HIGH RESOLUTION COLONY STAINING FOR THE DETECTION OF BACTERIAL GROWTH REQUIREMENT MUTANTS USING NAPHTHOL AZO-DYE TECHNIQUES

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Selective colony staining as a tool for distinguishing mutant bacterial colonies from wild type colonies has the advantage that it makes mutants directly and conveniently visible. Colony staining of fair selectivity has been described before only for carbohydrate nonfermenting mutant types (Eosin-Methylene Blue, Lederberg, 1947; tetrazolium salts, Lederberg, 1948). In this communication we describe a staining method which allows a visual distinction to be made between mutant colonies having any desired growth requirement or lacking the ability to ferment any desired carbohydrate or other energy source, and wild type colonies, provided the strain used can be induced for either alkaline phosphatase or β-galactosidase.

The staining reaction is based on the histochemical naphthol azo-dye technique for the localization of specific cell enzymes (cf. Burstone, 1962); α-naphthol compounds serving as substrates release free α-naphthol enzymatically, which is coupled in situ with diazonium salts to form insoluble azo-dyes. Levinthal (1961) was the first to apply this technique on bacterial colonies in staining selectively alkaline phosphatase positive colonies in fields of negative colonies in Escherichia coli using α-naphthol phosphate as substrate. Naphthol β-D-galactosides similarly allow differential azo-dye staining of lactose positive E. coli colonies (Vielmetter, unpublished). Because the dye does not diffuse but is localized within the colony, large numbers of colonies per plate can be screened.

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To utilize the high resolution of this technique for the selection of any type of growth requirement mutant, the fact is used that both alkaline phosphatase and β-galactosidase are inducible enzymes, the former being synthesized only at low external levels of inorganic phosphate (Torriani, 1959) and the latter only in the presence of inducing galactosides (cf. Monod, 1956). Either enzyme system can be used for the indication procedure in the following way: colonies are grown on "carrier" membranes resting on the agar surface of growth plates, allowing both mutants and wild type to grow. The carrier membranes are then transferred for some time onto starvation plates, still lacking growth requirements but providing conditions of induction for alkaline phosphatase or β-galactosidase. The enzyme is now formed only in colonies able to grow, but not in the starved mutant colonies. When treated with dye, the mutant colonies remain unstained while the wild type colonies stain normally.

Materials and Methods:

Media: TG: Tris-glucose medium (Echols et al., 1961). TG 0, TG 2 or TG 20 containing 0, 2 or 20 μg/ml P as orthophosphate. TL, TC: TG medium, with the glucose replaced by either 0.2% lactose or other carbohydrates. Tryp: 10 g tryptone, 9 g NaCl per liter. Tryp-L: Tryp with 0.2% lactose. For use in petri dishes the media are solidified with 1.2% Difco-Bacto-Agar. TRIS: 1 M Tris buffer, pH 8. NA: Difco Nutrient Agar. SA: 0.45% agar in 0.9% saline. SSA: 0.3% agar in H_2O.

Carrier membranes for colony transfer: Opaque: Black membrane filters MF 50 [1] or Millipore filters AABP [2], of 80 mm or 83 mm diameter fit in the usual 90 mm petri dishes. Filters are "ironed" onto the agar surface with a glass rod spreader, to soak them with the medium. Transparent: Cellophan

1) Membranfiltergesellschaft, 34 Göttingen, Germany.
2) Millipore Corp., Bedford, Mass., U.S.A.

PT 600 [3] discs are autoclaved immersed in SSA, removed from the hot agar by stripping off excess agar at the vessel edge and put on agar dishes, which are then dried for 12 hours by incubation at 37°C.

Plating and colony transfer: Bacteria are inoculated onto the petri dishes by pouring 1.5 ml of melted (45°C) SA containing a bacterial suspension. After solidifying, 1.5 ml SA without bacteria is poured on top of the first layer. The following plate types are used for different mutant selections: plating without carrier membranes on TG 2 for mutants lacking alkaline phosphatase (AP^-), on TG 20 for AP constitutives, on Tryp-L for mutants lacking β-galactosidase (lac^-), on Tryp for lac constitutives. For growth requirement mutants, the following petri dishes with carrier membranes are used. Growth plates: NA for any auxotrophs, TG 20 containing specific growth factors for restricted auxotroph classes. Starvation plates: TG 20 (or TC 20 for specific carbohydrate nonfermenting mutants). Induction plates: TG 0 inducing for alkaline phosphatase, TL 20 for β-galactosidase and TL 0 for both indicator enzymes simultaneously. Colonies are incubated on growth plates until a size of 0.1 - 0.5 mm diameter is reached, then the carrier membrane is transferred onto the starvation plate for 3.5 - 4 hours and then onto the induction plate for 3.5 - 4 hours. All incubations were at 37°C. Indicator staining (at room temperature): Substrates: NAP: 2 mg/ml naphthol AS-Cl phosphate [4] or naphthol AS-MX phosphate [5] in TRIS. NAG: 2 mg/ml bromo-naphthyl β-D-galactoside [4] or naphthol AS-LC β-D-galactoside [5] in H_2O. Substrates filtered sterile may be stored at -20°C for months. Azo-dyes: FB: 20 mg/ml Fast Blue RR salt [5][6] in H_2O. FR: 20 mg/ml Fast Red B salt [6] in H_2O. The solutions are stable for only 10 minutes at 4°C.

