Vitamin Requirements of Root Nodule Bacteria

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SUMMARY

The vitamin requirements of 63 strains of *Rhizobium* and 18 of *Agrobacterium* were examined. *R. trifolii*, *R. leguminosarum* and *R. phaseoli* responded in most cases to thiamine, biotin, and calcium pantothenate. Strains of *R. meliloti* and the slow-growing rhizobia sometimes responded to biotin, but not to the other vitamins. An unknown growth factor, active for the slow-growing rhizobia, was detected. Three strains of *Agrobacterium* required thiamine.

INTRODUCTION

Species of root nodule bacteria differ in their vitamin requirements. Biotin is essential for growth of strains of *Rhizobium leguminosarum*, *R. trifolii*, *R. phaseoli*, *R. meliloti* and *R. lupini* (West & Wilson, 1939*a*, *b*, 1940). In some instances it is required only to initiate growth (Allen & Allen, 1950). According to Jordan (1952), some strains of *R. meliloti* require neither biotin nor any other of the B group of vitamins. Biotin is apparently not required by cowpea or soybean rhizobia or by the common nodule contaminant *Agrobacterium radiobacter* (West & Wilson, 1939*a*, 1940). Thiamine and pantothenic acid stimulate growth of some strains of *R. trifolii* (West & Wilson, 1939*a*; Lilly & Leonian, 1945; Ljunggren, 1961), but in others they are either not required for growth (McBurney, Bollen & Williams, 1935; Jordan, 1952) or have not been tested (Allen & Allen, 1950). Synthesis of large amounts of vitamin B₁₂ by the rhizobia (Burton & Lochhead, 1952; Levin, Funk & Tendler, 1954) has been considered in relation to leghaemoglobin production in the nodule (Levin *et al.* 1954). A cobalt requirement has been established for both rhizobial growth (Lowe, Evans & Shaukat, 1960) and symbiotic fixation (Shaukat & Evans, 1961).

The experiments reported here form part of an investigation of rhizobial taxonomy and were suggested by Dr C. A. Parker and Mr A. E. Oakley following their development of a defined growth medium for strains of *Rhizobium trifolii*.

METHODS

Organisms. The organisms used in routine work were maintained on yeast extract mannitol (YM) agar and were subcultured at monthly intervals. They were preserved by the lyophilization method described by Annear (1956) and the ampoules were stored at 4°.

Media. The defined basal medium used throughout contained (g./l. distilled water): MgSO₄·7H₂O, 0.25; CaSO₄·2H₂O, 0.03; KH₂PO₄, 0.55; CuSO₄·5H₂O,

0.00008; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00016; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0035; NaCl , 0.25; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.20; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.0004; H_3BO_3 , 0.0005; urea, 0.5; glucose, 10.0; sucrose, 10.0; tyrosine, 0.015; aspartic acid, 0.015; lysine, 0.015; histidine, 0.015; glutamic acid-HCl, 0.10; arginine, 0.015. The medium (minus the phosphates) was sterilized by autoclaving for 35 min. at 5 p.s.i. Solutions and the phosphates were sterilized separately and added before use.

This medium, with thiamine-HCl (0.004 g.), calcium pantothenate (0.004 g.) and biotin (0.001 mg.) per litre added, had previously been developed for two strains of *Rhizobium trifolii*. It supported growth to about 2×10^{10} organisms/ml. (Dr Parker and Mr Oakley, personal communication). In the present work it was used either as a broth or solidified with 1.5 % agar previously washed for 3 weeks in repeated changes of distilled water.

Experimental procedure. Ten vitamins were tested. Requirements for thiamine, calcium pantothenate, nicotinic acid, riboflavin, inositol, folic acid, *p*-aminobenzoic acid, vitamin B_{12} and pyridoxin were studied by the agar disk method described by Ford & Hutner (1957). Tubes of basal agar medium, each containing 25 ml., were liquefied by autoclaving, allowed to cool to 45° and inoculated with a washed suspension of the appropriate organism; they were then agitated and poured. Sterile filter paper disks, each impregnated with 1 μg . of one of the vitamins under test, were placed on the agar plates. Plates were examined for the presence or absence of growth stimulation after 3 days at 28° . Biotin requirements could not be studied by this method because the biotin content of the agar, even after repeated washing, was sufficient to supply the needs of the organisms. Instead, liquid medium (20 ml.) was used in 100 ml. Erlenmeyer flasks loosely plugged with biotin-free cotton wool and covered with a 50 ml. beaker. The flasks were then inoculated with a washed suspension as before; duplicate flasks and positive controls were used. They were incubated for 3 days at 28° in a reciprocal shaking machine. After three successive transfers in the basal medium the flasks were examined for differences in growth.

