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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject as results come to hand. It is better to wait until a comprehensive paper can be written.

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.) as well as attempting to convey their meaning to their readers.

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Chemical Nomenclature. Follow the 1969 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 7 Warwick Court, London, W.C.1.

Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in the *Journal* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The correct name of the organisms, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in brackets when the name is first mentioned. Names of bacteria must conform with the Bacteriological Code and the opinions issued by the International Committee on Bacteriological Nomenclature. Names of algae and fungi must conform with the International Rules of Botanical Nomenclature. Names of protozoa must conform with the International Code of Zoological Nomenclature. Bacteriological Code, Iowa State College Press, Ames, Iowa, U.S.A. (1958); Botanical Code, International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952); Zoological Code, International Trust for Zoological Nomenclature, London (1961). One or two small changes have been made to these rules at later International Congresses.

The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

Ainsworth and Bisby's Dictionary of the Fungi, 5th ed. (1961). Kew: Commonwealth Mycological Institute.

Latin Names. The species name is in italics (underlined once in typescript) and is used in full at first mention in each paragraph, but in subsequent mention with the name of the genus abbreviated, single letter abbreviations being used where they are not ambiguous. The genus name is in italic when the whole genus is referred to. When used trivially, genus names are in Roman (not underlined). Anglicized versions are not underlined and are used without capitals. Strain names or numbers are in small capitals (underlined twice in the typescript).

Descriptions of new species should not be submitted unless an authentic specimen has been deposited in a recognized culture collection.

D. W. W. HENDERSON, C.B., D.Sc., F.R.S.

(*Facing p.* 145)

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Obituary Notice

D. W. W. HENDERSON, 1903–1968

President of the Society for General Microbiology 1963–64

David Willis Wilson Henderson, the only child of John Henderson, a chartered accountant, was born in Glasgow on 23 July 1903. He attended Hamilton Academy but was a rather rebellious student, working well at science and subjects which interested him but giving scant attention to Latin, which he disliked. At his own insistence he was articled to a farmer as a prelude to a career in agriculture, but the experiment was not a success. The streak of impatience with arbitrarily imposed authority which became a notable feature of Henderson's character in later life asserted itself at this juncture with the result that the young David, with somewhat sceptical parental agreement, left the farm to read for a degree at Glasgow University. He chose agricultural bacteriology as his major subject and enrolled under Professor J. F. Malcolm at the West of Scotland Agricultural College, where he graduated in 1926.

Henderson's first post was as lecturer in bacteriology at King's College, University of Durham, where in 1930 he received a M.Sc. degree and married Beatrice Mary Davenport, daughter of Sir Westcott Abell, K.B.E. Henderson entered the Lister Institute of Preventive Medicine, London, in 1931 with a Carnegie Research Fellowship; at the end of its second year he obtained a Beit Memorial Research Fellowship for the years 1932–35. In 1934 he was awarded a Ph.D. degree in the University of London for a thesis entitled 'Studies on the spore-bearing anaerobes with experiments on active and passive immunity'.

Henderson remained at the Lister Institute and at the approach of war in 1939 he was at the Institute's Serum Department at Elstree, dealing mainly with the immunology of Salmonella and Clostridium species. Soon after the outbreak of war he showed that mice could be killed by clostridial toxins administered in aerosols. His Director, Sir John Ledingham, F.R.S., considered this result of sufficient importance for official action and in the early summer of 1940 Henderson found his time divided between Elstree and the Chemical Defence Experimental Establishment, Porton, Wiltshire, which already had experience in handling toxic aerosols. In October 1940, as a result of instruction from the War Council, he joined a group of microbiologists which was being established at Porton to assess the feasibility of the use of biological agents against man and to devise methods of protection against such attacks. Henderson was a prominent member of this group, though he remained a member of the staff of the Lister Institute until 1946. In 1943 a small party crossed the Atlantic and when it returned Henderson remained behind to assist Americans with their efforts on biological defence. Thereafter, till the end of the war, he shuttled across the Atlantic, making experiments wherever better facilities were to be found. Henderson was the mainstay of the British–American liaison; he became a valuable and fruitful collaborator of a number of American microbiologists and made among them many lasting friendships.

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By the end of hostilities, Henderson was convinced that attack on man by biological agents was possible and that the provision of protection against it was rudimentary. However, the magnitude of the threat was not established and *ad hoc* experiments done during a 'war for survival', though justified at that time, were not likely to give a realistic evaluation. A long-term programme was needed to investigate the physical, chemical and biological factors which determined the survival of micro-organisms in aerosols, and the genetic and environmental factors which determined microbial virulence and antigenicity. Who was to lead this effort? The war-time group was dispersing and Henderson was determined to participate only on his own terms, namely in basic studies in microbiology based on the programme sketched above, coupled with the provision of tailor-made laboratories and the highest priority to recruit the requisite staff to man it. After much discussion, ultimately at Chief-of-Staff and Cabinet level, the Minister of Supply was instructed to implement a programme much on the lines that had been suggested, with the priority necessary to get the project moving. In January 1946 Henderson was offered the post of 'Chief Superintendent of the Biological Research Establishment' and characteristically accepted as 'Director, Microbiological Research Department'. A powerful advisory board was enlisted under the chairmanship of Lord Hankey, Minister without Portfolio in the War Cabinet; thus was Henderson's course set for nearly 20 years.

The new Department continued to occupy part of the animal house of the Physiology Section of the Chemical Defence Experimental Establishment supplemented by prefabricated huts built during the war. Additional laboratories of standardized unit construction were added to house the staff that Henderson was busy recruiting. This was not an easy business, partly due to the opprobrium associated with the work and an active lobby against it and partly because of Henderson's belief that top-quality research work could arise only from dedication. His demands and standards for recruits were very high. He tended to recruit individuals, rather than to man a programme, and to rely on his powers of persuasion and infectious enthusiasm to weld the sometimes ill-assorted bunch into co-operative effort.

Meanwhile, plans for the new laboratories went ahead. Henderson based his concept on the laboratory of the National Institute for Medical Research at Mill Hill, planned before the war but still under construction. He wished to cover the same wide spread of scientific disciplines from the physical sciences to the biological sciences and to make special provision for the growth and handling of pathogenic micro-organisms on a considerable scale. He often set specifications so stringent as to be unrealistic. These he would only relax when convinced that they were unattainable. Building started in June 1948 and Henderson drove it forward every inch of the way, unleashing thunderbolts on officials of the Ministry of Works or on their contractors every few weeks. In consequence the building was completed in 3 years against the estimate of 2 years 9 months which Henderson had forced on a sceptical Ministry of Works. He took possession of a laboratory, unique of its kind in the United Kingdom, which served for many years as a model for microbiological buildings in many parts of the world.

Now followed the harvest; by 1959 Henderson and his staff had established an international reputation in microbiology. Henderson's cardinal role was recognized by the award of a C.B. in 1957 and scientifically by his election to the Royal Society in 1959.

In October 1959 the Ministry of Supply was dissolved and Henderson fought to have his department transferred to civilian control, but in vain; it passed to the War Office. This change in direction and the increasing complexities of administration depressed Henderson, but he continued to foster active research throughout the Establishment. The strain, however, was beginning to tell. Hypertension, established in the early 1950s, was becoming troublesome and Henderson, who had always been a heavy smoker, was increasingly subject to attacks of respiratory infection. By the time he reached the age of 60 these attacks were beginning to make the winters miserable for him unless he could escape into sunshine for a couple of months after Christmas. Within a year he, who had never been able to understand why people retire early, was seeking a successor. No one was more pleased than he when Dr C. E. G. Smith succeeded him in August 1964. By the end of 1964 he had completed the handover, and avoiding involvements in matters of policy or management with an almost pedantic correctness, he went back to the laboratory. He was delighted to be back at the bench, but several minor thromboses from which he recovered almost completely left his walking slightly impaired. Another attack in July 1967 brought his active work to an end. He was in and out of hospital several times during the following year but his condition slowly deteriorated and he died quietly in his sleep on 16 August 1968.

Henderson's contributions to the advance of microbiology were threefold: personal research, creative work, and guidance of the Microbiological Research Department, and interest in the affairs of the Society for General Microbiology. By far the greatest contribution was the second; the influence of Henderson's department on contemporary microbiology has been considerable.

Henderson's published research work was not large; directing his department left little time for personal research. His work was characterized by ingenuity and thoroughness. Before the war at the Lister Institute he came under the influence of Dr Muriel Robertson, and studied the immunology of the obligate anaerobes, particularly *Clostridium chauvoei*, *C. oedematiens* and *C. welchii*, and he co-operated with Professor W. T. J. Morgan in investigating the antigens of *Salmonella typhi*. Because of this early work, Henderson always called himself an immunologist, but as a result of his experience at Porton during and after the war he was more an experimental pathologist with special expertise in the techniques for studying respiratory infection and the subsequent spread of infection in the host. During the war he evolved an apparatus, known familiarly as 'the piccolo', for exposing animals to aerosols of pathogenic bacteria such as *Bacillus anthracis* and *Pasteurella pestis*, at known concentrations. The details of this apparatus were published in 1952 (*Journal of Hygiene* (1952), **50**, 52); since then modifications of the 'piccolo' have been used throughout the world for controlled respiratory infections. With this apparatus and a more sophisticated sequel, 'the organ', Henderson and his colleagues studied the effect of particle size on respiratory infection with *B. anthracis*, *P. pestis* and *Brucella suis*; the importance of small particles in establishing infection deep in the lung was recognized. Next Henderson turned his attention to mixed infections by the respiratory route. He showed the profound effect that one infection could have on the course of another; for example, guinea pigs could be protected from anthrax and plague by first having brucellosis. Henderson summarized his work on mixed infections in the 14th Symposium of the Society for General Microbiology (1964). Towards the end of his life Henderson became interested

in the pathogenesis of virus infections, despite a healthy scepticism of some methods of their assessment epitomized in his oft repeated remark 'virologists can't count'. His last paper (*British Journal of Experimental Pathology* (1967), **68**, 228) showed the route with which a respiratory infection with Semliki Forest virus reaches the brains of hamsters with astonishing rapidity.

In guiding the research of the Microbiological Research Department, Henderson had three main themes. First, in studies of infectious disease emphasis should be on initiation and pathogenesis of infection and not, as in most medical research, on therapy. Secondly, all disciplines—biochemistry, pathology, genetics and immunology—should be concentrated on relatively few micro-organisms. *Bacillus anthracis*, *Pasteurella pestis*, *Brucella* spp. and pox viruses received concentrated treatment. Thirdly, there should be better methods for producing large quantities of microbes than by batch culture in large tanks. These themes, coupled with Henderson's flair for allowing the maximum freedom for individual scientists to pursue their ideas, led to first-rate work, much of it of international repute. The department was especially noted for work on microbial infection by the respiratory route, factors involved in microbial survival, virulence and antigenicity, methods for handling dangerous micro-organisms safely, and continuous culture techniques. One has only to consult the journals, the proceedings and symposia of the Society over the years of Henderson's stewardship at Porton to realize the tremendous influence he had on microbiology through the work of his department.

Henderson was an original member of the Society for General Microbiology. He was a member of the Committee which guided the affairs of the Society in its formative years, 1947–51. When elected to President in 1963 Henderson was already in failing health. Nevertheless, he was rarely absent from a Council meeting or a General meeting of the Society during his term of office. Much work was done under his chairmanship, and the Officers of the Society and Council members remember the charm and good humour with which he conducted their affairs. His flair for persuading individualists to work together was abundantly apparent.

At the personal level few could remain indifferent to Henderson. He had a keen analytical brain but his judgements were often intuitive and only subsequently sanctioned by reason. He was always forthright, sometimes rude, occasionally downright offensive. He was often a thorn in the flesh of his superiors but his technical assessments were well considered in 'high places'. And he had much personal charm and humour. One suspected that his broad Glasgow accent which got more pronounced when he became excited was sometimes 'tuned up' to enhance the effect. He evoked friendship, respect, admiration and, in some cases, devotion from his staff for whom he fought valiantly across the board. Only rarely did his partisanship outrun his discretion. Several research workers, now with international reputations, have good cause to thank him for the hours he spent in clarifying their papers for publication. As the status of the Establishment as a centre of excellence grew, a number of overseas visitors asked for the use of its specialized facilities and Henderson would go to much trouble to ensure not only that the attachment was agreed but that everything was prepared for the visitor to start work immediately he arrived.

His first wife died in 1952 and in 1953 he married Emily Helen, daughter of the late D. Theodore Kelly of New York, who was herself trained as a bacteriologist. They made their home in Great Durnford on the banks of the Avon between Amesbury

and Salisbury. Their XVth century cottage had a large garden in which Henderson expended much time and labour and from which he derived much satisfaction. And on the rare occasions when he had leisure and the weather was right he would fish in the small stream which formed the western boundary of his property. His second wife survives him.

L. H. K.
H. S.

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Studies on the Fatty Acid Composition of some Salmonellas

By M. J. MODAK, S. NAIR AND A. VENKATARAMAN
Department of Biochemistry and Nutrition, Haffkine Institute, Bombay-12, India

(*Accepted for publication 17 October 1969*)

SUMMARY

The available data on the fatty acid composition of Salmonella organisms is meagre and incomplete; the present work indicates that the three organisms examined have fatty acids ranging from C_{12} to C_{22} in their lipids. Changes in the growth medium may be responsible not only for quantitative differences but also for qualitative changes in the fatty acid spectrum of neutral compounds as well as phospholipids. The organisms can become avirulent possibly through fatty acid alterations of an endotoxin molecule.

INTRODUCTION

The development of our knowledge of bacterial lipids began with the work of R. J. Anderson on the tubercle bacilli in 1920. This work was continued for over 30 years and was concerned with the isolation and characterization of the bacterial fatty acids (including the complex mycolic acid), phosphatides and glycolipids. It is now known that the infective unit or endotoxin in many bacterial species has lipid as one of its constituents. In the early days, researches were mainly directed towards the study of the lipids of Gram-positive organisms which contain more than 15% lipid. Gram-negative organisms having lesser amounts of lipid (below 10%) are now studied to achieve a certain evaluation of the taxonomic approach towards the differences between Gram-positive and Gram-negative organisms. Some results have been obtained in that the Gram-negative organisms have a high proportion of even-numbered saturated and unsaturated acids and odd-numbered cyclic acids; while the Gram-positive organisms have high amounts of the saturated odd-numbered branch-chain acids and relatively low amounts of the straight-chain saturate acids. The occurrence of some unusual fatty acids in the microbial lipids has also stimulated efforts to correlate these lipid molecules with virulence and disease (O'Leary, 1962; Neilson, 1966). Several organisms have been investigated for their fatty acid and lipid composition (Kates, 1964, 1966). With the availability of thin-layer and gas–liquid chromatography, rapid and sensitive techniques have become available for otherwise cumbersome lipid work. Although gas chromatographic methods alone cannot be used for the identification of these components, tentative identifications can be made and used as taxonomic criteria (Abel, Deschmertzing & Peterson, 1963; Asselineau, 1961; Henis, Goult & Alexander, 1966; Steinhauer, Flentge & Lechowich, 1967). Differences in the cultural conditions have been shown to be responsible for the changes in the fatty acid composition by many workers (Kates, Kuchner & Janes, 1962; Marr & Ingraham, 1962; Gavin & Umbreit, 1965; Divakaran & Modak, 1968). As regards Salmonella, not much information on the lipid composition is available

except for a few reports by Cmelik (1952, 1953, 1954, 1955), MacFarlane (1962) and Gray (1962). The present work was to study the lipid composition of three strains of Salmonella and to see the variations, if any. A fatty-acid analyses of the phospholipid and neutral lipid fractions after separation on kieselgel-G has also been included.

METHODS

The cultures of *Salmonella typhi* (TY 2 strain), *S. paratyphi A* and *S. paratyphi B* were supplied by the Bacteriology Department of this Institute (these strains are used for the production of TAB vaccine). The bacteria were grown in 250 ml. Erlenmeyer flasks containing 50 ml. nutrient broth or defined medium (Modak, Nair & Venkataraman, 1968) on a rotary shaker (100 rev./min.) for 18 hr. The cell-mass after centrifugation at 10,000 rev./min. in the cold was washed with cold saline and dried in vacuum over KOH.

Lipid extraction. Weighed amounts of the dried bacteria (about 500 mg.) were suspended in 30 ml. of chloroform + methanol in a 50 ml. beaker and subjected to ultrasonic disintegration for 30 min. at maximum power (MSE ultrasonic disintegrator). The bacterial residue after centrifugation was once again extracted overnight with the solvent mixture at 30°. The combined extracts were filtered through a microsinter glass funnel and the non-lipid contaminants and methanol removed by saline washing as described by Folch, Lees & Sloane-Stanley (1957). The chloroform + methanol was distilled off under a gentle stream of nitrogen and the % lipid on dry weight basis calculated. The lipid extracts were divided into two parts. One part was used for the gross fatty acid pattern, while the other portion was applied on kieselgel-G for the separation of phospholipid and neutral lipid fraction. The kieselgel-G (E. Merck) was activated by heating to 105° for 18 hr before use (for adsorption). Neutral lipids were first eluted with chloroform, while the phospholipid was eluted with methanol. The individual fractions of these lipids were concentrated under nitrogen and transmethylated as described below.

Preparation of methyl esters. Fatty acids from total lipid, neutral lipid and phospholipid were transesterified directly as described by Peterson, Deschmertzing & Abel (1962) and Morrison & Smith (1964) by boiling the lipids for 5 min. in 5 ml. of 10% boron trifluoride in methanol (Applied Science Laboratories) followed by the addition of 3 ml. normal saline and extraction with ether. The solvent phase was evaporated under nitrogen.

Hydrogenation and bromination of unsaturated methyl esters. An apparatus like that described by Brian & Gardner (1968) was used. About 5 mg. samples of methyl esters of fatty acids were taken up in 20 ml. of ethyl acetate into a 50 ml. Quick-Fit flask and platinum catalyst (200 mg. of 5% platinum oxide on charcoal) added. The flask was connected to the hydrogen source (a cylinder) and the contents kept stirred with a magnetic stirrer, after flushing with hydrogen. When hydrogen uptake ceased (60 min.) the catalyst was filtered off and the methyl esters dried under nitrogen. A portion of this hydrogenated sample was used for gas–liquid chromatographic analysis while the rest was used for bromination at 50° under nitrogen (1:5 bromine solution in ether) as described by Brian & Gardner (1968). Bromination was necessary to break the cyclic acids which were converted to the straight chain variety.

Gas–liquid chromatography (g.l.c.). All g.l.c. work was done with a Panchromatograph

Table 1. *Fatty acid composition of salmonellas (gross pattern)*

	Organism and growth medium					
	S. typhi		*S. paratyphi A*		*S. paratyphi B*	
Fatty acids	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Total lipid (%)	5·8	5·4	5·6	4·9	5·8	5·2
12:0*	1·5	7·6	1·6	4·6	11·7	3·3
14:0	9·0	9·0	11·5	9·2	10·3	8·1
15:0	0·6	0·65	0·65	0·7	13·9	0·12
16:0	49·5	60·7	56·5	52·2	30·5	25·0
16:1	0·6	0·7	1·6	0·9	4·8	0·13
16:2	—	—	—	—	—	0·76
17:0	Trace	Trace	Trace	Trace	Trace	Trace
17:0 (Cyc.)	0·4	1·0	2·0	1·7	0·37	0·27
18:0	0·28	0·53	0·3	0·25	0·95	Trace
18:1	4·4	2·3	6·1	7·1	9·5	1·3
18:2	11·9	6·0	7·2	6·8	7·15	Trace
19:0	—	—	—	—	—	29·9
19:0 (Cyc.)	14·4	9·2	4·3	3·8	5·28	18·9
20:0	2·3	—	—	—	—	—
20:4	Trace	0·9	0·85	0·9	Trace	2·3
21:0	14·4	0·9	7·8	11·05	5·05	5·3
22:0	4·7	0·7	0·9	0·85	0·5	4·7

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

Table 2. *Fatty acid composition of neutral lipid of salmonellas*

	Organism and growth medium					
	S. typhi		*S. paratyphi A*		*S. paratyphi B*	
Fatty acids	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Neutral lipid (% of the total lipid)	37·6	42·3	38·4	44·2	35·2	40·3
12:0*	2·02	1·57	2·5	2·4	1·03	1·08
14:0	5·95	11·0	11·3	10·2	8·95	10·4
15:0	0·2	0·7	1·38	0·9	0·4	1·1
16:0	62·5	37·5	23·8	30·5	37·4	22·2
16:1	4·2	0·1	8·95	2·3	Trace	26·5
17:0	Trace	Trace	0·1	—	0·4	0·7
17:0 (Cyc.)	1·68	0·2	2·65	3·2	3·75	0·99
18:0	0·13	3·9	0·07	0·9	0·26	0·69
18:1	8·75	0·9	28·0	22·0	0·26	24·4
18:2	—	17·6	—	10·0	6·25	—
19:0 (Cyc.)	5·7	—	9·65	10·2	15·5	3·4
20:0	5·9	16·2	6·4	2·4	—	—
20:4	—	—	2·57	1·6	—	—
21:0	2·5	6·35	3·4	11·2	3·55	1·79
22:0	0·37	0·47	1·54	0·9	3·1	0·5

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

equipped with ^{90}Sr ionizing detector. The g.l.c. conditions were: column packing, 15% ethylene glycol succinate (EGS) polyester on chromosorb W (80 to 100 mesh); column length 5 ft. (118 cm.) × 4 mm. internal diameter; column temperature 184°; detector temperature 210°; argon flow rate 30 to 60 ml./min.

Identification of the g.l.c. peaks. Identification of the g.l.c. peaks was done by: (i) comparing the retention time with standard compounds (Sigma) of known purity; (ii) analysis of g.l.c. results before and after hydrogenation to fix the identity of unsaturated fatty acid methyl esters; (iii) plotting retention times against chain length relative to methyl palmitate and determining the chain length of the unknown peak from the curve, (iv) comparing the retention times relative to methyl palmitate and stearate to those reported in literature (Brian & Gardner, 1968). No differentiation was attempted as regards the position of the double bonds. The percentage composition was calculated by the triangulation method.

Table 3. *Fatty acid composition of phospholipid of salmonellas*

	Organism and growth medium					
	S. typhi		*S. paratyphi A*		*S. paratyphi B*	
Fatty acids	Nutrient broth	Defined medium	Nutrient broth	Defined medium	Nutrient broth	Defined medium
Phospholipid (% of the total lipid)	50·0	45·6	49·8	44·2	52·7	40·2
10:0*	—	—	—	—	—	3·75
12:0	0·33	1·19	6·0	5·8	2·27	5·25
14:0	6·6	7·15	12·0	13·2	7·72	5·25
15:0	1·39	5·6	5·0	6·1	27·2	61·0
16:0	56·5	51·0	68·0	62·0	38·5	0·05
16:1	2·38	—	—	0·9	7·1	0·1
16:2	0·69	0·67	—	—	—	1·2
17:0	1·66	Trace	—	—	—	1·67
17:0 (Cyc.)	Trace	Trace	Trace	Trace	Trace	Trace
18:0	12·3	6·12	2·3	0·9	1·79	4·46
18:1	6·72	9·35	4·1	3·2	3·0	5·25
18:2	3·57	1·52	2·5	—	—	—
19:0	—	—	—	—	4·3	—
19:0 (Cyc.)	Trace	Trace	Trace	10·8	Trace	7·2
20:0	Trace	Trace	Trace	Trace	1·57	4·8
20:4	—	5·6	—	Trace	1·5	Trace
21:0	—	7·6	Trace	Trace	3·0	4·2
22:0	1·49	4·24	Trace	Trace	—	4·8

* The number preceding the colon designates the number of carbons; the number following indicates the unsaturation.

RESULTS

Gross fatty acid composition. The results of the gross fatty acid analyses are given in Table 1. More than 60% of the total fatty acids were C_{16} (palmitic) and the lower acids, palmitic acid being maximum in all the cases. Though there were quantitative differences, all the three organisms showed more or less similar spectra of fatty acids ranging from C_{12} to C_{22}. The change in the growth medium did not affect the spectrum, except in the case of *Salmonella paratyphi B* grown in defined medium where C_{19} acid

appeared in appreciable amounts. This acid was absent in *S. typhi* and *S. paratyphi A* and also in *S. paratyphi B* grown in nutrient broth. Similarly, C_{20} acid was present only in *S. typhi* grown in nutrient broth. The major acids present included C_{14}, C_{16}, C_{21} normal saturated, C_{18} and $C_{18:2}$ normal unsaturated and C_{19} cyclic.

Fatty acid composition of neutral lipids. The neutral lipid fractions of the three organisms were qualitatively similar except for the absence of C_{20} from *Salmonella paratyphi B.* Major acids are C_{16}, $C_{16:1}$, $C_{18:1}$, C_{19} cyclic and C_{20}. In addition, detectable amounts of C_{17} cyclic and C_{21} were also noted (Table 2).

Fatty acids of phospholipids. The major acid in all the three organisms was palmitic acid; high amounts of C_{15} acid were noted in the phospholipids of *Salmonella paratyphi B.* C_{19} cyclic acid was absent (Table 3). A peculiar observation of these analyses was that in *S. typhi* grown on defined medium, $C_{20:4}$ and C_{21} were present to the extent of 5%, though these acids were not noted in the phospholipids of *S. typhi* grown in nutrient broth. C_{17} acid was detected in traces. More than 80% of the fatty acids of the total phospholipids of *S. paratyphi A* were palmitic (C_{16}) and lower.

DISCUSSION

Cmelik (1952, 1953, 1954, 1955) investigated the lipids of several members of the Enterobacteriacea. The total lipid content ranged from 1·5 to 10% of which 50 to 90% was neutral lipid. Fatty acids were reported as being mainly palmitic and C_{18} monoenoic acids. As against this report, MacFarlane (1962) found a lipid content of 5·6% in *Salmonella typhimurium* of which only 14% was neutral lipid while the rest was phosphatidyl ethanolamine. The fatty acids consisted mainly of palmitic (C_{16}), odd-numbered C_{17} and C_{19} cyclopropane, C_{16} and C_{18} monoenoic acids with small amounts of myristic and stearic acids (Macfarlane, 1962; Gray, 1962). Our results agree with those of Cmelik in that the neutral lipid content of the three Salmonella strains examined in the present study was around 40% and palmitic acid was the major fatty acid. Our values of total lipid are in good agreement with those of MacFarlane, for *S. typhimurium.* The fatty acid spectrum we found differs from MacFarlane's report in having lesser amounts of monoenoic hexadecanoic ($C_{16:1}$) and cyclic C_{17} acids. Stearic acid was below 1% while myristic acid was in quite appreciable quantities (about 10%). Amounts of $C_{13:2}$ and C_{21} have been noted in this study which were not detected by earlier workers.

Data on the fatty acid composition of neutral and phospholipids are not available in the literature for comparison. The neutral lipid we found, showed a similar spectrum of fatty acids to that of total lipid but seemed to be much affected by the change in the medium for growth. This effect is seen more prominently for *Salmonella paratyphi B.* The values for palmitic, palmito-oleic ($C_{16:1}$) and monoenoic-octadecanoic ($C_{18:1}$) may be cited as an example of this; with *S. typhi and S. paratyphi A* this effect was seen in C_{16}, $C_{18:1}$ and C_{20} acids. *Salmonella paratyphi A* contained higher amounts of monoenoic stearic acid as compared with the other two strains.

When the fatty acid composition of the phospholipid was examined, it was seen that *Salmonella paratyphi A* contained the highest concentration of palmitic acid (62 to 68%). Noticeable amounts of stearic acid were detected in this fraction. Cyclic and straight chain C_{18} were absent. Arachidonic ($C_{20:4}$) acid was present in *S. typhi* (5·4%) and *S. paratyphi B* (1·5%) and was absent in *S. paratyphi A.* The phospho-

lipid isolated from *S. paratyphi B* grown in defined medium showed an unusually high content of C_{15} (61 %) and an extremely small amount of palmitic acid.

Thus, though fatty acid composition of the total, neutral and phospholipids of the three organisms were examined quantitatively and in some instances qualitatively, nothing definite could be inferred about variations observed in the same organism when subjected to different growth medium. It is known that temperature, growth medium and growth conditions can alter the fatty acid composition (Kates, 1964). Hence such quantitative alterations in the fatty acid composition of the bacteria grown in the defined medium and nutrient broth were to be expected. However, the changes observed were not only quantitative but also qualitative, i.e. the introduction or elimination of one or more fatty acids in the original profiles. On the basis of the above observation, it may be suggested that the loss of virulence in the Salmonella or other such pathogenic species when grown on different media or transferred from natural medium to defined medium might be the result of the fatty acid alterations.

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Effect of Aliphatic Polyamines on Growth and Macromolecular Syntheses in Bacteria

By U. BACHRACH AND A. WEINSTEIN

Department of Bacteriology, Institute of Microbiology, The Hebrew University–Hadassah Medical School, Jerusalem, Israel

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SUMMARY

The effect of synthetic and naturally occurring polyamines on bacterial growth was studied. The tetra-amine spermine was the most potent antibacterial agent and showed maximal activity at high pH values. The antibacterial action of the triamines was dependent upon the number of the primary amino groups in the polyamine molecule and was maximal when it contained a $-N(CH_2)_7N-$ moiety. Polyamines, in low concentrations, stimulated the incorporation of [^{14}C]uracil into the nucleic acids of growing bacteria, whereas higher concentrations were inhibitory. In contrast the incorporation of [^{14}C]valine into bacterial proteins was inhibited by polyamines, even in low concentrations.

INTRODUCTION

The naturally occurring polyamines, spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ and spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, inhibit the growth of various microorganisms, mainly Gram-positive bacteria (Rozansky, Gurevitch, Brzezinsky & Eckerling, 1949; Rozansky, Bachrach & Grossowicz, 1954; Grossowicz, Razin & Rozansky, 1955). Previous work has shown that the antimicrobial action is maximal at alkaline pH values (Rozansky *et al.* 1954) and that polyamines inhibit protein synthesis in growing bacteria (Razin & Rozansky, 1959; Friedman & Bachrach, 1966; Mills & Dubin, 1966; Ezekiel & Brockman, 1968), in bacterial extracts (Hershko, Amoz & Mager, 1961; Mager, Benedict & Artman, 1962; Martin & Ames, 1962) and in mammalian cells (Goldstein, 1965; Ochoa & Weinstein, 1965). On the other hand, RNA synthesis is stimulated by polyamines in low concentrations, while high concentrations exert an inhibitory effect (Doerfler, Zillig, Fuchs & Albers, 1962; Krakow, 1963; Fox, Robinson, Haselkorn & Weiss, 1964; Fox & Weiss, 1964; Dykstra & Herbst, 1965; Goldstein, 1965; Mills & Dubin, 1966; O'Brien, Olenick & Hahn, 1966; Caldarera, Moruzzi, Barbiroli & Moruzzi, 1968; Moruzzi, Barbiroli & Caldarera, 1968; Petersen, Kröger & Hagen, 1968). Synthetic polyamines, resembling spermidine in their structure, behave like naturally occurring polyamines in that they stabilize RNA against thermal denaturation (Goldstein, 1966) and serve as growth factors for some lactobacilli (Guirard & Snell, 1964).

The object of the present work was to test the effects of synthetic polyamines on growth and macromolecular syntheses in bacteria. It will be shown that these synthetic compounds resemble naturally-occurring polyamines in their bactericidal activity. This activity is dependent upon the number of free amino groups and on the length of the polyamine molecule.

METHODS

Chemicals. Spermine tetrahydrochloride and spermidine trihydrochloride were supplied by Fluke AG, Buchs, Switzerland. 3,3′-Diaminodipropyl amine was obtained from Eastman, Kodak, Rochester, N.Y., U.S.A. The other synthetic polyamines were kindly provided by Dr E. F. Elsager (Parke, Davis & Co., Ann Arbor, U.S.A.). DL-[1-^{14}C]Valine (0·35 mg./0·1 mCi; 33·9 μCi/ml.) and [2-^{14}C]uracil (0·276 mg./0·1 mCi; 25 μCi/ml.) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Bacteria and media. Escherichia coli strain B and *Staphylococcus aureus* were grown in nutrient broth (Difco Laboratories, Detroit, Mich., U.S.A.). Antibacterial activity was determined by twofold dilutions in nutrient broth. To each dilution of the antibacterial agent (1 ml.) was added 0·05 ml. of a diluted bacterial suspension containing 5×10^5 organisms/ml. Results were recorded after incubation at 37° for 20 hr.

RNA and protein synthesis. The incorporation of [2-^{14}C]uracil into bacterial nucleic acids was determined by growing *Escherichia coli* in 20 ml. quantities of nutrient broth to 5×10^8 organisms/ml., whereupon radioactive uracil (1·2 μCi) and the required polyamine were added. At intervals, 2 ml. portions were withdrawn and added to 2 ml. 10% (w/v) trichloroacetic acid containing 0·01% (w/v) uracil. After standing in the cold for at least 30 min., samples were filtered through membrane filters (0·45 μ, Millipore Filter Corp., Bedford, Mass., U.S.A.) which were then washed with 25 ml. trichloroacetic acid, dried, mounted on planchets and counted by a Nuclear Chicago thin window gas flow counter.

The incorporation of radioactive valine into bacterial proteins was similarly determined. Radioactive valine (6·8 μCi) and the respective polyamine were added to 20 ml. portions of the bacterial cultures. Two ml. portions were withdrawn at various times and added to 2 ml. 10% (w/v) trichloroacetic acid containing 1% (w/v) casamino acids (Difco Laboratories Detroit, Mich., U.S.A.). Filters were washed, dried and counted as above.

Table 1. *Effect of polyamines on growth of Staphylococcus aureus and Escherichia coli*

Compound			*Staphylococcus aureus*	*Escherichia coli*
No	Formula	Name or abbreviation	Minimal inhibitory concentration (μg./ml.)	
1	a–$NH(CH_2)_4NH$–a*	Spermine	2	17
2	a–$NH(CH_2)_2NH_2$	C_2	45	365
3	a–$NH(CH_2)_2OH$	C_2-OH	100	1400
4	a–NH–a	C_3†	6	400
5	a–$N(CH_3)$–a	C_3-NCH_3	14	450
6	a–$NH(CH_2)_4NH_2$	Spermidine	12	390
7	a–$NH(CH_2)_5NH_2$	C_5	3	460
8	a–$NH(CH_2)_6NH_2$	C_6	4	270
9	a–$NH(CH_2)_7NH_2$	C_7	4	140

*a = $-(CH_2)_3NH_2$; † = 3,3′-diaminodipropylamine.

RESULTS

Effect on bacterial growth

Spermine and spermidine, the naturally-occurring polyamines, are known to be antibacterial. To find whether this inhibitory action is common to a variety of polyamines, various synthetic polyamines were used. Synthetic polyamines were also used to determine the optimal structure for an antimicrobial polyamine. *Staphylococcus aureus* was more sensitive than *Escherichia coli* to the inhibitory action of the polyamines; the tetra-amine spermine was the most potent antibacterial agent tested (Table 1). It is also evident from Table 1 that the two primary amino groups were essential for the antibacterial action; the substitution of an amino group by a hydroxy radical, decreased the antibacterial activity significantly (compound no. 3). The methylation of the secondary amino group of a triamine resulted in a slight decrease of activity (see compound no. 4, 5). Results shown in Table 1 lead us to suggest that maximal antibacterial activity is obtained when the triamine contains a $-N(CH_2)_7N-$ moiety. As expected, the antibacterial activity of the polyamines was dependent on the pH value of the medium and was maximal at alkaline pH values (Fig. 1).

Effect on macromolecular synthesis

The effect of polyamines on macromolecular synthesis was studied. Spermine, in low concentrations, stimulated the incorporation of [2-^{14}C]uracil into trichloracetic acid-insoluble material in growing *Escherichia coli*. Maximal stimulation was obtained with 100 μg. spermine/ml. Ribonucleic acid synthesis was inhibited by higher concentrations of spermine (Fig. 2). It is noteworthy that RNA synthesis was stimulated by spermine in concentrations which inhibited growth. Similar results were obtained with the synthetic polyamine no. C7, $NH_2(CH_2)_3NH(CH_2)_7NH_2$; again, RNA synthesis was stimulated by the polyamine in low concentrations inhibited by larger concentrations (Fig. 3).

When polyamines were added to growing cultures of *Escherichia coli* an inhibition of protein synthesis was noticed, even in the presence of low concentrations of polyamine. Spermine at 100 and 25 μg./ml. inhibited the incorporation of [1-^{14}C]valine by 50 and 15%, respectively (Fig. 4). Similar concentrations of spermine stimulated the incorporation of radioactive uracil into bacterial RNA (see Fig. 2). The synthetic triamine no. C7, exerted a similar inhibitory effect on protein synthesis in growing *E. coli*. Inhibitions of 100 and 75%, respectively, were produced by the polyamine in concentrations of 17 and 9 mg./ml. of medium (Fig. 5).

DISCUSSION

The results described in this paper clearly show that *Staphylococcus aureus*, a Gram-positive bacterium was more sensitive to the antibacterial action of polyamines than the Gram-negative *Escherichia coli*. This is in agreement with the results reported elsewhere for spermine and spermidine (Rozansky *et al.* 1954). Although the tetra-amine spermine was most effective in inhibiting the growth of *S. aureus* and *E. coli*, significant inhibitions of growth also resulted from addition of triamines. It appears that the number of the primary amino groups and the length of the aliphatic chains play a role in inhibiting bacterial growth. Thus, when one primary amino group of the

triamine was substituted by a hydroxyl group, a significant decrease of antibacterial activity was observed. However, the presence of a secondary amino group in the triamine molecule is not essential for growth inhibition. This is demonstrated by the fact that methylation of the secondary amine caused only a slight change in antimicrobial activity. An aliphatic chain with seven carbon atoms seemed to be optimal for the inhibition of the growth of *E. coli*. It is of interest that cadaverine ($NH_2(CH_2)_5NH_2$) was most effective in protecting protoplast-infecting agent against thermal inactivation (Fraser & Mahler, 1958). This has been attributed to the N to N distances (7·3 Å) in the cadaverine molecule which is similar to the distances between the phosphate oxygens in the DNA molecule.

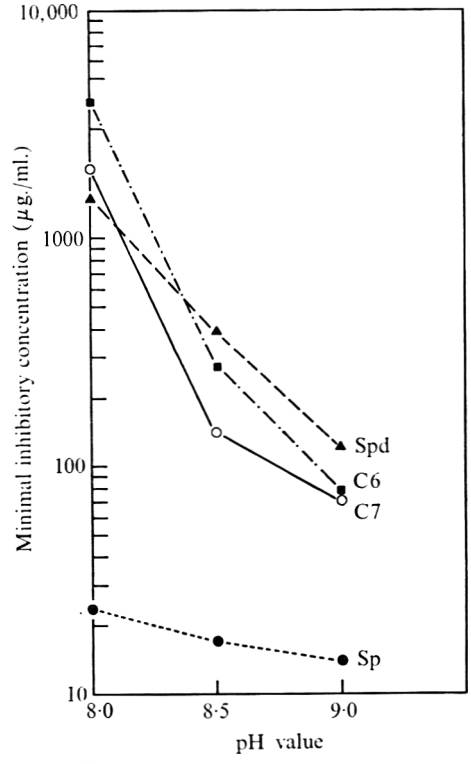

Fig. 1. Effect of pH value on the antibacterial activity of polyamines. Sp = spermine; Spd = spermidine; C_6 = *N*-3-aminopropylhexane,1,6-diamine; C_7 = *N*-3-aminopropylheptane,1,7-diamine.

Little is known about the mechanism of antimicrobial action of polyamines. Previous studies have shown certain similarities between the action of spermine on bacteria and that of another cationic antibiotic, streptomycin. Both agents are bactericidal, have early effects on protein synthesis and RNA synthesis as well as on potassium flux (Mills & Dubin, 1966). These compounds are presumably bound to the ribosomes on identical sites (Mager *et al.* 1962) and both cause extensive misreading of the genetic code (Davies, Gilbert & Gorini, 1964; Friedman & Weinstein, 1964; Davies, Gorini & Davis, 1965). However, the relationship between misreading, inhibition of protein synthesis and cell death has not been fully elucidated for streptomycin or spermine.

The stimulation of RNA synthesis by polyamines (Fig. 2, 3) is in agreement with earlier findings that RNA accumulated in polyamine-inhibited cells (Pirie, 1964; Goldstein, 1965). Raina & Cohen (1966) showed that spermidine stimulated RNA synthesis in arginine-starved *Escherichia coli* strain 15TAU, which requires thymine,

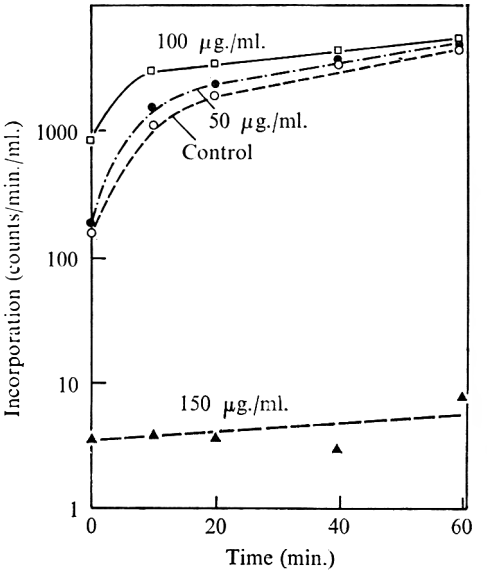

Fig. 2

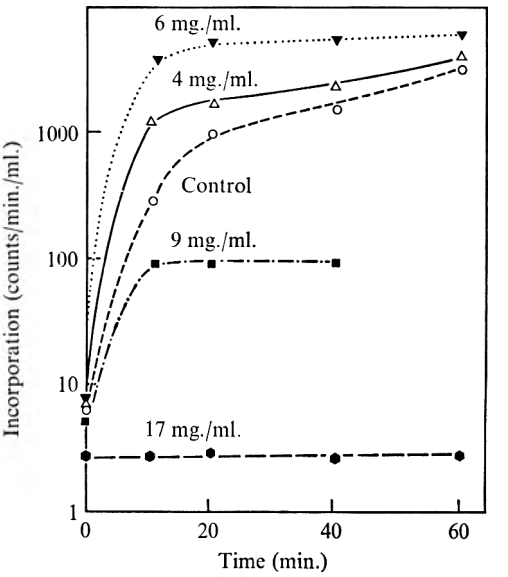

Fig. 3

Fig. 2. Effect of spermine on the incorporation of [^{14}C]uracil into growing *Escherichia coli*. The organism was grown in 20 ml. quantities of nutrient broth to 5×10^8 organisms/ml. [2-^{14}C]uracil (1·2 μCi) was then added in the presence of the polyamines at the concentrations indicated. Samples (2 ml.) were withdrawn at intervals added to equal volumes of 10% trichloroacetic acid, and the amount of incorporated isotope determined.

Fig. 3. Effect of *N*-3-aminopropylheptane,1,7-diamine on the incorporation of [^{14}C]uracil into growing *Escherichia coli*.

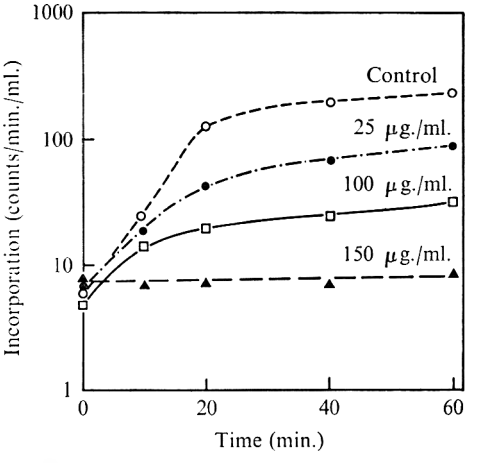

Fig. 4. Effect of spermine on the incorporation of [^{14}C]valine into growing *Escherichia coli*. Experimental conditions were as in Fig. 2, except that DL[1-^{14}C]valine (6·8 μCi) was added to each culture.

arginine and uracil for growth. They proposed that polyamines have a causal role in the control of RNA synthesis. It has, however, been claimed (Edlin & Broda, 1968) that the choice of *E. coli* strain 15TAU is not ideal because of the metabolic interrelationships of arginine, glutamate, putrescine and spermidine. Sparing of amino acids by polyamine in amino acid-starved bacteria has been offered as an alternative explanation for the stimulation of RNA synthesis (Ezekiel & Brockman, 1968) Results reported in the present paper show that RNA synthesis was also stimulated in

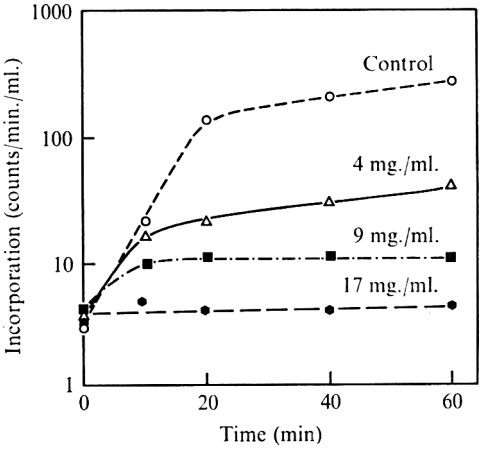

Fig. 5. Effect of *N*-3-aminopropylheptane,1,7-diamine on the incorporation of [^{14}C]valine into growing *Escherichia coli*.

non-starved bacteria by the synthetic polyamine no. C7 which does not bear any known metabolic relationship with amino acids. This finding does not support a sparing of amino acids by a polyamine but would favour a direct effect of the polyamine on RNA polymerase (Fox, Gumport & Weiss, 1965). It might also be explained by the stabilization of RNA and consequent protection against thermal denaturation (Goldstein, 1966) or enzymic degradation (Bachrach & Eilon, 1969). Stimulation of protein turnover by polyamines (Ezekiel & Brockman, 1968) might also explain the increased rate of RNA synthesis. At this stage it is difficult to distinguish between the alternative explanations and the reason for the stimulation of RNA synthesis in the presence of polyamines must await the results of further experiments.

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The Phycomycete *Catenaria anguillulae*: Growth Requirements

By R. A. NOLAN*

Department of Botany, University of California, Berkeley, California 94720, U.S.A.

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SUMMARY

The physical factors and nitrogen requirements for the growth of the aquatic phycomycete *Catenaria anguillulae* in liquid shaken cultures from measured zoospore inocula were examined. Growth was determined by measuring mycelial dry weight and rate of titratable acid production. The optimum pH range for growth was pH 8·2 to 8·7. The toxicity of the pH indicators used was assessed. The optimum temperature for growth was 25°. The organism was highly aerobic. Inorganic nitrogen sources were not utilized. When organic nitrogen sources were supplied individually, L-aspartic acid, L-asparagine, L-glutamic acid, L-arginine, DL-citrulline and L-ornithine were utilized. Asparagine was the best single source. When the N sources were supplied as Difco vitamin-free Casamino acids, only lysine, methionine and arginine were utilized during the lag phase. During the more rapid phase of growth, all of the amino acids detected in the Casamino acids were utilized. When the vitamin-free Casamino acids were replaced by an equivalent amino acid mixture, the subsequent decrease in yield was not corrected by adding NaCl, oleic acid or stearic acid. The optimum concentrations of various N sources were determined. The medium finally evolved contained L-asparagine + L-lysine as N sources.

INTRODUCTION

Catenaria anguillulae was classified as a member of the Order Chytridiales until Couch (1945) transferred it to the Order Blastocladiales. This change was made on the basis of similarities between the genus *Catenaria* and members of the Order Blastocladiales with respect to zoospore structure and behaviour, nature of hyphal septations, origin and germination of the resistant sporangium and life cycle. A review of nutritional studies of the aquatic Phycomycetes (Cantino & Turian, 1959) indicated that members of the Order Chytridiales could utilize inorganic nitrogen and sulphur sources and could either synthesize all vitamins or required thiamine for growth. Members of the Order Blastocladiales could neither utilize inorganic sulphur sources nor synthesize thiamine. Some isolates had additional vitamin requirements and, whereas most isolates could utilize inorganic nitrogen sources, one had lost this ability.

