THE NUTRITION OF PHYTOPATHOGENIC BACTERIA

II. The Genus Agrobacterium

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The nutritional requirements of the tumor-inducing phytopathogenic bacteria and soil saprophytes which Conn (1942) has placed in the genus *Agrobacterium* have received somewhat more attention than have the requirements of most of the other groups of plant-disease bacteria. It is clear from the literature (Sagen, Riker, and Baldwin, 1934; Riker, Lyneis, and Locke, 1941; Hofer, 1941) that the crown-gall bacterium, *Agrobacterium tumefaciens*, and the common soil saprophyte, *Agrobacterium radiobacter*, grow well in solutions containing only ammonium or nitrate nitrogen and any of a number of carbon sources.

On the other hand, the remaining two species at present in this genus, the hairy-root organism, *Agrobacterium rhizogenes*, and the cane-gall bacterium, *Agrobacterium rubi*, are reported to have somewhat more complex nutritive requirements. For example, Sagen, Riker, and Baldwin (1934) summarize their study of *A. rhizogenes* by stating that it "seems to lack the ability of *P. tumefaciens* and *B. radiobacter* to utilize the simpler nitrogenous compounds." Similar observations are recorded for *A. rubi* by Pinckard (1935) and Hildebrand (1940).

Inasmuch as knowledge of the exact nutrition of this group may be useful in interpretations of the systematics and general physiology of the genus, as well as provide source material for eventual evaluation on the possible interrelationship of microbial nutrition and virulence (McNew, 1938; Van Lanen, Baldwin, and Riker, 1940), a study of the four *Agrobacterium* species was undertaken. At the same time some observations were made on the nutritive requirements of *Bacterium pseudotsugae* and *Agrobacterium gypsophilae*, two species which have been placed in an Appendix to *Agrobacterium* in the forthcoming sixth edition of *Bergey's Manual of Determinative Bacteriology*. In general, the objective was prompt, moderate growth in simple solutions of known composition, rather than development as rapid and luxuriant as possible in complex media.

I

Representative cultures were secured from investigators who had specialized in this group; specific information concerning sources is presented in the sections which follow. The purity of all cultures was checked by microscopic examination and by streaking serially two or more times on yeast extract agar and on glucose, yeast extract, and $CaCO_3$ agar from dilute aqueous suspensions of cells.

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The technical details were similar to those in the previous study in this series (Starr, 1946). All glassware was acid-washed. Media were prepared from reagents of the highest available purity. The basal medium[2] was the same NH_4Cl, glucose, and salts solution used before. At times, a commercially prepared HCl hydrolyzate of "vitamin-free" casein (SMACO brand) was used. In order to minimize the carry-over of nutrilites with the inoculum, test media were inoculated, by means of a capillary pipette, via a dilution flask. Cultures were incubated, often on a shaking machine to increase the rate of growth, at 25 to 28 C. Quantitative estimations of "turbidity" were made in the Evelyn photoelectric colorimeter using the 620 mμ filter, and the results are expressed in terms of "optical density" (2 − log galvanometer reading).

II

Agrobacterium radiobacter and Agrobacterium tumefaciens. The following 5 cultures of *A. radiobacter* were used in this study:

TR1 (Hofer's R1-1a), received from A. W. Hofer, Geneva, N. Y., in 1941.
TR4 (Hofer's 36), an old culture from F. Löhnis, identified by M. W. Beijerinck but not the latter's original culture. Received from A. W. Hofer, Geneva, N. Y., in 1943.
TR5 (Hofer's S-192), isolated by N. R. Smith from corn soil in 1927. Received from A. W. Hofer, Geneva, N. Y., in 1943.
PG.1.2 (Leonard's 1911S) and PG.1.3 (Leonard's 2012), received in 1945 from the collection of C. B. van Niel, Pacific Grove, Calif. Originally from L. T. Leonard, U. S. Department of Agriculture.

