Phytochemistry, 1967, Vol. 6, pp. 977 to 982. Pergamon Press Ltd. Printed in England

ALKALOIDS OF BACTERIA

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(*Received* 4 *January* 1967)

Abstract—A method is given for determination of alkaloids in fresh bacterial cultures using two alkaloid reagents in conjunction with gas chromatographic analysis. In a screening of thirty species of bacteria, the following bacteria not previously known to contain alkaloids yielded base fractions that gave positive reactions with both Mayer's reagent and silicotungstic acid, and in addition contained peaks in their gas chromatograms indicating compounds with molecular weights greater than 175: *Corynebacterium pseudodiphtheriticum*, *Neisseria perflava*, *Proteus mirabilis*, *Pseudomonas mildenbergii*, *P. taetrolens*, and *Streptococcus lactis*‖ and the following gave a positive reaction with one reagent and had components with molecular weights greater than 175: *Aerobacter aerogenes*, *Acetobacter aceti*, *Bacillus circulans*, *Gaffkya tetragena*, *Micrococcus roseus*, *Pseudomonas putida*, *Proteus vulgaris*, and *Sarcina lutea*. The following bacteria previously reported to contain alkaloidal compounds gave positive reactions with both reagents and had peaks in their chromatograms representing compounds with molecular weights greater than 175: *Pseudomonas aeruginosa* and *Serratia marcescens*.

INTRODUCTION

IT HAS long been thought that alkaloids are virtually absent from the lower groups of plants.[1,2] For years the only noteworthy exceptions among the micro-organisms were a small group of fungi and bacteria.[3–6] Recently, however, the number of fungi reported to contain alkaloids has been increased substantially.[7–15] There has been no corresponding increase in the number of bacteria reported to contain alkaloids, although it has been stated that culture filtrates of *Bacillus anthracis* contain a substance with epinephrine-like activity.[16] Consequently, it was decided to undertake a screening program to determine the frequency of occurrence of alkaloids in certain representative species of bacteria.

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TABLE 1. CULTIVATION DATA

Micro-organism	Cultivation conditions				
	Source*	Media†	Time (hr)	Temp. (°)	Residual cell (wt., g) after extraction
Aerobacter aerogenes (Kruse) Beijerinck	L	D	44	30	1
A. cloacae (Jordon) Bergey *et al.*	L	D	48	30	2
Bacillus cereus Frankland and Frankland	L	D	20	37	3·1
Escherichia coli (Migula) Castellani and Chalmers	L	D	60	37	10
Pseudomonas aeruginosa (Schroeter) Migula	L	D	24	37	5·5
P. fluorescens Migula	L	D	26	37	13
P. fragi (Eichholz) Huss	V	D	45	31	4
P. fragi	C	D	45	30	0·5
P. mildenbergii Bergey *et al.*	V	D	28	31	8
P. putida (Trevisan) Migula	V	D	62	30	11·5
P. taetrolens Haynes	V	D	65	29	11
Serratia marcescens Bizio	L	D	20	37	13
Acetobacter aceti (Beijerinck) Beijerinck	C	Y	36	30	8·5
Bacillus cereus	L	Y	34	30	15
B. circulans Jordan	L	Y	45	30	10
B. cereus var. *mycoides* (Flugge) Smith *et al.*	L	Y	20	30	17
B. subtilis Cohn	L	Y	16	37	15
Corynebacterium pseudodiphtheriticum Lehmann and Newmann	L	Y	64	37	3·5
Flavobacterium aquatile (Frankland and Frankland) Bergey *et al.*	V	Y	42	30	2
Gaffkya tetragena (Gaffky) Trevisan	L	Y	40	37	0·5
Klebsiella pneumoniae (Schroeter) Trevisan	L	Y	21	37	10
Micrococcus lysodeikticus Fleming	L	Y	70	37	9
M. roseus Flugge	L	B	46	30	3
Mycobacterium phlei Lehmann and Newmann	L	Y	40	37	0·6
Neisseria perflava Bergey *et al.*	L	Y	68	37	1
Pseudomonas mephitica Claydon and Hammer	V	Y	19	30	4
Proteus mirabilis Hauser	L	Y	17	30	10·5
P. vulgaris Hauser	L	Y	28	37	9
Sarcina lutea Schroeter	L	Y	21	37	11
Staphylococcus epidermidis (Winslow and Winslow) Evans	L	Y	24	37	11
Streptococcus lactis (Lister) Lohn's	L	B	21	28	2·6
Vibrio percolans Mudd and Warren	C	Y	20	28	8·5