The first purpose of the present work was to examine the nutritional requirements of *Catenaria anguillulae* and to determine its nutritional relationship to members of the Orders Chytridiales and Blastocladiales. The second purpose was to analyse the nutritional behaviour of *C. anguillulae* to provide data for the interpretation of eco-

* Present address: Department of Biology, New Mexico State University, Las Cruces, New Mexico 88001, U.S.A.

logical information and for future studies on the enzymology of its amino acid metabolism. A brief abstract of earlier phases of this study has been published (Nolan, 1966).

METHODS

Origin and maintenance of the isolate used. All of the present work was done with an isolate of *Catenaria anguillulae* Sorokin (strain 58–12) obtained from Dr R. Emerson (Department of Botany, University of California, Berkeley, Calif., U.S.A.). The fungus had been isolated into axenic culture from material infecting nematodes (*Hemicycliophora arenaria*). The infected nematodes and their identification had been kindly supplied by Dr R. Mankau (Department of Plant Nematology, Citrus Experiment Station, Riverside, California). Stock cultures were maintained on slopes of a yeast-extract + soluble-starch medium (EYPSS; Emerson, 1941) and were transferred at intervals of 2 to 4 weeks. These cultures were incubated at $17 \pm 1°$.

Inoculum preparation. The method used to obtain a zoospore inoculum was a modification of the technique used by Carlile & Machlis (1965) for obtaining male gametes of Allomyces species. The concentration of zoospores obtained was 1 to 5×10^6/ml. One ml. of this suspension or a dilution of it was used to inoculate each flask in an experiment. Zoospore concentrations were determined with a haemacytometer. The viability of the zoospores used in each experiment was tested by adding 1 ml. inoculum to a small Petri dish containing EYPSS agar medium and by examination for subsequent germination and growth.

Chemicals and stock solutions. L-aspartic acid (A grade) was obtained from Calbiochem (Los Angeles, California), all other amino acids were obtained from Nutritional Biochemicals Corp., (Cleveland, Ohio). The pH indicators were obtained from Matheson, Coleman & Bell (Norwood, Ohio) The fatty acids were obtained from Nutritional Biochemicals Corp. The yeast extract and vitamin-free Casamino acids were obtained from Difco Laboratories (Detroit, Michigan). The micronutrients were of analytical grade where available.

All stock solutions were prepared with glass double-distilled water and stored at 4° in glass-stoppered bottles.

Preparation of glassware and media. All glassware was chemically cleaned (concentrated H_2SO_4 saturated with potassium dichromate) after having been machine-washed. The glassware was then rinsed several times with double-distilled water.

The experimental media were generally prepared and autoclaved in three portions: (*a*) the carbon source, (*b*) the calcium and magnesium salts, (*c*) the remainder of the medium. In some experiments, the nitrogen source was also autoclaved separately. All autoclaving was done for 15 min. and at 120°. The fatty acids were added in ether solution to each sterile experimental flask before adding the remainder of the separately sterilized medium. The combined volume of the components was 50 ml./flask. The initial pH value of a medium was always adjusted after mixing the separately autoclaved components and just before inoculation.

Growth of experimental cultures. All cultures were grown in 125 ml. Bellco flasks with stainless steel caps (Bellco Glass, Inc., Vineland, New Jersey). The inoculated flasks were placed on a horizontal rotary shaker (120 rev./min.) in a room maintained at $20 \pm 1°$ (for exceptions see below and text). These conditions provided vigorous mixing and aeration.

Two experimental regimes were maintained in Case glass anaerobic jars (19·5 inches (50 cm.) high × 7·75 inches (20 cm.) diameter; Case Laboratories, Inc., Chicago, Illinois). Anaerobic conditions were obtained by evacuating a jar to 6·5 cm. and then filling with 5% (v/v) CO_2 + 95% (v/v) hydrogen mixture. A catalyst, palladium-coated alumina pellets in a wire gauze sachet (Heller, 1954), and a reduced methylene-blue indicator (Baltimore Biological Laboratory, Cockeysville, Maryland; see Brewer, Allgeier & McLaughlin, 1966) were included in the jar. The 0·2% (v/v) oxygen concentration was obtained by evacuating a jar twice to 7·5 cm. Hg and filling each time with 'tank nitrogen'. The jars were placed on a low-speed horizontal, rotary shaker (60 rev./min.).

Adjustment of pH value. The pH value of a culture was maintained by the addition of sterile 0·5 N-KOH from a calibrated syringe. The amount of titratable acid produced in each culture flask was recorded as the amount of base required to return the culture medium to the original pH value; the pH value of the cultures was adjusted every 8 to 24 hr.

Yields of organism: dry weight. Cultures were harvested by vacuum filtration through previously tared filter papers. The papers were then placed in an oven (90°) for 24 hr, allowed to come to room temperature in a desiccator and weighed on a Mettler balance (Type H16, Mettler Instrument Corp., Princeton, New Jersey). All dry weights are given to the nearest mg. A constant dry weight (variation of less than 1 mg. for repeated weighings) was obtained for any single culture flask harvested.

Acid production and dry weight served as criteria for comparing the growth of the fungus under the different experimental conditions. By comparing the growth curve and the rate of acid production (Fig. 5, 6) in an experiment, two types of information were obtained by following acid production: (1) the initial observation of acid production indicated the end of the lag phase of growth; (2) the slight inflexion in the acid-production curve indicated when the peak of the growth curve had been reached.

The variation in dry weights between flasks in a single treatment was dependent upon the phosphate concentration in the medium. When the concentration of phosphate was 15 mM, the variation was ±20% or less; when the phosphate concentration was 10 mM, the variation was ±10% or less; at 1 mM phosphate the variation between the dry weights in a treatment was less than 1 mg.

Amino acid analyses. Culture filtrates were analysed for amino acid content according to the column chromatography technique of Moore & Stein (1963). All determinations were made on a Beckman-Spinco automatic amino acid analyser (Model 120B; Spinco Division, Beckman Instruments, Inc., Palo Alto, California) adjusted to a sensitivity such that 0·05 μM of an amino acid could give a full scale deflexion. The filtrates were frozen at the time of harvest and then thawed and diluted with buffer before being added to the column. All amino acid concentrations were determined from the chromatograms by using the 'height–width' method of integration (see manual for analyser). The accuracy of the column data was ±8%. The data for ammonium concentrations are not given because of sensitivity to external conditions.

Standard micronutrients. Many of the media used contained a group of micronutrients which were always added at a fixed concentration; these are referred to as standard micronutrients (Table 1). The concentrations of these ions were taken from Medium B (Machlis, 1953).

RESULTS

Initial experiments. Early attempts to grow *Catenaria anguillulae* at pH 7·0 on a defined medium containing ammonium-N (Medium B in Table 2) were unsuccessful even with incubation for 54 days. Subsequently Medium B was modified by decreasing the total phosphate to 1 mM and by changing the N source to $(NH_4)_2SO_4$. The possibility of ammonium-N utilization (at 1, 10, 30 mM-N) was then tested with this modified Medium B at pH 6·2, 7·2 and 8·2. After 25 days of incubation no growth had occurred.

Table 1. *Standard micronutrients of medium B (Machlis, 1953)*

		(mM)
Mn	As $MnCl_2.4H_2O$	0·009
Co	As $CoCl_2.6H_2O$	0·003
Zn	As $ZnSO_4.7H_2O$	0·001
Cu	As $CuSO_4.5H_2O$	0·001
Fe	As $FeCl_3.6H_2O$	0·018
B	As H_3BO_3	0·046
Mo	As $Na_2MoO_4.2H_2O$	0·002

Table 2. *The composition of experimental media*

The ratio of phosphates was adjusted to correspond to the initial pH value desired. All media contain the standard micronutrients (see Table 1), D-glucose (166 mM), $CaCl_2.2H_2O$ (0·5 mM), $MgCl_2.6H_2O$ (0·5 mM) and thiamine.HCl (150 μg.).

	Medium					
Components	B	1	4	5	6	7
Nitrogen source (g. N/l.)						
Difco Casamino acids	.	0·16	0·16	(see text, p. 172)		
$(NH_4)_2HPO_4$	10 mM	.	.	.	.	.
L-Asparagine	.	.	.	(see text, p. 176)		0·24
L-Lysine	.	.	.	.	.	100 μM
Sulphur source (mM-S)						
DL-Methionine	0·67	0·67	0·67	.	0·67	.
L-Methionine	.	.	.	0·53	.	0·53
Phosphate source (mM-P)						
K_2HPO_4	.	.	.	1·0	.	1·0
$K_2HPO_4+KH_2PO_4$	.	10	10	.	.	.
$K_2HPO_4+KH_2PO_4+(NH_4)_2HPO_4$	15	.	.	.	15	.
pH indicator (mg./l.)						
Bromocresol purple	2·0	.	.	.	2·0	.
m-Cresol purple	.	.	2·0	.	.	2·0
Other (see text)	.	2·0	.	2·0	.	.
Na_2EDTA (mM)	.	.	0·032	0·032	.	0·032

Growth occurred on Medium B only when the medium was supplemented with an organic nitrogen source. When L-glutamic acid was added to Medium B at 0·5 to 10 mM, growth was noted after 20 days. The isolate was also able to grow on a glucose + yeast-extract + salts medium (GY 5) (Emerson, 1958). A comparison of yeast-extract and vitamin-free Casamino acids (each at 1 g./l.) as N sources in Medium B indicated that yields were higher with Casamino acids.

pH indicators. The indicators selected (Table 3) were added to Medium B + Casamino acids (1 g./l.). The mean dry weights obtained in the different treatments were compared by using Duncan's multiple-range test (5% level, error degrees of freedom = 33; see Steel & Torrie, 1960). The means were in three distinct groups: indicators in group A (Table 3) were considered to be non-toxic; those in group B were slightly toxic; those in group C were highly toxic. There was no structural correlation among the members of any group; the chemical compounds present in the pH indicators used are far from being standardized and the possibility exists that phenolic intermediates might have been present in the products used (personal communication from Mr F. Green, Technical Director, Matheson, Coleman & Bell). To avoid the introduction of additional variables, the stock solutions of the non-toxic indicators which were prepared for this experiment were used throughout the subsequent work. A later experiment indicated that xylenol blue and alizarine yellow R were also non-toxic.

Table 3. *The effect of various pH indicators on the growth of Catenaria anguillulae*

The medium used was Medium B + Casamino acids (1·0 g./l.). Initial pH 7·0 maintained during the 7-day incubation period. The dry weights are the averages of 4 replicates.

Indicator (2·0 mg./l.)	Dry weight (mg.)
Group A	
Bromocresol purple	27
m-Cresol purple	23
Cresol red	23
Control (no added indicator)	22
Phenol red	21
Bromothymol blue	21
Group B	
Thymol blue	16
Bromophenol blue	14
Group C	
Methyl red	5
Bromocresol green	3
Chlorophenol red	1

Optimal pH range. Butler & Humphries (1932) studied the development of *Catenaria anguillulae* as it grew out from infected fluke eggs into various surrounding complex natural media. which varied from pH 6·5 to 6·9. These authors did not study the effects of pH value. I studied the effects of pH value on the growth of *C. anguillulae* in Medium 1 (Table 2). The results (Fig. 1) indicated that the optimum range for growth was pH 8·2 to 8·7.

Optimum temperature. The optimum temperature for the growth of *Catenaria anguillulae* strain 58–12 was determined using Medium 1 in static cultures. The results (Fig. 2) indicated that 25° was the optimum temperature under these conditions; above 25° and below 19° the growth rate decreased rapidly.

Oxygen relations. Buckley & Clapham (1929) investigated the viability of sporangia of Catenaria under a variety of conditions. From their experiments with the transfer of the growth of the fungus from infected fluke ova to previously uninfected ova in the presence of sewage with a high biological oxygen demand and in the presence of charcoal-treated water in paraffin-sealed tubes, they concluded that Catenaria could complete

its development under anaerobic or nearly anaerobic conditions. To study the effects of degree of aeration on the amount of growth *Catenaria anguillulae* was grown in shaken cultures and in static cultures, in Medium 1. An average dry weight of 112 mg./flask was produced in the shaken cultures, in contrast to an average dry weight of 28 mg./flask in the static cultures, after 9 days of incubation. This would seem to indicate *C. anguillulae* strain 58–12 is an aerobic organism.

Table 4. *Composition of the vitamin mixture*

Personal communication from Dr S. Hutner, Haskins Laboratories, New York, to Dr L. Machlis; 25 September 1965. The mixture is a dry mix, 50% of which is pentaerythritol. The concentrations listed below were obtained by adding 10 ml. of a stock solution to 1 l. medium.

Vitamin	Concentration (μg./l.)	Vitamin	Concentration (μg./l.)
Thiamine.NO_3	180	Putrescine.2HCl	60
Nicotinic acid	240	Spermidine phosphate.$3H_2O$	30
Ca pantothenate	300	Folic acid	12
Na riboflavin phosphate	60	DL-Carnitine.HCl	120
Riboflavin	30	Betaine.HCl	4800
Pyridoxamine.2HCl	60	Choline N_2 citrate	4800
Pyridoxal.HCl	60	Cystamine.2HCl	30
Inositol	3000	B_{12}	1·2
p-Aminobenzoic acid	30	Biotin	3

In another experiment two atmospheric regimes were produced to determine: (1) whether strain 58–12 could grow under more strict anaerobic conditions; (2) whether strain 58–12 could grow in the presence of 0·2% (v/v) oxygen. Medium 1 without the usual thiamine source but supplemented with the vitamin mixture listed in Table 4 was used. The flasks were incubated at 23° for 13 days for the anaerobic treatment and 7 days for the 0·2% (v/v) oxygen treatment. Five replicates were used in each treatment. After 13 days of incubation the anaerobic flasks showed no visible growth and microscopic examination of their contents indicated no trace of the inoculum. After 7 days of incubation the flasks under the 0·2% (v/v) oxygen treatment yielded an average dry weight of 2·0 mg./flask; microscopic examination of the contents of the flasks indicated that the fungus was growing, but very poorly. The 0·2% (v/v) oxygen treatment, because of the way it was obtained, also contained 3×10^{-4}% CO_2. It is clear that *Catenaria anguillulae* is neither anaerobic nor microaerophilic.

The optimum concentration of vitamin-free Casamino acids. The optimum concentration of vitamin-free Casamino acids was determined in Medium 6 (Table 2) but without ammonium phosphate. The results (Fig. 3) indicate that the optimum concentration of Casamino acids in this medium was about 2·0 g./l., equivalent to 0·16 g. N/l. Medium without added glucose was included to test availability of the Casamino acids at 1 g./l. as carbon sources. After 7 days of incubation only 4 mg. dry weight was produced.

Growth on an artificial Casamino acids mixture. An attempt was made to replace the casein hydrolysate (Casamino acids) with a mixture containing the same amino acids and in the same proportions (Table 5). The concentration of methionine used in the mixture was a departure from its concentration in the hydrolysate, in order to maintain the concentration at which methionine had normally been included in other

media. Medium 4 (Table 2) was used in this experiment but with the new N sources. The N concentrations in the various media were adjusted to correspond to 0 (control), 0·5, 1, 2, 3 and 4 times the total N concentration provided by the Difco product at the optimal concentration of 2·0 g./l. (Fig. 4). The decrease in yield can be seen by comparing the dry weight of 35 mg./flask obtained after 9 days of incubation on the artificial mixture with the dry weight of about 120 mg./flask obtained after the same period of time with the Difco hydrolysate (Fig. 5).

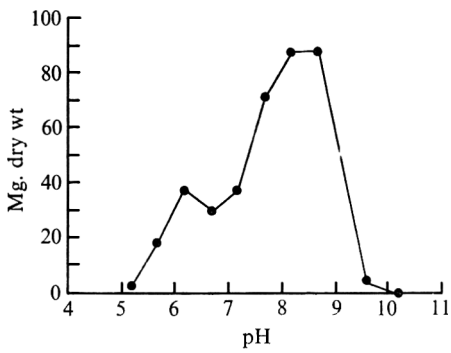

Fig. 1

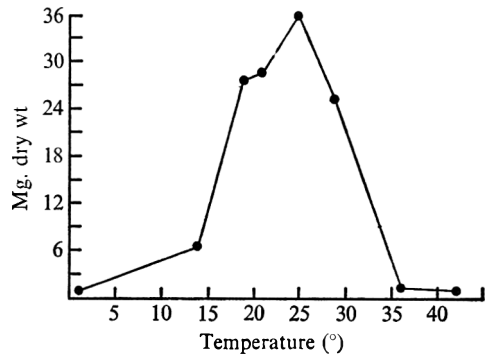

Fig. 2

Fig. 1. The effects of pH on the growth of *Catenaria anguillulae* in Medium 1. The incubation period of the experiment was 7 days. Each point on the growth response curve represents the average of five to six replicates.

Fig. 2. The effects of temperature on the growth of *Catenaria anguillulae* in Medium 1 adjusted to a pH of 8·7. The pH indicator was *m*-cresol purple. The incubation period of the experiment was 9 days. Each point on the growth response curve represents the average of five replicates.

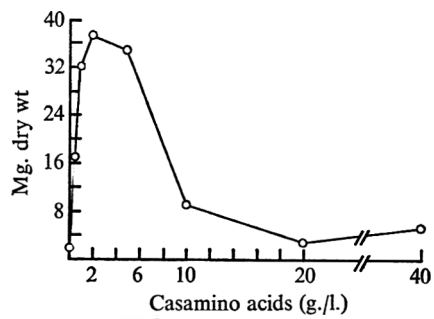

Fig. 3. The relation between growth of *Catenaria anguillulae* and the concentration of Difco Casamino acids in Medium 6 without the ammonium phosphate. The total phosphate was 15 mM; pH 6·9. Each point on the curve is the average of four replicate flasks. The incubation period 7 days.

Growth as measured by titratable acid produced is shown in Fig. 6. In an attempt to determine what critical factor(s) had been omitted from the medium in the change to a defined amino acid mixture two approaches were tried.

Effects of various 'Casamino factors'

NaCl. The Difco product is an acid (HCl) hydrolysate of casein; and since the final pH value of the hydrolysate is adjusted with NaOH, the final product contains 38% NaCl (Difco Manual, 1953). Various concentrations of NaCl were added to Medium 4 adjusted to pH 8·7. In this experiment the artificial amino acid mixture was the N source. Low concentrations of NaCl (0·00325, 0·0065, 0·013 M) were stimulatory to the

Table 5. *The composition of Difco vitamin-free Casamino acids (Column A) and of the amino acid mixture (Column B)*

Data on the Difco product supplied by Mr J. M. Willett (Technical Service, Difco Laboratories). All nitrogen (N) concentrations are for the L-isomer, even when the amino acid was supplied as a DL-mixture. The concentrations for the amino acids in the mixture are calculated as a substitute for Casamino acids 2 g./l.

Amino acid	A (%)	B (g. N/l.)
Arginine	2	0·0064 (L)
Aspartic acid	5	0·0160 (L)
Glutamic acid	15	0·0480 (L)
Glycine	1	0·0032
Histidine	1·5	0·0048 (DL)
Isoleucine	4	0·0128 (DL)
Leucine	5	0·0160 (L)
Lysine	5	0·0160 (DL)
Methionine	1	0·0080 (DL)
Phenylalanine	2	0·0064 (DL)
Threonine	2	0·0064 (DL)
Tyrosine	1	0·0032 (L)
Valine	4	0·0128 (L)
Total	48·5	0·1600

growth of strain 58–12 on this medium but did not account for the differences between the two nitrogen sources (Fig. 7).

Fatty acids. Arai & Kuwahara (1961) found that *Streptococcus hemolyticus* (S-8 strain) grew well on Difco vitamin-free Casamino acids but poorly on the ether-extracted Casamino acids; they concluded that the ether treatment removed a factor important for the growth of this streptococcal strain. The factor remained active in the ether extract and was found to be stearic acid which was present in the Casamino acids at 0·001 to 0·01%. Only saturated fatty acids (palmitic, arachidic) were able to replace the stearic acid. Demain, Hendlin & Newkirk (1959) with a Sarcina species found that on a defined amino acid medium the final yield was increased to one-half of the maximum yield obtained on a casein hydrolysate medium when they added; oleic acid 0·13 μg./ml., linoleic acid, 0·16 μg./ml., linolenic acid 0·32 μg./ml. or Tween 80 5·5 μg./ml. Thus unsaturated and saturated fatty acids were active in replacing, or actually may be, the active factor(s) lost in changing from a medium containing casein hydrolysate to one with a defined amino acid mixture as the nitrogen source.

For the experiment with *Catenaria anguillulae* oleic acid was selected as the unsaturated fatty acid and stearic acid as the saturated fatty acid. Medium 4 with the amino acid mixture (0·16 g. N/l.) as the N source was used. All three concentrations of oleic acid which were tested and stearic acid at 0·1 and 1·0 μg./ml. were stimulatory to *C. anguillulae* strain 58–12 in the medium containing the artificial amino acid mixture

(Fig. 8). The twofold increase in final dry weights was not, however, sufficient to account for the nearly fourfold difference between these results and those on the medium containing the Difco Casamino acids.

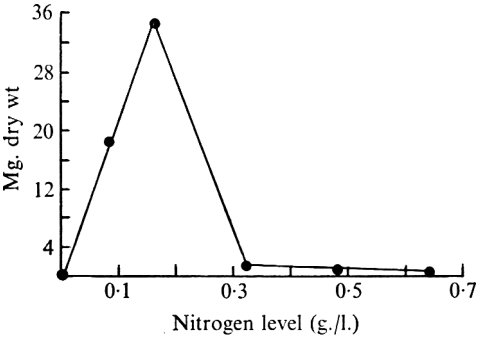

Fig. 4

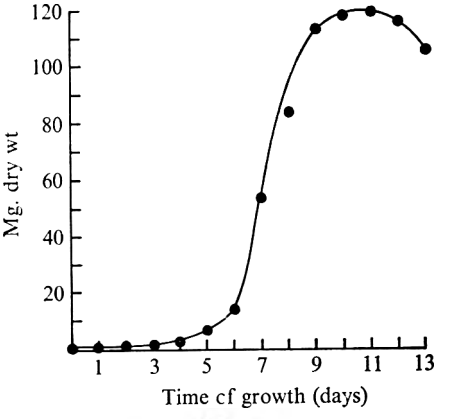

Fig. 5

Fig. 4. The relation between growth of *Catenaria anguillulae* and the concentration of the artificial amino acid mixture in Medium 4. The incubation period was 9 days, pH 8·7. Each point on the curve is the average of six replicates.

Fig. 5. The growth curve of *Catenaria anguillulae* on Medium 4. Each point on the curve is the average of three to four replicates.

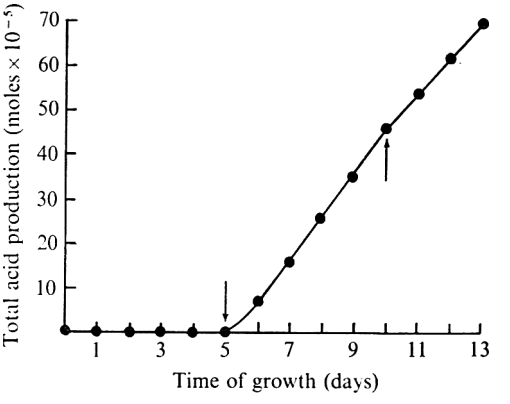

Fig. 6. Total titratable acid produced by *Catenaria anguillulae* when grown on Medium 4. Each point on the curve represents data from 10 pH adjustments or observations. The arrows indicate end of lag phase and peak of growth curve.

Utilization of single amino acids. The results (Table 6) indicated that the compounds in the ornithine + urea cycle, including glutamic acid, were utilized along with asparagine and aspartic acid. Because of the way in which the medium was prepared, the concentration of L-aspartic acid in the final medium was equivalent to 0·065 g. N/l., or one-half of the N concentration provided by the other compounds tested. Of the single N sources tested, L-asparagine gave the highest yield of organism for the 11-day incubation period; and this yield compared well with the yield obtained on the Difco Casamino acids at the same N concentration. L-asparagine was therefore used in further experiments.

The optimum concentration of L-*asparagine.* The amide L-asparagine was incorporated into Medium 5 at concentrations from 0 (control) to 0·48 g. N/l. The medium was initially adjusted to pH 8·7. The results (Fig. 9) indicated that the optimum concentration

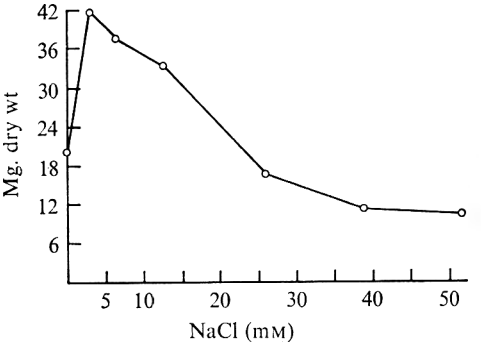

Fig. 7

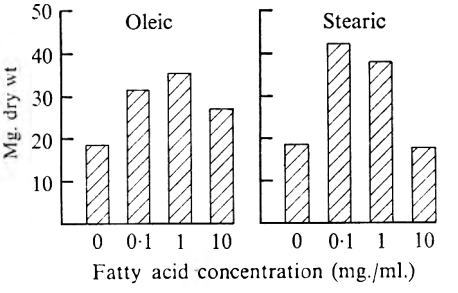

Fig. 8

Fig. 7. The relation between growth of *Catenaria anguillulae* and concentration of NaCl in Medium 4 with the amino acid mixture as the nitrogen source (0·16 g. N/l.). The incubation period 12 days; results of each treatment are the average of five replicates.

Fig. 8. The relation between the growth of *Catenaria anguillulae* and the concentration of oleic acid and stearic acid in Medium 4 with the amino acid mixture as the nitrogen source (0·16 g. N/l.). Incubation for 9 days; results of each treatment average of three replicates.

Table 6. *The growth of Catenaria anguillulae on organic nitrogen sources in Medium 5 at pH 8·7*

The incubation period 11 days; the dry weights averages of duplicate flasks. The N concentration was 0·13 g. N/l. except for L-aspartic acid (0·065 g. N/l.). For DL-mixtures the concentration is given for the L-isomer only.

Nitrogen source	Yield dry weight (mg.)	Nitrogen source	Yield dry weight (mg.)
Control (no added nitrogen)	2	Glycine, DL-histidine, DL-isoleucine, L-leucine, DL-lysine, DL-methionine, DL-phenylalanine, L-proline, hydroxy-L-proline, DL-serine, DL-threonine, L-tryptophan, L-tyrosine, L-valine,	all 2
Difco Casamino acids	85		
DL-Alanine	2		
L-Arginine	22		
L-Asparagine	91		
L-Aspartic acid	47		
DL-Citrulline	7		
L-Cystine	3		
L-Glutamic acid	38		
L-Ornithine	12		

of L-asparagine for the growth of *Catenaria anguillulae* on this medium was 0·24 g. N/l. The inoculum used was small in this experiment and accounted for the lower yield in comparison with the 11-day dry weight obtained with asparagine in the single N source survey.

The use of amino acids from a mixture. In studying the use of amino acids by an organism, an alternative to the method of supplying single sources is to provide the organism with a mixture of amino acids and then record the effects of the removal of

amino acids from this medium. One problem which arises in such studies is the possibility that the organism may remove an amino acid from a mixture even though it will not grow on that amino acid when it is supplied as sole N source. Inhibitory interactions between amino acids in a mixture also present a problem. Prescott, Peters & Snell (1953) found that alanine inhibited the growth of *Lactobacillus delbrueckii* and that this inhibition was annulled by serine or some of its peptides. The role of peptides in the present work was not assessed; peptides do occur in Difco Casamino acids. The early amino acid analyses were allowed to run for 24 hr, long enough for peptides to be indicated in the column chromatography; their total N concentration was not calculated and no attempt was made to identify specific peptides.

Table 7. *Amino acid concentrations in Medium 4 with Difco vitamin-free Casamino acids (2 g./l.) as the N source after growth of Catenaria anguillulae*

The analyses were made with a Beckman-Spinco automatic amino acid analyser; concentrations in μM/ml.

	Time of incubation (days)			
	0	5	6	8
Amino acid	Amino acid concentration in culture			
Lysine	0·744	0·500	0·451	0·085
Histidine	0·152	0·158	0·143	0·080
Arginine	0·118	0·118	0·056	Trace
Aspartic acid	0·657	0·630	0·638	0·277
Threonine	0·363	0·406	0·363	0·083
Serine	0·481	0·484	0·506	0·133
Glutamic acid	1·491	1·372	1·430	0·542
Proline	1·094	1·216	1·078	0·640
Glycine	0·257	0·238	0·264	0·101
Alanine	0·363	0·392	0·407	0·160
Valine	0·456	0·518	0·517	0·083
Methionine	0·753	0·644	0·418	0·139
Isoleucine	0·344	0·378	0·352	0·027
Leucine	0·620	0·644	0·649	0·051
Tyrosine	Trace	Trace	Trace	Trace
Phenylalanine	0·109	0·098	0·094	Trace

The disappearance of an amino acid from a mixture may not always mean that it has been taken up by the organism. Kihara, Klatt & Snell (1952) and Peters, Prescott & Snell (1953) reported for different bacteria the presence of an extracellular tyrosine decarboxylase which partially destroyed the free amino acid in the media before any utilization. Gale (1945) reported an instance in which a Streptococcus species converted free arginine to ornithine, but that the latter compound was not utilized for growth. Even when an amino acid is taken up by the organism it does not mean that it will be utilized for growth. Halvorson & Cowie (1961) reported the uptake of various isomers and analogues of amino acids which were not utilized by a yeast.

For the present study, *Catenaria anguillulae* was grown in Medium 4 with Difco Casamino acids (2·0 g./l.) as the N source. The growth curve (Fig. 5) and the curve for the production of titratable acid (Fig. 6) are from the present experiment. Through the lag phase of growth (5- and 6-day harvests; Table 7) the only amino acids removed from the medium in significant amounts (exceeding $\pm 8\%$ of the concentration

given in the 0-day column of Table 7) were lysine, arginine and methionine. Methionine was the only sulphur source of those detected in the analysis and was known to be an excellent sulphur source for *C. anguillulae* (Nolan, 1966). From the studies on utilization of single amino acids, methionine was not shown to be a readily utilized N source. Arginine was readily utilized as a N source; but was not the only one of those compounds in the medium available to strain 58–12. Of the compounds detected in the analysis, arginine, aspartic acid and glutamic acid were the ones utilized when given as

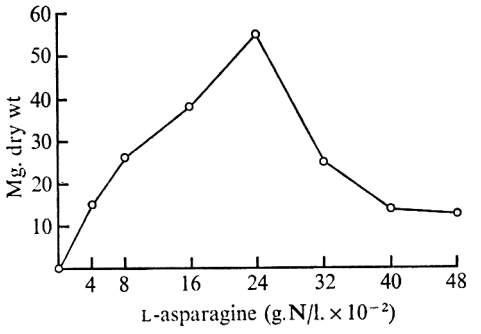

Fig. 9

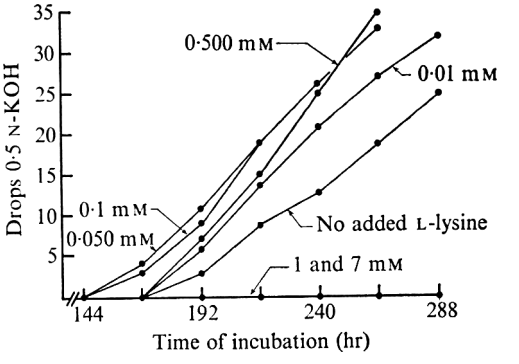

Fig. 10

Fig. 9. The relation between the growth of *Catenaria anguillulae* and the concentration of L-asparagine in Medium 5 at pH 8·7. Incubation for 11 days. Each point on curve average of three replicates.

Fig. 10. The relation between the concentration of L-lysine added to Medium 5 containing L-asparagine (0·24 g. N/l.). as the N source and the production of titratable acid by *Catenaria anguillulae*. Initial pH 8·7.

single sources; of these, arginine was the one utilized from the mixture. One anomaly, namely lysine, stands out in the early uptake from the medium; this amino acid was not utilized in the earlier study dealing with single sources. Once the organism had passed through the lag phase into the more rapid period of growth, all of the amino acids in the mixture disappeared from the medium in significant amounts and were presumably utilized by the fungus.

Effects of the addition of L-*lysine to a medium containing* L-*asparagine as the N source.* From the studies dealing with single N sources, L-asparagine emerged as the best source; this compound had the additional advantage that it was not utilized as a carbon source.

Because of the anomalous utilization of lysine from the Difco Casamino acids medium, the possibility that lysine might be a key to the problem of obtaining a shorter lag phase and a better yield was examined. Lysine might be a compound which was not utilized as a single source but which, when in a mixture with one or more other compounds which could be utilized as a N source, had a stimulatory effect. The medium used was Medium 5 adjusted to pH 8·7. L-asparagine was included in the medium at the optimum concentration (0·24 g. N/l.). L-lysine was added in concentrations varying from 0 (control) to 7 mM (Table 8). The concentrations of 0·1 and 0·5 mM were more in accord with the concentrations utilized during the lag phase on the Difco Casamino acids medium (0·244 and 0·293 mM during the first 5-day and 6-day harvests, respectively). The growth of these cultures was followed on the basis of

their production of titratable acid. The production of titratable acid was used to signify the end of the lag phase of growth, and hence the earlier production of such acid in the treatments to which 0·050 and 0·100 mM of L-lysine had been added was taken as evidence of the shortening of the lag phase under these conditions (Fig. 10). This was in comparison to the additional 24 hr required for the cultures in the control and other treatments (excluding the 1 and 7 mM treatments) to enter the phase of rapid growth. The rate of growth also appeared to be higher in all treatments with added lysine (except for the two highest concentrations) as compared to the control. Not only was the rate of growth higher, but the dry weights were considerably higher than those of the control (Table 8). From the dry weights at 240, 263 and 283 hr, it can be seen that growth in the flasks containing the lower concentrations (0·010, 0·050 and 0·1 mM) of added lysine had reached the peak of the growth curve after 10 or 11 days. At that time the control flasks were still growing rapidly and continued to do so through the 12th day of incubation when the experiment was terminated.

Table 8. *The effects of added* L*-lysine on the growth of Catenaria anguillulae in Medium 5 with* L*-asparagine (0·24 g. N/l.) as the nitrogen source*

Dry weights are the averages of duplicate flasks. (pH 8·7)

L-lysine level (mM)	Incubation period (hr)		
	240	263	283
	Yield dry weight (mg.)		
0	19	47	79
0·01	111	110	116
0·05	95	118	—
0·10	95	122	—
0·50	35	118	—
1·00	—	—	11
7·00	—	—	3

The final medium evolved. Medium 7 (Table 2) was adopted as best satisfying the growth requirements of the isolate strain 58–12 of *Catenaria anguillulae*.

DISCUSSION

Couch (1945) observed the morphological similarities between the genera Catenaria and Blastocladiella; representatives of these two genera are unique within the Order Blastocladiales in their inability to utilize inorganic nitrogen. The high pH optimum of the isolate strain 58–12 of *Catenaria anguillulae* used in the present work is unique among aquatic Phycomycetes; this is emphasized by its occurrence in regions of relatively alkaline pH value. For example, the fungus is found in the shallow margins of Lake George, Uganda, where the pH values often reach pH 10 (Dr L. G. Willoughby, personal communication to Dr R. Emerson; 29 November 1966) and in some barnyards (personal communication from Dr F. K. Sparrow, Department of Botany, University of Michigan, Ann Arbor, 1967).

The early studies of Buckley & Clapham (1929) and Butler & Humphries (1932) on the growth and development of Catenaria were made by using one of its natural substrates, the eggs of the liver-fluke *Fasciola hepatica* L. Qualitative information is available on the amino acid composition of liver-fluke egg proteins (Krvavica, Maloseja, Wagner & Martincic, 1964). Of those amino acids utilized by *Catenaria*

anguillulae in the present study, the eggs appear to contain proteins with high concentrations of arginine and lesser concentrations of glutamic acid and methionine; the eggs also contain stearic and oleic acids.

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An Electron Microscope Study of Structure and Germination of Conidia of *Cunninghamella elegans* Lendner*

By LILIAN E. HAWKER, BARBARA THOMAS AND A. BECKETT

Department of Botany, University of Bristol, Bristol, BS8 1UG

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SUMMARY

The spines projecting from the surface of the mature conidia of *Cunninghamella elegans* are hollow and are more or less circular in cross-section. The scanning electron microscope shows blunt tips and disc-like bases. The bases are partially embedded in shallow depressions in the outer wall of the spore. The spines are readily detached during processing for transmission electron microscopy.

Mature ungerminated conidia have 2-layered walls, and contain several nuclei, many mitochondria, numerous lipid bodies and sparse endoplasmic reticulum.

Shortly after being placed in water or a nutrient solution the conidia swell; irregular-shaped food vacuoles develop and the lipid bodies largely disappear. Germination takes place from 1 to 3 hr afterwards. A layer of material, denser than the original inner spore wall, develops over the germ-tube initial and later extends over the germ tube after emergence, tapering just behind the point of emergence to a thin dense line surrounding the entire spore. Both layers of the original spore wall are ruptured by emergence of the germ tube, apparently mechanically. These observations are relevant to the evolution of conidia.

INTRODUCTION

Hawker & Abbott (1963) and Ekundayo (1965, 1966) showed that mature sporangiospores of three Rhizopus species possessed a single-layered wall. Prior to germination a new inner wall similar in structure to the hyphal wall was formed. On germination the original cell wall was ruptured by the emerging germ tube which was enveloped in a thin extensible wall layer continuous with the newly formed inner spore wall. A similar sequence of events was reported by Bracker (1966) for *Gilbertella persicaria* and by Bartnicki-Garcia (1968) for a Mucor species. It is reasonable to assume that this formation of an extensible new inner wall just before germination is characteristic of the sporangiospores of those members of the Mucorales possessing multispored sporangia. *Cunninghamella elegans* lacks multispored sporangia or sporangioles and bears conidia singly on short sterigmata over the surface of an inflated conidiophore apex. These conidia, and those of some other members of the Zygomycetes, are usually interpreted as single-spored sporangia, possessing a double wall. A study of germination of such conidia is therefore desirable.

Preliminary electron micrographs (Hawker, 1966) showed that mature conidia of this fungus had a two-layered wall but that neither of these layers was continuous with

* See note on p. 189.

the germ-tube wall. It was suggested that a thin third wall layer had been formed within the original 2-layered wall of the conidium. Better methods of fixation and staining have, however, yielded preparations leading to a modified interpretation which is the subject of this paper.

METHODS

Samples of either unsoaked conidia or conidia which had been soaked for 2 hr in a 1% (w/v) glucose solution were briefly rinsed in a wetting agent and fixed by one of the following methods (all % values w/v except where stated):

1. Potassium permanganate; 2% unbuffered, on ice for 1 hr. 2. Glutaraldehyde + acrolein; 1 + 1 v/v mixture of 3% glutaraldehyde and 3% acrolein in 0·1 M-sodium cacodylate buffer in ice for 45 min., followed by post-fixation in 2% unbuffered osmium tetroxide on ice for 4 hr. 3. Freeze-etching; both soaked and unsoaked conidia were treated with 20% glycerol for 2 hr prior to fixation and preparation by the freeze-etching technique described by Moor (1966).

Chemically fixed material was stained in aqueous uranyl acetate, dehydrated in a graded ethanol series followed by propylene oxide and finally embedded in Epon. Sections were cut with a glass knife on an LKB Ultratome, stained in lead citrate and examined on an AEI EM6B electron microscope. Material for examination with the scanning electron microscope was prepared as previously described (Hawker, 1968).

RESULTS

The mature spore

Surface features. The scanning electron microscope and freeze-etched material show the spines on the spore surface to possess a blunt apex and an enlarged disc-shaped base (Pl. 1, fig. 2, 3), thus confirming the results obtained by Kawakami (1955) by observation of whole spores in silhouette with the transmission electron microscope and by Young (1968) both by the latter technique and by carbon replicas. Young's suggestion that the basal pads were hexagonal and that the spines themselves might be hexagonal in section, is not supported by the scanning electron microscope. The basal pads were circular discs except where they were unusually crowded or when the spore had contracted in processing, when the edges of the discs became compressed where two were in contact. The spines were smooth in outline, hollow and in transverse sections of chemically fixed material more or less circular. However, recent observations on cross fractured freeze-etched spines showed them to be doliform (barrel-shaped, Ainsworth, 1966).

The discs were flat at the base and raised on the upper surface, giving them a bun-like shape with the spine arising from the raised centre and tapering slightly towards the tip, the whole structure roughly resembling an inverted blunt tack (Pl. 1, fig. 2, 3). The basal discs were partially embedded in a shallow depression in the outer wall layer of the spore from which it was clearly differentiated. The spines were readily extracted during chemical fixation and left a shallow depressed scar (Pl. 2, fig. 12; Pl. 3, fig. 19).

Internal structure. Mature, ungerminated spores of many fungi are notoriously difficult to prepare satisfactorily for transmission electron microscopy. Opinions differ whether poor definition is a function of the contents of dormant spores or is due either to failure of the fixative to penetrate or to difficulty in sectioning a thick spore wall without distortion of the cytoplast (Madelin, 1966). Conidia of *Cunninghamella*

elegans were easier to prepare than were those of Rhizopus species but still presented some difficulty.

In sectioned material of mature ungerminated spores, the wall consisted of at least two layers (Pl. 1, fig. 4, 5). The outer layer was mainly composed of relatively electron-dense material of loose texture, was relatively thin (about 0·1 to 0·25 μ thick) and had an irregular outer surface. The inner wall of the spore was composed of homogeneous close-textured relatively electron-transparent material and was thicker (about 0·3 to 0·5 μ) than the outer one. At the interface between these two layers there was a smooth dense band, appearing as a black line in the electron micrographs, and varying in density and thickness according to the methods of fixation and staining used. We could not determine whether this dense zone was a distinct layer or part of the outer or inner walls. During sectioning tears frequently occurred along this band but on either side of it. This band may represent a fusion zone between the distinct outer and inner layers of the wall.

A similar wall structure has recently been figured for the aleuriospore wall of *Aspergillus carneus* by Pore, Pyle, Larsh & Skvarla, 1969. These authors suggest two possible interpretations of the complex wall.

The cytoplast contained many organelles and vacuoles but had only a few short segments and vesicles of endoplasmic reticulum most of which were situated just within the plasmalemma. Many mitochondria of various shapes, but with typical cristae, were present together with numerous spherical lipid bodies and several nuclei (Pl. 1, fig. 5).

The germinating spore

After a short period of soaking in 1% glucose solution (or in water, Ekundayo, 1965) the conidia swelled, the wall becoming stretched and consequently thinner, and one or more germ tubes emerged (Pl. 1, fig. 1). Plate 2, fig. 17 shows a section through a germinated conidium, but does not pass through the germ tube; in addition to the stretching already mentioned the dark zone on the inner side of the outer wall is less conspicuous. The two layers are still distinct and often separate under the stress of sectioning, confirming their separate identity. No clearly defined new innermost wall surrounding the body of the spore, as in Rhizopus, was seen but the wall was delimited on the innermost side by a thin zone which was not present in unsoaked spores. In material fixed with potassium permanganate, this zone was seen as an electron-dense line (Pl. 2, fig. 11 to 13, 15; Pl. 3, fig. 18 to 20). In glutaraldehyde-acrolein fixed conidia, the zone was less dense but readily visible (Pl. 2, fig. 16). Pieces of broken spines surround both the ungerminated and germinated spore and the bases of some may be seen embedded in the outer wall material (Pl. 3, fig. 18, 20). In both chemically fixed and freeze-etched material small vesicles were seen in the peripheral layers of the cytoplasm and similar ones occurred between the plasmalemma and the innermost layer of the wall (Pl. 2, fig. 13, 14). Lomasomes were seen in some earlier preparations but are not figured here. They were not seen in recent preparations and may have been artefacts due to slow fixation, there is, however, some indication of similar bodies in freeze-etched material. Multivesicular bodies were also occasionally seen in poorly fixed material and again may have resulted from slow penetration by the fixative. The nature of lomasomes and multivesicular bodies has recently been discussed by Bracker (1967) and Marchant & Robards (1968).

The number of nuclei did not greatly increase during soaking. However, mitochon-

dria were more numerous and more uniform in shape and size, suggesting that they had recently undergone division. The most striking change was the almost complete disappearance of the lipid bodies seen in the ungerminated spore and their replacement by irregular membrane-bound vacuoles containing granular material probably of phospholipid nature, and amorphous masses of non-sulphated slightly acid polysaccharide material (compare Pl. 1, fig. 4, 5 with Pl. 2, fig. 17). (Light microscope observations of stained conidia showed that this material, present only in germinated spores, was strongly periodic acid-leucofuchsin positive, β metachromatic in toluidine blue and with a methylene blue extinction of above pH 2·0, Pearse, 1953; Jensen, 1962.) The endoplasmic reticulum was still sparse and mainly confined to the peripheral zone. The endoplasm had a reticulate appearance which could be due to the hydrolysis of patches of food reserves. As the germ tube elongated the food vacuoles decreased in number but some polysaccharide reserves remained.

Plate 2, fig. 11, 12 show early stages in germ-tube formation and emergence. Fig. 11 shows the germ tube initial bulging into the spore wall, the outer layer of which is partially ruptured. The appearance suggests mechanical rupture rather than chemical dissolution since the tearing begins at the outside and not next to the emerging germ tube. The most striking feature of this figure is, however, the cap over the apex of the germ-tube initial consisting of a thin layer of relatively electron-dense material. This layer tapers off just behind the germ-tube apex to form a continuous dark line surrounding the spore. The thin inner line developing on soaking and the thicker dark zone over the germ tube could be a new wall layer, although not so distinct as that formed in Rhizopus, but its appearance is more consistent with a change in the chemical nature of the wall. If this is so, then the appearance of small vesicles and invaginations at the plasmalemma cell wall interface (Pl. 2, fig. 13, 14) may represent the sites of enzyme activity associated with such a change. Fig. 12 shows a slightly later stage in germ-tube emergence, the outer layer of the spore wall being completely ruptured and the inner one partially so. The apical cap again tapers away just behind the point of emergence.

Plate 3, fig. 18 to 20 show sections of two spores at a later stage of germination. Serial sections of these two spores indicated that the sections figured in fig. 18 and 19 were nearly median through the germ-tube apex, but that fig. 20 was not. The endoplasmic reticulum was less sparse than in the spore but was again mainly peripheral. Fig. 20 includes a tangential section through this peripheral layer of E.R. which is seen to consist of vesicles, cisternae and short sections of unit membrane. A nucleus is seen passing into the germ tube. The wall of the germ tube is clearly an extension of the original germ-tube cap and can again be seen tapering just behind the point of germ-tube emergence. This is shown most clearly in Pl. 4, fig. 21.

Transverse sections through young germ tubes (Pl. 1, fig. 8) showed that their structure was similar to that of a vegetative hypha (not illustrated), except that E.R. was more complex in the germ tube, probably correlated with rapid growth of the latter, and there were no vacuoles. The germ tubes increased in diameter after emergence (Pl. 1, fig. 1), and finally developed into normal vacuolated hyphae.

DISCUSSION

The evolution of the conidium from the multispored sporangium, by the gradual reduction in number of the spores to one, is a plausible hypothesis which has been favoured by many mycologists. On this interpretation the conidium wall was originally 2-layered, the outer corresponding to the sporangial wall and the inner to that of the sporangiospore. The genera of the Mucorales can be arranged in a series from those, such as *Mucor* and *Rhizopus*, possessing a sporangium containing numerous spores, to those with smaller sporangia containing fewer spores, to others with sporangioles containing only a few spores and finally to genera such as *Cunninghamella* in which the asexual stage produces only conidia.

Electron microscope studies have already produced evidence in favour of the evolution of conidia from multispored sporangia. The conidia of some higher fungi (e.g. *Botrytis cinerea*, Hawker & Hendy, 1963) have been shown to have a 2-layered wall, although these layers are less clearly differentiated from one another than are the two layers of the spore wall in Cunninghamella as described in this paper. One might claim that this is consistent with a greater evolutionary distance of Botrytis from a remote multispored ancestor.

