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Studies on the Plant Pathogenic Corynebacteria;

The Synthesis of B Group Vitamins by Plant Pathogenic Bacteria

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Corynebacterium 屬 植物病原細菌에 關한 硏究

植物病原細菌의 Vitamin B 群의 合成

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Abstract

The results of studies on the synthesis of B group vitamins by plant pathogenic bacteria indicate that most bacteria utilize thiamine, nicotinic acid, biotin and P-Aminobenzoic acid as growth factors.

Riboflavin (vitamin B_2) was produced by most bacterial genera including the Corynebacteria but with the exception of *C. rathay* and *C.fascians*. The results suggest that the ability to produce riboflavin is not a generic characteristic of *Corynebacterium*, and that the accuracy of the ultra-violet light method (one of the diagnostic tests for potato bacterial ring rot disease caused by *Corynebacterium sepedonicum*) must be reconsidered.

Introduction

There are many reports on the vitamin requirements of phytopathogenic Corynebacteria. Mueller[8] concluded that the nonexacting strains of *C.diphtheriae* required no accessory factors whereas the exacting strains required biotin, nicotinic acid, and pantothenic acid, or B-alanine for normal growth. Starr[14] found that most of the plant pathogenic Corynobacteria required thiamin, biotin, and nicotinic acid. Mohanty[7] noted the stimulatory effect of thiamin on *C.fascians*. McLachlan and Thatcher found that *C.sepedonicum* is able to grow in a vitamin-free medium and stated that only pantothenic acid is essential for growth since the synthesis of pantothenic acid is a limiting factor of the growth rate of the organism. Ramamulthi[10] reported that thiamin is essential to nearly all plant pathogenic Corynebacteria, and pantothenic acid is essental to *C. flaccumfaciens*. He also stated that biotin and niacin are required by *C. facians* and *C. michiganense*.

The ultraviolet light method is one of the diagnostic tests for potato ring rot caused by *C. sepedonicum*. It was first developed by Iverson, and Kelly[4] and is based on the presence of a byproduct of bacterial metabolism which emits a green fluorescent glow under ultravoilet light. This method has become a standard test in potato ring rot control programmes. Skaptason[13] reported that the byproduct of *C. sepedonicum* which fluoresced under ultraviolet light was riboflavin. It has also been reported from *C. diphtheria* (the type species of *Corynebacterium*) by Evans, Hardley, and Happoled[2] but studies have never been carried out on the synthesis of riboflavin by other plant pathogenic Corynebacteria.

On the basis of the above information, the authors considered that studies on vitamim B group synthesis by plant pathogenic bacteria were needed in order to

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reconfirm the accuracy of the ultra-violet test as a method of detecting infected potato tubers and to inv estigate the specific vitamin requirements of phytopathogenic bacteria.

Materials and Methods

(a) Test organisms

Table 1 shows the species and strains of bacteria used in the study. Thirty-eight species of bacteria were assayed for production and utilization of B vitamins[1,3,9,15] Ten species of Corynebacteria as the test organisms and 28 other species belong in each genera of Pseudomonas, Erwinia, Xanthomonas, Agrobacterium, Bacillus and Aerobacter as the control were samplified.

Table 2 shows the species of lactic acid bacteria used for the microbioassay of B vitamins.

Table. 1. Genera and species of bacteria assayed for synthesis and utilization of B group vitamins

Genus	Species
Corynebacterium	*insidiosum*
	sepedonicum
	michiganense (2 strains)
	rathay
	fascians (2 strains)
	poinsettiae
	tritici
	flaccumfaciens
	vesiculare
	oortii (3 strains)
Pseudomonas	*eriobotryae*
	marginalis
	syringae
	lachrymans (4 strains)
	coronafaciens
	conjac
	tabaci
	putida
	rodigiousus
	oryzicola
	schuylkilliensis
	mori
	caryophylli
Erwinia	*aroideae*
	milletiae
	carotovora
	herbicola
Xanthomonas	*pruni* (2 strains)
	oryzae (2 strains)
	citri
	hyacinthi
	vesicatoria
Agrobacterium	*tumefaciens*
	radiobacter
Bacillus	*subtilis*
	natto
	megaterium
Aerobacter	*aerogenes*

Table 2. Test organisms used for microbioassy of B group vitamins

Test organisms	Vitamins
Lactobacillus fermenti 36 ATCC 9338 (IAM 1083)	Thiamine
Lactobacillus casei ATCC 7469	Riboflavin
	Pyridoxal
	Folic acid
Lactobacillus arabinosus ATCC 8014(IAM 1084)	Nicotinic acid
	Pantothenic acid
	Biotin
	P-Aminobenzoic acid

