Methods of Preservation of Corynebacterium insidiosum Isolates in Relation to Virulence and Colony Appearance on a Tetrazolium Chloride Medium

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Contribution No. 609, Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication March 26, 1971, as Journal Series Paper No. 3945.

Accepted for publication 6 July 1971.

ABSTRACT

Three major colony types were detected when dilutions from single-cell isolates of *Corynebacterium insidiosum*, maintained for 1 year by three different methods, were streaked on a 2, 3, 5-triphenyl tetrazolium chloride medium. These were (i) a large butyrous colony with deep red center and a smooth, wide, white border; (ii) an intermediate pink-to-white colony sometimes showing a small, dark pink center and somewhat irregular border; and (iii) a small, green, round-to-oblong colony. Tests conducted on DuPuits alfalfa seedlings, grown under sterile conditions, showed that isolates derived from the pink-type colony were highly virulent, whereas those derived from the red- and green-type colonies were avirulent. A relationship was found between the method of storage and type of colonies produced on the tetrazolium chloride medium. A much higher percentage of the virulent pink colonies were recovered from isolates maintained in a sterile soil mix than from the same isolates maintained in sterile distilled water and on agar. Phytopathology 61: 1423-1425.

The vital dye, 2,3,5-triphenyl tetrazolium chloride (TTC), has been used in solid media for the detection of biochemical mutants and morphological variants of certain bacteria (9, 10). It was also reported to be highly useful in detecting colony variation in cultures of eight genera of bacteria (6). The mode of action of TTC in living cells has been described by Smith (13).

Kelman (7) employed a tetrazolium medium to detect mutants of *Pseudomonas solanacearum* differing in colony morphology from the normal type, and he related colony appearance on this medium to pathogenicity. Likewise, Smale & Worley (12) found that tetrazolium incorporated into potato-dextrose agar (PDA) was useful in separating highly pathogenic colonies from moderately and weakly pathogenic colonies of *Pseudomonas phaseolicola* obtained from stock cultures, although this relationship did not exist for isolates of *Xanthomonas phaseoli*. However, the medium was useful in obtaining highly pathogenic colonies of both bean pathogens when freshly isolated from infected plants. Friedman (4) also used tetrazolium in agar, along with carbon source, to separate virulent from weakly virulent colonies in two isolates of *E. carotovora* obtained from stock cultures or from infected host tissue. By contrast, Bordewick (1) states that seven highly virulent and four weakly virulent cultures of *C. insidiosum* all formed the same type of colonies on a TTC medium.

In a previous study (2), three methods of preservation of 45 single-cell isolates of *Corynebacterium insidiosum* (McCull.) H. L. Jens., causal agent of bacterial wilt of alfalfa, were compared. Results showed that the isolates persisted and remained virulent during a 1-year period when stored in a sterile soil mix at 4 or 21 C. This was in marked contrast to results for the same isolates maintained in sterile distilled water or by periodic transfer on beef-lactose agar (BLA).

A preliminary experiment indicated that three different colony types could be detected when the single-cell isolates of *C. insidiosum*, maintained by the different methods, were tested on a TTC medium. The investigation reported herein was conducted to confirm the presence of these colony types, and to determine whether they were related to method of storage and virulence.

MATERIALS AND METHODS.—Eight single-cell isolates of *C. insidiosum* were selected from the single-cell isolates used in the previous study (2). These had been maintained in sterile glass-distilled water, on BLA by periodic transfer, and in a sterile 3:1:1 soil:peat:perlite mix. Each isolate, maintained by the different methods for over 1 year at 21 C, was cultured on freshly prepared BLA plates and grown for 7 days at 21 C. Serial dilutions in sterile distilled water then were prepared and streaked onto a TTC medium as specified by Kelman (7). After 5 days' incubation at 21 C, these plates were examined under a dissecting microscope with obliquely transmitted light according to the method of Henry (5). A total of 150 colonies was characterized from replicate plates for each subisolate that had been maintained by the three methods. The percentage of different colony types was determined. This was repeated once.

Representatives of each colony type were isolated and tested for virulence on alfalfa seedlings grown under sterile conditions in large test tubes, and inoculated by the method previously given (2). Thirty 6-week-old DuPuits alfalfa seedlings were inoculated with each isolate. Fifteen seedlings treated with sterile distilled water served as controls. Disease evaluations based on a scale similar to that of

TABLE 1. Percentage of different colony types of *Corynebacterium insidiosum* on a tetrazolium chloride medium following different storage methods

Isolate no.	Storage method	Percentage of colony type[a]		
		Red	Pink	Green
G-1 K	Soil	4.0	94.7	1.3
	Agar	12.0	53.3	34.7
	H_2O	19.3	60.0	20.7
G-2 E	Soil	3.3	96.7	0.0
	Agar	12.7	64.7	22.6
	H_2O	16.0	76.0	8.0
G-3 B	Soil	6.7	93.3	0.0
	Agar	8.0	52.0	40.0
	H_2O	13.3	60.7	26.0
S-2 A	Soil	0.0	97.3	2.7
	Agar	3.4	57.3	39.3
	H_2O	14.0	61.3	24.7
S-2 D	Soil	10.0	86.0	4.0
	Agar	19.3	56.7	24.0
	H_2O	20.0	61.3	18.7
S-3 D	Soil	6.0	94.0	0.0
	Agar	22.0	64.7	13.3
	H_2O	26.7	55.3	18.0
S-5 C	Soil	5.3	92.7	2.0
	Agar	6.0	66.0	28.0
	H_2O	11.3	64.7	24.0
S-5 D	Soil	5.3	93.3	1.3
	Agar	14.0	69.3	16.7
	H_2O	16.0	72.0	12.0

[a] Percentage of 150 colonies characterized/isolate for each storage method.