A. tumefaciens was represented by the following 14 isolates:

TT2 (Braun's B2) and TT3 (Braun's B6), received from A. C. Braun, Princeton, N. J., in 1941.
TT4 (Hofer's SCA-2), TT5 (Hofer's SCT-5 fff3), and TT6 (Hofer's SCA-1), received from A. W. Hofer, Geneva, N. Y., in 1943.
TT7 (Williams' A-1), TT8 (Williams' B-3), TT9 (Williams' H-100), TT10 (Williams' 5 Gly Fe), and TT11 (Williams' T3-1C-3), from the collection of I. M. Lewis; received in 1945 from O. B. Williams, Austin, Texas.
A-6, Cor, Wellesley, and W-1, received from D. G. R. Wyckoff, Wellesley, Mass., in 1946.

Most of these *A. tumefaciens* strains induced typical galls when inoculated[3] by pin pricks into the crowns of sugar-beet plants (Suit, 1933); however, TT5, TT10, and TT11 did not cause gall formation in repeated trials.

All cultures of *A. radiobacter* and *A. tumefaciens* grew luxuriantly within a day or two in the NH_4Cl, glucose, and salts basal medium, except that culture TT10 grew somewhat more slowly than the others. Larger crops, and slight increases in growth rate (marked in the case of TT10), resulted from the addition of 0.5 per cent of "vitamin-free" casein hydrolyzate to the basal medium. This stimu-

[2] Per 100 ml of basal medium: NH_4Cl, 0.1 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; "trace" elements; distilled water; adjusted to pH 6.8 with NaOH. Separately sterilized, purified glucose (0.5 per cent) was added aseptically to the sterile salt solution.

[3] The co-operation of Dr. W. A. Campbell, Special Guayule Research Project, Salinas, California, in conducting the pathogenicity tests, is gratefully acknowledged.

latory effect is due, in part at least, to the maintenance of a favorable hydrogen-ion concentration, close to neutrality, in the casein hydrolyzate medium. Cultures in the unsupplemented basal medium become acid rapidly, the pH dropping to the presumably growth-inhibiting level of about 4.2 in a day or two. The addition of 0.1 per cent of synthetic *dl*-glutamic acid to the basal medium also resulted in larger crops, although the increase was not so marked as with casein hydrolyzate, and caused TT10 to grow as rapidly as the other cultures.

A mixture of seven B vitamins[4] added to the casein hydrolyzate medium similarly increased, very slightly, the initial growth rate of all these cultures, probably, as McIntire, Riker, and Peterson (1941) have shown, because of the content of thiamine, riboflavin, and pantothenic acid. However, no further attention was directed to the exact evaluation of these but slightly stimulating nutrilites.

Agrobacterium rhizogenes. Four isolates of *A. rhizogenes* were studied:

TR7 (Hofer's C-1), received from A. W. Hofer, Geneva, N. Y., in July, 1943.
TR12, received from E. M. Hildebrand, Ithaca, N. Y., in July, 1943.
TR16 (Williams' T37), from the collection of I. M. Lewis; received from O. B. Williams, Austin, Texas, January, 1945.
C-10, received from A. W. Hofer, Geneva, N. Y., in January, 1945.

The virulence of these cultures was tested (Suit, 1933) by inoculating them into the crowns of sugar-beet plants by means of pin pricks. From 4 to 6 plants were used per culture in each trial, and the series was carried out three times. Culture TR7 was able to induce typical hairy-root symptoms most readily of the four isolates; TR12 and C-10 caused somewhat less severe hairy root in only part of the plants used, and the symptoms were rather delayed; TR16 was pathologically more like the crown-gall organism and always yielded galls rather than hairy root on sugar beet. This last culture was received as number T37 and may be descended from the culture of the same designation with which Hendrickson, Baldwin, and Riker (1934) obtained similar intermediate results.