Further evidence is afforded by a study of the ornamentation of the sporangial and spore walls in Mucorales. Young (1968) shows by carbon replicas that the sporangial wall is spiny in *Mucor plumbeus*, *Gilbertella persicaria* and *Pilaira anomala*, that both conidia and sporangioles are spiny in Choanephora species and that conidia of Cunninghamella and of three Choanephora species are also spiny. The writers have examined sporangia of *Mucor mucedo* (Pl. 1, fig. 6, 7) and *M. hiemalis* with the scanning electron microscope. In both species the sporangial wall is covered with spines, having swollen bases as in the conidial spines of Cunninghamella. Only one of the species examined by Young, *Syzgites megalocarpus*, had spiny sporangiospores, those of other species being smooth or ornamented in a different pattern. The form of the spines varies with the species; most, however, have a swollen base as in Cunninghamella. We could not determine the exact shape of the Syzgites spines from Young's photograph.

This resemblance between the outer surface of sporangia, sporangioles and conidia of some of the Mucorales supports the suggestion that the outer wall of the conidium is homologous with the sporangium wall.

The methods of germination of sporangiospores and conidia are of particular significance in the interpretation of these spores. Bartnicki-Garcia (1968) recognized three 'basically different methods of vegetative wall formation during spore germination' and considered these to be characteristic of particular groups of fungi and perhaps to be correlated with the chemical composition of the walls. The results described in the present paper suggest that a fourth group or sub-group should be recognized.

(1) Electron microscopy has confirmed the well known fact that the originally naked zoospores of many aquatic Phycomycetes surround themselves with a cell wall before germination (e.g. Meier & Webster, 1954; Fuller, 1966). This *de novo* formation of a wall around a naked cytoplast is Bartnicki-Garcia's Type II.

(2) Bartnicki-Garcia's Type III is the '*de novo* formation of a vegetative wall under the spore wall' and, as already stated, has been seen in several members of the Mucorales. Bartnicki-Garcia suggests that it may be exclusive to fungi with vegetative walls of the chitosan–chitin type (viz. Zygomycetes) and might be the consequence of changes

in cell-wall composition during germination. He further points out that the formation of a new vegetative type wall is easy to determine in these fungi since the original spore wall is of distinctive architecture (Hawker & Abbott, 1963). He considers that it is more difficult to determine the exact layers concerned in germination in spores with a less distinctive original wall and suggests that Marchant's (1966*a*) claim that conidia of *Fusarium culmorum* germinate in this manner may be premature.

(3) Germination of the conidia of *Cunninghamella elegans* shows some features similar to those characteristic of the last group. The cap of relatively electron-dense material covering the emerging germ tube and continuous with a newly developed thin dark line on the inner side of the original wall could be interpreted as a new wall, as in sporangiospores of Rhizopus but fully developed only over the germ-tube initial. However, unlike the situation in Rhizopus this layer is not clearly distinct from the inner layer of the original spore wall. Its appearance is consistent with its being a chemically changed part of the original wall rather than an entirely new wall formed *de novo*. It does not show the clear structural difference from the spore wall which could be seen in Rhizopus. It has already been pointed out (p. 184) that numerous small vesicles present between plasmalemma and wall indicate enzyme activity at this site. This seems to be directed to changing the innermost zone of the existing wall rather than to the deposition of new and architecturally different material.

Marchant's (1966*a*, *b*) account of germination of conidia of Fusarium suggest a similarity between this fungus and Cunninghamella.

(4) In Bartnicki-Garcia's Type I the spore wall or the inner layer of it gives rise directly to the germ-tube wall with which it is continuous. This type is probably widespread among Higher Fungi and includes not only conidia (e.g. those of *Botrytis cinerea* Hawker & Hendy, 1963; *Byssochlamys fulva*, *Penicillium frequentans* and probably *Aspergillus niger*, Hawker, 1966; *Aspergillus oryzae*, Tanaka, 1966; *Penicillium megasporum*, Remsen, Hess & Sassen, 1967; *Neurospora crassa*, Bartnicki-Garcia, 1968; *Aspergillus fumigatus*, Campbell, 1968) but also uredospores of *Melampsora lini* (Manocha & Shaw, 1967) and ascospores of Neurospora (Sussman, 1966). The conidia of *Penicillium megasporium* possess a complex wall (Sassen, Remsen & Hess, 1967) consisting of several more or less distinct layers. The innermost layer is thin but continuous. Prior to germ-tube emergence (Remsen *et al.* 1967) this layer thickens over the area at which the germ tube is about to form. As the latter emerges the outer layer(s) of the spore wall are ruptured and the germ-tube wall is derived from the intact innermost one. This bears some resemblance to the events in Cunninghamella but in the latter the layer which forms the germ-tube wall cannot be distinguished in unsoaked spores.

Hawker & Hendy (1963) suggested that the 2-layered wall of Botrytis was consistent with the evolution of conidia from sporangia and discussed the absence of *de novo* wall formation in this fungus in comparison with the formation of such a new wall in the sporangiospores of Rhizopus. The germ-tube wall is obviously not homologous in these two genera.

The difficulty of clearly defining the exact relationship of the germ-tube wall of Cunninghamella to the inner layer of the original conidium wall is significant. It suggests a way in which the transition from the sporangiospore type of germination to that of the conidia of Higher Fungi could have taken place by the progressive loss of the ability to form a complete new wall during germination and the taking over by the

original spore wall or part of it of the function of germ-tube wall formation. Under favourable conditions germination of conidia of *C. elegans* is much more rapid than that of sporangiospores of Rhizopus or Mucor. The reduction in the amount of new wall formation could well result in an acceleration of the early stages of germination with consequent improved survival value. This might well have played a part in the evolution of the conidium.

Finally, the position of *Fusarium culmorum* is of interest. Marchant's (1966*a*) Plates are not convincing in support of his statement that a new wall forms as in Rhizopus (in which this new wall entirely envelops the protoplast). They do, however, suggest that a partial new wall may be produced or that chemical changes in the composition of the innermost layer of the spore wall may take place. Marchant refers to 'the deposition of a new electron-transparent wall layer on the inside of the conidium in the *region of germ-tube production*' (our italics). In a later paper (Marchant, 1966*b*) he figures germination of *F. culmorum* diagrammatically and shows the germ-tube wall tapering off behind the point of emergence as in Cunninghamella. It is of considerable interest to find such a 'relic' of the Zygomycete type of germination among the Higher Fungi. If other similar examples are discovered it may be that Bartnicki-Garcia's (1968) suggestion that the Rhizopus type of germination is probably exclusive to Zygomycetes with a chitosan–chitin cell wall may prove to be too rigid and will have to be reconsidered.

Thanks are due to Mr R. J. Hendy and Mrs M. A. Gooday for making available the results of preliminary studies, to Professor H. E. Hinton F.R.S. for allowing us to use the Stereoscan microscope provided for his use by the Science Research Council.

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EXPLANATION OF PLATES

Key to lettering: N = nucleus, M = mitochondria, P = plasmalemma, E.R. = endoplasmic reticulum, L = lipid bodies, V = vacuoles, E = point of emergence of germ tube, OW = outer wall layer of spore, DZ = dense zone on inside of inner spore wall, IW = inner wall layer of spore, GI = germ-tube initial, GW = germ-tube wall, S = spine, SO = socket from which spine has been torn in processing. All figures are of *Cunninghamella elegans*—except fig. 9 and 10 which are of *Mucor mucedo*.

PLATE 1

Fig. 1. Germinating conidia of *Cunninghamella elegans*. Photomicrograph by J. A. Ekundayo.

Fig. 2. Spores as seen by scanning electron microscope. Note swollen disc-like bases of blunt spines. White cylinder is broken part of conidiophore.

Fig. 3. A cross fracture of a freeze-etched conidium. The two layers of the wall can be seen at the points indicated by arrows, and some of the spines can be seen to have their bases embedded in the outer layer. The thick arrow indicates the direction of shadowing.

Fig. 4. Median section through ungerminated spore, fixed in glutaraldehyde-acrolein. Note thick wall and numerous lipid bodies.

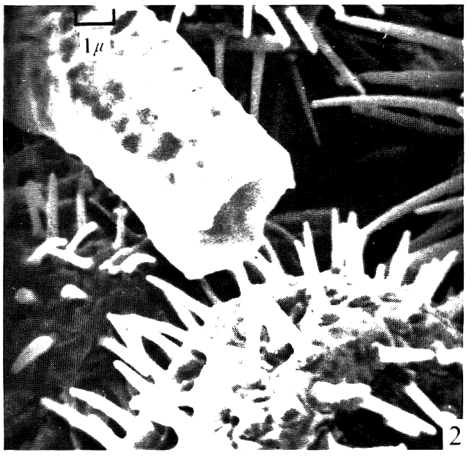
Fig. 5. A similar section through a spore fixed in potassium permanganate.

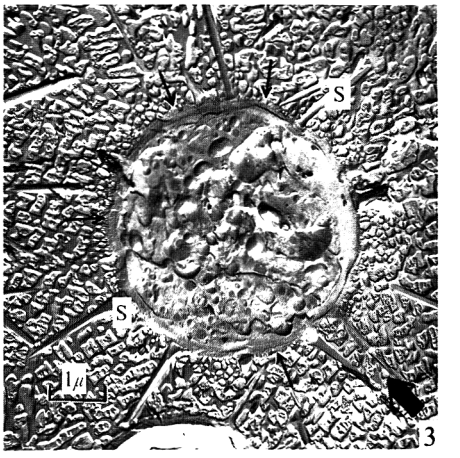
Fig. 6 and 7. Parts of the wall from the spores shown in fig. 4 and 5 respectively. The loose-textured outer layer readily separates along the interface between it and the inner layer (cf. fig. 7). The dense band may represent a fusion zone between the two layers.

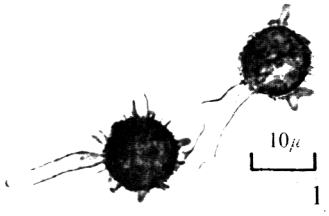
Fig. 8. T.S. of a germ tube.

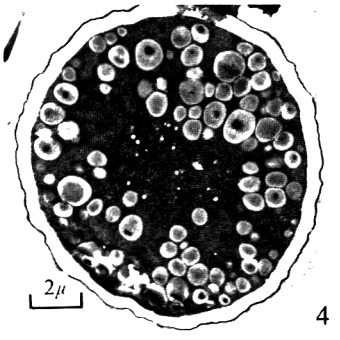
Fig. 9. Spiny sporangium of *Mucor mucedo*; scanning electron microscope.

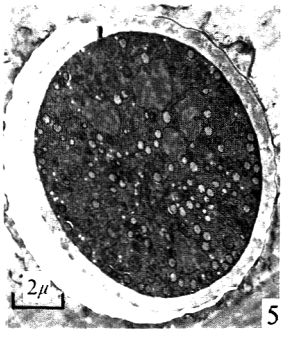
Fig. 10. Part of same enlarged, showing spines of essentially similar form to those on conidium of *Cunninghamella*.

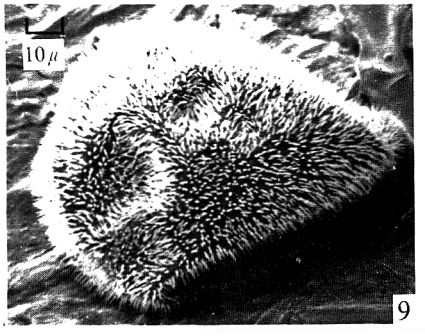


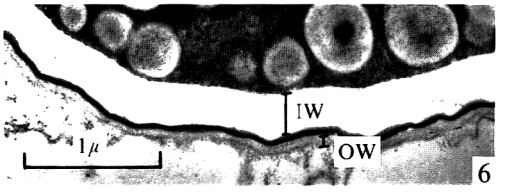


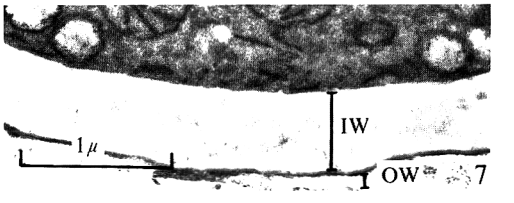


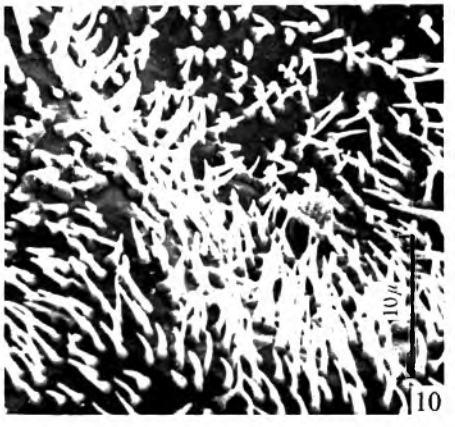


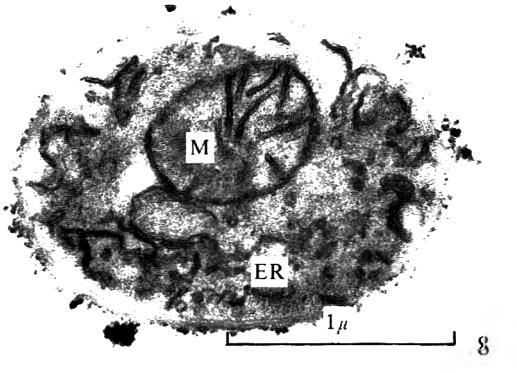












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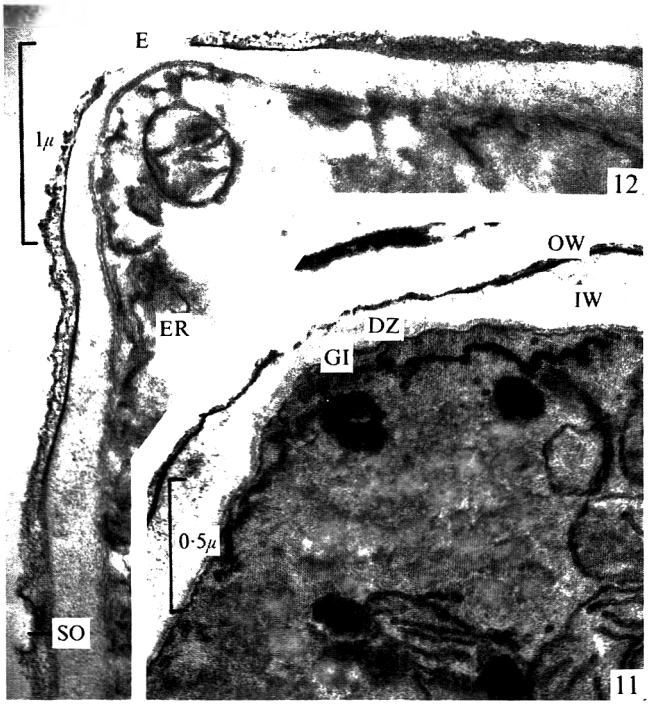
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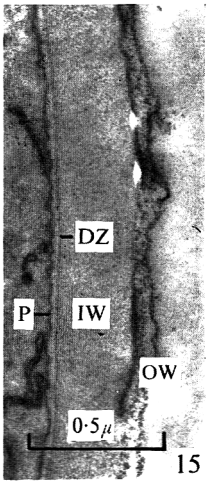
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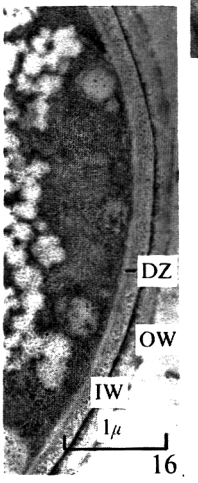
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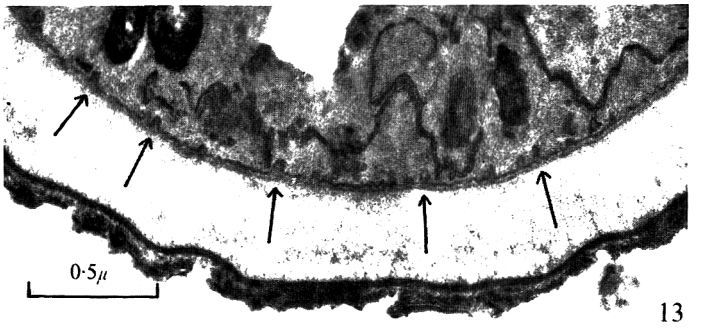
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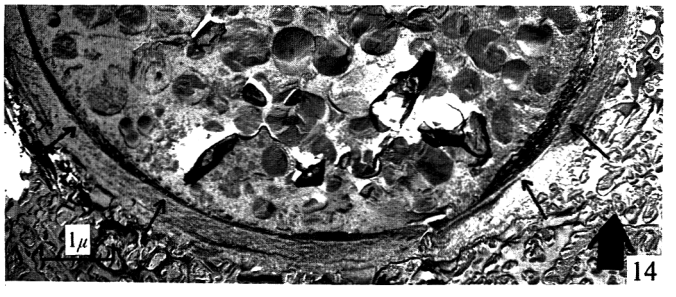
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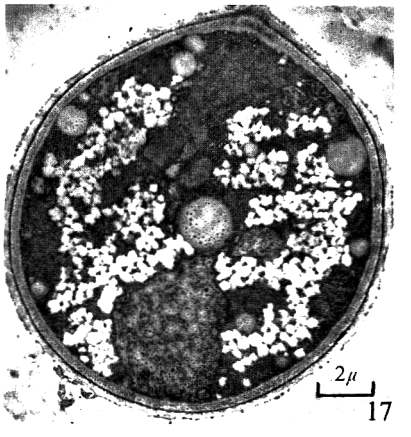
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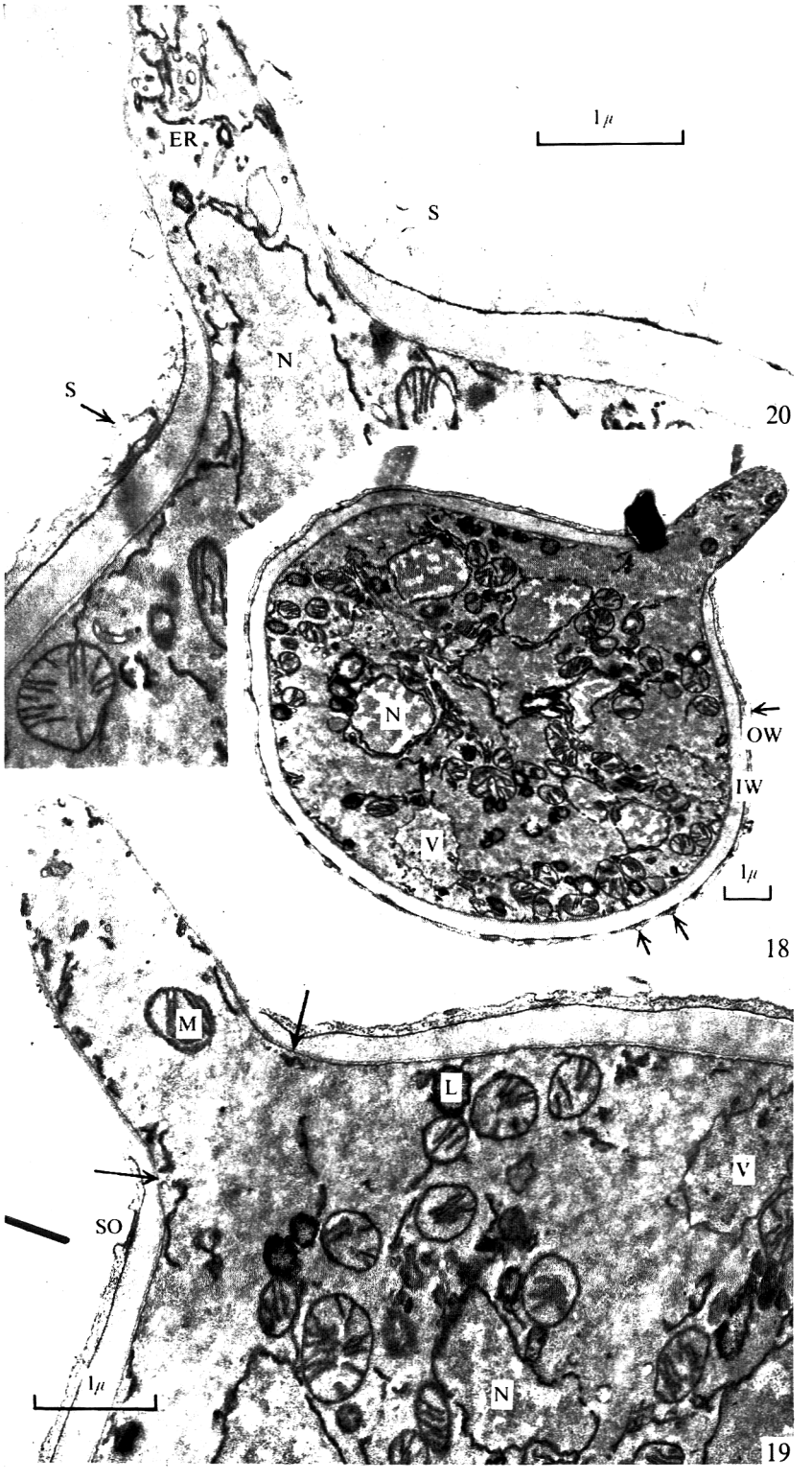

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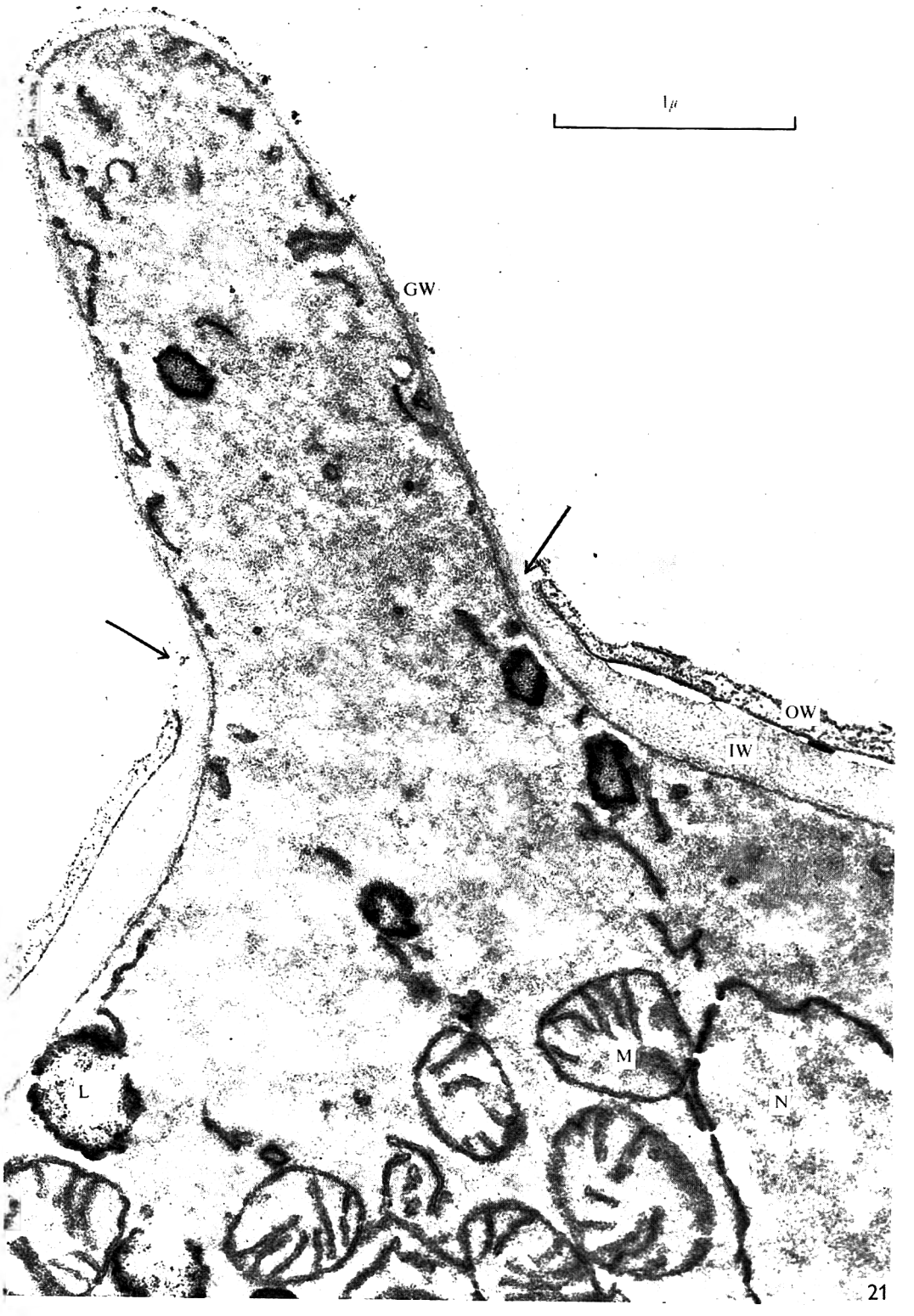

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PLATE 2

Fig. 11. Section through a conidium at the point where the germ-tube initial is forming. The outer layer of the spore wall is almost completely ruptured and the inner layer is stretched over the initial. A thin zone of electron-dense material can be seen also lying over the germ-tube initial, immediately outside the plasmalemma. $KMnO_4$ fixation.

Fig. 12. A slightly later stage in development of the germ-tube initial. The outer wall layer is completely ruptured and the inner layer partially so. The dense zone adjacent to the plasmalemma is readily seen in median section. $KMnO_4$ fixation.

Fig. 13. T.S. through part of a germinated conidium showing the accumulation of small vesicles at the plasmalemma–cell wall interface (arrows). $KMnO_4$ fixation.

Fig. 14. A cross fracture through a freeze-etched conidium showing similar vesicles (arrows) to those seen in fig. 13. The thick arrow indicates the direction of shadowing.

Fig. 15. T.S. through part of the wall of a germinated conidium showing the two distinct layers of the wall and the narrow dense zone which surrounds the spore on the inside of the wall. $KMnO_4$ fixation.

Fig. 16. As for fig. 15. Glutaraldehyde-acrolein fixation.

Fig. 17. T.S. through a germinated conidium showing the presence of amorphous masses of polysaccharide material. Lipids are absent (cf. Pl. 1, fig. 4). Glutaraldehyde-acrolein fixation.

PLATE 3

Fig. 18. Early stage in germ-tube development; nearly median section through emerging germ tube; Arrows indicate points where the bases of spines can be seen embedded in the outer layer of the wall. $KMnO_4$ fixation.

Fig. 19. Serial section of same spore as in fig. 18, showing ruptured spore wall and thin new wall covering germ tube and tapering to a thin line behind point of emergence (arrows). $KMnO_4$ fixation.

Fig. 20. Section through another spore, not passing through apex of germ tube. Note nucleus passing into germ tube $KMnO_4$ fixation.

PLATE 4

Fig. 21. Nearly median section through young sperm tube showing relation between germ-tube wall and conidium wall (arrows). $KMnO_4$ fixation.

Note added in proof

Since this paper went to press, we have received the following:

SAMSON, R. A. (1969). Revision of the genus *Cunninghamella* (Fungi Mucorales). *Proceedings of the Royal Netherlands Academy*, **72**, 322. The isolate described in the present paper was grown on potato extract agar or malt extract agar. On these media it does not fit exactly the description of any of the species listed by Samson.

J. gen. Microbiol. (1970), **60**, 191–197
Printed in Great Britain

A Comparison of Two Techniques for Counting Cellulolytic Rumen Bacteria

By N. O. VAN GYLSWYK

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, Republic of South Africa

(*Accepted for publication 23 October 1969*)

SUMMARY

Cellulolytic bacteria were counted by the 'direct' method: estimated from the number of clearings produced in films of cellulose-containing agar medium inoculated with high dilutions of rumen ingesta and by the 'indirect' method; numbers were calculated from 'total culturable' counts on films of a non-specific agar medium and from the percentages of bacteria found to be cellulolytic after isolation in pure culture. The two methods yielded similar results.

Two methods have been mainly used to determine the numbers of cellulolytic bacteria in the rumen. In the 'direct' method as used for instance by Hungate (1947, 1950, 1957), Kistner (1960), Gilchrist & Kistner (1962), Kistner, Gouws & Gilchrist (1962), Gilchrist (1965) and De Wet (1966), colony counts of cellulolytic bacteria are made on a selective medium containing finely divided cellulose. The cellulolytic colonies were identified by the circular clearings formed through the action of cellulase secreted by the bacteria. The number of clearings gives a direct estimate of the numbers of cellulolytic bacteria in the sample. In the 'indirect' method used for instance by Bryant & Burkey (1953*b*), Bryant, Small, Bouma & Robinson (1958) and De Wet (1966) an agar medium designed to support the growth of the widest possible spectrum of rumen bacteria was inoculated with high dilutions of rumen contents. Well-spaced colonies were picked non-selectively and transferred directly to agar slants of non-specific medium. All the isolates obtained from a number of samples, collected at intervals from an animal conditioned to a particular diet, were then examined for cellulolytic activity. From the percentage of isolates capable of hydrolysing cellulose and the mean colony count on the non-selective medium the mean level of cellulolytic bacteria in the rumen was calculated.

When comparing the counts of cellulolytic bacteria by the two methods, counts by the 'indirect' method were almost always higher than those obtained by the 'direct' method. In Table 1 the values for counts by the 'direct' method range from about 1×10^6 to 20×10^6 bacteria (colonies)/ml. whereas those by the 'indirect' method are within the range 1×10^6 to 500×10^6/ml. In no case, however, were counts by the two methods made on the same sample. Although De Wet examined samples from the same animals on the same diets, the samples were taken on different dates. To decide whether these differences are due to inherent differences in counting techniques, or are due to differences between animals or between diets, a comparison of the two methods was made on the same samples from sheep on three high-roughage

diets. This work was done in the course of studies on the cellulolytic flora of sheep on different diets. To get enough isolates in the case of the 'indirect' method in Expts. 1 and 3 it was necessary to obtain them from more than one sheep on a given ration. However, for the purpose of this study this was not considered to be important.

Table 1. *Numbers of cellulolytic rumen bacteria found by different authors using the 'direct' and 'indirect' counting methods*

Reference	Animals used	Ration	Numbers of cellulolytic bacteria/ml. or g. ($\times 10^6$)
Direct method			
Hungate (1957)	Cows	Timothy hay+different supplements	2·87 (12)* 5·57 (12) 3·00 (12) 2·12 (11) 2·27 (12) 3·14 (9) 1·88 (12) 0·83 (13)
Kistner (1960)	Sheep	Lucerne hay	8·2 (2–22)† (11) 18·0 (13·75–25·6) (5) 20·5 (19·25–21·5) (4)
Gilchrist & Kistner (1962)	Sheep	Poor teff hay	3·2 (0·1–10) (17)
Kistner *et al.* (1962)	Sheep	Lucerne hay	17 (1–39) (19)
Gilchrist (1965)	Sheep	Lucerne hay Teff hay Teff hay+urea +molasses	5 (4–6) (3) 2·03 (0·1–5) (3) 1·64 (0·06–3) (4)
De Wet (1966)	Sheep	Wheat straw Wheat straw+urea +molasses	0·87 (0·01–5) (20) 15 (2·5–50) (35)
Indirect method			
Bryant & Burkey (1953*b*)	Cows	Alfalfa hay+concentrates	113, 294
		Wheat straw	502
		Concentrates	338
		Alfalfa hay	258, 114
Bryant *et al.* (1958)	Cow		100
	Calves	Alfalfa hay+grain mixture	1·2 110 320 430 62
De Wet (1966)	Sheep	Wheat straw Wheat straw+urea +molasses	4·1 143

* Number of counts made. † Range.

METHODS

Animals. These were Merino wethers fitted with permanent rumen fistulas.

Diets. The diet for Expt. 1 consisted of 1500 g. of teff hay daily. For Expt. 2 the ration consisted of 1200 g. of teff hay, supplemented with 10 g. urea and 80 g. glucose in 1 l. water which was given *per fistula* at about 09.00 a.m. In Expt. 3 the sheep were fed 1200 g. of treated teff hay daily, the crude protein content of which had been raised

from 3·7 to 10·7% by application of spray-dried egg albumin. Each ration was supplemented with a balanced mineral+trace element lick and vitamin A. In all three experiments the hay was given to the sheep as one feed at about 08.15 a.m. The trace element lick (about 15 g.) was given separately at the same time while vitamin A was given once/week. The sheep were conditioned to the diets for at least 6 weeks before sampling of rumen ingesta began.

Sampling and treatment of rumen ingesta. Samples of rumen ingesta for bacterial counts were taken through the fistula with a sampling tube about 2·5 hr after feeding. On sampling days water was withheld from the sheep from the time of feeding until the sample had been drawn. Ingesta were generally not drawn more often than once/week from a sheep. In Expts. 1 and 2 about 150 ml. ingesta were withdrawn. The sample was blended with an Ultra Turrax, type TP 18/2 homogenizer (Janke & Kunkel, Staufen i. Br., West Germany) operating at 20,000 rev./min. for 30 sec. with the container cooled in ice. A 10 g. subsample was weighed out and then diluted with 90 ml. of an anaerobic diluting solution similar to that used by Bryant & Burkey (1953*a*) except that it contained indigo carmine instead of resazurin. This mixture was again treated with the homogenizer for 60 sec. to ensure maximal release of organisms from solid particles of ingesta and perhaps to decrease the size of aggregates of bacteria. For Expt. 3, in an attempt to obtain a truly representative sample from the rumen, about 600 ml. ingesta were withdrawn. This was mixed by shaking in a stoppered bottle; a 150 ml. sample, treated as described above, was used for making colony counts.

Cellulolytic counts by the 'direct method'. For these counts 1·2% (w/v) ball-milled Whatman no. 1 filter paper was substituted for the carboyhdrates in the medium of Bryant & Robinson (1961) for obtaining 'total culturable' counts. Indigo carmine (0·0005%) was substituted for resazurin. The filter paper cellulose was prepared as follows: 12 g. shredded filter paper and 600 ml. deionized water, were placed in a ball mill of about 1 l. capacity and this was milled with a mixed charge of porcelain balls for about 72 hr at 57 rev./min. Roll bottles of 7 ml. capacity, spun mechanically, were used as culture vessels (Kistner, 1960). Colonies of cellulolytic bacteria were counted after incubation for 4 weeks at 39°.

'Total culturable' and 'indirect' cellulolytic counts. Methods for obtaining colony counts of 'total culturable' bacteria were essentially those of Bryant & Burkey (1953*a*) as modified by Bryant & Robinson (1961). The medium (GCSX medium) contained 0·05% each of glucose, cellobiose, starch and xylan, with indigo carmine (0·0005%) instead of resazurin. Roll bottles were used as for the 'direct' cellulolytic counts. Colonies of 'total culturable' bacteria were counted after incubation for 1 week at 39°.

For the 'indirect' cellulolytic counts generally all the well-isolated colonies were picked from one or more roll bottles containing GCSX medium inoculated with the 10^{-7} or 10^{-8} dilutions of the samples. Inoculum was transferred to slopes of GCSX medium. The cultures were tested for ability to hydrolyse cellulose in a medium containing rumen fluid and 1·2% (w/v) ground α-cellulose prepared from teff hay. Inoculated bottles containing this medium were incubated for at least 4 weeks, after which samples of the well-mixed medium were placed in Wintrobe Hematocrit Tubes (Type A-2456, Clay Adams, Inc., N.Y.) and centrifuged for 30 min. at about 1500 *g*. Cultures showing more than 10% decrease in volume of cellulose when compared

with readings for uninoculated medium were considered to be cellulolytic. Ten% was considered a minimal value consistent with reliable interpretation.

Table 2. *Counts of 'total culturable' and cellulolytic bacteria made by 'direct' counting method on samples of rumen ingesta from sheep fed teff hay diets*

Experiment number*	Sheep	Number of colony-forming units/1 g. rumen ingesta: 'Total culturable' counts ($\times 10^8$)		Cellulolytic colony counts ($\times 10^6$)	
1	1	9		53	
	1	7		9	
	1	3		8	
	1	2		7	
	1	10	Mean = 5·2	4	Mean = 23
	1	8	$\sigma = \pm 3{\cdot}7$	60	$\sigma = \pm 21$
	1	1	$V\sigma = 71$	42	$V\sigma = 91$
	1	6		28	
	2	0·3		0·7	
	2	0·8		0·8	
	2	5		34	
	3	10		30	
2	4	9		8	
	4	10		7	
	4	4	Mean = 7·7	N.D.	Mean = 38
	4	0·5	$\sigma = \pm 4{\cdot}9$	0·4	$\sigma = \pm 54$
	4	14	$V\sigma = 63$	109	$V\sigma = 142$
	4	11		125	
	4	11		7	
	4	2		11	
3	5	11		37	
	5	37		18	
	5	38		24	
	5	26		24	
	5	32		28	
	5	36		42	
	6	19		43	
	6	13	Mean = 29	17	Mean = 24
	6	35	$\sigma = \pm 10$	19	$\sigma = \pm 11$
	6	28	$V\sigma = 35$	36	$V\sigma = 45$
	6	39		28	
	6	24		30	
	7	28		9	
	7	17		6	
	7	22		23	
	7	48		28	
	7	37		9	
	7	23		16	

* For diets see text. N.D. = not done.

RESULTS

The results obtained for the 'total culturable' and 'direct' cellulolytic counts for the three experiments are given in Table 2. Table 3 gives a comparison of the results for the 'direct' and the 'indirect' methods of counting cellulolytic bacteria. A remarkably close agreement existed in the results obtained with the two methods in

Expt. 3. The differences between the results for the methods in Expts. 1 and 2, although approaching twofold, were not considered to be excessive. The percentages of cellulolytic bacteria calculated from the 'direct' and 'indirect' counts were, respectively: Expt. 1, 4·4 and 7·2; Expt. 2, 4·9 and 3·0; Expt. 3, 0·8 and 0·8.

Table 3. *Derivation of 'indirect' cellulolytic counts and comparison with 'direct' cellulolytic counts made on samples of rumen ingesta from sheep fed teff hay diets*

		Indirect method					Direct method
Experiment number*	Sheep	Number of colonies picked from non-specific medium	Number of colonies found to be cellulolytic	Cellulolytic bacteria (% total number of colonies picked)	Mean 'total culturable' counts†	Mean number of cellulolytic bacteria/1 g. rumen ingesta (calculated)	Mean number of cellulolytic bacteria/1 g. rumen ingesta†
1	1, 2, 3	291	21	7·2	$5{\cdot}2 \times 10^8$	37×10^6	23×10^6
2	4	168	5	3·0	$7{\cdot}7 \times 10^8$	23×10^6	38×10^6
3	5, 6, 7	265	2	0·8	29×10^8	23×10^6	24×10^6

* For diets see text. † See Table 2.

DISCUSSION

In the present study, the 'direct' and 'indirect' methods of colony counts of cellulolytic rumen bacteria yielded essentially the same results when applied to the same samples. This does not exclude the possibility that the differences between the counts reported by various workers using one or other of the methods (Table 1) were partly due to differences in counting techniques. Both methods are subject to particular types of errors. In the case of the 'direct' method, the nature of the cellulose substrate may influence the counts. Certain forms of cellulose are not readily utilized by all strains of cellulolytic rumen bacteria (Halliwell & Bryant, 1963; Hungate, 1966; Shane, 1966). Moreover, if the cellulose substrate is not sufficiently ground the substrate is not homogeneously distributed throughout the medium. This can make detection of small clearings difficult, and it may also prevent the development of colonies from cellulolytic bacteria which are not near to the cellulose substrate. Low cellulolytic counts may also result from competition between cellulolytic bacteria for limiting amounts of accessory nutrients, or from accumulation of metabolic end-products, which prevent some of the bacteria from developing into visible colonies. This is often the case in cultures inoculated with lower sample dilutions.

In the case of 'indirect' counts, the method of determining cellulolysis may influence the results. The method described above showed up weakly cellulolytic strains which were previously missed when the method of Bryant & Burkey (1953*a*) was used for detecting cellulolysis. The extent to which the medium for the 'total culturable' counts supports the growth of all viable bacteria in the sample of rumen contents should not affect the 'indirect' counts of cellulolytic bacteria, provided that the medium meets the environmental requirements of all the cellulolytic species. However, the composition of the medium may affect the apparent percentage of cellulolytic bacteria. Thus the high percentage of cellulolytic bacteria in rumen contents of cows on various

diets reported by Bryant & Burkey (1953*b*) may be related to the fact that these authors included neither starch nor maltose in their medium for 'total culturable' counts and therefore excluded at least one important specifically amylolytic, organism namely *Bacteroides amylophilus*. The 'indirect' method becomes particularly inefficient when the cellulolytic organisms form a small percentage of the 'total culturable' bacterial population. Unless a very large number of isolations is made under these circumstances, considerable errors in cellulolytic counts may occur. However the 'indirect' method does give the proportion of the total population which is cellulolytic, although it is easier to obtain this information by making 'direct cellulolytic counts and total culturable' counts on the same sample. In the present work, percentage values for cellulolytic bacteria were obtained by both methods and the figures for the two methods were in close agreement.

Another factor which can profoundly influence the counts of cellulolytic bacteria obtained by different workers is the method of obtaining the sample. A large proportion of the cellulolytic bacteria in the rumen are attached to the food particles (Hungate, 1966) and may therefore be excluded by straining the ingesta. There is, however, no generally accepted method of collecting and preparing samples of ingesta for making counts. The influence of different procedures on the counts of cellulolytic and other groups of bacteria in the rumen is being investigated in this laboratory.

The time of day at which the samples are taken can influence the counts in two ways: (1) it may affect the proportion of organisms bound to food particles and hence enhance or decrease differences due to the method of sample preparation; (2) it may also reflect diurnal changes in the actual numbers of bacteria in the rumen. Thus the results of 'total culturable' counts on rumen contents of a cow fed a grain ration or a hay ration once a day, as reported by Bryant & Robinson (1961), showed marked diurnal variations with the former and very little change with the latter diet. Assuming that the cellulolytic counts showed a similar trend on each diet the time of sampling could exert a significant influence on the counts in the case of the grain ration, while it would have little effect with the hay diet. It is desirable that differences in counts associated with these different methods should be eliminated as far as possible, or at least be capable of assessment, so that the results of different workers with diets of different composition, in different localities, or with different species of ruminants can be compared with confidence.

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Taxonomy of the Aerobic Pseudomonads: *Pseudomonas cepacia*, *P. marginata*, *P. alliicola* and *P. caryophylli*

By R. W. BALLARD, N. J. PALLERONI, M. DOUDOROFF AND R. Y. STANIER

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720, U.S.A.

AND M. MANDEL

Section of Molecular Biology, Department of Biology, The University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025, U.S.A.

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SUMMARY

On the basis of phenotypic characterization and DNA–DNA homology studies of strains of phytopathogenic Pseudomonas species, it is concluded that *P. cepacia* is so similar to *P. multivorans* that the latter name should be regarded as a synonym. On similar grounds, *P. alliicola* appears to be a synonym of *P. marginata*. *P. caryophylli* is a readily distinguishable species. From the DNA–DNA hybridization studies all of these species seem to be related to each other and to the animal pathogens *P. pseudomallei* and *P. mallei*.

INTRODUCTION

As one outcome of their taxonomic study of aerobic pseudomonads, Stanier, Palleroni & Doudoroff (1966) proposed a new species, *Pseudomonas multivorans*, for a distinctive cluster of non-fluorescent strains. The first representatives of this species had been isolated from soil and water in Trinidad by Morris & Roberts (1959). *Pseudomonas multivorans* is characterized by its remarkable nutritional versatility. Most strains can use as sole sources of carbon and energy a substantially larger number of organic compounds than any of the fluorescent pseudomonads, previously considered the most omnivorous members of the genus *Pseudomonas*. The compounds utilizable by *P. multivorans* include a few—notably, D-fucose, cellobiose and L-threonine—that are not attacked by most other members of the genus. Many strains of *P. multivorans* produce pigments identified as phenazines (Morris & Roberts, 1959). Poly-β-hydroxybutyric acid (PHB) is accumulated as a reserve material, but cannot be utilized as an exogenous substrate. Redfearn, Palleroni & Stanier (1966) simultaneously characterized, by the same taxonomic methods, two Pseudomonas species pathogenic for animals: *P. pseudomallei* and *P. mallei*. These closely related organisms are also non-fluorescent pseudomonads that accumulate PHB as a reserve material and are nutritionally highly versatile. However, the substrates utilizable by the more versatile of the two species, *P. pseudomallei*, are fewer than those used by *P. multivorans*. Other characters which clearly distinguish *P. pseudomallei* and *P. mallei* from *P. multivorans* include the ability to denitrify, to utilize starch and maltose, and to hydrolyse and grow with exogenous PHB as carbon source. The DNAs of

P. pseudomallei and *P. mallei* also have a slightly higher G + C content than that of *P. multivorans* (Mandel, 1966).

Rogul, Brendle, Haapala & Alexander (1968) have recently demonstrated a close genetic relationship between *Pseudomonas pseudomallei* and *P. mallei* through experiments on DNA–DNA hybridization *in vitro*. Rogul *et al.* (1968) did not find evidence of genetic homology between the *P. pseudomallei–P. mallei* group and other species of aerobic pseudomonads with the exception of *P. multivorans*, which showed a relatively low but significant level of homology with both species. This work accordingly suggests that *P. pseudomallei*, *P. mallei* and *P. multivorans* constitute a species-cluster within the genus *Pseudomonas*, an interpretation that is consonant with the data of Redfearn *et al.* (1965) and Stanier *et al.* (1966) on the phenotypic characters of these three species.

There are many nomenspecies of phytopathogenic pseudomonads, most of which have been very inadequately characterized. They include aerobic pseudomonads which do not produce fluorescent pigments, and some known to accumulate PHB. In naming *P. multivorans*, Stanier *et al.* (1966) did not consider its possible synonomy with any of these species, primarily because there was no evidence to implicate *P. multivorans* as an agent of plant disease. However, Dr David Sands (personal communication), who has recently applied the methods of phenotypic characterization developed by Stanier *et al.* (1966) to a large number of phytopathogenic pseudomonads, found that a strain of the phytopathogen *Pseudomonas cepacia* Burkholder (1950) had a nutritional spectrum virtually identical with that of *P. multivorans*. Furthermore, an extensive study of the host ranges of bacteriophages for aerobic pseudomonads (Dr G. Cosens & Dr M. P. Starr, personal communication) has shown that the specific host range of certain bacteriophages encompasses both *P. multivorans* and a cluster of phytopathogenic nomenspecies: *P. marginata* (McCulloch) Stapp (1928) *P. alliicola* (Burkholder) Starr & Burkholder (1942), *P. cepacia* and *P. caryophylli* (Burkholder) Starr & Burkholder (1942). It appeared desirable to examine the possible relationships of *P. multivorans* to this group of phytopathogenic pseudomonads. We have therefore undertaken a complete phenotypic characterization of authentic strains of *P. marginata*, *P. alliicola*, *P. cepacia* and *P. caryophylli* by the methods used to characterize *P. multivorans* (Stanier *et al.* 1966). At the same time, we have extended the work of Rogul *et al.* (1968) on *in vitro* DNA–DNA hybridization. The strain labelled *P. marginata* (ATCC 17556), which was studied by Jessen (1965), Stanier *et al.* (1966) and Dr David Sands (personal communication), is misnamed. It is not a representative of this non-fluorescent species, and can be assigned to *P. fluorescens* biotype A (Stanier *et al.* 1966).