Cormack et al. (3) were made 4 weeks after inoculation. Isolations were made from seedlings to determine the presence of *C. insidiosum* and any possible contaminants.

RESULTS.—Three colony types were detected on the tetrazolium chloride medium: (i) red type, a large butyrous colony with deep red center and a smooth, wide, white border; (ii) pink type, a light-pink- to white-colony sometimes showing a small, dark-pink center and somewhat irregular border, about one-fourth as large as the red type; and (iii) green type, a very small greenish colony, round to oblong and about one-fourth as large as the pink type.

A high percentage of the pink-type colonies occurred in the dilutions made from the isolates maintained in soil, whereas only small percentages of the red and green types were detected (Table 1). This was in sharp contrast to the results for isolates that had been maintained on BLA and in sterile water. In the latter two cases, much higher percentages of the red and green colony types were detected. This occurred regardless of whether the single-cell isolates had been maintained at 4 or 21 C.

All the isolates derived from the pink colony type exhibited a high degree of virulence (Table 2). Seedlings began to wilt 5 days after inoculation, and usually were killed by 2 weeks. In contrast, isolates derived from the red and green colony types always gave ratings of such low magnitude that they easily could be designated avirulent.

All attempts to isolate *C. insidiosum*, on BLA, from seedlings inoculated with isolates derived from the pink-type colony were successful. In contrast, the bacterium could not always be isolated from seedlings inoculated with the isolates obtained from the red and green colony types. Contaminants were not detected during these isolations.

TABLE 2. Relationship of colony appearance on a tetrazolium chloride medium to virulence of *Corynebacterium insidiosum* to alfalfa seedlings

Colony type	Isolate no.[a]	Avg rating[b]
Red	G-1 K	0.1
	S-2 D	0.3
	S-5 C	0.2
	S-5 D	0.3
Pink	G-1 K	4.6
	S-2 D	4.2
	S-5 C	5.0
	S-5 D	4.8
Green	G-1 K	0.4
	S-2 D	0.2
	S-5 C	0.3
	S-5 D	0.2
	Controls	0.0

[a] Thirty seedlings tested/isolate.

[b] Based on scale ranging from 0 = no infection to 5 = dead or dying.

DISCUSSION.—The literature on various plant-pathogenic bacteria cultured on TTC indicates this medium is useful for the detection of avirulent mutants. Kelman (7) found that the most common mutant of *P. solanacearum* formed a round, butyrous,

deep-red colony with a narrow bluish border, whereas the normal virulent or wild type formed an irregularly round, fluidal, white colony with a pink center. In contrast, Smale & Worley (12) found colonies of *Pseudomonas phaseolicola* to be comparable in size and to vary in color from entirely red (virulent) to entirely white (avirulent), with various proportions of red and white between. In the latter category, the degree of virulence depended upon the proportion of red and white color in individual colonies. Some studies (8, 11) have reported a direct relationship of greater cell numbers and viability with increased reduction of TTC, and hence greater red color production.

Friedman (4) found colonies of a virulent strain of *E. carotovora* to be smooth and large, with large red centers and narrow colorless borders, whereas colonies of the weakly virulent strain were rough and small with small, red centers and narrow colorless borders.

Bordewick (1) reported that all virulent and avirulent cultures of *C. insidiosum* tested formed round, smooth, flat, glistening, light-pink colonies. This is in marked contrast to results obtained in the present study. Some of the differences may be because Bordewick did not employ the lighting method of Henry (5) for examination of colonies, and because his cultures were incubated for a longer interval (2-3 weeks) before examination. In the present study, the method of lighting appeared to be critical to the results obtained. Also, as the age of the cultures increased, the differences between colony types became less pronounced.

The presence of the green colony type has not been reported in similar studies utilizing TTC, and remains unexplained.

This study indicates that TTC can be used in the case of *C. insidiosum* to spot virulence. The pink-type colony detected on this medium is the virulent type of *C. insidiosum*, whereas the red and green colony types represent avirulent mutants.

Storage of bacteria in water has been accepted as a good method. However, in the present investigation, *C. insidiosum* produced many mutants when stored in sterile water. This may explain why so many cultures became avirulent, as researchers often preserve their cultures by mass transferring or by streaking from a culture stored in water, then continuing it as a mass transfer.

The relationship between the method of preservation of single-cell isolates and colony type indicates why the isolates maintained in the sterile soil mix retained a high degree of virulence in comparison to those in sterile water and on BLA (2). The small percentages of the red- and green-type colonies detected from the isolates maintained in the soil mix may have been partially due to their initial increase on BLA before storage and before making the dilutions to streak on the tetrazolium chloride medium. This evidence confirms our earlier report (2) on the advantage of the sterile soil mix as a method for maintaining *C. insidiosum* in a virulent state.

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