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Maintenance Procedures for the Curtailment of Genetic Instability: *Xanthomonas campestris* NRRL B-1459

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Characteristics are described of small-colony variants of *Xanthomonas campestris* NRRL B-1459 which are frequently encountered when routine culture maintenance procedures are employed. In contrast to the parental type, small-colony variants were shown to be resistant to a number of antibiotics, to acridine orange, and to phage which are virulent for the parent colony type. Sensitivity to ultraviolet radiation was similar in both colony types. A simple method for preservation of viable cells is described. The suitability of the method for providing reproducible inocula free from variant cell types is examined.

Frequently, maintenance of viable inocula by repeated culture is unsatisfactory for the preservation of desired genetic characteristics. To a certain extent such difficulty may be overcome by either storage at very low temperatures or lyophilization (3, 4, 7, 8). However, in some cases such measures are not feasible or are unnecessarily costly or troublesome. Lyophilization is a common compromise in balancing cost and convenience against preservation of desired genetic characteristics. This provides a preserved stock that can be drawn upon periodically for inocula assumed to possess the original genetic constitution. However, even this procedure is not entirely satisfactory with an organism subject to considerable genetic instability. It has been known that spontaneous mutation is related to the growth of bacteria since the classic paper of Luria and Delbrück (9), which demonstrated that mutations were spontaneous and occurred at random. Thus, it can be readily deduced that maintenance procedures which depend upon semi-continuous propagation must increase the possibility of altered cultural characteristics by a factor directly proportional to the number of generations involved. The same argument applies to the buildup of inocula for large-scale fermentor inoculations.

With the above principles in mind, we have examined alternative procedures for maintenance, propagation, and inoculum buildup of *Xanthomonas campestris* NRRL B-1459. The present report is of special significance to the use of this particular organism because of its importance in the production of polysaccharide for use in food and industrial applications (1, 5) and its known capacity to produce variant colony types (1, 11). However, the principles discussed are of general significance to procedures appropriate to industrial microbiology. We also report some of the characteristics of small-colony variants of strain NRRL B-1459.

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MATERIALS AND METHODS

Organisms. The parent culture of *X. campestris* NRRL B-1459 normally produces only large (L) mucoid colonies on nutrient agar plates. After repeated culture, two other types of colonies develop on the plates: an intermediate size (previously designated B-1459 S_m [1]) and a smaller size (S) (8). When observed on YM agar (3) plates after incubation for 48 h at 25°C, the diameters of these colonies are 4 mm for L, 2 mm for S_m, and 1 mm for S. L-type colonies are light yellow in color; S_m and S types are darker yellow. These colony types differ also in polysaccharide production (1): L (mucoid) produces an abundance of normal polysaccharide, S_m (mucoid) produces an abnormal polysaccharide in lesser amounts, and S (nonmucoid) produces none. L and S_m colonies have been investigated previously by Cadmus et al. (1).

Media and cultivation methods. For plate counts, antibiotic susceptibility testing, maintenance, and inoculum buildup for polysaccharide production, a standard YM medium (3) either with or without 2% agar was used. The medium components were as follows (g/liter): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; glucose, 10.0. Water was added to give a final volume of 1,000 ml. Autoclaving was performed at 121°C for 15 min. The medium pH after autoclaving was approximately 7.0. For acridine and ethidium sensitivity testing, this same medium was employed except that the pH was adjusted to pH

7.6 with sodium hydroxide after autoclaving. All additives to the basal medium were filter-sterilized. Polysaccharide production was examined either in Fernbach flasks or in a 14-liter Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) by use of a medium containing distillers' solubles (1).

The inoculation schedule for polysaccharide production was as follows when paper-strip inoculum was used. A single paper strip was aseptically transferred, by use of forceps, to 7 ml of YM broth in an 18-mm tube. After being shaken at an inclined angle (45°) at 28°C for 24 h, the entire culture was used to inoculate 43 ml of YM broth in a 300-ml Erlenmeyer flask which was similarly incubated but without inclination. As many flasks were used as were required to provide a 10% inoculum for the final fermentation.

