of colony growth occurred; agar coloration was still very weak, and very weakly pronounced halos were discerned after two days. No *P. espejiana* colonies were formed at MG concentrations of 0.2 and 0.5 mg/ml. At high concentration of salts, binding between MG and RNA [16] or denatured DNA [17] is known to be practically nonexistent. The weak agar coloration in BAL 50-10 medium correlates with these findings.

P. espejiana was plated on the medium with DNA and TB (approx. 10^2 CFU per plate and 10^7 CFU on the plate for smears). TB concentrations were 0.05 and 0.1 mg/l. No colony growth was detected even at the lowest TB concentration. Pink spots and individual colonies appeared on the smears; the colonies were probably formed by TB-resistant mutants. Massive growth, however, was not observed. Thus, TB is relatively toxic for *P. espejiana*, as well as for a number of other bacteria [18].

Since neither MG nor TB could be applied for staining *P. espejiana* colonies, certain other basic dyes were tested for their absorption shift in the presence of denatured DNA (1.5 mg/ml). The following vital dyes were used as solutions in BAL 50-10 broth: brilliant cresyl violet, Nile blue A, neutral red, and Janus green [19]. TB and congo red (CR), an anionic vital dye, were used as controls. Except for Nile blue A, all the solutions changed coloration upon the addition of DNA. The change in color was visually more pronounced in the case of red dyes (neutral red and, unexpectedly, CR). On addition of denatured DNA, the CR absorption maximum shifted from 490 to 515 nm (batochrome effect). This spectral shift indicates the formation of a DNA–CR complex.

At low ionic strength (in 20 mM *Tris*-HCl buffer, pH 7.6), the DNA–CR complex was shown to form only at relatively high concentrations of Mg^{2+} or Ca^{2+}



A. espejiana colonies on agar with congo red: agar with BAL 50-10, DNA, and CR prepared as described in Materials and Methods, two days of incubation (*1*); the same plate on the third day of incubation (*2*); the same medium with 1 ml water substituted for DNA, two days of incubation (*3*); the medium containing 5 ml of agarized (2%) LCM instead of BAL 50-10, 1 ml of 2% aqueous DNA solution, 4 ml of CR solution (1 mg/ml) in 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.6, two days of incubation (*4*). Black rectangles on photos *1–3* are pieces of black paper used to facilitate automatic focusing.

(for native DNA, the spectral shift was detected at 2 mM; saturation occurred at 20 mM). When the ionic strength of the solution was sufficiently high (in 20 mM *Tris*-HCl buffer, pH 7.6 with 10 mM EDTA), it was possible for the complex to form even without bivalent cations (for native DNA, the spectral shift was detected at NaCl concentration above 0.1 M; saturation occurred at 0.3 M). These results will be presented elsewhere in more detail.

Published data exist concerning the DNA–CR complex formation under certain conditions. In the work [20], CR was shown to bind with phage T7 DNA and with poly-[d-(AT)] in the medium with *Tris*-HCl (10 mM), $MgCl_2$ (10 mM), and KCl (0.1 mM) at pH 7.9. The band of circular dichroism indicated the presence of the complex. The presence of magnesium in this medium is the only possible explanation for the existence of the complex under these conditions. The role of magnesium, however, has not been discussed in this work. In [21], the formation of complexes between native DNA and CR in the buffer with 0.1 M *Tris*-HCl was found to occur only in the presence of cetyl trimethylammonium bromide, a cationic detergent. The spectrum of resonance light-scattering on CR molecules was used as an indicator of complex formation.

In order to reveal the colonies secreting BAL nuclease, agar plates with the addition of DNA and CR were used (see Materials and Methods). The plates

Kinetics of CR coagulation in media of various composition

Experiment no.	Major medium components*	CR, μg/ml	λ_m, nm	A_0	A_m					
					Time after the preparation of the medium					
					1 h	12 h	1 day	2 days	3 days	4 days
1	BAL salts	20	490	0.84	0.49	0.06	0.02	–	–	–
2	BAL 50-10	20	490	0.84	0.50	0.46	0.43	0.40	0.38	0.32
3	agar, BAL salts	200	490	1.39	1.13	0.47	0.50	0.39	–	0.42
4	agar, BAL 50-10	200	490	1.39	1.24	0.51	0.41	0.42	–	0.35
5	agar, BAL 50-10	400	490	2.78	(2.1)	–	0.69	0.68	0.61	0.61
6	agar, DNA, BAL 50-10	400	520–530	2.78	(2.0)	–	1.20	1.14	0.98	0.94

Notes: A_m is the optical density at the maximum of CR absorption spectrum (λ_m). A_0 is the calculated A_m value for the aqueous CR solution of the same concentration and the same light path; the Beer law is assumed to be valid. Since the spectra readings for media 5 and 6 (1 h) were off-scale at around λ_m, the estimated A_m values are given in parentheses.