* L, Dr. S. T. Lyles, Biology Department, Texas Christian University; V, Dr. C. Vanderzant, Animal Science Dairy Section, Texas A and M University; C, Carolina Biological Supply Co.

† D, defined; Y, yeast extract and salts; B, brain heart infusion.

All species of bacteria investigated were cultivated in 10-l. batches and harvested by centrifugation (Table 1). The fresh cells were suspended in ethanol, treated with ultrasonic energy,[17, 18] and extracted with acetic acid-ethanol. The ether-washed aqueous acid soluble part of the extract was made basic and extracted with chloroform. The chloroform extracts were freed of solvent, leaving a residue of crude bases which was tested with Mayer's reagent and analyzed by gas chromatography. If a positive reaction with the reagent was noted, or if the chromatogram contained significant peaks, then the crude bases were purified, tested with Mayer's reagent and silicotungstic acid; and re-analyzed by gas chromatography. Positive results with both reagents in these confirmatory tests,[12] or a positive reaction with one reagent combined with a chromatogram containing peaks with retention times representing compounds of molecular weight greater than 175 were considered presumptive evidence for the existence of alkaloids in the bacterial cells.

RESULTS AND DISCUSSION

The screening procedure used in this survey was a modification of that used by Wall *et al.*[19] This method was compared with several others by Euler and Farnsworth,[20] who found it to be one of the best from the point of view of specificity and gave a minimum of false-positive tests. Gas chromatography has been demonstrated to be a useful analytical technique for the detection, separation, isolation, and identification of alkaloids.[21–26] It is well adapted to a survey such as this because of its speed and sensitivity. However, it is limited to compounds that can be vaporized in the instrument, and it is essential that the base fraction be free of non-basic material. For this reason the purification process used in the Wall procedure was repeated twice more for our experiments.

The results from the screening of thirty species of bacteria for alkaloids are presented in Table 2. Of the bacteria examined ten species gave positive tests with both reagents and thirteen gave a positive test with one reagent. The approximate molecular weights of the compounds corresponding to each retention time reported in Table 2 can be roughly estimated from Fig. 1, which is a plot of the retention times of twelve known alkaloids vs. their molecular weights.[25] The molecular weights found, ranging from 150 to 400, indicate the "alkaloidal" nature of some of the bases as opposed to simple amine substances. The occurrence of alkaloidal compounds in two of the species studied, *Pseudomonas aeruginosa* and *Serratia marcescens*, had been previously reported.[28–30]