The inoculation schedule with inoculum grown on solid medium employed conditions for buildup of inocula and batch fermentation based on those used at the Northern Regional Research Center (6). Slants of 5 ml of YM medium were grown at 25°C for 16 h and then stored at 4 to 6°C for up to 14 days. Similar slants were then inoculated from these stored cultures and incubated at 25°C for 24 h. A single loop, constructed of 20-gauge wire with an inner diameter of 3 mm, of the latter culture was used to inoculate 7 ml of YM broth. The remainder of the schedule was identical to that described in the previous paragraph.

Final fermentations were conducted as follows. Fernbach flasks were incubated at 28°C for 48 h on a gyratory shaker with a 2-inch (5-cm) throw at 250 rpm. The stirred fermentor was operated in either batch or continuous modes with the conditions used by Silman and Rogovin (10). For polysaccharide production under batch conditions, the medium indicated was used. However, for continuous culture, the urea-containing medium of Silman and Rogovin (10) was employed.

Drug sensitivity. Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide, B grade, Calbiochem) was made to 10^{-5} M in either YM broth or solid medium adjusted to pH 7.6. Acridine orange (3,6-bis-dimethylaminoacridinium chloride, practical grade, Sigma Chemical Co.) was similarly prepared but was initially tested at 10^{-4} M. Because of the sensitivity observed for acridine orange, a highly purified preparation was also tested but was found to give results identical to the practical grade initially used. Sensitivity testing was performed either in 2-ml culture volumes contained in 18-mm tubes, shaken at an inclined angle (45°) at 28°C, or in solid medium at 28°C onto which had been spread 0.1 ml of a suitable dilution, in saline, of a 16-h broth culture.

Antibiotic susceptibility. Plates containing YM medium were spread with a 16-h culture to yield confluent growth. Difco sensitivity disks were added and inhibition zones were measured after 12, 24, and 36 h at 28°C.

UV sensitivity. Sixteen-hour cultures were washed and then resuspended to yield about 10^8 cells/ml in the following buffer (quantities are in g/liter and the components were dissolved in the order shown): Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 0.2. Ultraviolet (UV) irradiation was performed with a Sylvania germicidal lamp (model G15T8). Glass petri dishes containing 7 ml of the cell suspension were placed on a gently gyrating shaker at a distance of 41 cm from the lamp tube. After irradiation, diluted suspensions were plated as above for counting with as low a light level as possible used for all manipulations.

Bacteriophage sensitivity. Phages were isolated from raw sewage and tested at appropriate dilutions in 0.75% agar overlay plates at 28°C.

Polysaccharide production. Polysaccharide production was estimated by measuring viscosity with a Brookfield model LVT viscometer at 30 rpm (10).

Preservation of inoculum. Preliminary testing of adsorbent materials such as glass beads, polyethylene beads, polystyrene beads, and paper eliminated all materials but the thickest paper on the basis of the viable cell numbers which could be recovered from each material. Extensive testing was performed with Whatman 3M chromatography paper cut into strips 2.0 by 0.125 inch (5 by 0.3 cm). Fifty or more such strips fit comfortably into a small (2.5 cm in diameter, 10.5 cm high) screw-top bottle. The paper strips were first soaked overnight in 20 mM disodium ethylenediaminetetraacetic acid, as a precaution against inhibitory metals, washed in several changes of deionized water, and oven-dried at 100°C. The dry strips contained in the loosely capped bottle were sterilized at 121°C for 15 min. Suitable screw-top bottles were packed with desiccant (Zeolite molecular sieve beads, 0.4 nm pore size, 8 to 12 mesh, Davison Chemicals, Baltimore, Md.) which was covered with a layer of glass wool that had been washed and dried in the same manner as the paper strips. These bottles were then sterilized, oven-dried at 110°C, and stored over a desiccant ($CaSO_4$). Paper strips were loaded with a large number of viable cells by immersion in either log-phase (10-h) or stationary-phase (24-h) YM broth cultures. Excess culture not absorbed by strips was withdrawn by pipette, and the strips were transferred to the desiccant bottles. Slow drying of strips was effected by simply standing the bottles over $CaSO_4$ for 24 h at ambient pressure and a temperature of 20°C. Rapid drying, actually freeze-drying but not from prefrozen materials, was performed under vacuum by use of a dry ice/ethanol cold trap. The dried samples were then stored at 6°C, with caps tightly closed, and viability counts were regularly performed. It should not be overlooked that not only are the means of drying cells different but probably the degree of dryness finally achieved will also be different. Indeed, there is indirect evidence gained in this laboratory (unpublished data) which suggests that cells dried by the slow method reported in this paper should contain considerable free water. Strips were blended in a Vortex Jr. Mixer (Scientific Industries, Inc.) in 5 ml of 0.85% saline dilution blanks for 20 s to resuspend adsorbed cells.