Despite the varied pathogenicity, the four *A. rhizogenes* cultures behaved uniformly with respect to nutritive requirements. None of these cultures grew in the NH_4Cl, glucose, and salts basal medium, nor upon the addition of "vitamin-free" casein hydrolyzate. The addition of 0.5 per cent yeast extract to the casein hydrolyzate medium resulted in good growth. The yeast extract could be substituted by the mixture of seven B vitamins, and, by successively omitting one growth factor at a time from this mixture, biotin was inferred as the only active ingredient. In support of this inference, all four strains of *A. rhizogenes* grew when biotin was added to the casein hydrolyzate medium. In the presence of biotin the casein hydrolyzate could be replaced by 0.1 per cent of synthetic *dl*-glutamic acid. There was much slower growth in the basal medium supplemented only by biotin, and the final crops were decreased—probably, as indicated by pH measurements, because of acid production without a simultaneous neutral-

[4] *p*-Aminobenzoic acid, biotin, nicotinic acid, calcium pantothenate, pyridoxine HCl, riboflavin, and thiamine HCl. The vitamins and vitamin derivatives used in this study represent a generous gift from Merck & Co., Rahway, N. J.

ization by the alkaline decomposition products from the amino acids. Figure 1 shows the quantitative response to biotin in the glutamic acid medium. Biotin could be substituted by the hydrosulfate of 3,4-diamino-tetrahydro-2-thiophene-valeric acid, but not by pimelic acid.

The finding of an obligate biotin requirement for *A. rhizogenes* explains the difficulty encountered by Sagen, Riker, and Baldwin (1934), Riker, Lyneis, and Locke (1941), and Hofer (1941) in culturing this species in media which lacked peptone or yeast extract.

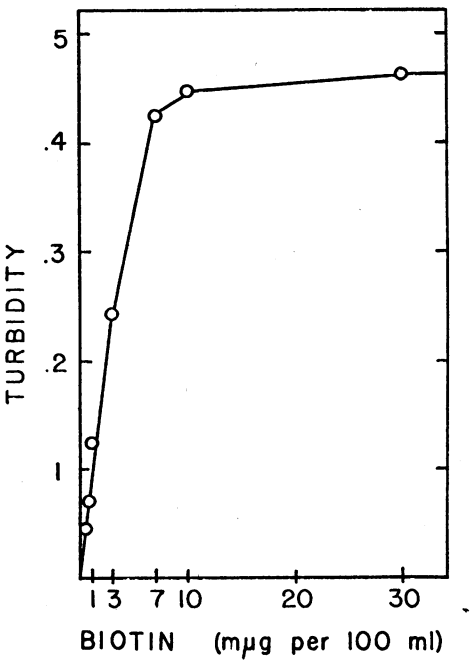

FIG. 1. RESPONSE OF AGROBACTERIUM RHIZOGENES TR12 TO BIOTIN IN THE GLUTAMIC ACID MEDIUM

The glutamic acid medium, supplemented by biotin in concentrations ranging from 0.1 to 100 millimicrograms (mμg) per 100 ml, was inoculated and incubated, with constant shaking, for 4 days at 28 C, at which time the "turbidity" was measured. The "optical density" at 100 mμg was the same as at 30 mμg.

The use of a calcium glycerophosphate medium for distinguishing *A. rhizogenes* from *A. tumefaciens* and *A. radiobacter* has been recommended. The hairy-root organism reportedly fails to grow (Riker, Banfield, Wright, Keitt, and Sagen, 1930; Sagen, Riker, and Baldwin, 1934), but the crown-gall bacterium and *A. radiobacter* make abundant growth. Inasmuch as it seemed likely that the failure of *A. rhizogenes* to grow in this biotin-free glycerophosphate medium merely mirrors its inability to develop in the absence of biotin and glutamic acid, the *A. rhizogenes* isolates were cultured from small inocula in the glycerophosphate medium[5] used by Riker *et al.* (1930), and with additions of biotin, of glutamic

[5] Calcium glycerophosphate, 0.8 g; mannitol, 20.0 g; KNO_3, 5.0 g; NaCl, 3.8 g; KCl, 0.1 g; $MgCl_2 \cdot 6H_2O$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; distilled water, 1,000 ml.

acid, and of both biotin and glutamic acid. These experiments may be summarized as follows: (1) There was no growth in the unsupplemented glycerophosphate medium or when glutamic acid alone was added. (2) There was slow growth when biotin alone was added. (3) There was good development in the glycerophosphate medium with both biotin and glutamic acid. The unsupplemented glycerophosphate medium supported good growth of all cultures tested of *A. tumefaciens* and *A. radiobacter*, entirely in accordance with the findings of previous investigators. There can be no doubt, then, that the *modus operandi* of the glycerophosphate medium test results from the fact that the nutrilite needs of *A. rhizogenes* are not satisfied by that medium.

