NOTES

Preliminary Observations on the Application of the Multiple Inocula (Replicator) Method for Carbon Substrate Utilization Studies of *Alcaligenes faecalis*

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Received for publication 15 December 1976

The use of the replicator technique in evaluating carbon source utilization by 55 cultures of *Alcaligenes faecalis* was examined. Six of the 20 substrates tested were utilized by the bacterial cultures. In this study, the reproducibility of the technique was 100%.

The identification of nonfermentative microorganisms is one of the primary concerns of the clinical microbiology laboratory. Hugh and Leifson (6), with the introduction of their oxidation-fermentation (OF) medium, provided an extremely useful technique, which facilitated the characterization of these bacteria. Currently, determining the ability to oxidize carbohydrates (produce acid oxidatively) forms an integral part of all schema for identifying gram-negative bacteria. Some of these organisms, however, do not detectably oxidize carbohydrates. They also demonstrate so few positive reactions in conventional tests that their identification is often incomplete and unsatisfactory. Moreover, problems with acid detection in media containing peptone have been noted by Park (7) and Snell and LaPage (9). They indicated that the oxidative production of acids from carbohydrates can occur in peptone-containing media without any corresponding indicator change.

In view of these problems, we decided to use the carbon substrate utilization (CSU) technique as an alternate system of evaluation. Stanier et al. (10) had success in differentiating *Pseudomonas* species by adapting the replica-plating technique to their needs in substrate utilization. Pinter and Kantor (8) also used CSU patterns in their study of the nonoxidative microorganisms *Alcaligenes faecalis* and *A. odorans* var. *viridans*. They incorporated the substrates in broth medium and determined utilization by comparing, with the naked eye, the amount of cell turbidity in tubes supplemented with substrate to that in control tubes. Both of these methods proved to be limited in

usage. Difficulty was experienced with the replica-plating technique in determining whether a substrate had stimulated growth. The broth tube method was not useful either in terms of accuracy or in dealing with mucoid strains.

Thinking that the reproducibility and accuracy of these techniques did not fulfill our expectations, we decided to investigate the apparatus that Fuchs (3, 4) and Chadwick et al. (1) used in biotyping bacteria, the Steers replicator (11). With it, they performed a number of biochemical tests very rapidly and with a high degree of accuracy. This report concerns the determination of the efficacy of the replicator plate method of Fuchs (3) in studying the CSU patterns of the nonoxidative microorganism *A. faecalis*.

The bacteria used in this study were isolated from clinical specimens. These cultures were taken from the stock culture collection of the Special Bacteriology Section at the Center for Disease Control. They were characterized and identified by the procedures of Weaver et al. (12).

The basal salts medium was prepared as described by Cohen-Bazire et al. (2), with some modifications. The solution of ammonium-D,L-malate was not included in the preparation. All of the substrate solutions were adjusted to pH 7 and filter-sterilized before being incorporated into the basal salts medium. The final substrate concentrations were at the rate indicated by Stanier et al. (10), 0.2% for carbohydrates and 0.1% for all other substrates.

Cultures were inoculated onto tryptone-glucose-yeast extract agar (5) slants and incubated at 35°C for 18 to 24 h. The cellular growth was

washed off the slants with sterile basal salts solution, which contained neither the added substrate nor Casamino Acids. After the cellular growth was suspended, the optical density of the suspensions was adjusted to 0.35 to 0.40 at a wavelength of 550 nm. These suspensions were then diluted 1/10 in sterile basal salts solution before being used to fill the replicator seed plate. After being dried, the substrate plates were inoculated in duplicate with the replicator. The inoculum on each plate's surface was allowed to dry before incubation at 35°C for 48 h. Four separate trials were performed on each substrate.

The *A. faecalis* bacteria proved to be a better test for the system than we had previously envisioned. This group included nonoxidative gram-negative rods, which were practically inert on routine test media (12); furthermore, about 20 to 25% of the cultures were also very motile and spread over the entire surface of the plate. This caused contamination among the cultures. This effect was obviated by reducing the concentration of the Casamino Acids in the basal medium from 0.50 to 0.25%.

