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IDENTIFICATION OF A CHLOROSIS-INDUCING TOXIN OF *PSEUDOMONAS GLYCINEA* AS CORONATINE

ROBIN E. MITCHELL and HARRY YOUNG
Plant Diseases Division, D.S.I.R., Private Bag, Auckland, New Zealand

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Key Word Index—*Pseudomonas glycinea*; bacterial pathogen; soybean; bean; chlorosis; toxin; coronatine.

Abstract—A toxin causing chlorosis in bean and soybean leaves has been isolated from liquid cultures of *Pseudomonas glycinea*, and purified. It has been identified as coronatine, a toxin produced also by *Pseudomonas coronafaciens* var. *atropurpurea*.

INTRODUCTION

Pseudomonas glycinea is thought to be closely related to *Pseudomonas phaseolicola* and it infects *Glycine* spp. (e.g. soybean) resulting in local and systemic chlorosis symptoms similar to those caused on *Phaseolus* spp. (bean) by *Ps. phaseolicola*. Culture filtrates of *Ps. glycinea*, when applied to leaves of soybean, give chlorosis symptoms like those of the disease itself, indicating the presence of a toxin. It has been presumed that *Ps. glycinea* and *Ps. phaseolicola* produce the same or closely related toxins [1], but this has no convincing experimental basis. However, biologically active culture filtrate from *Ps. glycinea* was recently found not to contain phaseolotoxin ([N^{δ}-phosphosulfamyl] ornithyl-alanylhomoarginine) [2], the toxin characteristic of *Ps. phaseolicola* [3]. We now report the isolation and characterization of a *Ps. glycinea* toxin.

RESULTS AND DISCUSSION

Our finding that the *Ps. glycinea* toxin had acidic character and was ethyl acetate extractable simplified its isolation and purification. The final PLC purification revealed a second biologically active component which has not yet been identified. The main toxin was homogeneous on TLC and by MS. The MS studies indicated a molecular formulation $C_{18}H_{25}NO_4$. The single product of methylation, which had no chlorosis-producing activity, was formulated as $C_{19}H_{27}NO_4$, and two fragment ions, $C_{12}H_{15}O_2$ and $C_7H_{12}NO_2$, were consistent with a molecular fragmentation at an amide bond. The *Ps. glycinea* toxin therefore had the same characteristics as coronatine, obtained from *Ps. coronafaciens* var *atropurpurea*, a pathogen of *Lolium multiflorum* Lam. (Italian ryegrass) [4]. Coronatine causes chlorosis on the leaves of Italian ryegrass. An authentic sample of coronatine co-chromatographed with the toxin during TLC (both UV absorbing), and gave the same MS under our conditions. We conclude that the *Ps. glycinea* toxin is coronatine (or a sterioisomer of coronatine), whose structure has already been reported [4].

This finding is of bacteriological interest since it suggests a relationship, not previously suspected, between *Ps. glycinea* and *Ps. coronafaciens* var *atropurpurea*. With respect to toxin production, these two organisms now form a fourth group of phytopathogenic Pseudomonads. The other three groups are the tabtoxin producers (e.g. *Ps. tabaci*, *Ps. coronafaciens*, and *Ps. coronafaciens* pv *zeae*)[5, 6], the phaseolotoxin producers (various isolates of *Ps. phaseolicola*) [3], and the syringomycin producers (various isolates of *Ps. syringae*) [7].

Although *Ps. glycinea* and *Ps. phaseolicola* are very similar in their bacteriological behaviour, with overlapping host ranges [8], they produce toxins having no chemical relationship. This difference is reflected in the different biological responses of bean leaves to each toxin. Thus phaseolotoxin causes chlorosis in 1–2 days with a sharp boundary between yellow and green tissue, whereas the *Ps. glycinea* toxin is slower to act (2–3 days) with a more diffuse boundary. Also phaseolotoxin causes a greatly elevated ornithine concentration in the chlorotic tissue [9] whilst *Ps. glycinea* toxin does not [10]. Thus the mode of action of the *Ps. glycinea* toxin may be completely different from that of phaseolotoxin. Gulya and Dunleavy [11] have recently reported that partially purified *Ps. glycinea* toxin was an inhibitor of production of aminolevulinic acid, a precursor of chlorophyll, whereas one known effect of phaseolotoxin is its inhibition of ornithine carbamoyltransferase [10]. Further testing of the biological activity of coronatine is required.