(b) Strain maintenance[5,9]

The strains used for the assay of B-vitamins were maintained in Snieszko and Bond's medium; Bacto peptone 0.3%, bacto tryptose 0.3%, bacto yeast extract 0.3%, dextrose 0.3% (percentages are W/V). The pH was adjusted to 7.0 with normal NaOH. This medium supported good growth of all the strains. The lactic bacteria were top cultured at 37°C for 20 hours on a medium containing bacto yeast 2%, proteose pepton 0.5%, dextrose 1%, KH_2PO_4 0.2% and were then maintained in a refrigerator at 5°C. They were recultured once every 3 days on the above medium.

(c) Assay test[11]

The test organisms were shake cultured at 160rpm at 25°C for 10 days in Snieszko and Bond's broth. The free type B-vitamins were then extracted by heat treatment at 80°C for 30 minutes.

(d) Microbioassay of bacteria[59,12]

A uniform medium for microbiological determination of the B-vitamins with various lactic acid bacteria (Table 3) was employed for the test. The double strength medium was added (5ml per test tube) to test tubes containing 5ml each of dilute standard vitamins or test materials. The tubes were then autoclaved at 10 p.s.i. for 5min, inoculated with the lactic acid bacteria and washed 3 times with 0.85% NaCl solution with the rotor running at 3,000rpm for 10min. After the inoculated microbioassay media had been incubated at 35°C for 20 hours, they were sterilized at 100°C for 20 hours, and the turbidity was then measured at 640 mu. The quantities of Bgroup vitamins were calculated from standard curves prepared from the microbioa ssay of the standard vitamin solutions.

Table 3. Double concentration basal medium for microbioassay of B group vitamins

Vitamins		Amino acids	
Thiamine-HCl	1 mg	Casein hydrolysate	10 g
Riboflavin	1 mg	L-Tryptophane	400 mg
Nicotinic acid	2 mg	L-Cysteine-HCl	1.6 g
Biotin	8 r	L-Asparagine	200 mg
Ca-pantothenate	1 mg	L-Tyrosine	400 mg
P-Aminobenzoic acid	200 r	Minerals	
Pyridoxine-HCl	400 r	KH_2PO_4	6 g
Pyridoxal-HCl	1 mg	$MgSO_47H_2O$	400 mg
Pyridoxamine-2HCl	400 r	$FeSO_47H_2O$	20 mg
Folic acid	20 r	$MnSO_44H_2O$	20 mg
Bases		NaCl	20 mg
Adenine sulfate	20 mg	Others	
Guanine-HCl	20 mg	Na-acetate	40 g
Xanthine	20 mg	Glucose	40 g
Uracil	20 mg		

(Amounts per 1 liter of double concentration medium, pH 6.5—6.8)

Table 4. Preparation of the standard B-vitamins

Vitamins	Solvent	Concentration/ml
B_1-HCl	0.02N acetic acid	200 r
B_2	0.3% acetic acid	50 r
NiA	0.02N acetic acid	200 r
Biotin	0.02N acetic acid	8 r
Ca-paA	25% ethanol	200 r
PABA	25% ethanol	40 r
PIN-HCl	25% ethanol	100 r
PAM-HCl	25% ethanol	100 r
PAL-HCl	25% ethanol	100 r
FA	25% ethanol	10 r

Results and Discussion

Results for the tests of 48 cultures of bacteria, representing 39 species classified in 8 genera, are presented in table 5 and 6. The quanities of B group vitamins are tabulated as mr of free type vitamin per 1ml of Snieszko and Bond's medium. The values obt ained by microbiological assay of the cultured media with various bacteria accurately indicate the B group vitamins synthesized by the bacteria and released into the medium after heat treatment.