The amount of growth visualized on the substrate plates is illustrated in Fig. 1. Stimulation of growth of the cultures by a particular substrate (Fig. 1A) can be easily distinguished from the blank plate (Fig. 1B), which was identical except that the substrate was absent. Conversely, where there was no stimulation of cellular growth, the growth on the substrate plate

was equal to or perhaps less than that on the blank plate (Fig. 1C). Moreover, in some instances, cellular growth was inhibited, as indicated by the plate supplemented with cetrимide as the substrate (Fig. 1D). Positive reactions were recorded only when there was obvious growth stimulation within 48 h after inoculation. About 80% of the substrates recorded as being utilized were detectable after 24 h of incubation, whereas the remaining 20% required an additional 24 h for growth enhancement.

The CSU patterns of the *A. faecalis* cultures were examined after an incubation period of 48 h (Table 1). Six of the substrates, glutamate, histidine, γ -amino-*n*-butyrate, L-malate, proline, and phenylalanine, were utilized by more than 90% of the cultures. Azelate was utilized by 23 cultures. Except for butyramide and ethanolamine being used by one culture, the remainder of the substrates were not utilized.

The observations made in this study support the previous comments as to the rapidity and accuracy of the replicator system (1, 3, 4). Of 4,400 recordings, we noted no variance in the results. Thus, the accuracy or reproducibility rate was 100%. The 55 bacterial cultures were inoculated onto 20 substrate media, in duplicate, in less than 30 min. The process, from the point of harvesting the organisms off the agar slants until the substrate had been inoculated, required approximately 1.5 h.

From these results we believe that the replicator technique provides a quick and reproduc-

FIG. 1. Comparison of the types of reactions observed with the *A. faecalis* cultures on substrate plates during CSU evaluation. All of the plates were incubated at 35°C for 48 h. (A) Stimulation of bacterial growth on glutamate substrate agar. (B) Growth on the blank plate (contains no added substrate) which served as a growth control. (C) Growth on the plate containing maltose, a substrate that was not utilized by the cultures. (D) Inhibition of cellular growth by the cetrимide substrate plate.

TABLE 1. Carbon source utilization profile of *A. faecalis*

Substrate	<i>A. faecalis</i> ^a	
	Sign	% +
Azelate	±	31
Butyramide	—	2
Cetrimide	—	0
Creatine	—	0
Glucose	—	0
Ethanolamine	—	2
Glutamate	+	100
Fucose	—	0
Histidine	+	100
γ-Amino- <i>n</i> -butyrate	+	94
Isophthalate	—	0
Maltose	—	0
L-Malate	+	100
Methionine	—	0
Hydroxy-L-proline	—	0
Proline	+	100
Phenylalanine	+	100
Ribose	—	0
Sarcosine	—	0
Suberate	—	0

^a Sign: +, 90% or more positive; —, 90% or more negative; ±, 50% or more negative.

ble approach to evaluating CSU profiles of various groups of bacteria. Its main forte, however, lies in the area of screening substrates to find those which may be indicators for a particular group. For this reason, the research laboratory and not the clinical laboratory will find the replicator most beneficial.

LITERATURE CITED

1. Chadwick, P., G. J. Delisle, and M. Byer. 1974. Biochemical identification of hospital enterobacteria by replica agar plating. *Can. J. Microbiol.* 20:1653-1664.
2. Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell Comp. Physiol.* 49:25-68.
3. Fuchs, P. C. 1975. The multiple inocula (replicator) method for bacterial speciation and biotyping, p. 70-125. *ASCP Technical Information Service* no. 22.
4. Fuchs, P. C. 1975. The replicator method for identification and biotyping of common bacterial isolates. *Lab. Med.* 6:6-11.
5. Haynes, W. C. 1951. *Pseudomonas aeruginosa*—its characterization and identification. *J. Gen. Microbiol.* 5:939-950.
6. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bacteriol.* 66:24-26.
7. Park, R. W. A. 1967. A comparison of two methods for detecting attack on glucose by pseudomonads and achromobacters. *J. Gen. Microbiol.* 46:355-360.
8. Pinter, M., and M. Kantor. 1974. Comparison of *Alcaligenes faecalis* and *Alcaligenes odorans* var. *viridans* by carbon source utilization test. *Acta Microbiol. Acad. Sci. Hung.* 21:293-295.
9. Snell, J. J. S., and S. P. LaPage. 1971. Comparison of four methods for demonstrating glucose breakdown by bacteria. *J. Gen. Microbiol.* 68:221-225.
10. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* 43:159-271.
11. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiot. Chemother.* 9:307-311.
12. Weaver, R. E., H. W. Tatum, and D. G. Hollis. 1972. The identification of unusual pathogenic gram negative bacteria (Elizabeth O. King). Center for Disease Control, Atlanta.