Our objective was to achieve the simplest possible procedure for supplying a level of inoculum comparable to that obtained with the traditional loop

transfer method. The minimal objective for preservation of high viability was 3 months; this corresponded to the time at which laboratory cultures, maintained by culture transfer every 14 days, are usually discarded in favor of fresh inocula from lyophilized stocks (6).

Quantitative investigation of conventional inoculation practice. To make any reasonable comparisons of nonpropagative maintenance procedures with conventional maintenance by culture transfer, it was necessary to determine the numbers of viable cells transferred by loop from slant cultures of various ages. It was also necessary to determine the minimal average number of generations occurring in slant cultures. Therefore, viable counts were performed as above for standard bacteriological loops and for 5-ml YM slants prepared in 18-mm tubes and inoculated in the conventional fashion. Cells were resuspended from slants by gently blending in a Vortex mixer after flooding with 5.0 ml of saline.

RESULTS AND DISCUSSION

Drug sensitivity. Cultures grown for 16 h in the presence of acridine orange which had been previously subjected to a number of transfers, and hence contained a number of S_m and S colonies in addition to the predominant L type, yielded cultures containing exclusively S colonies. This effect was not shown by ethidium bromide. It was subsequently established that low levels of acridine orange killed the L and S_m types preferentially (Fig. 1). The minimal inhibitory concentrations of acridine orange were found to be 2 and 50 μg/ml for the L and S types, respectively. One useful application of this finding was the testing of cultures for significant contamination by S colonies. Visible growth after overnight culture in the presence of 10 μg of acridine orange/ml was found to be a fairly sensitive indication of the presence of S-type cells. Neither acridine orange nor ethidium bromide was observed to cause interconversion of S and L types even at high rates of kill.

Antibiotic sensitivity. Of the antibiotics tested, only kanamycin was effective against the S colony type (Table 1). None of the other antibiotics produced even a small zone of inhibition. The L colony type was susceptible to a number of antibiotics, particularly to erythromycin and tetracycline, which produced zones of inhibition in excess of 2.5 cm.

Phage sensitivity. Phages forming a variety of plaque types were obtained by incubation of raw sewage filtrate with the L colony type. None of these phages produced plaques in the S colony type. One of the phages, P-14, which produced a large, clear entire plaque, was purified by repeated subculture and tested against a range of L and S isolates. In all instances, the L type was lysed but in no case was the S type lysed.

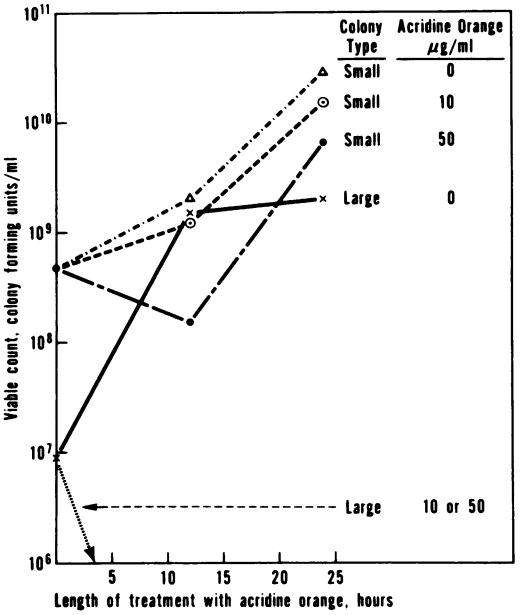

FIG. 1. *Influence of high and low acridine orange concentrations on viability of small (S) and parental large (L) colony types of X. campestris NRRL B-1459. After treatment with acridine orange, cultures were plated on YM agar, and viable-cell counts were made after 24 and 48 h of incubation at 25°C. Counts for L colonies treated with acridine orange concentrations of 10 and 50 μg/ml are below limits of scale on the graph.*

