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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)

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(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. Typescripts should also carry four key words for index purposes.

(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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the bibliography by the letters *a*, *b*, etc., following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper. Titles of journals, all books, reports and monographs should be set out in full and not abbreviated. References to books should include year of publication, title, edition, town of publication and publisher, in that order. When the reference refers to a particular page or chapter in a book, this should be given after the edition.

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Proprietary Substances and Materials. At first mention, the correct designation and the manufacturer's address should be given in the text.

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.

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Heretical Taxonomy for Bacteriologists*

By S. T. COWAN
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(*Accepted for publication 25 December 1969*)

SUMMARY

In taxonomy, the most subjective branch of a biological discipline, rigid application of the principles of the macrobiologists to bacteria has created puzzles and problems. Too much attention has been given to nomenclature, and too little to the bacteria themselves, their characters, and what they do. Different principles, which some will regard as heretical, should be applied to the classification and nomenclature of bacteria; ten of these principles are described briefly, but defy further summarization.

INTRODUCTION

A hitherto undetected similarity exists between Lewis Carroll's Alice and taxonomists, and bacterial taxonomists in particular. Alice lived (or dreamed) in a world of fantasy in which the eating or drinking of various delicacies gave her the ability to magnify or minimize characters, and she not only upset preconceived ideas of relationships in size, but also disturbed the significance or importance attached to hereditary factors (such as monarchy) and the normal (as distinct from the decapitate) state of the whole organism. Taxonomists also seem to do these things, though they do not need to bite mushrooms or drink magical concoctions in the laboratory. Like the people of Wonderland they tend to be argumentative, and one taxonomist seldom sees a problem through the same spectacles (whether rose tinted or not) as his colleagues. Again like Alice, the taxonomist makes an individual approach to the world around him, and while each is extremely conservative in his views about bacteria, he is by inclination a radical if not an anarchist, and an opportunist if not a dreamer. These may seem to be inconsistent and impossible combinations, but the proof of them should become apparent as I try to convince you that taxonomy can—and does—drive taxonomists to a topsey-turvey Wonderland.

I shall make two assumptions about readers who have got so far, namely, that they are all taxonomists at heart, and that they do not know anything about taxonomy (for anyone who thinks he does would have stopped reading before reaching this sentence). If these premises are true—and I believe they are true of most people—then an ignorance of the art or science of taxonomy should be an advantage to a taxonomist. And this is so, for the less one knows of one's subject, the easier it is to understand and accept its philosophies and conceits.

Because all taxonomists have different ideas on the subject it is necessary to remind readers what I mean by taxonomy (Cowan, 1965*a*). I distinguish it from systematics, which embraces all facets of biology intended to lead to the greater organization of

* Based on a seminar, 'Alice in Taxonomyland', at the University of Maryland, 5 May 1969.

organisms (Cowan, 1968). Taxonomy is divisible into three parts: first, there is the sorting of individuals into likes and unlikes; this is pigeon-holing or classification. Next, there is the labelling or naming of the groups sorted (nomenclature); the third part is the comparison of the unknown with the known and the identification, where possible, of the unknown with the previously recognized and named specimen, group, or population.

Although unrecognized as such, these three things are what everyone does, more or less subconsciously, from the cradle onwards, and explain my premise that everyone is a taxonomist at heart. Their recognition and acceptance lead to another very important point; we are not looking for a single classification of universal application, but for a series of classifications, each with a different objective. One set of objects can be classified in several ways for different purposes, so it is important to realize that we are not looking for THE classification of bacteria but for A classification bespoken for a particular purpose. It is surprising how many so-called microbiologists look upon the schemes published in *Bergey's Manual* (1923–1957) as if they were not only useful general classifications of bacteria, but ones that have received universal approval, both on earth and in heaven. I am assured by my colleagues that approval of *Bergey's Manual* is not universal, even on earth; I am not yet able to judge its reception in heaven.

It is now opportune to consider some of the absurdities of bacterial taxonomy, many derived from the macrobiologies, in the hope that by drawing attention to them we can make taxonomists think more deeply about the general implications of their work, and so avoid some rather stupid consequences.

Historical background

The history of bacteriology has been traced in detail by Bulloch (1938) and parts of historically important papers have been reprinted by Brock (1961) and it is from these useful books that much of this summary of historical background is taken. By listing these milestones of bacterial taxonomy we can hope to see the subject in proper perspective.

1675. van Leeuwenhoek first observed bacteria (he had seen protozoa in 1674); what he saw must have been some of the largest bacteria, for combinations of his best lenses magnified only about 300 times (Dobell, 1923).

1753. von Linné (Linnaeus) published his *Species Plantarum* and, although not the first to do so, he used a binominal nomenclature. His classification of plants was supported by names much shorter than the descriptive polynominals then in general use. Because of the convenience of 'binomials' the publication of this work became the official starting date for botanical nomenclature, and later of bacteriological nomenclature. What are called Linnaean classifications (those with binominal nomenclature) start from this date. But we should not blame von Linné for all the faults of what has become known as the Linnaean system.

1823. This was the year in which Bizio described, in a letter to a most eminent priest, the results of his microscopic examination of the phenomenon known as bleeding polenta in which, when the air was damp enough, a red colour appeared after 24 to 30 hr exposure. The importance of the date is that it was in this letter that Bizio gave the name *Serratia marcescens* to the 'many clusters of very small hemispheres' (translation by Merlino, 1924) which he thought were stemless fungi.

1881. Robert Koch described the plating technique by which he obtained colonies of bacterial growth on nutrient media solidified with gelatin. By this method he was able to obtain a pure culture of a bacterium more readily than by the earlier dilution methods in which cultures were (hopefully) expected to develop in broth from a single bacterial cell.

1887. The plating method was improved by Petri's invention of his dish, and at about the same time by the introduction of agar–agar as a gelling agent at the suggestion of Frau Hess, the wife of one of Koch's assistants. Thus began the era of pure cultures of which Perkins (1928) remarked 'The discovery of the principles of pure-culture study resulted in such a sudden burst of investigation that it was a lost month in which a new organism was not described, catalogued, and laid away, very frequently in the wrong grave.' But we do not assume that, thereafter, all cultures were pure; far from it, for as recently as the 1950s Muriel Robertson remarked that it was doubtful whether a strict anaerobe had ever been isolated in pure culture. Her pessimism was justified, for when she worked with anaerobes in the 1920s most of the descriptions of 'species' were really descriptions of the characters of mixed cultures.

1928. The year in which Griffith published his experiments in which he changed the serotype of pneumococci, a process now known as transformation. This was the beginning of the era that was to challenge the fixity-of-species concept, and was to make bacterial variation respectable and not a confession of poor technique.

Some bacterial heresies

This is the place to introduce a series of ideas about bacteria, taxonomy, and taxonomists that can, at best, be described as heretical, but will be thought by some to be anarchistic.

Bacteria are not plants. The least controversial of my heresies but one that needs to be stated to declare our complete independence of botany and the botanists. For a long time bacteria were regarded as plants and the earliest attempts at bacterial taxonomy were made by botanists such as Cohn (1872), who applied the principles and rules of botanical nomenclature to the bacteria. A more modern view, expressed by van Niel (1955), is that bacteria are different from both plants and animals, and are descendents of precursors of both. For those who like to put micro-organisms into a kingdom the name Monera has been used (for a detailed discussion see Whittaker, 1969). To me, living in a country with a monarchy, it seems quaint that the inhabitants of the great North American republic should accept kingdoms so readily and prefer them to the idea of separate, independent (taxonomic) states.

Hierarchical systems are unrealistic in biology. It is convenient and practical to catalogue spare parts for automobiles on a hierarchical basis, but misleading to do so with living units, because hierarchies imply phylogenies and phylogenies of bacteria are purely speculative. We think of hierarchies as groups broken down into subgroups, subgroups divided into sub-subgroups and these divided again into lesser groups, but in bacteriology this is literally stuff-and-nonsense. Exactly the opposite course is followed to create a hierarchy; individuals are said to be similar and to form a group which is labelled a species; several species with some common characters are combined to form a genus; several genera are combined to form a family, and so on. Hierarchies are built up, not broken down. Moreover the different groups are not sacrosanct; taxonomists build, with bricks of their own fabrication, walls, divisions,

and structures of their own design; they may build a pyramid but it is likely to be less enduring than those of the Pharaohs of the fourth dynasty.

The hierarchy is not appropriate for living things because it would presuppose that all are descended from one original living cell, whereas life almost certainly started in many different places by many independent syntheses. Apart from their evolutionary connotation, hierarchies are attractive to taxonomists because, by the separate listing of group characters, the descriptions of subgroups can be shortened.

Another (antihierarchical) view of the relations of living things is to regard them as different segments of a spectrum blending one with another but having patches or zones of greater concentration that form the smaller units such as species. The spectrum concept allows for, and indeed expects, differences between individuals, and identical units are the exception rather than the rule (Cowan, 1959).

Importance of variation. Because it directly opposed the basis of the typological concept of the ideal invariant form which made the unit, variation was generally ignored by bacterial taxonomists until fairly recently and was, in fact, regarded as a nuisance which introduced intermediate forms. An invariant unit was the foundation stone of the type concept (type method of botanists) by which a low category taxon such as a species was based on a single specimen or strain. Being derived from the botanical, the type of a species could be a drawing or other non-living specimen; bacteriology (and perhaps virology) stands alone in having living type specimens; in theory at least, the type specimens of the microfungi are dried-up specimens.

The *type method*, which once received my support (Cowan, 1956), is responsible for many of the faults of modern taxonomy. It makes a permanent link between a name and a single specimen, but inevitably the name becomes attached to a taxon of a particular rank so that the type strain (or specimen), intended as a name stabilizer, becomes the fixed point of a taxon. By the rules of nomenclature (for the type method is a purely nomenclatural device) the type specimen or strain should be designated as such by the original author of the name (who is not necessarily the first describer of the taxon); if he does not do this, the type (a neotype) can be designated by subsequent workers; originally a nomenclatural attachment, the type is now more often regarded as attached to a taxon such as a species or serotype. The important point is that the strain chosen as type does not need to be typical (in the sense of characteristic) of the taxon. This ridiculous situation can be overcome by ignoring the type method and treating the taxon as a population, as described below.

The population concept. Several strains are needed to represent a taxon above the individual, and Gordon (1967) believes that a species can be defined only as 'a group of newly isolated strains, old stock strains, and their variants that have in common a set of reliable characteristics separating them from other groups of strains'. This is a recognition of a taxon as a population and means that it can only be typified (if that word must be used) by several strains chosen, as far as possible, to show the normal limits of variability within the taxon. Even if the strains are not specially chosen but are a random sample, taken together they must be more representative of the taxon than any one strain could possibly be.

Overlapping of taxa, by which we mean that a taxon may belong to more than one taxon of the next higher category, is a concept unacceptable to the typologist or to the conservative taxonomist. It is a hypothesis that accepts the idea that micro-organisms can be arranged like a spectrum or as a series of intergrading forms (Cowan, 1955); to

be realistic the spectrum should be multidimensional as are the models made by Lysenko & Sneath (1959) to express visually the relations of the Enterobacteriaceae and of some pigmented bacteria. It can also be shown simply, but less convincingly, as a series of circles, the overlapping of which represents areas common to more than one taxon (Cowan, 1959).

Inadequate technique has been too much in evidence in bacteriological laboratories; like the car driver, no one questions his own ability or his own technique. We have had the era of the biochemist-turned-bacteriologist who never knew, or seldom cared, if he had a pure culture or was working with a glorious mixture or even a replacement culture. We now have the era of the molecular biologist and wonder (hopefully) if he is better than the ordinary biochemist.

As taxonomists we believe that our descriptions should be based on the examination of pure cultures, but the day has arrived when the possibility of a stable culture is questioned, and the older concept of a 'pure culture' is dying out. We accept the parasitization of our strains by minute bacteria, as *Bdellovibrio*, and even smaller biological units such as bacteriophages as normal events of everyday occurrence, but such acceptance is only possible when our techniques are beyond reproach. Yet it is only just over ten years since the publication of annotations to the Bacteriological Code (Editorial Board, 1958) in which a culture infected with a phage was given as an example of an 'abnormality' (annotation to Rule 24h).

Technical ability determines the facts on which descriptions are based; and good descriptions are essential to good taxonomy. One of the first principles of nomenclature concerns priority, which requires that the oldest validly published name should be used. This may seem sensible but its application can be vexatious because the interpretation is subjective. A requirement for valid publication of a name is that it should be accompanied by a description or a reference to a description. What passed for a good description in, say 1900, may not be recognizable now, but some workers will accept a name with even the vaguest description of what is named. The situation is complicated because the rules are said to be retroactive, so that the poor bacteriologist of 1900 had to anticipate rules—and changes of rules—made 50, 60, or 70 years later!

Too much attention is given to names, and not enough to what the bacteria do. Names are only labels and they can be replaced by numbers, or by a mixture of letters and figures (Cowan, 1965*a*, *b*). Names do not have any significance except as a means of communication; it is naïve to expect that the name of a bacterium will have a meaning, or that it is descriptive. Quite elaborate (many think too elaborate) rules have been drawn up in codes of nomenclature to try to regulate the formation and use of names, but these codes would delight the hearts of lawyers because they are too detailed and try to cater for all eventualities. In the event, they are confusing and self-contradicting; for example, Principle 4 of the Bacteriological Code says that names are taken from Latin or Greek words, but if taken from any other language or formed arbitrarily, they are to be treated as if they were Latin, and Latin terminations should be added. In practice many specific epithets are formed in an arbitrary manner, or are unaltered modern words or place names without any attempt at latinization; all these are acceptable because the Code gives permission to correct faulty latinization, improper connecting vowels or endings, without taking away any credit due (in an author citation) to the original author who had mishandled, or been frightened off, the Latin. (The zoologists, in their code, are much less permissive.) Since names for bacteria can

be formed in an arbitrary manner, all the rules about latinization, correction, and so on, are really unnecessary elaborations.

This heresy can be summarized thus: since scientific names may be formed in an arbitrary manner, all names printed in the appropriate letter-characters should be acceptable; there should not be a need to worry about latinization or Latin endings. The corollary is that names, as names, would cease to be centres of argument and debate, and this would free the taxonomist to devote more time to the more important characterization and description of the organism or taxon.

The heresy of importance. This heresy is not concerned with the weighting (or importance) of characters, a subject adequately discussed by Sneath (1957), but asks the question, What is important in taxonomy? Is it the name, the description, or the ability to recognize (identify) the organism? The last heresy dismissed the name as important only in communication; here we are considering the search for practical day-to-day characters that enable us to visualize and recognize the organism we have isolated. Two things are important: the ability to search out characters (i.e. technical acumen and prowess), and the literary ability to describe clearly and unambiguously the characters found. What the taxonomist and his colleagues need to know is the nature of the organism as revealed by the characterization.

The heresy of Serratia marcescens, or names of the pre-bacteriological era. What were the organisms seen by Bizio and described in 1823 as stemless fungi, hemispherical capsules occurring in clusters (Merlino, 1924); were they yeasts or some of the smaller bacteria? In trying to answer these questions we should bear in mind the quality of microscopes in the early part of the nineteenth century, and the fact that the bacterium later named *Monas prodigiosus* was so small that it became the standard test organism to detect faulty bacterial filters. Which, if either, of these organisms did Bizio see, a bacterium or a yeast? Mycologists unhesitatingly implicate a yeast as the cause of bleeding polenta; we also know that the late Dr R. S. Breed reproduced the phenomenon with a bacterial culture. It is reasonable to suppose that the cause of the phenomenon is non-specific, and may be a yeast or a bacterium; but it seems unlikely that Bizio saw a very small bacterium and more probable that the hemispherical capsules were yeast cells. Therefore, I believe that the name *Serratia marcescens* was not applied to a bacterium, but may have been applied to a yeast. However, Bizio's description is so vague that it is impossible for us to be sure what organism he saw; it follows that the name he applied to his ill-defined organism should not be accepted but should be rejected under Rule 24f of the Code, which says that a name is illegitimate 'If its application is uncertain'.

The fact that a name such as *Serratia marcescens* was ever considered by so practical a bacteriologist as Breed suggests that there is something wrong with the rules of nomenclature that allow such old names to be valid. It is an example of a name applied to something before bacteria were isolated or studied scientifically, but it serves a purpose in drawing attention to the scientific uncertainty that may arise from the use of names of the pre-Pasteurian era.

The starting date of bacteriological nomenclature, 1753, is much too early; it would be more realistic to consider a date in the present century.

Although Dobell (1923) described Leeuwenhoek as the first bacteriologist and the founder of bacteriology, it would be unwise to regard his observations as the beginning of scientific bacteriology. Fortunately Leeuwenhoek did not apply any latinized names

to his animalcules, but if he had done so his drawings would have been good enough to satisfy Rule 9d(1) Note 1 of the latest Code (Editorial Board, 1966), which says that 'For a species which cannot be maintained in laboratory cultures...the type is the original description, preparation or illustration.' Which shows that 'the wind of change' has not yet swept through the Bacteriological Code.

The starting date 1753 follows botanical practice, and is a relic of the days when scientific bacteriologists (as distinct from medical ones, who regarded the subject as a branch of pathology) accepted the Botanical Code in matters of nomenclature. Botany was, and is, the science of living plants, but its nomenclature is based on pressed, dead specimens, descriptions, and illustrations; bacteriologists are fortunate in being able to study and preserve living specimens so that our reference material is much more satisfactory than that of the botanists. We developed our own code, but only by modifying the botanists' code; why did we not start afresh with a less complicated, less ambitious code? And why did our predecessors accept a starting date before bacteria had been grown? Other dates have been suggested, and I think that a reasonable one would be 1920, when the Winslow Committee of the Society of American Bacteriologists (Winslow *et al.* 1920) issued its final report on bacterial classification; the nomenclature used in this report was obviously based on the work of one of the Committee's members, R. E. Buchanan, who had published ten papers on the subject between 1916 and 1918 (Buchanan 1916, 1917*a–c*, 1918*a–f*). The names were mostly those in use at the time the report was published, and it would have been reasonable to conserve them and to ignore (reject is the correct technical term) all other names published before 1920.

That the *Bacteriological Code is too detailed* and tries to anticipate every problem that may arise will seem to some to be a heretical statement, but it is justified by the failure of the Code, as shown by the frequent revision at almost every international congress for microbiology, to meet the needs of bacteriologists. The Code is divided into four parts: 1, General Considerations; 2, Principles; 3, Rules and Recommendations; 4, Provisions. Of these, Chapter 2, Principles, is much the best and most useful section; it is the part least like the Botanical Code and shows that bacteriologists would have done better for themselves and the discipline if they had paid less attention to the practices of the botanists.

This heresy is the expression of a wish for a much simplified Code, based on the Principles of the present Code together with a means of putting teeth into it; it could be improved by simpler language and less pseudo-legal phraseology. To achieve a measure of compliance some form of registration of names would be required and, if the conditions for registration were stringent enough, we should stop having names without descriptions or with only inadequate descriptions. This is not a new suggestion, it is merely the echo of one made for fungi by Ainsworth & Ciferri (1955).

My last heresy in this paper, number 13, should really not be regarded as a heresy. Taxonomists are lucky in that their work is personal, varied, and above all, full of interest; it is work that can and should be enjoyed by those who take part in it, whether as individuals or as members of a team. Taxonomy is a subject that tends to stimulate heated arguments and nomenclature is the most temperature-raising branch of all. For some unknown reason it seems to be impossible to discuss nomenclatural problems calmly and with the quiet detachment expected of scientists; arguments become acrimonious, tempers frayed, and blood pressures raised, but seldom do taxonomists

become spiteful or lose their dignity in personalities; that has happened, but fortunately only occasionally. So, while we argue at length and appear to be mortal enemies, we keep a sense of judgement and recognize that there is always more than one side to every argument. We know we are lucky in our work for it is work to be enjoyed and, what may surprise non-taxonomists, is not to be taken too seriously.

To round off my list of heresies I shall describe some principles of heretical taxonomy in the hope that readers will think about them, and compare them critically with the taxonomy learnt from the cradle onwards. In commending these principles let me remind you that they are Utopian and come, almost directly, from Alice in Wonderland.

TEN PRINCIPLES OF HERETICAL TAXONOMY

1. We should not attempt to classify bacteria in a rigid hierarchical system.

2. The relations between bacteria can be expressed only in a spatial concept, but can be visualized simply (if inaccurately) by a series of spectra, and of overlapping taxa.

3. The type method, as now practised, is not suitable for bacteriology; it should be replaced by a method in which several strains represent the taxon and indicate the limits of its variability or spread.

4. Good bacteriology is dependent on good technique and pure cultures; too much attention cannot be given to these qualities.

5. Names are important only as labels; they have no significance and cannot have a meaning.

6. Similar organisms cannot be recognized by the similarity of their names; they may have been mislabelled or misidentified. Clear, concise descriptions are the cornerstones of good taxonomy, and they alone enable similar organisms to be recognized and identified.

7. Accurate characterization and good descriptions are dependent on the use of good technique and pure cultures; clarity of description is dependent on literary adequacy.

8. The Bacteriological Code should be simplified by deleting the Rules and Recommendations. It should consist of Principles, and discretion should be given to bacteriologists to apply them intelligently.

9. The starting date of bacteriological nomenclature should be changed to some time when bacteriology was being done scientifically; I suggest that 1920, when the S.A.B. Winslow Committee reported, would be a suitable starting date.

10. Names of taxa should be acceptable only when (i) they are accompanied by an adequate description, and (ii) representative strains are deposited in at least two culture collections. Acceptance of names should be made by a committee which would sit to ensure that (i) and (ii) had been carried out, and that names were not duplicated. Accepted names should be registered

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The Isolation and Characterization of Mutants of *Bacillus subtilis* and *Bacillus licheniformis* with Disturbed Morphology and Cell Division

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SUMMARY

Mutants (*rod*) have been isolated from one strain of *Bacillus subtilis* and two of *B. licheniformis* after treatment with 1-methyl-3-nitro-1-nitrosoguanidine, by replica plating from media containing 0·8 M-NaCl to media of low salt content. When grown on the latter media these mutants appear as groups or strings of coccal bodies which, when examined in section under the electron microscope, show gross distortions in their walls and membranes; septum formation is greatly disorganized. When grown in media containing 0·8 to 1·0 M-NaCl, or KCl, or ample supplies of organic nitrogen, considerable correction of the morphology of one class of these mutants occurs. The other class of mutants is not changed to rods by growth in media of high salt content but is so changed by growth on rich media containing yeast extract. All the mutants revert to the parent type, but at very different rates. The physiological characteristics of the mutants and the parents are in most respects identical.

INTRODUCTION

The chemical structure and biosynthesis of the component polymers in bacterial cell walls are now well documented (Rogers & Perkins, 1968). Little is yet known about the regulatory relationships between wall and membrane formation which control replication of shape during cell division. A reasonable simile to illustrate the present state of knowledge about the interrelationship would be to regard it as a conversation between the membranes, the wall and the DNA. The DNA, at least in Bacillus, appears to be attached to the sac of the mesosomal membrane (Ryter, 1968), which in turn appears likely to be involved in the division of the DNA (Jacob, Ryter & Cuzin, 1966; Ryter, 1968). Meanwhile the membranes have fixed to them many of the enzymes and the intermediate carrier concerned with the synthesis of the wall polymers (Rogers & Perkins, 1968). The mucopeptide in the wall presumably defines the shape of the micro-organisms during growth and its inward growth into the septum divides the cell into two siblings. Disturbance in this conversation by lesions affecting either wall or membrane synthesis would be expected to lead to upsets in the morphology of the cell and in the division process. In one of the extreme forms of disturbance of wall synthesis, the wall is not formed at all by Gram-positive species, and then the shape and the normal division process are lost; such is the case with multiplying protoplasts (Landman & Halle, 1963; Landman, Ryter & Fréhel, 1968) and with L-forms. Failure to initiate DNA synthesis, on the other hand, also leads to filaments and other morphological disturbances (Mendelson & Gross, 1967; Hirota, Ryter & Jacob, 1968).

One way to select mutants capable of growing with weakened walls is to look for bacteria that are dependent on high concentrations of suitable substances, such as sucrose, in the growth medium. These substances act as osmotic supports for bacteria with inadequate wall structures. Mangiarotti, Apirion & Schlessinger (1966) isolated mutants of *Escherichia coli* dependent upon a high concentration of sucrose, and of five such mutants reported four of which grew as filaments without sucrose, whilst one was absolutely dependent upon the presence of sucrose in the growth medium. For one of the mutants growing as a filament, sucrose could be replaced by D-alanine, an essential component of the mucopeptide in the wall.

In the present work mutants of *Bacillus subtilis* and *B. licheniformis* have been selected which are partially dependent on the presence of high concentrations in the growth medium of sodium chloride rather than sucrose. Sucrose was unsatisfactory because of the nature of the colonies produced on media containing high concentrations of this carbohydrate. These mutants have a disturbed gross morphology which can be corrected to a large extent by the presence in the growth medium of low concentrations of certain amino acids and high concentrations of salts, or by rather high concentrations of the amino acids alone. One of these mutants examined in detail has a relatively uncrosslinked wall (Rogers, McConnell & Hughes, to be published), and examination of sections with the electron microscope shows that the organization of the membranes and division septa is greatly disturbed. The distribution of DNA also would seem to be abnormal. These disturbances in ultrastructure can be corrected by the same agents as those which correct the gross morphology.

A brief report on these mutants (naming them *rod* mutants) has already appeared (Rogers, McConnell & Burdett, 1968). Whilst preparing this paper a brief report on a temperature-sensitive mutant of *Bacillus subtilis* with a similar grossly disturbed morphology has been published (Boylan & Mendelson, 1969).

METHODS

Micro-organisms. Bacillus subtilis 168 *trp*, *B. licheniformis* 9945A *met cap*, *B. licheniformis* 9945A *ade cap* and *B. licheniformis* 6346 *lys* were kept as spore suspensions in water at 0°, except the latter strain, which was kept dried by the Stamp (1947) method. These suspensions were revived by inoculation into Penassay broth + 0·5% glucose contained in a conical flask five times the volume of the broth, and incubation at 35° overnight without shaking. This culture was then inoculated into a further volume of the broth also contained in a conical flask of the same relative size. This flask was shaken at 35° until the culture reached about the middle of the exponential phase of growth.

Treatment with mutagen. The exponentially growing cultures were centrifuged down and the bacteria were immediately suspended in 0·1 M-NaK phosphate buffer (pH 6·5) at 1 mg. dry weight/ml. as determined by optical density. This suspension was treated with 1-methyl-3-nitro-1-nitrosoguanidine at 35° with aeration until about 99% of the micro-organisms had been killed. The concentration of mutagen was adjusted to give the required killing within 30 to 60 min. For *Bacillus subtilis* 168 *trp* this was 0·02%; for *B. licheniformis* 9945A, 0·04%; for *B. licheniformis* 6346, 0·002%. The bacteria were then centrifuged down, suspended in Penassay broth of ten times the volume of the original, and incubated at 35° with shaking for 3 to 4 hr.

Isolation of mutants. The mutagen-treated culture was plated on the following

medium containing 0·8 M-NaCl: $FeCl_2$, 0·46 mg.; NH_4Cl, 0·535 g.; KH_2PO_4, 0·068 g.; Na_2SO_4, 0·106 g.; NH_4NO_3, 0·096 g.; $MgCl_2$, 0·004 g.; $MnCl_2$, 0·013 g.; Casamino acids, 1 g.; NaCl, 46·8 g. This solution was adjusted to 1 l. with distilled water. It was solidified with 1·5 % agar and sterilized. Glucose solution, sufficient to give final concentration of 0·5 % and the appropriate growth factors to satisfy auxotrophic requirements were added before pouring plates. The mutagen-treated culture was spread so that each plate would grow about 100 to 200 colonies after 2 days at 35°. The growth on the plates was replicated on to the same medium but *not* containing 0·8 M-sodium chloride; these plates were incubated for 1 day at 35°. Colonies were then picked which grew on the plate containing sodium chloride; these colonies either did not grow on the plate without the salt, or grew as very small colonies as compared with the remainder.

The isolations were all tested for their appropriate auxotrophic requirements and were streaked several times on the 0·1 % casein hydrolysate medium; and single colonies were isolated which were then dried by the Stamp (1947) technique and kept at room temperature.

Growth of mutants in liquid cultures. The following minimal medium was used: $(NH_4)_2SO_4$, 1 g.; NH_4Cl, 1 g.; KH_2PO_4, 13·6 g. This solution was adjusted to pH 7·0 with 1·0 N-NaOH and to 1 l. in volume with distilled water. After sterilization, enough $MgSO_4$ solution was added to make the final concentration 3 mM, and glucose solution to give a final concentration of 0·5 %. All incubation in liquid cultures was, with shaking, at 35°.

Table 1. *Effect of inoculum size on lag and growth rate of mutant rod-4 and the parent Bacillus subtilis 168 trp*

The inoculum was made by washing the growth from a minimal salts + 0·1 % casein hydrolysate + 0·5 % glucose plate which had been incubated overnight at 35°. The bacteria were washed once and suspended in minimal fluid medium; this suspension served as inoculum; its dry weight was determined from its optical extinction and 1 mg. dry weight/ml. corresponded to 3×10^7 living units/ml. For the parent strain, grown and treated in the same way, 1 mg. dry weight/ml. corresponded to 1×10^8 living units/ml. The number of living units shown under inoculum was added to 10 ml.

Organism	Inoculum (living units/ml.)	Lag (hr)	Doubling time (min.)	Time to full growth (hr)
rod-4	5×10^2	> 26	—	> 48–72
	5×10^3	> 26	—	36–72
	5×10^4	19	96	32
	5×10^5	17	96	26
Parent	2×10^3	> 18	—	24–48
	2×10^4	8	96	24–48
	2×10^5	7	120	24
	2×10^6	—	—	< 18

The original agar media used for isolating mutants from *Bacillus subtilis* 168 *trp* contained 0·1 % acid hydrolysed casein. With this present the mutants formed larger colonies when growing in the presence of 0·8 M-NaCl than when growing without the high salt concentration. They would, however, grow on the minimal agar medium in the absence of the sodium chloride and the casein hydrolysate. They would also grow in the liquid minimal medium described in Methods; the lag in growth in this latter

medium was inoculum-dependent. Results were concerned with the growth of the parent strain and mutants in the liquid minimal medium (Table 1). The lag in growth of the parent was also dependent on inoculum size; the principal difference between the mutant and the parent was in the length of the lag period. Attempts to overcome the lag in the growth of the mutant by the addition of heat-killed bacteria were unsuccessful; but it could be achieved by the addition of low concentrations of casein hydrolysate.

Preparation of cell walls

For lysozyme treatment. Cultures of one of the mutants and of wild-type *Bacillus subtilis* 168 *trp* were grown on salts + casein hydrolysate agar medium with and without the addition of 0·8 M-NaCl. After 2 days of incubation at 35°, the bacteria were harvested in a solution of 2 % (w/v) sodium dodecyl sulphate. They were then broken by shaking with Ballotini beads for about 20 min. in a Mickle (1948) disintegrator working at 0 to 4°. The resulting cell walls were washed with sodium dodecyl sulphate at room temperature until they were protein-free, then with M-sodium chloride and finally with water until they were free from chloride. The walls were then lyophilized.

For autolysis. Cultures of two of the mutants of *Bacillus subtilis* 168 *trp* were grown in liquid minimal medium containing casein hydrolysate with and without 0·8 M-NaCl. The bacteria were harvested towards the end of the exponential phase of growth, broken by shaking with Ballotini beads in a Braun cell homogenizer and then washed six times with water and lyophilized. The temperature was at about 2° throughout the treatment.

Electron microscopy

Colonies of bacteria were removed from the surface of agar plates with a platinum loop and placed in the fixative. In some cases the samples were fixed with 5 % glutaraldehyde in 0·05 M-cacodylate buffer (pH 7·2) containing 10 mM-$CaCl_2$ for 2 hr. Following overnight wash in cold buffer, the samples were fixed with 1 % OsO_4 by the method of Ryter & Kellenberger (1958); some samples were placed directly in the OsO_4 fixative without prefixation with glutaraldehyde. Similar results were obtained with these two fixation procedures. Before dehydration in ethanol the pellets of fixed bacteria were soaked for a minimum period of 2 hr in 0·5 % uranyl acetate dissolved in the Ryter–Kellenberger buffer. All samples were embedded in Epikote 812 resin. Sections were cut with glass knives on an LKB Ultrotome and picked-up on bare 400-mesh copper grids. The sections were stained with uranyl acetate (a saturated solution in 50 %, w/v, ethanol in water), or with uranyl acetate and lead citrate (Venable & Coggeshall, 1965). Some sections were lightly coated with carbon before examination in the electron microscope. Serial sections were picked up on slot Sjöstrand-type grids covered with a Formvar film, stained with uranyl acetate and then stabilized with carbon. Sections were examined in either a Philips EM-300 or a Philips EM-200 operated at 60 kV and 80 kV, respectively, with 50 μm. objective apertures and double-condenser illumination. In both microscopes the specimen chamber was surrounded by a liquid-nitrogen anticontamination trap.

Spore formation

Cultures of three of the mutants grown overnight in salts casein hydrolysate (0·1 %) liquid medium were used to inoculate media of the following composition: Evans Peptone, 10 g.; Lemco, 3 g.; sodium chloride, 2 g., made to 1 l. in ion-exchange water

and adjusted to pH 7·1 before autoclaving. Samples were withdrawn every 48 hr and appropriate dilutions heated at 75° for 15 min. The heated and unheated cultures were then plated on Hedley–Wright nutrient agar. The plates were incubated for 24 hr at 35° and the number of colonies developing from the heated cultures was taken as indication of the number of spores in the original broth cultures.

RESULTS

Mutants isolated

Most of the work reported here is concerned with mutants isolated from *Bacillus subtilis* 168 *trp*. In this strain mutants were recognized at the rate of about 1:4000 of those micro-organisms surviving the mutagen treatment. Thirty isolates were made which had the appearance under phase contrast (Pl. 1, fig. 2) when they were grown on minimal salts + glucose agar with no added sodium chloride. All of these isolates required tryptophan for growth and all showed some tendency to revert to the parent phenotype, which could be readily recognized on the plates by larger rough colonies. Of the 30 isolates, 13 were kept for further study. When these 13 were grown on the medium containing 0·8 M-NaCl, nine of them changed to rod-like forms (Pl. 1, fig. 3) but four did not change. Those that will change will be referred to as class A *rod* mutants; those that do not will be called class B. From *Bacillus licheniformis* 9945A *met cap*, six class A mutants were isolated. Three of these had the appearance shown in Pl. 1, fig. 1, and were very like those obtained from *B. subtilis*; three were less coccoid but of a similar type. All required methionine for growth and did not form polyglutamic acid. When grown in media containing 0·8 M-sodium chloride they also formed rod-like bodies. About 30 mutants were obtained from *B. licheniformis* 6346; all grew as rod-like forms in the presence of 0·8 M-NaCl. Some mutants of the rod type were also isolated from *B. licheniformis* 6346 *lys* and *lys str-r*, but the auxotrophic markers could not always be shown in the *rod* mutants; these will be disregarded in this paper, though morphologically they appeared identical to other *rod* mutants.

General physiological characteristics of the mutants from Bacillus subtilis

Fermentation characteristics. Apart from the slight fermentation of sorbitol by *Bacillus subtilis rod-4* and of maltose by the parent strain, the results (Table 2) for the parent and the two mutants were identical. In some instances fermentation by the mutants appeared to be slightly slower than for the parent; this is to be expected from their overall slower growth.

Sensitivity to antibiotics. The results obtained are shown in Table 3. The *Bacillus subtilis* mutants (*rod-4*, *rod-5*) showed qualitatively the same pattern of sensitivity to the four antibiotics tested. Both mutants are sensitive to all four antibiotics (Table 3) and *rod-4* quantitatively has the same pattern as the parent. Surprisingly, *rod-5* is considerably more sensitive to all the antibiotics except chloramphenicol.

Effects of lysozyme and autolysin. Both *Bacillus subtilis* and *B. licheniformis* have walls that are sensitive to egg-white lysozyme and the bacteria are in consequence lysed by this enzyme. The effect of lysozyme on the mutants was studied by growing them on the usual solid medium with and without 0·8 M-NaCl and then suspending the bacteria in 0·1 M-ammonium acetate buffer at pH 6·5. Egg-white lysozyme solution was added to half of each suspension to a final concentration of 50 μg./ml., whilst an

equivalent volume of water was added to the other half. Drops of cultures were examined under the phase-contrast microscope. The mutants were more sensitive than the parent strain and there was no obvious difference according to whether the mutants

Table 2. *The fermentation tests on the parent and rod mutants of Bacillus subtilis 168 trp*

Incubation was station. culture at 35° in test-tubes containing inverted Durham tubes. The symbols + to + + + indicate increasing degrees of acidity. o means no acid and Alk means that alkalinity was produced. The inoculum was a suspension of the organisms obtained by emulsifying a few colonies from plates of the 0·1 % casein hydrolysate salts glucose agar in liquid minimal medium. The basal medium for the sugar solutions was peptone water.

	Mutants					
	Parent strain		Class A *rod-4*		Class B *rod-5*	
Time (hr) ...	24	48	24	48	24	48
Carbohydrate	Degree of growth					
Arabinose	+ +	+ +	+ +	+ +	+	+ +
Dextrin	o	o	o	o	o	o
Dulcitol	o	o	o	o	o	o
Galactose	o	o	o	o	o	o
Glucose	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
Glycerol	o	+	o	+	o	+
Glycogen	+	+	+	+	+	+
Inositol	o	o	o	o	o	o
Inulin	o	Alk	o	Alk	o	o
Lactose	o	Alk	o	Alk	o	Alk
Fructose	+ +	+ + +	+ +	+ + +	o	+ +
Maltose	o	+	o	o	o	o
Mannitol	+	+ +	+	+ +	o	+
Raffinose	o	o	o	o	o	o
Rhamnose	o	o	o	o	o	o
Salicin	+	+ + +	+	+ + +	+	+ +
Sorbitol	o	o	+	+	o	o
Starch	o	o	o	o	o	o
Sucrose	+ + +	+ + +	+ + +	+ + +	+	+ +
Trehalose	+	+ +	+	+ + +	o	+
Xylose	+	o	+	o	+	o

Table 3. *The antibiotic sensitivity of rod mutants of Bacillus subtilis compared with the parent*

The growth medium was the salts+casein hydrolysate agar medium described in Methods. All inocula were first grown for 24 hr on the same medium. For testing the effect of benzylpenicillin the 24 hr cultures were subsequently grown in Penassay broth to about the middle of the exponential phase of growth and then diluted before plating to give about 300 colonies/plate. The figures recorded are those for the highest concentration (μg./ml.) showing growth and the lowest showing no growth after 4 days at 35°.

Antibiotic	Parent (μg./ml.)	*rod-4* (μg./ml.)	*rod-5* (μg./ml.)
Benzylpenicillin	0·01 to 0·05	0·01 to 0·05	0·005 to 0·01
Tetracycline	10 to 50	10 to 50	1 to 10
Erythromycin	1 to 10	1 to 10	0 to 0·1
Chloramphenicol	1 to 10	1 to 10	1 to 10

were grown in the presence or absence of NaCl. Cell walls of *rod-4* were similarly treated by suspending them to 1 mg./ml. in 0·1 M-ammonium acetate buffer (pH 6·5) containing 50 μg./ml. lysozyme. The optical density of the suspension was measured at intervals during about 2 hr. The rates of lysis of the wall of the wild type and of the mutant grown under the same conditions were very similar. Walls from bacteria grown on salts+casein hydrolysate medium containing 0·8 M-NaCl (Fig. 1) lysed more slowly. When suspensions of the mutants or of wall preparations from them were incubated at 35° without lysozyme in 0·03 M-phosphate buffer (pH 9·2), the *rod-4* and *rod-5* mutants lysed, providing they had been grown without NaCl. Free amino groups of L-alanine appeared, showing that an autolysin of the type previously studied (Young, 1966) in the parent strain was active in the mutants.

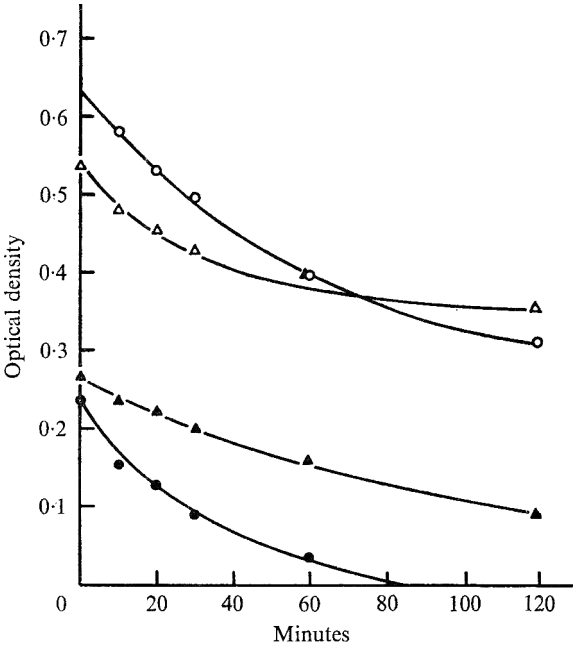

Fig. 1. Rate of lysis of cell walls of *Bacillus subtilis* 168 *trp* and of the mutant *rod-4*. The cell walls were suspended in 0·1 M-ammonium acetate buffer (pH 6·5) to approx. 1 mg./ml. Lysozyme was added to 50 μg./ml. At intervals the extinction of the culture was read. ○, Wall from *rod-4*; ●, wall from wild type, both grown on salts+casein hydrolysate medium; △, wall from *rod-4*; ▲, wall from wild type 168, both grown in salts+casein hydrolysate medium containng 0·8 M-NaCl.

The effect of temperature on growth. Plates of the usual salts+casein hydrolysate medium, with and without 0·8 M-NaCl, were inoculated from plates of the same media with *rod-4*, *rod-5* and the parent strain. The plates were incubated at 45°, 35°, 25°, room temperature (about 18°) and 4°. Growth was recorded after 3 days of incubation. Table 4 shows that the mutants differed from the parent in not growing at 45° in absence of NaCl. When 0·8 M-NaCl was present, a little growth occurred at 45° with *rod-4* and *rod-5*. There appeared to be some evidence that at the lower growth temperatures *rod-4* formed longer rods in the presence of NaCl than at the higher temperatures. An occasional rod was also seen in the mutant *rod-5* growing at room temperature in

the presence of 0·8 M-NaCl. During this work it was observed that the mutants were more susceptible than the parent strain to cold shock and that *rod-4* was more susceptible than *rod-5*. For example, live organisms of *rod-5* and the parent could be recovered from a plate streaked and stored at 0° up to 6 days; at 10 days there was no recovery from *rod-5*. No recovery was made from *rod-4* after 4 days without 0·8 M-NaCl, and after 5 days no recovery was made from this plate. The plates were removed from the refrigerator to room temperature each day for examination and subculture.

The effect of direct changes in osmolarity on the morphological form. Transfer of

Table 4. *The effect of the temperature upon rod mutants and the parent strain*

The agar medium was the salts medium + 0·1 % casein hydrolysate described in Methods with and without the addition of 0·8 M-NaCl. The degree of growth is the growth recorded as from 0 to + + +. The terms rod and round refer to the appearance of the growth under the phase-contrast microscope.