RESULTS

There was no growth response by any of the cultures to nicotinic acid, pyridoxin, folic acid, *p*-aminobenzoic acid, inositol, vitamin B_{12} or riboflavin. Strains of *Rhizobium trifolii*, *R. leguminosarum* and *R. phaseoli* were alike in their requirement for biotin, thiamine and calcium pantothenate (Table 1). Of the 31 strains of these 3 species, 5 responded to both biotin and calcium pantothenate, 8 to thiamine and calcium pantothenate and 10 to thiamine, biotin and calcium pantothenate. Only 5 of the 31 strains did not require calcium pantothenate for maximum growth. In several instances both thiamine and calcium pantothenate were essential to initiate growth (Fig. 1). Biotin was also required by strains of *R. lupini* and *R. meliloti*, but the frequency with which it was required appeared to be much less than that suggested by earlier workers (Allen & Allen, 1950). Thiamine was also required by one of 7 strains of *A. radiobacter* and 2 of 11 strains of *A. tumefaciens*.

Strains of *R. lupini*, *R. japonicum* and the cowpea rhizobia did not appear to require most of the vitamins studied in this work, although some unknown growth factor present in the yeast extract mannitol medium was absent from or deficient in the defined one. On transfer from YM agar medium these bacteria grew adequately

Table 1. *Vitamin requirements of Rhizobium and Agrobacterium species*

			Species								
			<i>R. trifolii</i>	<i>R. leguminosarum</i>	<i>R. phaseoli</i>	<i>R. lupini</i>	<i>R. japonicum</i>	Cowpea complex	<i>R. meliloti</i>	<i>A. radiobacter</i>	<i>A. tumefaciens</i>
No. strains tested	...	...	16	8	7	7	6	9	10	7	11
Vitamin required											
Biotin	—	—	—	1	—	3	—	2	4	—	—
Thiamine	—	—	—	—	—	—	—	—	—	1	2
Ca pantothenate	2	—	—	—	—	—	—	—	—	—	—
Biotin + thiamine	—	—	—	—	—	—	—	—	—	—	—
Biotin + Ca pantothenate	3	1	—	1	—	—	—	—	—	—	—
Thiamine + Ca pantothenate	3	2	3	—	—	—	—	—	—	—	—
Biotin, thiamine and Ca pantothenate	8	2	—	—	—	—	—	—	—	—	—

Fig. 1. Stimulation of the growth of *Rhizobium trifolii* strain 1 A 31 by thiamine (central disk) and calcium pantothenate (centre, top). Note the absence of growth unless both vitamins are supplied.

at first but in subsequent transfers were much diminished in growth. Certain contaminating fungi overcame this deficiency, but adding L-glutamic acid, DL-tryptophan, DL-methionine, DL-valine, DL-phenylalanine, DL-threonine, L-citrulline, L-cysteine HCl, or L-serine singly or in combination to the medium did not replace

the requirement. Guanine, adenine, thymine, uracil, choline, thioctic acid and ascorbic acid were similarly inactive in making the defined medium like the YM agar.

Bergersen (1961) considered that near-maximal growth of root nodule bacteria can be achieved in a medium in which the only growth-promoting substances are thiamine, biotin and sodium glutamate. It would appear that at least two additional substances, namely, pantothenic acid and an unknown growth factor(s) are important in the nutrition of some of these bacteria. Bergersen's results indicate the necessity for using numerous representative strains in work on *Rhizobium*.

Vitamin and amino-acid requirements have been used in the separation and classification of various soil-borne bacteria (Lochhead, 1952) and in Adansonian classification (Sneath & Cowan, 1958). The present results suggest that they could be equally valuable in classifying the root nodule bacteria. Further taxonomically useful characters are being sought.

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