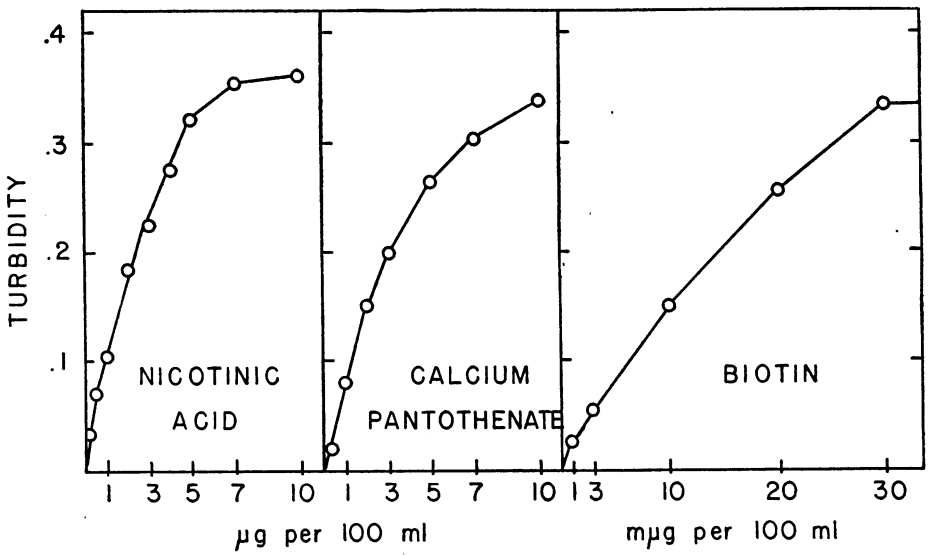

FIG. 2. RESPONSE OF AGROBACTERIUM RUBI TO NICOTINIC ACID, CALCIUM PANTOTHENATE, AND BIOTIN IN THE GLUTAMIC ACID MEDIUM

The response to nicotinic acid was measured in the presence of 10 μg per cent of calcium pantothenate and 100 millimicrograms (mμg) per cent of biotin; the response to calcium pantothenate, in the presence of 10 μg per cent of nicotinic acid and 100 mμg per cent of biotin; and the response to biotin, in the presence of 10 μg per cent each of nicotinic acid and calcium pantothenate. These media were incubated, with constant shaking, for 4 days at 28 C, at which time the "turbidity" was measured. The plotted values are the averaged "optical densities" of duplicate determinations.

This may be the place to comment on an experiment described by Hendrickson, Baldwin, and Riker (1934) in which the relation of the growth of *A. rhizogenes* to the oxidation-reduction potential of the medium was being considered. In one trial *A. rhizogenes* was cultured in an agar medium which was somewhat deficient in the necessary nutrilites, although sufficient growth factor was supplied by the agar so that some growth did occur. Hendrickson, Baldwin, and Riker (1934) go on to state that "when the oxidation-reduction potential of the medium was increased by the addition of 0.1 gram of potassium permanganate per liter . . . the hairy-root cultures either failed to grow or produced only slight growth below the surface." Inasmuch as biotin activity is destroyed by oxidizing agents (Melville,

1944), the inactivation of this needed factor might be considered as an alternative explanation of this experiment.

Agrobacterium rubi. Two isolates of this species were available for study; both (TR2, TR3) were obtained from E. M. Hildebrand, Ithaca, New York, in 1942, and both induced gall formation readily by inoculation, through pin pricks, into young stems of a bramble and into sugar-beet crowns. Neither of these cultures grew in the basal medium alone, or when "vitamin-free" casein hydrolyzate was added. By a process similar to that described above, it was learned that this species requires obligately three nutrilites for development in the casein hydrolyzate medium, viz., biotin, nicotinic acid, and calcium pantothenate. In the presence of these required growth factors, 0.1 per cent of synthetic *dl*-glutamic acid could replace the casein hydrolyzate. There was no growth in the basal medium plus the three vitamins alone. The response of *A. rubi* to each of the required vitamins in the glutamic acid medium is shown in figure 2. Biotin could be replaced by the hydrosulfate of 3,4-diamino-tetrahydro-2-thiophene-valeric acid, but not by pimelic acid; nicotinic acid was replaceable by nicotinamide; pantothenate was not replaceable by pantoyl 1-lactone, β-alanine, or a mixture of the two.