Temperature (° of growth)	Parent. No NaCl	Growth (NaCl)			
		rod-4		*rod-5*	
		No NaCl	+ NaCl	No NaCl	+ NaCl
45	+ + + (rods)	0	+ (short rods)	0	+ (round)
35	+ + + (rods)	+ + + (round)	+ + + (short rods)	+ + + (round)	+ + + (round)
25	+ + + (rods)	+ + (round)	+ + (long rods)	+ (round)	+ (round)
'18'*	+ + + (rods)	+ + (round)	+ + (long rods)	+ (round)	+ + (round)

* Room temperature.

Table 5. *The effect of increasing concentrations of sodium and potassium chloride upon morphology of the rod mutants of Bacillus subtilis 168 trp*

The basal medium was the 0·1 % casein hydrolysate + salts + agar medium described in Methods. Incubation was for 18 hr at 35°. The morphology was observed with the phase-contrast microscope. The words italicized indicate the concentrations at which morphological change was clear.

Conc. (M)	Salt	Mutants				
		Class A			Class B	
		rod-3	*rod-4*	*rod-7c*	*rod-7d*	*rod-8*
0	NaCl	Round	Round	Round	Round	Round
0·1		Round	Round	Round	Round	Round
0·2		Round	*Short rods*	Round + short rods	Round*	Round
0·4		*Short rods*	Short rods	*Short rods*	Round	Round*
0·6		Short rods	Short rods	Rods	Round	Round
0·8		Short rods	Long rods	Thin rods	Round*	Round*
1·0		Long thin rods	Long thin rods	Rods	Round*	Round*
1·2		Long thin rods	Long thin rods	Long rods	Round	Round
0·1	KCl	Round	Round	Round	Round	Round
0·2		Round	Round	Round	Round	Round
0·4		*Short rods*	Round	*Short rods*	Round	Round
0·6		Short rods	*Short rods*	Short rods	Round†	Round†
0·8		Short rods	Short rods	Long rods	Round†	Round†

* Occasional rod-like forms were seen in *rod-7d* and *rod-8* at these concentrations of sodium chloride.

† Some rods were seen in mutants *rods-8* and *rod-7c* at these concentrations of potassium chloride.

bacteria of *rod-4* grown as rods on a medium containing 0·8 M-NaCl to distilled water did not alter the morphology of the bacteria, and vice versa.

The effect of increasing concentration of salts in the growth medium. Table 5 shows the effect of increasing concentrations of sodium and potassium chloride added to 0·1 % casein hydrolysate-salts + agar medium. There appeared to be little difference between sodium and potassium chlorides. In the presence of both chlorides the class A mutants grew as rods at about 0·4 M. For *rod-4* the sodium salt appeared to be more effective, since the mutant appeared as rods at 0·2 M whereas 0·6 M, potassium salt was required. The class B mutants did not grow as rods in any of the media; an occasional rod-like form was seen with higher concentrations of both salts.

Spore formation. Two class-A and one class-B mutants were tested for spore formation. After 48 hr the cultures of the two A mutants consisted of about 50 % spores. There were no spores in the *rod-5* culture.

Viable counts. Attempts to see whether each individual round form was alive and capable of giving rise to a rod have so far been complicated by the occurrence of large groups or strings of round forms and by the formation of filaments during the transition from the round form to the rods (Rogers & McConnell, 1970). A relationship was established between the turbidity and the dry-weight content of suspensions of the mutant *rod-4* grown as a round form. Suspensions were then washed from 0·1 % casein hydrolysate-agar plates and living counts measured both on the same medium and on nutrient agar. The mutant grew on the former medium as a round form and on the latter as a rod. The same was done for the parent *B. subtilis* 168 *trp*. The viable counts for the parent strain growing on either medium were $9{\cdot}5 \times 10^7$ to $1{\cdot}05 \times 10^8$ colony-forming units/mg. dry weight. The counts for the mutant were again the same within 5 % on the two media but only $3{\cdot}3 \times 10^7$ colony-forming units/mg. dry weight were present. Measurements of the number of colony-forming units in a liquid minimal medium culture of *rod-4* incubated for 48 hr into the late exponential or early stationary phase gave $1{\cdot}9 \times 10^6$ colony-forming units/mg. dry weight. The discrepancies between the colony-forming units/unit mass bacteria may be due to different sizes of clumps or to the presence of a different proportion of living bacteria. The correspondence between the number of colonies appearing on poor and rich medium, respectively, suggests that any unit capable of forming a colony of round forms can also form a colony of rods (see later, this paper). The proportion of individual round forms that is viable either as round forms or as rods cannot so far be deduced.

Detailed electron microscopic observations on rod-4 derived from Bacillus subtilis

Appearance when grown in absence of added NaCl. The picture at low magnification (Pl. 2, fig. 4) illustrates the bizarre shapes, the variable thickness of the cell wall, the erratic beginnings of division septa and the apparently dispersed nuclear body. This picture may be compared with those of the parent *Bacillus subtilis* already published (e.g. van Iterson, 1961; Ryter & Landman, 1964). The cell walls may be fairly thin (approx. 135 Å in width) or very much thickened (up to 1800 Å). When thick, they appear to be multilayered (Pl. 3, fig. 6; Pl. 5, fig. 12, 13; Pl. 7, fig. 22), each layer being 50 to 85 Å wide. In most organisms the inner edge of the cell wall is composed of a densely staining zone (Pl. 4, fig. 8) which is 55 to 65 Å thick. This zone is usually absent from the outer surface of the wall but is present on both edges of septa. Occasionally there appear to be two distinct walls surrounding some cells (Pl. 4, fig. 7). The inner

wall member, presumably that most recently formed, bears a densely staining zone on both the inner and outer faces (Pl. 4, fig. 9). Irregular thickenings may occur in these walls (Pl. 4, fig. 10) and appear multilayered.

The surface layers of the cell wall usually appear frayed (e.g. Pl. 3, fig. 6). This fraying could be a preparative artifact, but preliminary examination of negatively stained organisms shows a system of fine fibrils and amorphous material to be associated with the walls. In section, the frayed-wall material is present as strands lying side by side, suggesting a breakdown of the multilayered structure (Pl. 5, fig. 13). Septa sometimes have a moth-eaten appearance (Pl. 5, fig. 11) where a lytic enzyme may have been active. Fine strands of material appear to extend across the septum in these areas. Serial sections show that these patches are apparently continuous throughout the septum and are not confined to discrete areas. They also show that the septa are arranged in an irregular manner (Pl. 6, fig. 14 to 21).

The cytoplasmic membrane is approximately 90 to 120 Å thick and bears a fringe of dense material up to 65 Å thick on its outer surface, and thus appears to be asymmetric (Pl. 4, fig. 8). The material on its outer edge is of similar density to the inner edge of the cell wall. The membrane may be slightly infolded at irregular intervals along its length (Pl. 4, fig. 8) or drawn into small vesicles facing the wall (Pl. 5, fig. 12). Mesosomes like those of the normal type were never seen, but sheets of membrane and vesicles were present, usually in association with the wall or with the septa (Pl. 7, fig. 22 to 24). Some of these vesicles appear to contain electron-dense material (Pl. 7, fig. 22, 24).

The nuclear body appears to be dispersed throughout the cytoplasm as fine fibrils in discrete areas of low electron-density (Pl. 7, fig. 25). There is some suggestion that the fibrils of the nuclear body may be dispersed in the form of a reticulum linking the apparently isolated areas of cytoplasm. No connexion of the nuclear body to a membranous structure has been observed.

Appearance when grown in media containing increasing concentrations of sodium chloride. Growth of the mutant in these media produces a number of striking changes. The most obvious of these is an alteration of shape from predominantly round to rod-shaped organisms. At the ultrastructural level, conspicuous changes concern the condensation of the nuclear body, the restoration of orderly division septa and a decrease in the thickness of the cell wall. Membranous structures in the cytoplasm also become more evident. In 0·1 M-NaCl the organisms are still round in shape. The cell wall is thinner but still appears layered. The outer surface of the wall is relatively smooth in outline and less frayed (Pl. 8, fig. 26, 29); the inner edge is again composed of a densely staining zone. The septa are considerably thickened, however, and rarely appear in apposition. Associated with the septa are a number of vesiculate bodies resembling mesosomes (Pl. 8, fig. 27). These structures are occasionally lamellar (Pl. 8, fig. 28) and appear to be free in the cytoplasm or possibly associated with the nuclear body. The latter is composed of bundles of fibrils aligned in parallel array, and thus appears condensed into long strands which are often lobed in appearance (Pl. 8, fig. 26). Different bacteria from the same sample show varying degrees of condensation of the nuclear body. Surrounding the nuclear body and scattered throughout the cytoplasm are areas of material most of which were amorphous though some appeared crystalline.

In 0·2 M-NaCl many of the bacteria were round but a few were rod-shaped. The wall was smooth in outline and evidence of layering was less apparent (Pl. 9, fig. 30). Between dividing bacteria the septum appeared to be chewed away, more particularly

at the middle and ends (Pl. 9, fig. 30). Few mesosome-like structures were seen in these bacteria. The nuclear body occupied a median position and was highly condensed. Unlike the bacteria grown in 0·1 M-NaCl, the nuclear body was more usually oval not lobed in outline. The fibrils were whorled in appearance and seemed clumped at certain positions, which may represent transverse sections of the fibrils. Amorphous material was again present throughout the cytoplasm.

The bacteria in 0·4 M-NaCl closely resembled those grown in media supplemented with 0·2 M-NaCl. In many bacteria, the nuclear body was surrounded by a distinct ring of cytoplasmic material containing ribosomes (Pl. 9, fig. 31) or fibrillar material (Pl. 9, fig. 32). In some sections plugs of cytoplasmic material appeared in the middle of the nuclear body, and the latter appeared to be woven around them (Pl. 9, fig. 31). The fibrils of the nuclear body were again aggregated at a number of points (Pl. 9, fig. 31). Peripheral to the cytoplasm, and within the plugs, were zones of amorphous material, and lamellar membranous structures were occasionally present in the cytoplasm.

Bacteria grown in 0·8 M-NaCl presented a complex array of variation in shape, cell wall structure and position of the nuclear body. Examination of a large number of sections suggested that these variants could be classified into three types. The most usual variant was rod-shaped, with orderly division septa and a highly condensed nuclear body (Pl. 10, fig. 33 to 34). The cell wall was approximately 270 Å wide and was much thickened at the septa and at the poles of the cell. At these positions the wall appeared to be layered and fibrous in texture (Pl. 11, fig. 44); the wall was frayed at some points along its length. Invaginations of the cytoplasmic membrane were associated with the walls and septa, but did not resemble normal mesosomes. Material of similar electron-density to the wall was present within these infoldings (Pl. 11, fig. 42; Pl. 12, fig. 48). Protrusions of the membrane near the septa were sometimes present (Pl. 11, fig. 42). The nuclear body was median in position and highly condensed, the fibrils appearing coarsely aggregated into bundles (Pl. 10, fig. 36). In some cells the DNA was less aggregated, but patches of amorphous material were lying scattered in the nuclear region. When present in the cytoplasm the amorphous material was in discrete crystalline regions with a repeat period of 56·7 Å (Pl. 11, fig. 43). The ribosomes were scattered throughout the cytoplasm. Certain bizarre organisms, highly irregular in shape but possessing the above characteristics, were sometimes present (Pl. 10, fig. 35).

The second type of variant was a rod-shaped organism bounded by a wall of very variable thickness and rarely possessing division septa (Pl. 10, fig. 37, 38). In this variant the nuclear body was not as condensed as in the above organisms but appeared to be closely associated with, or possibly linked to, the cytoplasmic membrane (Pl. 10, fig. 37, 38). The membrane was infolded and the pockets contained electron-dense material. The cytoplasm was restricted to a ring around the nuclear body by patches of amorphous material (Pl. 11, fig. 40). The third category included spherical bodies resembling protoplasts and spheroplasts. The latter type were continuously bounded by a thin layer of wall, or the wall was present only as short strips. In those resembling protoplasts the outer edge of the cytoplasmic membrane bore a fringe of dense material 35 to 70 Å wide (Pl. 10, fig. 36, 39). Many of these forms appeared to be lysing. The nuclear body was closely associated with the surface membrane (Pl. 10, fig. 36). Occasionally scattered patches of amorphous material were present in the cytoplasm.

Associated with bacteria grown in 0·8 M-NaCl were many vesicular membranous

bodies, pieces of wall material and strands of nuclear bodies (Pl. 10, fig. 33, 34). Negative-staining of *Bacillus subtilis* and *B. licheniformis* confirmed the presence of membranous and amorphous material associated with the organisms. The origin of the vesicles was uncertain. In some organisms vesicles could be seen closely associated with the cell wall in the region of the septum (Pl. 11, fig. 41). It is not known whether the vesicles actually emerged from the septum, but the wall tended to be frayed at these points.

All the organisms examined from samples grown in M-NaCl appeared to be rod-shaped, with some tendency to form filaments (Pl. 12, fig. 45, 46). The septa and poles of the cells possessed thickened clumps of wall (Pl. 12, fig. 49). The nuclear bodies appeared to be more aggregated than those of previously described samples. Associated with the wall were invaginations of the cytoplasmic membrane containing vesicular

Table 6. *Bacillus subtilis: the growth of rod mutants on solid media*

The abbreviations for the names of media are: MM = minimal salts medium + glucose + tryptophan + Mg^{2+}; CH = acid hydrolysed casein (Difco); CH/S/C = 1 % casein hydrolysate medium (see Janczura, Perkins & Rogers, 1960); CCY = acid hydrolysed casein, tryptic hydrolysed casein and yeast extract (see Gladstone & Fildes, 1940); HW = Hedley–Wright nutrient agar (Wright, 1933); YE = minimal medium + yeast extract. The symbols in the Table are: o = round in form; ○ = fat rod; — = normal rod; ō = rods and round.

		Medium					
Mutant no.	Class	MM	MM + 0·1 % CH	CH/S/C	CCY	HW	YE
		Morphology					
3	A	o	o o	○	—	—	○
4	A	o	o	—	—	—	—
5a	A?	o	o	o	○	○	o
5b	B	o	o	o	ō	ō	ō
7c	A?	o	o	o	—	—	ō
7d	B	o	o	o	—	o	o
8a	B	o	o	o	ō	o	ō

structures closely resembling mesosomes (Pl. 12, fig. 47). Tubular and vesicular membranes were sometimes present in the vicinity of the organisms (Pl. 12, fig. 49). Flagella were not detected by negative-staining or sectioning in any samples of organisms grown on solidified minimal medium or on this medium + NaCl. Examination of the mutant grown on the minimal liquid medium + 1 % (w/v) casein hydrolysate, when it grew as a rod, showed only well-ordered rods with fairly normal mesosomes and with discrete organization of the DNA.

Growth of the mutants from Bacillus subtilis on richer complex media

Mutants of class A and of class B were streaked on nutrient agar media, Hedley–Wright agar, C.C.Y. + 1 % glucose agar, minimal salts + 0·5 % glucose medium + yeast extract agar, 1 % acid casein hydrolysate + 0·5 % glucose medium (CH/S/C) + tryptophan agar. In all cases good growth was obtained after incubation for 18 hr at 35°. All the mutants showed some tendency to change to rod form in some of the rich

complex media (Table 6). The class-A mutants had less stringent requirements for the change from round form to rod than had class-B mutants. For example, on Hedley–Wright nutrient agar all the A strains grew completely or partially as rods, whereas two of the B strains remained as cocci and only a few rods appeared in the other. Subcultured on two simpler media, these rods grew as cocci. On media containing yeast extract (i.e. C.C.Y.+Y.E.), however, all the mutants tested showed some rods whilst on 1 % casein hydrolysate medium (CH/S/C) only two of the four A mutants were converted.

DISCUSSION

The gross morphological distortion of these mutants grown without additional salt or organic substances appeared to be associated with disturbed septum formation and membrane arrangement. The DNA also seemed to be more disperse than in any pictures of the parent *Bacillus subtilis*. This apparently abnormal distribution of the nuclear material was seen whether the bacteria were fixed with glutaraldehyde or treated by the Ryter–Kellenberger (1958) technique; further work is necessary to establish its significance. The condensation of nuclear material by growing the bacteria in higher salt concentrations was reported by Kellenberger (1960) and this phenomenon was very evident in the present work. Serial sections did not show a connexion between the membrane and the DNA in the round form, although such a connexion can readily be demonstrated in the cells of normal *Bacillus subtilis* and in the mutant grown in high salt concentrations. No mesosomes were found in the round form, although in the rod form shapes which looked like mesosomes were seen. The 'extracellular' membranous bodies in cultures grown in presence of the higher salt concentrations were very reminiscent of the pictures published of staphylococci growing in high salt concentrations (Cripps & Work, 1967) and may be of similar origin.

It would appear possible that the lesions in the *rod* mutants were due to blocks in the biosynthesis of the substance or substances in the rich media. Whatever these substances are, they would appear to be necessary for the orderly construction and division of the bacilli. If this is the true explanation, then the effect of salts might be an osmotic remedial effect like those reported for *Neurospora crassa* (Kuwana, 1961; Metzenberg, 1968) and *Saccharomyces cerevisiae* (Hawthorne & Friis, 1964; Baesel & Douglas, 1968) in which genetic defects were reversed by low molecular weight substances in the growth medium. The mechanism is not known; the application of this hypothesis will be discussed in the succeeding paper (Rogers & McConnell, 1970), where it is shown that one of the mutants (*rod-4*) can be corrected by the presence of glutamate or glutamine in the medium. For this mutant it would appear that the effects of high salt concentration are connected with the availability of this amino acid or its amide, rather than with any effect of the high ionic strength *per se*.

There is complete similarity between the morphological form of the A mutants reported in the present paper and in Rogers *et al.* (1968) and those mutants described by Boylan & Mendelson (1969). Whereas the latter author's organisms rounded up at unfavourable temperatures, ours did so under what were presumably unfavourable nutritional circumstances. It is not without interest that the mutants described here, unlike the parent strain, did not grow at 45° in the absence of salt, although they did so in its presence. This is the same temperature as that at which the mutant studied by Boylan & Mendelson (1969) changed to a coccus. Mutants of *Escherichia*

coli having a spherical form have also been described (Adler, Terry & Hardigree, 1968; Hirota *et al.* 1968; Normark, 1969). So far the physiological basis of these morphological mutants is not clear.

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EXPLANATION OF PLATES

PLATE 1

Appearance of *rod* mutants under the phase-contrast microscope.

Fig. 1. *Bacillus licheniformis* 9945A *met cap* grown on minimal salts + 0·1 % casein hydrolysate agar. × 1800.

Fig. 2. *Bacillus subtilis* 168 *trp* grown on minimal salts agar. × 1400.

Fig. 3. *Bacillus subtilis* 168 *trp* grown on minimal salts agar + 0·8 M-NaCl. × 1400.

PLATE 2

Fig. 4. Low-power electron micrograph of *rod* mutant grown in absence of added NaCl, showing the irregular shape, wall of variable thickness and erratic septa. The nuclear body is dispersed in areas of low electron-density. × 29,754.

PLATE 3

Fig. 5, 6. Mutant grown in absence of added NaCl, showing two organisms of very different relative wall thickness; the wall is layered in fig. 6. Fig. 5: × 46,701; fig. 6: × 60,496.

PLATE 4

Mutant *rod-4* grown without added NaCl.

Fig. 7. Section of a cell of *rod-4* in which there appear to be two layers of wall (a, b) separated by a gap. The arrows point to details enlarged in fig. 9 and 10. × 60,496.

Fig. 8. The inner edge of the cell wall (cw) densely stained, but the outer layer not. The stained edge is of similar density to the fringe of material present on the outer edge of the cytoplasmic membrane (m); f, an infolding of the membrane. × 289,345.

Fig. 9. Portion of the organism shown in fig. 7, showing an inner layer of wall densely stained on inner and outer margins (arrow); the outermost wall stained only on its inner face. × 208,375

Fig. 10. Portion of organism shown in fig. 7, showing irregular thickening on the inner wall; the two faces of this layer are densely stained (black arrows); evidence of layering indicated by white arrows. × 154,601.

PLATE 5

Mutant *rod-4* grown without added NaCl.

Fig. 11. Section of a septum showing areas of possible lytic enzyme activity (s); within these electron-transparent areas are strands of finely fibrillar material (arrows). The edges of the septum are bounded by densely staining zones. × 225,046.

Fig. 12. Enlargement of organism shown in fig. 6, showing presence of vesicular structures in contact with the outer edge of the cytoplasmic membrane (black arrows). The wall is layered (white arrows). × 241,975.

Fig. 13. Section of a wall apparently frayed into four strips (arrows) of similar width but at different stages of breakdown. × 192,897.

PLATE 6

Mutant *rod-4* grown in absence of added NaCl.

Fig. 14 to 21. Part of a sequence of serial sections showing irregular growth of septa (arrows). Note areas of low density within the septa are continuous. × 16,302.

PLATE 7

Mutant *rod-4* grown without added NaCl.

Fig. 22. Mesosome-like structure (white arrows), consisting of electron-dense vesicles associated with the cell wall. The wall is layered (black arrows). × 184,860.

Fig. 23. Mesosome-like structure consisting of large vesicles bounded by unit membranes lying near the junction of cell wall and septum. × 171,405.

Fig. 24. Vesicular mesosome-like structure; two of the vesicles (arrows) contain material of similar density to regions of the cell wall. × 109,305.

Fig. 25. High magnification micrograph showing fibrils of the nuclear body (arrows) linked to cytoplasmic material. × 443,190.

PLATE 8

Mutant *rod-4* grown in presence of 0·1 M-NaCl.

Fig. 26. Section of a pair of dividing organisms, both round in shape. Nuclear body of the organism on the left is condensed and lobed (white arrows). A mesosome-like structure is present on the right (black arrows). × 40,863.

Fig. 27. Enlargement of vesicular mesosome-like structure shown in fig. 26. × 143,952.

Fig. 28. Lamellar mesosome-like structure present in association with the nuclear body. × 195,000.

Fig. 29. Section of a rod-shaped dividing organism. Note apposition of septa and condensed lobes of nuclear body. The wall is relatively smooth in outline but thickened at some points. × 54,600.

PLATE 9

Mutant *rod-4* grown in presence of 0·2 M-NaCl (fig. 30) and 0·4 M-NaCl (fig. 31, 32).

Fig. 30. Section of a pair of dividing organisms; septum frayed at the ends and at middle. × 52,000.

Fig. 31. The nuclear body condensed into a ring around a core of cytoplasmic material containing ribosomes (r). Similar cytoplasmic material (r) present at outer boundary of nucleus. The fibrils of the latter are aggregated at several points (arrows). A membranous structure (m) is present in the cytoplasm. × 104,000.

Fig. 32. The nuclear body is surrounded by fibrillar material (f) external to which are clumps of amorphous material (a). × 72,800.

PLATE 10

Mutant *rod-4* grown in presence of 0·8 M-NaCl.

Fig. 33 to 34. Sections of rod-shaped organisms bounded by a wall of irregular thickness and containing condensed nuclear bodies. × 36,400; × 14,364.

Fig. 35. Irregularly shaped cell containing condensed nuclear bodies the fibrils of which are coarsely aggregated; wall much thickened in places. × 36,400.

Fig. 36. Protoplast-like organism, with nuclear body in contact with membrane (arrow). × 36,400.

Fig. 37 to 38. Thin-walled organisms without division septa, and nuclear body in contact with membrane (arrows). × 47,052; × 36,400.

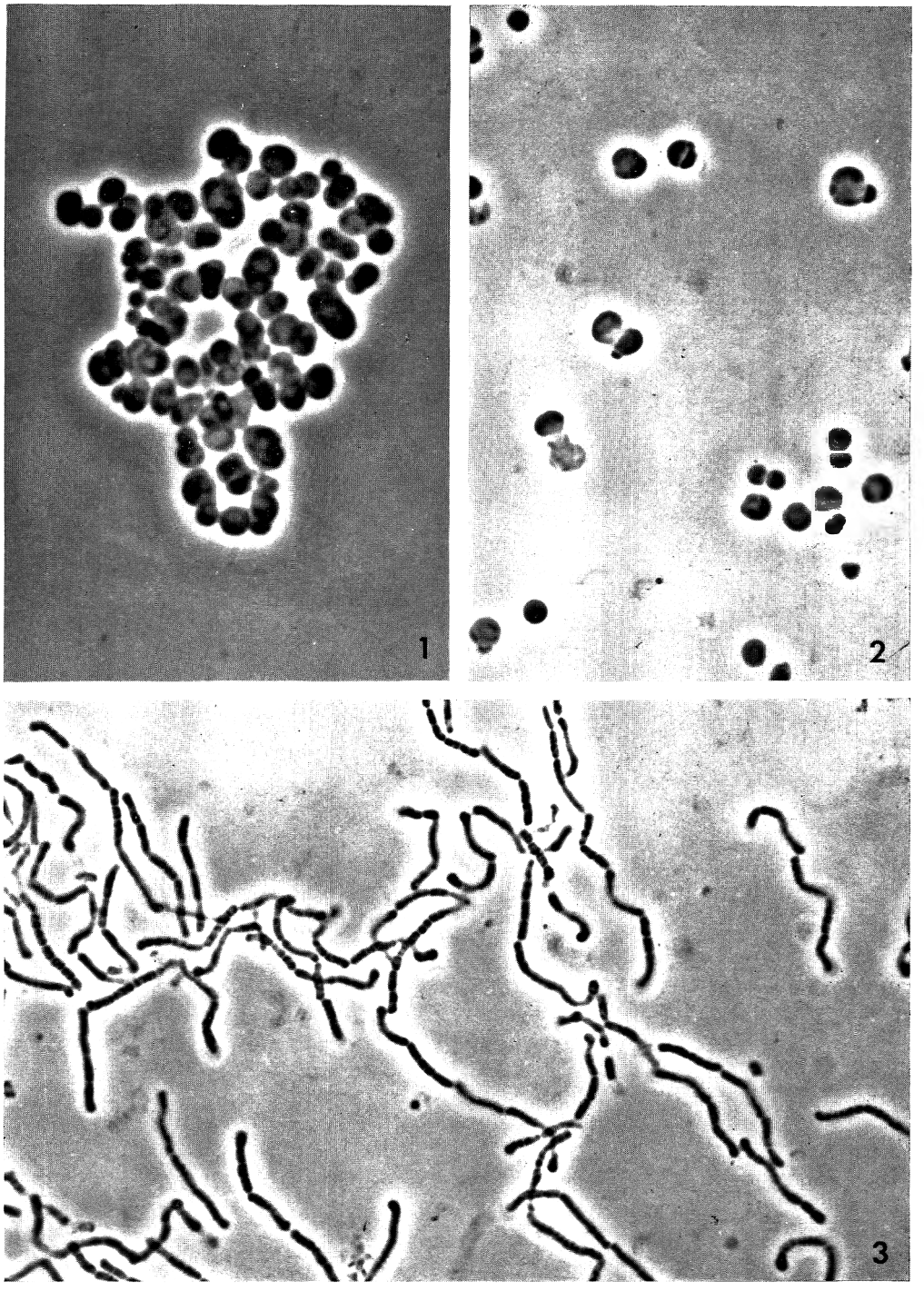
Fig. 39. Enlargement of part of fig. 36, showing presence of a dense fringe on the outer face of the cytoplasmic membrane of the protoplast-like cell (a). This fringe is also present on the membrane of the cell bounded by a wall (b). × 213,200.

PLATE 11

Mutant *rod-4* grown in presence of 0·8 M-NaCl.

Fig. 40. Section of a nuclear body, fibrils aggregated (arrows). Around the nucleus is a ring of fibrillar cytoplasmic material (f), around which is a zone of amorphous material (a). × 169,000.

Fig. 41. Section of a septum (s) showing presence of vesicles (v) linked with surface or confined to base of septum. × 269,093.



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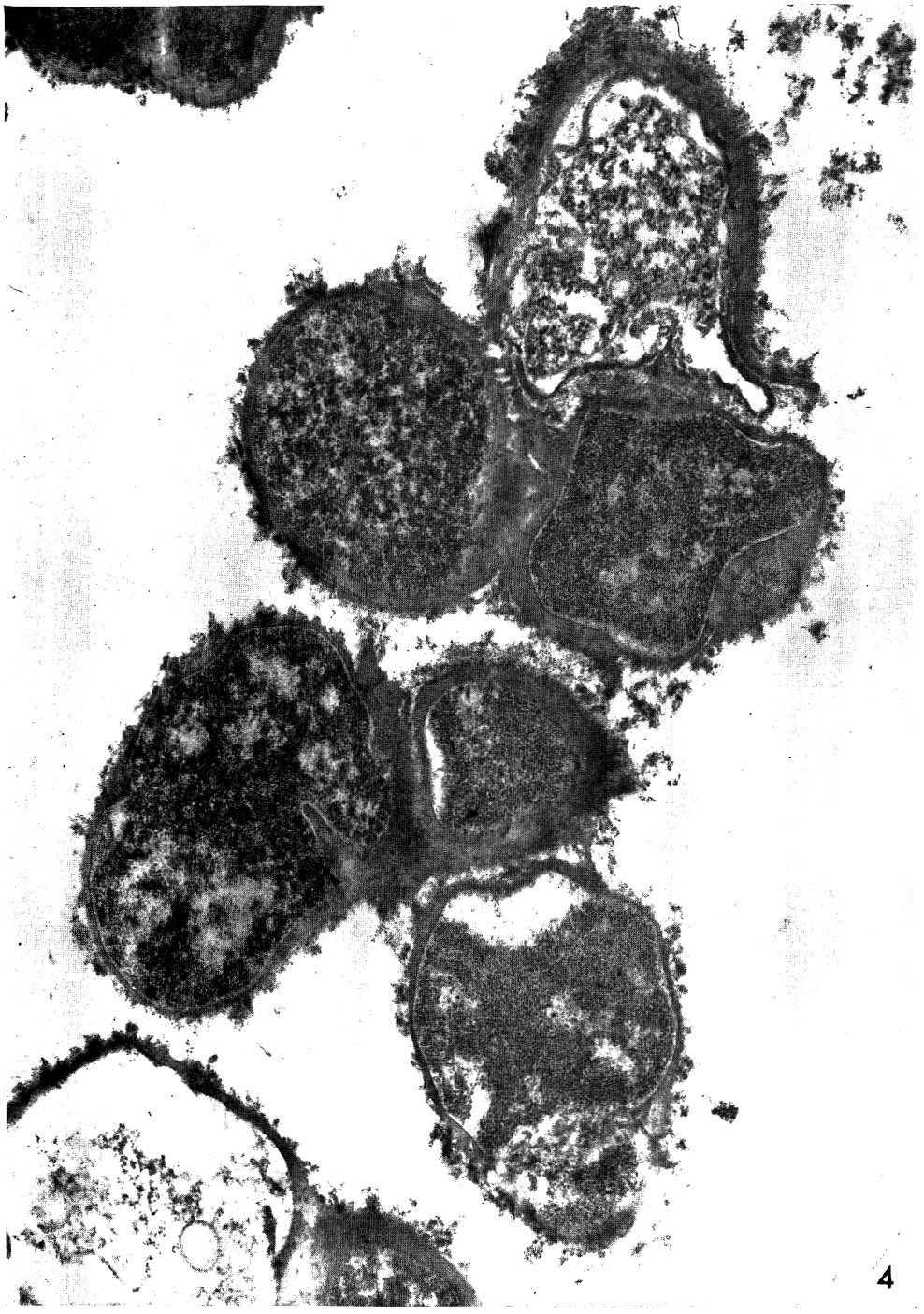

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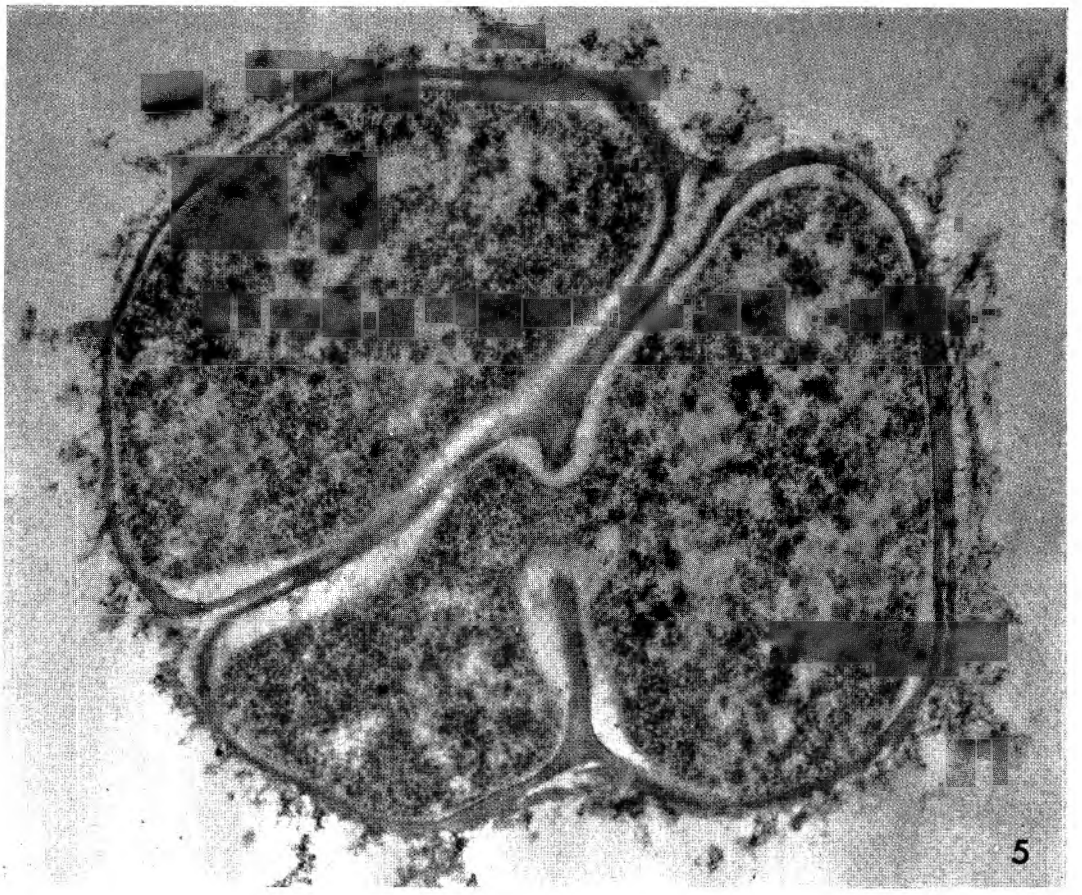

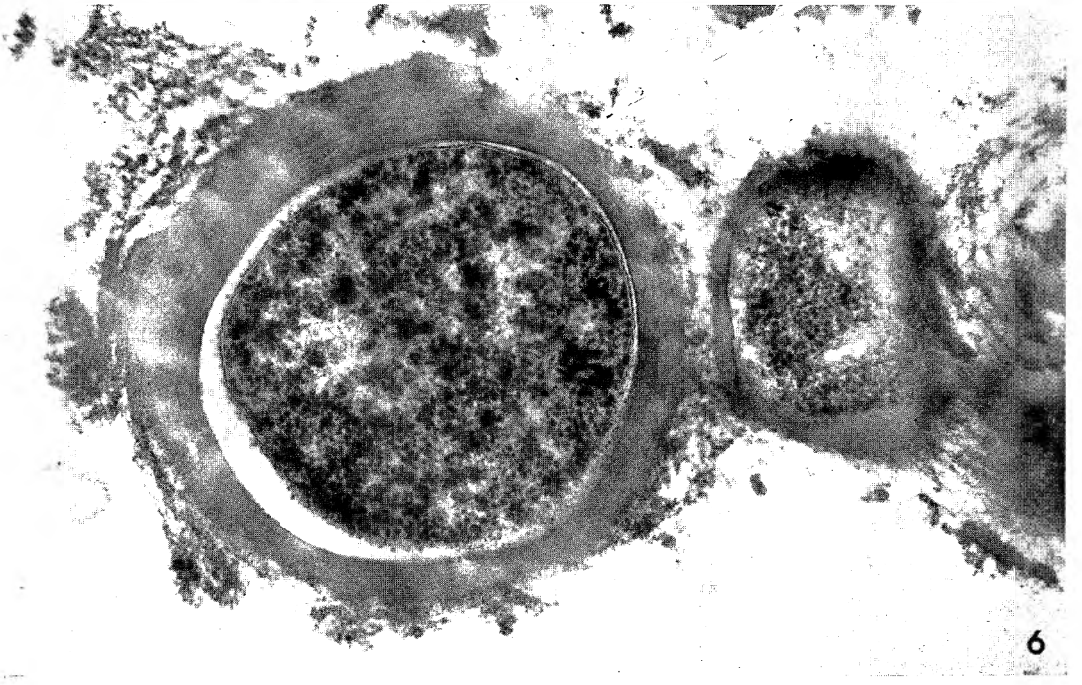

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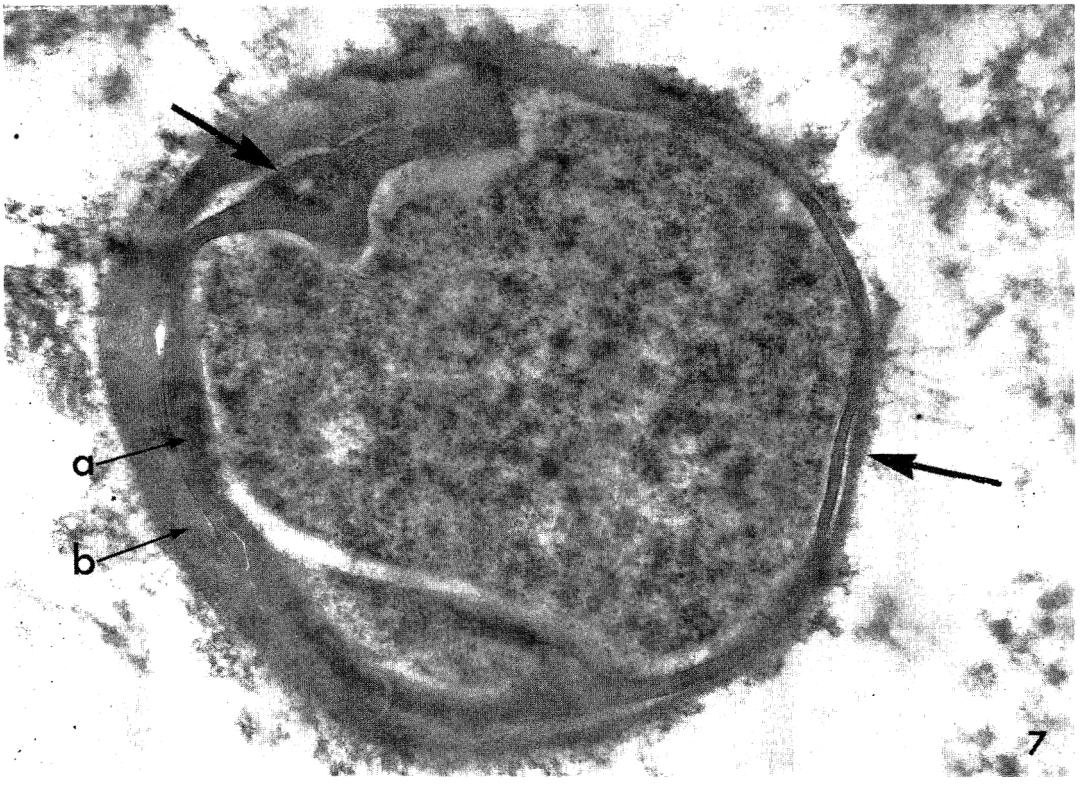

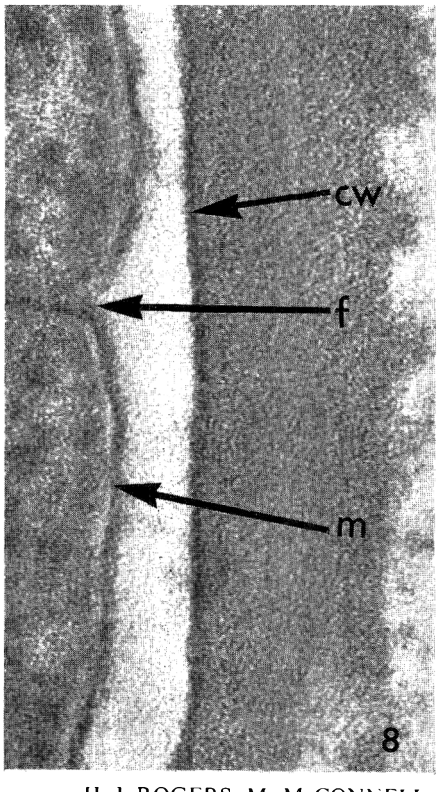

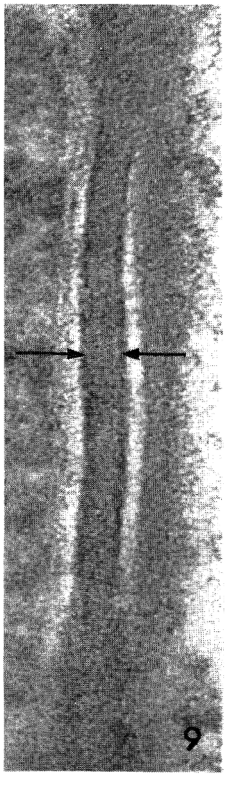

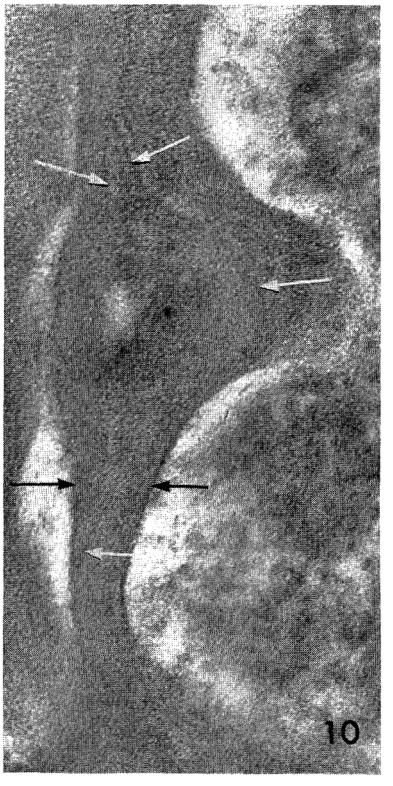

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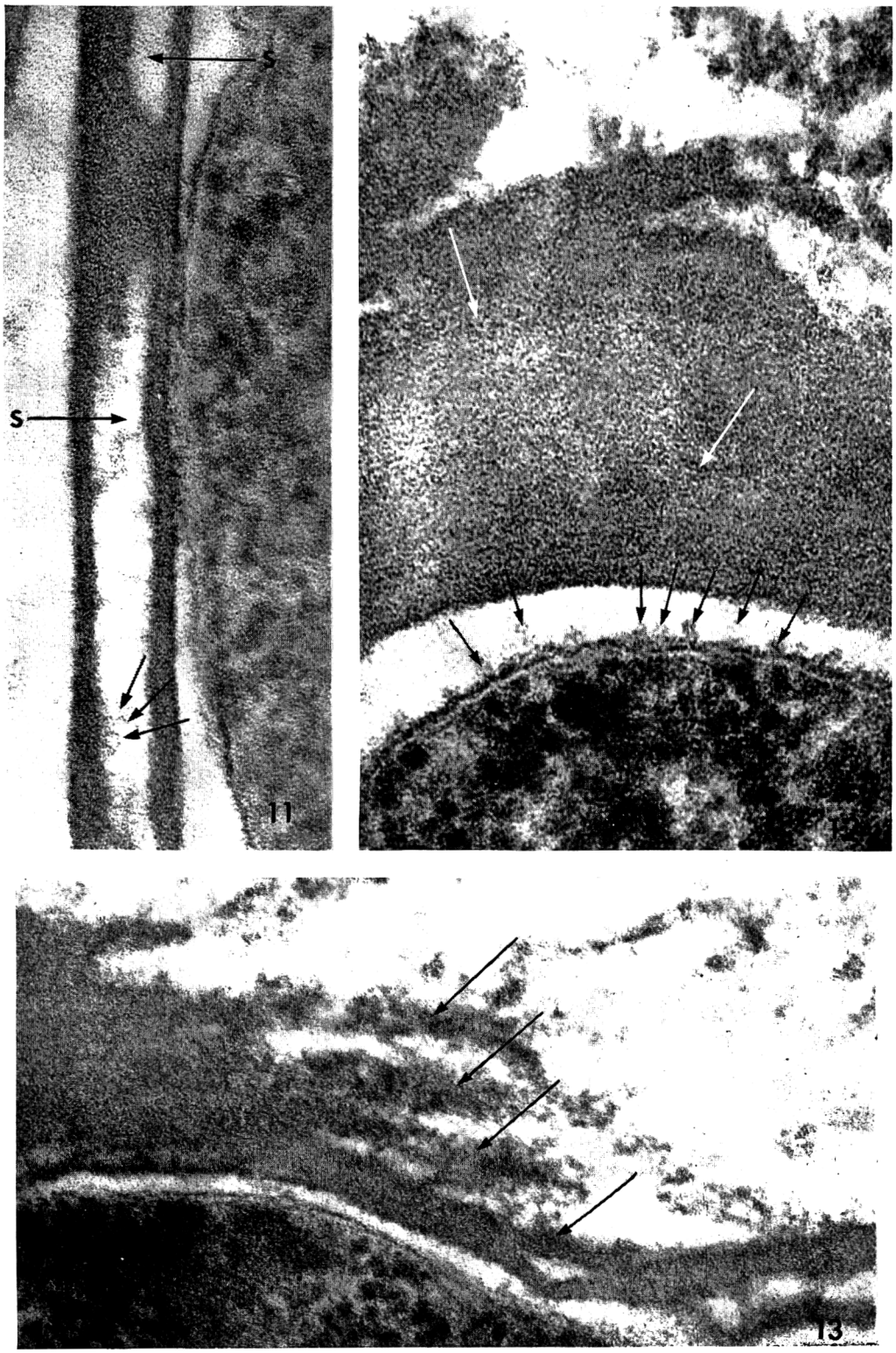