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TABLE 2. RESULTS

Family*	Species	Confirmatory tests: Initial test† Mayer's reagent	Mayer's reagent	Silicotungstic Acid	Gas chromatographic retention times (min)
Pseudomonadaceae	*Pseudomonas aeruginosa*	+	+	+	6·83, 9·83, 15·67
	P. fluorescens	+	+	+	—
	P. fragi	+	−	+	5·4
	P. mephitica	−	−	−	2·33, 5·5
	P. mildenbergii	+	+	+	5·4
	P. putida	+	+(weak)	−	5·4. 8·33
	P. taetrolens	+	+	+	—
	Acetobacter aceti	+	−	+	1·00, 2·42, 5·68
Spirillaceae	*Vibrio percolans*	+	+(weak)	−	8·0, 9·61
Mycobacteriaceae	*Mycobacterium phlei*	+	+(weak)	−	—
Achromobacteraceae	*Flavobacterium aquatile*	+	−	−	—
Enterobacteriaceae	*Escherichia coli*	+	−	+	—
	Aerobacter aerogenes	+	−	+	—
	A. cloacae	−	−	−	—
	Klebsiella pneumoniae	−	−	−	—
Serratieae	*Serratia marcescens*	+	+	+	2·22, 6·22, 9·95, 11·83, 12·4
Proteeae	*Proteus mirabilis*	+	+	+	2·66, 5·25
	P. vulgaris	+	+	−	2·66, 5·25
Micrococcaceae	*Micrococcus lysodeikticus*	+	−	−	—
	M. roseus	+	+	−	5·0, 5·42, 8·17
	Staphylococcus epidermidis	−	−	−	5·0, 5·35, 5·92
	Gaffkya tetragena	+	+(weak)	−	—
	Sarcina lutea	+	+(weak)	−	5·33
Neisseriaceae	*Neisseria perflava*	+	+	+	2·33, 5·25, 7·61
Lactobacillaceae	*Streptococcus lactis*	+	+	+	1·33, 1·88, 2·33, 5·5
Corynebacteriaceae	*Corynebacterium pseudodiphtheriticum*	+	+(weak)	+(weak)	2·68, 6·0
Bacillaceae	*Bacillus cereus*	+	+	−	1·21, 2·17, 6·3, 8·0
	B. cereus var. mycoides	+	+	+(weak)	8·0
	B. circulans	+	+	−	5·61
	B. subtilus	+	−	−	—

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† + and − designate positive and negative reactions.

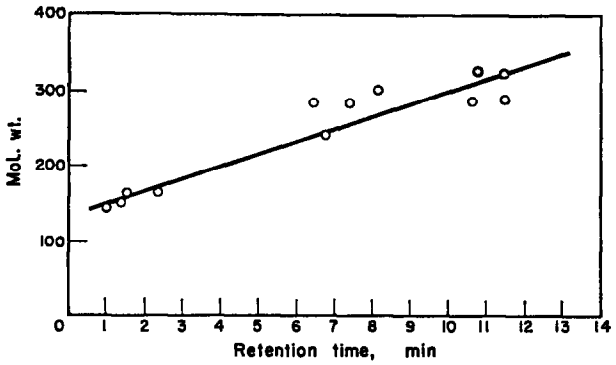

FIG. 1. PLOT OF THE RETENTION TIMES OF SOME KNOWN ALKALOIDS VS. THEIR MOLECULAR WEIGHTS, ADAPTED FROM REFERENCE 25.

Betaines, quarternary compounds, phenolic compounds, and certain non-quaternary substances that might be regarded as alkaloids (psilocybin, lysergic acid) but which are soluble in the alkaline-aqueous phase, would not be detected by the confirmatory tests. The possibility that positive reactions with the reagents might be caused by phenylethylamine or tryptamine (from decarboxylation of the respective amino acids) was eliminated by the absence of peaks at the retention times of the amines. The possibility of contamination from the yeast extract used as nutrient in some of the media was eliminated by the observation that chloroform extraction of 100 g of the extract used in aqueous solution yielded a negligible base fraction that did not give a reaction with the reagents in the chemical tests and gave only nominal background when gas chromatographed.

Repeated analyses of different batches of the same species of bacteria indicated that the results were reproducible. However, in some cases the relative amounts of the contained alkaloids could be varied simply by changing the media and/or cultivation conditions used. In the case of *Pseudomonas mildenbergii* it was observed that the media contained the same alkaloids as the cells, but in larger quantities, and we are attempting to isolate the individual alkaloids for structural analysis.

The high incidence of alkaloids observed in bacteria in this limited study (46·7 per cent) was unexpected. The incidence of alkaloids in higher plants is commonly believed to be about 5–10 per cent.[1, 2] The incidence of alkaloids in 254 species of higher fungi recently studied by Tyler and Stuntz was only 2·8 per cent.[12, 13]

This study indicates that alkaloids, or alkaloid-like compounds, occur in a wide range of bacteria, and that these micro-organisms are potential sources of new alkaloids. However, examination of a much larger number of additional species and families will be required before valid generalizations can be made concerning the overall distribution and significance of alkaloidal material in bacteria.