METHODS

Origins of the strains examined. We shall refer to the strains by the numbers assigned to them in the collection of pseudomonads maintained in the Department of Bacteriology and Immunology of the University of California, Berkeley. The majority of the phytopathogenic strains examined were obtained from the International Collection of Phytopathological Bacteria, Department of Bacteriology, University of California, Davis, through the courtesy of its curator, Dr M. P. Starr. Additional strains of *Pseudomonas cepacia* and *P. alliicola* were received from Dr R. Dickey, Department of Plant Pathology, Cornell University, New York. The strains of *P. solanacearum*

Table 1. *Origins of the strains*

	Strains received as *Pseudomonas cepacia*
714 Starr PC 22	Received from W. H. Burkholder, New York, 1949
715 Starr PC 23	Received from W. H. Burkholder, New York, 1949
716 Starr PC 24	Received from W. H. Burkholder, New York, 1949
717 Starr PC 25	Received from W. H. Burkholder, New York, 1949
725 Dickey 63–53	Isolated from decayed onion, Orange Co., New York, 1963
726 Dickey 63–46	Received from Dr D. C. Hildebrand; isolated from muck soil, Prattsburg, New York, 1963
727 Dickey 64–20	Isolated from muck soil, Orange Co., New York, 1964
738 Dickey 64–22	Isolated from muck soil, Orange Co., New York, 1964
739 Dickey 64–32	Isolated from muck soil, Orange Co., New York, 1964
740 Dickey 63–46	Isolated from muck soil, Prattsburg, New York, 1963
741 Dickey 61–52	Isolated from decayed onion, Orange Co., New York, 1961
742 Dickey 63–56	Isolated from decayed onion, Orange Co., New York, 1963
743 Dickey 63–56b	Isolated from 742 by Mrs V. Vitanza as a colony variant
744 Dickey 63–71	Isolated from decayed onion, Orange Co., New York, 1963
745 Dickey 63–71b	Isolated from 744 by Mrs V. Vitanza as a colony shape variant
746 Dickey 63–75	Isolated from decayed onion, Orange Co., New York, 1963
747 Dickey 63–75b	Isolated from 746 by Mrs V. Vitanza as a colony shape variant
748 Dickey 63–87	Isolated from decayed onion, Orange Co., New York, 1963
	Strains received as *P. marginata*
701 Starr PM 2	(Clara G40) Isolated by W. H. Burkholder from gladiolus leaves, 1938
702 Starr PM 12	(NCPPB 1888) Isolated from gladiolus leaves in Ithaca, New York, 1945
703 Starr PM 13	(NCPPB 1889) Isolated in Ithaca, New York, from a greenhouse, 1945
704 Starr PM 107	(NRRL B-793) (ATCC 10248) (NCPPB 1891) Received from W. C. Haynes, NRRL, Peoria, Ill., 1948
705 Starr PM 109	(Dowson 116) Received from W. J. Dowson, 1950
706 Starr PM 175	(NCPPB 1051) (Hayward B670) Isolated from gladiolus sp. in S. Rhodesia, 1961;
722 Starr PC 144	(Robbs 349) Received from C. F. Robbs as unidentified isolate, Brazil, 1965, isolated from rotted onion
723 Starr PM 106	(NRRL B-792) (NCPPB 1890) (ATCC 10247) Received from W. C. Haynes, NRRL, Peoria, Ill., 1948
737 Starr PM 11	(NRRL B-851) (NCPPB 1887) Isolated from gladiolus leaves in Lima, New York, 1945
	Strains received as *P. alliicola*
707 Starr PA 6	(NRRL B-823) Isolated by W. H. Burkholder, 1939
708 Starr PA 7	(ATCC 19302) Isolated by W. H. Burkholder, 1939
709 Starr PA 8	Isolated by W. H. Burkholder, 1939
710 Starr PA 9	Isolated by W. H. Burkholder, 1939
711 Starr PA 10	Isolated by W. H. Burkholder, 1939
712 Starr PA 15	Isolated as onion pathogen, 1945
713 Starr PA 16	(NRRL B-828) Isolated as onion pathogen, 1945
724 Dickey 61–3	Isolated from decayed onion, Orange Co., New York, 1961
	Strains received as *P. caryophylli*
718 Starr PC 101	Isolated by K. F. Baker from carnations at Sato greenhouses, Hawthorne, California, 1950
719 Starr PC 102	Isolated by K. F. Baker from carnations at Sato greenhouses, Hawthorne, California, 1950
720 Starr PC 113	Received from N. A. MacLean, Plant Pathology, University of California, Berkeley; isolated from carnation, white Sims variety, 1951
721 Starr PC 115	(Hellmers 89) Received from E. Hellmers, Denmark; isolated from Danish carnation 'Harvest Moon', 1959
	Strains received as *P. solanacearum*
729 Strain 139b	Received from Mrs V. Vitanza; isolated by I. Buddenhagen from banana plant, Honduras
730 Strain P-28	Received from Mrs V. Vitanza; isolated by I. Buddenhagen from a tomato plant, Honduras

were received from Mrs V. Vitanza, Department of Plant Pathology, University of California, Berkeley. The designations and histories of the phytopathogenic strains examined are listed in Table 1. The histories of the other strains of aerobic pseudomonads examined are given by Stanier *et al.* (1966), with the exception of *P. saccharophila* (Doudoroff, 1940).

Methods of phenotypic characterization. These have been described by Stanier *et al.* (1966). A few compounds included in the nutritional screening by Stanier *et al.* were omitted in the present study; they were: oxalate, eicosanedioate, methanol, isophthalate, terephthalate, L-kynurenine, *m*-aminobenzoate, *p*-aminobenzoate, methylamine, *n*-dodecane and *n*-hexadecane.

In vitro DNA–DNA hybridization experiments. The bacteria were grown in the standard mineral base described by Stanier *et al.* (1966) with the addition of Difco yeast extract 5 g./l. and sodium lactate 2 g./l. Radioactive DNA was obtained by the addition of ^{14}C-labelled adenine to a culture in a medium prepared by adding vitamin-free casaminoacids 2 g./l. and sodium lactate 2 g./l. to the standard mineral base. The adenine was added between 30 and 60 min. (about 0·5 generations) before harvesting the culture which was grown on a rotary shaker at 30°. Prior to DNA isolation, the bacteria were harvested by centrifugation, washed, and resuspended in saline-EDTA (saline-EDTA contains 0·15 M-NaCl and 0·1 M-EDTA, pH 8·0) to a concentration of 10% (wet weight/volume). Lysis was effected by the addition of sodium lauryl sulphate (15 g./l.) and heating the mixture in a water bath at 60° for 5 min. (Marmur, 1961). The mixture was cooled in ice, one-half volume of liquefied phenol was added, and the mixture shaken until homogeneous. The mixture was then centrifuged and the aqueous phase removed. The semisolid material at the aqueous DNA:phenol interphase was mixed well with 50 to 100 ml. of saline-EDTA and extracted with one-half volume of liquefied phenol. The pooled aqueous phases were precipitated with 2 vol. of 95% (v/v) ethanol. The DNA was redissolved in one-tenth strength SSC (SSC contains 0·15 M-NaCl and 0·015 M-Na citrate, pH 7·0) and dialysed against one-tenth strength SSC for 15 hr at 4°. The solution was then incubated with 50 μg./ml. ribonuclease (A-grade bovine pancreatic, Calbiochem, Los Angeles, Calif.) for 2 hr at 37°. Then 100 μg./ml. pronase (B-grade, Calbiochem, Los Angeles, Calif.) was added and the mixture incubated for an additional 3 hr. The salt concentration was increased to that of SSC and the DNA deproteinized by repeated extraction with chloroform + isoamyl alcohol, 24 + 1 (v/v) (Marmur, 1961). The DNA was then precipitated by adding 2 vol. ethanol, redissolved in one-tenth strength SSC, reprecipitated with isopropyl alcohol as described by Marmur (1961) and finally redissolved in one-tenth strength SSC. The DNA was stored until use at 4° in the presence of chloroform.

The DNA hybridization experiments were performed using the optimal conditions and techniques established by Johnson & Ordal (1968). Membrane filters (10·5 mm. diam.) contained from 25 to 35 μg. of immobilized denatured DNA. The filters were incubated in the mixture recommended by Denhardt (1966) made with double strength SSC for 2 hr at 65°. The filters were immersed singly in 0·25 ml. of solution which contained 1 μg. of denatured radioactive DNA, homologous to the DNA immobilized on the filter, and 150 μg. of denatured competitor DNA in double strength SSC. The DNA in solution was previously sheared to a mol. wt of about 500,000 by passage through a French pressure cell 15,000 lb./in.^{2} (McCarthy &

Bolton, 1963) and denatured by heating at 102° for 8 min. in one-tenth strength SSC. Controls with homologous radioactive DNA and without competitor were included. The vials were incubated 12 hr at temperatures of 72° (25° below the Tm of the reference DNA) and 80°. The amount of radioactive homologous DNA bound to the filters in the absence of competitors was generally between 22 and 27% of the initial input after incubation at 72° and between 12 and 16% at 80°. By using radioactive high mol. wt DNA from *Pseudomonas aeruginosa* (67% GC) and *P. stutzeri* (62% GC) immobilized on filters identical with those used in the present experiments, it could be shown that about 53 to 61% of the immobilized DNA was retained on the filter after 12 hr incubation at 70° and about 31 to 41% at 80°. No significant differences in percentage retention were observed with DNA from the two species, or between experiments without competitor and those with 150 μg. of either homologous or heterologous sheared preparations of competitor DNA. After incubation, the filters were washed in double-strength SSC at the temperature used for incubation, dried and counted in a Nuclear-Chicago Mark I liquid scintillation counter. All experiments were done in duplicate and the counts, which generally agreed within 5%, were averaged for the calculations. The results are expressed as 'percentage competition', i.e. the relative competition obtained in each case as compared to the competition with homologous DNA, which is arbitrarily assigned a value of 100%:

$$\text{Percentage competition} = 100 \times X - Y / X - Z.$$

$X = {}^{14}C$ retained without competitor.
$Y = {}^{14}C$ retained with heterologous competitor.
$Z = {}^{14}C$ retained with homologous competitor.

Preparations of DNA isolated from *Pseudomonas pseudomallei* and *P. mallei* were kindly furnished by Dr M. Rogul, Veterinary Immunology Section, Division of Veterinary Medicine, Walter Reed Army Medical Centre, Washington, D.C. 20012. These were isolated from *P. pseudomallei*, strains 4845 (ATCC 15582), NCTC 1691 and 295; and from *P. mallei* 4.

Determination of the base composition of DNA. The moles percentage G+C in the DNA of various strains was determined from buoyant density measurements in CsCl gradients (Mandel, 1966).

RESULTS

Phenotypic characterizations of phytopathogenic nomenspecies

Eighteen strains of *Pseudomonas cepacia* were examined. They are Gram-negative rods with polar multitrichous flagella as previously described by Burkholder (1950). None of the strains produces fluorescent pigments; however, all strains produce greenish yellow pigments, the chemical nature of which has not been ascertained. All strains accumulate poly-β-hydroxybutyric acid (PHB) as intracellular reserve material. They are oxidase-positive. They grow at 41° but not at 4°. Most strains produce slime in mineral media containing 2 or 4% sucrose (negative: 714, 715, 716, 745), give a positive egg-yolk reaction (negative: 714, 715, 716, 743, 747) and hydrolyse Tween 80 (negative: 743, 747). Ten strains (715, 717, 738, 739, 740, 741, 742, 745, 746, 747) hydrolyse gelatin. None hydrolyses starch or PHB. None produces gas anaerobically in a complex medium containing nitrate. None synthesizes the enzymes of the arginine

dihydrolase system constitutively. The cleavage of protocatechuate by toluene-treated cells grown with *p*-hydroxybenzoate is of the *ortho* type. The strains of *P. cepacia* are nutritionally homogeneous. They use a wide variety of organic compounds as sole sources of carbon and energy (Tables 2, 3). Fourteen of the strains can use at least 102 of the 136 substrates tested in the screening. The list of compounds used by

Table 2. *Substrates used by all strains of P. cepacia, P. marginata, P. alliicola and P. caryophylli*

D-Ribose, D-xylose, D-arabinose, L-arabinose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose, cellobiose, gluconate, 2-keto-gluconate, saccharate, mucate, salicin
Acetate, propionate, butyrate, isobutyrate
Malonate, succinate, fumarate
D-Malate, L-malate, *meso*-tartrate, DL-β-hydroxybutyrate, DL-lactate, glycerate, hydroxymethyl-glutarate
Citrate, α-keto-glutarate, pyruvate, aconitate
Mannitol, sorbitol, *meso*-inositol, glycerol
n-Propanol
p-Hydroxybenzoate, phenylacetate, quinate
L-Alanine, β-alanine, L-serine, L-aspartate, L-glutamate, DL-arginine, γ-aminobutyrate, L-histidine, L-proline, L-tyrosine, L-phenylalanine, L-tryptophan
Betaine, hippurate

Substrates not used by any strain of P. cepacia, P. marginata, P. alliicola or P. caryophylli

Maltose, lactose, starch, inulin
Maleate
Poly-β-hydroxybutyrate
Erythritol, ethyleneglycol
iso-Propanol, geraniol
D-Mandelate, phthalate, phenylethanediol, phenol, naphthalene
D-Tryptophan
Creatine, pantothenate

Table 3. *Substrates used by some strains studied*

Substrate	Names under which strains were received: *P. cepacia* (18 strains)	*P. marginata* (9 strains)	*P. alliicola* (8 strains)	*P. caryophylli* (3 strains)
D-Fucose	100*	89	100	100
L-Rhamnose	0	0	0	100
Trehalose	83	100	100	33
Valerate	100	100	87	100
Isovalerate	100	33	38	0
Heptanoate	100	89	100	0
Caprylate, caprate, caproate, pelargonate	100	100	100	0
Glutarate	100	44	38	0
Adipate, azelate, sebacate	100	100	100	0
Pimelate, suberate*	100	0	0	0
D-(−)-Tartrate	0	100	100	0
L-(+)-Tartrate	100	100	100	0
Glycollate	100	0	0	100
Levulinate	100	0	0	0
Citraconate	100	100	100	0
Itaconate	0	0	38	0
Mesaconate	0	100	100	0

* % strains positive.

Table 3 (*cont.*)

	Names under which strains were received			
	P. cepacia (18 strains)	*P. marginata* (9 strains)	*P. alliicola* (8 strains)	*P caryophylli* (3 strains)
Adonitol*	100†	100	100	0
Propyleneglycol*	0	22	25	0
2,3-Butyleneglycol	100	0	0	100
Ethanol	72	100	100	0
n-Butanol	94	44	87	100
iso-Butanol	22	0	12	0
L-Mandelate, benzoylformate	94	0	0	33
Benzoate	94	100	100	0
o- & *m*-Hydroxybenzoate	100	0	0	0
Testosterone	94	0	0	0
Glycine	0	22	38	0
D-Alanine	100	100	100	0
L-Threonine*	100	100	100	33
L-Leucine	33	100	100	33
L-Isoleucine, L-lysine	100	100	100	0
DL-Norleucine	0	33	0	0
D-Valine	94	100	100	33
DL-Ornithine*	100	89	100	0
DL-Citrulline	100	78	75	0
DL-α-Aminobutyrate	0	67	87	0
DL-α-Aminovalerate	0	33	0	0
δ-Aminovalerate	94	0	0	0
Kynurenate	100	100	100	0
Anthranilate	100	89	87	0
Ethanolamine	100	89	100	0
Benzylamine	89	0	12	0
Putrescine	94	0	0	0
Spermine	17	0	0	0
Histamine	94	0	0	0
Tryptamine, α-amylamine	100	0	0	0
Butylamine	94	0	12	0
Sarcosine	89	56	87	0
Acetamide	100	0	0	0
Nicotinate	0	100	100	0
Trigonelline	0	100	100	0

Strain numbers of negative strains for each substrate used by some strains of each species are listed below.

P. cepacia: trehalose (714, 715, 716), butanol (740), isobutanol (all except 725, 726, 738, 743), L-mandelate, benzoylformate and benzoate (725), testosterone (726), L-leucine (all except 714, 715 716, 717, 725, 727), L-valine (741), δ-aminovalerate (744), benzylamine (725, 738), putrescine (738), spermine (all except 715, 739, 745), histamine (725), butylamine (727), sarcosine (717, 727)

P. marginata: D-fucose (705), isovalerate (all except 701, 703, 737), heptanoate (705), glutarate (all except 701, 702, 704, 722), propyleneglycol (all except 722, 723), butanol (all except 704, 705, 722, 723), glycine (all except 702, 704), norleucine (all except 703, 706, 737), DL-ornithine (706), DL-citrulline (705, 706), α-aminobutyrate (705, 723, 737), α-aminovalerate (all except 703, 706, 737), anthranilate (723), ethanolamine (722), sarcosine (705, 706, 722, 723).

P. alliicola: valerate (711), isovalerate (all except 708, 709, 712), glutarate (all except 712, 713, 724), itaconate (all except 707, 710, 724), propyleneglycol (all except 710, 724), butanol (711), *iso*-butanol (all except 710), glycine (all except 708, 709, 712), DL-citrulline (709, 711), α-aminobutyrate (724), anthranilate (724), benzylamine (all except 724), butylamine (all except 724), sarcosine (724).

P. caryophylli: trehalose (719, 720), L-mandelate, benzoylformate (719, 720), L-threonine (719, 720). L-leucine (720, 721), L-valine (720, 721).

* These substrates were tested with only seven strains of *P. cepacia* (714, 715, 716, 717, 725, 726, 727) and with eight strains of *P. marginata* (all except 737). The percentages are calculated on the basis of the strains tested.

† % strains positive.

P. cepacia is identical with that reported by Stanier *et al.* (1966) to be utilized by *P. multivorans*. These two species cannot be distinguished by any of the phenotypic characters that we have examined.

Nine strains of *Pseudomonas marginata* and eight strains of *P. alliicola* were examined. These two species are Gram-negative rods, motile by polar multitrichous flagella, as reported by McCulloch (1921) and Burkholder (1942). None of the strains produces fluorescent pigments. All strains accumulate PHB as reserve material. They are oxidase-positive, although some strains give a very weak reaction. They grow at 41° but not at 4°. They produce slime in mineral media containing 2 or 4% sucrose, give a positive egg-yolk reaction and hydrolyse Tween 80 and gelatin. None hydrolyses starch or PHB. None of the strains produces gas anaerobically in a complex medium containing nitrate. None synthesizes the enzymes of the arginine dihydrolase system constitutively. The cleavage of protocatechuate by toluene treated cells grown with *p*-hydroxybenzoate is of the *ortho* type. The nutritional spectra of the strains of these two species are essentially identical (Tables 2, 3). They are versatile; all strains use at least 78 of the 136 substrates tested.

Four strains of *Pseudomonas caryophylli* were examined. They are Gram-negative rods motile by polar multitrichous flagella as described by Burkholder (1942). None of the strains produces fluorescent pigments. All strains accumulate PHB as reserve material. They are oxidase-positive. They grow at 41° but not at 4°. None of the strains produces slime in mineral media containing 2 or 4% sucrose. One strain (720) produces a positive egg-yolk reaction and two strains (720 and 721) hydrolyse Tween 80. None hydrolyses gelatin, starch or PHB. They produce gas anaerobically in a complex medium containing nitrate and synthesize the enzymes of the arginine dihydrolase system constitutively. The cleavage of protocatechuate by toluene-treated cells grown with *p*-hydroxybenzoate is of the *ortho* type One of the strains of *P. caryophylli* (722) requires unidentified growth factors, and hence cannot grow in the defined mineral medium used for nutritional screening. The nutritional patterns were thus determined only for the other three strains (Tables 2, 3). These three strains of *P. caryophylli* utilize from 61 to 65 of the compounds tested as sole sources of carbon and energy.

On the basis of these phenotypic characterizations, it appears that *Pseudomonas cepacia* and *P. multivorans* are indistiguishable species, as are *P. marginata* and *P. alliicola*. However, the *P. cepacia–P. multivorans* complex, the *P. marginata–P. alliicola* complex and *P. caryophylli* represent three phenotypically distinct clusters of strains, even though they do share many common properties.

Mean DNA base compositions

Table 4 summarizes data obtained by buoyant density measurements for the mean base composition (moles percentage G+C) of DNA from representative strains of four nomenspecies of non-fluorescent phytopathogenic pseudomonads. Also included are data for two additional strains of *Pseudomonas multivorans*; data for strains belonging to this species have been published previously by Mandel (1966). The mean value and standard deviation for the DNA base composition of *P. multivorans* shown in Table 4 have been calculated on the basis of data for all strains of this species so far examined. Data for *P. pseudomallei* and *P. mallei*, from Mandel's (1966) earlier work, are included.

It is evident that the mean DNA base compositions of *Pseudomonas cepacia*, *P. multivorans*, *P. marginata* and *P. alliicola* did not differ significantly. The values for *P. pseudomallei* and *P. mallei*, not significantly different from one another, were slightly higher, while the value for *P. caryophylli* was slightly lower.

Table 4. *Buoyant density and GC content of the DNA of Pseudomonas multivorans, P. pseudomallei, P mallei and some phytopathogenic nomenspecies**

Species	Strain	Mean density (g./cm.^{3})	Mean value and standard deviation calculated for each group	
			Density (g./cm.^{3})	GC (moles %)
P. cepacia	714	1·726	1·7256±0·0009	66·9±0·9
	715	1·7265		
	717	1·7265		
	725	1·725		
	726	1·725		
	727	1·725		
P. multivorans	85	1·7265	1·7263±0·0006	67·6±0·6
	104	1·727		
	249	1·7265		
	382	1·7255		
	383	1·7255		
	384	1·726		
	385	1·726		
	386	1·7265		
	387	1·7265		
	396	1·727		
	397	1·727		
	398	1·726		
	399	1·7265		
	424	1·726		
P. marginata	702	1·727	1·7271±0·0008	68·5±0·8
	704	1·7275		
	706	1·727		
P. alliicola	707	1·7275	1·7271±0·0010	68·5±1·0
	709	1·727		
	724	1·727		
P. caryophylli	719	1·724	1·7240±0·0006	65·3±0·6
	720	1·7235		
	721	1·7245		
P. pseudomallei	(6 Strains)	—	1·7281±0·0007	69·5±0·7
P. mallei	(7 Strains)	—	1·7276±0·0010	69·0±1·0

* Values given for mean density are raw averages of two determinations of CsCl buoyant density and include those previously reported by Mandel (1966).

Experiments on DNA–DNA hybridization in vitro

Genetic relationships among the species studied were examined by the technique of DNA–DNA hybridization *in vitro*, using as reference standards strain 382 of *Pseudomonas multivorans* (Table 5), strain 704 of *P. marginata* (Table 6) and strain 721 of *P. caryophylli* (Table 7). Competition experiments were conducted at two annealing temperatures, 72 and 80°. The former temperature, about 25° below the melting point of the reference DNA, is the annealing temperature commonly used in such

experiments. Competition at the higher temperature (80°) is a more stringent test of homology. Hence, similar competition values at both 72 and 80° indicate a close genetic relationship between the test strain and the reference strain, whereas a sharp decline in the competition value at 80° relative to that at 72° indicates a much looser genetic homology between the test strain and the reference strain.

Table 5. *DNA competition experiments using DNA of Pseudomonas multivorans 382 as reference standard*

DNA of	Strain no.	Per cent competition at	
		72°	80°
P. multivorans	85	59	58
P. multivorans	104	79	57
P. multivorans	249	52	20
P. multivorans	383	66	59
P. multivorans	385	54	40
P. multivorans	387	54	42
P. multivorans	396	46	31
P. multivorans	424	75	65
P. cepacia	727	76	79
P. cepacia	725	66	70
P. marginata	704	35	6
P. alliicola	709	30	18
P. alliicola	724	28	0
P. caryophylli	721	24	14
P. pseudomallei	295	19	14
P. pseudomallei	1691	43	4
P. pseudomallei	4845	24	10
P. mallei	4	23	16
P. solanacearum	729	0	—
P. solanacearum	730	0	—
P. saccharophila	—	0	—
P. aeruginosa	131	0	—
P. putida	90	0	—
P. fluorescens	31	0	—
P. fluorescens	38	0	—
P. stutzeri	222	0	—
P. stutzeri	224	0	—
P. acidovorans	105	0	—

The most extensive series of experiments was conducted using DNA of *Pseudomonas multivorans* 382 as the reference standard (Table 5). The competition values with DNA from eight other strains of this species ranged from 46 to 79 % at 72°. In most cases, the values did not decline significantly at 80°; the largest decline occurred with DNA from strain 249, for which the competition value fell from 52 % at 72° to 20 % at 80°. The DNA of two strains of *P. cepacia* have competition values in the same range (66 and 76 %) at 72°, and the values did not decline significantly at 80°. Lower but significant competition values (19 to 43 %) were obtained at 72° with DNA from strains of *P. marginata*, *P. alliicola*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*; in every case the values fell substantially at 80° (0 to 18 %). No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results provide independent confirmation of the specific identity of *P. cepacia* and *P. multivorans* They also show that there is a significant degree of genetic relationship between these

two nomenspecies and *P. marginata*, *P. alliicola*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*.

Table 6 presents the results of competition experiments using DNA of *Pseudomonas marginata* 704 as the reference standard. DNA from one other strain of this species and from two strains of *P. alliicola* showed high levels of competition (79 to 100%) at 72°, not significantly depressed (63 to 81%) at 80°. Lower levels of competition at 72° (17 to 46%) were shown by DNA from strains of *P. cepacia*, *P. multivorans*, *P. caryophylli*, *P. pseudomallei* and *P. mallei*. In nearly every case, the competition fell to a much lower level (0 to 17%) at 80°; however DNA of *P. cepacia* strain 727, which had given the highest value (46%) at 72°, also gave a high value (36%) at 80°. No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results provide independent confirmation of the specific identity of *P. marginata* and *P. alliicola*.

Table 6. *DNA competition experiments using DNA of Pseudomonas marginata 704 as reference standard*

DNA of	Strain no.	Per cent competition at	
		72°	80°
P. marginata	702	100	81
P. alliicola	709	79	79
P. alliicola	724	82	63
P. cepacia	727	46	36
P. cepacia	725	17	11
P. multivorans	382	35	17
P. multivorans	249	23	0
P. caryophylli	721	28	3
P. pseudomallei	4845	27	13
P. mallei	4	30	5
P. solanacearum	729	0	—
P. solanacearum	730	0	—
P. saccharophila	—	0	—
P. aeruginosa	131	0	—
P. putida	90	0	—
P. fluorescens	38	0	—
P. stutzeri	222	0	—
P. stutzeri	224	0	—
P. acidovorans	105	0	—
P. testosteroni	78	0	—

Table 7 presents the results of competition experiments using DNA of *Pseudomonas caryophylli* 721 as the reference standard. The DNA of two other strains of this species gave extremely high competition values (99 and 100% at 72°, 99 and 94% at 80°). The DNA of two strains of *P. pseudomallei* showed much lower competition at 72° (24 and 29%), but the competition values remained at similar levels at 80°. Significant competition (8 to 29%) was also obtained at 72° with DNA from strains of *P. mallei*, *P. cepacia*, *P. multivorans*, *P. marginata* and *P. alliicola*, but the values fell to much lower levels (0 to 13%) at 80°. No competition was detected at 72° with DNA from strains of *P. solanacearum*, *P. saccharophila*, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri* or *P. acidovorans*. These results confirm the phenotypic data, which suggest that *P. caryophylli* is a distinctive species.

Table 7. *DNA competition experiments using DNA of Pseudomonas caryophylli 721 as reference standard*

DNA from	Strain no.	Per cent competition at 72°	Per cent competition at 80°
P. caryophylli	719	99	99
P. caryophylli	720	100	94
P. pseudomallei	1691	29	24
P. pseudomallei	4845	24	24
P. mallei	4	24	13
P. cepacia	725	13	0
P. cepacia	727	20	8
P. multivorans	382	29	6
P. multivorans	249	16	0
P. marginata	702	9	0
P. marginata	704	19	0
P. alliicola	709	9	0
P. alliicola	724	8	0
P. solanacearum	729	0	—
P. solanacearum	730	0	—
P. saccharophila	—	0	—
P. aeruginosa	131	0	—
P. putida	90	0	—
P. fluorescens	31	0	—
P. fluorescens	38	0	—
P. stutzeri	222	0	—
P. stutzeri	224	0	—
P. acidovorans	105	0	—
P. testosteroni	78	0	—

DISCUSSION

The pseudomallei group: nomenclature and internal relationships

The 18 strains of the phytopathogen *Pseudomonas cepacia* that we examined cannot be distinguished phenotypically from the strains, isolated from soil, water and clinical material, which Stanier *et al.* (1966) placed in the new species *P. multivorans*. Furthermore, our studies on DNA–DNA hybridization *in vitro*, using DNA from the type strain of *P. multivorans* as the reference standard, reveal that two strains of *P. cepacia* share a level of genetic homology with the type strain of *P. multivorans* at least as high as that of any of the eight other strains of *P. multivorans* examined. The mean DNA base composition of six strains of *P. cepacia* fall in the range of *P. multivorans*. The sole reported difference between the two species is the ability of *P. cepacia* to cause a plant disease, a root rot of onion bulbs. Inoculation experiments with onion bulb slices, performed by Mrs Vilma Vitanza and Dr D. C. Hildebrand (personal communication) showed that some strains of *P. multivorans* studied by Stanier *et al.* (1966) were capable of producing such a rot. In view of this fact, the two species are without question synonymous; and the correct specific designation is *Pseudomonas cepacia* Burkholder (1950) emend. The specific description should be amended to exclude phytopathogenic ability as a specific character, since not all strains of *P. multivorans* produce a clear-cut rot of onion bulb slices, and to include the many distinctive phenotypic characters established by Stanier *et al.* (1966) and by the present study. Since a type strain was not chosen by Burkholder (1950), we propose his isolate *P. cepacia* 717 (ATCC 25416) as the lectotype strain.

The nine strains of *Pseudomonas marginata* and the eight strains of *P. alliicola* that we studied cannot be distinguished phenotypically. Studies on DNA–DNA hybridization *in vitro*, using DNA from a strain of *P. marginata* as a reference standard, show that two strains of *P. alliicola* share with it a level of genetic homology almost as high as that of the only other strain of *P. marginata* tested. The mean DNA base compositions of three strains belonging to each species cannot be distinguished. The sole reported difference between these two species concerns their specific phytopathogenic behaviour; *P. alliicola* was described as the causative agent of a rot of onion bulbs (Burkholder, 1942), whereas *P. marginata* causes a rot of Gladiolus (McCulloch, 1921). However, Mrs Vitanza and Dr Hildebrand (personal communication) have shown that some of the strains of *P. marginata* we studied can rot onion bulb slices; the alleged difference between the two species is, therefore, probably not significant, and simply reflects the particular plant sources from which strains were isolated. These two species should be considered as synonymous; the correct specific designation, on grounds of priority, is *P. marginata* (McCulloch) Stapp (1928). The description should be amended to exclude the particular phytopathogenic properties originally attributed to *P. marginata* as characters of the species, and to include the many distinctive phenotypic characters established in the present study. Since a type strain was not originally chosen (McCulloch, 1921, 1924) we propose *P. marginata* 704 (ATCC 10248) as the lectotype strain.

Although we have studied only four strains of *Pseudomonas caryophylli*, this species appears to be valid. It is readily distinguishable from both *P. cepacia* and *P. marginata* in phenotypic respects; *in vitro* DNA hybridizations show that the three strains examined share a high level of genetic homology, but share very low homology with either *P. cepacia* or *P. marginata*. Although *P. caryophylli* was originally described as the agent of a necrosis (wilt) of carnations, Dr V. Vitanza and Dr D. C. Hildebrand (personal communication) showed that the strains studied by us can cause a rot of onion bulb slices, like *P. cepacia* and *P. marginata*. The specific description should therefore be amended to exclude the particular phytopathogenic properties originally attributed to *P. caryophylli*, an omission which will not in any way impair recognition of this species, in view of its distinctive phenotypic characters, revealed by the present study. Since a type strain was not originally chosen (Burkholder, 1942) we propose *P. caryophylli* 720 (ATCC 25418) as the neotype strain.

Pseudomonas cepacia, *P. marginata* and *P. caryophylli* share many phenotypic properties with *P. pseudomallei* and *P. mallei*. Furthermore, the data on DNA–DNA hybridization *in vitro* of Rogul *et al.* (1968), which have been confirmed and extended in the present study, show varying degrees of genetic homology among all five species. Using DNA from *P. cepacia*, *P. marginata* and *P. caryophylli* as reference standards, we were unable to detect genetic homology with any of the other Pseudomonas species examined: *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. stutzeri*, *P. solanacearum*, *P. acidovorans* and *P. saccharophila*. For these reasons, it seems appropriate to regard *P. pseudomallei*, *P. mallei*, *P. cepacia*, *P. marginata* and *P. caryophylli* as a major and isolated species-cluster among the aerobic pseudomonads, which possibly deserves recognition at the generic or subgeneric level. Provisionally, we shall term this cluster the 'pseudomallei group'. The principal characters that serve to distinguish the five component species are summarized in Table 8.

The taxonomic significance of DNA homology groups

The aerobic pseudomonads constitute a large and varied sub-group among the eubacteria. Past attempts to create generic subdivisions among these organisms, as exemplified by such genera as *Hydrogenomonas*, *Comamonas* and *Xanthomonas*, have

Table 8. *Characters distinguishing the species of the pseudomallei group*

Character	*P. cepacia**	*P. marginata*	*P. caryophylli*	*P. pseudomallei*†	*P. mallei*†
Moles % G+C in DNA	67·4±1·4	68·5±1·0	65·3±0·6	69·5±0·7	69·0±1·0
Dinitrification	−	−	+	+	v
Arginine dihydrolase	−	−	+	+	+
Extracellular Gelatin	v	+	−	+	+
hydrolases PHB	−	−	−	+	+
for Starch	−	−	−	+	+
No. of flagella	> 1	> 1	> 1	> 1	0
Utilization of:					
L-Rhamnose	v	−	+	−	−
Maltose, starch	(−)	−	−	+	v
Saccharate, mucate	+	+	+	−	−
Malonate	+	+	+	−	+
Glutarate	+	−	−	v	(−)
Adipate	+	+	−	+	+
Azelate, sebacate	+	+	−	+	v
L(+), D(−) & *meso* Tartrate	−	+	−	−	−
Glycollate	v	−	+	−	−
Hydroxymethylglutarate	+	+	+	+	−
Aconitate	+	+	+	+	−
Levulinate	+	−	−	+	−
Citraconate	+	+	−	−	−
Mesaconate	−	+	−	−	−
Erythritol	−	−	−	+	−
2,3-Butyleneglycol	+	−	+	−	−
Benzoate	+	+	−	+	+
D-Alanine	+	+	−	+	+
L-Valine	v	+	−	+	v
DL-α-Aminobutyrate	−	+	−	−	+
δ-Aminovalerate	+	−	−	+	v
Kynurenate, anthranilate	+	+	−	+	v
Ethanolamine, butylamine	(+)	+	−	+	−
Benzylamine	+	−	v	−	−
Putrescine	+	−	−	+	v
Spermine	+	−	−	v	−
Acetamide	+	−	−	−	−
Nicotinate	−	+	−	−	−

* Data in part from Stanier *et al.* (1966) and Mandel (1966).
† Data from Redfearn *et al.* (1966) and Mandel (1966).
( ) Character possessed by 90% or more of the strains.

been of questionable value: each of these genera is defined in terms of a limited number of arbitrarily selected phenotypic characters. For this reason, Stanier *et al.* (1966) favoured a very broad definition of the genus *Pseudomonas*.

Recent work suggests that the genetic homology group, as established through experiments on nucleic acid hybridization *in vitro*, may eventually provide the best

basis for the circumscription of the bacterial genus. The pseudomallei group, as defined through the hybridization experiments described in the present paper, provides one example among the aerobic pseudomonads of such a homology group, embracing a considerable number of species. A second large homology group, clustered around the type species of the genus *Pseudomonas*, *P. aeruginosa*, is also evident from recent work; it includes at least three other species of fluorescent pseudomonads: *P. putida*, *P. fluorescens* and *P. syringae* (under which designation we would place most of the nomenspecies of fluorescent phytopathogenic pseudomonads). We have established (unpublished data) that these four species all share a substantial level of genetic homology. Furthermore, work performed at Berkeley has shown that non-fluorescent denitrifying pseudomonads of the *P. stutzeri* species-group, closely related to one another in terms of genetic homology, also show a more distant relationship in these terms to some members of the fluorescent group. None of the members of the fluorescent-stutzeri group shows detectable DNA homologies with any of the species of the pseudomallei group.

The bacterial genus, defined as a cluster of species which belong to a single genetic homology group, would be a taxonomic entity of considerable biological significance; in effect, its component species could be construed as offshoots of a single evolutionary branch among the bacteria. The practical feasibility of assigning generic status to each homology group within a large bacterial assemblage such as the aerobic pseudomonads is still uncertain. Since nucleic acid hybridization *in vitro* is unlikely to become a routine taxonomic tool in the near future, genera defined in these terms would be of little value unless they could also be distinguished from one another by easily determinable phenotypic traits. To ascertain whether the two large homology groups now recognizable among the aerobic pseudomonads can be distinguished from one another at the phenotypic level, we compared the known characters that are common to all species of each homology group. From this it was evident that many phenotypic characters of great value in the differentiation of species among the aerobic pseudomonads—for example, denitrification, the oxidase reaction, the utilization of starch and poly-β-hydroxybutyric acid, the ability to grow at 41°—are not distinctive of either homology group, since they are variable in the component species. Only five known characters sharply differentiate these two large homology groups: the formation of PHB as a reserve material, and the ability to utilize D-arabinose, D-fucose, cellobiose and L-threonine. All five characters are positive in the pseudomallei group, and negative in the fluorescent-stuzeri group. It does not seem prudent to propose a generic differentiation on such slim phenotypic grounds.

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Taxonomy of the Aerobic Pseudomonads: the Properties of the *Pseudomonas stutzeri* Group

By N. J. PALLERONI, M. DOUDOROFF AND R. Y. STANIER

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720, U.S.A.

AND R. E. SOLÁNES

Cátedra de Microbiología, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina

AND M. MANDEL

Department of Biology, The University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025, U.S.A.

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SUMMARY

Strains of *Pseudomonas stutzeri* and related denitrifying bacteria were compared in their phenotypic properties and mean deoxyribonucleic acid (DNA) base composition. On the basis of this comparison and of *in vitro* DNA hybridization experiments, it was concluded that, using practical diagnostic tests, no more than two nomenspecies can be recognized within the group. One, *P. stutzeri*, was extremely variable in phenotypic characteristics and in DNA base composition; we included in it the strains previously assigned to *P. stanieri*. The other was a new species, *P. mendocina* Palleroni, which was more homogeneous in phenotypic characters, and in DNA base composition and homology. The comparative properties of known denitrifying pseudomonads are tabulated.

INTRODUCTION

Pseudomonas stutzeri is a non-fluorescent, denitrifying pseudomonad, widely distributed in soil and water. It was discovered and described as *Bacillus denitrificans* II by Burri & Stutzer in 1895, and renamed *Bacterium stutzeri* by Lehmann & Neumann in 1896. Thereafter, the taxonomic and nomenclatural status of this well-characterized species became confused, and was cleared up by van Niel & Allen only in 1952, when they retraced the history of the species, and redescribed it. *P. stutzeri* can be distinguished from other *Pseudomonas* spp. by many properties, of which the salient ones are: vigorous denitrifying ability; use of starch and maltose as carbon sources; and a characteristic and unusual colony structure; colonies of freshly isolated strains are wrinkled, tough and coherent, and have a light-brown colour which reflects the unusually high cytochrome *c* content of the cells.

Stanier, Palleroni & Doudoroff (1966) extended the description of *Pseudomonas stutzeri*, on the basis of a detailed examination of 17 strains isolated in diagnostic laboratories in Denmark and France from clinical specimens. A concurrent determination of the buoyant densities of DNA from these strains by Mandel (1966)

revealed a marked bimodal distribution: six strains had a guanine+cytosine (GC) content of about 62 moles %, and 11 of about 65 moles %; he assigned the former strains to a new species, *P. stanieri* with strain 224 as holotype, and with the reservation that strains 223, 320 and 228 were atypical and might not belong to the species. He limited *P. stutzeri* to the strains of the higher GC content. Stanier *et al.* (1966) found few consistent phenotypic differences between the two groups of strains and they did not accept Mandel's taxonomic proposal. The number of strains examined in this study was relatively small, and the principal source from which they had been isolated (clinical specimens) was certainly atypical, as there is no evidence that these denitrifying bacteria are associated with vertebrates as parasites, pathogens or commensals. It therefore seemed desirable to enlarge the study of these organisms.

Two of us (N. J. Palleroni & R. E. Solánes) isolated many additional strains of non-fluorescent denitrifying pseudomonads by enrichment procedures from soil and water samples collected in the province of Mendoza, Argentina; most of these strains had the typical distinguishing features of *Pseudomonas stutzeri* outlined above. However, some failed to attack starch and maltose, and formed smooth colonies on primary isolation. Further study showed that these strains were representatives of a related but readily distinguishable species, which we shall name *P. mendocina*. In this paper, we shall describe the phenotypic and genotypic properties of *P. mendocina* and of the *P. stutzeri–P. stanieri* complex.

MATERIALS AND METHODS

Origins of the strains. In addition to the strains described by Stanier *et al.* (1966) the following new isolates from denitrification enrichment cultures were examined. The source material and the conditions of the enrichment (carbon source and temperature) are indicated for each strain in parenthesis.

Strains assigned to the *P.mendocina stutzeri–P. stanieri* complex: CH 19 (soil, ethanol, 40°); CH 29 (soil, succinate, 30°); CH 39 (soil, L-tartrate, 40°); CH 44 (soil, L-tartrate, 30°); CH 48 (water, sebacate, 30°); CH 49 (soil, ethanol, 30°); CH 52 (water, ethanol, 30°); CH 53 (water, succinate, 30°); CH 54 (water, succinate, 30°); CH 58 (soil, L-tartrate, 30°); CH 60 (water, L-tartrate, 30°); CH 62 (soil, L-tartrate, 30°); CH 63 (soil, L-tartrate, 30°); CH 64 (soil, L-tartrate, 30°); CH 69 (water, ethanol, 40°); CH 70 (water, ethanol, 40°); CH 80 (soil, succinate, 30°); CH 82 (water, succinate, 30°); CH 85 (soil, succinate, 30°); CH 88 (water, L-tartrate, 30°); CH 90 (soil, ethanol, 30°); CH 118 (soil, sebacate, 40°).

Strains assigned to *Pseudomonas* sp. nov.: CH 20 (water, succinate, 40°): CH 34 (soil, succinate, 40°); CH 35 (soil, L-tartrate, 40); CH 50 (soil, ethanol, 30°); CH 67 (water, L-tartrate, 40°); CH 74 (water, ethanol, 40°); CH 91 (soil, sebacate, 40°); CH 95 (water, ethanol, 30°); CH 110 (soil, ethanol, 40°); CH 113 (soil, ethanol, 40°); CH 120 (water, sebacate, 40°); CH 139 (soil, ethanol, 30°).

Phenotypic characterization. The phenotypic characterization of the strains was carried out by the methods described by Stanier *et al.* (1966).

DNA base compositions. DNA was isolated and the base composition determined as described by Mandel (1966).

In vitro DNA hybridizations. Extraction and purification of DNA was by the procedure of Marmur (1961). Radioactive DNA was obtained by addition of ^{14}C adenine

to a culture in a medium prepared with the standard mineral base described by Stanier *et al.* (1966), with the addition of 0·2% (w/v) vitamin-free Casamino acids, and 0·2% (w/v) of sodium lactate. Adenine was added 30 to 60 min. (about 1·5 generations) before harvesting the culture, which was grown on a rotary shaker at 30°.

DNA hybridizations *in vitro* were performed by the technique of Johnson & Ordal (1968), fully described by Ballard *et al.* (1970). By this technique, competition with the homologous DNA, rather than direct binding, was determined. The results were expressed as 'per cent competition', i.e. the relative competition obtained in each case compared with the competition with homologous DNA, arbitrarily assigned a value of 100%.

RESULTS

Isolation of Pseudomonas mendocina from enrichment cultures. The strains of *P. mendocina* described in this paper were isolated from water and soil by enrichment cultures with different carbon sources, either at 30 or 40°. Nine of the twelve isolates were from enrichments at 40°; in seven the same inocula yielded *P. stutzeri* when incubated at 30°, with the same carbon source.

Table 1. *Pseudomonas stutzeri–P. stanieri complex. Substrates used by 90% or more of the strains*

Carbohydrates and sugar derivatives
- Glucose*
- Maltose*
- Starch*

Fatty acids
- Acetate
- Butyrate*
- Caprylate*
- Caprate*

Dicarboxylic acids
- Succinate
- Fumarate*
- Azelate*
- Sebacate*

Hydroxyacids
- L-Malate*
- DL-β-Hydroxybutyrate*
- DL-Lactate
- Glycollate*

Miscellaneous organic acids
- Citrate*
- α-Ketoglutarate*
- Pyruvate
- Itaconate*
- Mesaconate*

Polyalcohols and glycols
- Glycerol*
- Ethylene glycol
- Propylene glycol*

Alcohols
- Ethanol*

Non-nitrogenous aromatic and other cyclic compounds
- (None)

Aliphatic amino acids
- L-α-Alanine*
- D-α-Alanine*
- L-Leucine*
- L-Glutamate*

Amino acids and related compounds containing a ring structure
- L-Proline*

Amines
- (None)

Miscellaneous nitrogenous compounds
- (None)

Paraffin hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are listed in Table 2.

Common properties of the stutzeri group. It may be useful to summarize the properties shared by the *Pseudomonas stutzeri–P. stanieri* complex and *P. mendocina* n.sp., and indicate those characters that set them apart from other major species groups among the aerobic pseudomonads.