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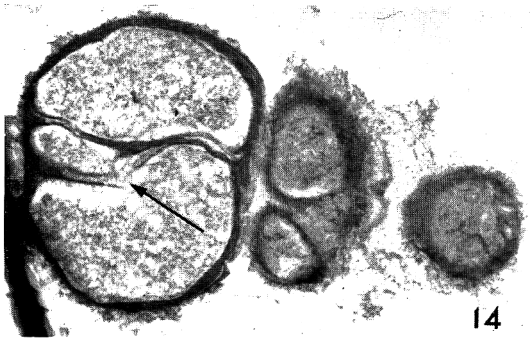
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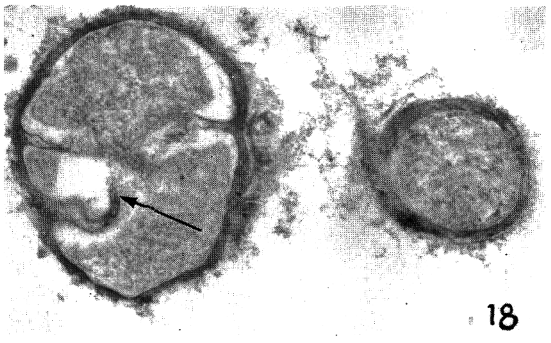
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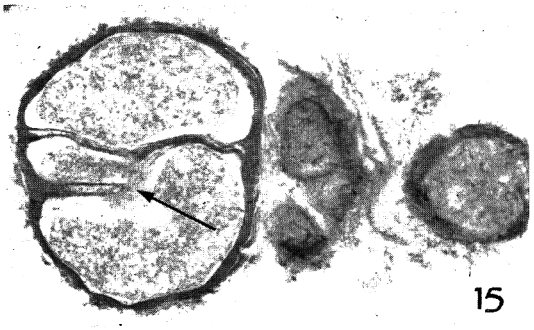
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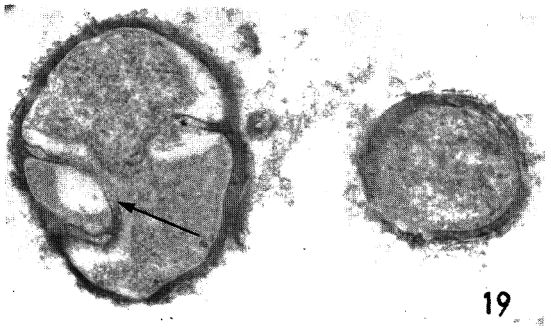
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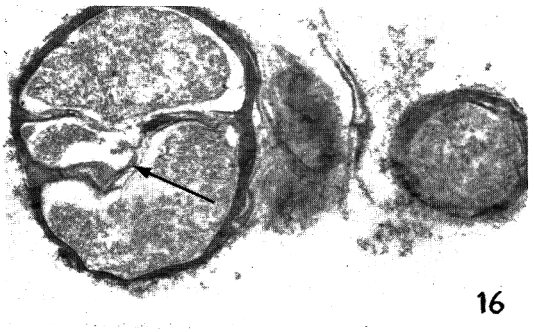
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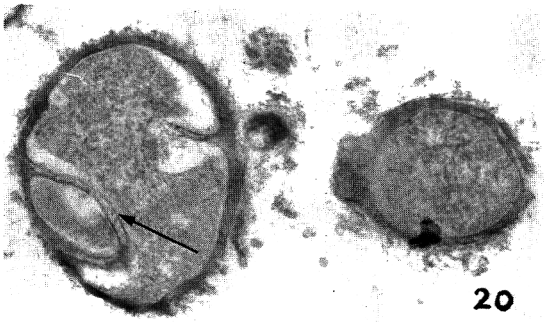
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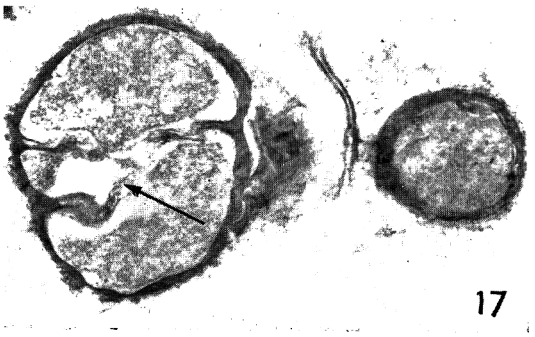
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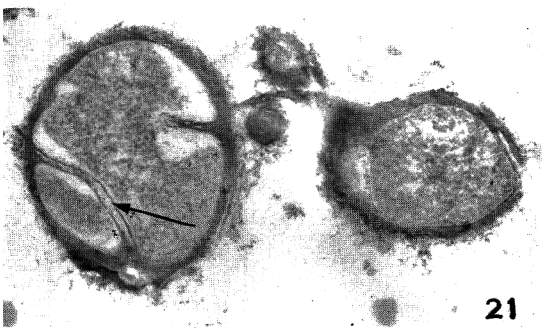
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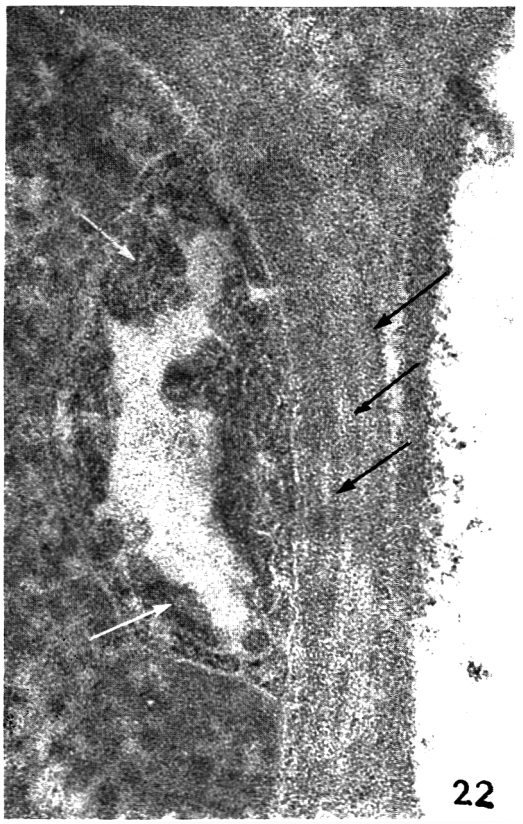
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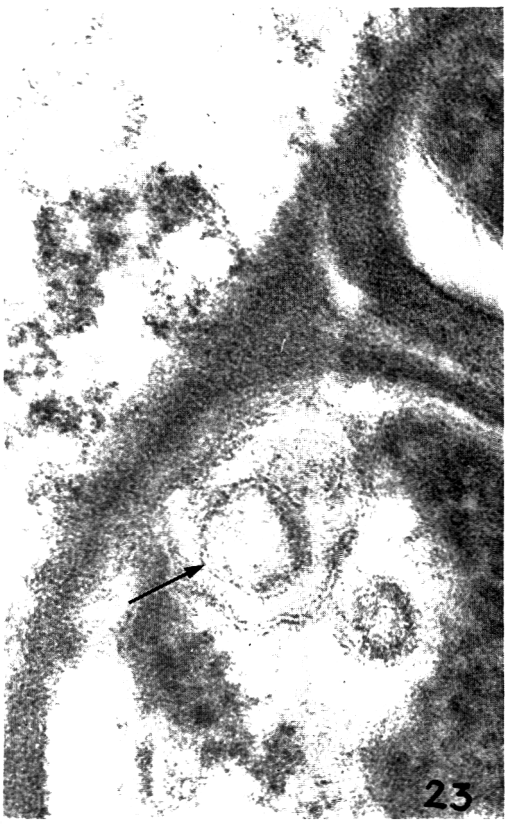
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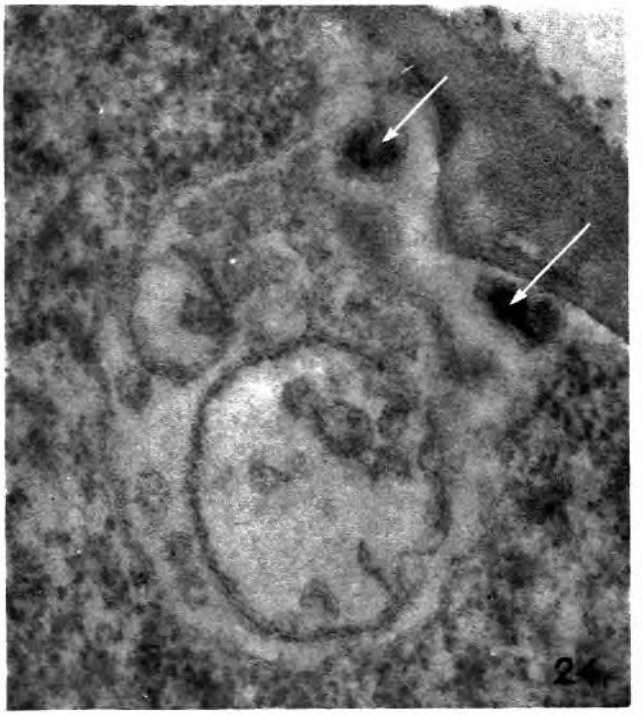
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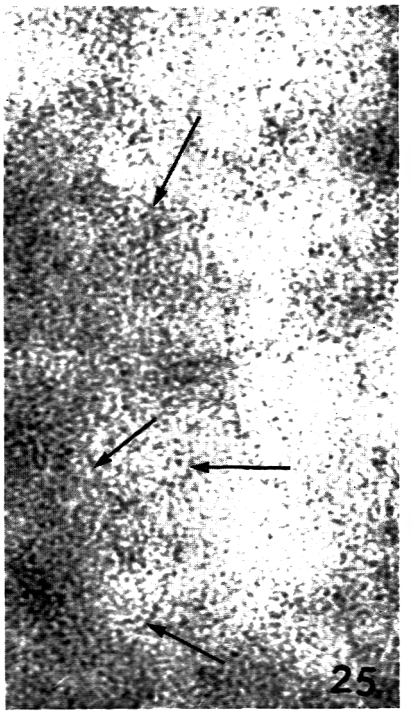
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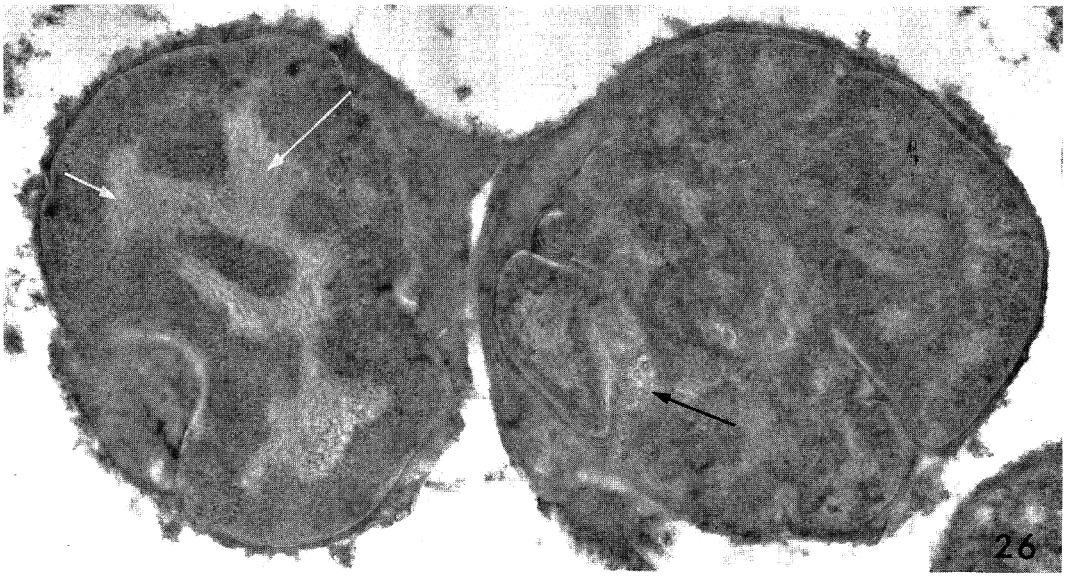
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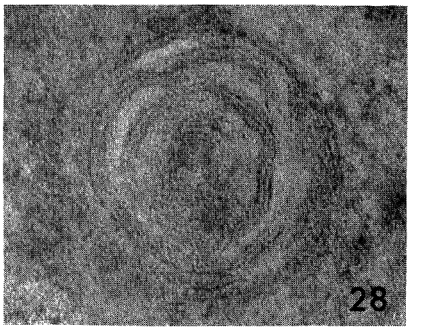
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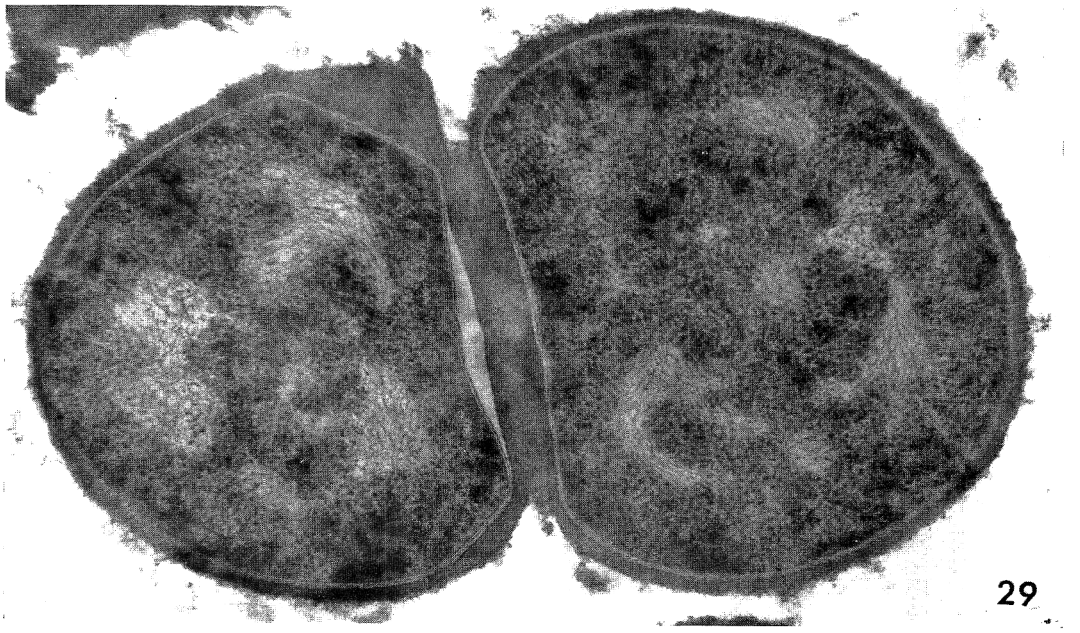
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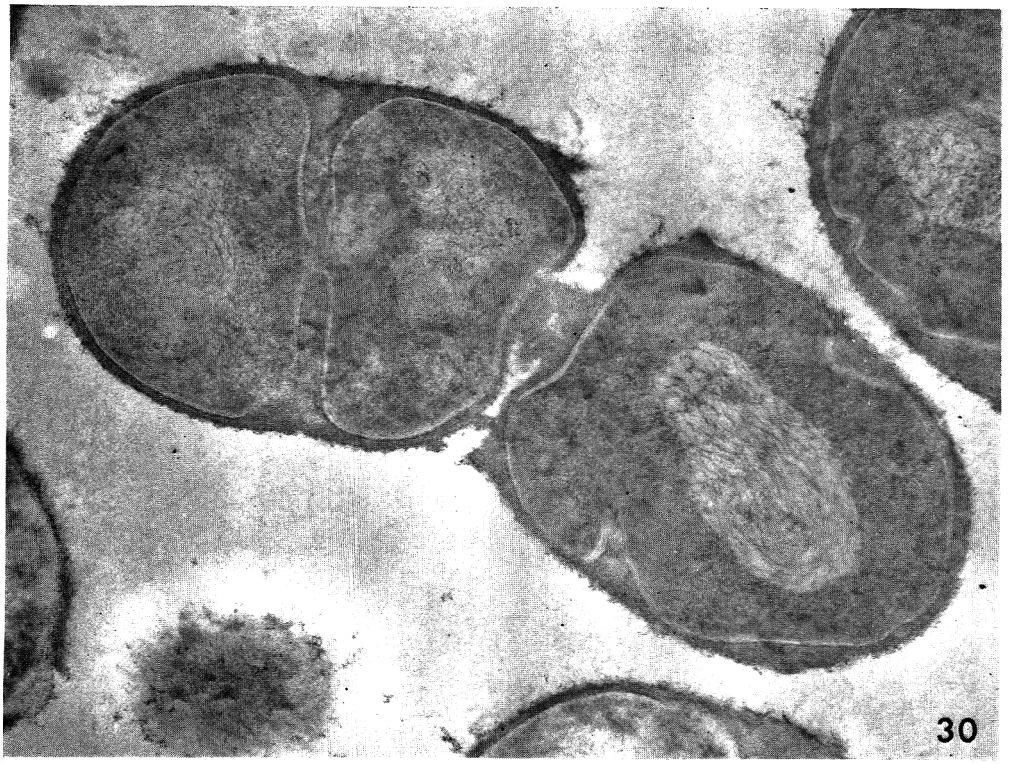

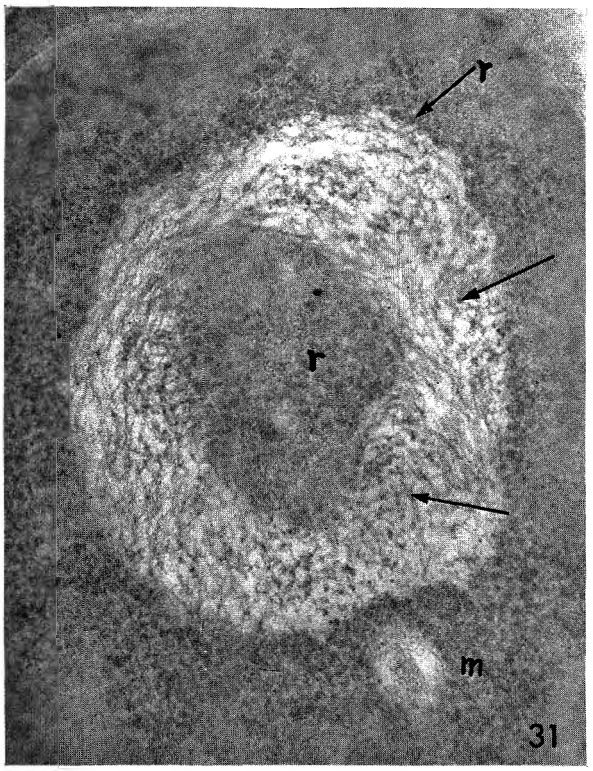

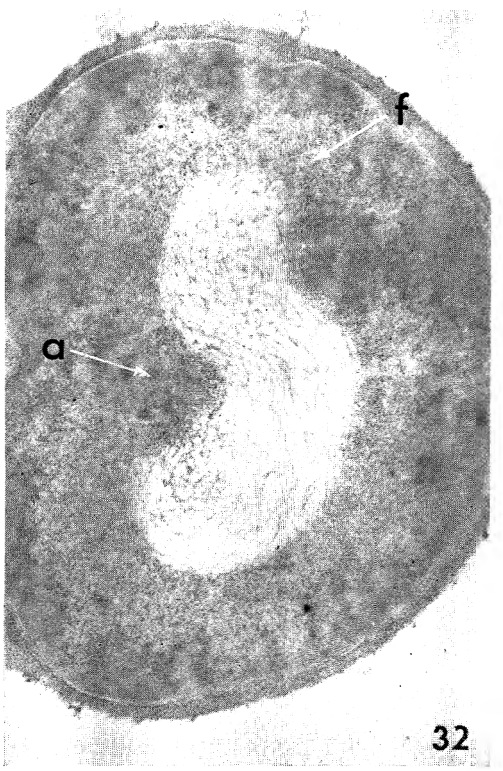

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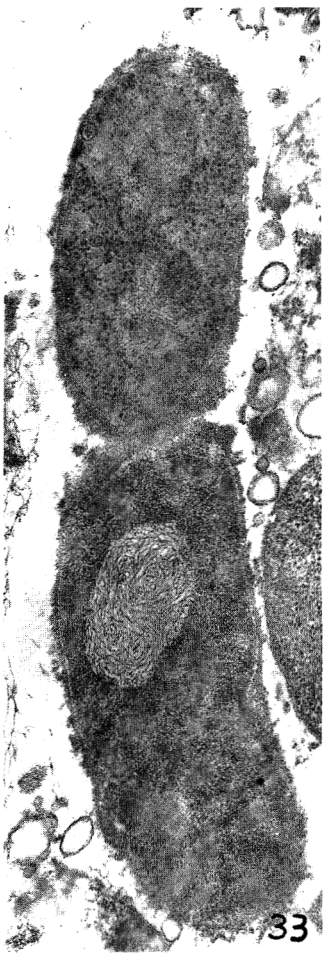

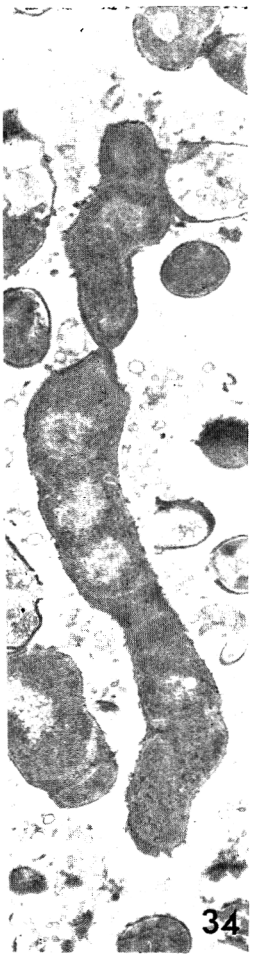

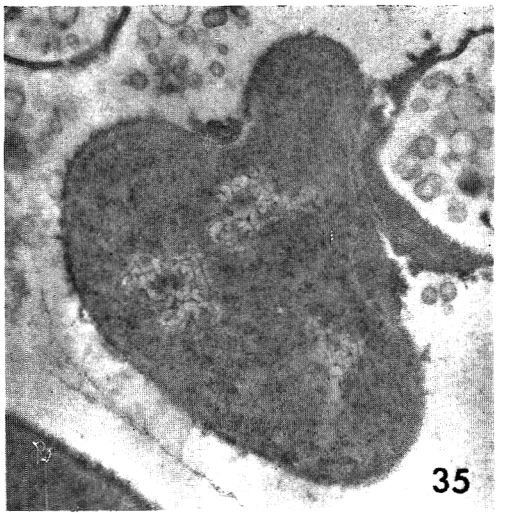

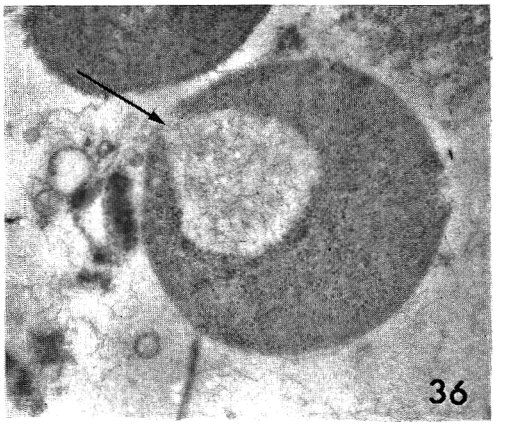

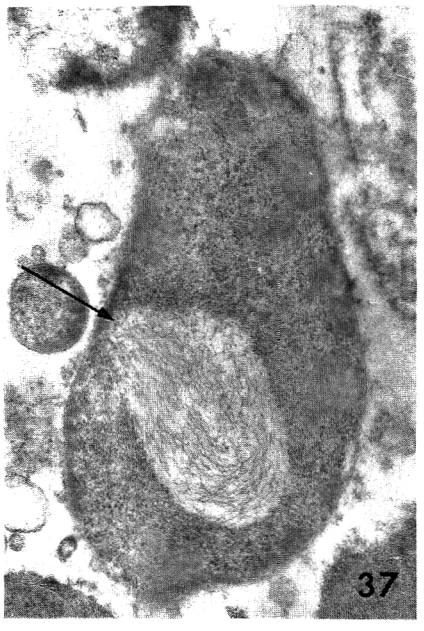

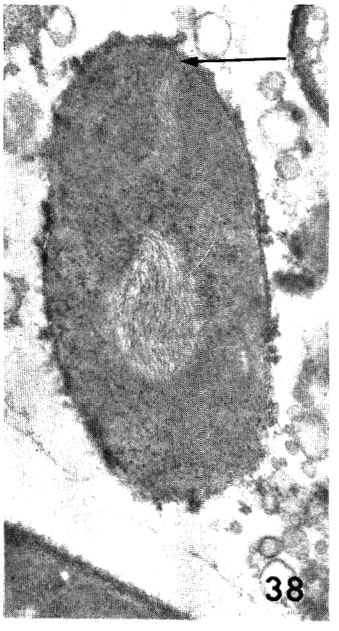

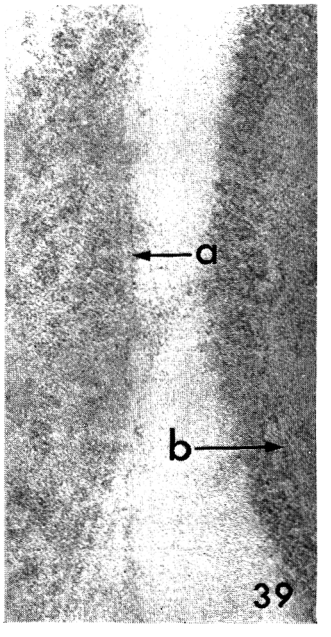

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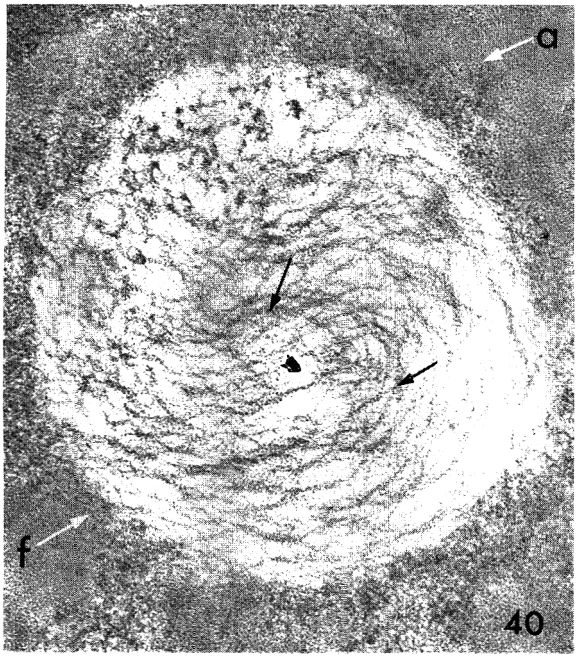
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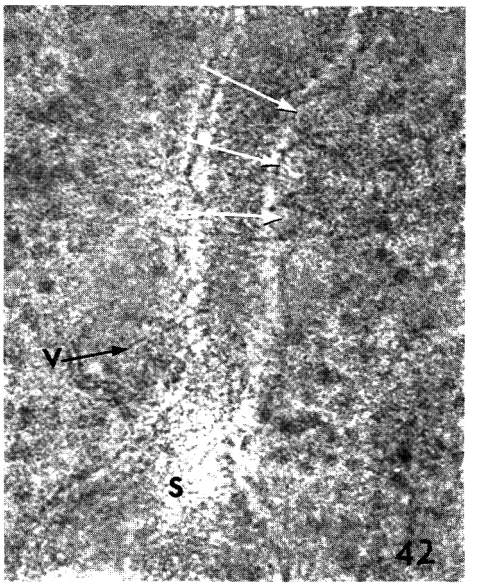
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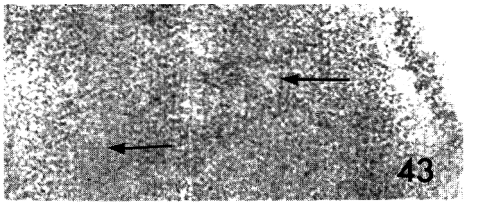
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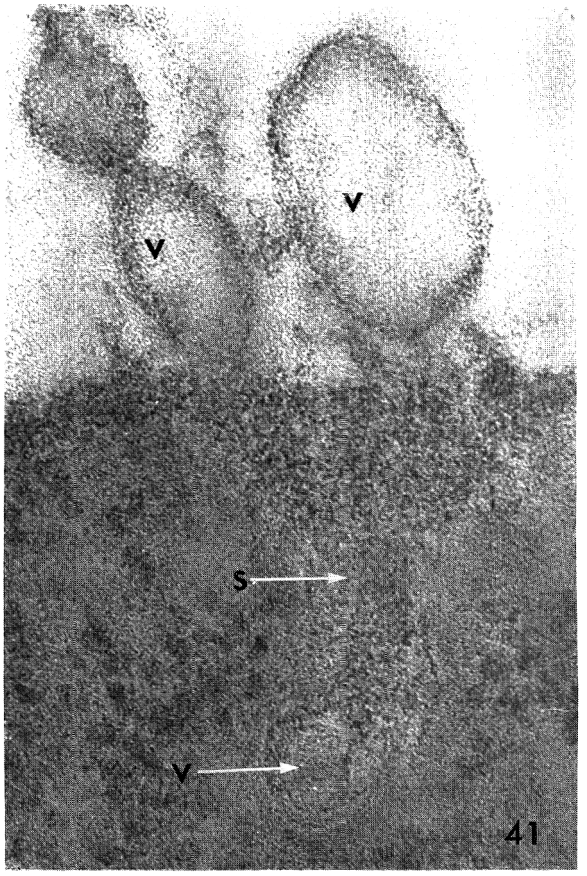
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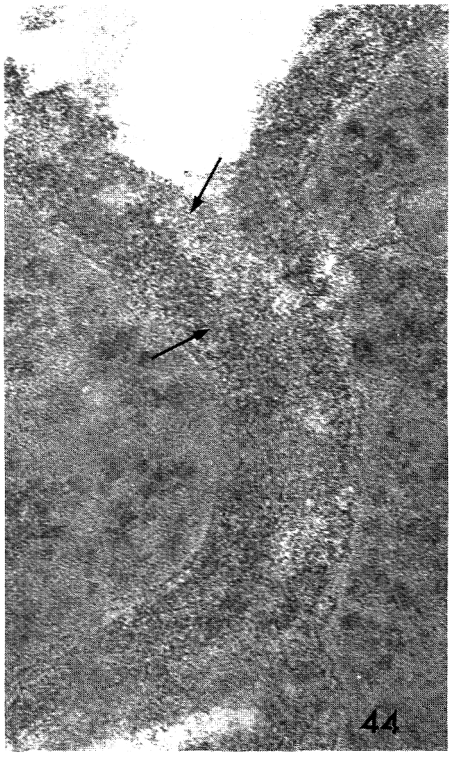
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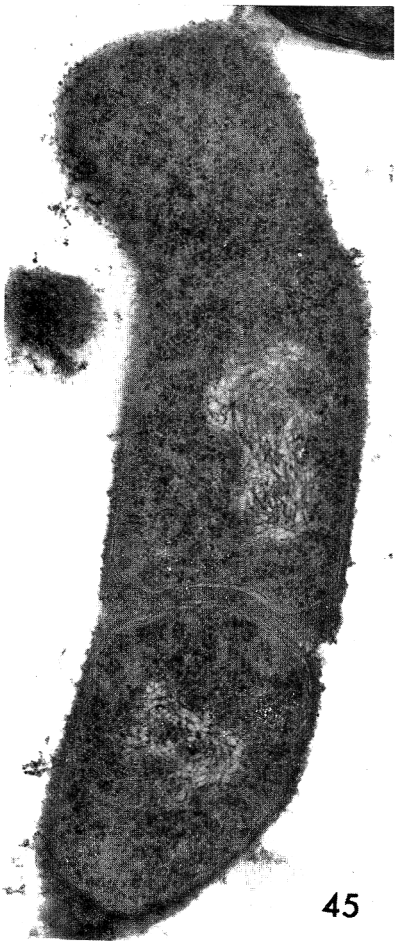
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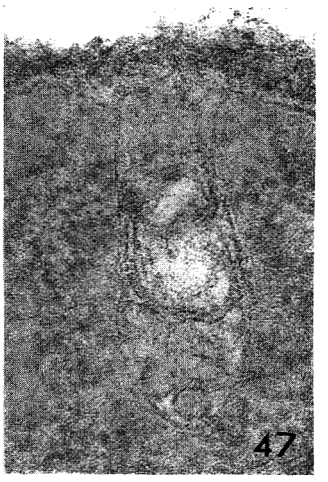
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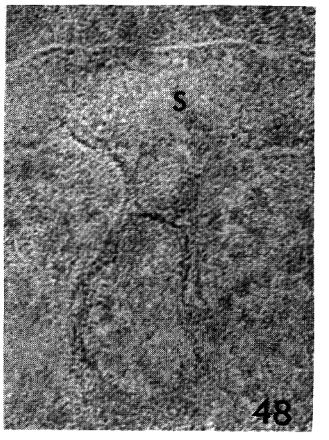
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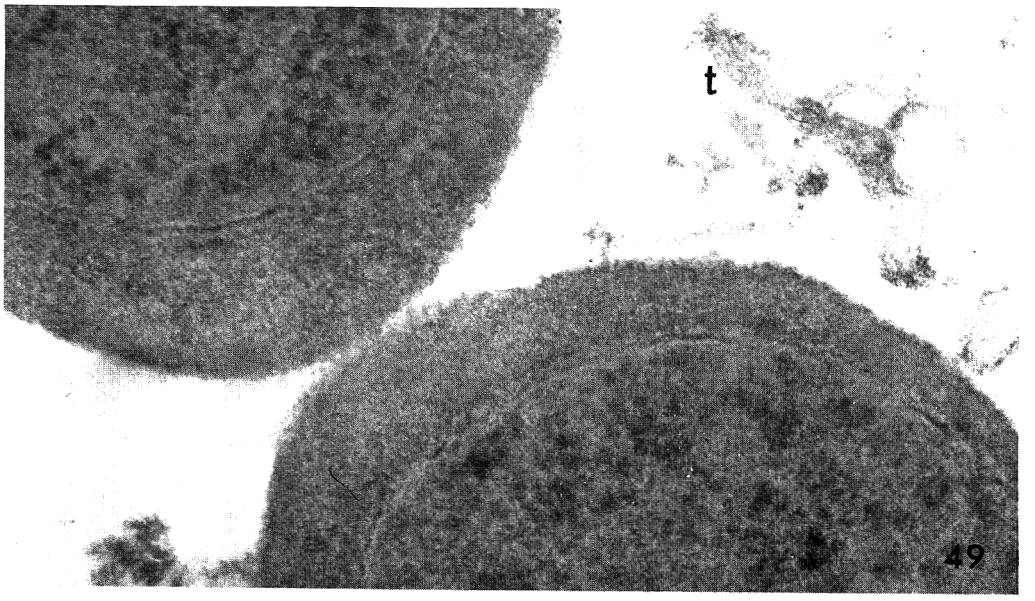
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Fig. 42. Section of a septum (s) showing infoldings of cytoplasmic membrane as vesicles (v) or as protrusions (white arrows). The vesicle contains material of similar density to the septum. Note thickened base of septum. × 176,000.

Fig. 43. Section of a crystalline region showing a marked periodicity (arrows). × 176,000.

Fig. 44. Section of a septum separating a pair of dividing organisms; septum composed of two zones of different density (arrows). × 169,000.

PLATE 12

Mutant *rod-4* grown in 0·8 M-NaCl (fig. 48) and 1 M-NaCl (fig. 45 to 47, 49).

Fig. 45 to 46. Rod-shaped organisms with thickened septa and highly condensed nuclei. × 42,809; × 27,242.

Fig. 47. Mesosome-like structure consisting of large vesicles within an infolding of the cytoplasmic membrane. × 135,200.

Fig. 48. Vesicular structures within an infolding of cytoplasmic membrane and in association with a septum (s). × 174,766.

Fig. 49. Section of thickened walls between a pair of organisms; associated with the organisms are tubular and vesicular membranes (t). × 157,316.

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With 1 *plate*
Printed in Great Britain

The Role of L-Glutamine in the Phenotypic Change of a *rod* Mutant Derived from *Bacillus subtilis* 168

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(*Accepted for publication 24 December 1969*)

SUMMARY

The morphological mutant *rod-4* derived from *Bacillus subtilis* 168 *trp* can be changed from a round form to a rod by the addition to the growth medium of sufficient acid-hydrolysate of casein. The hydrolysate can be replaced by a mixture of amino acids, and the only individual amino acids giving similar results are L-glutamic acid, L-proline, L-arginine and L-ornithine. Since the lag in the action of L-glutamate was less than for the other amino acids, this amino acid is likely to be responsible for the effect of the mixture. Experiments with the L-glutamine analogue, γ-L-glutamylhydrazide, strongly suggest that L-glutamine is the active metabolite rather than the amino acid itself. The correcting effect of high ionic strengths of the growth medium on the morphology of this mutant seems to be mostly due to the increased effectiveness of L-glutamate or L-glutamine in the presence of high concentrations of salts.

INTRODUCTION

In previous papers (Rogers, McConnell & Burdett, 1968, 1970) the isolation and characterization of *rod* mutants from strains of *Bacillus subtilis* and *B. licheniformis* were described. It was shown that the phenotypic transformation from a collection of coccal bodies to rods could be effected in one class of mutant, either by high ionic strength of the medium or by an increase in its organic-N content. The present paper examines the nature of the metabolite present in the richer medium which is necessary for the transformation of one of the mutants, *rod-4*, the most easily transformed of the mutants studied.

METHODS

Micro-organisms. The mutant *rod-4* derived from *Bacillus subtilis* 168 *trp* was stored at room temperature in the dried state and was revived as required by mixing with minimal liquid medium and inoculating a 0·1 % (w/v) casein hydrolysate + salts + glucose agar plate with a suspension. The mutant was subcultured every few days on the 0·1 % casein hydrolysate + salts + glucose agar medium previously described (Rogers *et al.* 1970); after overnight incubation at 35° the plates were stored at room temperature. The parent strain *B. subtilis* 168 *trp* was stored as a spore suspension.

Media. The basal liquid minimal medium was as previously described (Rogers *et al.* 1970). The acid-hydrolysed casein used was the dried Difco product.

Chemicals. The L-amino acids used were obtained from British Drug Houses and were shown to be chromatographically homogeneous when examined by one-dimensional paper chromatography with butanol + acetic acid + water (63 + 10 + 27 by vol.,

upper phase) as solvent. Two specimens of γ-L-glutamylhydrazide were used. One was a specimen synthesized and given us by Dr H. R. Perkins (Chemistry Division, this Institute); the other was obtained from the Mann Research Laboratories, New York, U.S.A.

RESULTS

The effect of acid hydrolysed casein on the morphology

Liquid minimal medium was supplemented with increasing concentrations of casein hydrolysate and inoculated with a loopful of a suspension of *rod-4* bacteria. The suspension was made by taking 3 or 4 colonies from an overnight culture of *rod-4* grown on the surface of the usual 0·1 % (w/v) casein hydrolysate + salts + glucose agar medium and emulsifying them in the minimal liquid medium. The cultures were incubated overnight at 35° with shaking. At concentrations above about 0·2 % casein hydrolysate the bacteria appeared as rods, whilst at lower concentrations most of the bacteria were round in form (Table 1). The acid-hydrolysed casein could be replaced at about the same concentration by a mixture of L-amino acids (Table 1); the relative concentrations of the amino acids in the mixture were about the same as those present in casein. It thus appeared that one or more amino acids were responsible for the morphological change.

Table 1. *Bacillus subtilis 168: effect of increasing concentrations of casein hydrolysate and amino acid mixtures on the morphology of rod-4*

The cultures (10 ml.) were incubated for 18 hr at 35° shaken in 50 ml. flasks. The basal minimal medium was as described by Rogers *et al.* (1970).

Concentration of casein hydrolysate (%)	Morphology	
	In casein hydrolysate	In amino acid mixture
0	Round	Round
0·05	Round	Round
0·1	Round	Round and oval
0·2	Short rods	Long rods
0·5	Short and long rods	Long rods

On repeated tests of the lower concentrations of casein hydrolysate or amino acid mixture, considerable variability was found in the morphology of the bacteria when cultures were first examined at 18 hr. Occasionally the bacteria appeared as rods at this time, but on further incubation (up to 48 hr) as round or oval forms. Examination every few hours after inoculation showed that a definite cycle of events frequently occurred in liquid culture, and always on the surface of solid media. Groups of the round forms first threw out filaments consisting of cells of five or ten times the length of the cells in the parent culture. These filaments extended in length to a degree which was related to the concentration of casein hydrolysate or amino acid mixture in the growth medium. Plate 1 shows such filaments photographed as part of a time-lapse film of the process (the film was made in collaboration with Mr M. R. Young of this Institute). In liquid medium very long filaments were occasionally not produced, but rods were broken off while the filaments were still quite short. This difference appeared to be correlated with the degree of motility of the resultant rods: when they were motile, long filaments were sometimes not formed. Eventually the extension of the

chains of very long forms slowed or stopped and the forms divided and separated to give rods. When very low concentrations of amino acids were originally present in the medium, swellings appeared in the filaments and round forms appeared.

