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NOTES

Phenotypic Recognition of Pyocyanine Mutants in *Pseudomonas aeruginosa*

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Certain classes of pyocyanine mutants in *Pseudomonas aeruginosa* escape detection when screened in the presence of wild-type or other mutant cells. A technique is described for recognizing mutant phenotypes after cells are in individual agar wells. The procedure eliminates cross-feeding phenomena as well as the masking of mutant phenotypes by diffusing pyocyanine produced by nearby clones.

The phenazine pigment, pyocyanine, of *Pseudomonas aeruginosa* is synthesized from chorismate, the major branch-point metabolite of aromatic amino acid biosynthesis (1, 4). Pyocyanine is a quantitatively substantial end product of aromatic synthesis. Hence, if the experimental system of biochemical genetics for aromatic biosynthesis is to be complete, mutant blocks for each enzyme of the pigment pathway branch are required.

Pyocyanine and its various phenazine precursors are water soluble and excreted into the agar medium, accumulating during growth. This presents the possibility that various pigment mutants may go unnoticed due to the presence of pigment or pigment precursors produced by surrounding clones being tested on the same plate. Indeed, this communication shows that some pigment mutants either (i) complement in the presence of wild-type clones to produce pyocyanine or (ii) exhibit a phenotype that is masked by the diffusing pyocyanine of wild type. Recognition of these pigment phenotypes depends upon the spatial separation of mutants on agar after mutagenesis.

The A medium of King et al. (3) provides excellent nutritional conditions for pigment formation. King medium was solidified with 1.5% agar (Difco) and was supplemented further with the following vitamins at a final concentration of 0.01%: thiamine, riboflavine, *p*-aminobenzoate, *p*-hydroxybenzoate, pyridoxine, folate, 2,3-dihydroxybenzoate, 3,4-dihydroxybenzaldehyde, nicotinic acid, pantothenate, choline, inositol, biotin, and vitamin K (all from Calbiochem or Sigma Chemical Co.). After autoclaving and aseptic addition of the vitamin supplement, a sterile 1.0-ml Cornwall syringe was used to deliver 0.2 ml of hot medium into individual wells of a sterile microtiter plate (Microtiter V plates, model 220-25A; Cooke Laboratory Products; Alexandria, Va.) The poured microtiter plates were dried at 37 C overnight. Cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (2) and plated at appropriate dilution on King agar medium for clonal isolation of survivors of the mutagenesis procedure. Individual isolates under test were transferred by means of a sterile toothpick to the separate wells of the microtiter plates. Mutant pigmentation was easily determined after overnight incubation at 37 C. Mutants were purified by repeated streaking of an isolated colony on blood agar base medium (BBL). The pigmentation of wild-type and mutant clones is extremely reproducible under these conditions.

Many of our newly isolated mutants exhibited pigment phenotypes that had not been noted in previous mutagenesis experiments in which about 50 survivors were routinely picked per agar plate. Reconstruction experiments were done to determine whether any mutants isolated by the microtiter plate technique would fail to display the mutant phenotype in the presence of diffusing pyocyanine and/or pyocyanine precursors from surrounding clones. About 25% (8 of 32) of the pyocyanine mutants isolated possessed a phenotype that was completely masked in the presence of diffusing pyocyanine