Table 5. The synthesis of B group vitamins by bacteria

Organism	B-vitamin(mr per 1ml of medium)			
	B_1	B_2	Pal	NiA
Corynebacterium				
insidiosum	18.0	450.0	40.0	0
sepedonicum	51.0	1150.0	74.0	−1300
michganense[1]	7.0	100.0	42.0	− 400
michiganense[2]	− 34.0	500.0	47.0	−1100
rathay	4.0	−100.0	49.0	0
fascians[1]	− 21.0	−400.0	40.0	1400
fascians[2]	− 35.0	0	47.0	−1400
poinsettiae	− 72.0	200.0	47.0	− 600
tritici	16.5	0	40.0	0
flaccumfaciens	626.0	450.0	257.0	1000
vesiculare	− 14.0	300.0	207.0	2100
oortii[1]	1776.0	500.0	9.5	− 450
oortii$_2$[]	976.0	550.0	9.5	− 500
oortii[3]	1256.0	100.0	9.5	− 400
Pseudomonas				
eriobotryae	14.0	100.0	162.0	300
marginalis	4.0	800.0	81.0	1800
syringae	− 28.5	550.0	167.0	7000
lachrymans[1]	2.5	450.0	25.0	800
lachrymans[2]	− 36.0	1200.0	122.0	3650
lachrymans[3]	− 37.5	1300.0	87.0	4100
lachrymans[4]	− 1.0	750.0	42.0	4250
coronafaciens	− 16.0	1700.0	25.0	3250
conjac	24.5	−500.0	65.5	500
tabaci	17.5	0	71.0	11100
putida	5.5	1950.0	172.0	0
rodigiousus	− 8.0	1350.0	73.0	−1600
oryzicola	976.0	350.0	112.0	3100
schuylkilliensis	426.0	250.0	167.0	2750
mori	31.0	−150.0	74.0	2450
caryophylli	22.0	550.0	32.0	2600
Erwinia aroideae	51.0	51.0	1650.0	0
milletiae	− 28.5	50.5	900.0	0
herbicola	8.0	41.0	50.0	− 850
carotovora	− 15.5	25.0	500.0	0
Xanthomonas pruni[1]	− 60.5	117.0	1300.0	13000
pruni[2]	− 18.0	—	—	—
oryzae[1]	4.0	44.0	500.0	650
oryzae[2]	− 4.0	48.5	500.0	1200
citri	− 64.0	—	—	—

hyacinthi	26.0	47.0	0	0
vesicatoria	18.0	54.0	—	1200
Agrobacterium				
tumefaciens	− 35.0	282.0	1650.0	350
radiobactor	− 52.5	47.0	1150.0	250
Bacillus subtilis	− 35.0	162.0	−750.0	− 300
natto	11.5	14.0	0	− 300
megaterium	14.0	25.0	100.0	− 600
Aerobacter aerogenes	− 6.5	42.0	350.0	0

Table 6. The synthesis of B group vitamins by bacteria

Organism	B-vitamin(mr per 1ml of medium)		
	Ca-PaA	Bio	PABA
Corynebacterium			
insidiosum	19250	6.80	− 260
sepedonicum	1400	4.20	− 120
michiganense[1]	6770	8.60	− 110
michiganense[2]	2970	0.20	− 80
rathay	7470	2.80	− 90
fascians[1]	3770	3.90	220
fascians[2]	3970	6.60	− 50
poinsettiae	820	8.60	− 220
tritici	12570	1.00	− 120
flaccumfaciens	− 530	4.70	420
vesiculare	12570	−4.18	− 175
oortii[1]	− 930	3.72	− 170
oortii[2]	− 830	3.92	− 185
oortii[3]	− 840	4.02	− 178
Pseudomonas eriobotryae	11970	0.20	− 160
marginalis	16170	0	− 145
syringae	12170	0	− 110
lachrymans[1]	1970	−1.10	− 220
lachrymans[2]	15970	−3.50	− 60
lachrymans[3]	15070	−1.80	− 10
lachrymans[4]	16070	−4.50	− 155
coronafaciens	6370	0.20	− 290
conjac	−1180	−4.80	240
tabaci	10270	−3.93	− 270
putida	5620	−4.40	120
rodigiousus	10570	−4.00	− 165
schuylkilliensis	2470	2.40	− 270
mori	11170	8.10	− 260
caryophylli	—	—	—
Erwinia aroideae	670	−4.90	− 90

milletiae	3870	−4.80	240
carotovora	670	−4.90	20
herbicola	3620	−4.80	− 40
Xanthomonas pruni[1]	6570	1.00	− 245
pruni[2]	—	—	—
oryzae[1]	3620	4.52	—
oryzae[2]	3320	2.70	− 70
citri	− 140	—	—
hyacinthi	—	19.10	− 40
vesicatoria	—	—	—
Agrobacterium			
tumefaciens	670	0.50	− 250
radiobacter	− 860	22.10	− 340
Bacillus subtilis	9070	18.10	− 300
natto	9770	0.30	− 300
megaterium	− 750	13.10	− 270
Aerobacter aerogens	5470	−3.50	− 290

Among the plant pathogenic Corynebacteria, *C.michiganense*, *C .fascians* and *C. poinsettiae* could not synthesize vitamin B_1 (thiamin). About half the spec ies from the other genera also could not synthesize thiamin and particularly those from the genus *Pseudomonas*.