Analysis of the effects of individual amino acids

Since an amino acid mixture could substitute for acid-hydrolysed casein, the possibility that one particular amino acid or metabolically related group of amino acids was responsible for converting the round forms through filaments to rods was tested. The examination of cultures at a single time interval was likely to be unreliable,

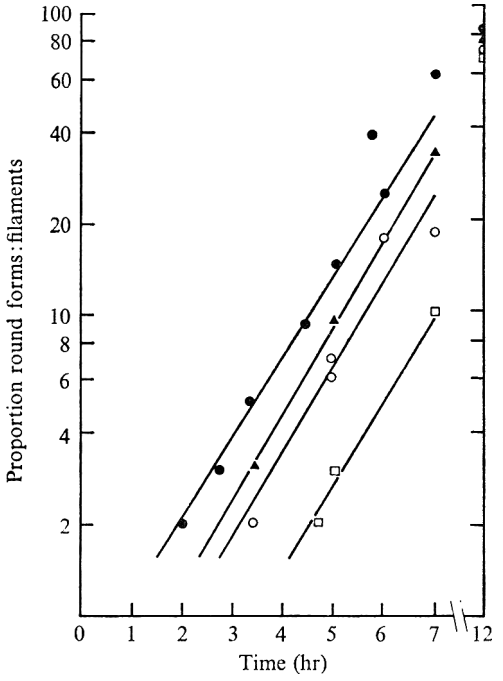

Fig. 1. The effect of L-amino acids on the appearance of filaments. The minimal liquid medium (10 ml. in 50 ml. flasks) supplemented with 0·2% of L-amino acids and inoculated with 0·5 ml. of a suspension of the *rod-4* mutant. The inoculum was prepared by suspending the growth from an 18 hr culture on a 2·5 in. Petri dish of the minimal + 0·1% casein hydrolysate + glucose agar medium (Rogers *et al.* 1970). The suspension (10 ml.) was centrifuged and the deposited bacteria washed once and then suspended in 5·0 ml. minimal medium. The original suspending fluid and washing fluids were minimal liquid medium. Such an inoculum gave a final concentration equivalent to 10^5 viable units/ml. culture. Incubation was at 35° with shaking. ●——●, Glutamic acid; ▲——▲, ornithine; ○——○, proline; □——□, arginine.

in the absence of knowledge of both the time and the concentration for any amino acid required for the filaments to divide into recognizable rods. The following technique was therefore used: flasks containing the basal liquid minimal medium were supplemented with the various L-amino acids, heavily inoculated and incubation continued, loopfuls of the culture being removed at intervals of a few hours. These samples were examined with the phase-contrast microscope (× 200) and the number

of groups of round forms showing the beginning of filament formation was measured as a percentage of the total. One hundred or more groups of organisms were counted for each sample. The increase in this proportion by using group A amino acids appeared to be exponential and the doubling-times were about the same for L-glutamic acid, L-proline, L-ornithine and L-arginine (Fig. 1). The following amino acids (group B) had no influence in producing filaments from the round forms: L-valine, L-methionine, L-leucine, L-isoleucine, L-lysine, L-phenylalanine, L-threonine, L-cystine, L-tyrosine, L-tryptophan (increased concentration) and glycine. The amino acids L-alanine, L-aspartic acid and *meso*-2,6-diaminopimelic acid (group C) gave variable results. Sometimes this latter group of amino acids gave rise to the start of filaments in up to 60 % of the groups of micro-organisms; these never became very long but rapidly divided and rounded up. In other experiments L-alanine and *meso*-2,6-diaminopimelic acid produced no filaments at all, and L-aspartate produced only 20 to 50 %. The main difference between the effect of amino acids in group A and group C was that whereas at a final concentration in the growth medium of 0·2 % all group A amino acids led to the maintenance of the rod form after 18 to 24 hr incubation at 35°, group C amino acids never did.

Table 2. *Bacillus subtilis 168: effect of different concentrations of sodium L-glutamate added to liquid and solidified minimal-glucose medium upon the morphology of rod-4*

The liquid cultures (10 ml.) were grown (shaken) for 18 hr at 35° and were in 50 ml. flasks. The solid media were incubated at 35° for the same time. The liquid and solid basal minimal media were as described by Rogers *et al.* (1970). No casein hydrolysate was present in the basal agar medium.

Concentration of L-glutamate (%)	Morphological form	
	Liquid culture	Solid medium
0·005	Round	Round
0·01	Round + rod	Round
0·02	Round + rod	Round + rod
0·04	Round + rod	Round + rod
0·10	Rod	Rod
0·20	Not done	Rod

The amino acids in group A are all on the direct path for the metabolism of glutamic acid. From the shorter delay in the appearance of filaments when L-glutamic acid itself was added, it was assumed that this amino acid was principally responsible for the effect of the amino acid mixture. Variation of the concentration of L-glutamic acid showed that increases between 0·1 and 1·0 % led to increases in the rate at which filamented groups of round forms began to appear. When shaken liquid cultures and cultures on solid agar medium supplemented with L-glutamate were examined after 18 hr at 35°, the results shown in Table 2 were obtained. D-Glutamic acid, in contrast, was somewhat growth-inhibitory, and at no concentration between 0·02 and 0·2 % did it convert the round forms to rods in 24 hr, and no filamentation was seen.

Interrelationships between salt concentration and glutamic acid

The observation of Rogers *et al.* (1968) was that the class of mutants which includes *rod-4* grew as rods at high salt concentrations on minimal salts agar medium, whether or not casein hydrolysate was present. The casein hydrolysate was usually included to

avoid the long periods of incubation necessary for growth of the mutants on unsupplemented minimal salts media. The interrelationship between the presence of salt and of casein hydrolysate or glutamic acid in liquid media was next examined.

The presence or absence of 0·8 M-sodium chloride in the medium was not relevant to the final morphology of the mutant grown in minimal liquid medium. When the mutant was subcultured into liquid minimal medium containing 0·8 M-sodium chloride but no Na L-glutamate the results shown in Table 3 were obtained. The essential rod-

Table 3. *Bacillus subtilis 168: effect of NaCl and sodium L-glutamate on the growth of rod-4 in liquid medium*

The cultures (10 ml.) were in shaken 50 ml. flasks incubated at 35°. Inoculated from solid medium with no added sodium chloride. The basal liquid medium was as in the previous experiments.

Medium	Growth (24 hr)	Morphology 4 hr	6 hr	11 hr	24 hr
Minimal	++	Round	Round	Round	Round
Minimal + 0·8 M-NaCl	+	Round	Not done	Not done	Round
Minimal + 0·1 % glutamic acid	+++	Round	Round + rods	Round + rods	Round
Minimal + 0·1 % glutamic acid + 0·8 M-NaCl	+++	Rods	Rods	Rods	Rods
Inoculated from solid medium + 0·8 M-NaCl:					
Minimal	++	Rods	Not done	Round	Round
Minimal + 0·8 M-NaCl	++	Rods	Not done	Rods	Round
Minimal + 0·1 % glutamic acid	+++	Rods	Not done	Rods	Rods
Minimal + 0·1 % glutamic acid + 0·8 M-NaCl	+++	Rods	Not done	Rods	Rods

forming factor in the liquid medium was the presence or absence of Na L-glutamate. In the absence of the amino acid, the addition of 0·8 M-sodium chloride did not effect the conversion to rods but only decreased the amount of growth. Moreover, when the inoculum was in the form of rods, being taken from the 0·1 % casein-hydrolysate medium containing 0·8 M-NaCl, it changed to a round form in 0·8 M-NaCl when glutamate was not present. High ionic strength in the medium considerably increased the effectiveness of the glutamate so that even at 0·01 % the mutant grew as a rod and remained so after 24 hr of incubation (Table 4). In media not containing 0·8 M-NaCl complete conversion (maintained for 24 hr) was not effected until a concentration of 0·1 % Na L-glutamate was reached. Even at this concentration the results were variable (e.g. see Table 3, line 3) and 0·2 % was required for the organism certainly to remain as a rod after 24 hr of incubation. The effect of high ionic strength seemed to be in preventing the subdivision and rounding-up of the long cells formed at first, during prolonged incubation, rather than in altering the effect of L-glutamate or in starting the conversion of the round form into a filament. The reason for the difference between the solid and liquid media is not understood.

The relative importance of L-glutamic acid and L-glutamine

A relatively high concentration of L-glutamic acid was required to effect and maintain the change from round forms to rods. It therefore seemed possible that L-glutamine formed from the added L-glutamic acid and the NH_4^+ in the medium was responsible rather than the amino acid itself. To test this, the technique for measuring the increase

in groups of round forms forming filaments was applied to cultures in which mixtures of Na L-glutamate and γ-L-glutamylhydrazide were added to the basal minimal medium. γ-L-Glutamylhydrazide has been shown (McIlwain, Roper & Hughes, 1948) to inhibit the hydrolysis of glutamine to ammonia and glutamic acid, and to inhibit the growth of *Streptococcus haemolyticus* and *S. faecalis* competitively with L-glutamine.

Table 4. *Bacillus subtilis 168: effect of different concentrations of Na L-glutamate upon the morphology of rod-4 growing with and without the addition of 0·8 M-NaCl*

The cultures (10 ml.) were in 50 ml. flasks and were incubated at 35° with shaking. The cultures were also inspected with the phase-contrast microscope at 4 hr, 6 hr and 11 hr.

Na L-glutamate concentration (%)	Morphology at 24 hr	
	+0·8 M-NaCl	No added NaCl
0	Round	Round
0·01	Rod (short)*	Round†
0·03	Rod*	Round†
0·05	Rod*	Round + rod†
0·10	Rod (variable)*	Rod
0·20	Rod*	Rod
0·50	Rod (long)*	Rod

* At 6 hr showed long twisted rods.
† About 40% of the round-form groups showed the beginnings of filamentation at 6 hr.

Table 5. *Bacillus subtilis 168: effect of γ-L-glutamylhydrazide on morphological change of rod-4*

Two of the readings of the experiment are recorded. The cultures (10 ml.) were contained in 50 ml. flasks and were shaken at 35°.

Additions to basal medium			% of groups with filaments at 10 hr	Morphological form after 22 hr
Na L-glutamate	L-glutamine	γ-L-glutamyl-hydrazide		
0	0	0	7	Round
0·2	0	0·025	2	Round
0·2	0	0·050	4	Round
0·2	0	0	>90	Rods
0	0·2	0·025	>90	Rods
0	0·2	0·10	>90	Rods
0	0·2	0	>90	Rods

At a molar ratio of Na L-glutamate : γ-L-glutamylhydrazide of about 10, complete inhibition of the transformation of the round forms was effected (Table 5). In other experiments inhibition occurred at ratios as high as about 50. Little or no inhibition of growth occurred at these ratios of Na L-glutamate to L-glutamylhydrazide, although no growth occurred at a ratio of about 2. In the presence of L-glutamine, inhibition of neither growth nor morphological transformation occurred at a ratio of 2·0. Thus it seemed likely that the utilization of glutamine was of importance in changing the round forms to rods.

Effect of different concentration of Na L-*glutamate and* L-*glutamine on rod-4*

It might be expected that, if L-glutamine is the active substance in effecting the change of the round form of the mutant into the rod-like form, then it would be functional at lower concentrations. On the other hand this might only be perceptible if the rate of synthesis of glutamine or the rate of penetration of glutamate into the organism were the limiting factors. If the lesion in the mutant involves the efficiency of the utilization of glutamine for one or more reactions within the organism, then an effect might not be seen. Also, if the mutant has an active glutaminase which rapidly hydrolyses glutamine into glutamic acid and ammonia, a difference might not be easily seen.

A small superiority of L-glutamine was shown both in the rate and degree of the permanence of the effect (Table 6). These effects were undoubtedly marginal; in several tests it was seen that whether or not the former was seen depended on readings being taken during the exponential phase of the change. There was no difference in the time of onset of the change (the lag phase) between cultures containing L-glutamine and those containing L-glutamate (Table 6).

Table 6. *Bacillus subtilis 168: comparison of Na* L-*glutamate and* L-*glutamine in causing the round form to change into rod-4*

The experiment was done as described in the text for the effect of L-amino acids on *rod-4*. Incubation was at 35° with shaking.

Concentration (%)		% of round forms showing filaments at hr:		Morphological form after 24 hr
L-glutamate	L-glutamine	4·5	10·5	
0·02	0	6	25	Round
0·10	0	10	44	Round
0·20	0	19	72	Rod
0	0·02	6	64	Round
0	0·10	14	90–100	Rod
0	0·20	18	90–100	Long rod
0	0	< 1	< 1	Round

*Effect of γ-*L-*glutamylhydrazide on parent strains*

The parent strain *Bacillus subtilis* 168 *trp*− was grown on the 0·1 % casein hydrolysate + minimal salts + glucose agar for 18 hr, washed from the plate and washed once in the minimal liquid medium. This suspension was then inoculated into the minimal liquid medium supplemented with mixtures of Na L-glutamate, L-glutamine and γ-L-glutamylhydrazide exactly as in Table 5. Examination after 18 hr incubation at 35° showed that there was an increase in the length of the bacilli with increasing concentration of γ-L-glutamylhydrazide in the presence of Na L-glutamate, but no such increase when the amino acid was replaced by L-glutamine. No rounding of the bacilli was seen although some of the long bacilli were twisted and bent.

DISCUSSION

L-Glutamine formed from L-glutamic acid seems likely to be the major, if not the only, factor concerned with changing this particular mutant from a round form to a rod, at least in liquid growth media. High ionic strengths simply rendered much lower concentrations of sodium glutamate effective. The change effected on solid media by high salt concentration alone may well have been due to the presence of small amounts of amino acid in the agar medium. This enhancement of the effect of glutamate may have several explanations which, in terms of simplicity, range from a decrease of the surface forces needed to make a rod as compared with those needed to make a sphere by osmotic support, through possible effects on the permeation of glutamate into the bacillus, up to sophisticated effects of salt on the enzymes involved in glutamate and glutamine metabolism. The latter type of effect would be akin to the osmotic remedying of mutations previously referred to (Rogers *et al.* 1968, 1970). At present we have no evidence from which to distinguish these possibilities.

A disturbance in the metabolism of glutamine might be expected to lead to widespread effects within the bacteria. Glutamine is known to be involved in the biosynthesis of purines, of pyrimidines, of folic acid and of amino sugars (see Meister, 1965, for a summary of these reactions); these compounds are concerned with vital cell structures. Also, an exceptionally high proportion of the carboxyl groups of membrane proteins is amidated (Maddy & Malcolm, 1965; Wallach & Zahler, 1968) and about 50 % of the free α-carboxyl groups of the D-glutamic acid in the mucopeptides of bacilli are probably amidated (Hughes, Pavlik, Rogers & Tanner, 1968; Mirelman & Sharon, 1968). Abnormalities in the fine structure in the walls, in the arrangement of the membranes and possibly in the DNA have been seen (Rogers *et al.* 1970) with the electron microscope in sections of the mutant. All these structures have components likely to be dependent on L-glutamine for their formation. However, although an analogue of L-glutamine (γ-L-glutamylhydrazide) can prevent glutamic acid from correcting the morphology of the mutant, it is not itself capable of causing abnormality in the parent, although it appears to some extent to interfere with division.

Two aspects of the disturbance in the mutant can be distinguished. The first is that in the presence of glutamic acid or glutamine, long filamentous forms were produced with regularity on solid media. These filaments appeared to be made up of individuals 5 to 10 times longer than those in the parent strain and were joined to form very long threadlike forms. These threads subdivided differently according to the supply of the amino acid. When the concentration was high (i.e. 0·1 to 0·2 % in media of low ionic strength) then the final length of most of the forms was like the bacteria in the parent strain. When the concentration was low (0·05 % or less), subdivision continued and the walls swelled until more or less spherical organisms were formed. When the mutant was growing on solid media, the final result was like a string of beads. Thus one disturbance in the *rod* mutant was clearly connected with the process of division, which appeared to be partially inhibited while a sufficient supply of L-glutamine was available. This situation is reminiscent of that reported by Walker & Pardee (1967) for the *lon* mutant of *Escherichia coli* K12 F^- strain AB1899NM. This mutant formed filaments after irradiation when grown on a yeast+peptone medium, but divided to form normal length bacteria when transferred to a minimal medium. Multiple septation of some cells of *Bacillus cereus* ATCC4342 has also been observed (Pfister & Lundgren,

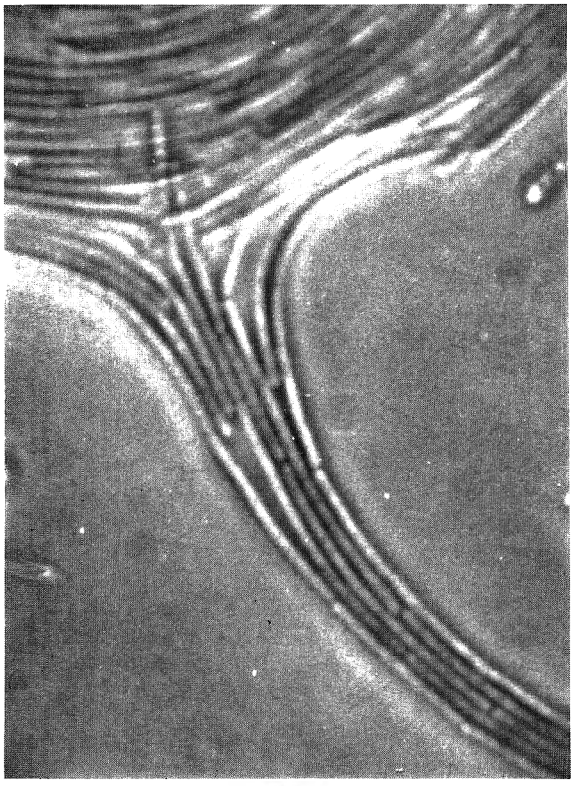

Fig. 1

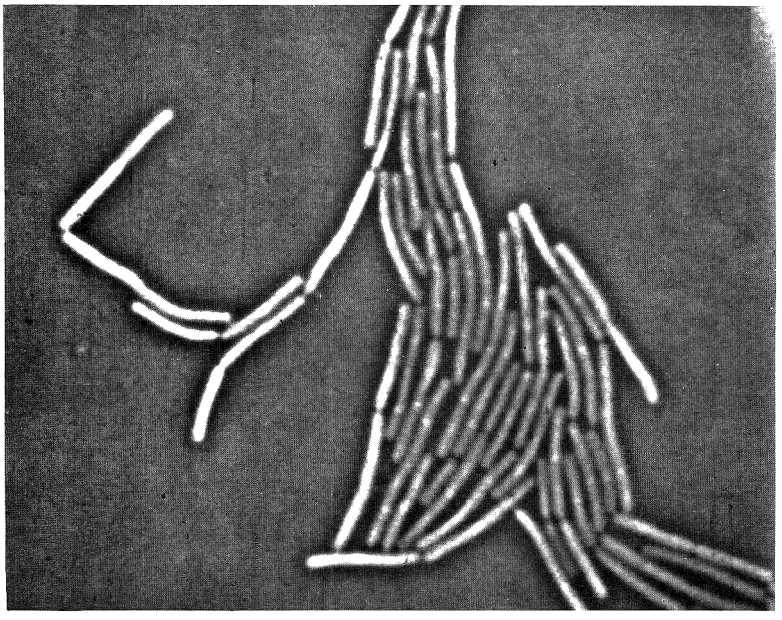

Fig. 2

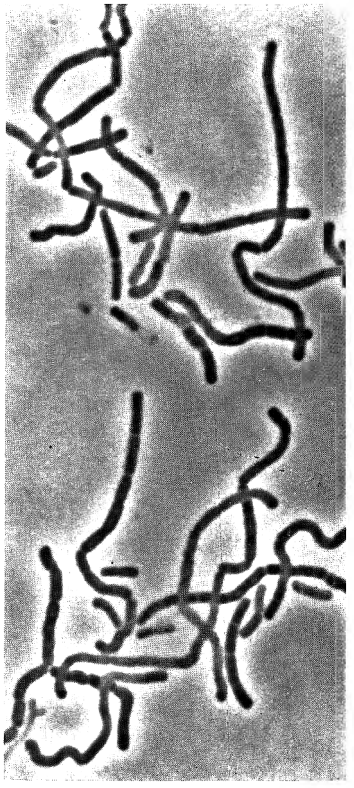

Fig. 3

(*Facing p.* 181)

1964; Remsen & Lundgren, 1965) in cultures undergoing sporulation and therefore likely to have been in some ways nutritionally deficient. These observations ought to be thought about in relation to the normal lengthening of cells that occurs at faster growth rates on richer media (Schaechter, Maaløe & Kjeldgaard, 1958). All these phenomena may be related to the regulation of the formation of a division protein.

The second disturbance in the *rod* mutants was concerned with the nature of the walls of the organism when growing in the coccal form. It would appear that these were weak and bulged when the bacteria were deficient in their supply of glutamine. This weakness is consistent with the presence of a relatively uncrosslinked mucopeptide in the wall as compared with that in the rod-like form of the mutant (Rogers, McConnell & Hughes, to be published).

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EXPLANATION OF PLATE

Fig. 1. *Bacillus subtilis* 168 *trp rod-4* growing on nutrient agar as filaments and very long forms.

Fig. 2. A later stage of growth of the same culture as in fig. 1 with the very long forms subdividing to form rods.

Fig. 3. An 18 hr culture of the parent strain (*Bacillus subtilis* 168 *trp*) growing on nutrient agar.

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Structure and Role of a Soluble Cytoplasmic Glucan from *Phytophthora cinnamomi*

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SUMMARY

A soluble glucan was isolated from the mycelium of *Phytophthora cinnamomi* and found to be a β-1→3-linked glucan with branches arising from residues substituted at both C-3 and C-6. This glucan is an important reserve material of the fungus; it accumulates in mycelium grown in glucose-rich medium and disappears upon incubation in media devoid of glucose.

INTRODUCTION

β-Glucans seem to be the principal polysaccharides elaborated by the fungus *Phytophthora cinnamomi*. Two different β-glucans comprise nearly 90 % of the mycelial walls of this oomycete (Bartnicki-Garcia, 1966): cellulose is the minor component while the major wall constituent is a very insoluble branched glucan, linked predominantly by β-1→3 bonds and β-1→6 links at the branching residues (Zevenhuizen & Bartnicki-Garcia, 1969). As described below, an important reserve carbohydrate found in the mycelium of *P. cinnamomi* is also a β-1→3 and β-1→6-linked glucan. Branched β-1→3-glucans with the branching residues substituted at both C-3 and C-6 are commonly found in fungi (see reviews: Clarke & Stone, 1963; Gorin & Spencer, 1968). The fine structure, physical properties, cellular location and probable function of these β-1→3–β-1→6-glucans vary: (1) Some are found dissolved in the culture medium (Bouveng, Kiessling, Lindberg & McKay, 1963; Wallen, Rhodes & Shulke, 1965; Buck, Chen, Dickerson & Chain, 1968) and their function has yet to be defined. (2) Others are highly insoluble constituents of the cell wall fabric (see Bartnicki-Garcia, 1968), where they probably play a key structural role. (3) Some occur in the cytoplasm and probably serve as C and energy reserves, e.g. the glucan from the sclerotia of *Sclerotinia libertiana* (Kitahara & Takeuchi, 1959) and the one from *P. cinnamomi*, whose isolation and properties are described in the present paper.

METHODS

Cultivation. Phytophthora cinnamomi strain SB-216-1 was studied. Mycelia were grown in 350 ml. prescription bottles containing 50 ml. of an asparagine+glucose medium previously described (Bartnicki-Garcia, 1966). The bottles were inoculated with 10 ml. of a homogenized mycelial suspension derived from a liquid culture, and incubated horizontally in a BOD incubator at 25°. At harvest time, the cultures were

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filtered on a coarse sintered-glass filter and the mycelial mat washed with distilled water several times.

Glucan isolation. Mycelium grown for 7 days was washed and broken in a Bronwill MSK cell homogenizer as described earlier (Zevenhuizen & Bartnicki-Garcia, 1969). The cell walls were centrifuged down, washed with water and weighed. The supernatant liquid was deproteinized by addition of trichloroacetic acid to 2·5 % (w/v). To the deproteinized solution, two volumes of 95 % (v/v) ethanol in water were added to precipitate the glucan. The resulting white precipitate was centrifuged down and dissolved in water; a small amount of insoluble residue was discarded. The glucan was again precipitated with 2 vol. of ethanol; a small amount of sodium acetate was added to facilitate precipitation. The glucan was washed successively with ethanol, and ethyl ether, and dried in air.

Chemical methods. Methylation analysis and periodate oxidation were done as before (Zevenhuizen & Bartnicki-Garcia, 1969). Determinations of methoxyl and total-N were performed by the Schwarzkopf Microanalytical Laboratory, Woodside 77, N.Y., U.S.A. Total hexose was determined by the anthrone reagent with glucose as a standard. The distribution of carbohydrate in mycelial fractions was also measured by the anthrone method. The fractions were obtained by extracting consecutively the washed mycelium harvested from each bottle with: (1) 20 ml. 70 % (v/v) ethanol in water in a boiling water bath for 30 min. (2) 20 ml. distilled water at 97° for 30 min. (3) 20 ml. 1 N-KOH at room temperature for 30 min. The final residue was washed with water.

Enzymic methods. The action of α-amylase was tested under conditions specified earlier (Zevenhuizen, 1966). Digestion with β-glucanases was tested with a crude mixture of β-1→3 and β-1→4 endoglucanases from *Streptomyces* sp. QMB 814 (a gift from E. T. Reese, U.S. Army Laboratories, Natick, Mass., U.S.A.) under conditions as used before (Bartnicki-Garcia & Lippman, 1967). After de-ionization on a mixed-bed column of Dowex-1-acetate and Dowex-50-hydrogen, the sugars were separated by descending paper chromatography with *n*-butanol + pyridine + water (6 + 4 + 3, by vol.) as solvent.

RESULTS

Properties of the cytoplasmic glucan. The glucan isolated from the cytoplasm of *Phytophthora cinnamomi* (see Methods) was a white powder soluble in water and also in hot 70 % (v/v) ethanol in water. It contained 96 % anhydroglucose residues as measured by the anthrone method. A 1·75 % total-N value suggested a protein content of about 10 %. Whether this protein was merely a contaminant or an integral part of a glycoprotein complex was not investigated. The glucan did not give the characteristic colours of glycogen or starch when treated with iodine and was not attacked by α-amylase. Its low specific optical rotation $[\alpha]_D^{25}$ -1·2 (about 5·0 in water) indicated a β-glucan. After digestion with a crude glucanase mixture from Streptomyces, a series of oligosaccharides was separated on paper chromatograms and corresponded in mobility to laminarin oligosaccharides; from glucose to the hexamer, a linear relationship was obtained between oligomer size and the log $1 - R_F/R_F$ (French & Wild, 1953). There was also an unresolved streak of higher oligosaccharides extending to the origin. Cellobiose was not detected.

Structural analysis of the glucan. The cytoplasmic glucan was dissolved in dimethyl

sulphoxide and methylated by the Hakomori (1964) procedure as described by Sandford & Conrad (1966). After methylation, the glucan showed a $-OCH_3$ content of 42·75 % and N content of 0·00 %. The methylated glucan was hydrolysed with 72 % (w/v) H_2SO_4 at room temperature for 1 hr. The acid was diluted to 8 % with water and the hydrolysis completed at 100° for 18 hr. The resulting mixture of glucose-O-methyl ethers was separated by gas–liquid partition chromatography (GLC) after conversion to the corresponding methyl glucosides. Three different anomeric pairs of O-methyl ethers were recognized (Table 1). Similarly, three different fractions of glucose-O-methyl ethers were separated by cellulose column chromatography corresponding to those resolved by GLC. From 400 mg. of hydrolysate applied to the cellulose column, the following fractions were obtained and characterized: (a) 2,3,4,6-tetra-O-methyl-D-glucose, 42 mg.; on crystallization from light petroleum (b.p. 30 to 70°) gave a product with m.p. 76° to 85°; (b) 2,4,6-tri-O-methyl-D-glucose, 258 mg.; on recrystallization from ethyl ether showed m.p. and mixed m.p. 123° to 125° and $[\alpha]_D^{25} + 105$ (1 min.) → +75 (24 hr) (about 0·1 in water); (c) 2,4-di-O-methyl-D-glucose, 49 mg.; this compound could not be crystallized as such; upon conversion into *N*-*p*-nitrophenyl-2,4-di-O-methyl-D-glucosylamine it afforded crystals from an ethyl acetate solution with m.p. and mixed m.p. 242° to 245°.

Table 1. *Methylation analysis of the soluble cytoplasmic glucan of Phytophthora cinnamomi*

Methylated derivatives prepared and analysed by gas–liquid chromatography, as previously described (Zevenhuizen & Bartnicki-Garcia, 1969).

Methyl-D-glucoside ethers	Mole (%)
2,3,4,6-tetra-O-methyl ether	11·3
2,4,6-tri-O-methyl ether	76·7
2,4-di-O-methyl ether	12·2

The total values of periodate consumed and formic acid released, after oxidation of the glucan with periodate, were calculated by extrapolation to zero time to correct for over-oxidation (Fig. 1). The glucan consumed 0·3 moles periodate and yielded 0·135 moles HCOOH/glucose residue. A complete Smith degradation of the periodate-oxidized glucan was done. The glucan was oxidized with periodate for 3 days at room temperature, then reduced with sodium borohydride and hydrolysed with 2 N-H_2SO_4 for 16 hr at 100°. After acetylation, two degradation products were separated by GLC, namely glycerol triacetate and glucose penta-acetate, in amounts corresponding, respectively, to 0·226 moles and 0·774 moles/mole glucose residue. Erythritol tetra-acetate was not detected.

Accumulation and utilization of cytoplasmic glucan. The amount of cytoplasmic glucan isolated from the mycelium of *Phytophthora cinnamomi* was very dependent on the glucose concentration in the culture medium (Table 2). The utilization of stored carbohydrate was shown in the following experiment. Two sets of five bottles of asparagine + glucose medium were inoculated with a suspension of mycelium homogenized in a Sorvall Omni-Mixer; after 4 days incubation one set of cultures was harvested for analysis. The liquid medium of the other set of cultures was replaced with glucose-free asparagine medium, and the incubation continued for 4 more days. The change of culture medium was accomplished *in situ* by using a sintered-glass

immersion filter and a water aspirator. All manipulations were done aseptically. After removing the spent culture medium the mycelium was washed three times with asparagine medium (glucose omitted), resuspended in 50 ml. of glucose-free asparagine medium and the incubation resumed for 4 more days. The mycelium from each bottle

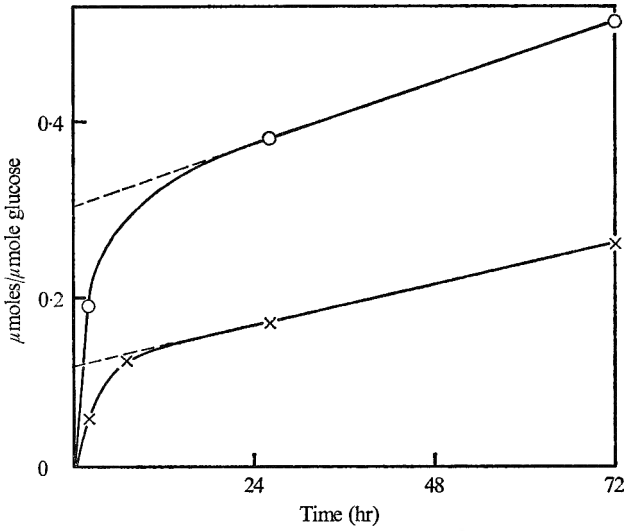

Fig. 1. Periodate oxidation of the cytoplasmic glucan of *Phytophthora cinnamomi*. ○——○, periodate consumption; ×——×, formic acid released.

Table 2. *Effect of two glucose concentrations on glucan formation by Phytophthora cinnamomi*

Values correspond to 500 ml. asparagine+glucose culture incubated for 7 days.

Initial glucose conc.	Cell yield (g. wet wt)	Cell walls (g. dry wt)	Cytoplasmic glucan (g. dry wt)
2·0%	21·3	1·43	0·36
0·5%	13·9	0·90	0·024

Table 3. *Changes in carbohydrate content following glucose deprivation of the mycelium of Phytophthora cinnamomi*

	Carbohydrate content*	
Fraction	Before deprivation† (mg./50 ml. culture)	After deprivation‡ (mg./50 ml. culture)
Hot 70% (v/v) ethanol in water-soluble	35·1	11·7
Hot water-soluble	1·3	1·2
N-KOH-soluble	0·4	0·3
Insoluble residue	14·8	20·1
Total	51·6	33·3

* Determined by anthrone method and expressed as mg. glucose/culture (50 ml.).
† Grown for 4 days in asparagine+glucose medium.
‡ Grown for 4 days in asparagine+glucose medium followed by 4 days in glucose-free asparagine medium.

was harvested, washed with water by centrifugation and extracted consecutively (see Methods). Table 3 shows the changes in carbohydrate content in the mycelial fractions caused by glucose deprivation. Glucose deprivation effected a substantial decrease in the total amount of carbohydrate in the mycelium and a marked redistribution of these carbohydrates. The most conspicuous changes were the large decrease in soluble carbohydrate (hot 70 % (v/v) ethanol in water soluble fraction), and the increase in cell-wall glucan (insoluble residue). The decrease in ethanol-soluble carbohydrate shows extensive consumption of cytoplasmic glucan which comprises 77% of the carbohydrate in this fraction. In another experiment glucose deprivation was prolonged for 19 days by which time the 70 % (v/v) ethanol in water soluble carbohydrates had been nearly all consumed; only 1 % of the initial value remained.

DISCUSSION

The methylation analysis indicated that the cytoplasmic glucan of *Phytophthora cinnamomi* is built predominantly (76·7 %) of glucose residues substituted at C-3, with branchings at some residues (12·2 %) substituted at both C-3 and C-6; the remaining glucose units (11·1 %) are non-reducing terminal groups. Theoretically, such glucan would consume 0·22 moles periodate, and yield 0·11 moles formic acid per mole of glucose residue; however, the experimental values were higher. The discrepancy probably resulted, despite the extrapolation correction, from underestimation of the large extent of over-oxidation. Also, over-oxidation probably accounted for the amount of Smith degradation products observed. Thus only 77 % of the initial glucose residues survived periodate oxidation, against the 89 % of periodate-resistant residues predicted from the methylation data; instead a corresponding large proportion of glycerol was obtained. There was, however, no erythritol indicative of 1→4-linked glucose residues, in agreement with the methylation data. The evidence from the methylation analysis, periodate oxidation, β-glucanase digestion and optical rotation support the view that the cytoplasmic glucan of *P. cinnamomi* is a branched β-1→3-linked glucan with 1→6 links at the branching residues. The methylation data also indicate that there are, on the average, eight glucose residues per branching point. The relative orientation of the 1→3 and 1→6 linkages on the branching glucose units and the length of, and spacing between, branches remains to be determined. The soluble cytoplasmic glucan of *P. cinnamomi* resembles in part the very insoluble glucan found in the cell walls of this fungus; both have mainly β-1→3 links, and 1→6 links at the branching points, but differ in the degree of branching and, seemingly, in the presence of 1→4 links which appear to be associated with the wall glucan only (Zevenhuizen & Bartnicki-Garcia, 1969).

Some *Phytophthora* species (Eschrich, 1956) and other related genera of Oomycetes (Mangin, 1894) have long been known to give positive staining tests for callose. The β-1→3–β-1→6 glucans found previously in the cell walls and now in the cytoplasm of *P. cinnamomi* are possible candidates responsible for this staining reaction, characteristic of certain β-1→3 glucans; but their actual involvement has not been demonstrated. The cytoplasmic glucan of *P. cinnamomi* appears to be a readily accessible reserve carbohydrate. It accumulates in mycelium grown in glucose-rich medium and is consumed upon omission of glucose from the growth medium. A substantial proportion of this soluble cytoplasmic glucan was eventually utilized for the synthesis of

insoluble cell wall glucan. Whereas glycogen is the conspicuous reserve polysaccharide of some fungi such as *Neurospora crassa*, *Saccharomyces cerevisiae*, etc. (see Gorin & Spencer, 1968), glycogen seems to be absent from the oomycete *Phytophthora cinnamomi*, the reserve polysaccharide being instead the β-1→3–β-1→6 glucan described here. So little is known about the nature and distribution of reserve polysaccharides among fungi that any generalization, with intrinsic phylogenetic value, appears premature (see Klein & Cronquist, 1967). Suffice it to suggest, for the time being, that the nature of the polysaccharide storage materials in *P. cinnamomi* might be one further example of the biochemical traits which make Oomycetes unique among fungi (see Bartnicki-Garcia, 1969).

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Characterization by Transformation of an Ampicillin-resistant Mutant of Pneumococcus

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SUMMARY

Mutagenesis of a transformable strain of pneumococcus by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produced several mutants resistant to ampicillin. One of these was resistant to 0·1 μg. ampicillin/ml., but a purified solution of its DNA transformed the sensitive parent to three different levels of resistance at frequencies compatible with single, double and triple transformants. The higher levels of resistance were dependent on the presence of the genes conferring the lower resistances. Furthermore, transformants to the higher levels of resistance can be obtained at single or double frequency by using as recipient the strain already possessing the genes conferring the lower resistances. The expression times of the ampicillin genes are short (approximately 25 min.), but the actual times were difficult to determine since there was a delay of some 15 to 20 min. before the ampicillin exerted an effect on the sensitive strain under these conditions.

INTRODUCTION

In the course of experiments designed to map the relative positions of genes on the pneumococcus chromosome, it became necessary to make and characterize new markers to add to those already available. For this purpose, mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG) was performed and mutants having auxotrophic properties or antibiotic resistances were looked for. Amongst these were mutants resistant to ampicillin, and the characteristics relating to the transforming properties of one of these are described below.

MATERIALS AND METHODS

Organisms. Diplococcus pneumoniae strain Cl 3, derived from strain R36A of Avery, MacLeod & McCarty (1944) by Ephrussi-Taylor (1951), sensitive to streptomycin and ampicillin. Stock cultures were kept as previously described (Butler, 1965).

Diplococcus pneumoniae strain r_2SQ, derived from strain r_2 of Ravin and Iyer (1961) by transformation, carrying the *ery-r2*, *str-41*, and *opt-r2* genes giving resistance to erythromycin, streptomycin and optochin respectively.

Media. The peptone media 'P' and 'NS' were prepared as previously described (Sicard, 1965; Butler, 1965), and the synthetic medium of Sicard (1965) was used in the mutagenic reaction with the following additions: glutamic acid 80 mg./l., aspartic acid 95 mg./l., tryptophan 100 mg./ml., serine 125 mg./l., proline 200 mg./l., phenylalanine 250 mg./l., adenine 18 mg./l. These compounds were added to allow the survival of auxotrophs.

Ampicillin. A fresh solution of ampicillin ('Penbritin', Beecham Research Laboratories, England) in 0·15 M-NaCl at the required dilution was prepared for each experiment.

Mutagenesis with NNG. Mutagenesis was obtained by allowing the NNG to act on an exponentially growing culture of strain Cl 3. The organism was first grown in 'P' medium, washed and resuspended in supplemented Sicard medium. An aliquot of 1·0 ml. of this suspension was inoculated into 20 ml. supplemented Sicard medium and incubated at 37°. NNG at a final concentration of 20 μg./ml. was added at 50 min. and treatment was continued for 30 min. Under these conditions the NNG allowed 50% survival of the culture. Samples were removed and plated in nutrient agar, incubated at 37° for 2 hr and a top layer of nutrient agar added containing ampicillin to give a final concentration of 0·025 μg./ml. The strain Cl 3 is sensitive to ampicillin at a concentration of 0·0125 μg./ml. A check that the conditions gave good mutagenesis was provided by testing for the appearance of aminopterin-resistant mutants; if conditions are good, aminopterin-resistant mutants are obtained in high numbers (A. M. Sicard, private communication).

Preparation of deoxyribonucleic acid (DNA). The appropriate strain was inoculated into 750 ml. of medium containing 1% (w/v) neopeptone (Difco), 0·85% (w/v) yeast extract (Difco), 0·85% (w/v) NaCl, 0·5% (w/v) glucose and 31·5 % (w/v) glutamine (pH 7·1) and incubated at 37°. After 2½ hr phenol red solution was added, and as growth proceeded the lactic acid produced was neutralized by the addition of sodium hydroxide solution. When about 40 ml. of N-sodium hydroxide had been added, 55 g. hydrated sodium citrate was added and when dissolved, the culture was lysed by adding 4·5 g. sodium desoxycholate dissolved in a little water and incubated at 37° for 30 min. One volume of ethyl alcohol was stirred into the lysed culture, and the threads collected. Protein was removed by repeated shaking with chloroform and *sec*-octyl alcohol (Sevag, Lackman, & Smolens, 1938), the DNA again precipitated with one volume of ethyl alcohol and the fibres dissolved in sterile 0·15 M-NaCl.

Preparation of lysates. The organism was grown for about 2 hr in 10 ml. 'P' medium supplemented with asparagine and glutamine, centrifuged and resuspended in 1·0 ml. of the same medium. To the homogeneous suspension was added 0·1 ml. of 5% (w/v) sodium desoxycholate and incubated at 37° for 30 min. The lysates were then kept at 4° until required, and their activity remained stable for several weeks.

Assay of transforming activity. The method used was essentially as described previously (Butler, 1965); differences in the technique which eventually arose will be described below.

RESULTS

Isolation of ampicillin-resistant mutants. As a result of the treatment with NNG, several colonies were isolated resistant to ampicillin at the challenging concentration of 0·025 μg./ml. Sensitivity curves were constructed, and the organisms were found to fall into two groups showing resistance to either 0·1 μg./ml., or 0·05 μg./ml. Strain Amp3, which fell into the first group, was selected for further study.

Transformation with Amp3 as donor. Transformation was carried out by using purified DNA prepared from the mutant strain Amp3 as donor and strain Cl 3 as recipient. Since mutants of lower resistance had been isolated it was of interest to ascertain whether the DNA from Amp3 could give transformants resistant only to

0·1 μg./ml. or whether it was also able to transform to lower resistances. After exposure to the DNA, aliquots of the culture were plated in nutrient agar and allowed to express for 2 hr before challenging with several concentrations of ampicillin. The results, given in Fig. 1, showed that the DNA transformed to three levels of resistance, 0·03 μg./ml., 0·06 μg./ml. and 0·09 μg./ml. Transformants at the 03 level were obtained at a frequency some 10^2 greater than those at the 06 level, which themselves occurred at a frequency some 10^2 greater than those at the 09 level, indicating that these three levels of resistance were the result of single, double and triple transforming events respectively.