The members of this group were similar in structure. The rod-shaped bacteria were

Table 2. *Pseudomonas stutzeri–P. stanieri complex. Substrates used by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
Xylose	1	All except CH 85
L-Arabinose	1	All except CH 85
Glucose	38	CH 62
Mannose	2	All except 275, CH 85
Fructose	25	223, 225, 275, 316, 319, 419, CH 19, CH 48, CH 52, CH 53, CH 54, CH 62, CH 80, and CH 82
Maltose	36	229, 316, CH 62
Starch	35	275, 229, 316, CH 62
Gluconate	28	228, 319, 320, 321, 419, CH 29, CH 53, CH 54, CH 62, CH 80 and CH 82
Saccharate	25	220, 221, 224, 225, 226, 227, 275, 316, 319, 419, CH 19, CH 53, CH 54 and CH 118
Mucate	18	All except 222, 223, 228, 229, 318, 230, 321, CH 39, CH 52, CH 58, CH 62, CH 63, CH 64, CH 69, CH 70, CH 85, CH 88 and CH 90
Propionate	24	220, 223, 224, 225, 228, 275, 319, 320, 321, 419, CH 49, CH 52, CH 58, CH 88 and CH 90
Butyrate	35	275, CH 29, CH 58, CH 60
Isobutyrate	3	All except CH 53, CH 70 and CH 80
Valerate	28	220, 229, 275, 318, 321, 419, CH 29, CH 49, CH 52, CH 58 and CH 63
Isovalerate	29	275, 320, 321, CH 29, CH 49, CH 52, CH 60, CH 62, CH 90 and CH 118
Caproate	30	275, 320, 321, CH 29, CH 49, CH 52, CH 58, CH 62 and CH 90
Heptanoate	34	223, CH 29, CH 49, CH 58 and CH 60
Caprylate	36	CH 29, CH 60 and CH 62
Pelargonate	33	223, CH 29, CH 49, CH 52, CH 60 and CH 90
Caprate	35	CH 60, CH 64, CH 70, CH 85
Malonate	33	228, 229, 320, 321, 419 and CH 58
Fumarate	37	CH 49 and CH 60
Glutarate	32	228, CH 29, CH 44, CH 49, CH 52, CH 58 and CH 60
Adipate	4	All except 223, CH 44, CH 58 and CH 85
Pimelate	1	All except CH 58
Suberate	2	All except 223 and CH 85
Azelate	35	228, 229, 275 and 419
Sebacate	37	275 and 419
D-Malate	4	All except 228, 316, 320 and 321
L-Malate	37	CH 19 and CH 48
L(+)Tartrate	2	All except 419 and CH 85
M-Tartrate	1	All except CH 85
DL-β-Hydroxybutyrate	35	CH 29, CH 49, CH 53 and CH 62
Glycollate	35	CH 29, CH 44, CH 58 and CH 60
Glycerate	16	All except 223, 275, 319, 320, 321, 419, CH 19, CH 48, CH 52, CH 58, CH 63, CH 69, CH 70, CH 80, CH 82 and CH 85
Hydroxymethylglutarate	4	All except 222, 226, 227 and CH 48
Citrate	38	318
α-Ketoglutarate	37	228 and CH 29
Aconitate	18	All except 220, 221, 223, 224, 225, 228, 275, 316, 319, 320, 321, 419, CH 48, CH 69, CH 70, CH 80, CH 82 and CH 118
Levulinate	2	All except CH 48 and CH 60
Itaconate	37	223 and CH 62
Mesaconate	37	223 and CH 62

Table 2 (*cont.*)

Substrate	Total of positive strains	Negative strains
Mannitol	24	226, 227, 316, 318, 319, 320, 321, 419, CH 19, CH 48, CH 53, CH 54, CH 80, CH 82 and CH 118
Glycerol	38	275
Propylene glycol	35	221, CH 53, CH 54 and CH 60
2,3-Butylene glycol	34	226, 227, 229, 275 and CH 48
Ethanol	36	CH 53, CH 54 and CH 60
Propanol	34	221, CH 53, CH 54, CH 60 and CH 64
Butanol	31	275, CH 49, CH 53, CH 54, CH 58, CH 60, CH 62, and CH 64
Isobutanol	31	226, 227, 229, 275, 320, 321, CH 58 and CH 60
D-Mandelate	1	All except CH 88
L-Mandelate	1	All except CH 88
Benzoyl formate	1	All except CH 88
Benzoate	29	220, 221, 223, 224, 225, 275, 318, 320, 321 and CH 85
p-Hydroxybenzoate	6	All except 226, 227, 318, 419, CH 69 and CH 85
Quinate	1	All except CH 48
Glycine	34	CH 29, CH 49, CH 58, CH 60 and CH 90
L-α-Alanine	37	CH 49 and CH 60
D-α-Alanine	35	CH 29, CH 44, CH 60 and CH 52
β-Alanine	3	All except CH 48, CH 69 and CH 70
L-Serine	4	All except CH 48, CH 69, CH 30 and CH 82
L-Leucine	38	CH 60
L-Isoleucine	34	275, 319, 419, CH 60 and CH 62
L-Valine	33	228, 229, 275, CH 39, CH 49 and CH 60
L-Aspartate	27	221, 229, 275, 419, CH 19, CH 39, CH 53, CH 54, CH 62, CH 69, CH 88 and CH 90
L-Glutamate	37	CH 49 and CH 60
α-Amino-butyrate	25	220, 221, 222, 224, 225, 228, 275, 316, 318, 320, 321, CH 19, CH 29 and CH 62
δ-Amino-valerate	21	221, 222, 223, 224, 225, 228, 229, 275, 316, 320, 321, CH 19, CH 29, CH 44, CH 52, CH 60, CH 88 and CH 118
L-Histidine	1	All except CH 85
L-Proline	37	419 and CH 62
L-Tyrosine	34	223, 229, 318, CH 29 and CH 60
L-Phenylalanine	9	All except 223, 275, 319, CH 19, CH 48, CH 70, CH 80, CH 82 and CH 85
Kynurenate	2	All except 316 and 319
Ethanolamine	8	All except 220, 221, 224, 225, 316, 319, 320 and 321
Putrescine	29	229, 316, 419, CH 44, CH 49, CH 53, CH 58, CH 60, CH 90 and CH 118
Spermine	8	All except 319, 320, 321, CH 48, CH 69, CH 70, CH 80 and CH 82
Betaine	3	All except 223, CH 48 and CH 85
Sarcosine	1	All except CH 48
Nicotinate	1	All except 319
Trigonelline	1	All except 319

0·75 to 0·85 μm. wide and 1·4 to 2·8 μm. long in exponentially growing cultures. All bore a single polar or subpolar flagellum with a wavelength of 1·75 to 2·0 μm. (Pl. 1). In several strains of *Pseudomonas mendocina* (notably CH 39, 110 and 120), lateral flagella of shorter wavelength (about 1 μm.) (Pl. 2) were also produced, particularly in young cultures on complex solid media. Two of ten strains of *P. stutzeri* (227 and

319) also had lateral flagella of short wavelength, although they occurred on fewer bacteria than in *P. mendocina*. In this group, the flagella of shorter wavelength were easily shed during the manipulations of flagella staining.

No member of the stutzeri group accumulated poly-β-hydroxybutyrate as a reserve material. Neither fluorescent nor phenazine pigments were produced. Growth factors were not required. A fairly wide, though variable, range of simple organic compounds could serve as sole sources of carbon and energy; the most distinctive substrates, which supported growth of nearly all strains of the group, but were rarely used by other *Pseudomonas* species, were ethylene glycol and glycollic acid. All strains grew well under anaerobic conditions, with abundant gas production, in media that contained sufficient nitrate. It was noted that the capacity for vigorous denitrification might be lost after prolonged cultivation of strains on nitrate-free media, and a period of adaptation might be required to restore this activity to a high level (Stanier *et al.* 1966). The temperature range was relatively large; all strains grew well at 40°, and none grew at 4°.

Phenotypic properties of newly isolated strains assigned to the Pseudomonas stutzeri–P. stanieri complex. Twenty-two strains of *P. stutzeri* from soil and water in Argentina isolated by enrichment techniques for denitrifying bacteria were characterized, and some of the nutritional properties of the strains previously examined (Stanier *et al.* 1966) were rechecked. The nutritional spectrum of all 39 strains is shown in Tables 1 and 2. The nutritional variability of the complex, evident from earlier work, became even clearer. Although 29 compounds among those tested could be utilized by 90% or more of the strains, only acetate, succinate, lactate, pyruvate and ethylene glycol were universal substrates (Table 1). Glucose, caprylate, caprate, fumarate, L-malate, glycollate, ethanol, L- and D-alanine and glutamate, universally used by the 17 strains studied by Stanier *et al.* (1966), were not attacked by some of the strains isolated in Argentina.

Phenotypic properties of Pseudomonas mendocina Palleroni sp. nov. Colonies were flat, smooth, butyrous, of brownish yellow colour. The wrinkled appearance of freshly isolated colonies of *P. stutzeri* was not observed. The yellow colour was due to the presence of carotenoid pigments in the cells.

Starch, gelatin and poly-β-hydroxybutyrate were not hydrolysed and the egg-yolk reactions were negative. All strains were arginine dihydrolase positive. Strains that grew with benzoate or *p*-hydroxybenzoate attacked the respective diphenolic intermediates (catechol and protocatechuate) by *ortho* cleavage, and therefore degraded aromatic substrates through the β-ketoadipate pathway. The temperature optimum was about 37°; all strains could grow at 41°, and none at 4°.

The nutritional spectrum of *Pseudomonas mendocina* is described in Tables 3 and 4. In nutrition, the species was far more homogeneous than the *P. stutzeri–P. stanieri* complex. Of the 67 compounds that can be used by the species as a whole, 46 were used by all twelve strains and 55 by at least eleven strains. The species differed from *P. stutzeri* in many nutritional respects; it failed to use starch, maltose, azelate and sebacate, used by most strains of *P. stutzeri*; it grew with spermine, betaine, sarcosine and geraniol, rarely used by strains of *P. stutzeri*. In nutrition, and in the possession of arginine dihydrolase, *P. mendocina* resembled species of the fluorescent group, particularly *P. aeruginosa*. Properties shared by these two species included: polar monotrichous flagella, denitrifying ability, capacity for growth at 41°, and ability to

use geraniol, an alcohol not attacked by other *Pseudomonas* spp. *Pseudomonas mendocina* and *P. aeruginosa* could, however, be readily distinguished from one another by differences in pigmentation, in attack on mannitol, azelate and suberate, and in gelatinase production.

Table 3. *Pseudomonas mendocina. Substrates utilized by 90% or more of the strains*

Carbohydrates and sugar derivatives
- D-Glucose
- Gluconate
- Saccharate
- Mucate*

Fatty acids
- Acetate
- Propionate
- Butyrate
- Valerate
- Isovalerate*
- Caproate*
- Heptanoate
- Caprylate
- Pelargonate
- Caprate

Dicarboxylic acids
- Malonate
- Succinate
- Fumarate
- Glutarate

Hydroxyacids
- DL-β-Hydroxybutyrate
- DL-Lactate
- Glycollate
- DL-Glycerate*

Miscellaneous organic acids
- Citrate
- α-Ketoglutarate
- Aconitate
- Levulinate*
- Itaconate*
- Mesaconate

Polyalcohols and glycols
- Ethylene glycol*
- Propylene glycol

Alcohols
- Ethanol
- Propanol
- *n*-Butanol
- Isobutanol
- Geraniol

Non-nitrogenous aromatic and other cyclic compounds
- Quinate*

Aliphatic aminoacids
- L-α-Alanine
- D-α-Alanine
- β-Alanine*
- L-Serine
- L-Leucine
- L-Isoleucine
- L-Valine
- L-Aspartate
- L-Glutamate
- DL-Arginine
- α-Aminobutyrate
- α-Aminovalerate

Aminoacids and related compounds having a ring structure
- L-Histidine
- L-Proline
- L-Tyrosine

Amines
- Putrescine
- Spermine

Miscellaneous nitrogenous compounds
- Betaine
- Sarcosine

Paraffinic hydrocarbons
- (None)

* Substrates not used by all strains. Negative strains are listed in Table 4.

Nutritional characters common to the two species of the stutzeri group, but rarely if ever encountered in species of the fluorescent group, included the ability to use ethylene glycol and glycollate, and the failure to use 2-ketogluconate.

DNA base composition of the stutzeri group. DNA base compositions were determined from buoyant density measurements for many of the strains isolated in Argentina (Table 5). The mean base composition of the DNA of *Pseudomonas mendocina* showed little variation from strain to strain; all values clustered closely around 63·2 moles % GC. However, values for the remaining 22 newly isolated strains ranged from 62·2 to 66·3 mole % GC; the distribution of values for the individual strains was about Gaussian, with a peak of 64·3% GC (Fig. 1). When these data were combined with the

Table 4. *Pseudomonas mendocina. Substrates utilized by a fraction of the strains*

Substrate	Total of positive strains	Negative strains
D-Fructose	9	CH 20, CH 34, CH 110
Mucate	11	CH 95
Isobutyrate	7	CH 20, CH 34, CH 35, CH 50 and CH 67
Isovalerate	11	CH 95
Caproate	11	CH 95
D-Malate	5	All except CH 20, CH 34, CH 35, CH 50 and CH 67
L-(+)-Tartrate	5	All except CH 35, CH 67, CH 95, CH 110 and CH 139
DL-Glycerate	11	CH 95
Hydroxymethylglutarate	9	CH 34, CH 50 and CH 95
Pyruvate	10	CH 91 and CH 95
Levulinate	11	CH 91
Citraconate	10	CH 20 and CH 120
Itaconate	11	CH 95
Glycerol	9	CH 91, CH 95 and CH 120
Ethylene glycol	11	CH 95
Benzoate	10	CH 74 and CH 110
Quinate	11	CH 67
Glycine	10	CH 91 and CH 95
β-Alanine	11	CH 91
L-Phenylalanine	5	All except CH 20, CH 34, CH 35, CH 50 and CH 67
Tryptamine	9	CH 74, CH 110 and CH 113

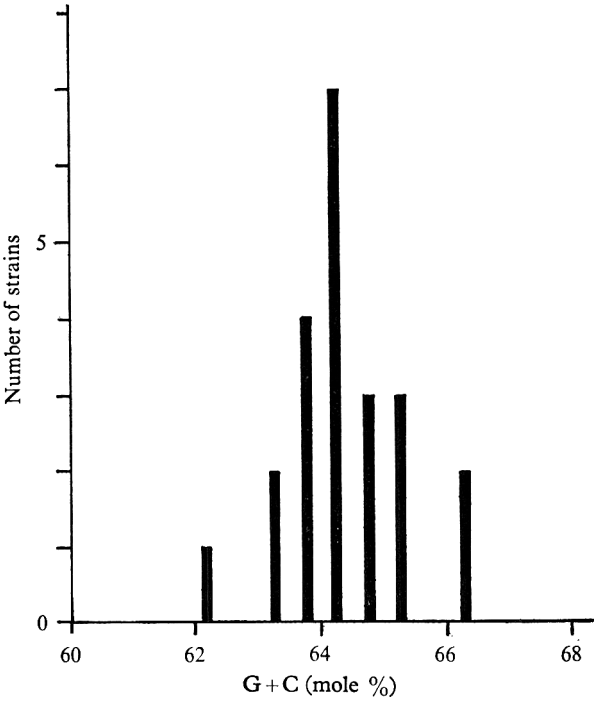


Fig. 1. Distribution of guanine + cytosine values (mole %) among the strains of *Pseudomonas stutzeri* isolated in Argentina.

data of Mandel (1966) on the 17 strains of *P. stutzeri* studied by Stanier *et al.* (1966), the bimodal distribution of GC values which then led Mandel to propose a separation into two species remained, but was less clear-cut (Fig. 2). The majority of values clustered between 64·3 and 65·3 mole % GC; there was still a minor peak at 62·2, but it was now connected by intervening values to the major peak. There is little probability that these values can be fitted to a single Gaussian distribution on the assumption that the standard deviation of the values is about 1 % GC, that is, no greater than that

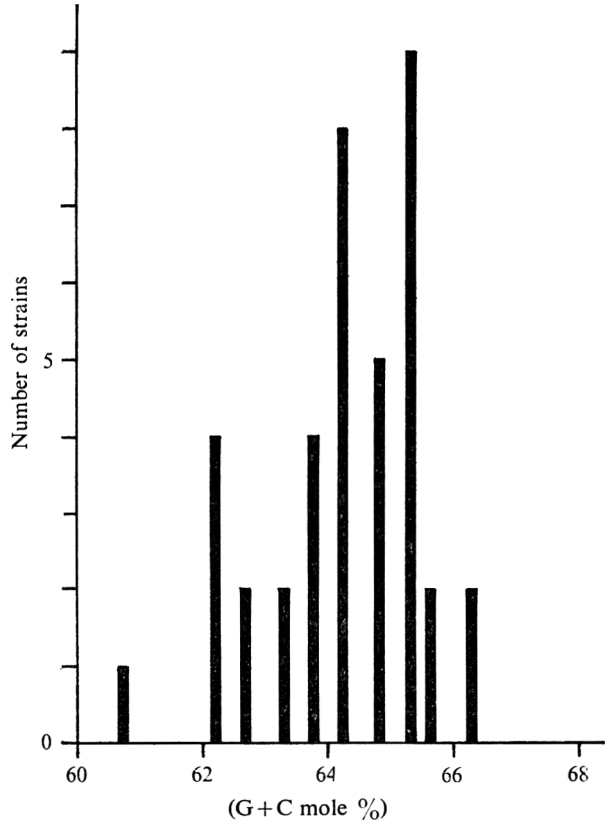


Fig. 2. Distribution of guanine+cytosine values (mole %) among all the strains of *Pseudomonas stutzeri* described in this paper.

found for other *Pseudomonas* species (Mandel, 1966). The extreme limits of base composition were represented by one strain with a value of 60·6 mole % GC, and two with 66·3. Mandel's (1966) data for other *Pseudomonas* species in which the DNA base composition of many strains had been determined, showed that well-characterized species generally had a remarkably constant mean base composition. The wide variation in the DNA base composition of the *P. stutzeri–P. stanieri* complex was exceptional among aerobic pseudomonads, although a comparable situation occurred in the *Acinetobacter* group (Baumann, Doudoroff & Stanier, 1968). The variation in GC content may be related to the very high degree of nutritional heterogeneity in the strains of this complex (see Table 2).

In vitro DNA hybridization in the stutzeri group. To obtain a further insight into the genotypic structure of the stutzeri group, we performed experiments on *in vitro* DNA hybridization, using several reference strains of the *Pseudomonas stutzeri–P. stanieri* complex, with different DNA base compositions, and one reference strain of *P. mendocina*. In most competition experiments, annealing temperatures of 70 to 72° (about 25° below the *Tm* of the DNA samples being compared) were used. A few competition

Table 5. *CsCl buoyant densities* and calculated GC (mole %) in the DNA of strains of the stutzeri group isolated in Argentina*

P. stutzeri–P. stanieri complex			*P. mendocina*		
Strain	ρCsCl	GC	Strain	ρCsCl	GC
CH 19	1·725	66·3	CH 20	1·723	64·3
CH 29	1·723	64·3	CH 35	1·7215	62·8
CH 39	1·7235	63·8	CH 47	1·7215	62·8
CH 44	1·7225	63·8	CH 50	1·723	64·3
CH 48	1·7225	63·8†	CH 67	1·723	64·3
CH 49	1·7225	63·8	CH 74	1·7215	62·8
CH 52	1·7235	64·8	CH 91	1·722	63·3
CH 53	1·724	65·3	CH 95	1·7215	62·8
CH 54	1·725	66·3	CH 110	1·721	62·2
CH 58	1·723	64·3	CH 120‡	1·7215	62·8
CH 60	1·723	64·3	CH 139	1·7215	62·8
CH 62	1·723	64·3	$\overline{X}$ = 1·7219 ± 0·0010		
CH 63	1·722	63·3	$\overline{GC}$ = 63·2 ± 1·0 mole % (22 determinations of ρ)		
CH 64	1·723	64·3			
CH 69	1·724	65·3			
CH 70	1·723	64·3			
CH 80	1·724	65·3			
CH 82	1·2735	64·8			
CH 85	1·721	62·2			
CH 88	1·723	64·3			
CH 90	1·722	63·3			
CH 118	1·7235	64·8			

$\overline{X}$ = 1·7230 ± 0·00093

$\overline{GC}$ = 64·3 ± 0·9 mole % (46 determinations of ρ)

* Mean of two determinations for each, except CH 48.

† CH 48: four values.

‡ Satellite at 1·7095, even in re-cloned population.

experiments were also made at a higher annealing temperature (80°), where conditions for DNA–DNA hybridization were much more stringent; the occurrence of detectable competition at this annealing temperature probably indicated some virtually complete base sequence homologies between the two organisms.

As one of our goals was to assess the genetic significance of the variable DNA base composition of the *Pseudomonas stutzeri–P. stanieri* complex, reference DNA was prepared from five strains: three (220, 224 and 320) had DNA containing about 62 mole % GC, and two (221 and 222) had DNA of about 65 mole % GC. In preliminary experiments, the homology of one reference strain from each group was determined with strains of *P. mendocina*, *P. aeruginosa*, *P. fluorescens* (denitrifying biotypes), *P. putida*, *P. acidovorans* and *P. testosteroni*. These experiments (Table 6) revealed that the *P. stutzeri–P. stanieri* complex was genetically well isolated from other species of the genus *Pseudomonas*, with the exception of *P. mendocina*. At an annealing

temperature of 70 to 72°, competition by DNAs from all species except *P. mendocina* was less than 20%, whereas competition by DNA of *P. mendocina* ranged from 20 to 40%.

Competition at an annealing temperature of 70 or 72° among DNA preparations from strains studied by Stanier *et al.* (1966) and Mandel (1966) is shown in Table 7. Strains could be subdivided into four groups in terms of GC content and their relative

Table 6. *DNA competition among strains of the Pseudomonas stutzeri group and other species of aerobic pseudomonads**

DNA competition within the *P. stutzeri* group	Range of % DNA competition at 70–72°	Range of % DNA competition at 80°
P. stanieri v. *P. stanieri*	81–100	76–92
P. stutzeri v. *P. stutzeri*	81–92	71–83
P. stanieri v. *P. stutzeri*	52–70	30–56
P. mendocina v. *P. mendocina*	98–100	85–95
P. mendocina v. *P. stanieri*	19–38	7–9
P. mendocina v. *P. stutzeri*	28–40	13
DNA competition between the *P. stutzeri* group and other *Pseudomonas* spp.		
P. stanieri (220, 224) v. *P. aeruginosa* (52)	0–18	
P. stanieri (220, 224) v. *P. putida* (90)	0–4	
P. stanieri (224) v. *P. fluorescens* (2, 50)	0–7	
P. stanieri (224) v. *P. acidovorans* (105)	0	
P. stanieri (224) v. *P. testosteroni* (78)	0	
P. stutzeri (221, 222) v. *P. aeruginosa* (52)	0–5	
P. stutzeri (221, 222) v. *P. putida* (90)	0–8	
P. stutzeri (221, 222) v. *P. fluorescens* (2, 50)	0–3	
P. stutzeri (221) v. *P. acidovorans* (105)	0	
P. stutzeri (221) v. *P. testosteroni* (78)	0	
P. mendocina (CH 20) v. *P. aeruginosa* (52)	9	
P. mendocina (CH 20) v. *P. fluorescens* (2, 50)	18–19	
P. mendocina (CH 20) v. *P. fluorescens* (31)	21	
P. mendocina (CH 20) v. *P. fluorescens* (83)	21	
P. mendocina (CH 20) v. *P. acidovorans* (14)	0	
P. mendocina (CH 20) v. *P. testosteroni* (78)	0	

* Values for homologous DNA (100%) are not included. The data for competition within the *P. stutzeri* group are collected from Tables 7, 8 and 10. For all other DNA competition experiments, the strain numbers are shown in parentheses, with the reference underlined.

and absolute competitions with high and low GC reference strains. Five low GC strains (group I) showed consistently high homology (80 to 100% competition) with the low GC reference strains, and a lower homology (58 to 78% competition) with the high GC reference strains. Six high GC strains (group II) showed the inverse pattern of homology. Repetition of some experiments at an annealing temperature of 80° gave essentially similar results (Table 8). The strains of groups I and II appear to constitute two intraspecific clusters, each characterized by comparatively high internal genetic homology. However, the correlations between GC content and genetic homology were by no means perfect. Five strains, one of low GC content (group III) and four of high GC content (group IV) showed relatively low competition values (20 to 65%)

Table 7. *Percentage DNA competition among strains of the Pseudomonas stutzeri–P. stanieri complex (annealing temperature: 70 or 72°)*

Group	Mean DNA base composition mole % GC	Competitor DNA strain number	Reference DNA strain number 220	224	320	221	222
I	62·2	220	—	100	81	68	58
	62·2	224	100	—	81	60	61
	62·7	225	90	90	82	68	59
	62·7	228	83	86	84	70	63
	62·2	320	81	83	—	70	64
II	65·2	221	60	64	60	—	81
	64·7	222	64	57	60	86	—
	64·7	226	67	57	60	88	83
	65·5	227	64	60	65	80	83
	65·2	318	52	59	62	89	87
	65·2	321	61	60	68	92	86
III	60·7	223	33	35	32	33	37
IV	64·2	275	20	38	47	29	36
	65·7	316	31	49	34	65	47
	65·2	319	46	35	33	49	49
	65·2	419	33	31	44	56	50

Table 8. *DNA competition among strains of the Pseudomonas stutzeri–P. stanieri complex (annealing temperature: 80°)*

Group (mole % GC)	Competitor DNA strain number	Reference DNA strain number 220	224	320	221	222
		DNA competition* (%)				
I (62 % GC)	220	(100)*	—	84	—	47
	224	—	(100)	80	55	48
	225	92	—	88	—	47
	228	—	—	84	—	40
	320	76	82	(100)	48	48
II (65 % GC)	221	33	30	54	(100)	71
	222	37	47	56	83	(100)
	226	—	—	52	—	—
	227	—	—	48	—	78

* Figures in parentheses are control values for homologous DNA.

Table 9. *DNA competition between strains of the Pseudomonas stutzeri–P. stanieri complex of the CH series, and strains of P. stanieri and P. stutzeri*

Strain	% GC	DNA competition with reference strain (%) 224 (62·2 % GC)	222 (64·7 % GC)
CH 63	63·3	57	77
CH 39	63·9	64	75
CH 48	64·3	60	74
CH 54	66·3	45	62

with all the reference strains; and in most of these cases, the values obtained with reference strains of similar GC content were not significantly or consistently greater than the values obtained with reference strains of markedly different GC content. It should be noted that these five strains (223, 275, 316, 319 and 419) are also aberrant in nutritional respects (Table 2). This analysis accordingly showed that the strains of high GC and of low GC content studied by Stanier *et al.* (1966) were genetically heterogeneous.

Reference DNAs from strains 222 (high GC) and 224 (low GC) were tested at an annealing temperature of 70 or 72° with DNA preparations from five strains of the *Pseudomonas stutzeri–P. stanieri* complex isolated in Argentina, selected for differences in GC content. The competition data are summarized in Table 9; the test strains are arranged in order of increasing GC content, but the clusters of strains that constitute groups I and II do not stand out sharply. *In vitro* hybridization data accordingly suggest that there may be considerable genetic homology among all strains of the complex, overlain by a substantial amount of genetic variation. The mean DNA base composition of a given strain is therefore not necessarily indicative of its genetic relatedness to other strains of the complex.

Table 10. *DNA competition between strains of the Pseudomonas stutzeri group and P. mendocina* CH *20 (annealing temperature: 70°)*

Competitor DNA	% GC	DNA competition (%)
P. stutzeri 220	62·2	33
P. stutzeri 223	60·7	35
P. stutzeri 224	62·2	20
P. stutzeri 225	62·7	38
P. stutzeri 228	62·7	32
P. stutzeri 320	62·2	19
P. stutzeri 221	65·2	40
P. stutzeri 222	64·7	34
P. stutzeri 226	64·7	40
P. stutzeri 227	65·5	28
P. stutzeri 319	65·2	29
P. mendocina CH 50	64·3	98
P. mendocina CH 120	62·8	99
P. mendocina CH 139	62·8	100

With *Pseudomonas mendocina* strain CH 20 as the source of reference DNA, virtually total competition was obtained with DNA from three other strains of the same species, indicating a high degree of intraspecific genetic homology (Table 10). DNAs from high and low GC strains of the *P. stutzeri–P. stanieri* complex showed considerably lower, but still substantial, competition at an annealing temperature of 70°. Even at an annealing temperature of 80°, some competition by these DNAs was evident (Table 6).

Experiments with DNA from other *Pseudomonas* species (Table 6) at an annealing temperature of 70° showed significant competition by biotypes B, C, D and F of *P. fluorescens*; in all these cases, however, competition fell to zero at an annealing temperature of 80°.

The hybridization studies accordingly suggested that *Pseudomonas mendocina* was a genetically uniform species, with some genetic relationship to the *P. stutzeri–P. stanieri* complex, and a lesser genetic relationship to some of the fluorescent pseudomonads.

DISCUSSION

The problem of nomenspecies in the Pseudomonas stutzeri group. Within the stutzeri group, the twelve strains assigned to *P. mendocina* constituted a uniform and readily differentiable species in terms of all the criteria that we applied: DNA base composition, *in vitro* DNA hybridization and phenotypic properties. Judged by the same criteria, the remaining 39 strains of the group constituting the *P. stutzeri–P. stanieri* complex, emerged as a heterogeneous array which presented a difficult problem in terms of species recognition. The GC contents of the DNA of these strains ranged from 60·7 to 66·3 mole %, and it was primarily because this range appeared too wide to characterize a single species, that Mandel (1966) proposed to limit the circumscription of *P. stutzeri* to strains with DNA of high GC content (about 65 mole %) and to create a second species for strains with DNA of low GC content (about 62 mole %). Such a specific separation could not be substantiated in terms of phenotypic differences among the 17 strains of the complex originally examined (Stanier *et al.* 1966), and the study of an additional 22 strains has not improved the situation. The apparently clear-cut bimodal distribution of DNA base compositions observed by Mandel (1966) in the 17 strains was not evident in the 22 newly examined strains, which showed an approximately Gaussian distribution of DNA base composition. When both sets of data were combined (see Fig. 2), the distribution of DNA base composition appeared to be polymodal, although the number of samples was still too small to permit any firm conclusion concerning the frequency distribution.

The variability of the nutritional characters of this group, evident from our original work (Stanier *et al.* 1966) became even more marked by the inclusion of data for an additional 22 strains (Tables 1, 2). A detailed analysis of nutritional data (not presented here) showed that strains of widely different GC content might be virtually indistinguishable in phenotypic respects; for example, strains 320 (62 mole % GC) and 321 (65 mole % GC) differed by only one of 80 nutritional characters variable within the group. We were unable to detect among our strain any clusters of differing GC content that showed sufficiently clear-cut phenotypic differences to justify their recognition as separate species.

The data on DNA hybridization with selected strains representative of the span of DNA base composition found in the complex indicated (as might be expected) that strains of similar GC content tend to show a higher level of homology with one another than with strains of different GC content. However, there were exceptions. Furthermore, all strains of the complex showed a considerable degree of genetic relatedness.

In short, we do not consider that a useful specific subdivision of this complex can be justified at present and we propose to include all strains of the *P. stutzeri–P. stanieri* complex in the single nomenspecies, *P. stutzeri.* This taxonomic treatment does not, of course, preclude the possibility of an eventual subdivision of the complex into two (or more) species, but work on a larger collection of strains, using an expanded phenotypic characterization, will undoubtedly be required to provide the necessary grounds for such a subdivision.

Problems associated with the use of flagellar insertion as a generic character. Only the polar insertion of flagella distinguishes the members of the genus *Pseudomonas* from the many other Gram-negative, rod-shaped eubacteria with DNA of relatively

high GC content. However, the use of this particular structural character as a generic one raises many difficulties, both practical and theoretical. One permanently immotile organism, *P. mallei*, was placed in *Pseudomonas* on account of its close phenotypic resemblance to the polarly flagellated *P. pseudomallei* (Redfearn, Palleroni & Stanier, 1966), and this assignment was confirmed by the demonstration of a substantial degree of nucleotide sequence homology between the two species (Rogul, Brendle, Haapala & Alexander, 1968). A different facet of the same problem is our observation on the modes of flagellar insertion characteristic of *P. stutzeri* and *P. mendocina*. Both species may possess lateral, as well as polar, flagella. Their characterization as polarly flagellated bacteria can be maintained only because the polar are distinguishable by their wavelength from the lateral flagella and predominate under certain conditions of cultivation. Comparable situations were described in *Aeromonas* and *Chromobacterium* (Rhodes, 1965). Such bacteria presumably possess two sets of genetic determinants that govern, respectively, polar and lateral flagellation, a supposition supported by Sneath's (1956) finding that the polar and lateral flagella of *Chromobacterium* are antigenically different, and also by the correlation between flagellar wavelength and insertion site. A bacterium in which polar and lateral flagella were of identical wavelength would, however, be characterized phenotypically as 'peritrichous' or 'degenerately peritrichous' even if it possessed two different sets of flagella subject to separate genetic control. The possibility that some apparently peritrichous Gram-negative bacteria may be related to one of the subgroups of *Pseudomonas* should be kept in mind by taxonomists.

Identification of denitrifying Pseudomonas spp. The early bacteriological literature on denitrification contains specific descriptions of many denitrifying organisms that are assignable to the genus *Pseudomonas*. No authentic strains of these species have survived, and the descriptions are so fragmentary that with the exceptions of *P. stutzeri*, *P. aeruginosa* and *P. fluorescens* it would be difficult to recognize a newly isolated strain as a representative of one of the named species. For this reason, we believe that most of the older names proposed for denitrifying pseudomonads should be treated as *nomina dubia*, and in this category we would place *Bacillus denitrificans* α and β Gayon & Dupetit (1886); *Bacillus denitrificans* Giltay & Aberson (1892); *Bacillus denitrificans* I Burri & Stutzer (1895); *Bacillus denitrificans agilis* Ampola & Garino (1896).

The present work, coupled with that of Stanier *et al.* (1966) and Redfearn *et al.* (1966), shows that the capacity for denitrification is characteristic of five well-defined *Pseudomonas* species that occur in soil and water. As an aid to their identification, we have prepared a tabular key of the characters that appear to have the greatest differential value (Table 11).

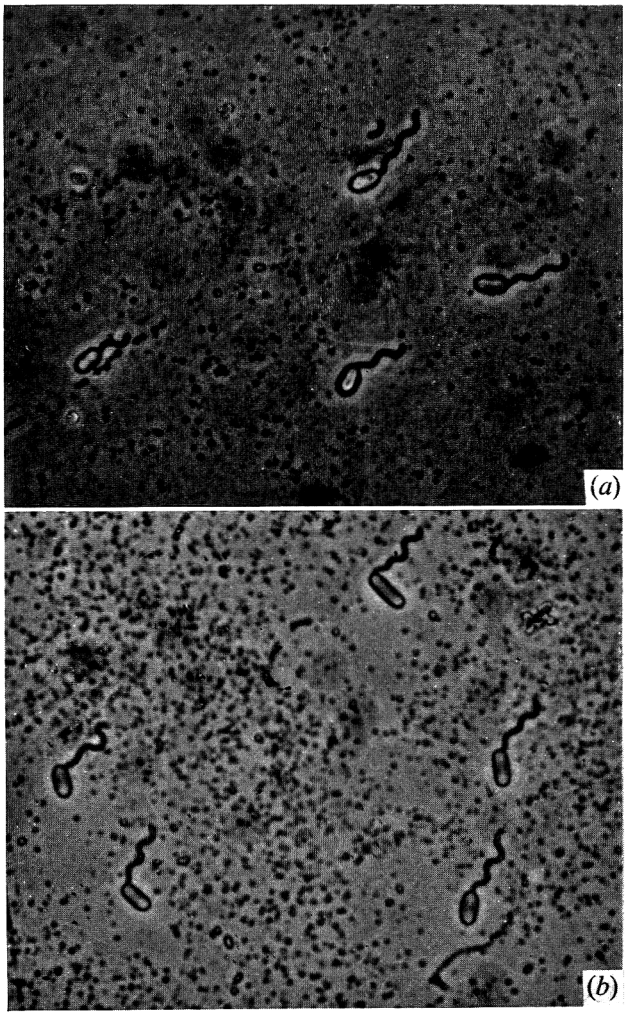
A sixth species (*Pseudomonas caryophylli*) which to our knowledge has not been reported to be a soil inhabitant, but which is pathogenic for plants, is also included in the table. This vigorously denitrifying and readily recognizable organism has been characterized by Mr R. W. Ballard in our laboratory and the information shown is abstracted from his data. Some strains of another plant pathogen, *P. solanacearum*, also cause denitrification (Hayward, 1964). We have confirmed Hayward's report, but have not yet studied this *Pseudomonas* species in sufficient detail to include data for it in Table 11. For each phenotypic character, we list the percentage of positive strains in each species; the values for *P. fluorescens* are based on the pooled phenotypic

data for the three denitrifying biotypes (B, C and D), and do not include data for non-denitrifying biotypes (A, E, F and G). The denitrifying biotypes of *P. fluorescens* can, in turn, be differentiated from one another in terms of additional characters (Stanier *et al.* 1966).

Table 11. *Characters of highest differential value for the identification of denitrifying aerobic pseudomonads*

Figures represent the percentages of strains which are positive for each of the characters

	P. stutzeri–P. stanieri complex (39 strains)	*P. mendocina* (12 strains)	*P. aeruginosa* (29 strains)	*P. fluorescens* (biot. B, C and D (44 strains)	*P. pseudomallei* (26 strains)	*P. caryophylli* (3 strains)
DNA (GC, mole %)	60·7–66·3	62·8–64·3	66·3–68·3	60·3–63·3	69·4–69·9	66
Structural characters						
Monotrichous flagellation	100	100	100	0	0	0
poly-β-Hydroxybutyrate accumulation	0	0	0	0	100	100
Temperature relationships						
Growth at 40°	100	100	100	0	100	100
Growth at 4°	0	0	0	100	0	0
Pigmentation						
Fluorescent pigment	0	0	72	84	0	0
Pyocyanine	0	0	86	0	0	0
Carotenoids	0	100	0	0	0	0
Biochemical characters						
Arginine dihydrolase	0	100	100	100	100	100
Starch hydrolysis	98	0	0	0	100	0
poly-β-Hydroxybutyrate hydrolysis	0	0	0	0	100	0
Nutritional characters						
Growth on:						
Xylose	3	0	0	34	0	100
Maltose	92	0	0	0	100	0
Saccharate	64	100	0	63	0	100
Mannitol	62	0	100	100	100	100
Ethylene glycol	100	92	0	2	8	0
2,3-Butylene glycol	87	0	93	73	0	100
Geraniol	0	100	100	0	0	0
Azelate	90	0	97	20	88	0
Levulinate	5	92	100	20	92	0
Glycollate	90	100	0	2	0	100
Serine	10	100	14	80	96	100
Arginine	0	100	100	100	100	100
Histidine	0	100	100	91	100	100
Betaine	5	100	100	100	100	100
Sarcosine	3	100	90	84	88	0



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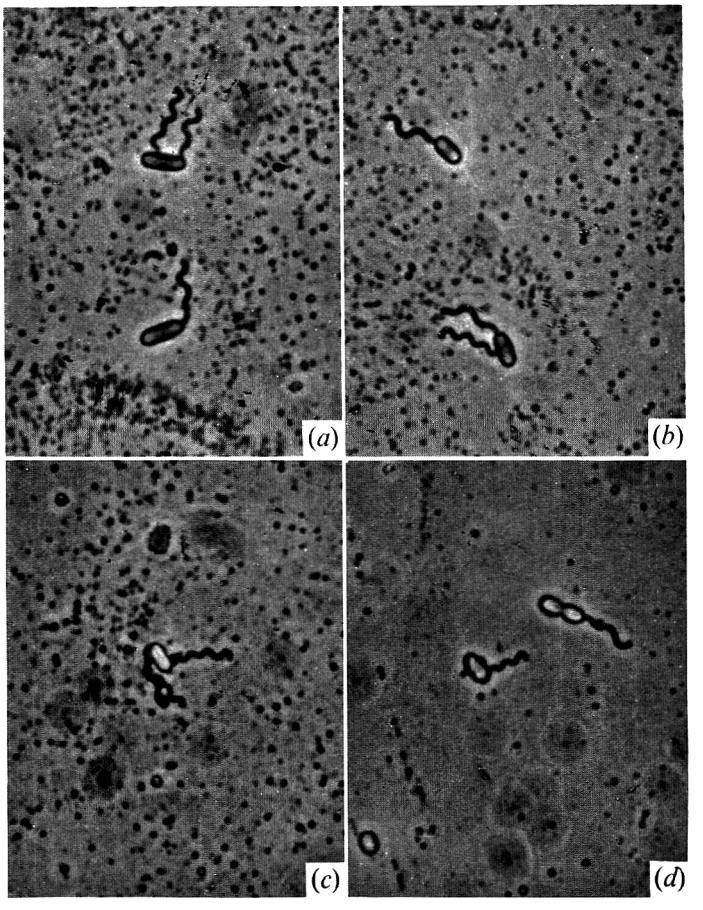
(a)
(b)
(c)
(d)

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EXPLANATION OF PLATES

PLATE 1

Flagella stain (Leifson's method) of *Pseudomonas stutzeri*, strain 227(*a*), and of *P. mendocina*, strain CH 110(*b*). × 2000.

PLATE 2

Flagella stain (Leifson's method) showing cells with lateral flagella of short wavelength: *Pseudomonas mendocina*, strain CH 110 (*a*) and (*b*); *P. stutzeri*, strain 319 (*c*), and *P. stutzeri*, strain 227(*d*).

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Recovery of Bacteria from Damages Induced by Heat

By P. MUKHERJEE AND S. B. BHATTACHARJEE
Biophysics Division, Saha Institute of Nuclear Physics, Calcutta 37, India

(*Accepted for publication 27 October 1969*)

SUMMARY

When *Escherichia coli* B was heated at 52° and subsequently incubated at 20° or at 37° in phosphate buffer, there was an increase in the number of viable organisms. No such increase was observed when the treated suspension was incubated at 0·5°. Untreated organisms did not increase in number when incubated in phosphate buffer, suggesting that the increase in viable number of heat-treated organisms was not due to division. The observed increase in number of viable organisms was time-dependent and complete in about 60 min., but could be stopped by exposing the treated organisms to X-ray doses greater than 1·5 krad., before or after the heat treatment. Growing the organisms in the presence of chloramphenicol 100 μg./ml. before heat treatment also blocked the repair mechanism, but this could be re-established by growth in the absence of chloramphenicol for 30 min. before heat treatment.

INTRODUCTION

The existence of a repair system in bacteria has been established and found to be of a general nature in that it operates on damage induced by various types of agents, such as X-rays, ultraviolet light, and radiomimetic agents (Setlow, 1966).

Recovery in *Escherichia coli* cultures damaged with heat has been shown by Heinmetz, Taylor & Lehman (1954), and the number of organisms surviving an exposure to heat found to be dependent on the use of different plating media (Busta & Jezeski, 1963; Heather & van der Zant, 1957*a*, 1958), the post-incubation temperature in liquid media and the pH value of the plating media (Heather & van der Zant, 1957*b*). In the present paper, recovery phenomenon in heated *Escherichia coli* populations in a medium not supporting growth has been examined.

METHODS

Escherichia coli B organisms were grown in the tris + glucose medium of Maaløe & Hanawalt (1961). Overnight cultures were diluted 50 times with the same medium and growth continued, with aeration, at 37°. Rapidly growing organisms were collected by centrifugation and freed from nutrients by three washings with ice-cold M 15-phosphate buffer (pH 6·9). The organisms were then suspended in warmed phosphate buffer and heated at 52° in a constant temperature waterbath for various times. After the selected periods of heating the suspension was divided into three parts, one of which was placed immediately at 0·5° and the others at 20° and 37° and held at these temperatures. The colony count of bacteria was determined at selected time intervals and the initial viable count of bacteria determined from an unheated sample. In different experiments, this concentration of bacteria varied in the range 5×10^8 to 8×10^8

colonies/ml. The number of viable bacteria was determined by plating suitable dilutions of each suspension on nutrient agar. Suspension (0·1 ml.) was spread on the agar surface and the visible colonies counted after incubation at 37° for 18 hr.

The influence of X-irradiation on the recovery of heated organisms was studied by irradiating the organisms with X-rays and then heating at 52° for the required time or, alternatively, first heating at 52° and then irradiating with X-rays. The samples were then incubated at 20° in phosphate buffer and the concentrations of viable bacteria determined at intervals.

X-irradiation of the bacteria was done in the cold in a special brass holder in a small Petri dish in contact with ice from a Muller MG 150 tube run at 80 kV, 9 mA. The X-ray doses at various positions of irradiation were measured by $FeSO_4$ dosimetry. The influence of protein synthesis on the recovery of bacteria was studied by growing organisms for 30 min. in the presence of chloramphenicol 100 μg./ml. This concentration of chloramphenicol was chosen because previous experiments showed that the increase in extinction at 450 mμ was completely stopped at such a concentration of chloramphenicol. After this incubation in the presence of chloramphenicol, organisms were washed free from chloramphenicol and exposed at 52° for 40 min. Subsequently, the organisms were incubated at 20° in phosphate buffer. This experiment was varied by allowing growth of bacteria to occur in the absence of chloramphenicol for 20 and 30 min. after the chloramphenicol treatment.

RESULTS

The influence of incubation at 20° on the survival of heat-exposed *Escherichia coli* organisms is shown in Fig. 1. For convenience, the initial % survivors in all cases was calculated to 100. The actual number of survivors under different conditions of treatment is shown in Table 1. Clearly there was an increase in the number of survivors during incubation; this increase was very steep for the first 30 min. and reached a maximum value in about 60 min.

When the organisms were exposed to heat for brief periods of time, the subsequent increases were small. The increase in number of survivors on incubation was greater when the number of heat survivors was small; however, an increase in number was observed in all cases. Unheated bacteria remained fully viable in control experiments under identical post-heating conditions. In all cases the maximum increase in number of survivors was reached in 60 min. and thereafter there was no change for the rest of the 3 hr period of the experiment. In Table 1, the results of holding the heated organisms at different incubation temperatures in phosphate buffer are given. No increase in survivors was observed when heated organisms were incubated at 0·5°; at 37 and 20° the results were similar. However, the recovery was prominent at 20° for the organisms exposed to shorter periods of heating. Later experiments on the recovery were therefore done at the incubation temperature 20°.

The effect of inhibition of protein synthesis on the observed increase in number is shown in Fig. 2. In this experiment, the initial heat survival was 0·008% in all cases. The organisms that were exposed to chloramphenicol for 30 min. before exposure to heat showed no subsequent increase or decrease in the number of survivors on incubation at 20°; but by allowing growth in the absence of chloramphenicol for 20 and 30 min. before heat-exposure, organisms again showed the increase in survival

number on subsequent incubation in phosphate buffer, at 20°. However, the increase in number was small for the organisms that were allowed to grow in the absence of chloramphenicol for only 20 min.

Thus there was a gradual increase in number of survivors on incubation of bacteria grown in the absence of chloramphenicol for 0 to 30 min. The organisms grown for

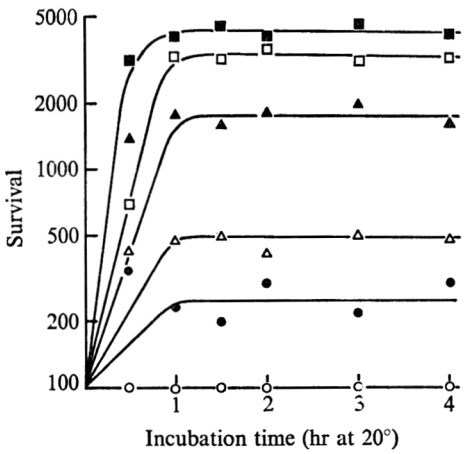

Fig. 1

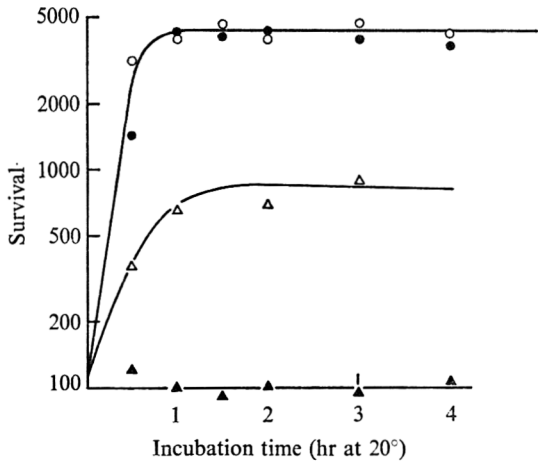

Fig. 2

Fig. 1. Effect of incubation in buffer at 20° on the survival of heat-damaged bacteria. Initial heat-survivors: ■, 0·008%; □, 0·01%; ▲, 0·07%; △, 1·4%; ●, 2·2%; ○, 100% (unheated).

Fig. 2. Influence of protein synthesis on the recovery of heat-damaged bacteria. ○, Protein synthesis not inhibited, prior to heating. ▲, Protein synthesis inhibited by growing cells in presence of 100 μg./ml. of chloramphenicol for 30 min. prior to heating. △, Protein synthesis inhibited, but prior to heating, 20 min. of growth in absence of the inhibitor (chloramphenicol) was allowed. ●, Protein synthesis inhibited but prior to heating 30 min. of growth in absence of the inhibitor (chloramphenicol) was allowed.

Table 1. *Post-incubation of heated bacteria at 0·5, 20 and 37°*

Temperature of post-incubation (°C)	Initial heat survivors (%)	Percentage surviving at incubation times (hr)					
		½	1	1½	2	3	4
0·5	2·76	2·4	2·9	2·5	2·86	2·7	2·5
0·5	0·17	0·19	0·15	0·17	0·16	0·15	0·17
0·5	0·027	0·026	0·028	0·027	0·020	0·027	0·027
0·5	0·0015	0·002	0·0016	0·0016	0·002	0·0011	0·001
20	2·20	7·6	5·2	4·4	6·4	4·8	6·4
20	1·4	6·0	6·7	7·0	5·8	7·0	6·7
20	0·07	1·1	1·27	1·14	1·28	1·4	1·14
20	0·01	0·07	0·33	0·316	0·35	0·31	0·32
20	0·008	0·25	0·32	0·37	0·32	0·37	0·33
37	1·5	6·0	6·1	7·0	5·8	6·0	6·8
37	0·07	0·29	0·43	0·43	0·33	0·5	0·47
37	0·015	0·077	0·12	0·12	0·11	0·16	0·12
37	0·008	0·25	0·17	0·2	0·3	0·2	0·18
0·5	100	99	98·9	100	100	101	99·5
20	100	100	98	98·8	99·2	100	100
37	100	98·4	99	100	100	102	98·8

o min. before heat treatment showed no subsequent increase in number; organisms grown for 20 min. showed some increase; but only the organisms grown for 30 min. after chloramphenicol treatment showed the same increase as that given by organisms not exposed to chloramphenicol.

