QUANTITATIVE ASPECTS OF TRANSFORMATION OF VIRULENCE IN AGROBACTERIUM TUMEFACIENS

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In the last decade, a number of bacterial species were shown to possess the ability to transmit morphological and biochemical characters to deficient strains. Tumor-inducing ability or virulence in crown-gall bacteria is such a biochemical character, and the acquisition of virulence is here taken to be the acquisition by avirulent strains of bacteria of the ability to synthesize a tumor-inducing principle active in a given host (Klein and Link, 1955). In an earlier paper, (Klein and Klein, 1953) we reported that a bacterin, a culture filtrate, and the deoxyribonucleic acid (DNA) of virulent crown-gall bacteria were each capable of transmitting the property of host-specific virulence to avirulent strains of Agrobacterium tumefaciens. The property of virulence, once acquired, was permanent and it was concluded that crown-gall bacteria are capable of undergoing transformation reactions.

Since our first paper appeared, a quantitative bioassay for crown-gall tumor formation was developed. This new test system has been used to reinvestigate and to extend various aspects of the transformation of virulence in crown-gall bacteria.

MATERIALS AND METHODS

A number of strains of A. tumefaciens varying in virulence from high through moderate to avirulent were used in this study. Maintenance of cultures, conditions of growth, and the media used were, unless otherwise noted, identical to those previously reported (Klein and Klein, 1953). The transforming principle for virulence (here designated TP) was in all experiments obtained from the culture fluid of donor strains of A. tumefaciens grown as shake cultures in modified McIntire's medium (McIntire et al., 1940) for 18 hr at 26 C. The culture was centrifuged at 10,000 x G in a Servall angle-head centrifuge. The TP-containing supernatant was sterilized by filtration through 0.02 and 0.03 Sela porcelain filter candles and was usually used without further treatment. Five-ml aliquot portions of this TP were added to 5 ml of synthetic media in 50-ml flasks and were inoculated with 0.1 ml of an 18-hr culture of the test bacteria grown in McIntire's medium. These cultures were incubated for various lengths of time at 26 C. Sterility checks were made at all stages of the work, and the integrity of the filters was demonstrated many times.

At the end of the incubation period, 0.05 ml of the resulting cultures (ca 10⁶ cells) was placed on the freshly cut, cambial-adjacent surface of standardized "strong-reactor" carrot discs (Klein and Tenenbaum, 1955). Resulting tumors were excised and weighed 15 days after inoculation. Replicates of 8–10 discs gave results significant at the 5 per cent level of confidence. The sensitivity of the carrot bioassay with respect to numbers of virulent bacteria was examined. It was found (table 1) that about 1 × 10⁴ virulent bacteria per disc were required for maximum tumor response. This is true even when 1 × 10⁶ cells constituted as little as 1 per cent of the total inoculum (table 2). Altered cultures were stored on potato-glucose agar and subs cultures made in nutrient broth (Difco). Chymotrypsin was kindly supplied by Professor Hans Neurath (University of Washington, Seattle) and crystalline deoxyribonuclease was purchased from the Mann Research Laboratories, New York.

RESULTS

Donor-recipient combinations. The interrelations of various donor and recipient strains have,
TABLE 1
Effect of numbers of bacteria per inoculum on eventual weight of crown-gall tumors

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Tumor Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>cells/disc</td>
<td></td>
</tr>
<tr>
<td>2 × 10⁴</td>
<td>100</td>
</tr>
<tr>
<td>2 × 10⁵</td>
<td>102</td>
</tr>
<tr>
<td>2 × 10⁵</td>
<td>105</td>
</tr>
<tr>
<td>2 × 10⁵</td>
<td>105</td>
</tr>
<tr>
<td>2 × 10⁴</td>
<td>78</td>
</tr>
<tr>
<td>2 × 10¹</td>
<td>12</td>
</tr>
</tbody>
</table>

TABLE 2
Effect of relative dilution of virulent crown-gall bacteria on weight of tumors. Number of virulent cells maintained at level (1 × 10⁴/disc) required for good tumor response

<table>
<thead>
<tr>
<th>Virulent Bacteria in Total Inoculum</th>
<th>No. Cells in Inoculum</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virulent (B)¹</td>
<td>Avirulent (SP)</td>
</tr>
<tr>
<td>%</td>
<td>0</td>
<td>10⁴</td>
</tr>
<tr>
<td>1</td>
<td>10³</td>
<td>10⁴</td>
</tr>
<tr>
<td>5</td>
<td>10³</td>
<td>5 × 10⁴</td>
</tr>
<tr>
<td>10</td>
<td>10³</td>
<td>10⁴</td>
</tr>
<tr>
<td>50</td>
<td>10³</td>
<td>10⁴</td>
</tr>
<tr>
<td>100</td>
<td>10³</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Actual number = 0.9 × 10⁴ cells/ml.
† Actual number = 0.85 × 10⁴ cells/ml and dilutions thereof.