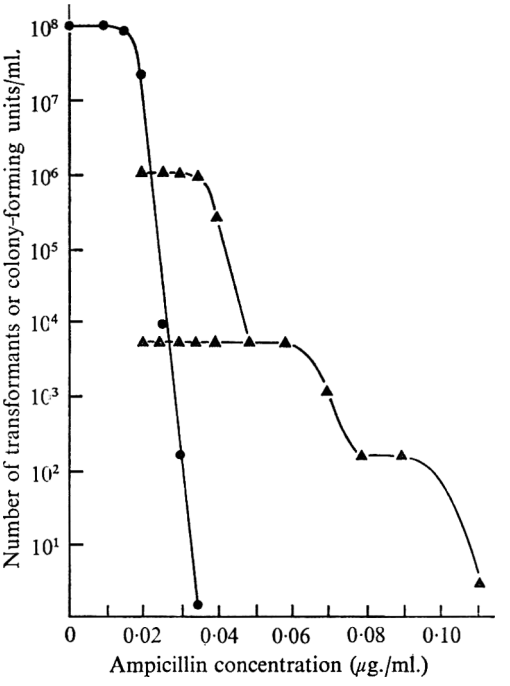

Fig. 1. Transformation of strain cl 3 to ampicillin resistance by DNA from strain Amp 3. ●, Sensitivity curve of strain cl 3; ▲, ampicillin-resistant transformants.

At dilutions which allowed both 03 (i.e. Amp 03) and 06 (Amp 06) transformants to form colonies on the same plate, Amp 06 could be distinguished from Amp 03 by their much larger colony size. This explains the projection back, in Fig. 1, of the Amp 06 curve to concentrations below 0·03 μg./ml.

Expression time. In the first instance, the expression times at the 03 and 06 levels were investigated by crossing strain cl 3 with 09-DNA. The double layer technique always gave the full number of transformants even at zero time, and hence expression was allowed in the reaction tube after a 4 min. exposure to the DNA followed by treatment with DNAse for 1 min. Samples were then plated at intervals directly into nutrient agar containing ampicillin at concentrations of 0·03 μg./ml. and 0·05 μg./ml. Full expression was complete at about 4 to 5 min. for the 03 transformants and 9 min. for the 06 transformants. The curves obtained are shown in Fig. 2.

These expression times are very short, and it was considered that they may be artifacts. It seemed possible that the ampicillin was not exerting an immediate effect

on the organism, either because (*a*) competent organisms or organisms being transformed were insensitive to ampicillin, or (*b*) the ampicillin required a period of time before the bacteriocidal action occurred with perhaps a period of bacteriostatic activity, allowing expression to occur.

Action of ampicillin on transforming organisms. To test the effect of ampicillin on a culture which was in the process of being transformed, strain Cl 3 was exposed to r_2SQ-DNA for 4 min., treated with DNAse for 1 min. and ampicillin at concentrations of

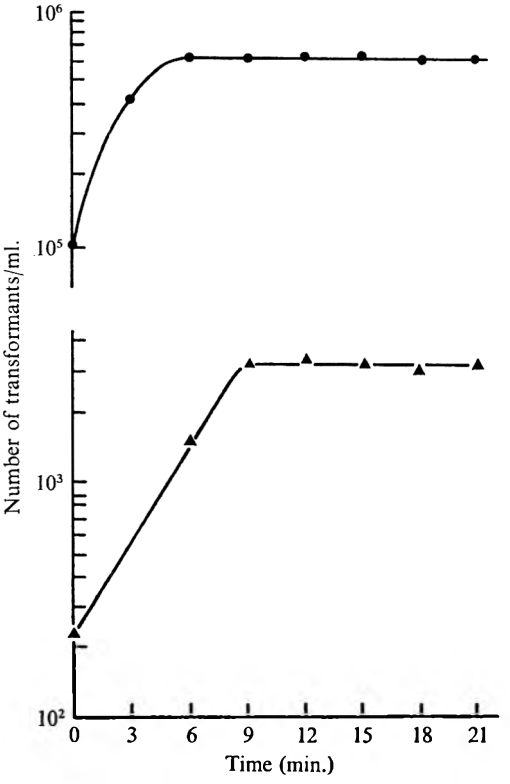

Fig. 2. Apparent expression curves of genes conveying ampicillin resistance to the 03 μg./ml. and 06 μg./ml. levels in the cross Cl 3 × (Amp 3). ●, Transformants to Amp 03 as singles; ▲, transformants to Amp 06 as doubles.

0·03 μg./ml., 0·06 μg./ml. and 0·09 μg./ml. were added. Samples were then plated in agar at intervals, given full expression for streptomycin resistance (2 hr) before overlaying with streptomycin agar. Maximum numbers of streptomycin transformants were obtained until 15 to 20 min. exposure to the ampicillin, when the number of transformants dropped, indicating that the organisms were insensitive to the bacteriocidal action of the ampicillin up to that time. Furthermore, there was no effect by the ampicillin during the first 5 min. after the end of the DNAse treatment so that the rise seen at the early part of the curve in Fig. 2 was a true increase in the number of transformants with increase in the time of expression. It was possible that a bacteriostatic action of the ampicillin occurred which was negated on plating in the ampicillin-free medium. However, a culture of strain Cl 3 prepared in exactly the same way (i.e. to produce

competence) but not treated with DNA gave a growth curve showing a doubling of the number of colony-forming units for the first 15 to 20 min. followed by a fairly rapid drop. This showed that there was no bacteriostatic action at the earlier exposure times, and that the bactericidal action occurred only after 20 min. exposure. This culture was competent at the time of exposure to the ampicillin but a non-competent culture tested in the same way gave essentially similar results.

From these results it can be concluded that the expresson times of 4 to 5 min. for the 03 transformants and 9 min. for the 06 transformants are apparent times and must be increased by the 15 to 20 min. necessary for the ampicillin to exert its effect under these conditions. The procedure adopted for scoring for ampicillin transformants is to allow exposure of the recipient organism to the DNA for 15 to 30 min. and then to plate aliquots directly into nutrient agar containing ampicillin.

Table 1. *Resistance levels in μg./ml. of ampicillin transformants appearing at frequencies corresponding to singles, doubles and triples*

Recip.	Donor DNA	Resistance level (μg./ml.) of ampicillin transformants: Singles	Doubles	Triples
cl 3	Amp 09	0·03	0·06	0·09
cl 3	Amp 06	0·03	0·06	—
cl 3	Amp 03	0·03	—	—
Amp 03	Amp 06	0·06	—	—
Amp 03	Amp 09	0·06	0·09	—
Amp 03	Amp 03	—	—	—
Amp 06	Amp 09	0·09	—	—
Amp 06	Amp 03	—	—	—

Table 2. *Apparent expression times of the 06 and 09 genes as single or double transformants*

Cross	Apparent expression time (min.): Singles	Doubles
cl 3 × (Amp 09)	4–5(03)*	9(06)
Amp 03 × (Amp 09)	4–5(06)	10(09)
Amp 06 × (Amp 09)	5(09)	—

* Figures in parentheses give the resistance level of the transformants.

Crosses between the ampicillin transformants. The single, double and triple transformants obtained by the cross cl 3 × (Amp 09) were isolated and lysates of the strains prepared. Crosses were then made using these lysates as donors and strain cl 3 or the resistant transformants as receptors. The results obtained are summarized in Table 1. The higher levels of resistance could not be obtained as single transformants from cl 3, but could be obtained by using as recipient the transformant having a resistance one step lower than that of the donor DNA. Similarly, resistances at the 09 level could be obtained at doubles frequency in the cross Amp 03 × (Amp 09). Hence, the higher resistances are the result of the organism possessing two or more of the genes conferring ampicillin resistance. The 06 level requires two genes, and the 09 level requires three genes.

Expression of the 06 and 09 levels as singles and doubles. The apparent expression times for transformation to the 06 and 09 levels as single or double transformants were

determined in the same manner as described above. The results are summarized in Table 2. Both the 06 and 09 genes when transformed as singles have the same apparent expression time as the 03 gene transformed as a single into Cl 3, whilst the 09 gene transformed as a double had the same time as the 06 gene with Cl 3, as recipient.

DISCUSSION

The results show that Amp 3 contained three loci conveying resistance to ampicillin which, although transmitted independently (i.e. are unlinked), are dependent on each other in a sequential fashion for the manifestation of the higher resistance levels. The lowest level of resistance, 03, was obtained at the frequency of single transformants in the cross Cl 3 × (Amp 09), whereas 06 was obtained at doubles frequency and 09 at trebles frequency. Furthermore, in the cross Amp 03 × (Amp 09), 06 was obtained at singles frequency and 09 at doubles frequency. Hence resistance at the 06 level could not be obtained in the absence of the 03 gene, and the 09 level could not be obtained in the absence of both 03 and 06 genes.

Amp 06 transformed Cl 3 to give both Amp 03 and Amp 06 as transformants, and Amp 09 transformed Amp 03 to give both Amp 06 and Amp 09. Hence, the pneumococci show the presence of three unlinked genes conferring resistance to ampicillin, namely (*a*) *amp-A* conferring resistance to 0·03 μg./ml., (*b*) *amp-B*, which in the presence of *amp-A* confers resistance to 0·06 μg./ml. and (*c*) *amp-C* conferring resistance to 0·09 μg./ml. provided the organism also possesses *amp-A* and *amp-B*. Strains possessing the latter two systems are similar to strain r_4 of Schaeffer (1956), a pneumococcus which, although resistant to 50 μg. streptomycin/ml., gave a transformant in the first instance resistant to 10 μg./ml. which was then transformed by the same DNA to the full resistance of 50 μg./ml. The sulphonamide system described in pneumococcus by Hotchkiss & Evans (1958) however is very different; in this case the three individual markers *a*, *b*, and *d* potentiated each other to give higher resistances but each marker itself could be transformed as single units. The penicillin-resistant mutants of pneumococcus described by Hotchkiss(1951) were similar to our ampicillin-resistant mutants in the sense that the DNA from the penicillin-resistant mutants transformed to resistance levels lower than that of the donor DNA. However, they were very different in that no transformations to the highest resistance level possessed by the donor DNA were described even after further transformation on a resistant transformant. Also, the expression time of the penicillin mutants was more than 90 min.

It is worth noting that the three resistance loci were produced in the same organism by a single mutagenic treatment by NNG.

As suspected, the very short expression times of 4 to 5 min. were found to be artifacts due to the delay in the manifestation of the ampicillin action. Even so, an expression time of 20 to 25 min. is still short when compared to the other resistance markers known in pneumococcus, such as 70 min. for *opt-r2*, 90 min. for *ery-r2* and 120 min. for *str-41*. It is interesting to note that the apparent expression times become the same for all three loci when being transformed as singles or as doubles, the time for the singles being about half that of the doubles. Hence it would appear that the second marker in the double transformants could not be expressed until after the first marker had completed an essential step in its expression.

Nester (1964) showed that competent cultures of *Bacillus subtilis* were insensitive to penicillin, from which it was deduced that such cultures were not dividing, and it is believed that they are in a latent state. The results concerning the effect of ampicillin on the pneumococcal competent culture show that these cultures are not similar to the *B. subtilis* cultures in this respect since a doubling in the colony-forming units of the competent culture occurred first, followed by a rapid drop to 1 % survival after 50 min. exposure to the ampicillin.

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The Sensitivity of Gram-negative Bacteria, Recovered from Aerosols, to Lysozyme and Other Hydrolytic Enzymes

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SUMMARY

The susceptibility of five strains of bacteria, recovered after storage in the aerosol state, to certain hydrolytic enzymes has been examined. Aerosolized *Escherichia coli* organisms, strains B and JEPP, rapidly became susceptible to the bactericidal effects of lysozyme, ribonuclease, deoxyribonuclease and trypsin. *E. coli* strain COMMUNE and *Serratia marcescens* strain 8 UK organisms remained insensitive to all four enzymes, *Aerobacter aerogenes* strain H organisms developed sensitivity to lysozyme only. Raffinose, dextran, glucose, glycerol or sodium glutamate added to the bacterial suspensions increased their survival time as aerosols and decreased the sensitivity of survivors to lysozyme. These results support the hypothesis that changes in the outer wall structure of bacteria precede, and possibly contribute to, the death of organisms in the aerosol state.

INTRODUCTION

Bacterial survival in aerosols depends on a number of factors including relative humidity, composition of the gaseous environment and the compositions of both spray and collecting fluids (Webb, 1960; Cox, 1966*a*, *b*; Benbough, 1967; Cox & Baldwin, 1967). Comparatively little is known about the effect of aerosolization upon the integrity of bacterial walls and membranes.

The Gram-negative bacterial wall comprises an inner rigid layer of mucopolysaccharide, which can be depolymerized by lysozyme, and a more flexible lipoprotein-lipopolysaccharide component (Salton, 1957; Weidel, Frank & Martin, 1960) which overlays the mucopolysaccharide and protects it from the action of lysozyme, possibly by impeding penetration of the enzyme into the wall. Salton (1957) and Noller & Hartsell (1961) showed that Gram-negative bacteria can be made sensitive to lysozyme by certain pretreatments which appear to act by disrupting the outer wall complex (Voss, 1967; Wilkinson, 1968). It seemed possible, therefore, that changes in the structure of the bacterial wall, following aerosolization, might be detected by the development of sensitivity to various hydrolytic enzymes. Some indication of such a change has been observed by Maltman (1969), who studied the effects of lysozyme, ribonuclease and deoxyribonuclease on *Klebsiella pneumoniae* organisms that had been recovered from aerosols.

A number of substances improve the survival of bacteria in an aerosol and it has been suggested that these act by either limiting water loss from the organism (Cox, 1966*a*), replacing structural water (Webb, 1960) or through interaction with specific

intracellular sites (Benbough, 1969). The work described here was designed to test the propositions that damage to the bacterial wall preceded, and possibly contributed to, the death of bacteria atomized from aqueous suspension, and that certain 'protecting' agents acted by preventing these changes in structure.

METHODS

Organisms and growth conditions. Stock cultures of *Escherichia coli* (strains B, JEPP and COMMUNE), *Aerobacter aerogenes* (strain H) and *Serratia marcescens* (strain 8UK) were maintained as frozen pellets at $-70°$ (Cox, 1968). For use, the pellets were thawed, streaked on tryptone agar plates (Cox, 1966*a*) and cultures grown from individual colonies. For each experiment, cultures of *E. coli* (strains B, JEPP and COMMUNE) and *A. aerogenes* H were grown in shaken conical flasks (750 ml.) containing 30 ml. of the chemically defined medium of Benbough (1967) for 16 hr at 37°; *S. marcescens* 8UK was grown in the same way, but at 28°.

Preparation of suspensions for spraying. Cultures were harvested by centrifuging in an MSE bench centrifuge (3000 *g*, 10 min.), washed twice with distilled water and finally resuspended in distilled water to about 10^{10} organisms/ml. Protecting agents were added immediately before aerosol generation. The substances used, and final concentrations were: raffinose (0·3 M), glucose (0·3 M), glycerol (0·3 M), dextran (mol. wt 115,000, 5% w/v) and sodium glutamate (0·13 M).

Generation and collection of aerosols. Aerosols were generated using a 1- or 3-jet Collison spray (Green & Lane, 1957), mixed with a secondary air supply of controlled relative humidity (RH) and stored in a 75 l. rotating drum (Goldberg, Watkins, Boerke & Chatigny, 1958). Aerosol samples were collected by raised impingers (May & Harper, 1957) containing 1 M-sucrose in 20 mM-potassium phosphate buffer (Cox, 1966*a*). The sucrose in the collecting fluid minimized loss of viability of bacteria on rehydration without affecting enzyme activity.

The survival of bacteria in aerosols. Viability of aerosolized bacteria was estimated as described by Cox (1966*a*).

The sensitivity of aerosolised bacteria to enzymes. 2·0 ml. of a suspension (10^8 organisms/ml.) of bacteria recovered from aerosols, or diluted suspension which had not been aerosolized, were added to 1·0 ml. phosphate buffer containing lysozyme, ribonuclease, deoxyribonuclease or trypsin, 10 μg./ml. (The enzymes were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, England.) The suspensions were then incubated for 1 hr at 37°. Controls contained no added enzyme.

Estimation of survivors. Suspensions were diluted in phosphate buffer containing sucrose (0·3 M) and the numbers of colony-forming units from known volumes on tryptone agar plates were counted. Survivors were estimated before and after incubation with enzymes.

RESULTS

The sensitivity of bacteria recovered from aerosols to some enzymes

Prior to aerosolization, washed suspensions of *Escherichia coli* (strains B, JEPP and COMMUNE), *Serratia marcescens* 8UK and *Aerobacter aerogenes* H were all resistant to the bactericidal effects of lysozyme, ribonuclease, deoxyribonuclease and trypsin. Even after incubation with each of the enzymes for 1 hr at 37° the maximum decrease

in viability of each suspension was less than 2 %. Fig. 1 *a* shows that viability of *E. coli* B organisms stored in aerosols decreased from about 85 % after 1 sec. storage to 14 % after 10 min. and 6 % after 30 min. The effect of enzymes upon the viability of *E. coli* B organisms recovered from aerosols is shown in Fig. 1 *b*. Lysozyme consistently

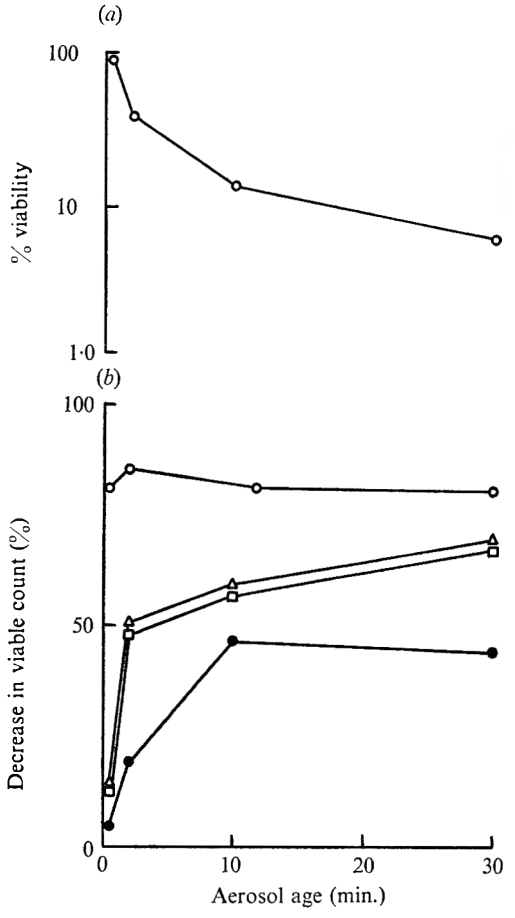

Fig. 1

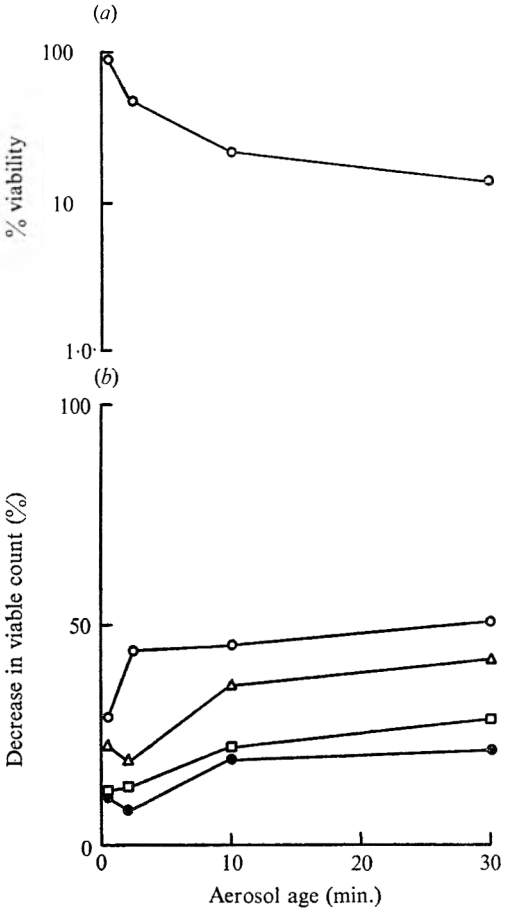

Fig. 2

Fig. 1 (*a*). The death rate of *E. coli* B sprayed from distilled water into air at 75 % RH.

Fig. 1 (*b*). The effect of some enzymes on the viability of *E. coli* B recovered after spraying into air at 75 % RH. Organisms were incubated with each enzyme (10 μg./ml.) for 1 hr at 37°. ○, Lysozyme; △, ribonuclease; □, deoxyribonuclease; ●, trypsin.

Fig. 2 (*a*). The death rate of *E. coli* JEPP sprayed from distilled water into air at 75 % RH.

Fig. 2 (*b*). The effect of some enzymes on the viability of *E. coli* JEPP recovered after spraying into air at 75 % RH. Organisms were incubated with each enzyme (10 μg./ml.) for 1 hr at 37°. ○, lysozyme; △, ribonuclease; □, deoxyribonuclease; ●, trypsin.

decreased the viable population by about 80 %, this value being independent of aerosol age. The decreases in viable counts following treatment with ribonuclease, dexoyribonuclease and trypsin were less marked with organisms from aerosols aged 1 sec. but the lethal effect of these enzymes became marked when bacteria were kept in the aerosol state for longer periods.

With *E. coli* JEPP organisms the effects of enzymes were less marked than with suspensions of *E. coli* B. Fig. 2*a* shows that survival of *E. coli* JEPP was nearly 100% after 1 sec. in aerosols (95% viability) but decreased subsequently (16% viability after 30 min.). The increased susceptibility of aerosolized *E. coli* JEPP organisms to treatment with enzymes is shown in Fig. 2*b*. Lysozyme decreased the viability of organisms collected from aerosols by about 50%. The other enzymes were always less effective than lysozyme but still showed marked effects on the viability of recovered bacteria.

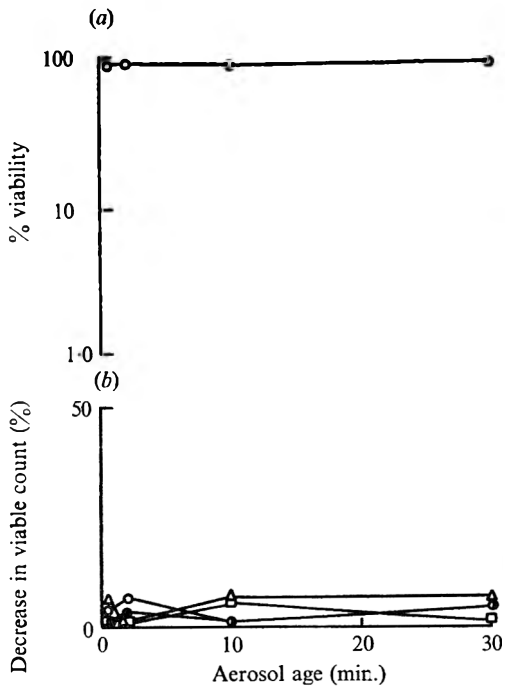

Fig. 3

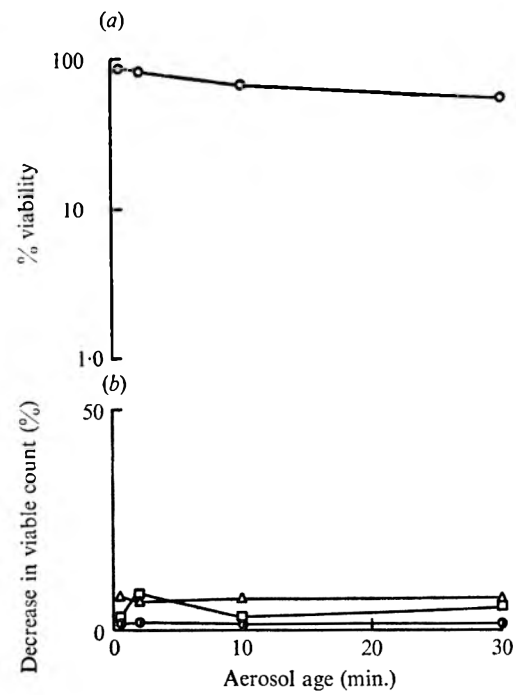

Fig. 4

Fig. 3(*a*). The death rate of *E. coli* COMMUNE sprayed from distilled water into air at 75% RH.

Fig. 3(*b*). The effect of some enzymes on the viability of *E. coli* COMMUNE recovered after spraying into air at 75% RH. Organisms were incubated with each enzyme (10 μg./ml.) for 1 hr at 37°. ○, lysozyme; △, ribonuclease; □, deoxyribonuclease; ●, trypsin.

Fig. 4(*a*). The death rate of *S. marcescens* 8UK sprayed from distilled water into air at 75% RH.

Fig. 4(*b*). The effect of some enzymes on the viability of *S. marcescens* 8UK recovered after spraying into air at 75% RH. Organisms were incubated with each enzyme (10 μg./ml.) for 1 hr at 37°. ○, Lysozyme; △, ribonuclease; □, deoxyribonuclease; ●, trypsin.

Escherichia coli COMMUNE organisms, unlike strains B and JEPP, survived well in the aerosol state (Fig. 3*a*), viability was 90% after storage for 30 min. The organisms recovered from aerosols were resistant to enzymes (Fig. 3*b*) and the decreases in the numbers of viable organisms, following various enzyme treatments, never exceeded 8%.

With *Escherichia coli* (strains B, JEPP and COMMUNE) there was a correlation between the survival of organisms in the aerosol state and their subsequent resistance to the lethal action of some enzymes. This relationship was also evident with *Serratia mar-*

cescens 8UK bacteria which survived well in aerosols (Fig. 4*a*) and when recovered from the aerosol state were resistant to the bactericidal effect of lysozyme, ribonuclease, deoxyribonuclease and trypsin.

Aerobacter aerogenes H organisms had survival characteristics that closely resembled those of *Serratia marcescens* 8UK (Fig. 5*a*). Fig. 5*b* shows that *A. aerogenes* bacteria

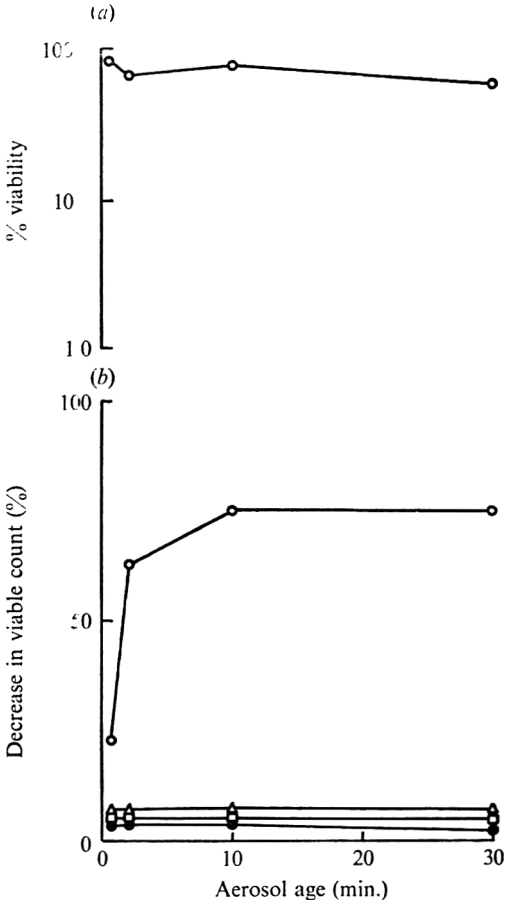

Fig. 5(*a*). The death rate of *A. aerogenes* H sprayed from distilled water into air at 75 % RH.

Fig. 5(*b*). The effect of some enzymes in the viability of *A. aerogenes* H recovered after spraying into air at 75 % RH. Organisms were incubated with each enzyme (10 μg./ml.) for 1 hr at 37°. ○, Lysozyme; △, ribonuclease; □, deoxyribonuclease; ●, trypsin.

collected from aerosols were unaffected by ribonuclease, deoxyribonuclease and trypsin. However, the viable count of organisms after 1 sec. in aerosols was decreased by about 20 % by lysozyme treatment and this susceptibility increased as the age of aerosols increased.

The effect of composition of the spray fluid on the sensitivity of bacteria recovered from aerosols to some enzymes

Suspensions of *Escherichia coli* strain B were sprayed from solutions containing raffinose (0·3 M), dextran (5 %, w/v), glucose (0·3 M), glycerol (0·3 M) or sodium

glutamate (0·13 M) and the sensitivities of surviving organisms to some enzymes were examined (Table 1). Clearly, all the additives lowered the sensitivity of bacteria recovered from aerosol to lysozyme; raffinose (which was present in the greatest mass concentration) and dextran were the most effective substances tested. Similarly, organisms sprayed from solutions containing these protecting agents and recovered were less susceptible to ribonuclease and deoxyribonuclease. Glucose, glycerol, sodium glutamate and dextran were less effective than raffinose in decreasing the susceptibility of recovered bacteria to trypsin.

The protecting agents did not act simply by inactivating the enzymes since the sensitivities of bacteria aerosolized from water to enzyme treatments were not affected by the addition of these protective substances to the collecting fluid.

Table 1. *The effect of composition of spray fluid on the sensitivity to some enzymes of Escherichia coli* B *collected after storage in aerosols at 75% RH for 10 min.*

Escherichia coli B organisms were aerosolized from various spray fluids into air at 75% RH. Samples were collected after storage in aerosols for 10 min. and incubated with each of the enzymes (10 μg./ml.) for 1 hr. at 37°. Numbers of viable organisms were estimated before and after incubation with the enzymes.

	Decrease in viable count (%)			
Spray fluid	Lysozyme	Ribo-nuclease	Deoxyribo-nuclease	Trypsin
Distilled water	81	61	55	52
Raffinose (0·3 M)	8	7	7	14
Dextran (5%, w/v)	7	4	0	40
Glucose (0·3 M)	21	0	2	57
Glycerol (0·3 M)	32	0	0	43
Sodium glutamate (0·13 M)	22	9	7	48

DISCUSSION

Although the walls of Gram-negative bacteria contain mucopeptide, generally these organisms are resistant to lysozyme. This probably is due to the lipoprotein-lipopolysaccharide outer wall layer which prevents access of the enzyme to the mucopeptide (inner wall) substrate. Sensitivity of Gram-negative bacteria to lysozyme can be induced by a variety of pretreatments which apparently alter the bacterial surface, exposing the lysozyme substrate (Salton, 1957; Voss, 1967; Wilkinson, 1968; Work, 1967). Thus, the development of sensitivity to lysozyme of organisms may be used as an indicator of disruption of the outer layers of the bacterial wall. This study shows that changes in the structure of some Gram-negative bacterial envelopes, manifested as the susceptibility of organisms to some enzymes, occurred following aerosolization of bacteria in air at 75% RH. The viabilities of *Escherichia coli* strains B and JEPP organisms recovered from aerosols were significantly decreased by lysozyme, ribonuclease, deoxyribonuclease and trypsin.

The viabilities of organisms of all the bacterial strains examined were, following recovery after 1 sec. in the aerosol state, invariably greater than 85%. There were significant differences in the sensitivities to enzymes of organisms recovered from aerosols within 2 min. of spraying. This sensitivity correlated with the subsequent survival patterns of bacteria in aerosols. For example, *E. coli* B which did not survive well in aerosols, were susceptible to the bactericidal action of lysozyme, ribonuclease,

deoxyribonuclease and trypsin on recovery. *Escherichia coli* JEPP which survived in aerosols better than *E. coli* B was generally more resistant to the lethal effect of the enzymes; *E. coli* COMMUNE and *Serratia marcescens* organisms, which were very stable in aerosols, were not susceptible to any of the enzymes. Since this correlation existed for organisms recovered from aerosols aged less than 2 min. where the number of viable organisms were similar for all the strains of bacteria examined, it is reasonable to suppose that the differences observed in the susceptibility of organisms surviving in aerosols for 30 min. to enzymes were valid, despite the fact that there then were marked differences in the proportions of viable organisms of each strain recovered.

The resistance of aerosolized *Aerobacter aerogenes* to ribonuclease, deoxyribonuclease and trypsin fitted into the pattern outlined above. However, these organisms became increasingly sensitive to lysozyme with aerosol age. This suggests that, whilst there was some change in structure of the envelope of aerosolized *A. aerogenes*, the changes were not as extensive as those occurring in the wall and membranes of aerosolized organisms of *Escherichia coli* strains B and JEPP.

The substances used as spray additives differ in their ability to penetrate the bacterial wall (Cox, 1963, 1966*a*, 1967). Under the conditions employed raffinose and dextran did not penetrate the wall. Glucose entered only very slowly, as did sodium glutamate, which does not permeate the bacterial membrane. Glycerol alone was capable of penetrating the organism rapidly. In the aerosol, these protecting agents form supersaturated solutions around the bacteria (Cox, 1966*a*) and as such they probably protect the wall from the otherwise damaging effects of aerosolization. All the substances studied protected the airborne bacteria from the lethal effect of extracellular enzymes. These results support the view that effective concentrations of some protective compounds in the spray fluid aid survival of aerosolized bacteria by preventing changes occurring in the bacterial walls and membranes.

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Enrichment, Isolation and Some Properties of Methane-utilizing Bacteria

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SUMMARY

More than 100 Gram-negative, strictly aerobic, methane-utilizing bacteria were isolated. All used only methane and methanol of the substrates tested for growth. The organisms were classified into five groups on the basis of morphology, fine structure, and type of resting stage formed (exospores and different types of cysts) and into subgroups on other properties. Methods of enrichment, isolation and culture are described.

INTRODUCTION

The existence of methane-oxidizing bacteria has been known for many years (see Leadbetter & Foster, 1958) but attempts to isolate them in pure culture have generally been unsuccessful. Dworkin & Foster (1956) and Leadbetter & Foster (1958) isolated a number of methane-utilizing organisms and considered them all to be variants of the one species, *Pseudomonas methanica*. Two more species have since been isolated, a rod, *Methanomonas methanooxidans* (Brown, Strawinski & McCleskey, 1964) and a coccus, *Methylococcus capsulatus* (Foster & Davis, 1966). All three species were Gram-negative aerobes utilizing only methane and methanol as combined carbon and energy sources. Other reports of methane-oxidizing bacteria have been too brief to indicate whether they were new species or strains of known species. Reports of Gram-positive bacteria provide no evidence of methane utilization in pure culture and generally mention the difficulty of maintaining the organisms on mineral salts media and methane, with one exception (Perry, 1968). This organism, a strain of Brevibacterium, grew on all *n*-alkanes tested (C_1 to C_{22}) and the disappearance of methane during growth was measured. Some reports relate the difficulties of obtaining pure cultures and describe results of studies on methane oxidation by mixed cultures (for instance, Vary & Johnson, 1967).

The problems encountered in the isolation and culture of methane-oxidizing bacteria and the lack of cultures available for study, prompted the investigation we report here. Simple methods of enrichment, isolation and culture of methane utilizers, some of which are unique, are described.

METHODS

Media. Two basal media were routinely used and differed only in the nitrogen source added. They both contained in distilled water (%, w/v): $MgSO_4.7H_2O$, 0·1; $CaCl_2$, 0·02; sequestrene iron complex (Geigy, Johnsons of Hendon, Ltd.), 0·0004;

either NH_4Cl, 0·05 (ammonium mineral salts—AMS); *or* KNO_3, 0·1 (nitrate mineral salts—NMS); agar if added, 1·25. Trace element solution (Pfennig, 1962), 0·05 % (v/v) was added and the pH value of the media adjusted to 6·8 before autoclaving. Finally, 2 ml. of a sterile phosphate buffer solution (a mixture of KH_2PO_4 and $Na_2HPO_4.12H_2O$; 15 g. in 300 ml. distilled water, pH value 6·8) was added to media cooled to 60°.

Maintenance of cultures. Organisms were subcultured every 2 weeks on NMS or AMS agar slopes in tubes (plugged with cotton-wool) and incubated either in glass desiccators or bucket-shaped 'Tupperware' polythene containers (about 5 l. capacity) which had lids with airtight seals and, in the lids, small ports sealed with airtight caps. Desiccators were partially evacuated and filled with methane from a football bladder to give an approximate mixture of methane to air 30:70 under slightly less than atmospheric pressure. Polythene containers were gassed with methane by squirting a football bladder full (about 2 l.) through the ports in the lids, the final mixture of methane to air ranging from 30:70 to 50:50. Incubation was at 30°. Many cultures survived in these containers for 3 months at 4°, but frequently became contaminated with fungi as occasionally were cultures transferred at 2-weekly intervals.

The high humidity which developed in the enclosed containers stimulated fungal spores present in the unsterile atmosphere to germinate and grow on the inside surfaces of the containers and on the tube racks. Cultures incubated in containers in this state were frequently contaminated with fungi. Microscopical examination of cotton-wool plugs in culture tubes at intervals revealed that fungal mycelia grew on the outer top surface of the plugs and gradually penetrated down to the underside surface of the plugs in the tubes, where they released spores into the culture atmospheres. The spores subsequently contaminated the bacterial culture. This problem was overcome by (*a*) swabbing out the container and the tube racks with ethanol between transfers, and (*b*) maintaining a duplicate set of cultures. Cleaning the containers regularly with ethanol reduced fungal contamination to an infrequent occurrence. Replacement of contaminated cultures with pure cultures from the duplicate set dispensed with the problem of having to purify the contaminated cultures. Liquid media were usually dispensed into conical flasks (generally 250 ml. narrow-necked Quickfit flasks, 19/26). After inoculation, cotton-wool plugs were replaced by 'Suba-seal' caps. Tubes and 'Universal' vials were also used.

For stoichiometric and bacterial dry weight determinations methane content was reduced to a ratio of methane to oxygen (in air) 1:2 or 1:4.

Screw-cap bottles of 250 ml. capacity were used in growth yield experiments. 'Suba-seal' caps were fitted, methane inserted and metal caps screwed tightly over the 'Suba-seal' caps. Methane loss from these bottles was less than 1·0 % of that added (after a week).

In all sealed cultures, medium usually occupied 20 % of the container volume and the methane to oxygen (in air) ratio was usually 2:1. Air was not withdrawn before inserting methane by syringe so growth began at about 1·4 atmospheres pressure. Air-lift fermenters, stirred batch systems and conventional continuous culture vessels were adapted for use with methane. Tubing and stoppers were of silicone rubber as red rubber released toxic substances when in contact with liquid medium. As silicone rubber is permeable to methane, silicone rubber tubing was used to sleeve glass tubes together leaving as small an area as possible of silicone rubber in direct contact with

methane. Losses of methane from these systems during experiments involving measurements of gas utilization were less than 1·0 % (v/v). Closed-circuit systems were used in many cases, using aspirators as gas reservoirs and Watson–Marlow H.F. flow inducers as circulation pumps. Sampling devices, for both liquid and gas phases, were made from syringes inserted into silicone rubber bungs. All such systems were operated for long periods without contamination.

Routine production of large amounts of batch culture. A 5 l. Quickfit culture vessel, with a 5-port lid, containing 200 ml. liquid medium and 20 ml. inoculum was stirred magnetically at 30°. Two of the ports were sealed with 'Suba-seal' caps. The initial inoculum was pipetted through one of these ports. A third port was fitted with a glass stopcock which was connected with silicone rubber tubing to a medium reservoir. A fourth port was fitted with a glass tube to which was attached a football bladder containing 2 l. methane. Methane was sterilized on entering the vessel by a cotton-wool filter in the glass tube. The centre port was fitted with a stirrer gland converted to a siphon of adjustable depth. After 24 hr the culture was harvested aseptically by siphon leaving about 20 ml. behind to inoculate fresh medium transferred from the medium reservoir by peristaltic pump after flushing out the vessel with sterile air. Methane was added as before. Batteries of such systems were set up in a hot room.

Inocula. Inocula were taken either from 2-day-old slope cultures or, when many vessels were to be inoculated with the one strain, from 20 ml. flasks containing 5 ml. liquid culture (plugged with cotton-wool) incubated in desiccators.

Growth on methanol and other substrates. Conventional methods were used except when growth yields and changes in the culture atmosphere were being determined when sealed systems were used.

Slide cultures. Molten agar medium was poured on to sterile glass slides and a sterile coverslip dropped on to the molten agar to cast a flat surface. The coverslip was removed when the agar solidified and the surface inoculated by streaking. After the surface had dried, a central channel of agar was removed and a coverslip placed on the agar. The slide cultures were incubated in a polythene container to which methane was added. Growth of the cultures was observed under a phase-contrast microscope at intervals.

Analyses. A katharometer isothermal head on a Pye series 104 gas chromatograph was used to analyse for CH_4, CO_2, O_2 and N_2. Gas samples in which the content of CH_4 and CO_2 were being compared, were passed through a 5 ft column containing 80 to 100 mesh silicagel at 85°, and samples in which CH_4, O_2 and N_2 were being compared, were passed through a 5 ft column containing 60 to 80 mesh molecular sieve at 85°. Helium, the carrier gas, flowed at 60 ml./min. Signals were recorded on a Leeds and Northrup Speedomax 'w' recorder. Samples (1·0 ml.) were extracted and 0·5 ml. was inserted by syringe, or 0·5 ml. samples were removed from closed atmospheres by a gas sampling loop attached to the chromatograph. Changes which occurred in the composition of gas mixtures over stirred batch cultures were assayed at short intervals by the latter method.

Methane (when O_2, CO_2 and H_2 were not being assayed), ethane, methanol, ethanol and acetaldehyde were assayed with a flame ionization detector on a Pye series-104 gas chromatograph using a 5 ft column packed with 'Phasepack Q', treated with 'trimer' acid (Phase Separation Ltd., Flintshire), at 100°. Gas samples containing acetylene and ethylene were assayed on a 5 ft column containing 'Phasepack R' at

100°. Nitrogen, the carrier gas, was flowed through both columns at 60 ml./min. Gas samples (0·5 ml.) and liquid samples (1·0 μl.) were extracted and 0·2 ml. and 0·4 μl., respectively, inserted by syringe. Results were recorded on a Vitatron Manual Linear Recorder UR 402.

Gases. Methane, biologically produced, was obtained from Greater London Council, Mogden Works, Isleworth, Middlesex, and was used routinely. Methane and other gaseous *n*-alkanes of high purity were from Air Products Ltd. and Cambrian Chemicals Ltd. Natural gas, originating from the Sahara and from the North Sea, was from Air Products Ltd.

Reduction of acetylene. Batch cultures (25 ml. in 250 ml. bottles sealed with a 'Suba-seal' cap and screw-capped) grown in AMS liquid medium, in which NH_4Cl had been lowered to 0·01 % (w/v), under a methane-air mixture, were screened for ability to reduce acetylene to ethylene. When ammonium ion was no longer detectable, the cultures were flushed with helium and resealed with 'Suba-seal' caps. Helium (20 ml.) was withdrawn and gases (28 ml.) added (O_2, 10 ml.; CH_4, 10 ml.; N_2, 4 ml.; C_2H_2, 4 ml.). Metal caps were screwed over the seals and the stagnant cultures were incubated at 30°. Samples of gas were withdrawn at 7 and 14 days and analysed.

Assay of poly-β-hydroxybutyrate. The method of Law & Slepecky (1961) was used.

Nitrite estimation. The method of Rakestraw (1936) was used.

Incubation. Cultures were incubated either statically or in shaking incubators at 30, 37 or 45°.

RESULTS AND DISCUSSION

Enrichment and isolation

Mud and water (from ponds, rivers, streams and ditches), and soil samples, obtained from the U.K., the European continent (France, Germany and Russia), North America, South America, East and North Africa and Egypt, were used as inocula. Approximately 1 g. was added to 25 ml. of AMS or NMS liquid medium in a 250 ml. bottle, which was sealed with a 'Suba-seal' cap and then injected with 20 ml. of methane. The enrichments were incubated statically at 30, 45 and 55°. Turbidity, often accompanied by a pellicle, was taken to be presumptive evidence of the growth of methane-utilizers and usually appeared after 3 or 4 days.