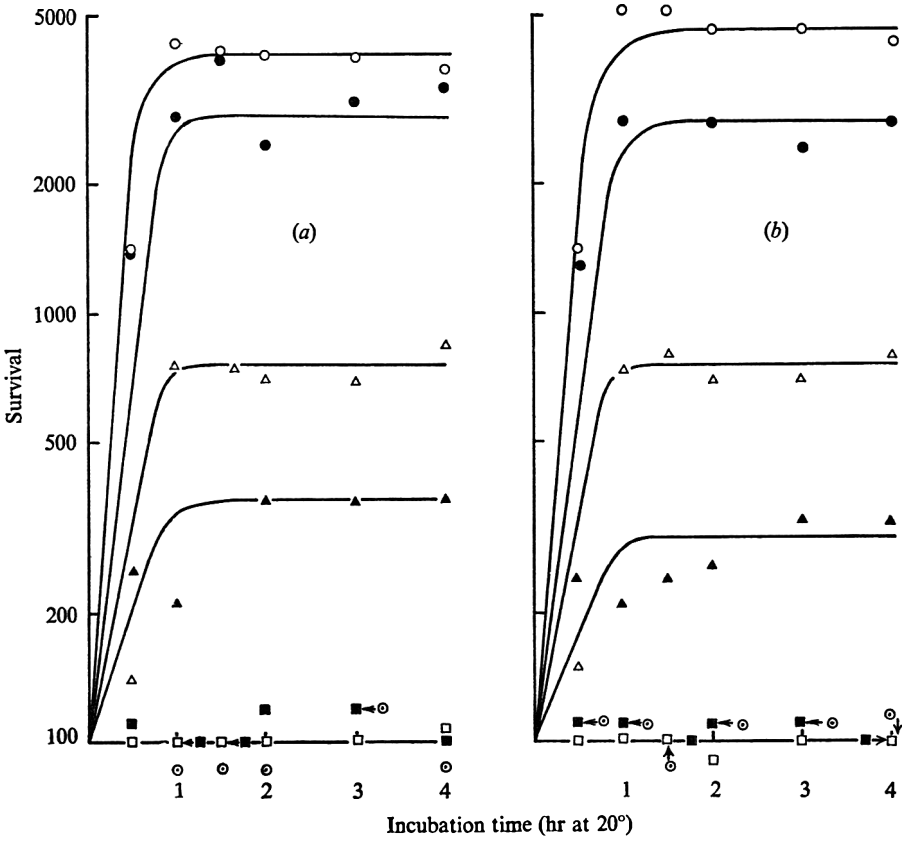

Fig. 3. Effect of X-irradiation on the recovery of heat-damaged bacteria. Curves: (*a*) irradiated before heating and (*b*) irradiated after heating. Doses used; ○, 0·268 krad.; ●, 0·804 krad.; △, 0·877 krad.; ▲, 1·072 krad.; □, 1·608 krad.; ■, 4·021 krad.; ⊙, 6·432 krad.

In Fig. 3, the influence of X-irradiation on the observed increase in number of survivors of heat treatment is shown. In all cases, survivors were at the level of 0·008%. The organisms were exposed to various doses of X-rays either before or after the heat treatment and then they were incubated in phosphate buffer at 20°. Then X-irradiation affected the increase in number of heat survivors. When the X-ray doses were greater than 1·5 krad., no increase was observed. This was true both for pre- and post-X-ray treatment. At X-ray doses up to 2 krad., there was a gradual suppression in the observed increase in number of post-heat survivors.

DISCUSSION

The observed increase in number of survivors after heating at 52° and incubation at 20 and 37° was not likely to be due to limited division of the organisms. This follows from the fact that under identical conditions of incubation organisms not heated did not increase in number. Thus the observed increase must have been due to repair of the lesions induced by heating the organisms. In other experiments, bacteria after heating were freed from the media in which they had been heated and were incubated at 20 and 37° in fresh phosphate buffer. The results obtained were identical with those in which the medium was not changed. This clearly indicated that recovery was not due to assimilation of products which leaked from the bacteria after heat treatment. However, in absolute terms, the number of organisms recovering from the damage was not high. In all cases, the observed recovery was in a medium which could not sustain growth, and in this respect the experiments presented here differ from those reported by Iandolo & Ordal (1966). These authors made experiments in complete growth media and the observed recovery was very high; but the present experiments show that even in the absence of growth nutrients a limited amount of recovery was possible. This recovery was suppressed at 0·5°, indicating that some metabolism was necessary; this was further supported by the observation that growth in presence of chloramphenicol (100 μg./ml.) for 30 min. stopped the process of recovery. The recovery phenomenon was fully re-established when the organisms were subsequently allowed to grow in the absence of chloramphenicol for a period of at least 30 min. before exposure to heating. Thus a direct involvement of protein in the mechanism is indicated. There is no evidence that chloramphenicol inhibits the synthesis of RNA (Maaløe & Kjeldgaard, 1966). Thus even if the involvement of RNA is postulated (Iandolo & Ordal, 1966), it cannot be direct.

A feature of the repair phenomenon is that it was completely inhibited by a small dose of X-rays. This X-ray dose was of the order at which colony formation was affected. Also, whether the exposure was made before or after the heat treatment was immaterial. So it would seem that the radiation acted on a system which had the same sensitivity to radiation before and after heat treatment. Since inhibition of protein synthesis stopped the recovery process completely, it is tempting to suggest that irradiation also acts through the same pathway. However, the ultimate results of protein inhibition and of X-irradiation were capable of suppressing the recovery. No effect on any of the synthetic abilities of cell were observed to follow these low doses of X-rays. The division ability of heat survivors was more sensitive to radiation than that of the control bacteria. Thus the process of recovery may be unrelated to the division ability of the bacteria because, in these experiments, pre- and post-X-ray exposures had the same effect on the recovery of heat-damaged organisms. The bacteria which recovered from heat-induced lesions had the same sensitivity to X- and u.v.-irradiation as of untreated organisms. They also showed photo-reactivation and liquid-holding recovery, indicating that the recovered organisms were not different from the untreated bacteria in these respects.

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Changes in Constituents and Ultrastructure of Hyphal Compartments during Autolysis of Glucose-starved *Penicillium chrysogenum*

By A. P. J. TRINCI AND R. C. RIGHELATO*
Microbiology Department, Queen Elizabeth College, Campden Hill, London, W. 8

(*Accepted for publication 30 October 1969*)

SUMMARY

The dry weight of *Penicillium chrysogenum* starved of an energy source decreased exponentially. The protein, RNA, DNA and endogenous respiration decreased rapidly during the first few hours of starvation and reached about 25% of their original values after 5 days starvation. The carbohydrate content of the mould only decreased after the first 2 days of starvation. About 60% of the carbon lost during autolysis was oxidized to carbon dioxide. Lysis of organelles in individual hyphal compartments occurred synchronously and did not seem to involve autophagy. Autolysis of separate compartments in the same hyphae was not synchronized. Ribosomes were rapidly degraded but membranes were particularly resistant to breakdown. Intra-hyphal hyphae were observed. In the final stages of autolysis the culture consisted largely of empty hyphal walls. A proportion of cytologically normal hyphal compartments were present at all stages, suggesting that maintenance and/or cryptic growth of some hyphae (or compartments) occurred at the expense of others.

INTRODUCTION

Righelato, Trinci, Pirt & Peat (1968) described the cytology of *Penicillium chrysogenum* in growing chemostat cultures and the maintenance of the structural integrity of non-growing cells by adding glucose at the maintenance rate. The cytology and metobolism of the non-growing mould under conditions in which endogenous material provides the substrates for cell maintenance or growth are reported here.

Although several studies have been made of the endogenous metabolism and survival of bacterial cultures starved of one or more essential nutrients (see Dawes & Ribbons, 1964; Postgate, 1967) comparable investigations with moulds are lacking. The viability of a population of unicellular organisms can usually be assessed without difficulty by the criterion of growth of individual cells. With filamentous systems such determinations are not possible and cytological criteria of the metabolic integrity of hyphal units must be used. A survey of the literature has not revealed any studies of the cytological changes accompanying starvation of moulds. Lysosomal systems form part of the cellular organization of higher animal (DeDuve & Wattiaux, 1966), protozoan (Elliott, 1965), algal (Brandes, Buetow, Bertini & Malkoff, 1964) and possibly higher plant cells (McLean & Gahan, 1968) but are not found in procaryotic cells. One function of this system is degradation of organelles, such as mitochondria, by autophagy under starvation conditions (Brandes *et al.* 1964). The presence of an

* Present address: Glaxo Laboratories Ltd., Ulverston, Lancs.

analogous system in fungi is not definitely established but Thornton (1968) identified autophagic vesicles in *Phycomyces blakesleeanus* sporangiophores on morphological criteria. Lysosome-like vesicles have also been implicated in the secretion of extracellular enzymes by moulds (Matile, 1966). However, it may be significant that golgi, which are usually involved in lysosome formation in animal and protozoan cells (DeDuve & Wattiaux, 1966) are absent from many moulds (Bracker, 1967).

METHODS

The organism was *Penicillium chrysogenum* WIS 54-1255. The chemostat growth conditions and analytical methods were as described by Righelato *et al.* (1968) unless otherwise stated. Dry weight and cell polymers are all expressed in terms of mould harvested from a unit volume of the culture.

Shake flask cultures. The medium was the DAN medium described previously (Trinci, 1969). The mould was grown in 250 ml. conical flasks containing 25 ml. of medium. Each flask was inoculated with 1 ml. of conidial suspension (final spore density, 1 to 2×10^6/ml.) and incubated at 25° on a rotary shaker (200 rev./min.). Growth was followed by harvesting three flasks at intervals in the manner described by Trinci (1969). The dry weight of the conidial inoculum was also determined.

Electron microscopy. Mould samples were fixed in the following ways: (1) 2% (w/v) aqueous, unbuffered $KMnO_4$ for 1 hr at 18 to 20°. (2) 6% (v/v) acrolein in cacodylate buffer at pH 7·2 for 1 hr at 18 to 20°. (3) 4% (v/v) glutaraldehyde in 0·05M-cacodylate buffer at pH 7·2, overnight at 4° or at 18 to 20°. Samples fixed in acrolein or glutaraldehyde were post-stained in 1 or 2% (v/v) aqueous osmium tetroxide for 1 hr at 18 to 20°. The mould was dehydrated in ethanol–water mixtures and embedded in araldite. Sections were cut with an LKB ultramicrotome with glass knives. Sections of glutaraldehyde and osmium tetroxide fixed hyphae were stained with 2% (w/v) aqueous uranyl acetate for 30 min. followed by an alkaline solution of lead citrate for 5 min. (Venable & Coggeshall, 1965). Measurements of cytological features were made directly from the photographic plates.

Acid phosphatase histochemistry. Hyphae fixed in 4% (w/v) aqueous formaldehyde at 18 to 20° were used to demonstrate the location of acid phosphatase. The method described by Jensen (1962) was followed except that 0·1M-ATP was used as the substrate instead of β-glycerophosphate. The mould samples were incubated in the substrate mixture for 1 to 2 hr at 37°.

RESULTS

Changes in hyphal constituents, respiration and dry weight during glucose starvation

Batch, shake-flask culture. Growth of *Penicillium chrysogenum* at 25° could be divided into lag, exponential, stationary and autolytic phases (Fig. 1). Autolysis of the mould commenced when the glucose in the medium was exhausted. The dry weight of the fungus decreased exponentially during autolysis; the 'half life' of the mould dry weight was about 91 hr. The time at which the mould started to autolyse was estimated and is designated zero time. Periods before the onset of autolysis are prefixed with a minus sign, and those afterwards with a plus sign (Fig. 1). This culture will subsequently be referred to as the batch culture.

Chemostat-batch culture. The medium supply to a steady state, glucose-limited culture (specific growth rate 0·051 hr^{-1}, doubling time, 14 hr) was stopped and only

air and the pH control agents were subsequently fed to the culture vessel. All other essential nutrients were in excess throughout the period of glucose starvation. Immediately after termination of the medium feed the concentration of glucose in the vessel was about 0·001 g./l. Lipids and glycogen or other carbohydrate reserves did not accumulate during glucose-limited growth, thus it was expected that when the medium supply was stopped the mould would become rapidly starved of an exogenous carbon and energy source.

The decrease in dry weight of this culture also approximated to an exponential curve and the 'half life' of the mould dry weight was about 62 hr (Fig. 2). Rapid changes occurred in the nucleic acid and protein content of the mould when the medium feed was stopped (Fig. 2, 3). From 0 to +9 hr there was a linear decrease in

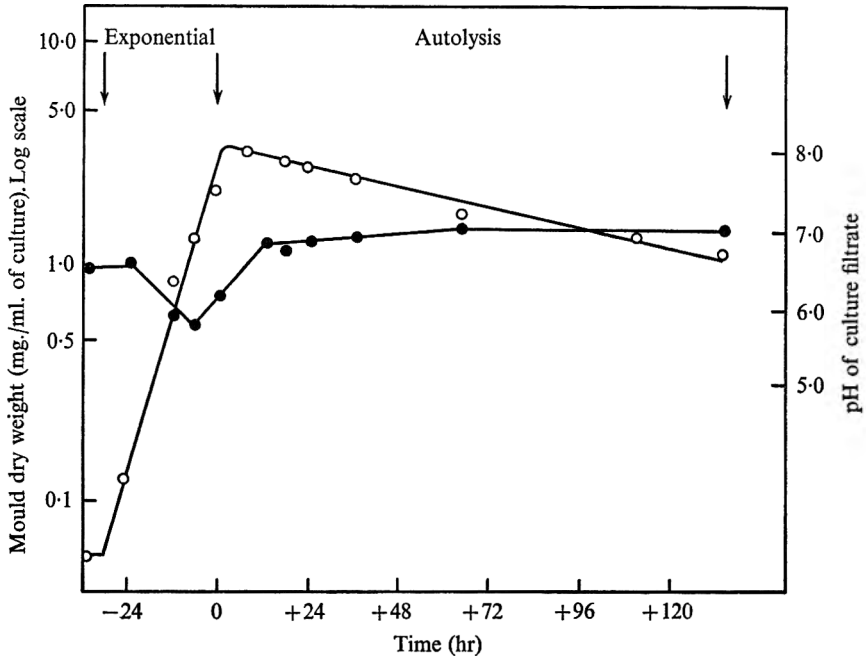

Fig. 1. Batch culture of *Penicillium chrysogenum* grown at 25°. ○, dry weight; ●, pH of culture filtrate.

the DNA content until it was about 20% of its original value, corresponding to a rate of decrease of about 9%/hr. The RNA and protein content of the mould decreased linearly at rates of 5·0 and 5·7%/hr respectively, during the period 0 to +6 hr after which the rates of RNA and protein loss became slower (Fig. 2, 3). Ammonia accumulated in the culture medium probably as a result of deamination of amino acids released during protein degradation. Considerable quantities of 260 nm. absorbing material also accumulated in the culture medium. The shift of this absorption band to about 285 nm. at pH 12 suggests that most of this was nucleotide bases and probably indicates that ribose was further metabolized (Beaven, Holiday & Dawson, 1965). The carbohydrate content of the mould was approximately constant during the first 2 days of starvation (Fig. 2).

The general shape of the endogenous q_{O_2} curve (Fig. 4) is almost identical to that

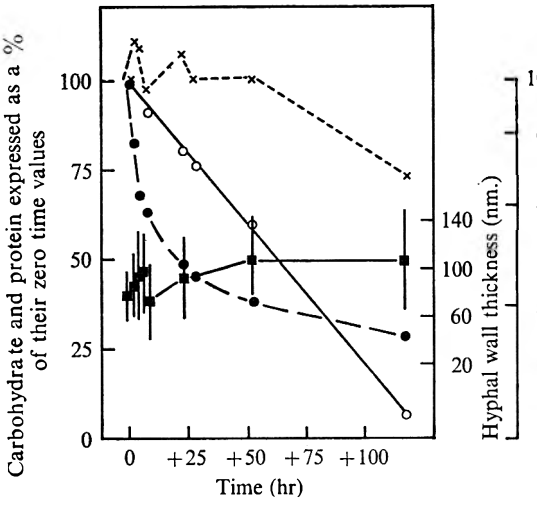

Fig. 2

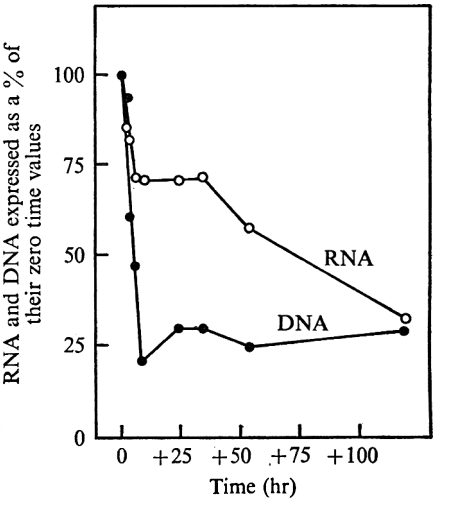

Fig. 3

Fig. 2. Chemostat-batch culture of *Penicillium chrysogenum* at 25°. Medium feed terminated at zero time. ×, carbohydrate; ○, dry weight; ●, protein; ■, wall thickness (the vertical lines represent the standard deviation).

Fig. 3. Chemostat-batch culture of *Penicillium chrysogenum* at 25°. Medium feed terminated at zero time. ●, RNA; ○, DNA. DNA present after 5 hr but too low for precise estimation.

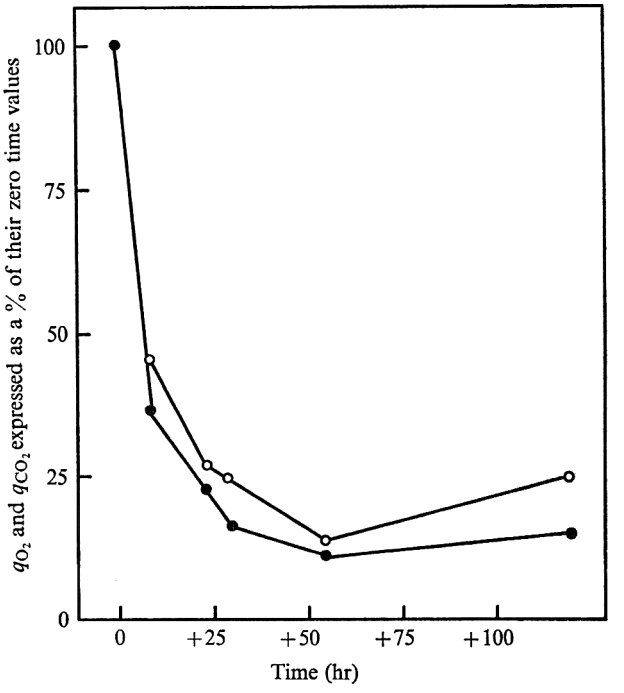

Fig. 4. Chemostat-batch culture of *Penicillium chrysogenum* at 25°. Medium terminated at zero time. ●, q_{CO_2}; ○, q_{O_2}.

obtained with starving cultures of *Aerobacter aerogenes* (Postgate & Hunter, 1962). These workers suggest that the upward inflexion of the q_{O_2} value towards the end of the curve may be attributable to cryptic growth. The quantity of carbon lost from the culture as carbon dioxide was equivalent to about 60% of the total carbon loss, assuming that carbon constitutes 47% of the dry weight (Righelato *et al.* 1968).

Cytological changes during glucose starvation

The same pattern of cytological development was observed in the batch and 'chemostat-batch' cultures. The ultrastructural changes were not synchronous; some hyphae with a 'normal' cytological appearance, i.e. identical with growing cultures, were observed after 5 days starvation (Pl. 1, fig. 1, 2), whilst a small proportion of hyphae sampled from steady-state, growing cultures showed clear cytological signs of autolysis. Thus the cultures were cytologically heterogenous at all stages of development, and culture age could not be used as an absolute guide to the sequence of morphological events during autolysis.

Cytological observations suggest that the autolysis of some hyphae might have provided sufficient nutrients for the maintenance or growth of the remaining hyphae (Pl. 1, fig. 3). This phenomenon has been observed with starving bacteria and is referred to as cryptic growth (Ryan, 1955) or re-growth (Strange, Dark & Ness, 1961). The proportion of maintained or growing hyphae decreased progressively during the starvation period. In fungi cryptic growth may, in part at least, involve the growth of hyphae within existing hyphae which are autolysing. This type of intrahyphal growth was observed in the present study (Pl. 2, fig. 4). In a few instances hyphae were observed with up to three concentric wall layers with cytoplasmic debris between them.

The 'normal' appearance of growing hyphae is shown in Pl 2, fig. 5, 6. Multimembrane, myelin-like figures, usually within vacuoles, were observed in some hyphae (Pl. 3, fig. 7). It was not definitely established whether these are normal features of hyphae or represent early stages in cellular disorganization.

When this investigation was initiated it was thought that one response of the mould to carbon starvation might be the sequential degradation of cytoplasmic organelles in autolysosomes after the fashion observed in starving cultures of Euglena (Brandes *et al.* 1964) and other cells (DeDuve & Wattiaux, 1966). Autolysosomes have been defined as 'membrane-lined vacuoles containing morphologically recognizable cytoplasmic components' (DeDuve & Wattiaux, 1966). Such structures have also been called cytolysosomes or autophagic vacuoles. Structures resembling autolysosomes were observed in the present study (Pl. 3, fig. 8; Pl. 4, fig. 12) but they were never very frequent and in only a few instances were nuclei observed surrounded by extra membranes (Pl. 3, fig. 9), whilst vesicle bound mitochondria were never observed. Histochemical demonstration of hydrolytic enzymes is commonly used to demonstrate lysosomes, however, ATPase activity was not demonstrated at the ultrastructural level although some positive results were obtained using light microscopy. The cytological disorganization of individual hyphal compartments (= the coenocytic cell between adjacent septa) proceeded in a synchronous manner (Pl. 4, fig. 10) rather than by sequential breakdown of individual organelles. Frequently the cytoplasm in one compartment was in a late stage of autolysis whilst that of an adjoining compartment had a normal appearance or was in an earlier stage of disorganization (Pl. 4, fig. 11). Thus autolysis of the compartments of a hyphal filament was not synchronized.

Ribosomes

One of the early changes in the cytology of starved hyphae was that the groundplasm of glutaraldehyde and acrolein fixed hyphae became less electron-dense and ceased to have a granular texture (Pl. 4, fig. 12).

Nuclei

The nucleoplasm of starved hyphae became less electron-dense and lost its granular texture except for a region which was probably the nucleolus (Pl. 4, fig. 12; Pl. 5, fig. 13). The shape of the nuclei and the appearance of their membranes did not seem to be affected during the early stages of nuclear disorganization (Pl. 4, fig. 12). Although nuclei were occasionally observed within vesicles, nuclear disorganization was not commonly preceded by this kind of envelopment.

Mitochondria

Mitochondria commonly retained a 'normal' appearance in starved hyphae for a longer period than ribosomes or nuclei (Pl. 4, fig. 12; Pl. 5, fig. 14) although mitochondrial matrices eventually became less electron-dense. Due to this difference in the rates at which various cytoplasmic components became disorganized, mitochondria appeared more sharply delineated in starved (Pl. 4, fig. 12) than normal hyphal compartments (Pl. 1, fig. 2; Pl. 2, fig. 6). Spherical, electron-dense bodies accumulated in the hyphae with prolonged starvation (Pl. 4, fig. 10, 12; Pl. 5, fig. 15). These were sometimes membrane-bound but were too large to be Woronin bodies. Cytological evidence suggests that these electron-dense bodies may be involved in the process of mitochondrial degeneration (Pl. 5, fig. 15). Alternatively they may be lipid globules. In some hyphae, mitochondrial disorganization appeared to precede ribosome breakdown. The groundplasm of these mitochondria was electron-transparent and contained vesicles which may have resulted from the disorganization of cristae (Pl. 5, fig. 16). Mitochondria with disorganized cristae were also observed in hyphae which had already lost most of their ribosomes (Pl. 5, fig. 15). In many cases the inner mitochondrial membrane appeared more electron-dense than the outer one (Pl. 5, fig. 13).

Late stages of hyphal autolysis

In the last stages of autolysis the hyphae contained only an accumulation of disorganized membranes (Pl. 1, fig. 3; Pl. 6, fig. 17 to 19). In some cases the latter retained their characteristic 'unit membrane' appearance (Pl. 6, fig. 19). It was possible to discern the previous history of some of these membranes (Pl. 4, fig. 10). Membranes thus contain polymers, probably the phospholipids, which are particularly resistant to breakdown. There is only one reported instance of the breakdown of structural lipid during bacterial starvation (Thomas & Batt, 1969). As autolysis proceeded, the hyphae contained fewer and fewer residual membranes (Pl. 1, fig. 3) until eventually only empty hyphal walls remained. Cytological evidence indicates that membrane loss may occur through release from ruptured hyphae rather than by enzymic degradation (Pl. 1, fig. 3; P. 6, fig. 18). Certainly detached membranes may be detected in the medium (Pl. 1, fig. 3; Pl. 6, fig. 18) and breaks in the hyphal walls were frequently observed after prolonged starvation (Pl. 1, fig. 3; Pl. 6, fig. 18). Membrane dispersal

may also occur as a result of the breakdown of septa (Pl. 6, fig. 20). It is not known if wall lysis was caused enzymically or mechanically but *N*-acetylglucosaminidase, an enzyme probably involved in chitin degradation, is certainly present in *Penicillium chrysogenum* (Brightwell, personal communication) and may be involved in cell-wall rupture.

Conidia were not formed at any stage during the starvation period. Optimum conditions for conidia formation require a slow feed of energy source (Righelato *et al.* 1968).

DISCUSSION

Under conditions of starvation due to limitations of exogenous carbon-and-energy source, *Penicillium chrysogenum* must derive its maintenance energy from endogenous material. Where no reserve material is present this involves the breakdown of functional polymeric cell components and catabolism of the monomers. Two distinct mechanisms may be envisaged; (i) the progressive utilization of components of each hyphal compartment and (ii) the rapid autolysis of some hyphal compartments providing substrates for the maintenance or growth of others.

The early loss of protein, RNA and DNA from starved *Penicillium chrysogenum* cultures probably took place in the majority of hyphal compartments and were similar to those shown by this organism when changing from a growing to a non-growing state in the presence of glucose supplied at the maintenance rate (Righelato *et al.* 1968). In the latter case the fall in protein, RNA and DNA concentrations halted after 5 hr, whereas in the glucose-starved culture the decline continued, albeit less rapidly. The decrease in electron density and granularity of the groundplasm of starved hyphae is probably indicative of a decrease in ribosome concentration; ribosome concentration in bacteria is directly proportional to growth rate and changes rapidly with changes in growth rate (Tempest & Strange, 1966). Protein and RNA are commonly degraded during bacterial starvation (Postgate & Hunter, 1962; Ben-Hamida & Schlessinger, 1966; Schlessinger & Ben-Hamida, 1966) and, as in the present study, the rate of RNA degradation is usually fastest in the early stages of starvation (Postgate & Hunter, 1962; Burleigh & Dawes, 1967; Thomas & Batt, 1969). The ribose derived from RNA degradation is used as an energy source in bacteria (Dawes & Ribbons, 1964). Starved cultures of *Aerobacter aerogenes* (Strange *et al.* 1961) or *Euglena gracilis* (Blum & Buetow, 1963) can lose up to 50% of their intracellular RNA without loss of viability.

The rapid degradation of DNA in starved *Penicillium chrysogenum* cultures was unexpected, because in bacteria this component is either stable (Mandelstam, 1960; Burleigh & Dawes, 1967; Thomas & Batt, 1969) or decreases by only a small amount (Postgate & Hunter, 1962) during starvation; however, *Euglena gracilis*, another eucaryotic organism, also degrades its DNA during starvation (Blum & Buetow, 1963). The decrease in the electron density and granularity of the nucleoplasm of 'starved' nuclei of *P. chrysogenum* is presumably related to the observed loss of DNA, a conclusion which is supported by the fact that they were cytologically very similar to liver nuclei which had been treated with deoxyribonuclease prior to fixation (Georgiev & Chentzov, 1963).

The lack of carbohydrate degradation during the first 2 days of starvation would seem to be consistent with the fact that the thickness of hyphal walls did not decrease

during the 5-day starvation period (Fig. 2). In bacteria it is generally believed that wall polymers (Dawes & Ribbons, 1964) and capsular polysaccharides (Wilkinson, 1958; Duguid & Wilkinson, 1961) do not serve as substrates for endogenous metabolism during starvation.

The degradation of cell polymers during starvation is presumably enzymic. Ribonucleases and deoxyribonucleases are present in autolysing *Aspergillus niger* cultures (Nagasaki, 1968), proteases in nitrogen-starved *Penicillium griseofulvum* cultures (Morton, Dickerson & England, 1960) and *N*-acetylglucosaminidase in autolysing cultures of a *Cunninghamella* sp. (A. P. J. Trinci & Dennis, unpublished results). In *Escherichia coli* the ribonucleases responsible for RNA degradation are constitutive and activated by starvation conditions (Ben-Hamida & Schlessinger, 1966).

Autolysis of most *Penicillium chrysogenum* hyphae occurred but some compartments retained the appearance of normal growing cells even after 5 days starvation, presumably these latter utilize the substrates released by autolysis. The fact that autolysis of adjacent compartments was not synchronized would imply that the septal pores were plugged, isolating individual compartments. There is no indication from the present work as to what determined whether a hyphal compartment would autolyse or retain its structural integrity. Autolysing hyphal compartments show a sequence of events beginning with reduction in the electron density and granularity of the groundplasm and nucleoplasm and ending in empty hyphal compartments. It would be interesting to know if there was a point of no return in this cytological and physiological sequence. The occurrence of intrahyphal growth has been reported previously (Lowry & Sussman, 1966; Chan & Stephen, 1967; Calonge, 1968). Calonge suggests that intrahyphal growth may occur in response to unfavourable conditions, however, in the present study intrahyphal hyphae were also observed in exponentially growing cultures. Multi-membrane, myelin like figures similar to those observed in this study have been found in other fungi (Berliner & Duff, 1965; Hyde & Wilkinshaw, 1966; Osumi & Katoh, 1967; Thomas & Isaac, 1967; Smith & Marchant, 1968; Ruinen, Deinema & van der Scheer, 1968). These structures may be artefacts (Revel, Ito & Fawcett, 1968) and unconnected with cell lysis.

Lysosomes containing several hydrolytic enzymes have not yet been isolated from fungi. The evidence that fungi possess an autophagic system similar to that present in animal cells is wholly cytological (Bracker, 1966; Thornton, 1968). Structures which could be interpreted as autolysosomes have been observed in the present study but similar 'multi-vesicular bodies' have also been implicated in cell-wall synthesis (Marchant, Peat & Banbury, 1967) and enzyme secretion (Calonge, Fielding & Byrde, 1969), suggesting that the function of such structures should be interpreted with considerable caution. The present study indicates that even if autophagy occurs in *Penicillium chrysogenum* it does not play a dominant role in cell lysis, however, autophagy may be of considerable importance in polymer turnover during differentiation (Bracker, 1966; Thornton, 1968).

Finally we hope that as a result of this study the detection of autolysing cells in fungal cultures will be facilitated. Electron micrographs may be found in the literature which purport to be of growing hyphae but nevertheless these cells show clear signs of autolysis as judged by the cytological criteria established in this study.

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EXPLANATION OF PLATES

Abbreviations used: *AH* = autolysing hypha; *C* = cell wall; *DM* = disorganized membranes; *D.Mit* = disorganized mitochondria; *ER* = endoplasmic reticulum; *EDB* = electron-dense body; *G* = groundplasm; *IH* = intrahyphal hypha; *M* = mitochondria; *MVB* = multivesicular body; *MM* = multi-membrane complex; *N* = nucleus; *NM* = nuclear membrane; *NP* = nucleoplasm; *NU* = nucleolus; *PM* = protoplasmic membrane; *RW* = ruptured wall; *S* = septum; *SP* = septal pore plug; *SR* = septum remains; *UM* = unit membrane; *V* = vacuole; *VM* = vacuolar membrane.

PLATE 1

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 1. Chemostat–batch culture + 120 hr. Hypha with 'normal' cytological appearance. $KMnO_4$ fixation.

Fig. 2. Batch culture + 116 hr. Hypha with 'normal' cytological appearance. Glutaraldehyde fixation.

Fig. 3. Batch culture + 116 hr showing the cytological heterogeneity of the culture. Hyphae with a 'normal' cytological appearance and others which have autolysed. Some hyphal walls are ruptured and detached membranes are present in the medium. Glutaraldehyde fixation.

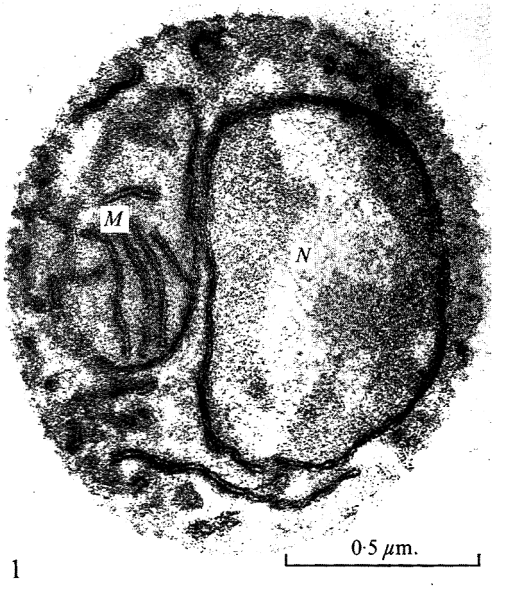
PLATE 2

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 4. Batch + 16 hr showing one hypha growing within the autolysed compartment of another. Glutaraldehyde fixation.

Fig. 5. Chemostat–batch 0 hr. Cytologically 'normal' hypha. $KMnO_4$ fixation.

Fig. 6. Batch + 2 hr. Cytologically 'normal' hypha. Glutaraldehyde fixation.



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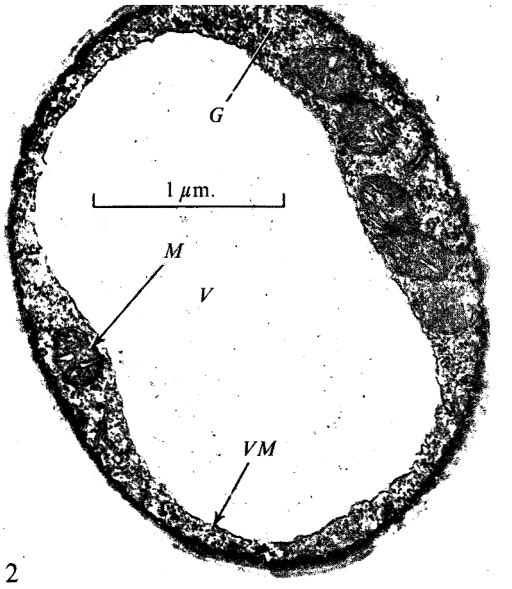

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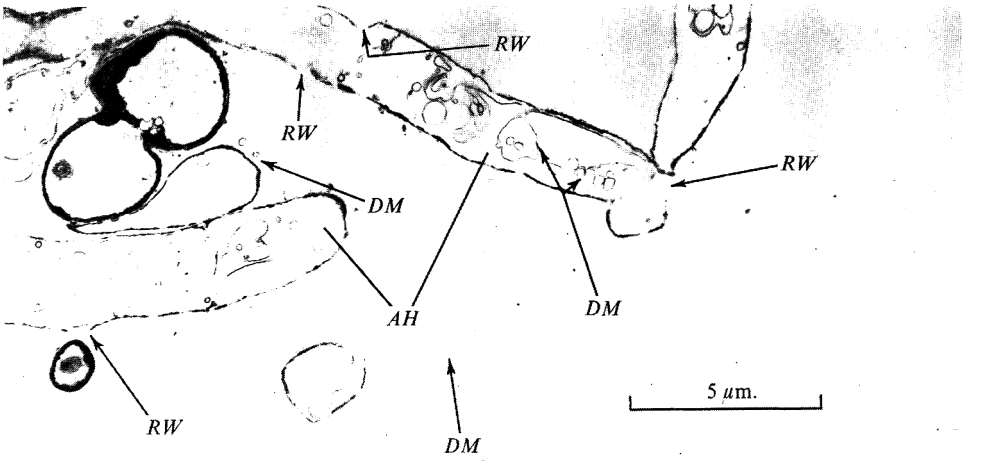

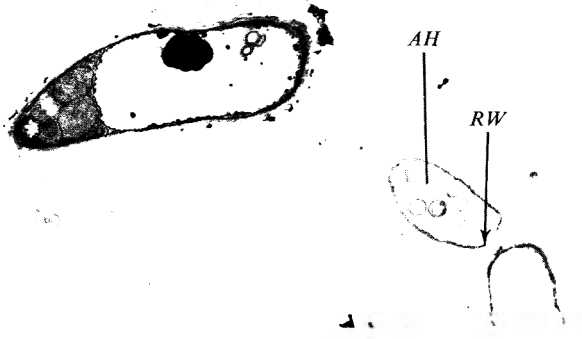

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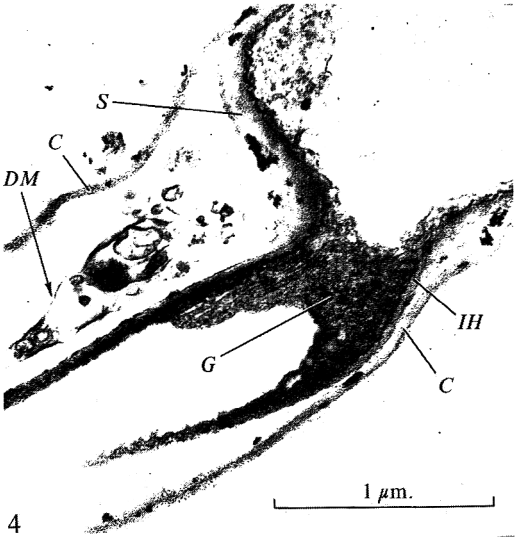

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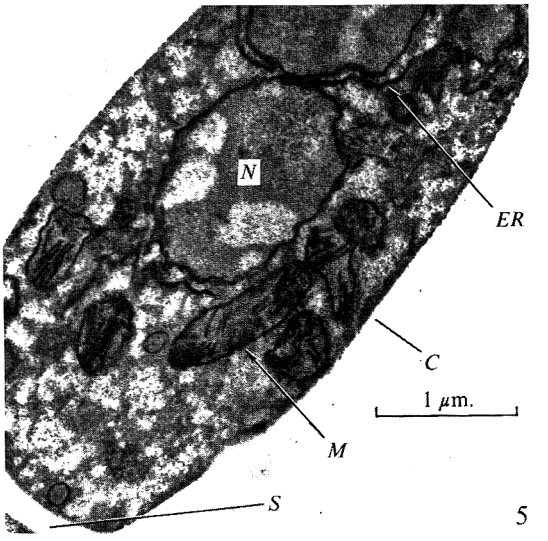

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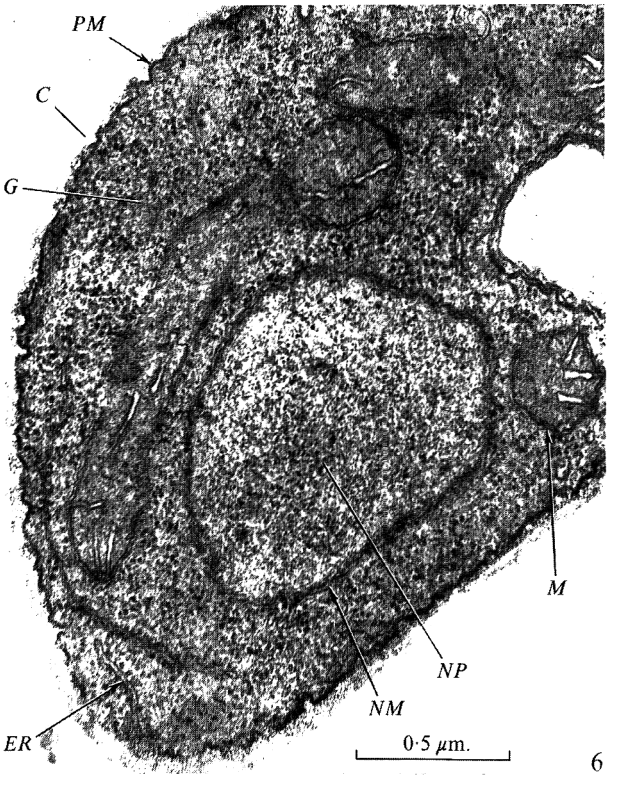

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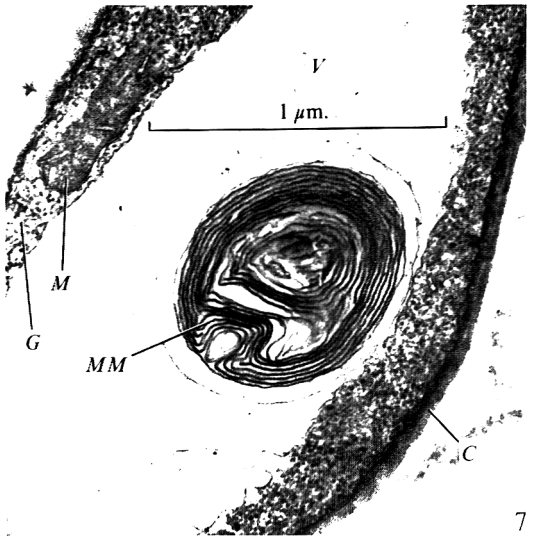

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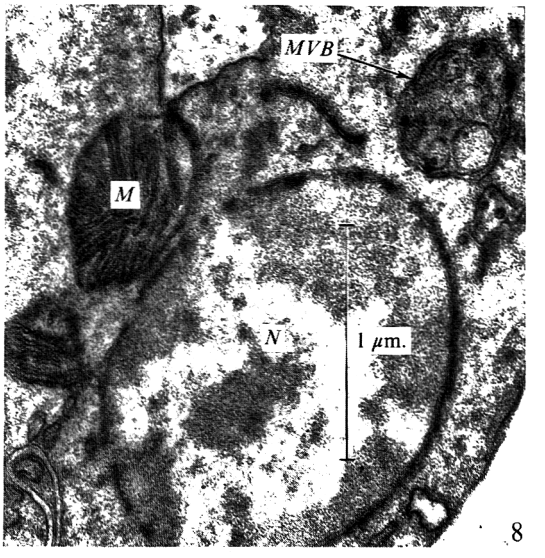

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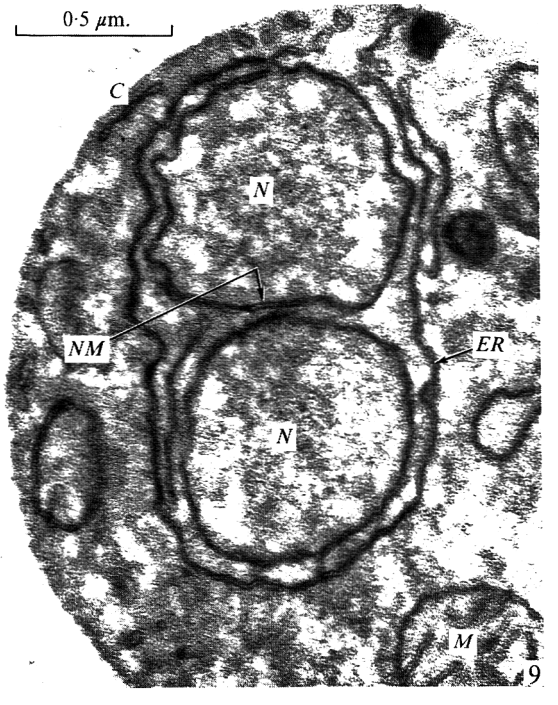

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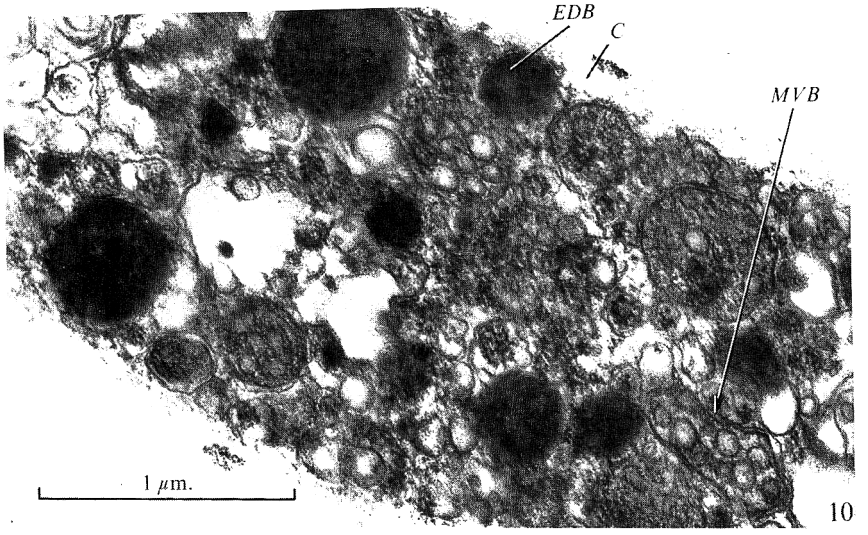

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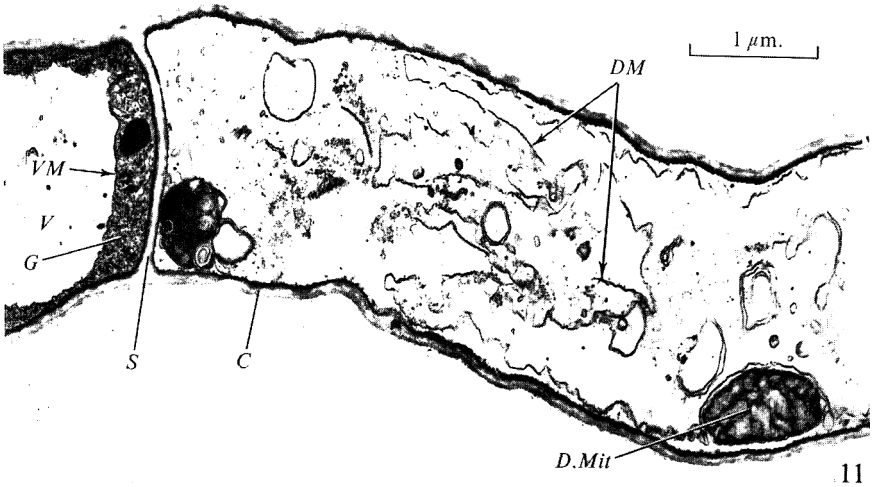

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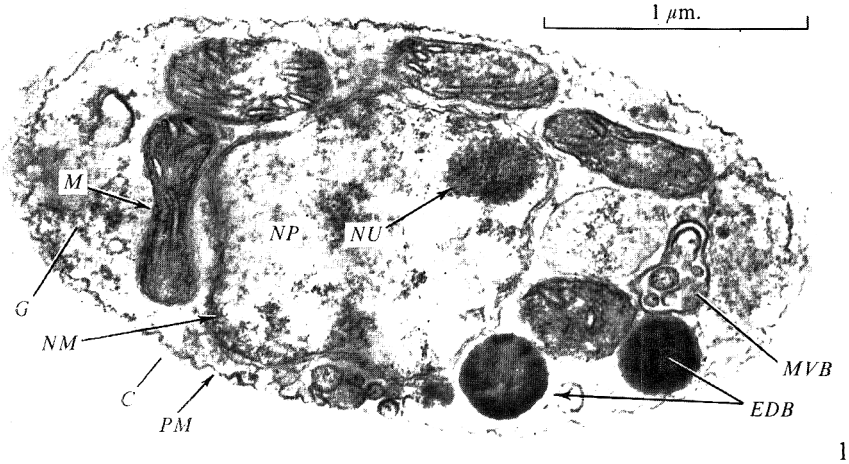

