PYOCYANIN FORMATION BY SOME NORMALLY APYOCYANOGENIC STRAINS OF *PSEUDOMONAS AERUGINOSA*

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Although several procedures (Haynes, J. Gen. Microbiol. **5**:939, 1951; Bühlmann, Vischer, and Bruhin, J. Bacteriol. **82**:787, 1961) have been suggested for the identification of apyocyanogenic strains of *Pseudomonas aeruginosa*, the demonstration of formation of pyocyanin is the most conclusive method of identification of this species. Hence, a number of media have been formulated to enhance pyocyanin production, i.e., Klinge's medium, (Arch. Mikrobiol. **33**:1, 1959). Pseudomonas Agar P (Difco Supplementary Literature, p. 281, 1962), and Sellers' medium (Sellers, Wynne, and Graber, Bacteriol. Proc., p. 129, 1961). Despite these media, there are still apyocyanogenic strains of *P. aeruginosa*.

We became concerned with pyocyanin production when a psychrophilic mutant of *P. aeruginosa* produced by ultraviolet irradiation (Azuma et al., J. Dairy Sci. **45**:1529, 1962) lost the ability to form pyocyanin on any of the above media after initially showing this ability. But the apyocyanogenic psychrophilic mutant recovered the ability to produce pyocyanin on Klinge's medium when it was previously grown in a medium consisting of 0.2% carbobenzoxy-DL-alanine, 0.9% DL-alanine, 0.5% glucose, 0.09% K_2HPO_4, 0.09% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.002% NaCl, and 0.002% $MnSO_4$, in distilled water for 4 days at 20 C and stored for 3 weeks at 5 C. This organism again became apyocyanogenic after three transfers on Klinge's medium or on Trypticase Soy Agar, but could consistently be made pyocyanogenic again by repeating the above procedure. The carbobenzoxy-DL-alanine could be replaced by carbobenzoxy-L-lysine or carbobenzoxy-DL-valine, but not by carbobenzoxychloride.

Six strains of *P. aeruginosa* known to be apyocyanogenic (Northern Regional Research Laboratory strains numbered B-7, B-12, B-241, B-275, B-282, and B-822) were supplied by W. C. Haynes. Each of these strains was grown for 4 days at 20 C in the medium described above, stored at 8.5 C in the same medium, and examined daily for the ability to form pyocyanin by culture at 30 C in Klinge's medium, Sellars medium, and Pseudomonas Agar P. None of these strains produced pyocyanin on either Sellers medium or Pseudomonas Agar P even after extended storage at low temperature. Strains B-241 and B-275, however, produced a blue pigment on Klinge's medium after storage for only 48 hr at 8.5 C. The blue pigment was confirmed to be pyocyanin by extraction with chloroform, the appearance of a red water-soluble pigment in the acidified aqueous layer and by paper chromatography (Frank and DeMoss, J. Bacteriol. **77**:776, 1959) compared with a known pyocyanin preparation.

The procedure described above increased the number of strains of *P. aeruginosa* that could be identified by pyocyanin formation. Also, the enhancement of the ability to form pyocyanin by growth in the presence of carbobenzoxy-amino acids suggests an interesting approach to the study of the mechanism of pyocyanin formation.

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PROTOCOL FOR MICRO ANTISTREPTOLYSIN O DETERMINATIONS

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This note gives a protocol for the adaption of the micro Plexiglas plate and wire diluting loop to antistreptolysin O determinations. The micro technique using Plexiglas plates and a wire loop