Such cultures were serially diluted in sterile tap water and spread on to AMS or NMS agar plates which were incubated in a methane-air mixture in vacuum desiccators or polythene containers. The plates were examined at 3-day intervals over 3 to 4 weeks. Colonies which proved to be non-methane utilizing reached their maximum size in about 3 days. Methane-utilizing colonies began to appear after about 5 to 7 days and were frequently 10 to 100-fold fewer in number than non-methane-utilizing colonies which presumably were using dissolved organic materials in the agar component of the medium; colonies, similar in size and number, appeared on duplicate plates incubated in the absence of methane. Under a plate microscope colonies which later proved to be composed of methane-utilizing organisms were opaque and on continued incubation increased in size over 2 to 3 weeks. Non-methane-utilizing colonies remained small and relatively transparent.

Isolation was most successful at the small colony stage (0·2 mm. diam.). With the aid of a plate microscope, colonies were transferred with a straight wire to AMS or NMS agar slopes, which were incubated for 2 weeks. The subsequent growth was

streaked on to AMS or NMS agar plates, single colonies picked and restreaked on to fresh medium. Isolates were considered to be pure if colonies were similar in appearance, consisted of morphologically similar organisms, failed to grow on nutrient agar and yeast extract agar, and grew on NMS and AMS agar only when methane was present in the culture atmosphere. Ability to utilize methane was checked by analysing gas samples at intervals.

The two main problems encountered in isolation were the scavenging activities of amoebae (cross-contaminating and consuming colonies) and the contamination of methane-consuming colonies with barely visible colonies of actively motile, small Gram-negative rods which did not utilize methane.

Properties

General. All the organisms were strictly aerobic, catalase and oxidase-positive, Gram-negative bacteria, growing only on methane and methanol of the substrates tested (below). Methanol was extremely toxic to many strains when added to the medium, even at 0·01 % (w/v), and they only grew on methanol when incubated on slopes of NMS agar in sealed 5 l. containers containing methanol vapour given off slowly from 4 or 5 slopes of uninoculated agar containing 0·1 % (w/v) methanol. Other strains tolerated up to 1·5 % (w/v) methanol in the medium. Formate, although oxidized by suspensions of organisms, was not utilized as a growth substrate, nor were other organic acids (gluconate, citrate, malate, acetate, fumarate, pyruvate and lactate), sugars and related compounds (glucose, mannose, fructose, arabinose, xylose, sucrose, maltose, cellobiose, lactose, glycerol, sorbitol and mannitol), and amino acids (L-glutamic acid, L-lysine, L-aspartic acid, L-serine, L-alanine, L-arginine, L-tryptophan and Casamino acids). Nutrient agar and yeast extract (0·5 % w/v) agar inhibited the growth of all organisms in the presence of methane. All strains grew over a pH range of 5·8 to 7·4 and a pH value of 6·6 to 6·8 appeared to be the optimum for growth rate and yield.

The final composition of the mineral salts media routinely used was chosen to suit all strains. Concentrations of phosphates greater than 0·2 % (w/v) and ammonium chloride greater than 0·05 % (w/v) inhibited many strains. Iron concentrations, as sequestered iron, were critical; a 10-fold increase or decrease of the concentration used diminished the growth rate of a number of strains. Calcium was essential and a trace element mixture was routinely included in the medium as some strains behave erratically in its absence.

All organisms were subcultured at 2-weekly intervals at 30°.

Nitrogen sources. Ammonium salts were used by all the organisms, nitrite and nitrate salts by the majority, and urea, casamino acids and yeast extract by some. All formed non-inhibitory concentrations of nitrite from ammonia, an activity previously recorded for methane-utilizers by Hutton & ZoBell (1949). Organisms using nitrate reduced some to nitrite, but were unable to grow anaerobically on methane with nitrate as an alternative electron acceptor to oxygen. Nitrate appeared to be preferred to ammonium salts by these organisms as a nitrogen source, as reported by Leadbetter & Foster (1958) for their organisms, but this proved to be an effect of pH change in the growing cultures. While the pH value in nitrate cultures remained around neutrality, in ammonium salts cultures it fell to 5·0 or lower and growth was partially inhibited. When pH value was controlled automatically by titration with KOH, growth rates

were similar with nitrate and ammonium salts and growth yields higher with ammonium salts.

No direct assay of nitrogen-fixing ability has been made, but all strains were screened for their ability to reduce acetylene to ethylene, a standard assay for nitrogenase (Dilworth, 1966; Schöllhorn & Burris, 1967). Only one organism actively reduced acetylene (25 % of that available compared with less than 1·0 % or none by the other organisms). This organism, strain '*Methylosinus trichosporium*' PG was isolated from a culture originating from V. F. Coty (Coty, 1967), kindly provided by Professor J. R. Postgate.

Methane and methanol utilization. A comparison of the stoichiometry of methane and methanol utilization, and the efficiency with which they were converted into bacterial dry weight, was carried out on a number of strains. The two most efficient organisms were non-capsulate, non-slime-forming, did not normally form detectable lipid inclusions, and were not inhibited by methanol at the concentrations used. The following results apply to these two strains. Organisms and carbon dioxide were the only end-products of methane and methanol utilization detected. The stoichiometry of methane oxidation was similar when measured in batch cultures, in which all the methane had been consumed, and when sampled at hourly intervals in a culture growing exponentially in a closed, circulating atmosphere of methane and air (see Methods). Results are expressed in the following two equations:

$$\text{(Methane) } 1\ CH_4 + 1{\cdot}0 \text{ to } 1{\cdot}1\ O_2 \rightarrow 0{\cdot}2 \text{ to } 0{\cdot}3\ CO_2$$

$$\text{(Methanol) } 1\ CH_3OH + 1{\cdot}0 \text{ to } 1{\cdot}1\ O_2 \rightarrow 0{\cdot}5 \text{ to } 0{\cdot}6\ CO_2.$$

The finding that more carbon dioxide was formed from methanol than from methane correlated with the bacterial dry weight yields, 0·4 g. dry wt/1·0 g. methanol and 1·0 to 1·1 g. dry wt/1·0 g. methane. On a molar basis methanol yielded about 20 % less dry weight organisms than did methane, implying that energy useful to the organisms is released in the initial oxidation of methane. Estimated carbon balances, based on the stoichiometry and bacterial dry weights obtained and assuming 47 % of the bacterial dry weight is carbon (Johnson, 1967) were about 100 % and suggested that carbon-containing products other than organisms and carbon dioxide were not formed under these cultured conditions.

Bacterial dry weights obtained with slime-forming strains were less than 1·0 g./1·0 g. methane, and carbon dioxide production higher per mole of methane than that given above. Variations in the stoichiometry of methane utilization also occurred under nitrogen limitation and with non-growing bacterial suspensions. In the former case, oxygen consumption and carbon dioxide production were lower per mole of methane utilized and the organisms became packed with lipid inclusions, the major portion being identified as poly-β-hydroxybutyrate. In the latter case oxygen consumption and carbon dioxide production increased per mole of methane utilized.

The pattern of oxygen consumption and carbon dioxide production by organisms growing on methane reported here is very similar to that recorded by Brown *et al.* (1964) but not to that reported by Leadbetter & Foster (1958). Their observation, 0·4 mole of O_2 utilized/1·0 mole of methane, seems impossibly low, especially on the assumption they make that 0·5 mole of O_2 is required in the initial conversion of 1 mole methane to methanol.

The effects of ethane, ethanol and methanol on methane oxidation. Leadbetter &

Foster (1958) observed that ethane and ethanol, while not supporting growth, were oxidized by methane utilizers. Most of the methane utilizers studied by us behaved similarly, non-growing suspensions of bacteria oxidizing ethane or ethanol to acetaldehyde, as recorded for *Methylococcus capsulatus* (Patel, Hoare & Taylor, 1969). One strain did not oxidize ethane but was inhibited by it, failing to oxidize methane, methanol or ethanol in its presence. A survey of the effects of methane, ethane, methanol and ethanol on the oxidation of each other revealed both stimulatory and inhibitory effects represented by the examples in Table 1. Methane oxidation by all strains was inhibited to different degrees by ethane and frequently by methanol and by ethanol. The inhibitory effect of ethane on methane oxidation was reflected in the growth rates of methane-utilizers on Sahara natural gas (containing 7·7 % v/v ethane) which proved to be inferior to biologically produced methane (free of other hydrocarbons) as a substrate. Organisms either failed to grow, or had 25 to 50 % longer generation times on Sahara natural gas than on ethane-free methane. Preliminary experiments revealed that *n*-propane and *n*-butane were at least as inhibitory as ethane.

Table 1. *Oxidation of methane, methanol, ethane and ethanol (single and in pairs) by methane-utilizing bacteria*

Conical flasks (25 ml.) contained 5 ml. suspensions of bacteria (equiv. dry wt, 3 mg./ml.) in NMS medium (see Methods) and were sealed with 'Suba-seal' caps through which substrate (45 μmoles), in liquid or gas phase, was inserted by syringe. Ratio of air to gaseous hydrocarbons, volumetrically, was approximately 20:1 (single hydrocarbon) or 20:1:1 (two hydrocarbons); suspensions were shaken for 4 hr at 30° and gas and liquid samples were removed by syringe for analysis (see Methods). Samples from controls (flasks with no bacteria) were taken at the beginning and end of the experiment.

		μmoles of substrate oxidized alone or in the presence of second substrate				
Strain	Substrate	Alone	$+CH_4$	$+CH_3OH$	$+C_2H_6$	$+C_2H_5OH$
'*Methylomonas agile*' A 30	CH_4	13	—	9	0	1
	CH_3OH	23	21	—	19	21
	C_2H_6	0	0	5	—	NT
	C_2H_5OH	12	16	12	NT	—
'*Methylococcus capsulatus*' MC	CH_4	26	—	15	21	10
	CH_3OH	31	23	—	28	NT
	C_2H_6	5	12	12	—	—
	C_2H_5OH	27	28	23	NT	—
'*Methylosinus trichosporium*' OB3B	CH_4	42	—	3	7	8
	CH_3OH	39	36	—	36	42
	C_2H_6	13	10	16	—	NT
	C_2H_5OH	42	42	23	NT	—
'*Methylomonas albus*' BG 8	CH_4	17	—	19	5	5
	CH_3OH	32	32	—	32	28
	C_2H_6	8	11	16	—	NT
	C_2H_5OH	30	31	28	NT	—

Effect of ammonium ion on methane oxidation. In cultures containing ammonium chloride above 0·05 % (w/v) growth rates of many organisms were lowered. The rate of methane oxidation by suspensions of bacteria (Table 2) decreased with increasing ammonium ion concentration. Strains differed in their sensitivity towards ammonium ion; the examples given in Table 2 represent the behaviour of most organisms, some

were less and others more sensitive to ammonium ion. The possibility that nitrite (formed by all strains) might be accumulating to inhibitory levels was discounted, as a concentration 10-fold greater than the maximum detected did not affect either methane oxidation or growth rates when added to AMS and NMS media. These results suggest the possibility of ammonium ion being an analogue of methane and perhaps a competitive inhibitor of its oxidation.

Table 2. *Ammonium ion inhibition of methane oxidation by strain 'Methylosinus trichosporium' OB3B*

Conical flasks (25 ml.) contained 5 ml. suspensions of bacteria (grown on NMS medium equiv. dry wt, 3 mg./ml.) in AMS medium (see Methods) containing different concentrations of ammonium chloride, and were sealed with 'Suba-seal' caps through which methane (45 μmoles) was inserted by syringe. Suspensions were shaken for 4 hr at 30° and gas and liquid samples were withdrawn by syringe for analysis of methane and nitrite content (see Methods). Control, uninoculated flasks, were sampled at the beginning and end of the experiment.

Ammonium chloride (%, w/v)	Methane oxidized (μmoles)	Nitrite formed (μmoles)
0	30	0
0·005	28	0·2
0·05	22	0·7
0·1	17	Trace
0·5	2	Trace

Similar results were obtained with equivalent amounts of ammonium ion as ammonium sulphate.

Morphology. Strains differed in morphology; rods, cocci, vibrioid and pear-shaped organisms, of various sizes and dimensions, were observed, some possessing capsules and flagella. Many formed resting stages; three distinct types were detected, exospores and two types of cyst, one of which was morphologically similar to those formed by Azotobacter species. All were desiccation-resistant (viable after 3 days air-drying on glass slide), the exospores being also heat-resistant (85° for 15 min.). A fourth type of cell resembling an immature Azotobacter-type cyst was formed by some strains and was considered to be a resting stage in that such organisms survived the absence of methane but not desiccation. Resting stages and their properties are described in another report (Whittenbury, Davies & Davey, 1970).

Fine structure. All strains possessed complex membranous organelles similar in complexity to those found in nitrifying and photosynthetic bacteria. Two basic membrane arrangements were observed, a system of paired membranes running throughout the bacterium or aggregated at its periphery (type I), and a series of bundles composed of disc-shaped membrane vesicles distributed throughout the cell (type II). These structures are described in detail in another report (Davies & Whittenbury, 1970).

Classification

Attempts at a formal classification are premature at this stage; conventional tests provided little information of differential value. However, variation in morphology, fine structure and type of resting stage formed, indicated fundamental differences between strains and served as a basis for a provisional classification (Table 3) of the organisms into two vibrioid groups, 'Methylosinus' and 'Methylocystis', and three

rod/coccoid groups, 'Methylomonas', 'Methylobacter' and 'Methylococcus'. These groups were subdivided on the basis of various characteristics (Table 4).

Group 'Methylosinus' (subgroups 'trichosporium' and 'sporium'). In the non-sporing stage the organisms were generally rod-shaped (Pl. 1, fig. 1), but occasionally of bizarre form (Pl. 1, fig. 7), and possessed a polar tuft of flagella (Pl. 3, fig. 31, 32). Rosettes (Pl. 1, fig. 4, 5) were frequently formed, the organisms being anchored at their non-flagellated poles by 'holdfast' material discernible microscopically (Pl. 1, fig. 4) and stained by the polysaccharide stain of Hotchkiss (1948). The capsules formed by these organisms did not respond to the polysaccharide stain. Exospores (Pl. 1, fig. 1, 2), which were heat-resistant, were budded off the non-flagellated poles of the organisms which assumed a pear-shape ('*Methylosinus trichosporium*') or vibrio-shape ('*Methylosinus sporium*') (Pl. 1, fig. 1, 2), at the onset of sporulation. All strains possessed a type I membranous system and divided at 5 to 6 hr under optimal growth conditions. Main differences between the two subgroups were cell-shape and size, spore morphology ('*Methylosinus trichosporium*' spores were capsulated) and pigment production.

Table 3. *Properties characterizing groups of methane-utilizing bacteria*

Group	Resting stage	Membrane type	Morphology	Rosettes formed	Colloquial description
'Methylosinus'	Exospore	I	Rod or pear-shaped cells	+	Vibrioid groups
'Methylocystis'	'Lipid' cyst	I	Rod or vibrioid cell	+	
'Methylomonas'	'Immature* Azotobacter-type' cyst	II	Rod	–	Rod/coccoid groups
'Methylobacter'	Azotobacter-type cyst	II	Rod	–	
'Methylococcus'	'Immature* Azotobacter-type' cyst	II	Coccus	–	

* Not all organisms form an identifiable resting stage.

Group 'Methylocystis' (subgroup 'parvus'). Only one strain was isolated (Pl. 1, fig. 3). It was similar in fine structure and rosetting habit (Pl. 1, fig. 6) to 'Methylosinus' strains, but differed from them in being non-motile and non-spore-forming. A cyst, however, was formed which was desiccation but not heat-resistant.

Group 'Methylomonas' (subgroups 'methanica', 'albus', 'streptobacterium', 'agile', 'rubrum' and 'rosaceus'). All the organisms were rod-shaped (Pl. 1, fig. 8 to 13) and possessed a type II membranous system. Many were capsulated (e.g. Pl. 3, fig. 26). Motile strains were polarly flagellated (Pl. 3, fig. 33, 34). In all subgroups some strains formed a resting stage (similar to immature azotobacter-type cysts) which was not desiccation resistant but survived the absence of methane for 4 to 5 weeks; vegetative cells only survived 3 to 4 days. The failure to detect the presence of desiccation-resistant forms in cultures of these organisms may be a result of inadequate cultural conditions.

Subgroup 'methanica' strains (Pl. 1, fig. 13) were identified as *Pseudomonas methanica* as described by Leadbetter & Foster (1958). They were isolated from samples from all areas examined and were the most commonly encountered methane-utilizing bacterium. On isolation colonies were both yellow-ochre and pink-coloured, the yellow-ochre colour disappearing as the culture aged and not appearing at all after

Table 4. *Properties of subgroups of methane-utilizing bacteria*

Group and subgroup	No. of strains	Growth at 37°	Growth at 45°	Growth on methanol (0·1 % w/v)	Growth on CH_4 enhanced (0·1 % w/v) Yeast extract	Malate	Acetate	Succinate	Shortest division time (hr)	Motility and flagellation	Capsule formed	Colony colour	Water soluble pigment
'Methylosinus'													
'*trichosporium*'	9	+	−	−	−	−	−	−	5	+PT	+*	W-Y	−
'*sporium*'	12	+	−	−	−	+	+	+	5	+PT	+*	W-Bu	Br-Bl‡
'Methylocystis'													
'*parvus*'	1	+	−	−	−	−	−	−	5	−	+*	W	−
'Methylomonas'													
'*methanica*'	30	−	−	+	+	+	−	+	3·5	+P	+	Oc-Pi	G-S‡
'*albus*'	3	+	−	+	+	−	−	+	3	+P	−	W	−
'*streptobacterium*'	5	−	−	−	−	−	−	−	4	−	+	W	−
'*agile*'	4	+	−	+	+	−	−	−	3·5	+P	−	W	−
'*rubrum*'	7	−	−	+	−	−	−	−	4	+P	+	R	−
'*rosaceus*'	2	−	−	−	−	−	−	−	4	+P	+	PP	−
'Methylobacter'													
'*chroococcum*'	9	−	−	−	−	−	−	−	5	−	+	PP	−
'*bovis*'	5	+	−	−	−	+	−	−	4	−	−	W-Br†	Y
'*capsulatus*'	4	−	−	−	−	−	−	−	4	+P	+	W-Br†	−
'*vinelandii*'	5	+	+	−	−	−	−	−	3	+P	+	W-Br†	−
'Methylococcus'													
'*capsulatus*'	3	+	+	−	−	−	−	−	3·5	−	+	W	−
'*minimus*'	5	−	−	−	−	+	−	−	3·5	−	+	W	−

PT = Polar tufts of flagella; P = polar flagellum; W-Y = white to yellow; W-Bu = white to buff; W = white; Oc-Pi = yellow-ochre to pink; R = red; PP = pale pink; W-Br = white to brown; Br-Bl = brown to black; G-S = green to sapphire.

* Capsules under electron microscope consisted of short fibres radiating from cell wall. No structure was seen in capsules of other organisms.

† Brown colour restricted to colonies containing cysts.

‡ Pigment produced on iron-deficient medium.

several subcultures. Carotenoid spectra were similar to those recorded by Leadbetter & Foster for *P. methanica*; no strain has yet lost its pigmentation as did some of Leadbetter & Foster's organisms. Colony texture varied with the nitrogen source. In agar medium containing ammonium salts, colonies were tough and difficult to remove, the organisms being embedded in slime (Pl. 3, fig. 23), and in liquid medium organisms grew in clumps which were difficult to break up. When nitrate was used, a slimy variant colony appeared on agar, easy to subculture, and after two or three subcultures became dominant, but colony type reverted to the tough form on subculture back to ammonium salts-containing agar media. In liquid media containing nitrate, copious amounts of slime were found, but the organisms were dispersed. Similar growth behaviour was observed by Leadbetter & Foster (1958) for their pink strains of *P. methanica*.

In static liquid cultures, the 'methanica' strains formed pellicles; most of the other subgroups did not.

Group 'Methylobacter' (subgroups 'chroococcum', 'bovis', 'capsulatus' and 'vinelandii'). Most strains were rod-shaped at all stages in their growth cycle, many resembling 'Methylomonas' strains morphologically. Some, however, were remarkably similar to the large-cell-forming species of Azotobacter, changing from rod form to coccal and intermediate forms and back to rod form (Pl. 2, fig. 14 to 17). In coccal form, tetrads were produced (Pl. 2, fig. 18). The largest organism (Pl. 2, fig. 19) measured 4·5 μm. $\times$ 3·2 μm. but was fragile and burst easily under pressure applied by thumb to a coverslip on a glass slide (Pl. 2, fig. 20). The similarity of all 'Methylobacter' strains to Azotobacter species extended to slime and capsule formation (Pl. 3, fig. 24, 28, 29), formation of desiccation-resistant cysts (e.g. Pl. 3, fig. 30) and production of pigment on cyst formation. None, however, grew in nitrogen-free liquid media and no Azotobacter species in the National Collection of Industrial Bacteria (Aberdeen) oxidized methane.

When isolated, all the large organisms of the 'chroococcum' subgroup formed multiple-bodied cysts but on continued subculture ceased to do so and diminished in size, finally dying out after 8 to 12 months. No medium has yet been devised which was able to maintain them for more than 3 to 4 weeks in the form in which they were isolated. The remaining strains of 'Methylobacter' were more robust than 'chroococcum' strains and were easily maintained. They have continued to form single and multiple-bodied cysts for 2 years. Subgroup 'vinelandii' strains were the most commonly isolated methane utilizers from enrichments at 37 to 55°. The first of the subgroup 'bovis' strains was isolated from a cow's mouth.

All 'Methylobacter' strains possessed a type II membranous system. Some were polarly flagellated. Under optimal growth conditions, the generation time was about 4 hr for all strains.

Organisms were divided into subgroups on the basis of morphology, pigment, motility and growth temperature.

Group 'Methylococcus' (subgroups 'capsulatus' and 'minimus'). These organisms were non-motile cocci (Pl. 2, fig. 21, 22) possessing capsules (e.g. Pl. 3, fig. 27) and a type II membranous system. Both types formed resting stages similar to immature azotobacter cysts. Shortest generation times were 3·5 to 4 hr. Organisms were divided into subgroups on morphology ('*Methylococcus minimus*' strains formed chains) and ability to grow at 37 and 45°. Subgroup 'capsulatus' includes a strain of *Methylococcus capsulatus* isolated by Foster & Davis (1966).

Comparison of isolates with previously described species

Some strains were considered to be identical with the previously described methane-utilizing species, *Pseudomonas methanica* (Leadbetter & Foster, 1958) and *Methylococcus capsulatus* (Foster & Davis, 1966). None were identified as *Methanomonas methanooxidans* (Brown *et al.* 1964). The 'Methylosinus' strains may be similar to *Pseudomonas methanitrificans* (Coty, 1967), as one strain was isolated from a culture originating from Coty. Other strains described here may have been encountered by Leadbetter & Foster (1958), particularly the '*Methylobacter vinelandii*' strains, which seem very similar to the organisms isolated by Leadbetter & Foster from enrichments at 37°.

CONCLUSION

Methane-utilizing bacteria were found to be a more diverse group of organisms than was implied in earlier reports. All utilized only methane or methanol of the substrates tested and all possessed other unusual properties (a complex fine structure and the ability to form cysts and exospores). As this report is only an introduction to the organisms, no attempt will be made to speculate on the significance of their unusual properties. A curious result of this investigation was the failure to isolate any Gram-positive organism, bacterium or yeast, able to utilize or oxidize methane. In this laboratory Mr A. G. McLee isolated a variety of Gram-positive bacteria which utilized C_2 to C_4 *n*-alkanes, but did not oxidize methane.

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EXPLANATION OF PLATES

PLATE 1

Phase-contrast micrographs, magnification × 1900. The bar (fig. 1) equals 5 μm.

Fig. 1. Indian ink preparation of '*Methylosinus trichosporium*' showing a pair of vegetative organisms (top right) and sporulating organisms budding off spores (centre). Spores possess larger capsule than sporulating or vegetative organisms.

Fig. 2. '*Methylosinus sporium*' preparation showing occasional vegetative cell and many sporulating organisms (vibrioid) and spores.

Fig. 3. '*Methylocystis parvus*' vegetative organisms.

Fig. 4. '*Methylosinus trichosporium*' rosette. Organisms anchored by visible 'holdfast' material.

Fig. 5. '*Methylosinus sporium*' rosettes made up of vegetative and sporulating organisms.

Fig. 6. '*Methylocystis parvus*' rosette of organisms.

Fig. 7. '*Methylosinus trichosporium*' malformed vegetative organism.

Fig. 8. '*Methylomonas rosaceus*' organisms.

Fig. 9. '*Methylomonas rubrum*' organisms.

Fig. 10. 'Methylomonas' organisms.

Fig. 11. '*Methylomonas streptobacterium*' organisms.

Fig. 12. '*Methylomonas agile*' organisms.

Fig. 13. '*Methylomonas methanica*' organisms.

PLATE 2

Phase-contrast micrographs, magnification × 1900. The bar (fig. 15) equals 5 μm.

Fig. 14. '*Methylobacter chroococcum*' strain HP organisms.

Fig. 15, 16. '*Methylobacter chroococcum*' strain P in coccal form and rod and rod-coccal form.

Fig. 17. '*Methylobacter chroococcum*' strain F organisms.

Fig. 18. '*Methylobacter chroococcum*' strain G in filamentous form.

Fig. 19. '*Methylobacter chroococcum*' strain L organisms.

Fig. 20. '*Methylobacter chroococcum*' strain L lysing organisms.

Fig. 21. *Methylococcus capsulatus* organisms.

Fig. 22. '*Methylococcus minimus*' organisms.

PLATE 3

Fig. 23 to 29. Phase-contrast micrographs of Indian ink preparations. Fig. 31 to 34. Brightfield flagella stain preparations. All micrographs magnification × 1900. The bar (fig. 25) equals 5 μm.

Fig. 23. '*Methylomonas methanica*' organisms embedded in slime.

Fig. 24. Capsules of '*Methylobacter chroococcum*' strain P organisms.

Fig. 25. Capsules of *Methylobacter capsulatus* organisms.

Fig. 26. Capsules of '*Methylomonas streptobacterium*' organisms.

Fig. 27. Capsules of '*Methylococcus minimus*' organisms.

Fig. 28. Capsules of '*Methylobacter chroococcum*' strain L organisms.

Fig. 29. Capsules of '*Methylobacter chroococcum*' strain HP organisms.

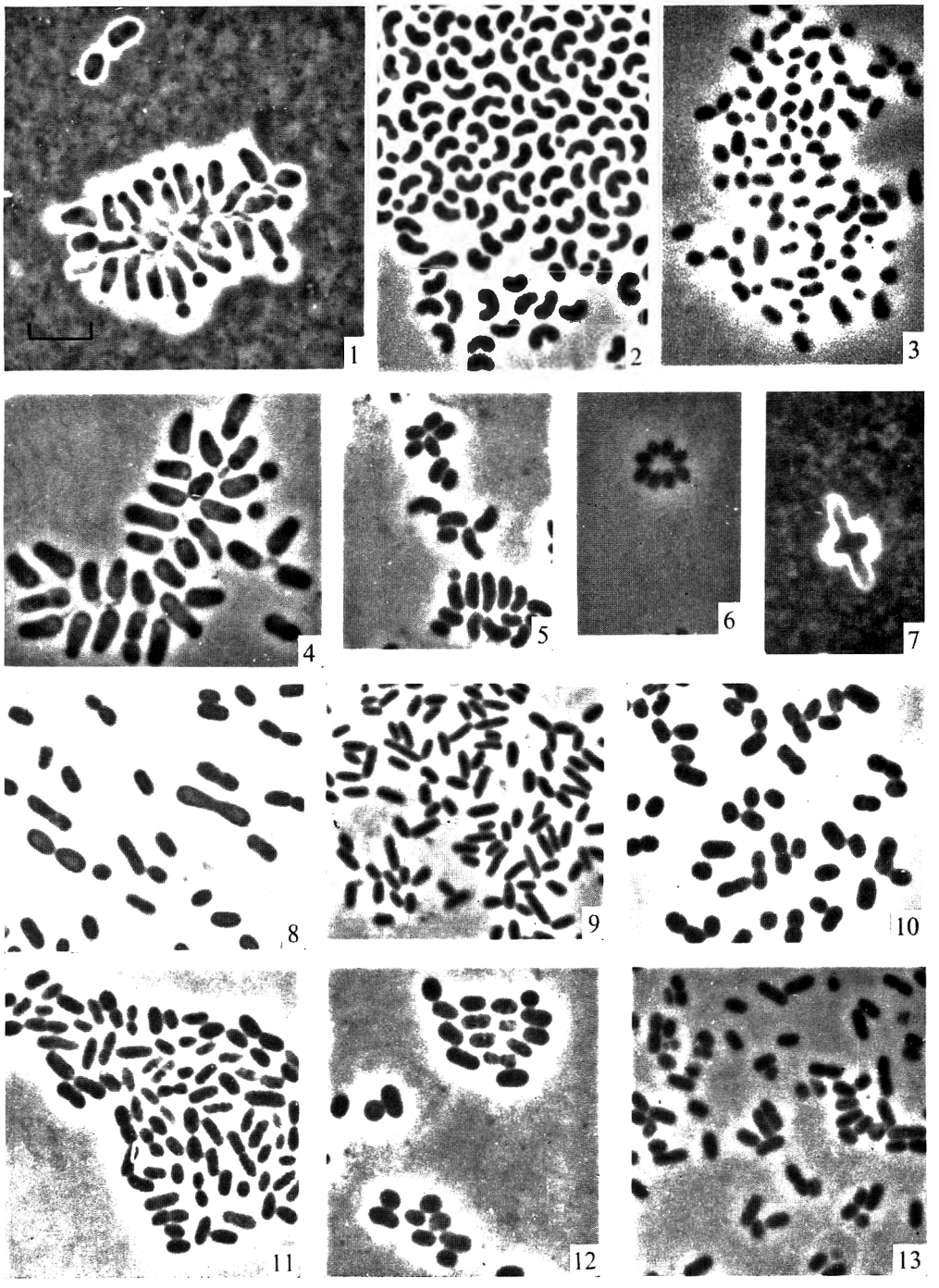
Fig. 30. Preparation of '*Methylobacter vinelandii*' showing immature cysts (large dark cells) and mature cysts (refractile cells) and vegetative rods.

Fig. 31. '*Methylosinus trichosporium*', polar tuft of flagella.

Fig. 32. '*Methylosinus sporium*', polar tuft of flagella.

Fig. 33. '*Methylomonas albus*' single polar flagellum.

Fig. 34. '*Methylomonas methanica*' single polar flagellum.



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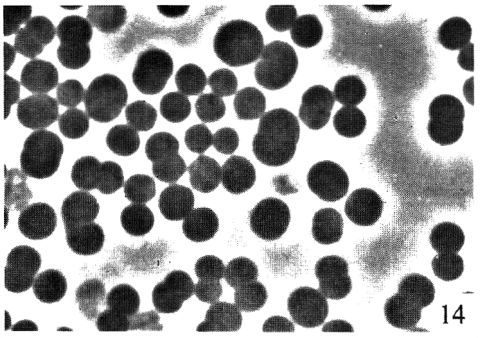
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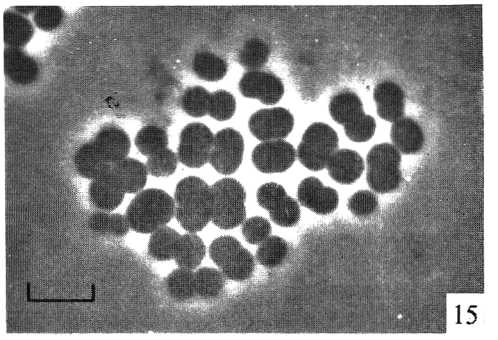
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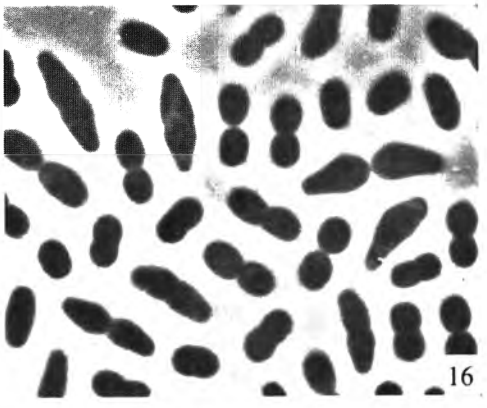
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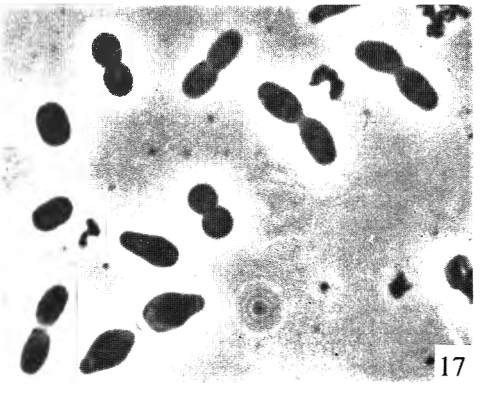
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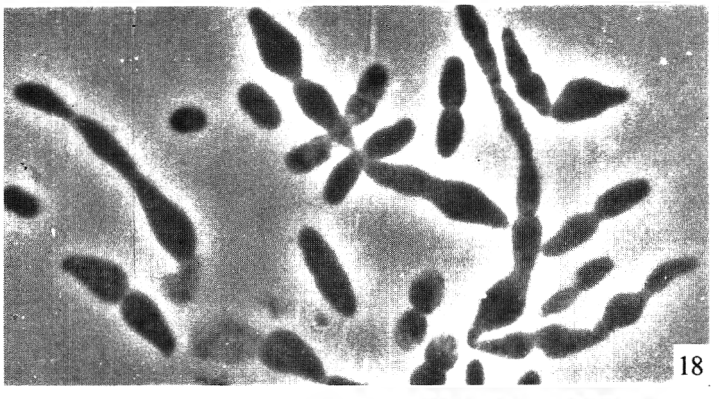
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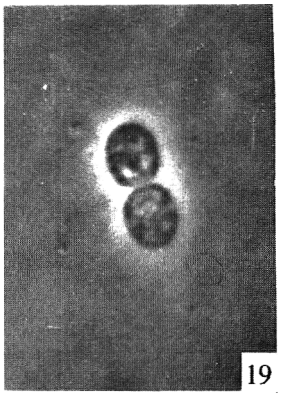
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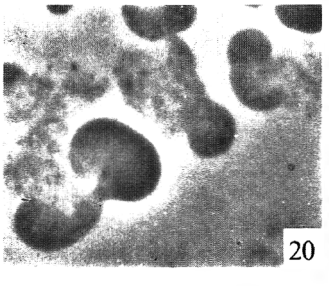
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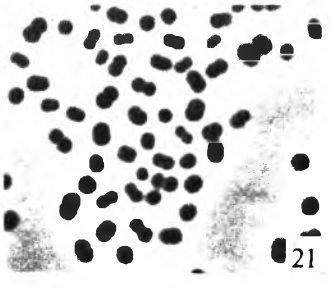
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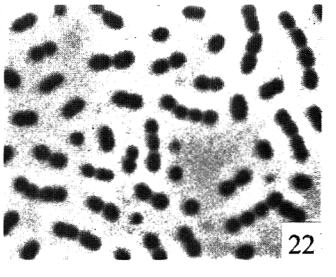
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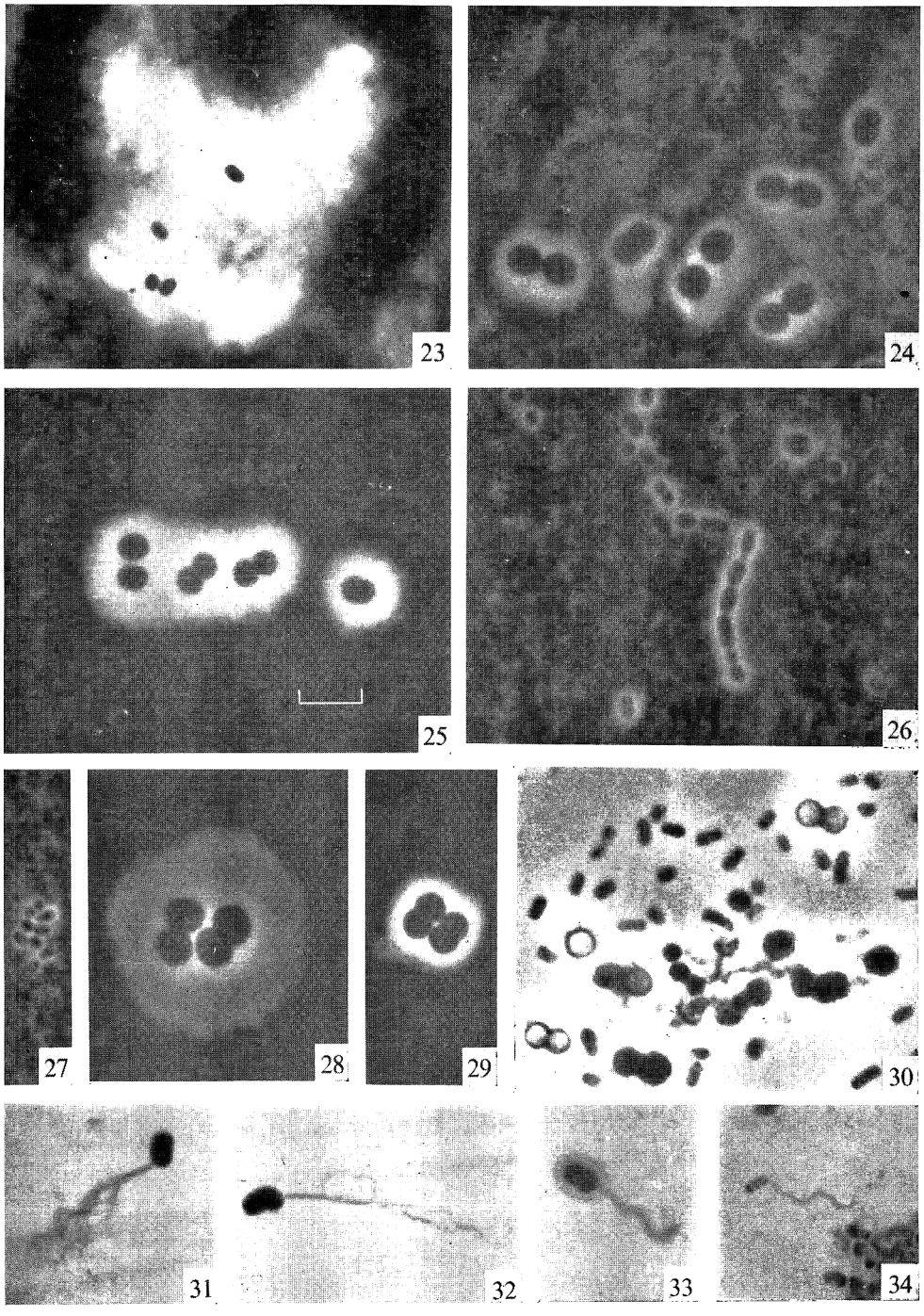

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Exospores and Cysts Formed by Methane-utilizing Bacteria

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SUMMARY

Three varieties of resting stage, an exospore and two types of cyst, were formed by methane-utilizing bacteria. Exospores were budded off by two types of organism, both of which underwent a change in morphology prior to spore formation. Exospores possessed some properties in common with endospores: staining properties, some structural features, mode of germination, desiccation and heat resistance, but contained no detectable dipicolinic acid. Some organisms formed desiccation-resistant cysts similar in morphology, staining properties and fine structure to cysts formed by Azotobacter species. Other strains formed non-desiccation-resistant bodies considered to be similar to immature azotobacter-type cysts. A desiccation-resistant cyst formed by one strain differed in structure and appearance from Azotobacter-type cysts and was referred to as a 'lipid' cyst.

INTRODUCTION

Methane-utilizing bacteria are a diverse group of organisms (Whittenbury, Phillips & Wilkinson, 1970). Many form resting stages, exospores and cysts, which are described in this report.

METHODS

Organisms. The organisms studied and the media and methods used for growth were described by Whittenbury *et al.* (1970).

Electron microscopy. The methods were those used by Davies & Whittenbury (1970) in studies on fine structure of vegetative cells of methane-utilizers. In addition, thin sections were made of organisms embedded in 'Epon' (Luft, 1961) and whole organisms were examined in platinum-shadowed preparations (Bradley, 1965).

Resistance tests. Tests were performed on suspensions of vegetative organisms and vegetative organisms + spores or cysts, in nitrate mineral salts medium (NMS). For heat resistance, 0·1 ml. suspension was added to preheated tubes of NMS medium in a constant-temperature water bath. Tubes were removed at various times into crushed ice before serially diluting and plating. To assess desiccation resistance, 0·1 ml. suspension was spread on to sterile glass slides, allowed to dry and stored at 30°. Dried preparations were resuspended at intervals and the proportion of vegetative cells to resting stages estimated microscopically before serially diluting and plating or adding to tubes of agar medium.

Assay of components. Dipicolinic acid content of organisms and spores was assayed by the methods of Perry & Foster (1955) and Janssen, Lund & Anderson (1958). Assay of poly-β-hydroxybutyrate was by the method of Law & Slepecky (1961).

RESULTS

Many strains of methane-utilizing bacteria formed bodies considered to be resting stages in that they possessed survival properties not found in vegetative organisms. Two categories of resting stage were recognized, exospores and cysts.

Exospores

Exospores were formed by two varieties of methane-utilizer, the '*Methylosinus trichosporium*' and '*Methylosinus sporium*' strains. Although the exterior morphology of the vegetative organisms and spores of these two varieties differed, the manner of formation and germination of the spores, their fine structure and resistant properties, were similar.

Spore formation and exterior morphology. In the exponential growth phase, bacteria multiplied by constrictive binary fission, no morphological difference being observed between the resultant two organisms. As the culture entered the stationary phase, however, a proportion of the organisms (5 to 95 % of the culture depending upon the strains) elongated and tapered either to a pear-shape ('*Methylosinus trichosporium*', Pl. 1, fig. 3) or to a comma-shape ('*Methylosinus sporium*', Pl. 1, fig. 30). This change was irreversible and signalled the onset of spore formation at the tapered end of the organism (opposite to the flagellated pole). The spore, a rounded body (Pl. 1, fig. 8, 24), was finally budded off (Pl. 1, fig. 7, 24, 29). Organisms which formed the spores failed to form additional spores or to divide, but became granulated and frequently lysed, opening at the tapered end of the organism (Pl. 1, fig. 28). Unlysed organisms which had formed spores failed to grow when transferred to fresh medium.

The onset of spore formation was most easily discerned in wet Indian ink preparations of '*Methylosinus trichosporium*' strains (Pl. 1, fig. 1 to 9, 25 to 27). The first sign of spore development was the appearance of a second capsule at the tapered end of the organism (Pl. 1, fig. 4). This capsule, larger and less well defined than the capsule of the vegetative organism (Pl. 1, fig. 1), increased in size as the spore developed and remained attached to the budded-off spore (Pl. 1, fig. 7, 8). Negatively stained and platinum-shadowed preparations examined in the electron microscope (Pl. 2, fig. 32 to 34) revealed the fine fibrous nature of the spore capsule which contrasted with the stubby more coarse fibrous nature of the vegetative cell capsule detected in thin sections (Pl. 2, fig. 36). Both capsules remained unstained when treated with conventional capsule stains, including the polysaccharide stain of Hotchkiss (1948), and remained attached after being ultrasonicated for 10 min.