for many cases of transformation, proved to be an important consideration in the success of these experiments. The TP-containing filtrates were obtained from five strains of crown-gall bacteria possessing various grades of virulence. *A. tumefaciens* strain B₄ is probably the most virulent for carrot, and strain BP is only slightly less virulent. *A. tumefaciens* strains A₄, S 5-6 and B₄(b) are moderately virulent on carrot. Three of the recipient strains, *A. tumefaciens* IIBNV₆, B₄(a) and SP, are avirulent on all tested hosts and the other two strains, H100 and Eu6, are weakly virulent on carrot and moderately virulent on some other hosts.

When the recipient bacteria were grown in the TP from the donor strains, transformations occurred as determined by the acquisition or enhancement of tumor-inducing ability. Only certain donor-recipient combinations were effective in transformation of virulence (table 3). For example, the TP from donor strain B₄ altered recipient strains IIBNV₆ and B₄(a) but not strains SP, H100, or Eu6. The TP from donor strain BP, on the other hand, was effective only on strain H100. In confirmation of our previous finding, *A. tumefaciens* strain S 5-6 was an effective donor of virulence for strain IIBNV₆. A bacterin preparation of this donor strain was just as effective as the filtrate. It is of interest that strain B₄(b), the parent strain from which strain B₄(a) was isolated, was not an effective donor for the derived culture.

*Increase in virulence in altered populations.* In our earlier paper it was noted that the virulence of populations containing altered cells increased with successive periods of active growth in media free of TP. These observations were tested quantitatively by examining the virulence of strain H100 initially altered by the TP of donor strain BP. At the end of the initial transformation experiment, a subculture of the recipient population, now containing altered cells, was made on potato-glucose agar. After 3 weeks, a subculture was made from the slant into nutrient broth and incubated at 26°C for 18 hr. This population of actively growing cells was assayed for virulence on carrot discs and again subcultured on potato-glucose agar. This procedure was repeated at 3-week intervals.

Successive periods of active growth had no significant effect on the low grade of virulence of recipient strain H100 nor on the high grade of virulence of donor strain BP. The altered H100, however, became progressively more virulent.

TABLE 3
Responses of recipient strains of Agrobacterium *tumefaciens* to transforming principle from the culture liquid of various donor strains of the same species

<table>
<thead>
<tr>
<th>Donor Strains</th>
<th>Mg Tumor Tissue/Std. Carrot Disc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>B₄</td>
<td>100</td>
</tr>
<tr>
<td>BP</td>
<td>95</td>
</tr>
<tr>
<td>A₄</td>
<td>60</td>
</tr>
<tr>
<td>S 5-6</td>
<td>55</td>
</tr>
<tr>
<td>B₄(b)</td>
<td>50</td>
</tr>
</tbody>
</table>

* Actual number = 0.9 × 10⁴ cells/ml.
† Actual number = 0.85 × 10⁴ cells/ml and dilutions thereof.
through 3 successive periods of active growth, and the virulence of this population stabilized at a level about twice that of the initially altered culture and about 3 times that of the unaltered, recipient strain (figure 1). When this stabilized and now moderately virulent culture was exposed to a second application of the TP from donor strain BP, its virulence was further boosted but to a lesser extent than in the initial transformation reaction.

This suggests that the relative effectiveness of a transformation reaction is, in part, a function of the initial virulence of an efficient recipient strain. This concept was tested. A very weakly virulent single cell isolate of strain H100 here designated H100/1, was altered by the TP of donor strain BP into a population which was as virulent as the parent strain H100 altered by the same TP (table 4).

Single cell isolates. A direct plate scoring procedure for the determination of the efficiency of transformation is impossible for the characteristics—being studied. To obtain information on this important point 100 single cell isolates each of the unaltered recipient strain H100 and of the stabilized, altered population were tested for virulence by the carrot disc bioassay.

**TABLE 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mg Tumor Tissue/Std. Carrot Disc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H100</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
</tr>
<tr>
<td>TP (from BP)</td>
<td>37</td>
</tr>
</tbody>
</table>

**Figure 1.** Increase in virulence of a population containing transformed bacteria with successive periods of active growth and a second exposure to transforming principle.