	WELL	1	2	3	4	5	6	7	8	9	10	11	12
Step 1:	Drops of ASO buffer (0.025 ml/drop)	See diag.	1	1	1	1	1	1	1	1	1	1	1
Step 2:	Loops of serum (0.025 ml/loop)	1	Make a 2-fold dilution of serum through 12 dilutions										
Step 3:	Drops of ASO buffer (0.025 ml/drop)	0	1	1	1	1	1	1	1	1	1	1	1
Step 4:	Drops of streptolysin O (0.025 ml/drop)	0	1	1	1	1	1	1	1	1	1	1	1
Step 5:	MIX by gentle bumping . . . . . Incubate at 37°C for 30 minutes . . . . . (Cover with scotch tape or acetate tape to prevent evaporation)												
Step 6:	Drops of 2% washed rabbit rbc (0.025 ml/drop)	0	1	1	1	1	1	1	1	1	1	1	1
Step 7:	COVER with scotch tape or acetate tape. . . . . Mix, incubate at 37°C for 45 minutes with mixing at 15-minute intervals. THOROUGH MIXING IS NECESSARY.												
Step 8:	TODD UNITS: Dilution #1 -------- 8 16 32 64 128 256 512 1024 2048 4096 8192 Dilution #2 -------- 12 24 48 96 192 384 768 1536 3072 7144 14288 REMOVE from incubator . . . . Centrifuge at 1500-2000 rpm for 3-5 minutes . . .												
Step 9:	READ the highest dilution that shows no hemolysis as the titer of the serum. The titer then is reported as Todd Units.												

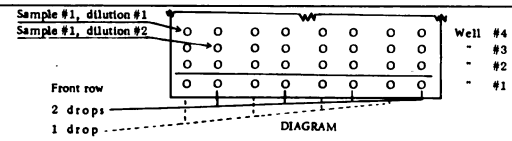

FIG. 1. *Micro antistreptolysin O protocol. Controls: serum control of a known titer, treated as an unknown serum. Hemolytic control: 2 drops (0.05 ml) of antistreptolysin O buffer, 1 drop (0.025 ml) of lysin, and 1 drop (0.025 ml) of 2% red blood cells. Cell control: 3 drops (0.075 ml) of antistreptolysin O buffer and 1 drop (0.025 ml) of 2% red blood cells. In step 2, each serum sample is diluted twice. One dilution starts at 1:2, and the second dilution starts at 1:3. This makes a dilution of each serum in 0.5 $\log_2$ increments. The front row is merely to make dilution and is not used in the test.*

(both available from Cooke Engineering Co., Alexandria, Va., or Intercontinental Scientific Corp., New York, N.Y.) as a diluting device has been adapted to several laboratory procedures for the determination of antibody levels (Takatsy, Acta Microbiol. Acad. Sci. Hung. **3**:191, 1955; Sever, J. Immunol. **88**:320, 1962; Rosenbaum, et al., Proc. Soc. Exptl. Biol. Med. **113**:224, 1963). Studies comparing the accuracy and reproducibility of the wire loop with the 0.2- and 1-ml pipettes have shown the loop to be equal to the pipette in making dilutions for the measurement of antibody (Edwards and Peck, Bureau Rept. MR 005.09-1300.1, Bureau of Medicine and Surgery, Navy Department, Washington, D.C., 1964). Although a number of micro techniques for the determination of the antistreptolysin O titer have been described (Crawford and Robinson, Am. J. Clin. Pathol. **24**:1103, 1954; Jablon and Saslow, Am. J. Clin. Pathol. **30**:83, 1958; Edwards and Heist, Public Health Lab. **20**:88, 1962), only one preliminary attempt (Rust et al., Bacteriol. Proc., p. 80, 1962) has been made to adapt the Plexiglas plate and wire diluting loop to the determination of antistreptolysin O titer.

In using the protocol (Fig. 1), a titration of an unknown serum from a dilution of 1:8 through 1:14, 288 at 0.5 $\log_2$ increments can be made with a minimal amount of reagents and effort. For these 22 tube dilutions (Fig. 1) in each serum titration, only 0.6 ml of streptolysin O reagent and rabbit or human red-cell suspension was used, as compared with 11 ml of streptolysin O reagent and rabbit or human red-cell suspension used in the standard procedure utilizing the same dilution scheme.

These savings, plus the several-fold increase in the number of determinations any one laboratory technician can perform in a day, make this microtechnique feasible for routine testing and mass screening. In our hands, the procedure is rapid, reproducible, and comparable to the conventional tube method of Rantz and Randall (Proc. Soc. Exptl. Biol. Med. **59**:22, 1945).

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