Although the spores of '*Methylosinus sporium*' did not possess a capsule, the surface of the spores of this variety and of '*Methylosinus trichosporium*' strains was similar (Pl. 2, fig. 32, 33, 35).

When freshly formed (2 to 3 hr), spores were not refractile and stained with Gram and other conventional simple stains. On ageing (1 to 7 days) many spores became refractile and only stained with endospore stains and became acid-fast. Mature spores (refractile) were routinely counted by using the malachite green spore stain (Cruickshank, 1965).

Fine structure. Thin sections of mature spores were difficult to prepare, but some were sufficiently clear in definition to show that both types of spore possessed a similar structure (Pl. 5, fig. 64 to 67) which bore some resemblance to that of endospores. An

outer coat (derived from the wall of the vegetative organism), a laminated inner coat, an area possibly corresponding to the cortex of the endospore, a protoplasmic membrane, and a protoplast were discernible (in that order) in one or more of the sections.

Spore properties. Spores formed by all stains were desiccation-resistant, surviving at least 18 months in a dried state in the absence of methane, and heat-resistant, surviving 85° for 15 min. Desiccation and heat-resistance correlated with refractility; survival rates of spores increased with an increase in the proportion of refractile to non-refractile spores which, in turn, was a function of increasing age of the spore. Spores were not separated to a significant degree from vegetative organisms by the methods employed

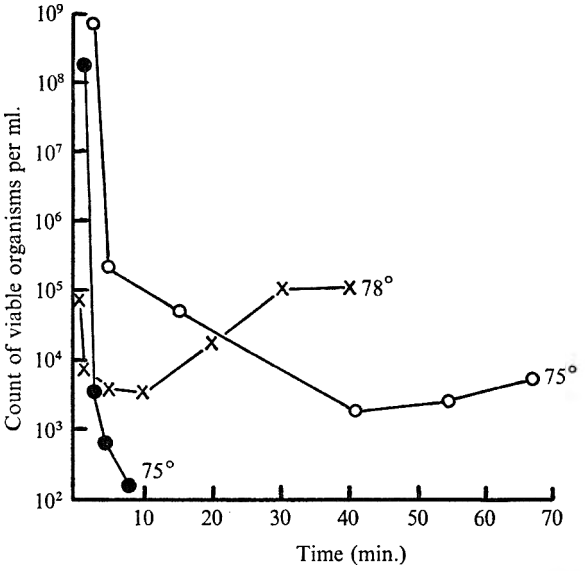

Fig. 1. Heat resistance of '*Methylosinus sporium*' strain 5 (see Methods). ●●, preparation of vegetative organisms. ○○ and × ×, preparations of vegetative organisms and exospores; the sharp decline in viable count in first few minutes is attributed to death of vegetative organisms, and the increase in viable count after a period of heating is attributed to heat stimulation of spore germination.

to separate endospores from vegetative organisms. Consequently survival tests were performed on cultures (24 hr), which contained none or a few spores and on cultures (7 or more days) which had spored profusely (Fig. 1). This example, a survival curve of spores and vegetative organisms heated at 75 and 78°, also suggested that spore germination was stimulated by heat.

Dipicolinic acid was not detected in spored cultures, either chemically or indirectly by fluorescence under a u.v. microscope. No respiratory activity was detected in spores, in the presence or absence of methane. Mature spores survived ultrasonication for 10 min.

Spore germination. Freshly formed spores (1 to 24 hr) germinated within 2 to 3 days, but old, refractile, spores (7 days to 18 months), heat-treated spores and dried spores germinated within 7 to 15 days. Occasionally spores germinated only in patches on uniformly spread plates. Attempts to find a factor which either shortened germination time or promoted uniformity of germination have failed; L-alanine, methanol and

formate did not alter germination behaviour in the presence of methane and spores did not germinate in the absence of methane.

Germination sequences, followed microscopically on slide cultures, were similar for both varieties of spores and were best demonstrated in Indian ink preparations of a germinating culture of a '*Methylosinus trichosporium*' strain (Pl. 1, fig. 9 to 18) sampled at various times. The capsular coat, only present on '*Methylosinus trichosporium*' spores, was frequently shed (Pl. 1, fig. 19 to 21) before germination and appeared to play no part in heat and desiccation-resistance properties of the spore. The first visible change in the germinating spore was the loss of refractility (accompanied by loss of acid-fastness). Swelling of the spore was not observed: the next visible change was the emergence of a rod which increased in length and either divided or, rarely, formed another spore (Pl. 1, fig. 22, 23). The inner spore coat (but not the capsulated outer wall) remained an integral part of the germinating organism (Pl. 1, fig. 15, 16) for at least two divisions. The germination process, from loss of spore refractility to the first division, lasted for 5 to 7 hr.

Spore crop. Spore crops varied from strain to strain (both varieties). On first isolation, all strains spored profusely, but after being subcultured 2 to 3 times, many strains spored sparsely (5 % or less of the organisms in a culture sporulated within 7 days). Other strains, however, continued to spore profusely (90 % of the organisms in a culture sporulating within 7 days). Some of the strains which spored poorly (3 to 5 spores/100 vegetative organisms in 7 days) were stimulated to a higher rate of spore production (2 to 10-fold increase) by transferring them to NMS medium from which nitrate had been omitted.

Cysts

Many methane-utilizers which did not form exospores formed cysts of which there were two distinct varieties, a 'lipid' cyst and an 'Azotobacter-type' cyst. A third variety of resting stage was also recognized, provisionally termed an 'immature' cyst.

'*Lipid*' *cyst.* This was formed by only one organism, strain '*Methylocystis parvus*', an organism similar in many respects to '*Methylosinus trichosporium*' and '*Methylosinus sporium*' strains (Whittenbury *et al.* 1970) and which might have been expected to form an exospore. In the exponential growth phase, the organisms were small rods, free of inclusions and stainable by Gram and other conventional stains. As the organisms entered the stationary phase a proportion of them (varying between less than 5 % to more than 90 %) formed large lipid inclusions (consisting mainly of poly-β-hydroxybutyrate) and increased in size (Pl. 4, fig. 54). Old organisms in this phase (7 to 14 days) were not stained with Gram or other simple stains, but were stained by the Ziehl-Neelson acid-fast stain (Cruickshank, 1965). The fine structure of these lipid organisms differed from that of the vegetative organisms (Pl. 4, fig. 55 to 57). The main differences were an increase in the complexity of the wall of the 'lipid' organism, compared with the vegetative organisms, and the absence from the 'lipid' organism of the complex membrane system seen in the vegetative organism.

A preliminary survey of the resistant properties of the two phases of organism revealed that neither survived 65° for 30 min., but that the 'lipid' organism survived desiccation, over 80 % remaining viable after 1 week when dried on a glass slide. When transferred to fresh medium, the lipid inclusion disappeared and organisms lost their acid-fastness and desiccation resistance. No other changes have yet been detected and nothing is known about the factors influencing cyst formation.

'*Azotobacter-type*' *cyst.* All varieties of 'Methylobacter' ('*Methylobacter chroococcum*', '*Methylobacter capsulatus*', '*Methylobacter bovis*', '*Methylobacter vinelandii*' and strains not subgrouped) formed multiple, double and single-bodied cysts (Pl. 3, fig. 37 to 49), indistinguishable in microscopic appearance from cysts formed by Azotobacter species. In the exponential phase of growth the organisms were rod or rod/coccal shaped, but as they entered the stationary growth phase many of the organisms (sometimes over 90 %) rounded up, generally increased in size (Pl. 3, fig. 42, 43) and eventually became refractile. No factor has been found which stimulated cyst production, with the exception that increases in encystment rate have been observed occasionally in cultures growing in the presence of low levels of oxygen. Cyst formation in some strains was prevented by the omission of magnesium ions from the medium. In many cases, profuse encystment was accompanied by the production of a pigment ranging in colour from yellow, to rust to brown, to dark-brown; non-encysted cultures remained creamy white.

Mature, refractile cysts (Pl. 3, fig. 44), stained by the method of Vela & Wyss (1964), were typical in appearance to stained, mature Azotobacter cysts (a green central body, a clear inner wall space, and a brown outer coat or exine). The structure of the cyst coat (Pl. 4, fig. 58; Pl. 5, fig. 60 to 63) closely resembled that of Azotobacter cysts described by Socolofsky & Wyss (1961) and Beaman, Jackson & Shankel (1968).

Initial experiments on resistance properties revealed that the cysts were not heat-resistant but were desiccation-resistant; mature cysts of some strains survived at least 18 months in a dried state in the absence of methane but others only survived 3 to 4 months. Viability tests of organisms dried on glass slides revealed that 60 to 90 % of the mature cysts survived 1 week, whilst vegetative organisms did not survive 24 hr.

'*Immature*' *cysts.* Strains of methane-oxidizing bacteria which did not form desiccation-resistant cysts were classified into 'Methylomonas' (rods) and 'Methylococcus' (cocci) groups. One or more strains in all subgroups (Whittenbury *et al.* 1970) formed rounded bodies (Pl. 4, fig. 52, 53) which appeared to be immature forms of the 'Azotobacter-type' cyst. Such bodies survived the absence of methane for 5 to 6 weeks in contrast to vegetative organisms which survived only 2 to 3 days and possessed a wall structure (Pl. 4, fig. 59) intermediate between that of a vegetative cell (e.g. Pl. 5, fig. 60) and a mature cyst (e.g. Pl. 4, fig. 58).

DISCUSSION

Methane-utilizing bacteria use only methane and methanol as energy sources but seem adapted to use only methane in their natural environment as methanol, even in low concentrations (0·01 % w/v) is toxic to many of them (Whittenbury *et al.* 1970). The precarious existence the organisms would seem to lead by relying on only one growth substrate, is relieved by the ability of many organisms, probably all, to form resting stages which survive considerable periods of time in the absence of methane. At least three distinct varieties of resting stage are formed, exospores and two types of desiccation-resistant cyst, a 'lipid' cyst and an 'Azotobacter-type' cyst.

The exospores, budded off by two varieties of organism, share some features in common with endospores of Bacillus species. Structurally the exospores are similar to endospores in many respects, with the exception that they lack a well-defined cortex, though this may be a reflexion of inadequate sectioning and staining technique. Heat

resistance, desiccation resistance, staining properties and mode of spore germination also relate exospores to endospores but the absence of dipicolinic acid in exospores distinguishes them from endospores. Clearly further work is necessary to define more closely the relationship of exospores to endospores.

The 'lipid' cyst, characterized by large lipid inclusions and complex wall structure, bears little resemblance to other bacterial cysts apart from its ability to resist desiccation. 'Azotobacter-type' cysts (although 'Myxococcus-type' would describe them equally well) are similar to those formed by Azotobacter species both in structure and desiccation resistance. A fourth type of resting stage, an 'immature' cyst, may prove to be an incompletely formed 'Azotobacter-type' cyst.

Clearly much remains to be discovered about the nature of these resting stages. Any structural and biochemical properties common to exospores and endospores, cysts formed by these organisms and Azotobacter cysts, should help to identify features specifically concerned with their survival properties.

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EXPLANATION OF PLATES

PLATE 1

Phase-contrast micrographs. Fig. 1 to 23, 25 to 27 are Indian ink preparations, magnification × 1900, the bar (fig. 25) equals 5 μm.

Fig. 1 to 9. Stages in exospore formation by '*Methylosinus trichosporium*' strain PG. Capsulated vegetative organism, rod-shaped (fig. 1) becomes pear-shaped (fig. 3) and a second capsule develops at the tapered end of the organism (fig. 2 to 3). A spore is formed (fig. 4 to 6) and is finally budded off (fig. 6 to 8) becomes refractile (fig. 9) and retains the capsule seen developing in fig. 4.

Fig. 10 to 18. Stages in germination of the exospore of '*Methylosinus trichosporium*' strain PG. The spore loses refractility (fig. 10), a rod begins to emerge and the capsule is distorted (fig. 11). Rod increases in size and divides (fig. 12 to 15), the spore coat (but not the capsule) remaining an integral part of the organism after division (fig. 15, 16). The second organism of the pair is a vegetative organism of normal appearance (fig. 17) and grows on to divide and form two morphologically similar organisms (fig. 18).

Fig. 18 to 21. Exospores of '*Methylosinus trichosporium*' strain PG losing capsule which is probably attached to outer wall of spore (see Pl. 5, fig. 66) and therefore indicates that outer wall is being shed.

Fig. 22 to 23. Germinated exospore of '*Methylosinus trichosporium*' strain PG forming an exospore.

Fig. 24. Preparation (Indian ink omitted) of sporing organism ('*Methylosinus trichosporium*' strain PG).

Fig. 25 to 27. Rosettes of organisms of '*Methylosinus trichosporium*' strain PG in different stages of spore formation.

Fig. 28. Rosette of lysed organisms of '*Methylosinus trichosporium*' strain PG. Organisms appear to open at spore bearing pole.

Fig. 29. Exospores, vegetative and sporulating organisms of '*Methylosinus sporium*' strain 5.

Fig. 30. Vegetative organisms and organisms in early stage of sporulation, '*Methylosinus sporium*' strain 5.

Fig. 31. Free exospores of '*Methylosinus sporium*' strain 5.

PLATE 2

Electron micrographs.

Figs. 32, 33. Negatively stained preparations of whole organisms of '*Methylosinus trichosporium*' strain PG, showing fine fibres emanating from developing and completed exospore and wrinkled appearance of exospore surface. × 16,000.

Fig. 34. Platinum-shadowed negative of developing exospore of '*Methylosinus trichosporium*' strain PG, showing fibres radiating from spore and appearance of spore surface. × 16,000.

Fig. 35. Negatively stained preparation of whole exospore of '*Methylosinus sporium*' strain 5. Spore surface is similar in appearance to exospore of '*Methylosinus trichosporium*', except that no fine fibres radiate from spore surface. × 40,000.

Fig. 36. Negatively stained thin section of vegetative organism of '*Methylosinus trichosporium*' strain PG, showing short fibrous material radiating from wall. This was not seen in preparations of negatively stained whole cells. × 60,000.

PLATE 3

Fig. 37 to 49. Phase-contrast micrographs of cysts and vegetative organisms of 'Methylobacter' strains, magnification × 1900, bar (fig. 43) equals 5 μm.

Fig. 37 to 40. *Methylobacter capsulatus* strain 1521, multiple bodied cysts (arrowed) with part of exine visible and apparently in process of germinating. Cysts in different stages of development (dark round bodies, refractile bodies), empty exines and vegetative organisms (rods) are also present.

Fig. 41 to 43. '*Methylobacter bovis*' strain CM, different stages of cyst formation. Mixture of mature cysts (refractile bodies with distinctive outer coats) and cysts apparently released from exine (fig. 41); vegetative organisms and maturing cysts (fig. 42); and maturing cysts (fig. 43).

Fig. 44 to 45. '*Methylobacter vinelandii*' strain MEXICO, mature refractile cysts and vegetative organisms (fig. 44); maturing cysts (refractile) and vegetative organisms (fig. 45).

Fig. 46 to 49. 'Methylobacter' strain PROVENCE VINE, vegetative organisms and immature cysts (fig. 46); mature cysts (fig. 47); multiple-bodied cyst and vegetative organisms (fig. 48); and large multiple-bodied cyst (fig. 49).

PLATE 4

Fig. 50 to 54. Phase-contrast micrographs, magnification × 1900, bar (fig. 53) equals 5 μm. Fig. 55 to 59. Negatively stained thin sections.

Fig. 50 to 51. '*Methylobacter bovis*' strain CM, germinating cysts. Exines and emerging organisms (singly and in pairs).

Fig. 52. *Methylococcus capsulatus* strain BATH. A partially refractile pair of organisms (considered to be immature cysts) amongst vegetative organisms.

Fig. 53. '*Methylococcus minimus*' strain TMC. Refractile bodies (considered to be immature cysts) and vegetative organisms.

Fig. 54. '*Methylocystis parvus*' strain OBBP. Vegetative organisms (small rods) and lipid cysts (large organisms with inclusions).

Fig. 55. '*Methylocystis parvus*' strain OBBP; negatively stained thin section of a lipid cyst. × 60,000.

Fig. 56. '*Methylocystis parvus*' strain OBBP, negatively stained thin section of lipid cyst showing wall structure. × 90,000.

Fig. 57. '*Methylocystis parvus*' strain OBBP, negatively stained thin section of vegetative organism showing wall structure. × 90,000.

Fig. 58. '*Methylobacter bovis*' strain CM, negatively stained thin section of mature cyst. × 60,000.

Fig. 59. '*Methylococcus minimus*' strain TMC, negatively stained thin section of 'immature' cyst. × 60,000.

PLATE 5

Negatively stained preparations of thin sections.

Fig. 60. '*Methylobacter bovis*' strain CM, part of vegetative organism showing wall structure. All 'Methylobacter' strains possessed a similar structure in vegetative state. × 90,000.

Fig. 61. *Methylobacter capsulatus* strain 1521, a cyst. × 45,000.

Fig. 62. '*Methylobacter vinelandii*' strain MEXICO, part of a cyst showing laminated coat structure. × 90,000.

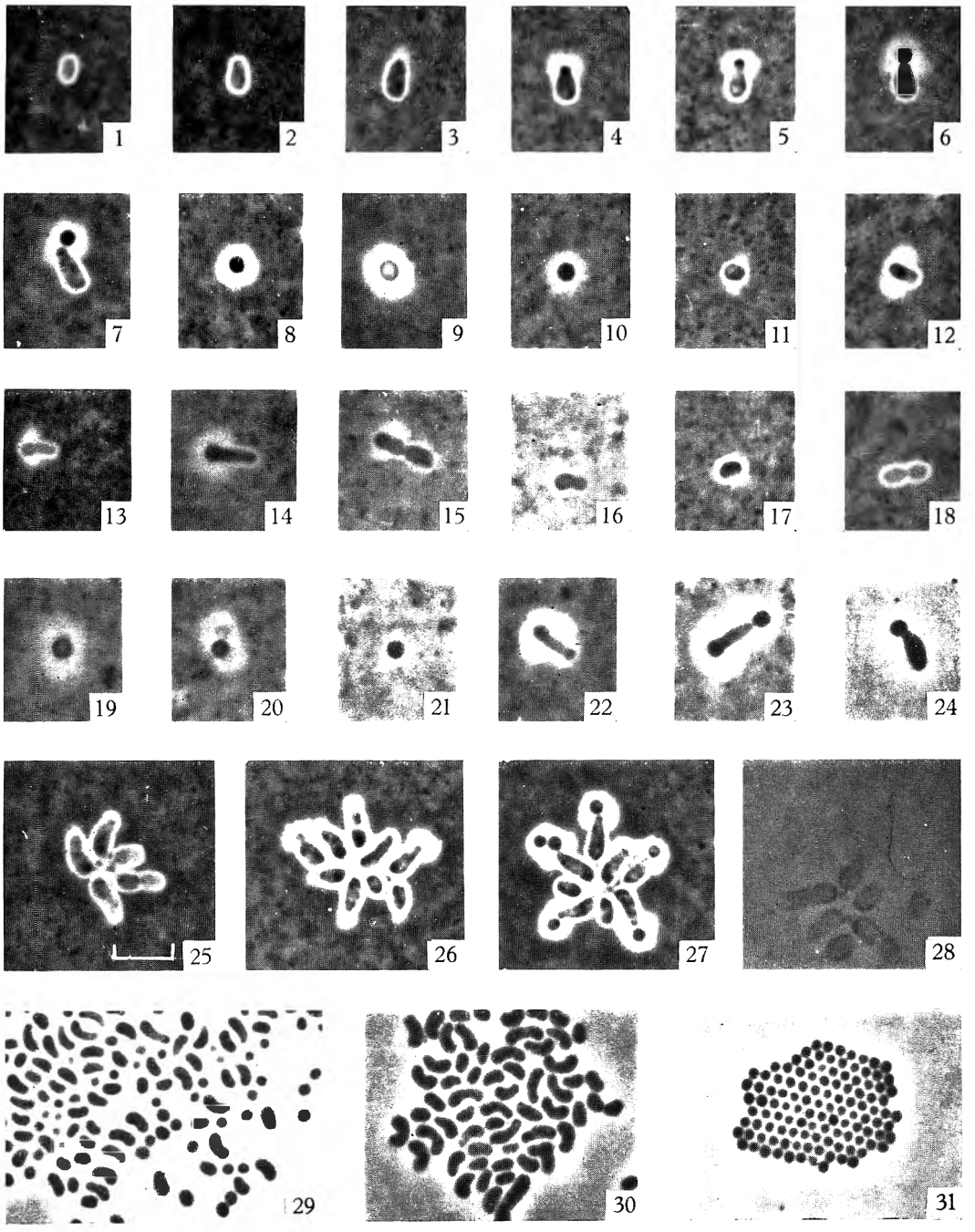
Fig. 63. '*Methylobacter bovis*' strain CM, part of a mature cyst showing wall structure. × 90,000.

Fig. 64. '*Methylosinus sporium*' strain 5, spore showing wall structure. × 80,000.

Fig. 65. '*Methylosinus trichosporium*' strain PG spore showing wall structure. × 80,000.

Fig. 66. '*Methylosinus trichosporium*' strain PG, part of mature spore. The outer layer (*w*) derived from vegetative organism in budding process, laminated inner wall (*l*), area (*c*) possibly corresponding to cortex of Bacillus endospore, structure (*m*) possibly corresponding to membrane, and protoplast (*p*) are evident. The wall layer (*w*) partially detached in thin section, probably corresponds to the capsulated area detached in whole preparations (Pl. 1, fig. 19 to 21). × 120,000.

Fig. 67. '*Methylosinus trichosporium*' strain PG, part of spore possibly showing an early stage in wall development. × 120,000.



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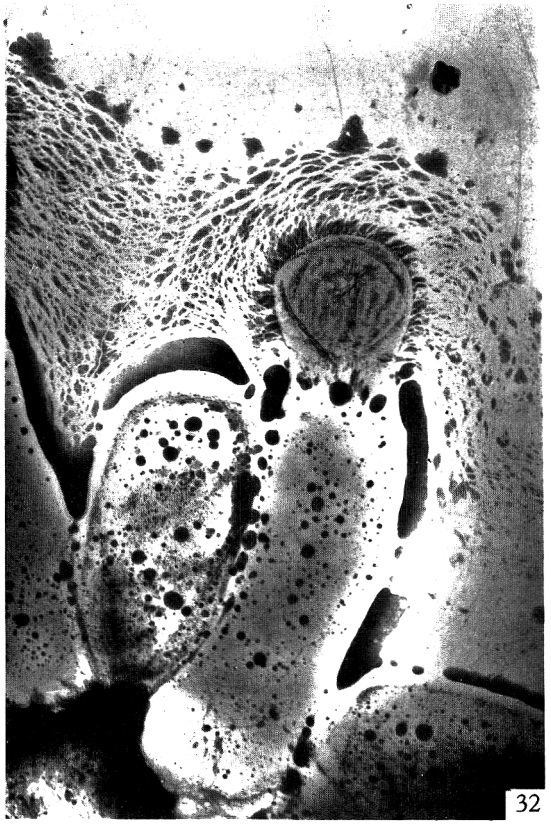
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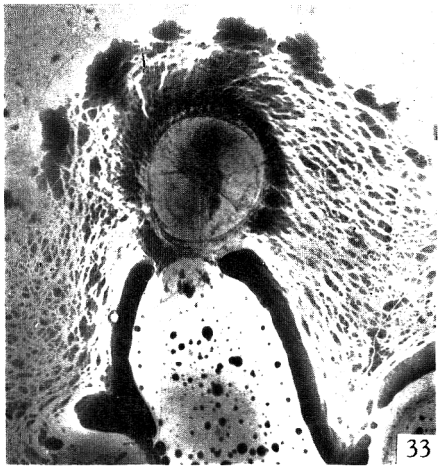
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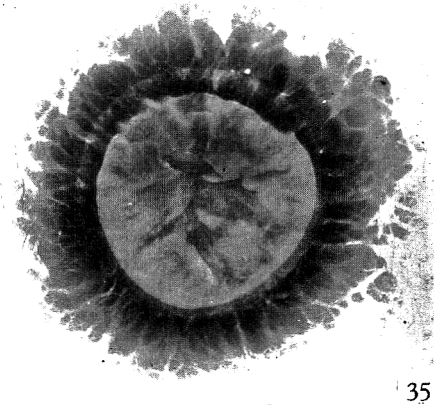
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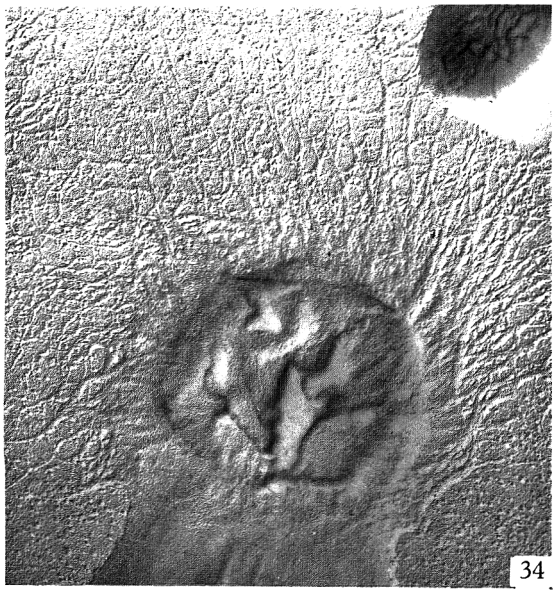
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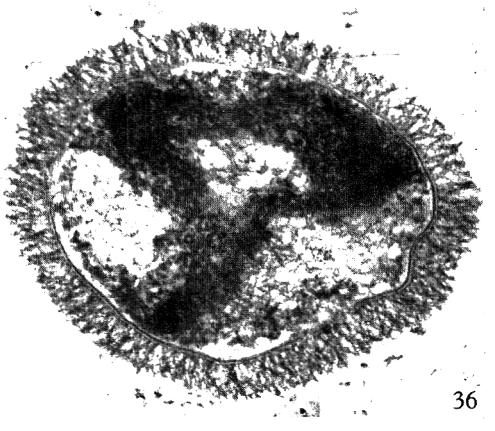
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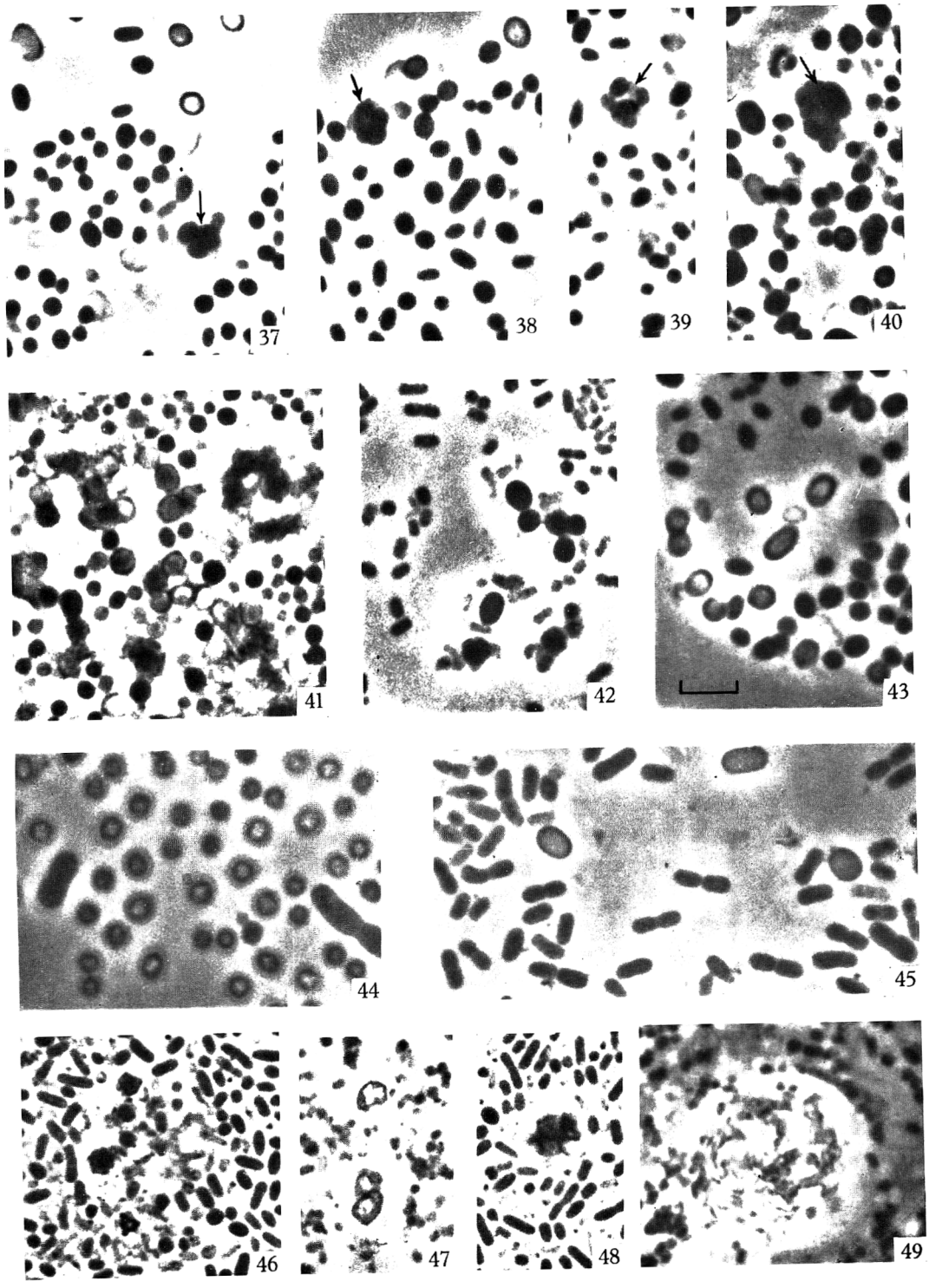

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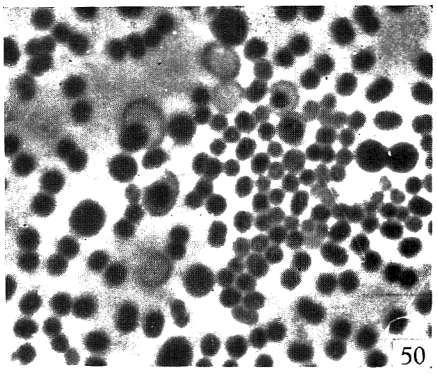
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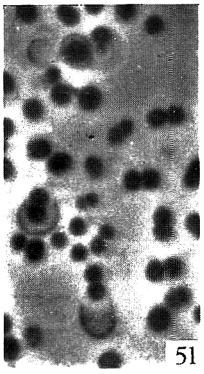
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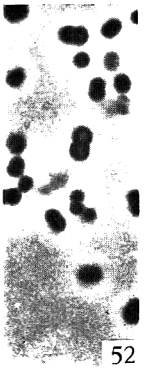
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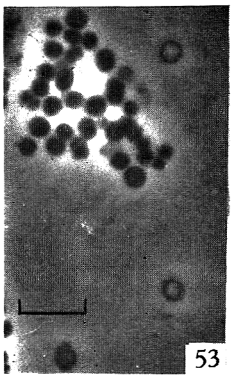
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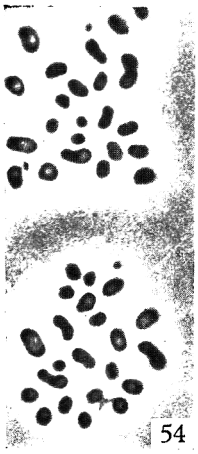
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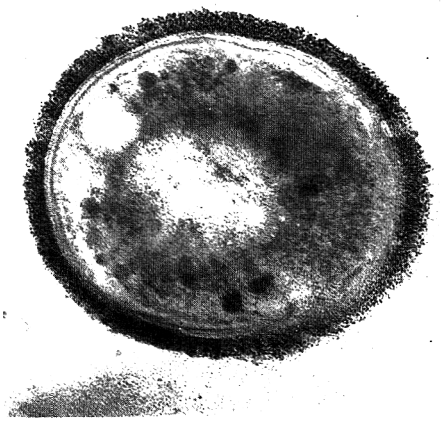
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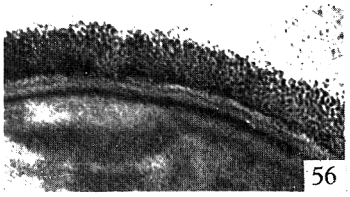
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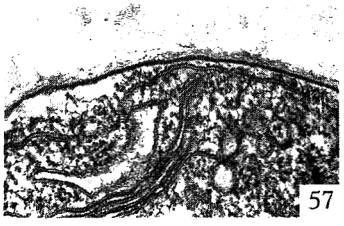
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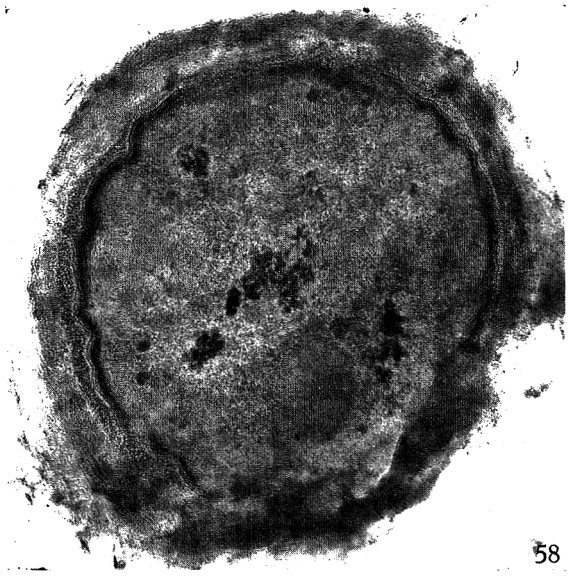
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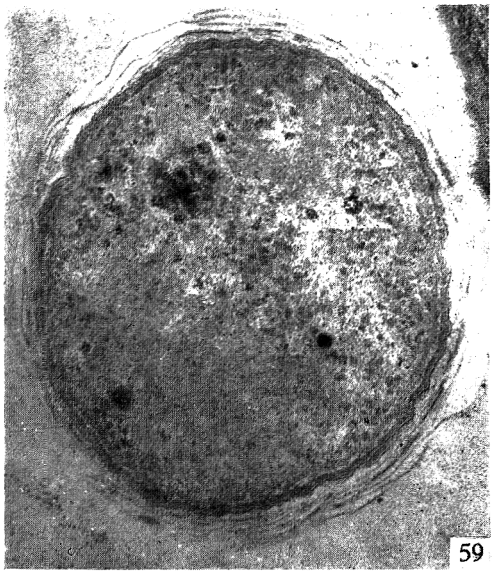
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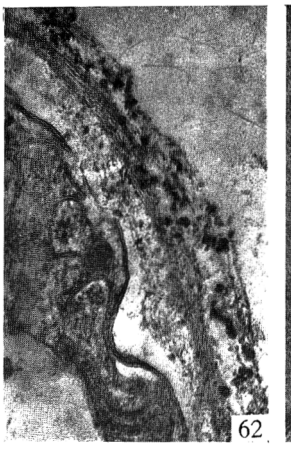

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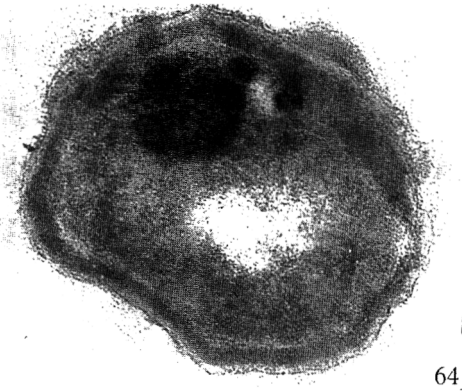

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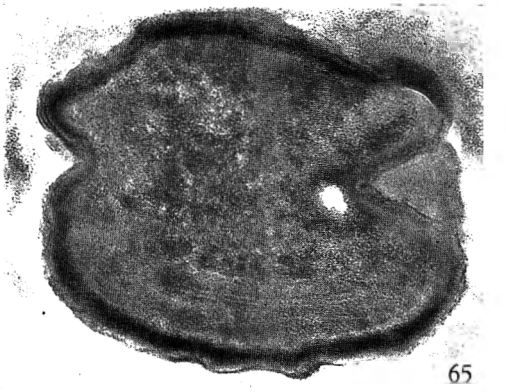

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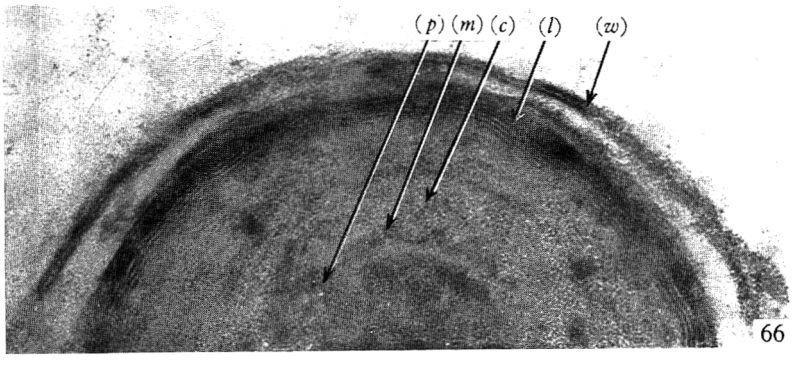

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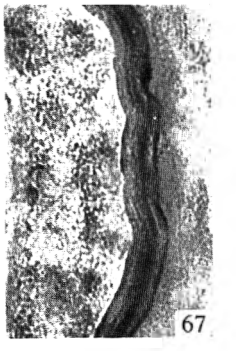

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Fine Structure of Methane and Other Hydrocarbon-utilizing Bacteria

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SUMMARY

Methane-utilizing bacteria were examined by electron microscopy and found to possess complex membranous structures within the cytoplasm. Two types of membrane organization were recognized. One type consisted of pairs of membranes which either extended throughout the organism or were arranged at the periphery where they ran parallel to the cytoplasmic membrane. The other type consisted of vesicular discs of membrane organized into bundles which were distributed throughout the organism. Bacteria utilizing C_2 to C_4 gaseous *n*-alkanes and C_{11} to C_{18} liquid *n*-alkane mixture did not possess such extensive membranous structures. The former contained membranous bodies of the mesosome type, whilst the latter possessed only a cytoplasmic membrane. These structural differences add to the growing list of properties separating CH_4-utilizing bacteria from those utilizing C_2 and higher *n*-alkanes.

INTRODUCTION

Complex internal membrane systems, in contrast to the relatively simple intracytoplasmic bodies classified as mesosomes, are restricted in the bacteria to a few groups such as the photosynthetic bacteria (Pfennig, 1967) and the nitrifying bacteria (Murray & Watson, 1965; S. W. Watson, private communication). Their presence in methane-utilizing bacteria, briefly described by Whittenbury (1969) and later by Proctor, Norris & Ribbons (1969), seems to be the first instance of such complex systems in obligate heterotrophs. These membrane systems are described in more detail in this paper and are compared with the type of internal organization found in bacteria utilizing higher hydrocarbons.

METHODS

Organisms and culture. The methane-utilizing bacteria studied, and the methods and inorganic media used, were those described by Whittenbury, Phillips & Wilkinson (1970). Strains of the *Mycobacterium–Corynebacterium* complex utilizing gaseous hydrocarbons, and Gram-negative cocci utilizing liquid *n*-alkanes were isolated in this laboratory by Mr A. G. McLee. They were cultured on the inorganic medium used for methane utilizers in an atmosphere of air and gaseous *n*-alkanes (C_2, C_3 or C_4), and in an atmosphere of air and vapour given off from filter paper impregnated with a C_{11} to C_{18} *n*-alkane mixture, respectively. All cultures were grown at 30° and the organisms harvested after 2 days.

Electron microscopy. The bacteria were fixed and embedded following the procedure

described by Bradley & Dewar (1967). After fixation in 6·25 % (w/v) glutaraldehyde and then in 1 % (w/v) osmic acid the procedure was modified by suspending the bacteria in 2·0 % (w/v) agar. Some of the suspension was transferred to the fine tube of a Pasteur pipette, allowed to solidify and then extruded. Small blocks, about 1 mm. long, were cut from the agar and dehydrated in acetone. Staining with uranyl acetate was carried out in the initial stages of dehydration. The agar blocks were finally embedded in 'Vestopal'. Thin sections were cut with a diamond knife on a Huxley microtome and mounted on grids. The sections were stained with lead citrate for 2 min. (Reynolds, 1963) before examination in the electron microscope.

For negative staining, harvested bacteria were washed in 0·1 M ammonium acetate solution containing sucrose, 0·2 % (w/v), and centrifuged. They were resuspended in ammonium acetate-sucrose solution and a drop was mixed with a drop of neutral potassium phosphotungstate, 2 % (w/v). A thin film of this mixture was allowed to dry on a carbon-coated grid.

RESULTS AND DISCUSSION

Representative strains of five groups, 'Methylosinus', 'Methylocystis', 'Methylomonas', 'Methylococcus' and 'Methylobacter' (Whittenbury *et al.* 1970), were found to possess complex internal membranous structures. Two patterns of organization were recognized, type I being found in the rod-coccoid organisms ('Methylomonas', 'Methylococcus' and 'Methylobacter' groups) and type II in the vibrioid organisms ('Methylosinus' and 'Methylocystis' groups).

Type I. Organisms in this category possessed structures resembling those in Pl. 1, fig. 1, 2. The membranes, cut across in these sections, were aggregated into bundles, which were a constant feature of these bacteria when grown on either methane or methanol. The individual membranes, which were about 90 Å thick, consisted of two outer electron-dense layers and an inner electron-transparent layer, and were similar in size and appearance to the cytoplasmic membrane. The parallel close-packing of the membranes within a bundle gave rise to triplet structures which were composed of pairs of adjacent membranes. The membrane units were in the form of flattened vesicles, the inner spaces of which usually contained material that appeared similar to the surrounding cytoplasm. The vesicular conformation of the membranes was seen more clearly in a fragment of a bacterium (Pl. 1, fig. 3) where the breaking of the wall appeared to have released the membranes from their usual close-packed arrangement. The membrane vesicles were seen in a side-on position in bacteria where the plane of sectioning was parallel to the side walls of the vesicles (Pl. 1, fig. 4). In such cases the membranes appeared as areas of relatively electron-dense material which were circular or ovoid in outline. Each bundle of membranes, therefore, appeared to consist of a number of disc-shaped vesicles in a highly oriented arrangement.

This interpretation was supported by negatively stained preparations of lysing bacteria. Although whole bacteria were not penetrated by the stain, the lysing organisms gave a three-dimensional impression of the numerous disc-shaped vesicles found within the bacteria (Pl. 2, fig. 5). This arrangement of vesicles bears a marked similarity to the smaller vesicular systems described in *Rhodospirillum molischianum* by Giesbrecht & Drews (1962). In Pl. 2, fig. 6 some of the closely-packed membranes are in an end-on position while others are in a side-on position. In fragments from disrupted bacteria, membrane discs tended to remain in groups accompanied by pieces of cytoplasmic

membrane (Pl. 2, fig. 7). This arrangement of discs and cytoplasmic membrane fragments suggests that there might be an attachment between the internal and the cytoplasmic membranes. Invaginations of the cytoplasmic membrane (Pl. 2, fig. 8) and connexions between the membrane bundles and the cytoplasmic membrane (Pl. 2, fig. 9) were seen in a number of sections, indicating that the membrane vesicles arose from invaginations of the cytoplasmic membrane.

