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

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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-oblung 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 subsolate 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

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Storage method</th>
<th>Red</th>
<th>Pink</th>
<th>Green</th>
</tr>
</thead>
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<tr>
<td>G-1 K</td>
<td>Soil</td>
<td>4.0</td>
<td>94.7</td>
<td>1.3</td>
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<tr>
<td></td>
<td>Agar</td>
<td>12.0</td>
<td>53.3</td>
<td>34.7</td>
</tr>
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<td></td>
<td>H₂O</td>
<td>19.3</td>
<td>60.0</td>
<td>20.7</td>
</tr>
<tr>
<td>G-2 E</td>
<td>Soil</td>
<td>3.3</td>
<td>96.7</td>
<td>0.0</td>
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<tr>
<td></td>
<td>Agar</td>
<td>12.7</td>
<td>64.7</td>
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</tr>
<tr>
<td></td>
<td>H₂O</td>
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<td>76.0</td>
<td>8.0</td>
</tr>
<tr>
<td>G-3 B</td>
<td>Soil</td>
<td>6.7</td>
<td>93.3</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>8.0</td>
<td>52.0</td>
<td>40.0</td>
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<td></td>
<td>H₂O</td>
<td>13.3</td>
<td>60.7</td>
<td>26.0</td>
</tr>
<tr>
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<td>Soil</td>
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<td>86.0</td>
<td>4.0</td>
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<td>Agar</td>
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<td>56.7</td>
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<td></td>
<td>H₂O</td>
<td>20.0</td>
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<td>18.7</td>
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<tr>
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<td>94.0</td>
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<td>H₂O</td>
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<td>S-5 C</td>
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<td>72.0</td>
<td>12.0</td>
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</tbody>
</table>

* 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.

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, fluid, 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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