EXPERIMENTAL

Culture and bioassay. Ps. glycinea Plant Diseases Division Culture Collection 3482 was grown as for *Ps. phaseolicola* in [2]. Solns were adjusted to pH 6.0–6.5, then bioassayed on bean (*Phaseolus vulgaris*) leaves as in [9]. Beans were used in preference to soybeans because they were easier to grow and use. However the ability of the toxin to produce chlorosis in soybean leaves was confirmed when appropriate.

Chromatographic methods. TLC (0.25 mm, Si gel PF_{254}) was with iPrOH–HOAc–H_2O–EtOAc (8:1:1:390), with UV light and I_2 stain detection. PLC (0.5 mm) used UV detection. Paper electrophoresis (PE), or PC used Whatman 3 MM paper. PE was at pH 7.9 as in [2]. Sephadex LH 20 and QAE columns were prepared and run as in [2].

Isolation and characterization of the toxin. In preliminary studies, the purification sequence was as in [2]: charcoal adsorption, chromatography on QAE Sephadex followed by LH 20 Sephadex. The toxin activity in this preparation after PE had moved 6 cm towards the anode and after PC with BuOH–HOAc–C_5H_5N–H_2O (5:1:4:4) had moved close to the solvent front. As the toxin was showing the characteristics of an organic acid, the main isolation procedure was simplified with an EtOAc extraction step. Supernatant from 9 l. of culture was

stirred (2 min) with activated charcoal (40 g), filtered and toxin extracted from the charcoal as in [2]. Charcoal extracts from 2 batches, combined in 50 ml H_2O with the pH adjusted to 8.5 (Na_2CO_3), were extracted with EtOAc (4 × 50 ml). The aq. soln was adjusted to pH 2.5 (2M H_2SO_4) and again extracted with EtOAc (4 × 50 ml). This second EtOAc fraction, which contained organic acids and biological activity, was dried (Na_2SO_4) and evaptd; the residue in 10 ml MCA was chromatographed on Sephadex LH 20, the active component cluting in 36 ml, commencing after 215 ml eluent was collected. The combined active fractions, when subjected to PLC as the free acids, separated into 3 components: the major (R_f 0.65) was not biologically active (discarded), the second yielded pure toxin (TLC R_f 0.40), the third (minor, R_f 0.25), also biologically active, was collected for further study. The toxin MS had *m/e* 319 (M^+), 301 ($M^+ - H_2O$), 259, 191, 163 and 145, all of similar intensities in the MS recorded for authentic coronatine [4]; mass measurements: 319.1797, calcd. for $C_{18}H_{25}NO_4$: 319.1784; 191.1054, calcd. for $C_{12}H_{15}O_2$: 191.1072. The toxin was methylated with CH_2N_2, affording a single TLC homogeneous product (R_f 0.50) after elution with EtOAc–$CHCl_3$ (1:4), MS *m/e* 333 (M^+), 301 (M^+ – MeOH), 191, 163, 145 and 142; mass measurements: 333.1934, calcd. for $C_{19}H_{27}NO_4$: 333.1940; 191.1078, calcd. for $C_{12}H_{15}O_2$: 191.1072; 142.0855, calcd. for $C_7H_{12}NO_2$: 142.0868; consistent for methylcoronatine.

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2,3-DI-*O*-PHYTANYL-*sn*-GLYCEROL AND PRENOLS FROM EXTREMELY HALOPHILIC BACTERIA

S. C. KUSHWAHA and M. KATES

Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

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Key Word Index—Halophilic bacteria; lipids; ether lipids; prenols; di-*O*-phytanyl glycerol.

Table 1. Contents of 2,3-di-*O*-phytanyl-*sn*-glycerol, geranylgeraniol and phytanol in extremely halophilic bacteria

Species and Strain No.*	% Content of† 2,3-di-*O*-phytanyl-*sn*-glycerol	Geranylgeraniol	Phytanol‡
Halobacterium cutirubrum, 54001	8.5	11.3	0.30
H. halobium, 34020	7	13	0.40
H. halobium M, 34014	10	9	0.20
H. salinarium, 34002	9	13	0.35
Amoebobacter morrhuae, 51001	20	3	0.06
Sarcina litoralis, 16006	12	10	0.25

* Details of the microorganism used and their numbers from the National Research Council of Canada culture collection are given in our previous communication [1].
† % Content is given as % by weight of the total neutral lipids.
‡ Determined by GLC using methyl palmitate as internal standard.

Several strains of extremely halophilic bacteria have been shown to contain carotenoids, MK-8, retinal and squalenes [1]. This paper reports on the remaining neutral lipid components which have been isolated and characterized.

From each of the strains listed in Table 1, three colour-