For alkaline phosphatase staining, petri dishes are rinsed with TRIS and then treated with a mixture of 4 ml NAP + 1 ml FB, or pretreated for 10

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3) Kalle and Co., 62 Wiesbaden-Biebrich, Germany.
4) Koch-Light Laboratories, Colnbrook, Bucks., England.
5) Sigma Corp., St. Louis 18, Missouri, U.S.A.
6) Serva Entwicklungslabor, 69 Heidelberg, Germany.

minutes with 5 ml NAP and then posttreated with 4 ml TRIS + 1 ml FR. For β-galactosidase staining, petri dishes are treated with a mixture of 4 ml NAG + 1 ml FB (or FR) + 0.5 ml TRIS (final pH of about 7). Substrate-dye mixtures are poured on the plates immediately.

The development of color is followed by observation on the cross-stage of a stereomicroscope with oblique incident illumination against a dark background and after sufficient staining (5 - 20 minutes), plates are rinsed with saline. Colonies may be isolated with glass capillaries (0.05 mm diameter).

Results and Discussion: The advantage of the described selection technique rests on its high resolution, allowing 2 x 10^4 colonies/plate for quantitative work and up to 10^5 colonies/plate for mutant isolation. The dye localization reaches beyond the cellular level: Fig. 1 shows that for phosphatase stained cells, the dye is distributed in a granular pattern over the cell surface, the size and number of granules depending on the degree of induction. Repressed or AP^- cells do not show any staining even when treated in dense pellets of AP^+ and AP^- cells. Therefore the method is useful for

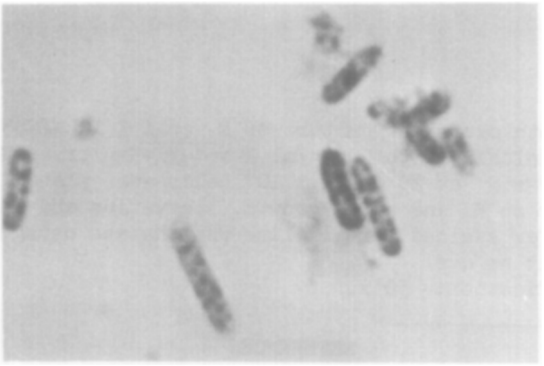

Fig. 1 TG 2 grown E. coli K 10 cells were treated in 1 mg/ml naphthol AS-MX phosphate plus 2 mg/ml Fast Blue RR salt diss. in 0.9% saline buffered with 0.1 M Tris buffer pH 8 for 45 min. at 25°C, washed and concentrated in 0.9% saline and mounted in glycerol gelatine.
Magnification: 2000 x.

visualizing details of mixed mutant colonies. This can be seen in the sectored colonies of auxotrophic mutants in Fig. 2.

The method has been applied successfully to the isolation of large numbers of various auxotrophic and carbohydrate nonfermenting mutants applying both indicator enzyme systems in E. coli strains B, C, ML and K 12 and Bacillus subtilis strains.

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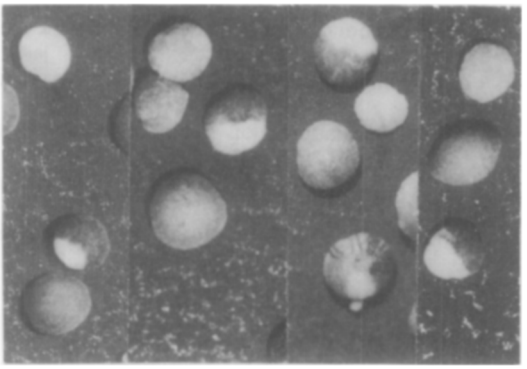

Fig. 2 Broth overnight culture of E. coli K 12 W1485 treated mutagenically with 20 μg/ml N-methyl-N-nitroso-N'-nitroguanidine for 80 min. at 20°C. 2 x 10^4 cells were plated on black MF 50 filter on NA and after growth, starvation and induction transfer stained for alkaline phosphatase using Fast Blue RR salt as dye.
Magnification: 50 x.

REFERENCES

Burstone, M. S., Enzyme Histochemistry, Academic Press, New York, 1962.
Echols, H., Garen, A., Garen, S., and Torriani, A., J. Mol. Biol., 3, 425 (1961).
Lederberg, J., Genetics, 32, 505 (1947).
Lederberg, J., J. Bacteriol., 56, 695 (1948).
Levinthal, C., personal communication (1961).
Monod, J., Enzymes: Units of Biological Structure and Function, Academic Press, New York, 1956, p. 7.
Torriani, A.M., Biochim. Biophys. Acta, 38, 460 (1959).