* Medium composition: 1. BAL 50-10 salts; *Tris*-HCl, 20 mM, pH 7.6; NaN_3, 2 mM. 2. BAL 50-10, NaN_3, 2 mM. 3. Medium 1 with 1.25% agar. 4. Medium 2 with 1.25% agar. 5. NaCl, 0.42 M; $MgSO_4$, 25 mM; $CaCl_2$, 5 mM, peptone, 4 g/l, agar, 1%. 6. Medium 5 with 2 mg/ml of denatured DNA.The absorption spectra of media 1 and 2 were determined against water in 1-cm cuvettes. Agarized media were dispensed into petri dishes (agar thickness, 1.65 mm). The dishes (uncovered) were then used to determine the spectra against air.

were inoculated with dilutions of the bacterial suspension and incubated for several days at 29–30°C.

The colonies of *P. espejiana* were found to form colonies on the medium with DNA and CR; congo red did not inhibit growth. *P. espejiana* colonies were stained with congo red. Binding of CR to bacterial cells usually indicates hydrophobicity of the cell wall; it is sometimes also the marker of pathogenitciy [22].

By the end of the first day, BAL nuclease-secreting *P. espejiana* colonies became discernible against the crimson-colored background due to the formation of surrounding pale pink halos. The halos increased in size with colony growth and merged at later stages of incubation (see Figure: photos 1 and 2 demonstrate the halos after two and three days of incubation). Three tests were applied in order to confirm the role of BAL nuclease secretion in the halo formation. No halos were formed on CR-containing agar without DNA (photo 3). Since BAL nuclease is irreversibly inactivated in the absence of calcium [5], *P. espejiana* was plated on a low-calcium medium (LCM) supplemented with DNA and CR. We have previously demonstrated that *P. espejiana* can grow on LCM, although the cell morphology changed (chains of rods and spherical cells emerged (see [14]); the complete results will be published later). The colonies on LCM agar did not develop halos after two days of incubation (see Figure, photo 4). *E. coli* AB 1157 was plated on BAL 50-10-based agar with DNA and CR; this strain can grow on this medium but does not secrete nuclease [8]. *E. coli* colonies were not surrounded by halos.

Bright halos, which are formed around BAL nuclease-secreting colonies of *P. espejiana*, are quite contrast against the dark crimson-colored background of the regions where DNA remained intact. Such halos certainly may be not the result of congo red absorption shift alone. Small dark particles can be discerned within the agar in the halos. The halos may therefore be formed as the result of precipitation (coagulation) of the dye in the zones where DNA was decomposed by the nuclease. Congo red has long been known to be a strongly aggregating (colloidal) dye [23, 24]. The high ionic strength, as well as high calcium and magnesium concentrations in the BAL 50-10 medium can significantly promote CR tendency for coagulation. Since the optical density (or, rather, extinction) of congo red in its absorption maximum (A_m) decreases with time due to coagulation, the kinetics of CR coagulation in solutions and agar gels of various composition has been determined (see Table).

In Bal 50-10 without peptone (experiment 1), an almost complete CR precipitation occurred after 12 h. In complete BAL 50-10 broth (experiment 2) with the same initial CR concentration, A_m decreased by 40% after three days. Thus, peptone stabilizes dissolved CR to a certain extent. Agar in the presence of salts also has a stabilizing effect (see experiments 1 and 3). Higher CR initial concentrations in BAL 50-10 broth (results not shown) or in BAL 50-10 agar resulted in decreases in 10 A_m to the level comparable with experiment 2 (experiments 4 and 5). The introduction of DNA had an additional stabilizing effect and shifted the absorption spectrum (experiment 6). The halos can be made more contrast due to the decrease in peptone concentration caused by peptone utilization by bacterial colonies. Bacteria, however, secrete proteins and other metabolic products into the medium; their effect on halo formation is difficult to assess. The results obtained indicate dye precipitation as the cause of halo formation.

The proposed method for visualization of nuclease-producing colonies can probably be applied to all marine bacteria and to halophilic bacteria as well.

ACKNOWLEDGMENTS

The authors are thankful to S.V. Vasil'eva for providing the strain *E. coli* AB1157.

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