**Figure 2.** Distribution histogram of the virulence of 100 single cell isolates each of recipient strain H100 and of the stabilized, transformed population of the same strain.

The isolates from the unaltered recipient culture showed a limited range of virulence with only one culture possessing virulence even slightly greater than the population as a whole. Of the tested cultures, 94 per cent were in the range inducing 10 to 25 mg of tumor tissue per disc (figure 2). Single cell isolates from the altered population possessed a much greater range of virulence. Over 30 per cent were significantly more virulent than were any of the isolates from the unaltered recipient population. It is of interest that 4 per cent of the altered population possessed the moderate grade of virulence found in the stabilized altered population as a whole. Without further qualification these data might suggest an unusually high efficiency of transformation. This question will be discussed later in this paper.

**Host range.** The ability of transformed H100 to induce tumor formation on host plants other than those of the primary test system, the carrot, was tested by inoculation into stems of sunflower (var. Yellow Gold), tomato (var. Mar-

**TABLE 5**

<table>
<thead>
<tr>
<th>Host</th>
<th>No. Plants</th>
<th>Days After Inoculation</th>
<th>Percentage Increase of Tumor Diameter over Diameter of Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower</td>
<td>10</td>
<td>30</td>
<td>168 132 46 62</td>
</tr>
<tr>
<td>Tomato</td>
<td>5</td>
<td>18</td>
<td>161 132 94 124</td>
</tr>
<tr>
<td>Bryophyllum</td>
<td>3</td>
<td>30</td>
<td>246 249 196 239</td>
</tr>
</tbody>
</table>
TABLE 6
Influence of time of contact of recipient strain H100 with transforming principle from Agrobacterium tumefaciens strain BP

<table>
<thead>
<tr>
<th>Time of Contact (Hr)</th>
<th>Mg tumor tissue/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  0.5  1  2  3  4  6  8  24  48  72</td>
</tr>
<tr>
<td>Donor (BP)</td>
<td>90 90 90 90 91 92 90 90</td>
</tr>
<tr>
<td>Recipient (H100)</td>
<td>22 21 22 22 23 18 21 20</td>
</tr>
<tr>
<td>Recipient (H100) + TP</td>
<td>22 33 37 37 37 36 36 36</td>
</tr>
</tbody>
</table>

TABLE 7
Effects of deoxyribonuclease, chymotrypsin, and heat on the activity of transforming principle from virulent crown-gall bacteria, strain BP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mg Tumor Tissue/Std. Carrot Disc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BP 95  H100 24</td>
</tr>
<tr>
<td>TP (from BP)</td>
<td>BP 90  H100 40</td>
</tr>
<tr>
<td>TP + Deoxyribonuclease</td>
<td>BP 94  H100 23</td>
</tr>
<tr>
<td>TP + Chymotrypsin</td>
<td>BP 97  H100 42</td>
</tr>
<tr>
<td>TP + 30 C for 30 min.</td>
<td>BP 97  H100 42</td>
</tr>
<tr>
<td>TP + 60 C for 30 min.</td>
<td>BP 91  H100 21</td>
</tr>
<tr>
<td>TP + 90 C for 30 min.</td>
<td>BP 92  H100 22</td>
</tr>
</tbody>
</table>

Properties of the transforming principle for virulence. Several characteristics of the transformation system for virulence in crown-gall bacteria were examined. There were no differences in the effectiveness of the TP obtained by growing the donor strains in two different synthetic media in spite of one (McIntire et al., 1940) containing glutamic acid, and the other (Braun, 1950) containing nitrate, as the sole nitrogen source.

The time relations of transformation were investigated in some detail (table 6). In summary, 1/2 hr of contact of the TP from donor strain BP with recipient cells was sufficient to give maximal transformation as determined by the virulence of the altered population. Longer periods of contact, up to 72 hr, were no more effective.

The filterable transforming agent for virulence appears to be a deoxyribonucleic acid. DNase-(0.05 mg/ml), free of both RNase and proteinase, completely inactivated the TP (table 7). Crystalline chymotrypsin (0.05 mg/ml) was without effect on this TP. The TP was stable to heating at 30°C for 1/2 hr but was inactivated in the same period of time at 60°C and at 90°C.