The obligate requirement of *A. rubi* for the three vitamins and glutamic acid throws some light on the poor growth obtained by Pinckard (1935), Hildebrand (1940), and Starr and Weiss (1943) in what can now be interpreted as inadequate media. It also explains the difficulty experienced by Hildebrand (1940) in attempting "fermentation" tests of this organism using the vitamin-free basal medium of the *Manual of Methods for Pure Culture Study of Bacteria* (Comm. Soc. Am. Bact., 1923–1936).

Agrobacterium gypsophilae and Bacterium pseudotsugae. As noted above, cultures of these species were included in this study because of their incorporation by H. J. Conn in an Appendix to the genus *Agrobacterium* in the sixth edition of *Bergey's Manual of Determinative Bacteriology.* The single culture of *A. gypsophilae* (TG1) was received from H. J. Conn, Geneva, New York, and was originally from N. A. Brown, Washington, D. C. Culture TG1 grew well in the unsupplemented NH_4Cl, glucose, and salts basal medium and was not particularly stimulated by the addition of casein hydrolyzate or yeast extract. These results are in accordance with the original description of this species, in which Brown (1934) reported good growth in several simple synthetic media.

Three cultures of *B. pseudotsugae* were used. These were isolated by the writer in 1945 from typical stem galls on Douglas fir material collected by H. N. Hansen, Berkeley, California, one of the describers of this species. The cultures isolated from this material were kindly identified by Dr. Hansen, but no pathogenicity tests were performed. These cultures did not grow in the unsupplemented basal medium, nor when casein hydrolyzate was added. By a process similar to that used for the foregoing species, it was determined that *B. pseudotsugae* requires biotin obligately for growth in the casein hydrolyzate medium. The casein hydrolyzate could not be replaced completely by glutamic acid alone, and no further study of amino acid requirements has been made.

III

Application of these findings to the taxonomy of this group would depend upon examining a larger sampling of these species to discover the range of variation with respect to exact nutritional requirements. In any case, the use of a nutrilite requirement as a determinative character in systematic microbiology must take into consideration the mutability of microorganisms in this regard. The potentialities in this group, however, appear promising in view of the consistent, reproducible results. Inasmuch as the literature recommends that the general fastidiousness of *A. rhizogenes* and *A. rubi* can aid in the identification of these species, it appears likely that the present disclosure concerning the exact nature of the specific nutrilite requirements would possess even more value in this connection.

The incorporation of the required nutrilites in basal media would make possible a study of the carbon metabolism of the fastidious species without possible interference from the yeast extract supplement used by Conner, Riker, and Peterson (1937) and Hofer (1941). Also, it would now be possible to determine, in adequately composed basal media, whether specific differences exist between the ability of the representatives of the genus *Agrobacterium* to utilize certain carbon compounds.

This knowledge might be used in the isolation of these species from plant materials, by preparing isolation media containing only the minimal nutrients for the species under investigation. In this way interference from contaminating organisms having more complex and different nutrient requirements would be avoided.

SUMMARY

The nutritive requirements of members of the genus *Agrobacterium* were determined under specified experimental conditions. By this means the previously reported ability of *A. radiobacter*, *A. tumefaciens*, and *A. gypsophilae* to grow in simple media was verified by using a medium containing NH_4Cl, other inorganic salts, and separately sterilized, purified glucose.

The inability of *A. rhizogenes*, *A. rubi*, and *Bacterium pseudotsugae* to grow in this medium was traced to obligate nutrilite requirements, viz., *A. rhizogenes*, biotin and glutamic acid; *A. rubi*, biotin, nicotinic acid, calcium pantothenate, and glutamic acid; *B. pseudotsugae*, biotin and some as yet unidentified component of "vitamin-free" casein hydrolyzate.

The utility of these findings in the systematics of the group is discussed.

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