TABLE 1. *Antibiotic susceptibility of small and large colony types of X. campestris NRRL B-1459*

Antibiotic	Susceptibility[a]	
	Large colony	Small colony
Penicillin G, 10 U[b]	S (2.0)	R (0.0)
Erythromycin, 15 μg	S (2.6)	R (0.0)
Ampicillin, 10 μg	S (1.8)	R (0.0)
Nitrofurantoin, 300 μg ..	R (0.0)	R (0.0)
Kanamycin, 30 μg	S (1.6)	S (1.6)
Sulfisoxazole, 300 μg	S (1.0)	R (0.0)
Chloramphenicol, 30 μg .	S (1.4)	R (0.0)
Tetracycline, 30 μg	S (2.7)	R (0.0)
Bacitracin, 2 U	R (0.0)	R (0.0)
Nafcillin, 1 μg	R (0.0)	R (0.0)

[a] S = Sensitive; R = resistant. Inhibition zone diameters in centimeters are given in parentheses.
[b] Quantity per disk.

UV sensitivity. Both L and S colonies showed sensitivity to UV irradiation, especially at high doses. An 80-s dose under experimental conditions gave an identical percentage survival (0.005%) for both colony types (Fig. 2). How-

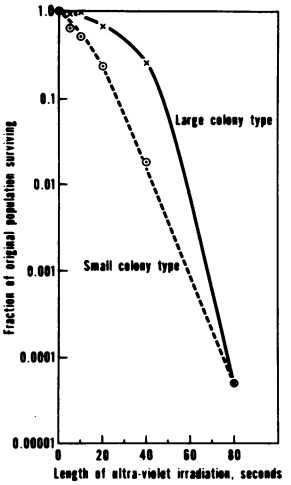

FIG. 2. *Survival of S and L colony types of X. campestris NRRL B-1459 as a function of exposure time to ultraviolet irradiation. After irradiation, cultures were plated on YM agar and colonies of survivors were counted after 40 and 60 h of incubation at 25°C.*

ever, the L colony type does appear to have a slight plateau in the low dose range which is essentially absent from the S type. Several generally known factors that influence UV sensitivity have been discussed elsewhere (2). In no case was interconversion of colony type observed.

Correlated characteristics of S colony type. The absence of polysaccharide production in S variants is correlated with failure to lyse with phages virulent for the L type and with resistance to acridine orange and a number of antibiotics. No other obvious differences between the L and these S variants have been found. However, Sutton and Williams (11) reported immunological differences and loss of virulence in what would appear to be (Sutton, personal communication) similar S variants. Preliminary nutritional studies indicated similar dependencies upon added nutrients of both L and S colonies. It has also been established that the intermediate colony size variants S_m (1), which produce abnormal polysaccharide, share the acridine orange, antibiotic, and phage sensitivity of the L type. An altered cell envelope in the S colonies could account for the resistance displayed towards chemicals and phages and is compatible with the other findings. It is equally possible that these variants may harbor a temperate phage which could prevent further infection. Indeed, in one S-colony isolate, tiny plaques were occasionally observed, but these could not be demonstrated in any of the other variants when either acridine or raised-temperature induction was used.

Preservation of inoculum. Investigation of the variables, growth phase, and drying procedure (Fig. 3) indicated that viability was very rapidly lost in cells which were freeze-dried on paper strips. Cells subjected to slow drying, at room temperature, showed sustained viability. The addition of cryoprotective agents might have diminished the damaging effect of rapid drying, but it was the intention of this study to examine the simplest possible procedure for preservation. It was also evident that log-phase cells retained viability much better than did stationary-phase cells.