EXPERIMENTAL

Micro-organisms. The organisms examined are listed in Table 1. Cultures labeled L were obtained from Dr. S. T. Lyles, Texas Christian University Biology Department; cultures labeled V were obtained from Dr. C. Vanderzant, Texas A and M University Animal Science Dairy Section, College Station, Texas; and cultures labeled C were obtained from Carolina Biological Supply Company, Burlington, North Carolina.

Cultivation of micro-organisms. The bacteria were cultivated in a 14-l. New Brunswick MF 114S Microferm fermenter equipped with an automatic foam control system (New Brunswick Scientific Company, Inc., New Brunswick, New Jersey). Sterile media (10 l.) were inoculated with 100 ml cultures of bacteria. Both the

initial starting cultures and the final 10·1 cultures were routinely screened for contaminating organisms. The chemically defined media of Gavin and Umbreit[27] was used when possible. The media contained 7 g K_2HPO_4, 1 g NaCl, 0·7 g $MgSO_4$, 0·5 g sodium citrate, 4 g $(NH_4)_2SO_4$, 1 g DL-phenylalanine, 0·1 g L-aspartic acid, and 10 g glucose per liter of distilled water. Organisms which did not grow on this medium were cultivated either on the same medium with yeast extract (Baltimore Biological Laboratory) replacing the amino acids and glucose, or on brain heart infusion broth (BBL). The media were aerated with filtered air at a rate of 2–4 l./min and agitated at 200 rpm in all cases. Cells were harvested with a Sharples centrifuge.

Cell extraction. The collected cells were suspended with 95% ethanol, treated with a Bronwill Biosonic Probe (Will Corp., Rochester, N.Y.) for 15–30 min, and then extracted with 95% ethanol–1% acetic acid in a Soxhlet for 24–48 hr. The alcohol extract was concentrated under vacuum, 5% HCl (50 ml) added, and the remaining alcohol evaporated.

Alkaloid fractionation and testing procedure. The filtered aqueous solution was washed with ether (3 × 100 ml), made basic with Na_2CO_3 and the crude bases extracted with $CHCl_3$ (3 × 100 ml). This extract was washed with water, reduced to dryness, and the residue dissolved in 1 ml $CHCl_3$. A 0·1 ml portion of this solution was placed in a test tube and evaporated to dryness. As much of the residue as possible was dissolved in 1 ml 5% HCl and 2–3 drops Mayer's reagent added.[2] The appearance of a distinct turbidity was taken as a positive test.

If a positive reaction was obtained in this initial test the remaining crude bases were purified by partitioning between $CHCl_3$ (2 ml) and acidic and then basic water (2 × 3 ml) twice more, or until the residue on evaporation of $CHCl_3$ appeared to be completely soluble in 5% HCl (1 ml). The acid soluble residue was then re-tested with Mayer's reagent and 12% silicotungstic acid solution.

The alkaloid containing residues were examined by gas chromatography.[25] A Barber–Colman Model 5000 gas chromatograph equipped with a linear temperature programmer, an automatic cool down module, and a flame-ionization detector was used. The column was 180 × 0·3 cm o.d. aluminum tubing packed with 1% JXR on 100/120 mesh silanized Gas Chrom P (Applied Science Laboratories, State College, Pa.). The flow rate of nitrogen carrier gas was 58 ml/min. The column temperature was programmed from 100 to 300° at 15°/min; the injection port and the detector bath were kept at 300°. The retention times reported were measured from the emergence of the solvent front to the top of the peak.

Acknowledgements—This investigation was supported in part by grants from Research Corporation (101865) and from the Texas Christian University Research Foundation (C6561). The bacteria were cultivated by R. H. Scott. The authors wish to thank Dr. S. T. Lyles and his staff for their assistance during the course of this investigation.