Nicotinic acid, pantothenic acid, P-aminobenzoic acid and biotin were generally utilized by the bacteria tested in this study. The synthesis of nicotinic acid was detected from 3 species of Corynebacteria; *C. fascians* (rough type), *C.flaccumfaciens* and *C. vesiculare*, and pantothenic acid from all except *C.poinsettiae*, *C. flaccumfacines* and *C. cortii*. *C. vesiculare* was the only Corynebacterium which reduced the con centlation of biotin in the culture medium below that of the standard medium. Most bacteria of other genera utilized biotin (except genus *Xanthomonas*. *Agrobacterium* and Bacillus. P-amino benzoic acid was utilized not only by Corynebactera (except of rough type *C. fascians* and *C. flaccumfaciens*) but also by most bacteria of the other genera. This facts indicate that most bacteria require at least one of the following anino acid for their normal growth; B_1, nicotinic acid, pantothenic acid, P-amino-benzoic acid or biotin.

Similar results have been found from previous studies of plant pathogenic Corynebacteria by other workers. Stare[14] studied the vitamin requirements of phytopathogenic Corynebacteria and showed that most of them required thiamin, biotin and nicotinic acid. Mohanty[7] noted the stimulatory effect of thiamin on *C. fascians*. MeLachlan and Thatcher[6] found that *C. sepedonicum* could synthesize all the vitamins required for its nutrition but that the rate of synthesis of pantothenic acid is a factor limiting the rate of growth of the organism. Ramamurthi[10] reported that thiamin is essential for the growth of most plant pathogenic Corynebacteria, pantothenic acid in addition to thiamin is essential to *C. flaccumfaciens*. He also found that biotin and niacin are required by *C. fascians* and *C. michiganense*.

Pyridoxal was synthesized by all the bacteria studied except *Bacillus subtillis*.

The ultraviolet light test method is based on the fluorescence under ultravoilet light of a green pigment which is a byproduct of bacterial metabolism. The test is one of the diagnostic tests for potato bacterial ring rot disease caused by *C. sepedonicum* and is used in control programmes for the detection of this disease. Skaptason[13] reported that this byproduct fluorescent compound was riboflavin. The synthesis of riboflavin by other plant pathogenic Corynebacteria has never been studied although its synthesis has been reported from *C. diphtheria*, the type species of *Corynebacterium* (Evans, Hardley and, Happold)[2]

The present study was carried out to determine whether the synthesis of riboflavin is a generic char-

acteristic of *Corynebacterium* or a specific characteristic of *C. sepedonicum* and *C. diphtheria.* It was found that riboflavin was produced by all bacteria studied with the exception of *C. rathay*, *P. conjae* and *P.mori.* The ability to synthesize riboflavin, therefore, is not a generic characteristic of *Corynebacterium.* The value of the ultra-violet light method as a diagnostic test of potato bacterial ring rot disease must be reassessed since the absence of fluorescence would indicate that the tubers are free of the disease but presence of fluorescence is not confirmation of the disease since other species of *Corynebacterium*, and indeed of the other genera, also produce riboflavin. Similar resultswere also obtained by Skaptason in 1942[13].

In general, it was found that the ability to synthesize B group vitamins by bacteria was a characteristic of the species or of an individual strain of the species and was not related to its classification.

Corynebacterium 屬 植物病原菌에 關한 硏究

—— 植物病原菌의 Vitamin B群의 合成 ——

적 요

본실험은 *Corynebacterium* 속 식물병원세균에 관한 연구의 일환으로서 *Corynebacterium* 속 식물병원세균의 종 및 속으로서의 특성을 구명함과 동시에 *C. sepedonicum*이 생성하는 Rumiflabin에 기초를 둔 ultra-violet method의 재검토를 위하여 행하여 진것으로 vitamin B群의 정량은 미생물 정량에 의하였다.

대다수의 식물병원세균은 Thiamine (vitamin B_1), Nicotinic acid, Biotin 및 안식향산을 생장물질로서 사용하였다. Riboflavin은 *corynebacterium* 속 식물 병원세균 (*C. rathay* 및 *C. fascians*는 제외)은 물론 타속의 식물병원세균도 생성하는 점으로 *Corynebacterium* 속 식물병원 세균의 특성이 아니라는 점이 구명되었으며 *C. sepedonicum*에 의하여 발생되는 감자의 윤부병과 청고병과의 감별에 사용되어온 Ultra-violet light법은 재검토 되어야 한다.

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