To assess the applicability of this preservation method to polysaccharide production, it was necessary to demonstrate that effective inocula could be derived from paper strips stored in excess of 3 months. Essentially two criteria were used for this purpose: (i) freedom from S-colony variants in the final fermentation and (ii) polysaccharide production at least equal to that obtained by the best available alternative inoculation scheme. The latter requirement was confirmed by culture both in Fernbach flasks and in a stirred fermentor. Viscosities in excess of 6,000 centipoises and equal to or exceeding control inoculum viscosities were reached within 48 h in each case. Continuous culture was also carried out for 5 days to see whether such additional selection pressure would lead to S-colony variants. No S-type cells

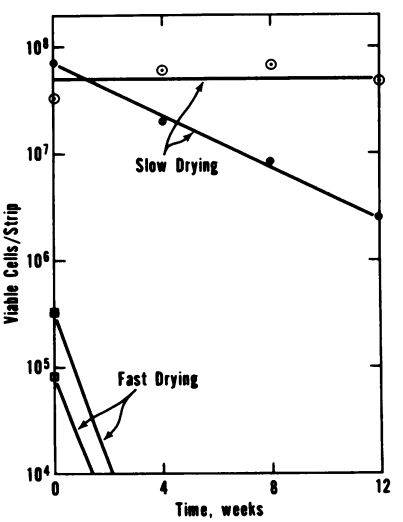

FIG. 3. *Survival of L colony type X. campestris NRRL B-1459 on dried paper strips as a function of culture age and speed of drying. Stationary phase: rapid drying, ■; slow drying, ●. Logarithmic phase: rapid drying, □; slow drying, ⊙.*

were observed. Since it has been demonstrated that the conventional scheme for culture maintenance produces an inoculum containing S-type cells (1) and that their numbers are further increased by the continuous culture procedure used in the present study (10), no further comparison was attempted. These observations, although of limited extent, indicate that preserving cells in this manner produced a viable inoculum which showed no tendency to degenerate during the course of a normal fermentation.

Quantitative investigation of conventional inoculation practices. Although it is often sufficient to establish some essentially empirical procedure such as that described above, it is also of interest to be able to suggest why such a procedure should be better than others. To this end, an examination of the previous method of culture maintenance and inoculation schedule was conducted.

The previous method consisted of routine transfers onto solid medium every 2 weeks. Cultures were incubated overnight at 25°C and then stored at 4°C. Preinoculum was obtained by further culture on solid medium as required. After several weeks, or whenever the cultures showed evidence of deterioration, fresh inoculum was obtained from lyophilized stocks (6).

The numbers of cells involved in the various manipulations are shown in Table 2. As a reasonable approximation it may be stated that the standardized loop transfers an inoculum of 5×10^8 cells from a slope which contains 10^{10} cells. Thus, each maintenance transfer involves in excess of four generations. Added to this is the effect of additional transfer to provide preinoculum. Even a fresh lyophilized stock culture will have undergone some 10 to 12 generations by the preinoculum stage, and a culture maintained by transfer over a 12-week period will have undergone in excess of 30 generations.

It can readily be seen that, irrespective of the rate of appearance of variants due to mutation and the influence of selection, the probability of culture degeneration will be directly proportional to the number of generations through which the culture has been grown. An inoculum derived from six maintenance transfers may be expected to harbor about 20 times the number of variants found in a fresh culture. This calculation is, of course, based upon the present example in which maintenance involves multiplication through not less than four generations with transfer of about 1 cell in 20 at each subculture. The calculation assumes no selective advantage of these variants.

TABLE 2. *Numbers of viable cells recovered from loops and slants of X. campestris NRRL B-1459*

Time period (h)		Viable cells[a]	
Culture	Storage	Loop	Slant
16	0	ND[b]	8.1×10^9
24	0	4.5×10^8	8.6×10^9
16	336	7.3×10^8	ND

[a] Independent counts of each of four replicates gave counts within a range of ±25%. Mean values are given.

[b] Not determined.

When such variants possess a significant competitive advantage, such as a higher growth rate, which appears to be the case with the S-colony variants, serious culture deterioration may occur with great rapidity.

In conclusion, it is suggested that serious consideration should be given to substituting nonpropagative maintenance techniques whenever this is possible. The usually small investment in effort required to establish a particular preservation schedule will be amply repaid by the possession of a very reliable inoculum. The specific preservation procedure used in the current study has much to recommend it in terms of cost, speed, simplicity, and reliability.

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