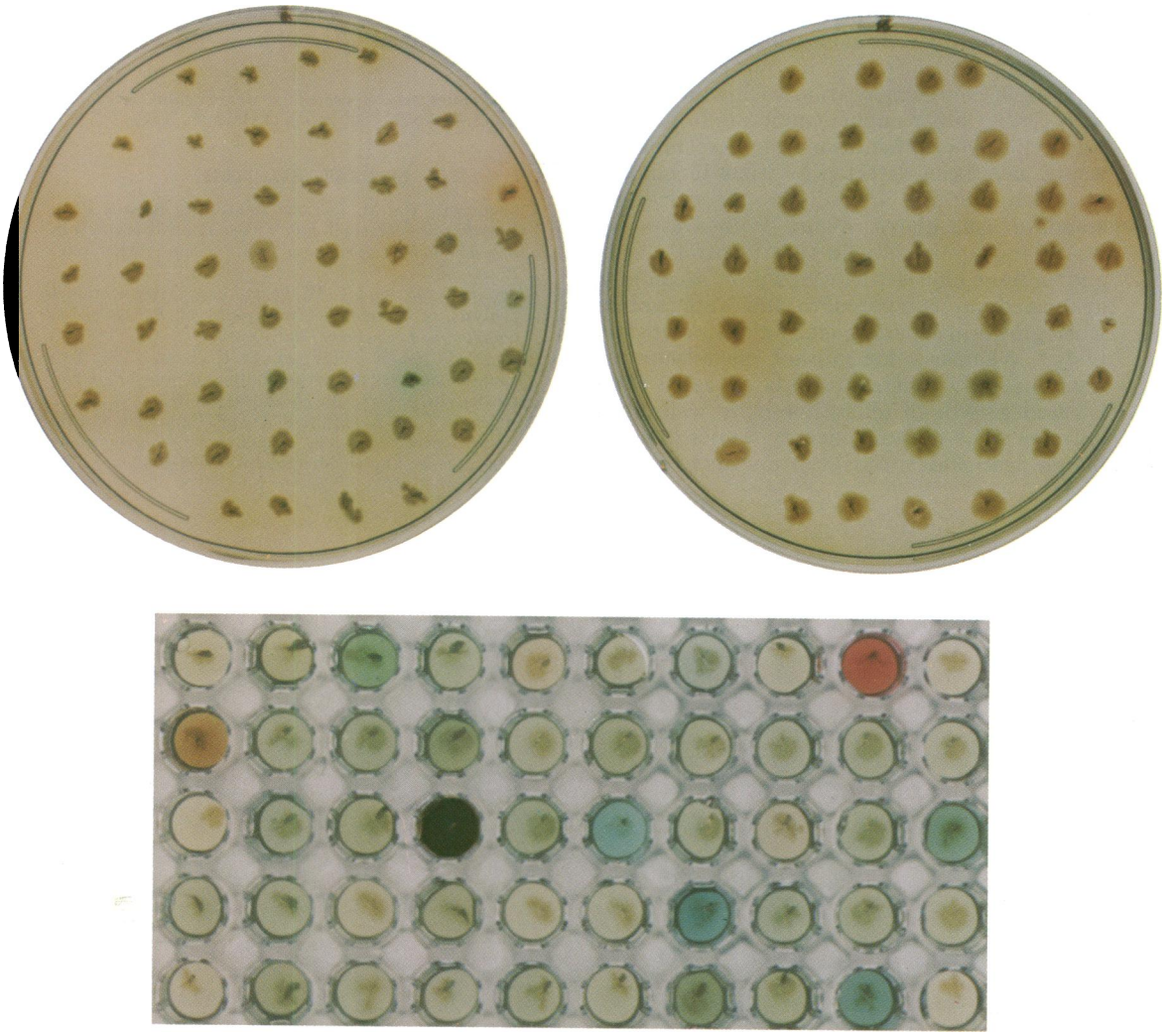

FIG. 1. *Comparison of pyocyanine mutant appearances when cloned individually or on a common agar surface. Twelve mutants and wild-type cells were seeded by means of sterile toothpicks to 52 equally spaced positions on the surface of a king A medium agar (3) plate and photographed after 24 h of growth at 37 C (upper left). It was again photographed after 48 h of growth (upper right). Mutant positions of various pigment mutants are identified by number from left to right and top to bottom as follows: (7) PGM 3, (9) PGM 8, (12) PGM 9, (18) PGM 12, (22) PGM 13, (24) PGM 20A, (28) PGM 23, (34) PGM 39, (38) PGM 53, (40) PGM 81, (43) PGM 135, and (51) PGM 147. All other positions are occupied by wild-type clones. The same mutants were grown on the agar surfaces of the microtiter plate wells for 24 h at 37 C (bottom). The well numbers corresponding to various pigment mutants are identified from left to right and top to bottom as follows: (3) PGM 3, (5) PGM 8, (7) PGM 9, (9) PGM 12, (11) PGM 20A, (24) PGM 23, (26) PGM 39, (28) PGM 13, (30) PGM 53, (37) PGM 81, (41) PGM 135, (49) PGM 147. All other positions are occupied by wild-type clones. Mutant pigment colors include reds, yellows, browns, and intermediate shades. Mutants displaying more intense blue or green coloration than the wild-type parent are commonly observed.*

produced by nearby wild-type clones when all were grown on a common agar plate. Each of three mutants currently in our collection is capable of complementation with wild type or with certain other pigment mutants to produce wild-type pyocyanine. None of these mutants would have been detected by using techniques in which mutants are initially recognized on standard agar plates. Figure 1 illustrates the visual appearance of wild type (control) clones and mutant clones when grown on a common agar medium compared to their appearance when grown in separate agar wells of microtiter plates. Whereas some mutant phenotypes are unambiguous, others are only revealed after isolated growth in microtiter plate wells. Not only are some mutant phenotypes not detectable on agar plates, but even mutants that are recognized after 24 h of growth are masked by diffusing pigment after 48 h.

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