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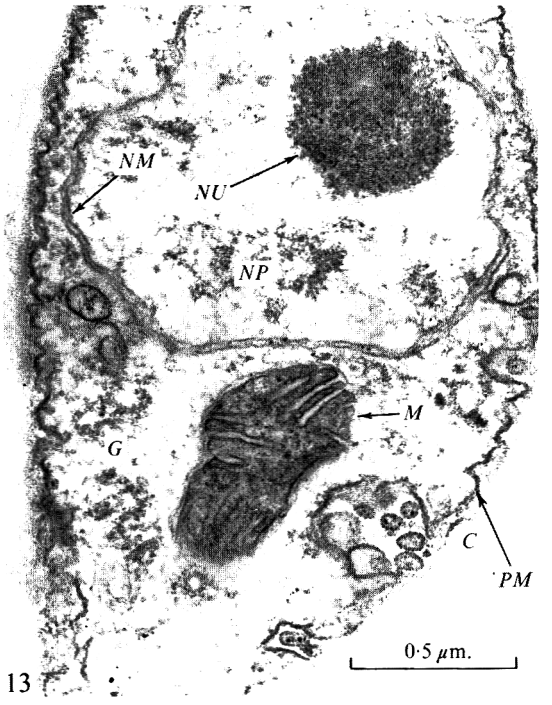

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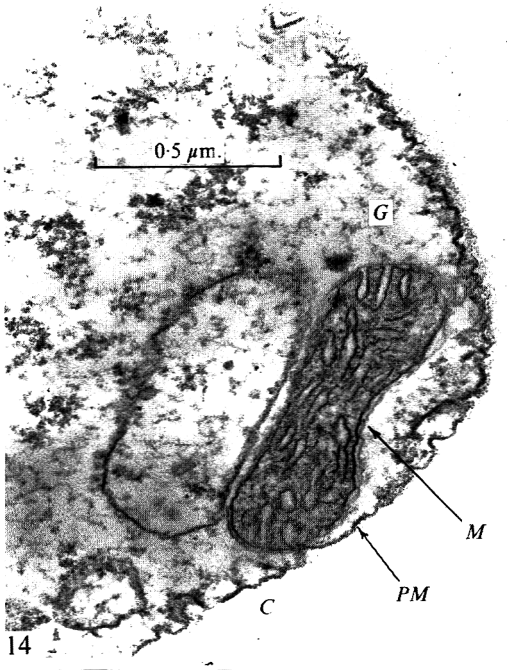

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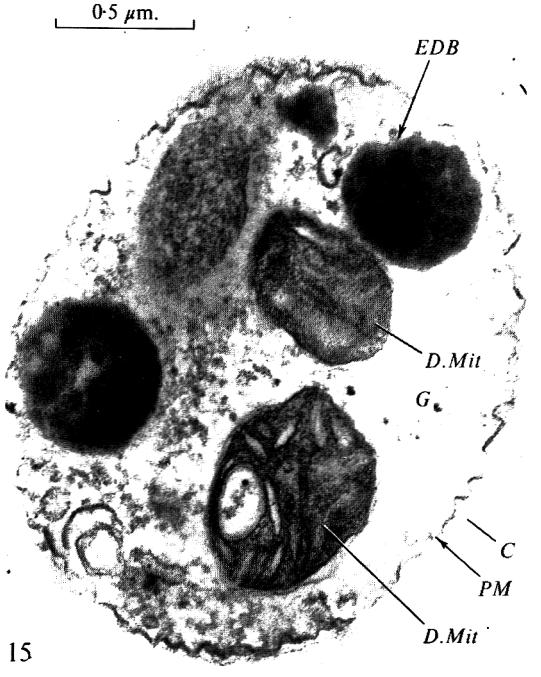

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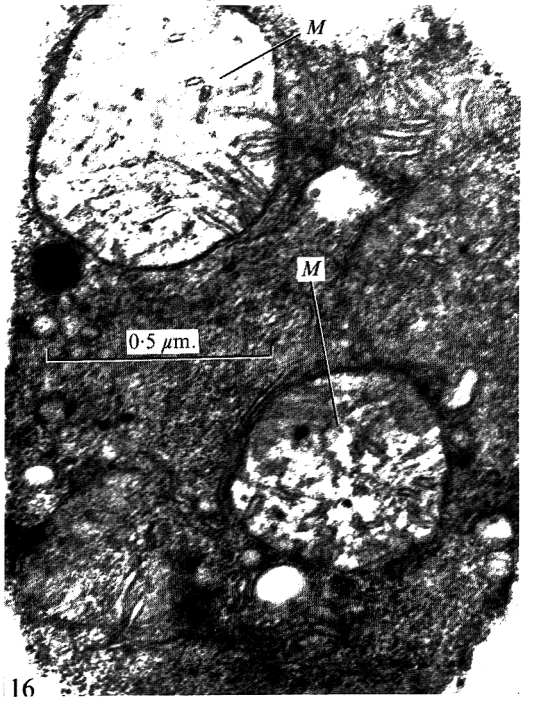

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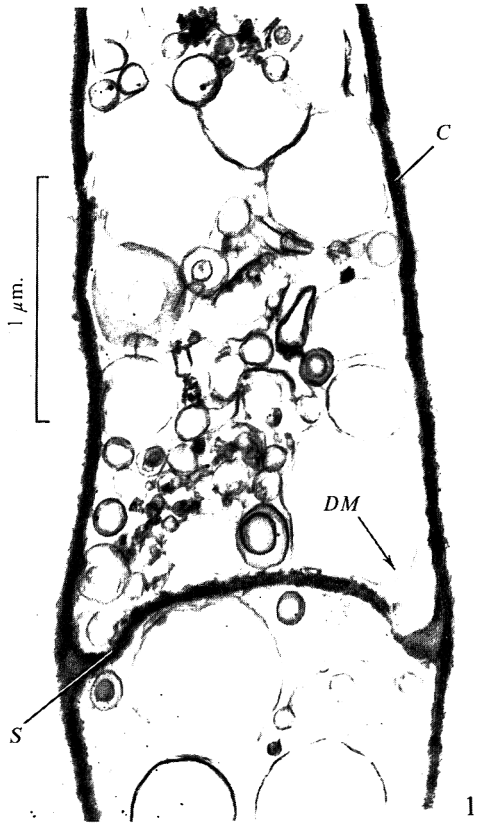

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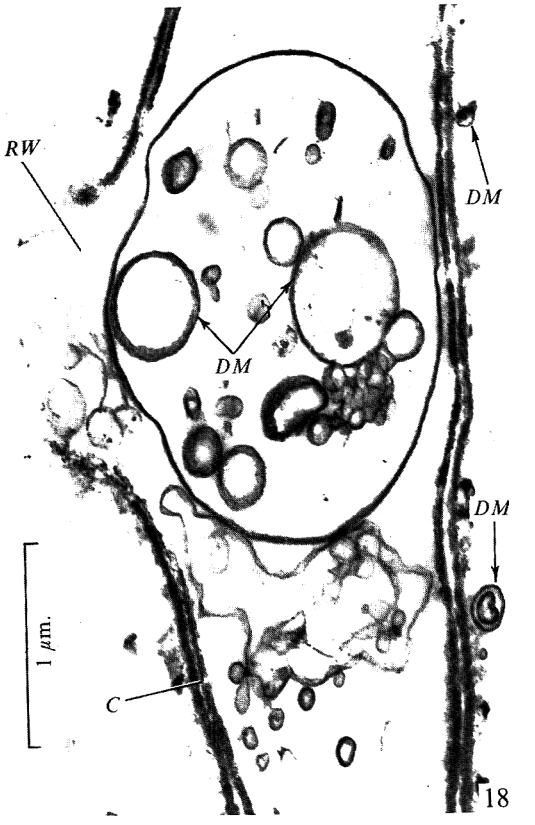

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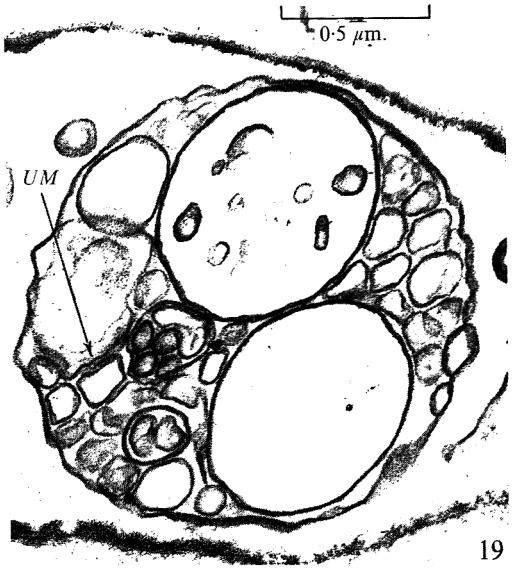

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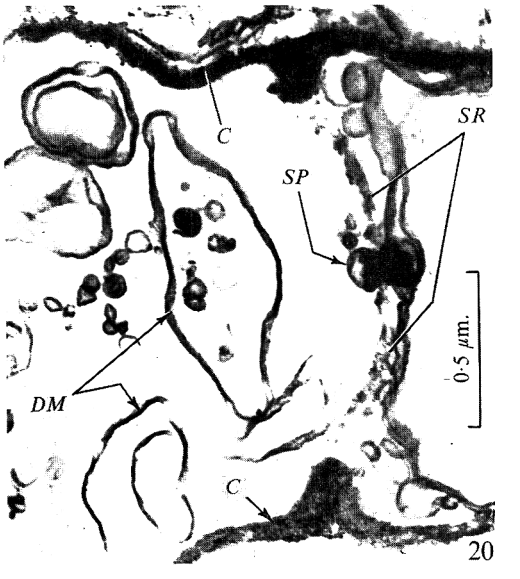

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PLATE 3

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 7. Batch + 116 hr showing a multimembrane complex within a vacuole. Glutaraldehyde fixation.

Fig. 8. Chemostat–batch 0 hr showing a multivesicular body which resembles an autolysosome. $KMnO_4$ fixation.

Fig. 9. Chemostat–batch 0 hr showing two nuclei partially enclosed by membranes. $KMnO_4$ fixation.

PLATE 4

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 10. Chemostat–batch + 1·4 hr showing a hyphal compartment which is autolysing synchronously. Acrolein fixation.

Fig. 11. Batch + 16 hr showing an autolysing hyphal compartment adjacent to a compartment with a 'normal' cytology. Glutaraldehyde fixation.

Fig. 12. Batch + 2 hr showing a hypha with an electron transparent nucleoplasm and groundplasm with few or no ribosomes. The mitochondria are more sharply delineated than in 'normal' cells. Glutaraldehyde fixation.

PLATE 5

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 13. Batch + 2 hr showing a hypha with an electron-transparent nucleoplasm and groundplasm lacking ribosomes. Some indication of a widening of the gap between the inner and outer nuclear membrane. Well-delineated nucleolus. Glutaraldehyde fixation.

Fig. 14. Batch + 2 hr. Apparently 'normal' mitochondrion and protoplasmic membrane but groundplasm lacking ribosomes. Inner mitochondrial membrane more electron-dense than outer. Glutaraldehyde fixation.

Fig. 15. Batch + 2 hr. Disorganized mitochondria in a groundplasm lacking ribosomes. Glutaraldehyde fixation.

Fig. 16. Chemostat–batch + 1·4 hr. Disorganized mitochondria in a groundplasm with ribosomes. Acrolein fixation.

PLATE 6

Electron micrographs of *Penicillium chrysogenum* WIS 54-1255.

Fig. 17. Batch + 24 hr. Autolysed hypha with an accumulation of disorganized membranes. Glutaraldehyde fixation.

Fig. 18. Batch + 116 hr. Autolysed hypha with an accumulation of disorganized membranes some of which appear to be released *via* a rupture in the hyphal wall. Detached membranes present outside the hypha. Glutaraldehyde fixation.

Fig. 19. Batch + 116 hr. Autolysed hypha with an accumulation of disorganized membranes some of which have a unit membrane structure. Glutaraldehyde fixation.

Fig. 20. Batch + 116 hr. Autolysed hypha with an accumulation of disorganized membranes. The septal wall between two compartments appears to be in the process of breakdown although the septal plug remains. Glutaraldehyde fixation.

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Phospholipids and Cellular Division of *Escherichia coli*

By J. STÁRKA AND JIŘINA MORAVOVÁ
Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, Marseille 9e, France

(*Accepted for publication 5 November 1969*)

SUMMARY

Low doses of penicillin which inhibited division but permitted filament formation in *Escherichia coli* did not influence the total rate of phospholipid formation but altered the ratio of individual phospholipids to that characteristic of resting organisms: the filaments contained more carciolipin and less phosphatidyl glycerol than normal exponentially dividing organisms. Addition of penicillinase to growing filaments restored both normal division and the normal phospholipid ratio. Filaments induced by u.v.-irradiation showed similar changes in phospholipid ratio. These findings suggest that phospholipids or their derivatives are directly involved in division.

INTRODUCTION

Filamentous forms of certain bacteria induced by different inhibitors of division can be used to study certain aspects of bacterial cytokinesis. We have described the properties of filaments formed by *Escherichia coli* B with a low concentration of penicillin (Stárka & Moravová, 1967). Penicillin (2·5 units/ml.) inhibit division completely but the synthesis of protein and nucleic acids continues at a normal rate. The filaments formed under these conditions contain several well-separated nuclei but septa are completely absent according to (1) electron microscopy of ultrathin sections of filaments and (2) the fact that each filament gives only one spheroplast with lysozyme and EDTA. Chemical composition of filament envelopes change as a result of penicillin action: after 60 min. cultivation with penicillin the contents of diaminopimelic acid and of hexosamines was about 25% lower than in untreated organisms. On the other hand, the kinetics of lysis of normal bacteria and filaments by lysozyme and EDTA were similar (J. Stárka, unpublished observations).

After addition of penicillinase, the filaments divide into rods of normal length. The reversion of filaments to normal rods by penicillinase can be stopped by chloramphenicol, mitomycin C, actinomycin D, nalidixic acid and other inhibitors of protein and nucleic acid synthesis. These results led us to the conclusion that (1) *de novo* synthesis of protein is essential for the division of filaments after removal of penicillin and that (2) the signal for septation is given by the replicating DNA (Stárka & Moravová, 1967).

There are serious reasons to suppose that not only the wall but also the cytoplasmic membrane is involved in the final stage of division of bacteria. Cronan (1968) described an increase of cardiolipin accompanied by a decrease of phosphatidyl glycerol after the transition from exponential to stationary phase of growth of *Escherichia coli*. These phospholipids are present almost exclusively in the cytoplasmic membrane. Similar

changes were reported by Bertsch, Bonsen & Kornberg (1969) in the early stationary phase preceding the spore formation of *Bacillus megaterium*. These variations might either be expressions of metabolic shifts occurring in stationary cells, or they might reflect direct function of the membrane in the division process. We therefore thought it useful to see whether any change in phospholipid content occurs in growing but non-dividing cells, i.e. during formation of filaments of *E. coli* which may be induced with penicillin or u.v.-radiation. The results are reported in this communication.

METHODS

Organisms and media. Escherichia coli B was maintained on nutrient agar (Difco). For each experiment an exponentially growing culture in glycerol-lactate (GL) medium (g./l.: NaCl, 3·0; $MgSO_4 \cdot 7H_2O$, 0·12; NH_4Cl, 1·0; $CaCl_2$, 0·001; KH_2PO_4, 0·15; K_2HPO_4, 0·35; neopeptone (Difco), 1·0; glycerol, 2·0; sodium-lactate, 8·0; distilled water; pH 7·2) was inoculated into the same medium and incubated at 37° on a reciprocal shaker.

Growth measurements. Extinctions in a 1 cm. cell at 450 mμ were measured in a Jean & Constant spectrophotometer. A Petit-Salumbeni chamber, depth 0·04 mm., calibrated with 0·10 units, corresponded to $0{\cdot}9 \times 10^8$ normal organisms/ml.

Induction of filamentous growth by penicillin. Exponentially growing bacteria were supplemented with 2·5 units sodium benzyl penicillin/ml. (Spécilline G, 1660 units/mg., kindly donated by Rhône-Poulenc Ltd., Paris). Samples for counting and extinction measurement were fixed with formol (0·2/5 ml. culture). To reverse penicillin action, penicillinase (Bacto Penase, Difco) was added to about 100 units/ml. Filament formation and growth, as well as division after addition of penicillinase, was monitored by phase-contrast microscopy.

Extraction and estimation of phospholipids. Exponentially growing cells were inoculated in 200 ml. of GL medium containing about 2 μC [^{32}P]labelled phosphate/ml. (obtained from CEA, Gif-sur-Yvette). Samples (15 ml.) were precipitated with 5 ml. 50% (w/v) cold TCA, and the precipitate was washed twice on a membrane filter (Millipore, porosity 0·45 μm.) with 5% TCA and finally with water. The filters, with precipitate, were placed in 5 ml. of chloroform + methanol (2 + 1, v/v) and allowed to stand at room temperature overnight. The extract was separated by filtration through membrane filter, placed in a scintillation vial, evaporated and redissolved in toluene scintillation solution (4·0 g. PPO and 0·5 g. POPOP/l). The samples were counted in a Mark I Nuclear Chicago instrument.

Separation of phospholipids. Phospholipids for chromatographic separation were extracted by two variant methods:

Extraction of intact bacteria (Kanemasa, Akamatsu & Nojima, 1967). The sample culture was rapidly refrigerated, centrifuged at 7000 *g* in a refrigerated centrifuge and the sediment was extracted with chloroform + methanol (2 + 1) three times. The combined supernatant fluid was evaporated *in vacuo*, redissolved in chloroform + methanol (2 + 1) and subjected to Folch's diffusion procedure (Folch, Lees & Sloan-Stanley, 1957). Dialysed extract was supplemented with methanol to original volume, partly evaporated and used for chromatography.

Extraction of perchloric acid-precipitated bacteria (Lubochinski, Meury & Stolkowski, 1965). A sample of culture was precipitated with cold perchloric acid (final conc.

0·3 N) and rapidly cooled, bacteria were centrifuged at 7000 *g* at 4°, washed twice with 50 mM-phosphate buffer pH 7·0 and the precipitate extracted and dialysed as in the first variant.

In all extracts total phosphorus content/ml. was then determined.

Thin-layer chromatography. Individual phospholipids were separated by thin-layer chromatography with Silica Gel G (E. Merck, A. G., Darmstadt, Germany) plates 0·25 mm. thick and prepared as recommended by the manufacturer. Plates were air-dried, activated by heating at 110° for 30 min. and stored overnight in a dessicator over P_2O_5 or $CaCl_2$. The sample applied to the plate never contained more than 0·3 μmole phosphorus. For quantitative determinations, two-dimensional separations were most useful. The first solvent was chloroform + methanol − water (70 + 25 + 4); after development plates were air-dried for 30 min., placed in a dessicator with P_2O_5 overnight, and run laterally in chloroform + methanol + 7 M-NH_4OH (60 + 35 + 4). Dried plates were detected with iodine vapour and then sprayed with ninhydrin or with molybdenum blue reagent (Dittmer & Lester, 1964). In some cases, radioactive phospholipids were located by autoradiography, using Kodak 'no screen' X-ray film. The spots were scraped from the plate and transferred quantitatively to scintillation vials containing scintillation liquid.

Phosphorus estimation. Total phosphorus in extracts containing phospholipids was carried out by the method of Bartlett (1958).

Ultraviolet irradiation. A 50 ml. sample of bacteria in GL medium was irradiated for 30 sec. 24 cm. from a Mineralight UVS 11 lamp. Bacteria (u.v.-irradiated) were cultivated in the dark.

Identification of phospholipids. The phospholipids were identified by their chromatographic behaviour compared with commercial standards. Cardiolipin (diphosphatidyl glycerol) was purchased from N.B.C., Cleveland, Ohio; phosphatidyl ethanolamine and phosphatidyl serine were from Koch–Light Laboratories, Ltd., Colnbrook, England. Phosphatidyl glycerol was a gift from Dr Ailhaud, C.N.R.S., Marseille. The solvents for chromatography were all reagent grade and were purified by the methods recommended by Rouser & Flescher (1967).

RESULTS

Incorporation of $^{32}[P]$ in lipid fraction of normal organisms and penicillin-induced filaments of Escherichia coli. Exponentially growing bacteria were incubated in 400 ml. of GL medium containing 1 μC [^{32}P]/ml. After 100 min., when OD = 0·20, the culture was divided into two parts. One was supplemented with 2·5 units penicillin/ml., the other served as control. Samples (20 ml.) were withdrawn at 20 min. intervals for extraction of lipids, O.D. determination and counting. After 60 min., penicillinase was added to induce division of filaments. Fig. 1 shows the amount of [^{32}P] incorporated in chloroform-methanol fraction of lipids from samples of the two cultures: the rate of incorporation was not altered either by penicillin or by its removal with penicillinase. The rate of incorporation of phosphorus into the unfractionated phospholipids had the same slope as the rate of increase of optical density and both rates remained constant during the experiment.

Composition of phospholipids in filaments of Escherichia coli. Although the total

phospholipids/turbidity unit did not change during inhibition of division by penicillin or its reversal by penicillinase, this experiment did not exclude the possibility that the relative amounts of individual phospholipids might have changed. Isotopically labelled

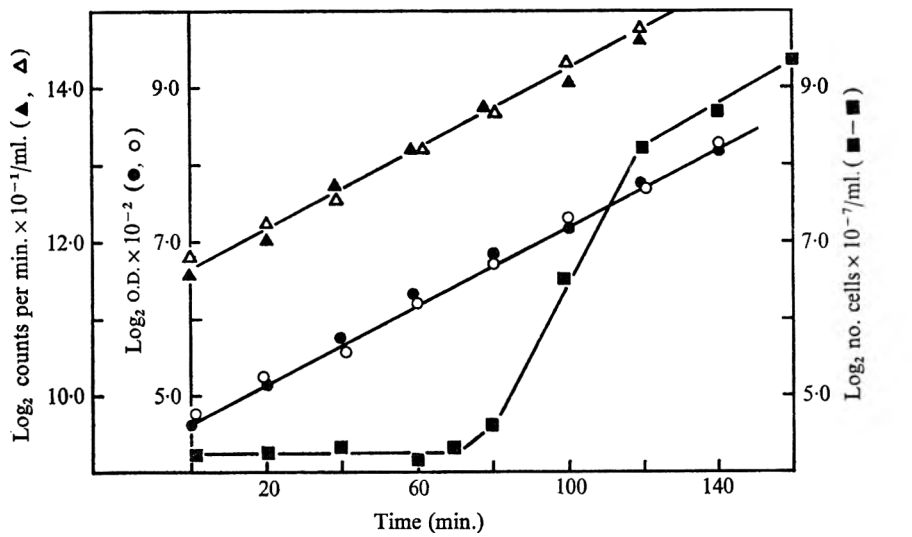

Fig. 1. Incorporation of radioactive phosphorus in phospholipids of normal cells of *Escherichia coli* B (empty triangles) and of penicillin-induced filaments (full triangles). Penicillin was added at 0 min., and penicillinase at 60 min. Optical density was measured in untreated control (empty circles) and in filaments (full circles). The number of cells treated with penicillin and with penicillinase was determined in a counting chamber (full squares).

Table 1. *Relative amounts of phospholipids detected in perchloric acid-treated extracts of Escherichia coli* B

Per cent of total ^{32}P counts in phospholipids separated by thin-layer chromatography

	Cardiolipin	Phosphatidyl glycerol	Phosphatidyl ethanolamine	Phosphatidyl serine
Exponential bacteria	0.35	22·90	72·31	4·44
Stationary bacteria	3·39	12·0	81·46	3·17
Filaments after 60 min. of penicillin action	4·82	16·75	74·0	4·43
Filaments after 60 min. of penicillin + 25 min. of penicillinase	3·65	17·30	74·55	4·50
Filaments after 60 min. of penicillin + 50 min. of penicillinase	0·28	22·62	71·95	5·15

phospholipids were therefore extracted from different phases of growth of filaments and normal rods. The arrangement of this experiment was essentially the same as described for Fig. 1 but 2 μC of [^{32}P]/ml. was present. Samples of untreated bacteria for extraction were withdrawn at O.D. = 0·35 (exponential phase) and in the stationary phase. Filaments were analysed after 60 min. of penicillin action. At that time penicillinase

was added and samples of reverting filaments were examined after 25 and 50 min. of further incubation.

After 60 min. with penicillin all cells appeared as filaments about 10 μm. long; reversion of filaments to normal rods was practically completed 60 min. after adding penicillinase. The rate of division was then the same as in the untreated control. Table 1 demonstrates that the relative amounts of radioactive phosphorus incorporated into phosphatidyl glycerol, cardiolipin, phosphatidyl ethanolamine and phosphatidyl serine in normal non-growing cells from the stationary phase and in exponentially growing filaments were practically identical. On the other hand, exponential normal cells contained relatively more phosphatidyl glycerol and less cardiolipin.

Table 2. *Relative amounts of phospholipids extracted from Escherichia coli* B *without precipitation by perchloric acid*

Per cent of total ^{32}P counts in phospholipids separated by thin-layer chromatography

	Cardiolipin	Phosphatidyl glycerol	Phosphatidyl ethanolamine	Phosphatidyl serine
Exponential bacteria	0·60	25·0	74·40	—*
Stationary bacteria	5·37	14·73	79·90	—*
Filaments after 60 min. of penicillin action	5·82	16·20	76·50	1·68
Filaments after 60 min. of penicillin + 50 min. of penicillinase	1·34	24·40	73·0	1·26

* No spot detected.

The values given in Table 1 are in good agreement both with the results of Lubochinski *et al.* (1965) who found about 20% of phosphatidyl glycerol and 1·5 to 3·0% of cardiolipin in exponential *Escherichia coli*, and with the observations of Kanfer & Kennedy (1963) who found 21% of phosphatidyl glycerol in exponential organisms and 7% in stationary organisms of the same species.

In dividing filaments treated with penicillinase the relative amount of phosphatidyl glycerol increased markedly and after 50 min. it was similar to that of exponentially growing cells. The content of cardiolipin also decreased gradually. Table 2 shows the results obtained with extracts when precipitation with perchloric acid was omitted. No substantial differences in relative amounts of individual phospholipids which could be attributed to the method of extraction were found. Nevertheless, all samples treated with perchloric acid before extraction contained more phosphatidyl serine.

Composition of phospholipids from u.v.-irradiated Escherichia coli. Exponentially growing *Escherichia coli* B in GL medium containing 2 μC ([^{32}P]/ml. was irradiated by u.v. and incubated for 60 min. in the dark while shaking. The majority of cells had by then formed filaments. The extract of $HClO_4$-precipitated cells was chromatographed and it contained 3·11% of cardiolipin, 14·07% of phosphatidyl glycerol, 77·6% of phosphatidyl ethanolamine and 5·19% of phosphatidyl serine, values typical of stationary rather than exponential bacteria, cf. Table 1. Thus it appears that the inhibition of division by u.v. had similar effect on relative content of phospholipids as had inhibition by penicillin.

DISCUSSION

Differences in the ratio of cardiolipin to phosphatidyl glycerol in exponential and stationary *Escherichia coli* B were observed earlier by several authors (Kanfer & Kennedy, 1963; Randle, Albro & Dittmer, 1969), in *E. coli* K 12 (Cronan, 1968) and in *Bacillus megaterium* (Bertsch *et al.* 1969). Another observation in good agreement with our results was reported by Cavard, Rampini, Barbu & Polonovski (1968). *E. coli*, after 30 min. in a mineral medium with no source of carbon, had a significantly higher content of cardiolipin and lower content of phosphatidyl glycerol; a similar situation was observed in organisms incubated in the same medium containing 1000 units of penicillin/ml. The present work shows that synthesis of total phospholipids in bacteria treated with penicillin occurs at the same rate as in exponentially growing control organisms even though the ratios of cardiolipin and phosphatidyl glycerol are different. Penicillin may interfere with the turnover of individual phospholipids or, more likely, with their further utilization in the mechanism of division. Phospholipids are not only structural; they are involved in the electron transport chain of mitochondria and bacterial membranes (Bruni & Racker, 1968; White & Tucker, 1969) and also in the synthesis of the peptidoglycan of the wall (Anderson, Matsuhashi, Haskin & Strominger, 1965). Isolated phospholipids from membranes of *Staphylococcus aureus* and *Micrococcus lysodeikticus* are essential for the synthesis of glucosaminyl-acetyl-muramyl-oligopeptide (Dietrich, Colucci & Strominger, 1967).

These observations and our results indicate that phospholipids have a direct rôle in wall synthesis and that their relative amounts reflect the situation of the membrane as far as division is concerned. The participation of phospholipids in mucopeptide synthesis may be generally valid but the relation between their metabolism and the mechanism of division is far from clear. Logically, two eventualities may be considered. The change in phospholipid ratios observed in our experiments may be the consequence of inhibition of wall synthesis, yet be of secondary importance for the process of division. The inhibition of wall synthesis, e.g. by penicillin, is in fact accompanied by changes in the level of phospholipid metabolism and by inhibition of division. It would not matter whether the inhibition of the wall synthesis were complete or only partial. Alternatively, phospholipids may have a distinct and direct function in the mechanism of division. In that case an intervention in their metabolism could result in inhibition of normal division or, *vice versa*, in reinitiation of division where this process is arrested.

The authors, on leave from the Department of Microbiology, Charles University, Prague, would like to express their appreciation for the kind hospitality enjoyed in Professor Senez's laboratory and to acknowledge specially the stimulating interchange of ideas with Drs G. Ailhaud and E. Azoulay, who helped them also with preliminary experiments. One of the authors (J. M.) received EMBO and ICRO short-term fellowships.

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Fatty Acid Requirements of Human Cutaneous Lipophilic Corynebacteria

By R. F. SMITH

Department of Microbiology, Temple University School of Pharmacy, Philadelphia, Pennsylvania 19140, U.S.A.

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SUMMARY

Thirty-nine human cutaneous and eight bovine obligate-lipophilic corynebacteria were examined for their fatty acid requirements for growth. Twenty-five saturated and five unsaturated compounds did not support growth. Oleic acid in *cis*, *trans* and *iso* forms supported various degrees of growth. Erucic acid was also utilized but not ricinoleic and nervonic acids. Linoleic acid supported the growth of some strains but linolenic acid did not. These acids were not inhibitory to most strains tested. Three short-chain acids (acetic, lactic, propionic) and glycerol did not potentiate growth of the strains in the presence of oleic acid. In oleic acid broth, palmitic acid which did not support growth alone, stimulated 14 strains and inhibited three others. Strains of *Corynebacterium bovis* differed from the human strains in their growth response.

INTRODUCTION

Normal human skin supports an abundance of lipophilic corynebacteria (Marples, 1969). These organisms require Tween 80 or oleic acid for growth *in vitro* (Pollock, Wainwright & Mansion, 1949). Tween 80 is known to contain from 0·4 to 0·8% unesterified fatty acids including myristic, myristoleic, palmitic, palmitoleic and oleic acids (Stinson & Solotorovsky, 1969); other Tween compounds also contain mixtures of fatty acids (Weary, 1968). Except for general growth support, Tweens cannot be used to determine accurately the specific fatty acid requirements of bacteria. The cutaneous corynebacteria have not been previously examined for the spectrum of their fatty acid requirements with the exception of oleic acid; the results of such a study are reported here.

METHODS

Organisms. Obligate-lipophilic corynebacteria (LC) from human skin were isolated and classified by the methods of Smith (1969*a*, *b*). The maintenance and sources of reference stains were reported previously (Smith, 1969*a*). Strains of *Corynebacterium bovis* were used in some experiments with the human strains.

Compounds. Lipids and other chemicals were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio and Sigma Chemical Co., St Louis, Missouri, U.S.A. All compounds were 99% pure except for vaccenic, erucic and elaidic acids and soybean lecithin which were 95% pure. Water-soluble compounds were prepared in aqueous solutions and sterilized by filtration. Water-insoluble compounds were dissolved in 95% (v/v) ethanol in water. All stock compounds were stored at —20°.

Basal medium and supplements. Nutrient broth (Difco) was prepared in concentrated form (24 g./100 ml.) and washed 3 times with light petroleum. This lipid-deficient concentrate was adjusted so that 30 ml. was diluted to yield 1 l. of broth. Three lots of concentrate were prepared. Each lot made 9 l. broth at final pH 7·0, obtained without adjustment. Previous experiments showed that human LC strains were incapable of growing in this medium unless supplemented with certain lipids. Growth response of non-lipophilic organisms was identical in each of the three test lots. Compounds were added to the autoclaved basal medium to give final concentrations of 10^{-4} M. Basal medium + 0·1 % Tween 80 was used as a growth control. Broth was dispensed aseptically in 5 ml. amounts.

Growth measurements. Cultures were grown on nutrient agar slopes (Difco) containing 0·05 % Tween 80 for 48 hr. The bacteria were washed from the slopes with sterile 0·1 M-phosphate buffer (pH 7·4) containing 0·05 % Triton X 100. Cell suspensions were adjusted to an extraction reading of 0·5 with a Bausch and Lomb Spectronic-20 colorimeter (650 nm.) and samples of 0·05 ml., delivered from serological pipettes, were used to inoculate the tubes of medium. Using conventional plating methods on to agar (Smith, 1969*b*) the inoculum was shown to contain about 100,000 viable bacteria. Cultures were incubated at 35° for 7 days with intermittent shaking by hand. Growth response was considered positive when cultures reached a turbidity of 50 % transmittance or less (a minimum of 1×10^7 bacteria/ml.).

RESULTS

Thirty-nine human cutaneous and eight bovine lipophilic corynebacterial (LC) strains were examined for ability to utilize and initiate growth on 38 different fatty acids and related compounds. Twenty-five saturated and five unsaturated compounds did not support the growth of any of the human or bovine strains (Table 1). Seven unsaturated fatty acids and lecithin supported growth of the 39 human LC strains in varying degrees (Table 2). Elaidic (*trans* oleic) and *cis* oleic acids were the most effective compounds, followed by vaccenic (*iso* oleic) and erucic acids. Several strains grew equally well on more than one of the active compounds. With increased desaturation of the *cis* oleic acids (linoleic, linolenic), decreased growth response resulted. Six of eight *Corynebacterium bovis* strains did not grow with any of the seven unsaturated fatty acids or lecithin. Strains NIRD 66 and 126 were the only *C. bovis* strains that grew substantially in the presence of four of the seven unsaturated fatty acids (Table 2). One strain each of *C. striatum*, *C. minutissimum*, *C. xerosis*, *C. diphtheriae*, *Brevibacterium linens*, *Enterobacter aerogenes* and 3 strains of *Staphylococcus epidermidis* grew in the basal lipid-deficient broth and in the media containing each of the 38 individually tested compounds. There were no appreciable stimulatory or inhibitory effects by any of the compounds on these cultures. Basal medium + Tween 80 was generally adequate for growth.

Experiments were made to determine whether linoleic, linolenic and ricinoleic acids were inhibitory rather than not utilized. Each of these fatty acids was separately added to basal medium containing Tween 80 or oleic acid; Tween 80 and oleic acid media without supplement were used as controls for comparison (Table 3). Six strains were completely inhibited by linoleic acid and five strains by linolenic acid. Ricinoleic acid was not inhibitory. Sixteen strains grew in oleate broth containing separate

supplements of 10^{-3} M lactic, acetic or propionic acids and glycerol. None of these short-chain compounds potentiated growth of the 16 strains in oleic acid broth. Finally, 37 of 39 human LC strains were grown in broth containing 10^{-4} M oleic and palmitic acids, and compared with growth in broth containing only oleic acid. Fourteen strains were stimulated in oleic broth by the addition of palmitic acid and three strains were inhibited.

Table 1. *Fatty acids and other compounds not supporting growth of human and bovine lipophilic corynebacteria*

Compounds tested at 10^{-4} M in basal lipid-free medium.

Saturated compounds:
Ethanol, *n*-butanol, ethylacetate, glycerol, monoacetin, triacetin, testosterone propionate
Acids:
acetic (C_1), proprionic (C_2), *n*-butyric (etc), valeric, caproic, caprylic, capric, lauric, myristic, palmitic, margaric, stearic, arachidic, nonadecanoic, behenic, pyruvic.
Sebacic. Lactic acid. Poly-β-hydroxyl butyric
Unsaturated acids: Crotonic, undecylenic, nervonic, ricinoleic

Table 2. *Utilization of unsaturated fatty acids and lecithin by human and bovine lipophilic corynebacteria*

	Response of 39 human strains			Response of eight *Corynebactrium bovis* strains†		
Compound	Positive	Weak positive	Negative	Positive	Weak positive	Negative
fatty acids						
cis oleic $C_{18:1}$	32*	5	2	2	3	3
cis linoleic $C_{18:2}$	7	17	15	0	1	7
cis linolenic $C_{18:3}$	0	3	36	0	0	8
iso oleic $C_{18:1}$ (vaccenic)	25	13	1	2	0	6
trans oleic $C_{18:1}$ (elaidic)	34	3	2	2	0	6
Erucic $C_{22:1}$	27	12	0	3	2	3
Palmitoleic $C_{16:1}$	8	9	22	0	0	8
Lecithin	29	8	2	0	1	7

* Number of strains growing of total tested.

† American Type Culture Collection, ATCC 7715; National Institute for Research in Dairying, NIRD 37, 65, 66, 112, 126, 128.

Table 3. *Inhibitory effect of certain fatty acids on the growth of human lipophilic corynebacteria*

Tween 80 used at a concentration of 0.05 % and fatty acids at 10^{-4} M.

Supplements added to basal medium	No. of strains tested	No. of strains inhibited
Tween 80 + linoleic acid	36	6
Tween 80 + linolenic acid	36	5
Tween 80 + ricinoleic acid	29	2
Oleic acid + linoleic acid	16	0
Oleic acid + linolenic acid	16	0
Oleic acid + ricinoleic acid	24	1

DISCUSSION

The 39 human cutaneous lipophilic corynebacterial (LC) strains examined represented groups II to VII of the scheme proposed by Smith (1969*a*). There were no observed relationships between the fatty requirements of the strains and their tentative grouping, which were based on fermentation reactions. Considering the array of compounds tested, the 39 strains fall into a relatively narrow range in terms of the fatty acids required to initiate growth. Long chain fatty acids, particularly C_{18} oleic acids, are essential. *Trans*, *cis* or *iso* oleic acid supported growth but ricinoleic acid did not. This may reflect the rôle of specific configurational changes in fatty acid utilization. Erucic acid $C_{22:1}$ supported growth but nervonic acid $C_{24:1}$ was not effective. Increased desaturation, as in linoleic and linolenic acids, resulted in decreased utilization by the majority of the human strains but few of the strains were inhibited. In the presence of oleic acid, compounds such as acetic, lactic and propionic acid did not enhance growth but longer chain acids such as palmitic acid did enhance growth. Many Gram-positive bacteria are inhibited by the *cis* form of linoleic or linolenic acid (O'Leary, 1962). Pillsbury & Rebell (1952) observed that cutaneous diphtheroids were inhibited by some fatty acids but in their tests, these authors used paper discs soaked with 10% solutions of the fatty acids; such a concentration is not physiological.

The samples of vaccenic, elaidic and erucic acids used here were only 95% pure but at the concentration used, impurities in the form of other fatty acids would not have exceeded 1·8 μg./ml.; at this concentration, oleic acid alone did not support growth of the LC strains. The lipid-deficient basal broth may have been depleted of other metabolites which limited growth but with this medium it was shown that certain fatty acids were needed to initiate growth.

Some investigators recognize that various human LC stains resemble *Corynebacterium bovis* (Marples, 1969). Characterizations of human and bovine LC bacteria have shown numerous biochemical, physiological and nutritional differences between the two groups (Smith, 1969*a*; Jayne-Williams & Skerman, 1966). Skerman & Jayne-Williams (1966) reported that vaccenic, erucic, palmitoleic, ricinoleic and oleic acids supported the growth of *C. bovis* to various degrees. The failure of six of the eight *C. bovis* strains to respond in a way similar to the human LC stains may provide further evidence to distinguish between LC strains of human and animal origin.

The rôle of fatty acid utilization in the ecology of human cutaneous bacteria is a perplexing problem. Sarkany & Gaylarde (1968) showed the localization of sebum to have characteristic patterns on skin. This could limit the distribution on skin of obligate lipophilic diphtheroids. Moreover, freshly collected sebum from intact sebaceous glands does not contain free fatty acids (Kellum, 1967) although some lipolytic strains of *Corynebacterium acnes* living in the pilosebaceous follicles or ducts liberate the fatty acids found in sebum (Reisner, Silver, Puhvel & Sternberg, 1968). The LC organisms are highly esterolytic but do not appear to contain true extracellular lipases (Smith, 1969*a*). In this respect, they appear to be similar to leptospires that are lipase-negative and require long-chain fatty acids (Johnson & Harris, 1968). Jayne-Williams & Skerman (1966) reported *C. bovis* strains to be lipolytic. More detailed studies of lipophilic-lipolytic relationships among the Corynebacteria are required.

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Chromosomal Location of Host Specificity in *Salmonella typhimurium*

By C. COLSON, ANNE M. COLSON AND ALINE VAN PEL
Laboratoire de Cytogénétique, Institut Carnoy, Université de Louvain, 24 Vaartstraat, Louvain, Belgium

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SUMMARY

The chromosomal location of the genes for host specificity in *Salmonella typhimurium* has been investigated by F-mediated conjugation using host specificity mutants isolated previously. It was found that the sites of mutations leading to two distinct phenotypes, $r^-_{LT}m^+_{LT}$ and $r^-_{LT}m^-_{LT}$, are closely linked to each other and are located near the marker *proC*.

INTRODUCTION

We have recently isolated a series of mutants of *Salmonella typhimurium* which are defective in a system of host-controlled restriction and modification that controls the acceptance of foreign phage P22 DNA and bacterial DNA (Colson, Colson & van Pel, 1969). These mutants closely resemble those isolated in *Escherichia coli* K and B in which recombination and complementation studies have shown that the functions of restriction and modification are governed by a cluster of at least three genes situated at about one time unit to the left of the marker *thr* (Colson, Glover, Symonds & Stacey, 1965; Wood, 1966; Glover, 1968; Arber & Linn, 1969; Boyer & Roulland-Dussoix, 1969; Glover & Colson, 1969). In view of the extensive homology observed between the genetic maps of *E. coli* and *S. typhimurium* (Sanderson & Demerec, 1965), one may consider *a priori* that this locus is a good candidate for the chromosomal location of the host specificity genes in *S. typhimurium*. On the other hand, the loss of the genetic determinants for host specificity in *S. typhimurium* was first observed in *lac*$^+$ 'hybrids' obtained from conjugation experiments between a Hfr *E. coli* and *S. typhimurium* LT7 *mut* (harbouring a mutator gene). Therefore, it was thought that these genetic determinants were lost by exchange of genetic material with *E. coli* in the *pro-lac* region of the map (Zinder, 1960). However, recent experiments show that crosses between *E. coli* Hfr and *S. typhimurium* tend to select host specificity deficient mutants in the recipient strain (Colson & Colson, 1967; Okada, Watanabe & Miyake, 1968).

The experiments presented in this paper define the map position of the genes for host specificity in *Salmonella typhimurium* by means of bacterial conjugation with host specificity mutants isolated in F$^-$ and Hfr strains.

METHODS

Bacteria. The bacterial strains used in this study are listed in Table 1, and the map position of the relevant markers is shown in Fig. 1.

Media. The media have been described previously (Colson, Colson & van Pel, 1969).

Scoring of host specificity phenotype. Restriction and modification were scored with a *c2* mutant of phage P22. The restriction test was performed by inoculating small broth cultures of the strains in soft agar overlays on plates and then adding drops of

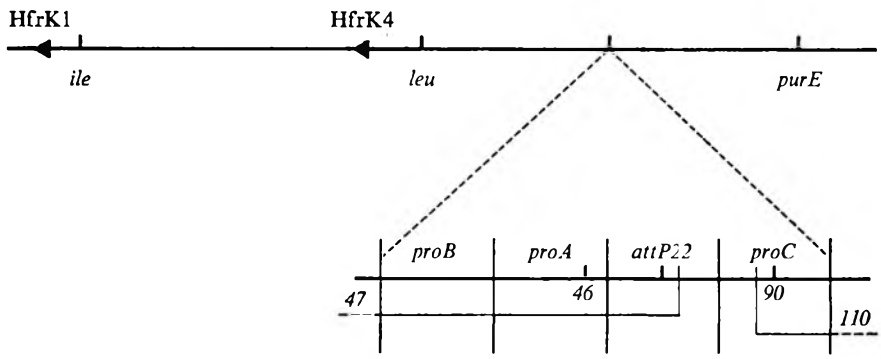

Fig. 1. *Partial linkage map of Salmonella typhimurium.* The point of origin and the direction of transfer by HfrK 1 and HfrK 4 are indicated by arrows. The details of the *pro* region are from Itakawa & Demerec, 1968. *proA 46* and *proC 90* are point mutations, *proC 110* is a deletion covering most of *proC*, *proAB 47* is a deletion extending across *proA*, *proB*, and the attachment site of phage *P22* (*attP* 22). The orientation of the whole *pro* region with respect to outside markers is not certain (Smith, 1968).

Table 1. *Bacterial strains*

Symbol	Genotype*	Host specificity mutations†	Source
SA 534 HfrK 4	*serA 13*	*hsr-51* *hss-53* *hss-91*	K. E. Sanderson
SA 464 HfrK 1	*serA 13*	—	K. E. Sanderson
SA 195	*proAB 47 purE 66*	*hsr-113* *hss-8* *hss-617*	K. E. Sanderson
SB 106	*ile-405 rha-461 leu-1003 proA-46 purC7 str-r*	*hsr-37*	P. E. Hartman
—	*proC 90*	*hsr-185* *hss-4* *hss-16* *hss-64* *hss-800*	H. O. Smith
—	*proC 110*	*hss-6* *hss-20*	H. O. Smith

* The map position of the markers used in this study are indicated in Fig. 1.

† The gene symbols used for host specificity are in agreement with the terminology of Arber & Linn, 1969. Mutants which have lost the capacity to restrict foreign DNA while retaining the capacity to modify it specifically (phenotype $r_{LT}^{-}m_{LT}^{+}$) are designated *hsr*. Mutants which have lost both the capacities to restrict and to modify DNA as the result of a single mutation (phenotype $r_{LT}^{-}m_{LT}^{-}$) are designated *hss*. The isolation and the phenotypic characterization of these mutants have been described previously (Colson, Colson & van Pel, 1969).

serially diluted suspensions of P 22 *c 2*.LT (having the LT modification) and P 22 *c 2*.0 (lacking the LT modification). After incubation, strains able to restrict (r^+_{LT}) show 10^4 less plaques of P 22 c *2*.0 than of P 22 *c 2*.LT, while strains unable to restrict (r^-_{LT}) accept both phages with the same efficiency. For scoring large numbers of recombinants a quicker method was utilized. Single colonies of the restreaked recombinants were resuspended in drops of buffer and cross-streaked on nutrient agar against streaks of suspensions containing 10^5 plaque-forming units/ml. of P 22 *c 2*.LT and P 22 *c 2*.0. After incubation, r^-_{LT} strains show confluent lysis at both phage streaks while r^+_{LT} strains are lysed only by P 22 *c 2*.LT. The modification test was performed by adding

Table 2. *Linkage between ile, leu, pro, and hsr in the cross: HfrK 1 × SB 106 ile leu proA hsr-37 str-r*

Exponentially growing cultures of the donor and recipient strains were mixed in equal volumes, centrifuged and kept at 37° for 60 min. without resuspending the pellets. The mixture was resuspended, diluted and plated on selective minimal media containing 100 μg./ml. of streptomycin. The recombinants were purified by single colony isolation on the same media and then tested individually for unselected markers.