DISCUSSION
The transforming principle for virulence in crown-gall bacteria appears, like other transforming principles (Hotchkiss, 1955), to be a deoxyribonucleic acid. Deoxyribonuclease specifically destroyed the biological activity of isolated DNA (Klein and Klein, 1953) and the transforming ability of the filterable transforming agent used in this study. Proteinase was without effect. A temperature of 60°C, which does not usually inactivate bacteriophage (Luria, 1953), and frequently denatures protein, inactivated the transforming principle for virulence. It is very unlikely that the acquisition of virulence by crown-gall bacteria involved a typical bacteriophage-transduction reaction.

The release of DNA into the culture medium of actively growing bacteria does not appear to be a unique property of A. tumefaciens. Borek et al. (1955) have shown that Escherichia coli excreted nucleic acid components into culture medium and, in fact, Weil and Binder (1947) obtained type-specific transformations in Shigella paratyphosa with filtrates of broth cultures of their donor strains.

The quantitative confirmation of the previously noted increase in the virulence of an altered population with successive periods of active growth (Klein and Klein, 1953) cannot be adequately explained at this time. Since as few as 10⁴ virulent bacteria gave a maximum tumor
response, and the average inoculum used in our present work was over $10^4$ cells, a 1 per cent efficiency of transformation would result in a sufficient number of altered cells to give a maximum response in the carrot disc assay. Further, since the 1 per cent level of transformation permits maximum tumor response, it is improbable that any selective growth advantage of altered cells in a mixed population would increase the virulence of the population as a whole. One can only suggest, following Hotchkiss (1955), that the transforming principle introduces its own enzymatic or phenotypic response within a short time and that it only later comes to be fully incorporated into the existing genome of the recipient cell.

The finding that 30 per cent of the altered cells in a stabilized transformed population possessed virulence greater than any of the recipient cells may be explained by several mechanisms. In descending order of probability these are: (a) an unusually high efficiency of transformation, (b) a selective growth advantage of transformed cells, or (c) the subsequent transformation of unaltered cells by previously altered cells during active growth. Hotchkiss (1955) has found as many as 17 per cent of the cells used for mannitol-utilization transformations to be susceptible. It is possible that, in crown-gall bacteria already possessing a low grade of virulence, the acquisition of higher grades of virulence might be quite efficient. This problem, however, cannot be resolved with the relative cumbersome scoring system used.

It is of interest to speculate on the genetics of transformation of virulence in crown-gall bacteria. On the basis of our data, it is here postulated that virulence in the bacteria is controlled by multiple loci regulating the synthesis of a tumor-inducing principle effective in a given range of susceptible plants (Klein and Link, 1955). The relative grade of virulence of the bacteria in a given host is ultimately controlled by multiple alleles at the host range loci. Grades of virulence may represent the quantity or quality of tumor-inducing principle synthesized by the bacteria in the host tissues under the influence of substances found in the cell sap. It was shown that additional host ranges may be acquired by transformation reactions without modification of the original host range or grade of virulence (Klein and Klein, 1953). Since neither substitution nor replacement of host ranges occurred in these experiments, multiple loci for different host ranges appears probable. Further, our data on the virulence of altered populations showed that the acquired or enhanced virulence was not general but was host-specific. The concept of multiple alleles controlling relative grades of virulence within a host range is supported by the finding that virulence may be increased in stepwise fashion by an additional exposure to transforming principle. It would appear that the lower grades of virulence or new host ranges were acquired with relative ease, but the addition of higher numbers of alleles became increasingly difficult. The wide range of virulence found in single-cell isolates of a stable, altered population is in line with this concept.

ACKNOWLEDGMENT

The authors are indebted to Dr. W. J. Robbins and Professor F. Ryan for their suggestions and to Mrs. I. L. Tenenbaum and Mrs. G. M. Groves for technical assistance.

SUMMARY

Transformation of virulence in the crown-gall bacterium *Agrobacterium tumefaciens* was studied, utilizing a quantitative bioassay for crown-gall tumor formation. The transforming principle was a deoxyribonucleic acid present in filtrates of growing donor cultures. It is not a bacteriophage. Only certain donor and recipient combinations were effective in transformation. Virulence of an altered population increased to a maximum with successive periods of active growth and could be further boosted by an additional exposure to transforming principle. Over 30 per cent of the isolates of a stabilized, altered population possessed grades of virulence significantly greater than any of the isolates from the unaltered, recipient population. Acquisition of virulence appeared to be host or host-range specific. It is proposed that virulence is controlled by multiple loci regulating the synthesis of a tumor-inducing principle effective in plants of a given host range. Grades of virulence in a given host-range may ultimately be controlled by multiple alleles at each host range locus.

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