Selected marker	Unselected markers (%)				No. of colonies tested
	ile$^+$	*leu*$^+$	*pro*$^+$	*hsr*$^+$	
ile$^+$	—	6	4	3	97
leu$^+$	41	—	35	32	99
pro$^+$	41	49	—	63	99

Table 3. *Inheritance of hsr$^+$ from HfrK 1 among various classes of recombinants in the leu-pro-purE region*

Same procedure as in Table 2, except that the purified recombinants were first classified with respect to the inheritance of unselected nutritional markers before being tested for their restriction phenotype.

Recipient strains	Classes of recombinants	*hsr*$^+$ (%)	No. of colonies tested
SB 106 *hsr-37*	*leu*$^+$ *pro*$^+$	56	83
ile leu proA str-r	*leu pro*$^+$	66	50
	leu$^+$ *pro*	23	64
SA 195 *hsr-113*	*pro*$^+$ *pur*$^+$	97	99
proAB purE str-r	*pro*$^+$ *pur*	88	75
	pro pur$^+$	< 2	46

drops of serially diluted suspensions of P22*c2* grown on the strain to be tested to plates seeded with soft agar overlays of known r^+_{LT} and r^-_{LT} strains. After incubation, an equal number of plaques is observed on both indicators if the phage carried the modification, indicating that the test strain was m^+_{LT}. On the contrary, the phage from a m^-_{LT} strain form 10^4 less plaques on the r^+_{LT} indicator than on the r^-_{LT}. For the scoring of a large number of recombinants, this test was simplified by using drops of buffer instead of tubes and calibrated loops instead of pipettes for diluting and spotting the phage suspensions.

RESULTS

Mapping the hsr mutation. About 100 *ile*$^+$, *leu*$^+$ and *proA*$^+$ recombinants were selected independently from a cross between HfrK 1 and the multiple auxotroph SB 106 recipient strain carrying a presumed *hsr* mutation (phenotype $r^-_{LT}m^+_{LT}$). These recombinants were analysed for the inheritance of unselected donor markers including the wild-type host specificity marker. The results show that *hsr*$^+$ is inherited together with *pro*$^+$ (63%) while its linkage to *leu*$^+$ (32%) and *ile*$^+$ (3%) is almost the same as that of *pro*$^+$ to those markers (Table 2). The close linkage of the *hsr* gene to the *pro* region was confirmed by the results of two other crosses performed using HfrK 1. In the first cross with SB 106 *hsr*, *leu*$^+$ and *proA*$^+$ recombinants were selected while *proAB*$^+$ and *purE*$^+$ recombinants were selected in the second cross to SA 195 *hsr*. From the first cross *leu*$^+$ *pro*$^+$, *leu pro*$^+$ and *leu*$^+$ *pro* recombinants were obtained and the inheritance of *hsr*$^+$ was scored among each class of recombinants. Similarly in the second cross *pro*$^+$ *pur*$^+$, *pro*$^+$ *pur* and *pro pur*$^+$ recombinants were obtained and the inheritance of *hsr*$^+$ was scored among each class. In both crosses whenever *pro*$^+$ was inherited by the recombinant class the inheritance of *hsr*$^+$ was high. When selection was made against the inheritance of *pro*$^+$ the inheritance of *hsr*$^+$ was low (Table 3).

In an attempt to locate the site of *hsr* mutations more precisely with respect to the *pro* cistrons, two interrupted mating experiments were conducted using HfrK 4 and selecting *proA*$^+$ recombinants in one cross while selecting *proC*$^+$ recombinants in the other. Both recipient strains were *hsr* mutants and the inheritance of *hsr*$^+$ was scored among *pro*$^+$ recombinants selected at 2 min. intervals. The percentage of *hsr*$^+$ among *proA*$^+$ increased with the time allowed for transfer, rising to 88% at a blending time of 36 min., while the percentage of *hsr*$^+$ among *proC*$^+$ was at the maximum level even at the earliest time of blending (Table 4). This means that when *hsr*$^+$ is transferred by

Table 4. *Inheritance of pro*$^+$ *and hsr*$^+$ *in interrupted mating experiments with HfrK 4*

1 ml. samples of the exponentially growing recipient strains were mixed with equal volumes of 1/10 dilutions of exponentially growing cultures of the donor and the cells were immediately collected by filtration on a millipore filter. The filters were kept for 10 min. on the surface of a soft nutrient agar plate at 37°. The filters were then transferred to large flasks containing 20 ml. of prewarmed broth and gently agitated in a shaking water bath at 37°. At intervals, a 1 ml. sample was diluted into 9 ml. of buffer, shaken vigorously for 30 sec. Samples of 0·1 or 0·5 ml. of the blended mixtures were added to 3 ml. of melted soft agar containing 100 μg./ml. of streptomycin and immediately poured over the surface of the selective media containing the same concentration of streptomycin. About 50 *pro*$^+$ recombinants from each sample were purified and scored for their host specificity phenotype.

	Recipient strains			
	SB 106 *proA 46 hsr-37*		*proC 90 hsr-185*	
Time of blending (min.)	No. of recombinants/ml.	*hsr*$^+$(%)	No. of recombinants/ml.	*hsr*$^+$(%)
24	$1{\cdot}3 \times 10^3$	56	$5{\cdot}8 \times 10^3$	90
26	$4{\cdot}3 \times 10^3$	56	$8{\cdot}8 \times 10^3$	>98
28	$8{\cdot}0 \times 10^3$	60	$2{\cdot}1 \times 10^4$	90
30	$1{\cdot}5 \times 10^4$	81	$3{\cdot}4 \times 10^4$	>98
32	$2{\cdot}0 \times 10^4$	62	$6{\cdot}3 \times 10^4$	98
34	$6{\cdot}5 \times 10^4$	78	$1{\cdot}1 \times 10^5$	92
36	$6{\cdot}4 \times 10^4$	88	$1{\cdot}9 \times 10^5$	98

HfrK 4 it enters the recipient cell after *proA*$^+$ and either before *proC*$^+$ or so closely after it that it cannot be separated from it by blending. Thus *hsr* maps either between *proA* and *proC* or just to the right of *proC*. In addition, this experiment suggests that the orientation of the *pro* region is: *leu-proB proA proC-purE*, since it is the only orientation compatible with the fact that *hsr* is closer to *proC* than to *proA*, while distal to *proA* when transferred by an Hfr injecting its chromosome in the order *leu-pro-purE*.

Linkage between hsr and hss mutations. The experiments presented in the previous section involved *hsr* mutants only. No conclusion can therefore be drawn from them about the chromosomal location of the presumed *hss* mutation leading to the $r^-_{LT}m^-_{LT}$ phenotype, although by analogy, one might expect to observe clustering of the genes

Table 5. *Host specificity among pro*$^+$ *recombinants in crosses between hss and hsr mutants*

The *pro*$^+$ recombinants were obtained by the method described in Table 2 and scored for their restriction and modification phenotype.

Expt. no.	Donors	Recipients	No. of recombinants with genotype			Donor type (%)
			hss$^+$*hsr*$^+$	*hsr*	*hss*	
1	HfrK 4 *hss-53*	*proC 90 hsr-185*	2	5	193	96
		proAB 47 hsr-113	0	8	192	96
2	HfrK 4 *hss-91*	*proC 90 hsr-185*	18	49	344	83
		proAB 47 hsr-113	1	14	83	85
3	HfrK 4 *hsr-51*	*proC 110 hss-6*	0	35	13	73
		proC 90 hss-4	0	48	2	96
		proC 90 hss-16	0	24	12	66
		proC 90 hss-64	0	38	10	79

for host specificity in *Salmonella typhimurium* similar to that found in *Escherichia coli*. To locate the *hss* mutation *proC*$^+$ and *proAB*$^+$ recombinants were selected in crosses between *hss* mutants of HfrK 4 and *hsr* mutants of SA 195 and *proC 90*. In each cross more than 80% of the *pro*$^+$ recombinants had inherited the donor $r^-_{LT}m^-_{LT}$ phenotype (Expts. 1 and 2, Table 5). Similarly, in crosses between an *hsr* donor and *hss* recipients most *pro*$^+$ recombinants expressed the $r^-_{LT}m^+_{LT}$ phenotype of the donor (Expt. 3, Table 5). This shows that both *hsr* and *hss* mutations are closely linked to *pro*. Moreover, the small number of recombinants expressing wild-type host specificity (3% or less) indicates that the sites of the *hsr* and *hss* mutations are closely linked since recombination between them to restore wild-type host specificity is a rare event (Table 5).

Crosses were also performed between an Hfr expressing wild-type host specificity and *hss pro* recipients. Surprisingly no inheritance of wild-type host specificity among *pro*$^+$ recombinants was observed for most of them (Table 6). Some *hss*$^+$ recombinants were found in only two cases (*hss-6* and *hss-4*) and at frequencies of 11 and 78% respectively. These results may reflect the existence of several mutational sites leading to the $r^-_{LT}m^-_{LT}$ phenotype scattered along the chromosome and in most cases not transferred at a measurable frequency from HfrK4. Nevertheless, the following observations render this hypothesis unlikely. First, on repetition of the crosses in which a measurable linkage between *hss* and *pro* had been observed, the values

obtained proved to be very variable (less than 2% instead of 11% for *hss-6* and 8% instead of 78% for *hss-4*, see Table 6). This indicates that these linkage values cannot be regarded as a true reflexion of map distances. Second, four *hss* recipient strains in which the linkage between *hss* and *pro* was variable or absent were crossed with a *hsr* Hfr. All of them inherited the donor host specificity phenotype at a high frequency among *pro*$^+$ recombinants indicating that the *hss* mutation they carry is closely linked to the *hsr* mutation (see Table 5).

Table 6. *Inheritance of hss*$^+$ *from HfrK 4 among pro*$^+$ *recombinants*

Same procedure as in Table 2; for each cross the results of two independent experiments are listed.

Recipient strains	No. of *hss*$^+$	No. of colonies tested
proC 110 hss-6	12	104
	0	50
proC 110 hss-20	0	24
proC 90 hss-4	78	100
	4	50
proC 90 hss-16	0	124
	0	50
proC 90 hss-64	0	24
	0	50
proC 90 hss-800	0	104
	0	50
proAB 47 hss-617	0	24
	0	50
proAB 47 hss-8	0	32

DISCUSSION

Mapping experiments using F-mediated conjugation in *Salmonella typhimurium* have shown that the sites of the *hsr* and *hss* mutations of the LT host specificity system are very close to each other and map in the vicinity of the marker *proC*. It must be pointed out that the use of the gene symbols *hss* and *hsr* was dictated only by the close phenotypic resemblance of the mutants in the LT system to those of *Escherichia coli* K and B, and that the experiments presented here give no information about the number and the functions of the genes. However, our experiments enhance the similarity between the *S. typhimurium* LT and the *E. coli* systems in showing that mutations leading to distinct phenotypes are clustered. Crosses between mutants have yielded recombinants between *hss* and *hsr* at a higher frequency than between similar mutants in *E. coli* (Lederberg, 1966; Glover & Colson, 1969). It is thus possible that the genes for the LT host specificity are less clustered than in *E. coli* or, perhaps more likely, it reflects the better resolving power of recombination analysis by conjugation in *S. typhimurium* which results from the lower rate of DNA transfer by *S. typhimurium* donors (Sanderson & Demerec, 1965).

On the other hand, our experiments show some differences between *Salmonella typhimurium* LT host specificity and host specificity in *Escherichia coli*. The almost complete absence of recombinants expressing the donor type host specificity in crosses between wild-type donors and *hss* recipients was not observed in similar crosses in

E. coli K and B. The explanation of this phenomenon remains obscure although the fact that it occurred *only* in crosses between wild-type donors and *hss* recipients suggests that this phenomenon may be related to the special situation in zygotes in which the resident DNA is not modified and which have freshly inherited a set of genes governing the ability to degrade this DNA.

The difference in map position between the LT host specificity genes and those of *Escherichia coli* suggests that this host specificity system may have a different origin. Moreover, experiments to be published have revealed that *Salmonella typhimurium* harbours another host specificity in addition to the LT system, the genetic determinants of which behave as allelic to those of the *E. coli* host specificities.

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The Nature of the Stimulation of Fungal Growth by Potato Extract

By R. E. BEEVER*
Botany Department, University of Auckland, Auckland, New Zealand
AND E. G. BOLLARD
Plant Diseases Division, DSIR, Auckland, New Zealand

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SUMMARY

Growth of four fungi on a medium of potato tuber extract and glucose was considerably higher than on a defined medium of glucose, asparagine, mineral salts, biotin and thiamine. Modification in turn of the carbon, nitrogen and growth factor components of the defined medium to resemble the extract medium did not indicate that any single component was responsible for growth stimulation; stimulation appeared to be due to the combined effects of all components. A synthetic medium of glucose, citric acid, amino acids, mineral salts and growth factors resembled the extract medium in composition and approached it in the ability to support growth of the test fungi.

INTRODUCTION

Marked responses to the inclusion of water extracts of potato tubers in growth media are obtained with many fungi, but little is known about the factors responsible. These responses are usually obtained despite differences in variety, maturity and conditions of growth of the tubers. Such extracts may supply both major and minor nutrients and different fungi may respond to different constituents or groups of constituents. In this study the effectiveness of a potato extract in stimulating growth of four plant pathogenic fungi was investigated.

METHODS

Organisms. Isolates of *Diplodia pinea* (Desm.) Kickx., *Mycosphaerella melonis* (Pass.) Chiu & Walker, *Peniophora sacrata* G. H. Cunn. and a *Rhizoctonia* sp. were used. The fungi did not sporulate under the experimental conditions.

Preparation of potato extract. A single bulk aqueous extract of potato tubers (*Solanum tuberosum* var. Ilam Hardy) was used in all the work reported. The extract was prepared, at a final concentration of 400 g. peeled tubers/l., as follows:

Tubers were peeled, minced into threequarters of the final quantity of water and boiled with constant stirring for 5 min. The mixture was frozen at −10° overnight and, after thawing, was filtered through 'Miracloth' (Chicopee Mills, Inc., New Jersey, U.S.A.) in a basket centrifuge. The filtrate was stored in plastic bottles at

* Present address: Department of Genetics, The University of Leeds, Leeds, LS2 9JT.

– 10°. When required, portions were thawed and filtered (Whatman no. 50 filter paper) to give a faintly opalescent extract.

Culture media. Compositions of the various media used are given in Table 1. Medium pH was adjusted to pH 6·4 (with 0·1 N-HCl or -KOH) before autoclaving (15 lb./in.^{2} for 30 min.). Autoclaving was chosen as the method of sterilization as it is normal practice to sterilize potato extract media in this way. Three flasks of the autoclaved medium were mixed and the pH measured (initial pH).

Table 1. *Composition of media*

Component and final concentration	Medium A	B	C	D	E
Glucose 10 g./l.	×	×	×	×	×
Citric acid 0·5 g./l.	—	—	×	—	—
L-Asparagine 1·06 g./l. (8 mM)	—	×	—	—	—
L-Asparagine 0·26 g./l. (2 mM)	—	—	—	×	—
KNO_3 2·32 g./l. (23 mM)	—	—	—	—	×
Amino acid mixture (1)	—	—	×	—	—
Inorganic salts (2)	—	×	×	×	×
Trace elements (3)	—	×	×	×	×
Thiamine (0·1 mg./l.)	—	×	×	×	—
Biotin (0·005 mg./l.)	—	×	—	×	—
Growth factor mixture (4)	—	—	×	—	—
Potato extract 500 ml./l.	×	—	—	—	—

(1) Amino acid mixture (mM concentration): L-Asn (2·75), L-Gln (1·75), 4-Aminobutyric acid (0·90), L-Arg (0·50), L-Val (0·50), L-Glu (0·40), L-Ala (0·25), L-Asp (0·25), L-Lys (0·25), L-Ile (0·20), L-Phe (0·20), L-Ser (0·15), Gly (0·10), L-Leu (0·10), L-Met (0·05), L-Pro (0·05), L-Thr (0·05).

(2) Inorganic salts (mM concentration): K_2HPO_4(1), K_2SO_4 (1), $CaSO_4$ (2), $MgSO_4$ (2).

(3) Trace elements (mg./l. final medium): H_3BO_4 (2·86), $MnCl_2.4H_2O$ (1·79), $ZnCl_2$ (0·10), $CuSO_4.5H_2O$ (0·08), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0·04). Iron was added as the EDTA complex to give 3 mg. Fe/l. final medium.

(4) Growth factor mixture (mg./l. final medium): *p*-Aminobenzoic acid—K salt, (0·1), D-biotin (0·005), *i*-inositol (10), nicotinic acid (0·1), pantothenic acid-Ca salt (0·1), pyridoxine HCl (0·1), riboflavin (0·1).

Growth of fungi. Inoculations, except for the experiment of Table 2, were made with one wire loopful (loop diam. 5 mm.) flask of a mycelial suspension prepared by homogenizing washed colonies in 10 ml. sterile glass-distilled water. For the experiment of Table 2 inoculum was pregrown on agar medium in Petri dishes and single plugs (diameter 2 mm.) were inoculated into each flask. The fungi were grown in static culture in the dark at 25° in 50 ml. Erlenmeyer flasks containing 10 ml. medium.

Fungi were harvested near the time of maximum dry weight yield on medium A (7 days for *Diplodia pinea*, *Mycosphaerella melonis* and *Rhizoctonia* sp.; 14 days for *Peniophora sacrata*) by filtering through preweighed glass gooch crucibles (porosity 3). The mycelium was washed three times with distilled water, dried at 105 ± 5° for 24 h and weighed. Yields are reported as the mean dry weight of mycelium/flask (three replicates). Filtrates from the three flasks were pooled and the pH measured (final pH).

Analytical methods. Unless otherwise stated analyses were carried out on autoclaved media. Total nitrogen was measured using a semi-micro Kjeldahl method without reduction before digestion because nitrate levels of the media analysed were low. Nitrate was measured by the method of Humphries (1956). Ammonium was estimated with ninhydrin (Yemm & Cocking, 1955) following microdiffusion (Conway, 1957).

Amino acids were estimated colorimetrically following separation by thin-layer electrophoresis and chromatography. The method used was essentially that of Bieleski & Turner (1966). Organic acids were separated by the method of Cook & Bieleski (1969) employing thin-layer electrophoresis and chromatography, except that sodium formate (0·05%, w/v) and bromophenol blue (0·015%, w/v) were added to the chromatographic solvent. Acids showed up as yellow spots on a blue background after drying in a cool air stream and were estimated approximately by visual comparison with standards.

Treatment of results. Means of yields have been compared using a form of the '*t*-test' (Bailey, 1959). All tables present data from single experiments.

RESULTS

The extent of the responses to potato extract shown by the text fungi was measured in a preliminary experiment where growth on a glucose/KNO_3 medium was contrasted with growth on the same medium to which potato extract had been added (Table 2).

Table 2 *Growth of test fungi on a glucose/KNO_3 medium with and without potato extract*

Growth medium was medium E. Potato extract was added at a rate of 500 ml./l. Inoculum pregrown on potato dextrose agar (medium A + agar 15 g./l.). Yields are in mg./flask.

	Without potato extract (initial pH 4·3)		With potato extract (initial pH 5·6)	
	Yield	Final pH	Yield	Final pH
D. pinea	6	7·9	44 (*s*)	7·5
M. melonis	31	8·2	46 (*s*)	6·3
P. sacrata	0	5·3	44 (*s*)	5·2
Rhizoctonia sp.	17	7·4	46 (*s*)	6·7

(*s*) Differs significantly ($P = 0·01$) from value without potato extract.

Following other preliminary work a standard potato extract medium (medium A) and a defined glucose/asparagine medium (medium B) were selected for further work. Asparagine, the sole nitrogen source in medium B, is known to be a major constituent of the soluble nitrogen fraction of potato tubers (Burton, 1966) and to be used widely as a sole nitrogen source by fungi. Thiamine and biotin were included in medium B as these are the two growth factors most commonly required by fungi (Cochrane, 1958). Growth on medium B was appreciable but for each fungus it was less than on medium A (Table 3). This difference in response to the two media was found to be independent of whether the inoculum was pregrown on a defined medium (medium D) or on potato extract medium (medium A).

Further work involved chemical estimations of certain components of potato extract, followed by attempts to modify the defined medium, medium B, so that it resembled the extract medium, medium A, more closely both in composition and in ability to support the growth of the test organisms.

Carbon. The major carbon source in media A and B was the added glucose. Growth of the test fungi on a potato extract medium without added glucose was in all cases less than 25% of that with glucose. Further carbon compounds probably present in the

extract medium include other sugars, probably some starch, amino acids and organic acids. Amino acids present are described later. Four organic acids were identified in the extract medium, medium A: citric acid (0·5 to 1·0 g./l.), 2-pyrrolidone-5-carboxylic

Table 3. *Growth of test fungi on a potato extract medium (medium A) and a glucose/asparagine medium (medium B)*

Inoculum pregrown for 12 days on medium D. Yields in mg./flask.

	Medium A (initial pH 5·4)		Medium B (initial pH 5·9)	
	Yield	Final pH	Yield	Final pH
D. pinea	66	8·3	45 (*s*)	7·4
M. melonis	60	8·1	43 (*s*)	6·5
P. sacrata	81	5·8	50 (*s*)	5·4
Rhizoctonia sp.	79	8·2	63 (*s*)	4·4

(*s*) Differs significantly ($P = 0{\cdot}01$) from value for Medium A.

Table 4. *Growth of test fungi on a glucose/asparagine medium with and without citric acid*

Growth medium was medium B. Citric acid was added at 1·0 g./l. Inoculum pregrown for 10 days on medium D. Yields are in mg./flask.

	Without citric acid (initial pH 5·3)		With citric acid (initial pH 6·0)	
	Yield	Final pH	Yield	Final pH
D. pinea	35	7·8	45 (*s*)	8·0
M. melonis	44	7·8	50 (*s*)	9·1
P. sacrata	50	6·3	57 (*ns*)	7·1
Rhizoctonia sp.	69	4·8	69 (*ns*)	8·4

(*s*) Differs significantly ($P = 0{\cdot}01$) from value for the left-hand treatment.
(*ns*) Not significantly different.

acid (0·25 to 0·5 g./l.), malic acid (trace) and succinic acid (trace). The 2-pyrrolidone-5-carboxylic acid is probably largely produced from glutamine during autoclaving. Citric acid (1·0 g./l.) stimulated growth of *Diplodia pinea* and *Mycosphaerella melonis* when added to medium B (Table 4). Added at this level citric acid represents about 7·5% of the total carbon available in the medium. This alternative form of carbon may account for some of the observed stimulation but an indirect effect of citric acid on availability of metal ions or in influencing pH changes may also be important.

Nitrogen. Estimation of nitrogen components accounted for about 75% of the total nitrogen in the extract medium (Table 5). The only significant change apparent on autoclaving was the disappearence of glutamine presumably due to its conversion to ammonium and 2-pyrrolidone-5-carboxylic acid. Asparagine was present in medium B at 8 mM (224 mg. N/l.). As the nitrogen in the extract medium (medium A) was 247 mg. N/l. the total nitrogen levels of both media were essentially the same. A number of reports, however, suggest that a mixture of amino acids may be a better nitrogen source for fungi than single amino acids (Nicholas, 1965; Harris, 1958). A mixture of amino acids of similar composition to those in the extract medium before autoclaving (note 1, Table 1) was therefore substituted for asparagine in medium B. The yields of the test fungi were, however, not significantly different from those on asparagine

although the final pH values of the medium were all much less than that of the asparagine medium.

Mineral salts. Analysis of the mineral salts in the potato extract was not attempted. From analyses of potato tuber ash (Burton, 1966) and potato dextrose agar (Miller, 1956) an estimate of the likely inorganic composition of medium A was made. Comparison of this with the mineral salts present in medium B suggested that differences were small and unlikely to be of significance for fungal growth. Although the extract medium (medium A) almost certainly contained elements not present in medium B none of these has been shown to be required by more than one or two fungi.

Table 5. *Nitrogen compounds in a potato extract medium (medium A)*

Analyses are means of at least two determinations (tr means less than 1 mg. N/l.).

Compound	Quantity in medium (mg. N/l.)	
	Non-autoclaved	Autoclaved
Nitrate	16	16
Ammonium	6	17
Asn	66	72
Arg	26	24
4-Aminobutyric acid	13	16
Lys	6	6
Glu	6	6
Val	7	6
Asp	3	5
Ala	3	4
Gln	49	3
Ser	2	2
Phe	2	2
Ile	3	2
Thr	tr	tr
Pro	tr	tr
Met	tr	tr
Tyr	tr	tr
Leu	tr	tr
Gly	tr	tr
Total of above components	205	181
Total nitrogen		247

Growth factors. The growth factors in the extract medium were not measured. However, from published analyses (Burton, 1966) extracts from potato tubers may be expected to contain all the main fungal growth factors. The response of the test fungi, especially *Peniophora sacrata*, to added thiamine when grown on defined media (Table 6) confirmed that thiamine was probably present in the potato extract. By similar reasoning Milton & Isaac (1967) concluded that biotin was present in potato extract.

To test whether other growth factors probably present in potato extract could stimulate fungal growth, a mixture of the commonly required growth factors (note 4, Table 1) was added to medium B at levels considered optimal for most deficient fungi (Cochrane, 1958). Despite the instability of some of these growth factors the medium was autoclaved as the growth response being studied was obtained with autoclaved media. None of the fungi showed any significant response. The independence of pregrowth conditions of the growth responses of media A or B supports the conclusion that no growth factor other than thiamine was involved in the responses of the test fungi.

A defined medium resembling potato extract medium. On the basis of the modifications described above a synthetic medium (medium C) was designed as a defined substitute for potato extract medium (medium A). Growth of three of the test fungi on medium C was not significantly different from growth on medium A (Table 7). Growth of other fungus, *Rhizoctonia* sp., was significantly less. All fungi showed better growth on medium C than on medium B.

Table 6. *Growth of test fungi on a glucose/asparagine medium with and without thiamine*

Growth medium was medium B. Inoculum pregrown for 11 days on medium D minus thiamine and biotin. Yields are in mg./flask.

	Without thiamine (initial pH 5·2)		With thiamine (0·1 mg./l.) (initial pH 5·3)	
	Yield	Final pH	Yield	Final pH
D. pinea	25	7·3	38 (*ns*)	7·3
M. melonis	49	6·1	47 (*ns*)	5·3
P. sacrata	0	3·9	35 (*s*)	5·4
Rhizoctonia sp.	21	—	66 (*s*)	4·3

(*ns*), (*s*)—See Table 4 footnote.

Table 7. *Growth of test fungi on a potato extract medium (medium A), a glucose/asparagine medium (medium B) and a defined substitute for potato extract medium (medium C)*

Growth media as described in Table 1. Inoculum pregrown 14 days on medium A. Yields are in mg./flask.

	Medium A (initial pH 5·8)		Medium B (initial pH 5·3)		Medium C (initial pH 5·9)	
	Yield	Final pH	Yield	Final pH	Yield	Final pH
D. pinea	60	7·3	38 (*s*)	7·3	54 (*ns*)	7·3
M. melonis	57	7·7	46 (*s*)	5·6	50 (*ns*)	6·8
P. sacrata	86	5·6	46 (*s*)	5·5	82 (*ns*)	5·5
Rhizoctonia sp.	78	7·8	59 (*s*)	6·2	64 (*s*)	7·4

(*ns*), (*s*)—See Table 4 footnote.

DISCUSSION

Growth of the four test fungi on potato extract and glucose (medium A) showed that potato extract supplies suitable sources and quantities of nitrogen, mineral salts, and growth factors. The good growth of fungi frequently observed on potato dextrose agar (medium A with agar) suggests that such stimulation is widespread.

Although the test fungi grew on the defined glucose/asparagine medium (medium B), in no case was growth as good as that on medium A. The experiments, where components of medium B were modified in turn to resemble the extract medium, indicated that this growth difference was not in general due to differences in carbon, nitrogen or growth factor sources. When, however, growth was tested on a medium incorporating such modifications simultaneously (medium C) all test fungi were found to grow better than on medium B and in three cases growth was not different from that on medium A. This suggests that there was a synergistic effect on growth between components of medium C. It would be interesting to compare growth of a wider range

of fungi on this defined medium. Medium C resembles medium A chemically in the major carbon source (glucose,) in the presence of citric acid, in the organic nitrogen constituents and in the growth factors present. Also the pH changes in this medium during fungal growth were similar to those in medium A. Growth yields on medium C suggest that above a certain level the effect of potato extract in stimulating growth lies in a combination of a number of factors, which may be different for different fungi, rather than in any single factor.

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SHORT COMMUNICATIONS

Some Effects of Light on the Vegetative Growth of *Pilaira anomala*

By H. J. FLETCHER

School of Pharmacy, Robert Gordon's Institute of Technology, Aberdeen, AB9 1FR

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Pilaira anomala exhibits a number of light-dependent phenomena (Fletcher, 1969*a*, *b*). There appear to have been no previous studies on its vegetative growth. Growth has been shown to be retarded by light in the related organisms Pilobolus (Page, 1952) and Phycomyces (Carlile, 1962). This paper describes the effect of light on its growth and the production of a vegetative 'growth ring', a phenomenon of limited occurrence in fungi (Jerebzoff, 1965). Such a ring was described by Stevens & Hall (1909) in *Ascochyta chrysanthemi* but this can now be interpreted as due to an endogenous rhythm and not directly related to environmental influence.

Pilaira anomala (I.M.I. 109387) was isolated from deer dung and maintained on 'Oxoid' Sabouraud Maltose Agar. Experimental cultures were grown on a synthetic basal medium containing glucose (10 g./l.) and salts (Page, 1952). Agar media contained in addition an amino-acid mixture (Fletcher, 1969*b*), yeast extract (Difco) at either 0·05 or 10·0 g./l. and 'Oxoid Ionagar' (12 g./l.). Liquid medium contained, in addition to the basal medium, vitamin-free Difco-Casamino acids (5·0 g./l.) and thiamine (1·0 μg./ml.). Plastic Petri dishes (90 mm.) containing 15 ml. of agar medium were used. Liquid cultures were grown in 100 ml. 'Monax' rimless Erlenmeyer flasks containing 20 ml. of medium. The inoculum for agar was a 3 mm. diam. disc of agar bearing mycelium cut from the margin of a young colony and for liquid cultures was a dense filtered spore suspension. Four replicates were used in each experiment. Cultures were incubated at 25° either in complete darkness or in an illuminated incubator fitted with an 'Atlas' miniature 13 W fluorescent tube. The light was filtered through a 1% $CuSO_4$ solution to reduce infrared radiation (Trinci & Banbury, 1969), and was at 750 lx at the level of the Petri dishes. Measurements of radial expansion were made on two diameters of each colony at right angles. Cultures maintained in darkness were examined under red photographic safelight (Kodak Wratten no. 1). Liquid cultures were harvested after 6 days into perforated 20 ml. 'Oxoid' aluminium caps lined with filter paper discs, and dried overnight at 105°.

To determine the effects of exposure to continuous light, agar cultures were grown in continuous light (controls in continuous darkness) and radial expansion was measured at intervals for 3 days. The rate of growth with 0·05 g. yeast extract/l. (colourless) was 0·7 mm./hr. in light and 1·1 mm./hr in darkness. With 1·0 g. yeast extract/l. (coloured brown) it was 1·2 mm./hr in light and darkness. With 0·05 g. yeast extract/l., marginal staling became marked in the light and growth ceased before the colony reached the edge of the plate. In static liquid cultures the dry weights obtained from a colourless medium were $163 \pm 2{\cdot}4$ mg./100 ml. medium in darkness but only $123 \pm 0{\cdot}6$ mg./100 ml. medium in light.

The retardation of Pilaira on colourless but not on coloured media is similar to that of Pilobolus on synthetic agar but not on dung agar (Page, 1952). However, Page (1965) cited Willoughby as having shown that dung did not exercise a mere shading effect. The medium containing only 0·05 g. yeast extract/l. might be nutritionally suboptimal for Pilaira, leading to the depression of growth in the light (Carlile, 1965).

To investigate further the retarding effect of light on 0·05 g. yeast extract/l., cultures incubated in darkness for 36 hr were exposed to light for 1 hr and then returned to darkness, while reciprocal transfers were made from light to darkness for 1 hr, followed by return to light. The most conspicuous effect of the transfer from darkness to light and back to darkness, was the production of a vegetative growth ring, the inner limit of which was the diameter reached by the outer edge of the colony margin at the moment of exposure to light (Pl. 1, fig. 1). Growth rate in darkness declined following 1 hr exposure to light. A growth ring was not formed as a result of light–darkness–light transfer.

Photomicrographs were made of the margin of the same colony before (Pl. 1, fig. 2) and 1 hr after (Pl. 1, fig. 3) exposure to light. The marginal zone in darkness was a region of sparse hyphae where only the minor branches of the main radial hyphae overlap. Behind this zone there was a much denser felt of hyphae. Exposure to light changed the spatial distribution of the hyphae in the marginal zone. The number of lateral branches was reduced and open spaces appeared between the radial hyphae, which grew erratically. These phenomena combined to give the macroscopic appearance of the growth ring. The marginal zone was narrower after light exposure. The shape of the hyphal tips did not alter through exposure to light. This behaviour is morphologically similar to that observed by Stevens & Hall (1909) in Ascochyta.

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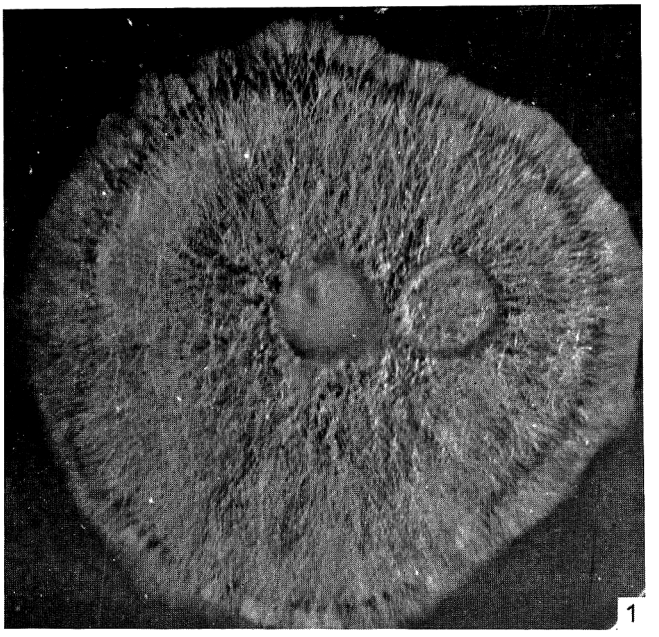
EXPLANATION OF PLATE

Fig. 1. Whole colony, showing macroscopic appearance of growth ring, × 2½.

Fig. 2. Marginal zone of colony grown in complete darkness, × 80.

Fig. 3. Marginal zone of colony after exposure to light for 1 hr, following return to darkness for a further 1 hr, × 80.



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Axenic Growth and Development of the Cellular Slime Mould, *Dictyostelium discoideum*

By M. SCHWALB* AND R. ROTH
Department of Biology, Brandeis University, Waltham, Massachusetts 02154, U.S.A.

(*Accepted for publication 21 October 1969*)

Dictyostelium discoideum has been used in numerous studies of development; it has been grown on living or dead bacteria (Gezelius, 1962) but Sussman & Sussman (1967) reported that it could be adapted to grow in a medium without bacteria or bacterial fractions. The use of *D. discoideum* in molecular studies of development has increased the need for an axenic medium for its growth. The present paper concerns simplification of the previous axenic medium, observations on growth kinetics and the kinetics of appearance and disappearance of a developmentally-controlled enzyme in axenically grown organisms.

METHODS

Axenic cultivation. Organisms were grown in 7 ml. of medium in 125 ml. Erlenmeyer flasks; when larger amounts were required, 20 ml. of medium in 125 ml. flasks were used. The medium previously reported (Sussman & Sussman, 1967) was adjusted to the following composition: 10 g. Proteose peptone (Difco) and 5 g. yeast extract (Difco) were dissolved in 650 ml. distilled water. Sixty-five ml. samples of this solution were autoclaved separately (121° for 20 min.). To each of the above samples was added 4 ml. of sterile glucose solution (27 g./100 ml.) and 1 ml. of sterile phosphate buffer (6·7 g. $Na_2HPO_4.7H_2O + 3{\cdot}4$ g. KH_2PO_4 in 100 ml. distilled water). Flasks were inoculated with organisms from a previous culture to 1 to 5×10^5/ml. and incubated on a reciprocating shaker (110 cycles/min.) at 22°.

Organisms were counted with a haemocytometer or a Coulter electronic counter. Dilutions for counting were made with a salt solution (NaCl 0·6 g., KCl 0·75 g., $CaCl_2$ 0·3 g./1000 ml. water; Bonner, 1947).

Cloning procedure. Axenically-grown organisms were cloned monthly (seven to eight transfers), by diluting organisms in axenic medium to an appropriate concentration, and plating them on SM/5 agar medium (Sussman & Lovgren, 1965) in association with *Klebsiella* (*Aerobacter*) *aerogenes*. This method was used since the organisms grew poorly on semisolid axenic medium and did not fruit on any medium which they could not deplete of nutrients. Spores from a clone which produced typical fruiting bodies were placed in a culture tube containing 5 ml. axenic medium + 500 μg. streptomycin sulphate (sterilized by filtration)/ml. These tubes were incubated without shaking at 22° for 2 to 4 days until microscopic examination revealed abundant growth of the amoebae. These were then transferred to 125 ml. flasks with 7 ml.

* Present address: Department of Microbiology, New Jersey College of Medicine and Dentistry, Newark, New Jersey 07107, U.S.A.

axenic medium and shaken. Growth rate was determined on the second passage. The cloning procedure prevented the accumulation of poorly-fruiting organisms which often become a significant proportion of the population after many passages without cloning.

Fruiting. Axenically-grown organisms were made to fruit synchronously by a modification of a technique already reported for cells grown with bacteria (Sussman & Lovgren, 1965). Late log phase organisms were collected by centrifugation, then washed once in axenic medium without glucose. The organisms were diluted in this solution to 5×10^7/ml. One-half ml. of this suspension was distributed on a membrane filter (Millipore) or on a 4·25 cm. filter paper disc (Whatman no. 50) supported by two filter pads containing 3·2 ml. pad diluting fluid in 0·04 M-phosphate buffer (pH 6·4) (Sussman, 1966). After 15 min. the filter with organisms was transferred to fresh pads and diluting fluid; this transfer removed most of the axenic medium. The filters were then incubated at 22° in a moist chamber.

Assays. UDP-galactose polysaccharide transferase was assayed by the method of Sussman & Osborn (1964) as modified by Yanagisawa, Loomis & Sussman, (1967). Organisms were broken by ultrasonic treatment. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Composition of the medium. It was soon evident that the foetal calf serum and liver concentrate present in the prevously reported medium (Sussman & Sussman, 1967) were not required for maximum growth rate or yield. Except for the increase in glucose concentration noted in METHODS, no short-term improvements were observed after adjusting the relative concentration of the constitutents or by using other peptones or sugars. Addition of powdered milk or lecithin, which were useful in the axenic cultivation of *Polysphondelium pallidum* (Sussman, 1963), did not aid *Dictyostelium discoideum*. The high concentration of buffer used previously was not necessary. Dialysis of the yeast extract and proteose peptone and reconstitution in differing proportions, adjustment of pH over the range 4·5 to 7·0, and addition of trace elements did not result in any improvement in growth.

Kinetics of growth. Fig. 1 shows the kinetics of growth of *Dictyostelium discoideum* in the present axenic medium. No changes in growth rate or yield have been observed over 1 year. The mean doubling time after 12 hr of growth was about 12 hr. During the first 12 hr after a transfer from late log phase, the doubling time was 8 hr and the protein doubling time 12 hr. It appears that the protein content/organism of an inoculum of late log. organisms decreases during the first 12 hr of growth on fresh medium (e.g. from 0·12 to 0·06 mμg. protein/organism).

When early log. phase organisms (about 12 hr) were used as an inoculum, there was no initial growth phase, and the initial generation time was 12 hr. An examination of the size distribution of axenically-grown organisms with the Coulter counter showed an increase in cell size during late log. phase growth which was lost soon after transfer to fresh medium. Analysis of the ploidal composition of the spores from axenic organisms (Sussman & Sussman, 1962) revealed a low grade, metastable, haploid condition.

Development of axenically grown organisms. Organisms grown axenically and placed on a filter support at a density of $2{\cdot}5 \times 10^7$ had the same developmental pattern both

morphogenetically and temporally over 24 hr as organisms grown with bacteria (Sussman & Lovgren, 1965) developing from a higher density (10^8 organisms/filter). Increasing the concentration of axenic developmental organisms to approach the optimal concentration for bacteria-fed organisms caused morphological abnormalities and loss of synchrony.

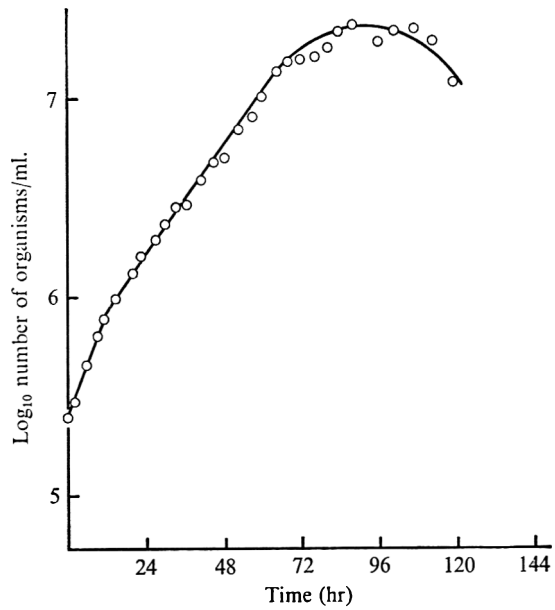

Fig. 1. Kinetics of growth of *Dictyostelium discoideum* amoebae in axenic medium. Inoculum from late log. phase organisms.

Several enzymes have been shown to be under developmental regulation in *Dictyostelium discoideum* (Sussman & Osborn, 1964; Ashworth & Sussman, 1967; Roth & Sussman, 1968). We examined the activity of UDP-galactose polysaccharide transferase during the development of axenically-grown organisms; the transferase activity was quantitatively and temporally identical to that for organisms grown with bacteria (Sussman, 1966).

DISCUSSION

Although the 12 hr doubling time of axenically grown organisms is significantly slower than the 3 to 4 hr found for growth with bacteria (Sussman, 1961; Sussman & Sussman, 1963), it is still fast enough to be of practical use. The total yield of organisms is the same for growth with bacteria in liquid medium. During late log. phase the axenically grown organisms accumulate an excess of protein, perhaps because of the slowing in rate of cell division at this time. When transferred to fresh medium they divide rapidly, presumably until they reach the proper protein content for balanced growth. The over-all rate and sequence of morphogenetic events and the accumulation and loss of a developmentally-regulated enzyme of axenically grown organisms were the same as those of cells grown with bacteria. These results indicate that many of the basic aspects of vegetative growth in *Dictyostelium discoideum*, including growth rate, type of growth and type of medium, do not affect the subsequent events associated with starvation-induced development.

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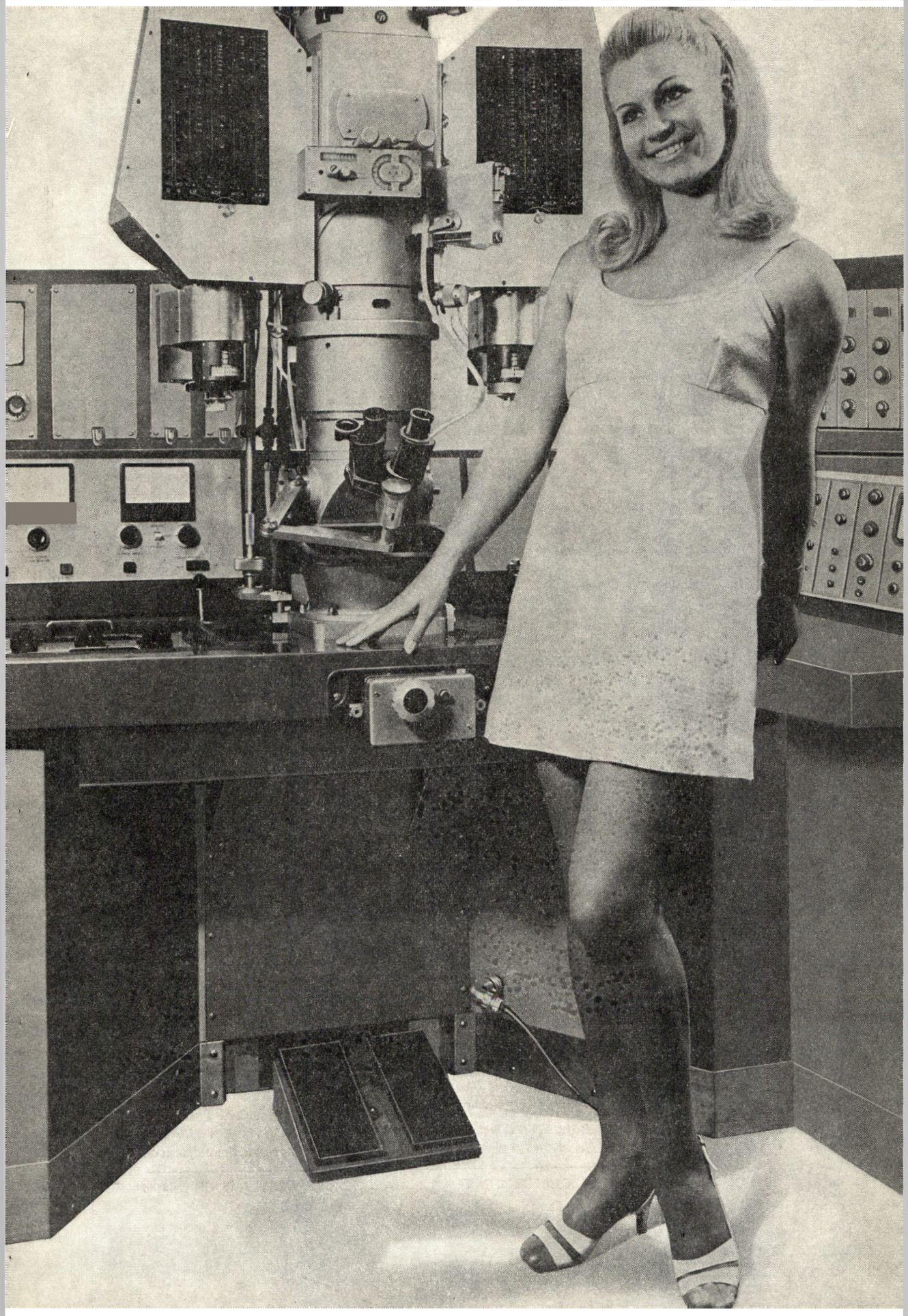

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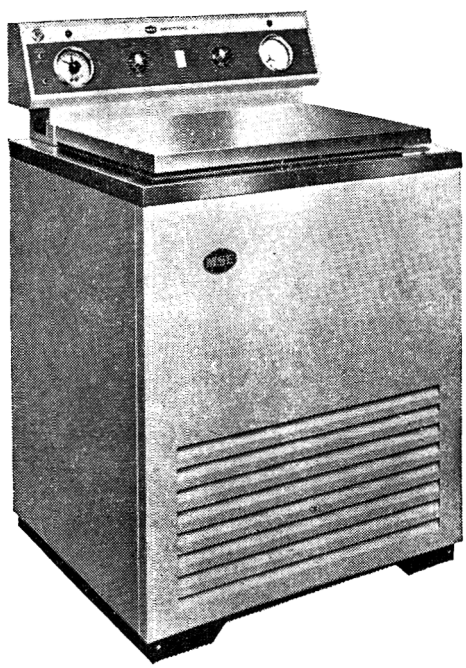

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