Most strains contained bundles of uniformly graded vesicles, but some contained a variant type of bundle (Pl. 2, fig. 9) composed of irregularly sized vesicles.

Type II. The internal arrangement of membranes in organisms of this group, when grown on either methane or methanol, was more variable and less ordered than that of the rod-coccoid strains. The individual membranes, which were about 80 Å thick and similar to the cytoplasmic membrane in both size and appearance, had the same three-layered structure as the type I membranes (Pl. 3, fig. 10). The membranes were always in pairs and bounded a lumen of varying dimensions which was usually filled with material noticeably less electron-dense than the surrounding cytoplasm.

In a few of the strains, paired membranes were usually found throughout the organism (Pl. 3, fig. 11), although in some sections the membranes tended to be concentrated at the periphery (Pl. 3, fig. 12). The membrane system in these bacteria may have been in the form of tubes and irregularly shaped vesicles or of flattened, more lamella-like tubes. In the same strains the membranes occasionally appeared in a more regular, peripheral arrangement (Pl. 3, fig. 13, 14) and branching was sometimes apparent. In other strains the membranes were always found at the periphery, an arrangement that is similar to those described in *Rhodopseudomonas palustris* (Whittenbury & McLee, 1967) and in *Nitrobacter agilis* and *Nitrosomonas europaea* (Murray & Watson, 1965). In these methane utilizers the membrane system is probably in the form of a series of concentric vesicles which more or less completely enclose the inner part of the organism.

The three-dimensional structure of the two variants of the type II membrane system could not be confirmed with negatively stained preparations of glycerol-lysed cells (Eimhjellen, Steensland & Traetteberg, 1967) as the membranous material appeared as a dense and convoluted mass.

Higher hydrocarbon-utilizing bacteria

Bacteria utilizing the *n*-alkanes, ethane, propane and butane, possessed membranous structures of the mesosome type. These were observed in thin sections and in negatively stained preparations of whole organisms. Each bacterium contained a number of convoluted membranous organelles of varying size (Pl. 4, fig. 15) with larger bodies occurring at sites of cross-wall formation (Pl. 4, fig. 16). Both types of mesosomes are very similar to those reported in *Caulobacter crescentus* (Cohen-Bazire, Kunisawa & Poindexter, 1966). Some bacteria also contained an array of small convoluted structures around their periphery (Pl. 4, fig. 17) which were similar to those reported in *Lactobacillus lactis* (Hurst & Stubbs, 1969). Bacteria growing on liquid *n*-alkanes contained no detectable membranes other than the cytoplasmic membrane (Pl. 4, fig. 18).

Discussion of the role of the membranous organelles

The electron micrographs reveal a marked gradation in the complexity of membrane arrangement in the various groups of hydrocarbon-utilizing bacteria, with extensive membrane systems occurring only in the methane utilizers. The role of these extensive membrane systems has not yet been elucidated, but the probable significance of various functions can be assessed by comparison with other organisms.

Low solubility of the substrate and metabolic stress resulting from the synthesis of components from C_1 compounds are unlikely to account for the development of such organelles. The higher hydrocarbon-utilizers have the same substrate solubility problem and yet do not differ greatly in structure from many heterotrophs growing in the presence of ample nutrients. The importance of these two functions in this context is further discounted by a consideration of the structure of Hydrogenomonas species. These organisms apparently possess only a cytoplasmic membrane (Schlegel, Gottschalk & Von Bartha, 1961) and yet utilize H_2, which has the same order of solubility as CH_4, and, unlike the methane utilizers, Hydrogenomonas species also have to fix CO_2 at the expense of reduced NAD and ATP. The unimportance of methane solubility in relation to membrane development also seems to be demonstrated by the behaviour of the organisms growing on methanol. Growth rates on methane and methanol were similar (Whittenbury *et al.* 1970) despite the fact that the concentration of methanol was sometimes 100 to 1000× greater than that of methane.

Evidence is beginning to accumulate (Patel, Hoare & Taylor, 1969; Whittenbury *et al.* 1970) which suggests that methane oxidizers have physiological properties more in common with autotrophs than with heterotrophs and a comparison with organisms of similarly complex structure, such as the nitrifiers, may eventually provide an explanation of the significance of the extensive membrane systems. CH_4, NH_4^+ and NO_2^- appear to have thermochemical properties in common, in that electrons released in the oxidation of these compounds are likely to be at too high a potential to reduce NAD, and their subsequent oxidation at some later point in the electron transport chain would result in a low efficiency of coupling of phosphorylation to oxidation. Extensive membrane development in these organisms would therefore result in an increased capacity for respiratory activity with which to compensate for low phosphorylation efficiency. In this context an approximate estimation of the increase in membrane surface area per organism afforded by the internal membranes is of interest. In the rod-coccoid organisms with the type I membrane system a total of four bundles, each composed of 15 vesicles, has been calculated to be a conservative average per bacterium. Such an arrangement provides a surface area of about eight times that of the cytoplasmic membrane. The vibrioid organisms with the type II membranes in a peripheral arrangement contain, on average, two to three concentric vesicles per bacterium, resulting in a surface area of about four to six times that of the cytoplasmic membrane. Thus, although both membrane systems result in a considerable increase in the total membrane surface area per organism, there appears to be a marked difference between the two systems in the extent of that increase. A difference of a similar magnitude is found between the fastest generation times of the two groups of organisms, 3·5 hr in the rod-coccoids and 5 to 6 hr in the vibrioids (Whittenbury *et al.* 1970), implying that the growth rate is a function of membrane area.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1, 2. Sections of a 'Methylococcus' (subgroup 'minimus') strain showing the type I membrane system. The triplet structure of membrane pairs is apparent. × 80,000.

Fig. 3. Fragment of a dividing 'Methylobacter' (subgroup 'vinelandii') strain showing the vesicular conformation of the membranes. × 60,000.

Fig. 4. Section of a 'Methylococcus' (subgroup 'minimus') strain with two membrane bundles (M) in a side-on position. × 90,000.

PLATE 2

Fig. 5. Negatively stained preparation of a lysing 'Methylomonas' (subgroup 'rubrum') strain showing closely packed, disc-shaped membrane vesicles. × 50,000.

Fig. 6. Negatively stained preparation of a lysing 'Methylomonas' (subgroup 'rubrum') strain showing membrane discs in end-on and side-on positions. × 50,000.

Fig. 7. Negatively stained membrane discs and cytoplasmic membrane (arrowed) from a lysed 'Methylococcus' (subgroup 'minimus') strain.

Fig. 8. Section of a 'Methylomonas' (subgroup 'albus') strain showing invagination of the cytoplasmic membrane. × 100,000.

Fig. 9. Section of a 'Methylomonas' (subgroup 'BG4A') strain with variant type of membrane bundle in section and side (M) view, and connexion (arrowed) between membrane bundle and cytoplasmic membrane. × 50,000.

PLATE 3

Fig. 10. Fragment of a 'Methylocystis' (subgroup 'parvus') strain showing the three-layered structure of the membranes. Arrows indicate bacterial wall. × 90,000.

Fig. 11. Section of a 'Methylosinus' (subgroup 'sporium') strain with the type II membrane system ramifying throughout the organism. × 60,000.

Fig. 12. Section of a 'Methylosinus' (subgroup 'sporium') strain showing a concentration of the membranes towards the periphery of the organism. × 90,000.

Fig. 13, 14. A more regular, peripheral arrangement of the membranes in a 'Methylosinus' (subgroup 'sporium') strain. The membrane system shows some branching (arrowed area). × 80,000; 60,000.

PLATE 4

Fig. 15. Negatively stained preparation of a strain of the *Mycobacterium–Corynebacterium* complex grown on propane. Each bacterium contains numerous mesosomes. × 60,000.

Fig. 16. Negatively stained, propane-utilizing bacterium of the *Mycobacterium–Corynebacterium* complex showing large mesosome at site of cross-wall formation. × 60,000.

Fig. 17. Negatively stained, propane-utilizing bacterium of the *Mycobacterium–Corynebacterium* complex with peripheral membrane bodies. × 60,000.

Fig. 18. Section of a Gram-negative coccal-rod grown on C_{11} to C_{18} *n*-alkane mixture. × 75,000.

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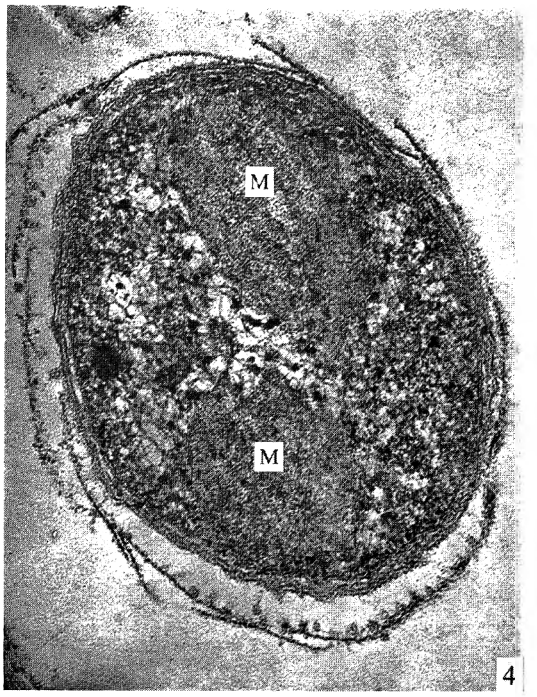

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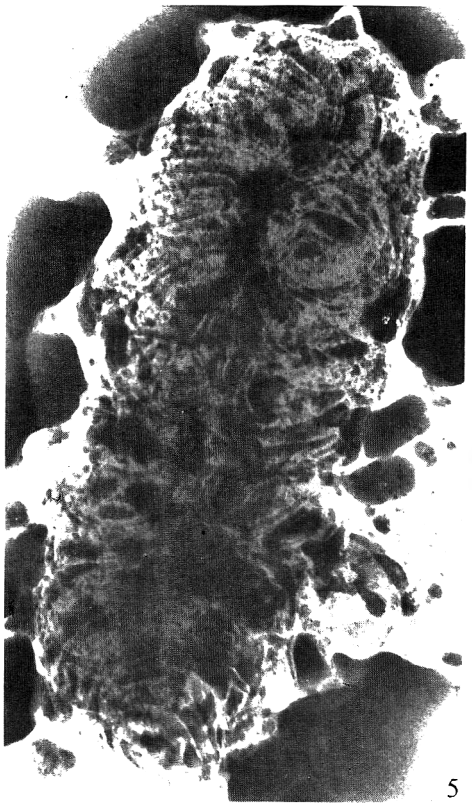

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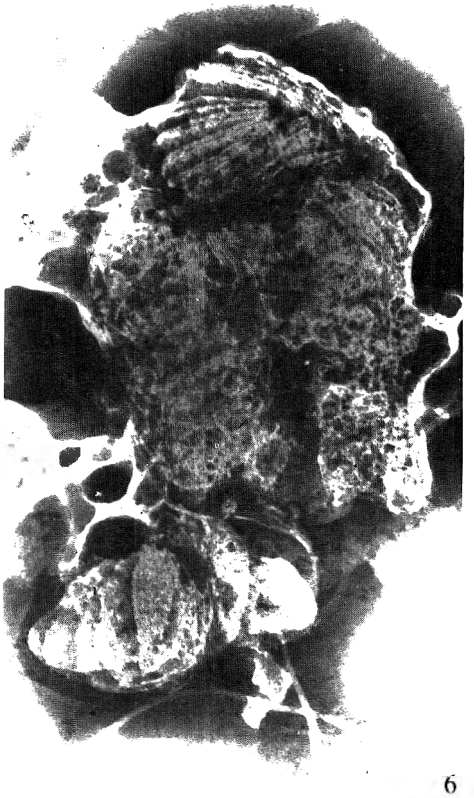

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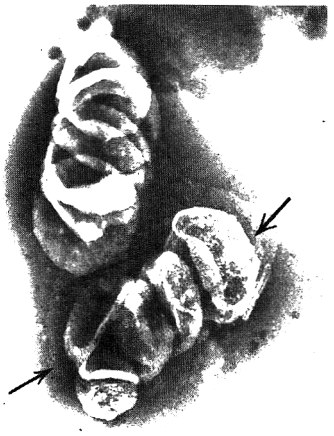

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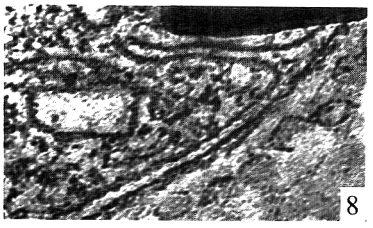

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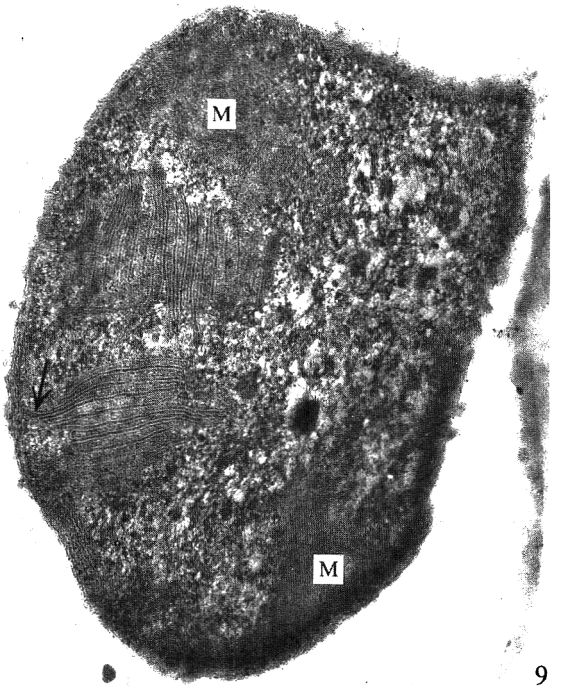

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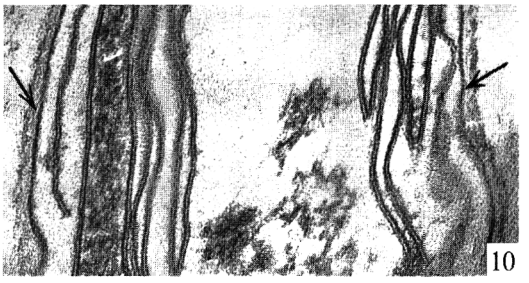

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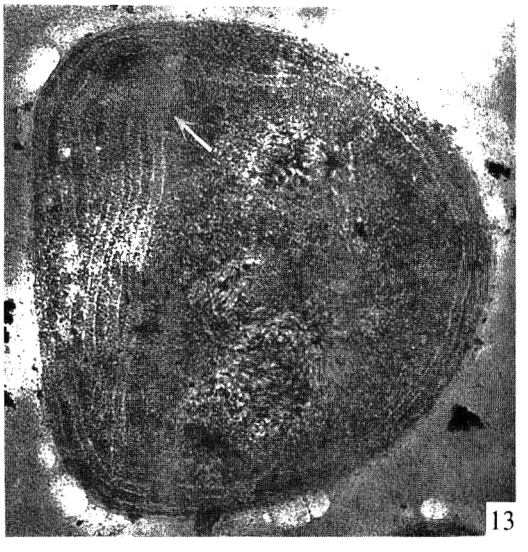

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Reversion of *Bacillus subtilis* Protoplasts to the Bacillary Form Induced by Exogenous Cell Wall, Bacteria and by Growth in Membrane Filters

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SUMMARY

Methods were developed for growing protoplasts or L bodies of *Bacillus subtilis* into L colonies on membrane filters. Only some types of filters permitted growth; for optimum growth the filters usually had to be extracted with 2 % (v/v) ethanol in water. Scoring was greatly improved by staining. Growth on the filters induced reversion of the protoplasts to the bacillary form. Reversion was greatly enhanced when *B. subtilis* wall was added to the filter. A lesser enhancement occurred when wall was added to protoplasts growing in agar. The stimulation of reversion by wall was non-specific since similar stimulation could be obtained by intact autoclaved *B. subtilis*, *Escherichia coli*, pseudomonads and yeast. Stimulation of reversion probably depended on physical contact between the naked protoplasts or L bodies and the surface provided by the filter, wall, or killed organism. The present status of induced reversion in *B. subtilis* is discussed.

INTRODUCTION

Bacteria usually synthesize cell wall at a rate commensurate with the increase in cytoplasmic mass. Partial removal of the wall through enzyme action, or through growth in presence of inhibitors, presents only a temporary challenge to these cells. Thus, spheroplasts of Gram-negative species and the 'reverting' L forms obtained through multiplication of such spheroplasts (these morphologically mostly exhibit '3B' forms; see Dienes & Weinberger, 1951) promptly synthesize more wall as soon as lysozyme, or the inhibitory antibiotic, is removed (Landman, Altenbern & Ginoza, 1958; Lederberg & St Clair, 1958; Altenbern, 1963). Similarly, 'quasi-spheroplasts' of *Bacillus subtilis*, produced by partial removal of the wall by lysozyme, can apparently repair wall without difficulty (Miller, Zsigray & Landman, 1967; reviewed, Landman, 1968).

A sharply different situation is encountered when all the wall is removed: naked protoplasts, or L forms derived from them, are generally unable to re-initiate formation of new wall, especially in liquid media. On soft agar plates these wall-less forms give rise to L colonies consisting of irregularly-sized and -shaped, wall-less L bodies. Such L colonies have been called 'stable L colonies' to emphasize the heritably stable persistence of the wall-less state (Landman & Halle, 1963).

Protoplasts of *Bacillus subtilis*, or 'stable' L forms derived from such protoplasts can, however, be induced to initiate wall formation—i.e. to revert to the bacillary form—in media possessing special physical characteristics. Detailed studies of reversion

in *B. subtilis* induced by 25 % gelatin media have been published (Landman, Ryter & Fréhel, 1968; Miller, Wiebe & Landman, 1968; Landman & Forman, 1969). An account of the induction of reversion by hard agar was given by Landman & Halle (1963). Induction of reversion by membrane filters has also been mentioned (Landman, 1968).

Despite the fact that the L state is passed on heritably from one cell generation to the next, no loss or gain of nucleic acid-borne genetic information occurs during commitment to the L state or during reversion. This conclusion is implicit in the fact that each of these processes can occur in 1 hr with about 100 % efficiency (Landman & Halle, 1963; Landman & Forman, 1969). Instead, it has been postulated that the heritable persistence of the L state is due to the interruption of a feedback system which, in normal organisms, is responsible for the continuity of wall biosynthesis. According to this view, reversion of protoplasts or 'stable' L forms entails a special priming process which triggers re-initiation of wall biosynthesis in these wall-less forms (McQuillen, 1956; Landman & Halle, 1963; Martin, 1963; Landman, 1968).

In gelatin medium, reversion requires an initial period of protein synthesis (Landman & Forman, 1969). This is followed by rapid deposition of a thin, apparently uniform, wall layer which then gradually thickens. Early during the wall deposition period, physical disturbance of the system by 3 min. of warming (enough to melt the gelatin) markedly retards reversion (Landman *et al.* 1968). This observation points to a requirement for a solid gelatin barrier in close proximity to the organism. Furthermore, the evidence indicates that this barrier is required at the time when priming of wall synthesis occurs. The presence of a solid physical structure very near the membrane seems to be the sole feature common to all the reversion-stimulation environments: solid gelatin, hard agar and membrane filters. Such a barrier is present during wall synthesis in partially and normally walled organisms in the form of pre-existing wall. The work reported in this paper was initiated to investigate more closely the nature of this requirement for a physical barrier. Was direct contact between organisms and the barrier necessary? Would a barrier closely resembling *Bacillus subtilis* wall be more effective than a barrier of very different chemical constitution? Our experiments show that direct contact between the protoplasts and the surface is important, if not essential, and that wall preparations from *B. subtilis* are effective at inducing reversion, but that the effect is probably non-specific since preparations of wall material from other species are also effective.

METHODS

Microbial strains. In all experiments but one, *Bacillus subtilis* strain 168 (tryptophan-requiring) was used. The other organisms used are listed in Table 4.

Membrane filters. The various types of filters used are shown in Tables 1 and 2.

Extraction of inhibitory material from membrane filters. Three stacks of ten 4·7 cm. filters, separated by Gelman type E glass fibre filters, were extracted in 5·0 cm. diameter Soxhlet extraction thimbles (Kimax). The stack of filters was weighted down with a 4·5 cm. diameter, 4 cm. tall glass cylinder. After 4 hr extraction with 750 ml. 2 % (v/v) ethanol in water, followed by 4 hr extraction with distilled water, the filters were dried between paper towels. Weights were placed on the paper towels to prevent wrinkling. The dried filters were then sterilized by 10 min. exposure of each side to a General Electric G15 T8 15 W u.v. lamp at a distance of 30 cm.

Media. Most media were slightly modified versions of those used by Landman & Halle (1963).

SS plating medium. 530 ml. distilled, de-ionized water containing 7·5 g. Noble agar (Difco) and 1·0 g. NH_4NO_3 was brought to the boil and poured over 20 g. Purified

Table 1. *Viability and reversion of wall-less forms of B. subtilis on membrane filters of various characteristics*

Manufacturer	Type	Chemical composition	Pore size (μ)	Extracted	Side up	Viability* L	Viability* P	Revertants‡ (P-inoculum) (%)
Schleicher & Schuell	B-6	Nitrocellulose	0·45	−	Concave	4	2	—
				−	Convex	4	2	—
				+	Concave	—†	4	40
				+	Convex	—	5	30
Millipore	HA	Cellulose—mixed esters	0·45	−	Gridded	4	4	20
				−	Plain	4	5	20
				+	Gridded	—	5	20
				+	Plain	—	5	20
Oxoid	—	Cellulose—acetate	0·45	−	Gridded	3	1	—
				−	Plain	3	1	—
				+	Gridded	—	5	10
				+	Plain	—	5	10
Millipore	AA	Cellulose—mixed esters	0·80	−	Gridded	2	0	—
				−	Plain	—	0	—
				+	Gridded	—	1	—
				+	Plain	—	0	—
Millipore	DA	Cellulose—mixed esters	0·65	−	Gridded	3	—	—
				−	Plain	3	—	—
Gelman	GM-6	Cellulose—di-acetate	0·45	−	A§	0	0	—
				−	B	0	0	—
				+	A	—	3	10
				+	B	—	0	—
Gelman	GA-8	Cellulose—tri-acetate	0·20	−	Glossy	0	1	—
				−	Dull	0	4	10
				+	Glossy	—	3	10
				+	Dull	—	3	10
Gelman	GA-6	Cellulose—tri-acetate	0·45	−	Gridded	0	0	—
				−	Plain	0	0	—
				+	Gridded	—	1	—
				+	Plain	—	1	—

* The viability of either protoplast inocula or inocula of L bodies on membrane filters was compared to viability of the same inoculum on the same medium without membrane filters. SA or SS medium was used and viable counts were the combined totals of L colony counts—the majority of colonies—and bacillary colony counts. Growth response is coded as follows: 5 = 80 to 100% of controls; 4 = 50 to 80%; 3 = 20 to 50%; 2 = 10 to 20%; 1 = 1 to 10%; 0 = no L growth.

† Not done.

‡ Much variation was noted between experiments, but not within experiments. Rounded off average values are shown only for filter types where viability exceeds 20%. Reversion was scored on the third day following inoculation.

§ Both sides of filters looked the same. Both sides were tested by cutting filters in two and turning one of the halves over.

Pigskin Gelatin (Eastman). When the gelatin was dissolved, this mixture was autoclaved for 15 min. at 121° and cooled to about 52°. Sterile, pre-warmed concentrated stock solutions of various reagents were then mixed in gently, in the order listed, to

give the indicated final concentrations in 1 l.: sodium succinate, pH 7·3 to 0·5 M; K_2HPO_4 and KH_2PO_4 to 3·5 and 1·5 g./l., respectively; glucose to 2·0 g./l.; $MgSO_4$ to 5×10^{-3} M and L-tryptophan (Nutritional Biochemicals) to 0·02 g./l. Plates were poured to a thickness of about 6 mm. and stacked in piles of 30 during pouring to minimize differences in gelling conditions. They were then dried at room temperature for 3 days and used without further storage.

Table 2. *Membrane filters incapable of supporting colony formation by plated wall-less forms*

All filters shown in this table were tested under the same set of conditions as the filters in Table 1. None supported L colony growth whether ethanol extracted or not.

Membrane filter			
Manufacturer	Type	Chemical composition	Pore size (μ)
Gelman	GM-4	Cellulose di-acetate	0·80
	VM-1	Poly-vinylchloride	5·0
	VM-4	Poly-vinylchloride	0·80
	VM-100	Poly-vinylchloride	10·0
	VNW-800	Nylon-poly-vinylchloride	0·80
	Alpha 6	Regenerated cellulose	0·45
	Versapor 6424	Reinforced epoxy	5·0
	Versapor 6429	Reinforced epoxy	0·90
	Glass Fibre type E	Glass microfibres	?
Selas	FM 47	Silver	0.20
	FM 47	Silver	0·45
	FM 47	Silver	0·80
Millipore	NC	Nylon	14·0
	NR	Nylon	1·0
	EH	Cellulose acetate	0.50
	VM	Cellulose—mixed esters	0·05
	BC	Information not available	?
	RH	Information not available	?

Table 3. *Some characteristics of the stimulation of reversion by exogenous wall*

The crude wall preparation used in these experiments was a suspension in DF of a washed 1000 to 10,000 *g* fraction of sonicated *Bacillus subtilis*. All platings were done in triplicate.

Plating surface	Inducer added	Reversion frequency (%)
Soft agar (SS medium)	None	1
	Unheated wall	4
	Heated wall	8
Ethanol extracted membrane filter resting on SS medium	None	4
	Unheated wall	27
	Heated wall	49

SA plating medium. This medium was the same as SS medium except that 2 g./l. bovine serum albumin (Fraction V, Armour) was substituted for the gelatin, the agar was 8 g./l. Bacto Agar (Difco), and 5×10^{-3} M-$MgCl_2$ was used instead of $MgSO_4$. The serum albumin was filter-sterilized and added last.

TPM liquid medium. This medium was used for preparing protoplasts and was a modification of the SL 2 medium of Landman & Halle (1963). The source of Mg^{2+} is

5×10^{-3} M-$MgSO_4$; tryptophan 0·0075 g./l.; acid-hydrolyzed casein (vitamin-free, Difco) was 0·1 g./l. and EDTA 5×10^{-5} M.

DFC dilution fluid. This medium, used in dilution and plating of protoplasts and L bodies, contained: sodium succinate (pH 7·3) 0·25 M; sucrose 0·25 M; K_2HPO_4 3·5 g./l.; KH_2PO_4 1·5 g./l.; $MgCl_2$ 5×10^{-3} M; EDTA 10^{-4} M. Concentrated stock solutions were autoclaved separately and added to sterile distilled water in the order listed.

Preparation and handling of protoplast suspensions. The initial stages of protoplast preparation were as described by Landman & Halle (1963). Organisms were shaken in SL 1 medium (Landman & Halle, 1963) for 4·5 hr at 37°, diluted 1/9 into TPM and shaken for another 1·5 hr at 37°. Then lysozyme was added to 300 μg./ml., the cultures transferred to Erlenmeyer flasks in a water bath at 32° and incubated in shallow layers without agitation for another 1·5 hr. At this time formation of protoplasts was about 99·99% complete in that only about 1 cell in 10^4 formed a bacillary colony on SS medium; the rest formed L colonies. Survival was between 60 and 100 %: 60 to 100 L colonies were formed for every 100 bacillary colonies formed by the original bacterial suspension. The organisms were centrifuged at 8000 *g* at room temperature for 12 min., resuspended in DFC and centrifuged and washed 3 times more to remove lysozyme. The final resuspension was in one-tenth the original volume. Some loss of viability occurred: in an average experiment the viable count of the washed protoplast suspension was about 25 % of that of the initial bacillary suspension. The washed protoplast suspension was dispensed in 1 ml. samples in test tubes, plunged into a dry-ice+acetone mixture for at least 10 min. before storage in a deep freeze at −20°. When melted immediately the viability had fallen to about 15 % of the original count. During storage viability declined slowly to 5 % of the original value in 2 months. For a series of experiments, a stock suspension of frozen protoplasts was prepared and samples of closely predictable viability withdrawn from storage for use.

Preparation of L-body suspensions. Protoplasts were inoculated on SS plates and incubated at 30° for about 24 hr. Sterile DFC (0·1 ml.) was then dispensed on each plate and the growth re-spread. This procedure was repeated on the second and third day after inoculation. Occasional contaminating bacillary colonies were carefully excised before re-spreading. Finally, the plates were incubated at 30° for 2 more days. DFC (5 ml.) was added to the near-confluent growth of L colonies so obtained and much of the growth was suspended in it by gentle rubbing with a spreader. This suspension was pipetted off, diluted and used within 1 hour.

Preparation of crude wall suspensions. Bacillus subtilis strain 168 *trp*$^-$ was grown overnight in Brain Heart Infusion (BHI, Difco), centrifuged, resuspended, and washed once in cold 0·1 M-potassium phosphate buffer (pH 5·0) to decrease autolytic enzyme activity (Young, 1966). The suspension was then disrupted for 5 to 10 min. in a Branson Sonifier (Model S 75), at maximum settings; breakage was monitored by phase-contrast microscopy. The treated suspension was centrifuged for 15 min. at 1000 *g*, the pellet discarded, and the supernatant fluid centrifuged for 15 min. at 10,000 *g*. The pellet from this step was resuspended in cold 0·1 M-potassium phosphate buffer (pH 5·0) and the cycle of centrifugation at 1000 *g* followed by 10,000 *g* was repeated twice to improve the homogeneity of the preparation. The final pellet was suspended in DFC and stored at −20° or below. Undiluted wall suspension (0·1 ml.) was plated in each experiment and was always sterile.

Preparation of heat-killed intact-organism suspensions. The various micro-organisms were grown in BHI to late exponential phase, autoclaved, and washed three times in sterile 0·85 % (w/v) NaCl. The final pellet was resuspended in DFC, steamed at 100° for 5 to 10 min., and then stored at −20° or below. Undiluted suspensions were plated in each experiment and were always sterile.

Standardization of suspensions for use in experiments on induction of reversion. Suspensions were adjusted to 10 % transmission at 670 mμ in the Bausch and Lomb Model 20 Spectrophotometer. About 66 μg. dry matter in 0·1 ml. was spread on a membrane filter or agar plate and allowed to dry. Then the protoplasts were inoculated on to the same surfaces.

Staining for visual differentiation of bacillary and L colonies on membrane filters. A few drops of a 1/1 mixture of Loeffler's alkaline methylene blue (Pelczar, 1965) with a 0·25 % solution of safranin in 10 % ethanol were introduced beneath the filter. About 30 min. later the colonies on the membrane filters were scored with a dissecting microscope.

RESULTS

Viability and reversion on membrane filters

Although previous experiments in our laboratory had shown that L forms could grow and revert on membrane filters, results were initially very variable. This situation has now been much improved because differential staining greatly improved scoring of L colonies (see methods) and plating efficiency was often enhanced by the removal of inhibitors from the membrane filters through extraction with 2 % (v/v) ethanol in water (Cahn, 1967).

The growth of protoplast and L form inocula was studied on extracted and on unextracted filters. About 100 viable bodies were spread on filters resting on SA medium or, in the case of the controls, directly on SA medium. After 3 to 4 days at 30° bacillary (B) and L colonies were counted. The two surfaces of the membrane filters did not always behave similarly (Tables 1 and 2) and only membrane filters made from substituted celluloses supported L growth. The effect of ethanol extraction was not predictable: with Oxoid and with Schleicher & Schuell filters, extraction improved plating efficiency, whereas extraction had little effect on Millipore HA filters. Not all batches of filters of a given manufacture behaved identically and these differences persisted after extraction.

The effect of exogenous wall on reversion

The observation that growth and reversion of L colonies was restricted to filters made of polysaccharide material suggested that similarities between these filters and normal cell wall might be important. In the case of reversion, earlier observations implicated wall in the continued synthesis of more wall and in the re-initiation of new wall biosynthesis (Landman, 1968; Miller *et al.* 1967). A logical test of this view was, therefore, to attempt to stimulate reversion by added exogenous wall.

Wall preparation (0·1 ml. containing 66 μg. dry wt in DFC) was spread directly on to extracted Schleicher & Schuell B6 filters resting on SS medium or on to equivalent areas of SS medium. After drying, about 300 protoplasts suspended in DFC were spread on the inoculated surfaces and on to control filters and plates without wall. The plates were scored for B and L colonies following incubation at 30° for 3 days

(Table 3). Clearly, wall stimulated reversion considerably. Boiled wall was more effective than untreated wall, presumably because boiling inactivated an autolytic enzyme. Reversion occurred much more freely when the protoplasts were in contact with the wall on the membrane than in the agar environment, suggesting that a sustained and firm contact between reverting protoplasts and the stimulating wall fragments was necessary.

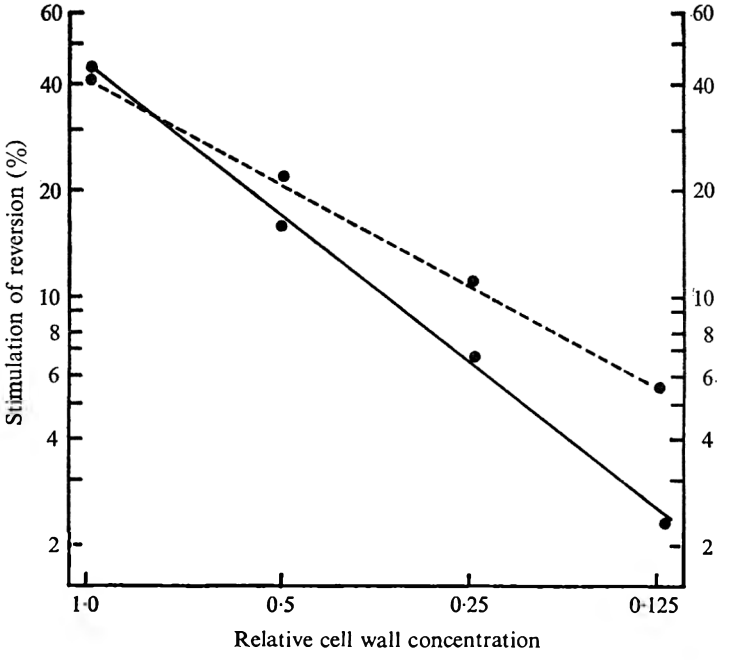

Fig. 1. Relationship between wall concentration and reversion. Twofold serial dilutions of a *Bacillus subtilis* crude wall preparation were spread on to ethanol-extracted Schleicher & Schuell B-6 membrane filters resting on SS medium. The greatest amount plated corresponded to the wall content of 5×10^8 whole cells. If spread in a single layer over the filter surface these walls would cover about 42% of the total surface area of the filter. The lower concentrations would cover proportionately smaller fractions of this area (broken line). About 500 protoplasts were spread on top of these preparations, the plates were incubated for 3 days at 30° and then scored for B and L colonies. The percentage reversion figures shown (solid line) are corrected for background reversion (7·1%) observed on membrane filters without added cell wall.

The effect of wall concentration on reversion

Serial dilutions (0·1 ml.) of boiled wall suspension in DFC (660 μg. dry wt/ml.) were spread on membrane filters and dried. Protoplasts were added and the plates scored for B and L colonies after 3 days at 30°. Reversion appeared to be an exponential function of the concentration of added wall despite the fact that the wall of 10^5 to 10^6 organisms had to be added to obtain a single reversion (Fig. 1). This result suggests that the physical wall itself rather than an associated diffusible product stimulated reversion. The approximate proportion of the filter surface covered by the added wall suspensions has been calculated and included in Fig. 1; the similarity between the two curves is consistent with the hypothesis that physical contact between wall fragment and protoplast is required to trigger reversion.

Reversion in the presence of heat-killed Bacillus subtilis. Time of appearance of revertant colonies

To avoid the time-consuming task of preparing wall fractions, we tested whether reversion could be induced by heat-killed bacilli. Autoclaved, washed *Bacillus subtilis* induced reversion with an efficiency comparable to a wall fragment preparation. Fig. 2 shows the time of appearance of revertants in this system. A suspension of killed cells in DFC (0·1 ml. containing 250 μg. dry wt) was spread on to extracted Millipore

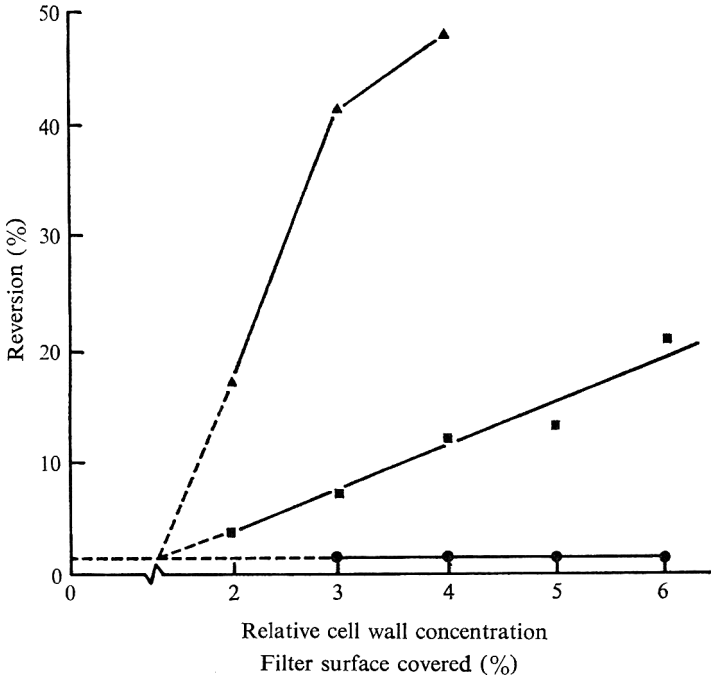

Fig. 2. Time course of the induction of reversion by killed, intact *Bacillus subtilis* and by membrane filters alone. About 500 protoplasts were spread on either SS plating medium alone (●——●), ethanol-extracted Millipore HA filters resting on SS medium (■——■), or extracted filters previously spread with 5×10^8 autoclaved *B. subtilis*, resting on SS medium (▲——▲). The plates were incubated at 30° and at the times indicated three randomly chosen plates from each group were counted, using a differential stain to distinguish reverted and non-reverted colonies.

HA filters, resting on SS medium. After drying, about 500 protoplasts were spread on the filters with or without killed cells, and also on plain SS medium. All plates were incubated at 30° and a selection withdrawn and scored for B and L colonies after 2, 3, 4, 5 and 6 days. Besides displaying marked stimulation by autoclaved bacilli Fig. 2 also shows that the time course of appearance of revertants was approximately linear on Millipore HA filters without killed bacilli. Since multiplication of L bodies and a consequent change in the area of surface contact between L bodies and membrane filter occurred during much of the period, the apparent simplicity of these results may well be deceptive.

Stimulation of reversion by other killed micro-organisms

Since heat-killed *Bacillus subtilis* and wall induced reversion, the question of specificity became important. Autoclaved suspensions of a wide range of Gram-positive and Gram-negative bacilli, Gram-positive cocci and yeast, all at about 2·5 mg. dry wt/ml., were compared with a suspension of *B. subtilis* of similar concentration. Table 4 shows clearly that reversion stimulated by autoclaved bacteria is non-specific; no matter what the chemical nature of their surface, all bacteria were about equally effective. Even killed yeast suspensions exerted a significant stimulatory effect, despite the fact that they suppressed about 50 % of the L colony growth.

Table 4. *Stimulation of reversion by killed micro-organisms of various types*

Cultures were grown overnight in Brain Heart Infusion medium, autoclaved, washed 3 times in sterile saline, resuspended in DFC to about 5×10^9 organisms/ml. and finally steamed for 10 min. One-tenth ml. samples were spread on Schleicher & Schuell B-6 filters resting on SS medium and allowed to dry before about 500 *Bacillus subtilis* protoplasts were added. After 3 days at 30° the plates were scored for B and L colonies. The stimulatory capacity of *B. subtilis* was taken as 1·0. Means and standard deviations of the relative stimulatory capacities were calculated from a minimum of 3 filters for each inducer.

Organism	Relative stimulatory capacity	Standard deviation
Bacillus subtilis, strain 168 *trp*⁻	1·0 (Reference)*	0·2
B. cereus, strain 569 S^r	1·2	0·4
B. megaterium, ATCC 9885	1·1	0·4
B. stearothermophilus, strain Schmidt	1·0	0·3
Escherichia coli, ATCC 11303	1·3	0·3
Proteus mirabilis, strain PMI (Baron)	1·1	0·3
Salmonella typhimurium, strain 1334-57 (CDC)	0·9	0·1
Pseudomonas aeruginosa, ATCC 14216	1·1	0·0
Staphylococcus aureus, ATCC 6020	1·1	0·4
Saccharomyces cerevisiae, ATCC 9763	0·8	0·3

* 42 % revertants. In the absence of any wall, the frequency of reversion averaged 9 %.

DISCUSSION

Partial or complete removal of the wall generally deprives bacteria of their ability to septate (Altenbern & Landman, 1960; Lederberg & St Clair, 1958). Accordingly, spheroplasts and protoplasts are unable to divide in liquid media. Only solid media possessing very special supporting properties can provide a substratum where these soft wall-deficient forms can continue to multiply as they 'ooze between and subdivide themselves among the fibres' (Landman & Halle, 1963). Among the standard bacteriological media, only soft agar is suitable, not hard agar or gelatin. Our present findings and earlier reports (Dienes, 1968; Dienes & Madoff, 1966; Landman, 1968) show that some types of membrane filters can also support L colony growth. The fact that only a few of the filter types can provide the required substratum for multiplication is not surprising in the light of the results with standard bacteriological media: the finding that growth of L forms appears to be restricted to filters in a certain range of porosities (Tables 1 and 2) is quite analogous, for example, to the earlier observation that an increase in agar concentration can interdict L colony formation.

By using filters of appropriate pore size, composition and manufacture, extracting with 2 % (v/v) ethanol in water and scoring with the aid of the differential stain, a useful advance in the technology of use of membrane filters for the growth of L colonies of *Bacillus subtilis* has been achieved.

Five types of environment have now been found to stimulate reversion of *B. subtilis* protoplasts or L bodies to the bacillary form: gelatin, hard agar, membrane filters, exogenous wall or heat-killed intact micro-organisms; to which should, perhaps, be added the residual endogenous wall of 'quasi-spheroplasts'. These reversion-inducing materials have little in common besides their particulate or solid state. In the case of washed membrane filters, it seems particularly clear that their ability to stimulate reversion must be due to the physical nature of the insoluble filter rather than to any soluble chemical constituent.

The effect of the chemical composition of the filters on reversion is less clear-cut. With cellulose filters, changes in the chemical substituents of the cellulose have little effect on their ability to induce reversion. The chemical nature of the non-cellulose based membranes may be much more important in the reversion process, but the lack of protoplast growth obtained with these filters precludes their investigation. In the exogenous microbial (or wall) system, the results are unambiguous: stimulation of reversion depends markedly on the amount of wall or killed microbial mass present and on firm contact between exogenous material and protoplasts (Table 3) and scarcely at all on the chemical constitution of the reversion-inducing organism surface.

Since the main factors affecting the growth of protoplasts on membranes are physical rather than chemical, we may now ask how these properties allow the re-initiation of wall synthesis. There are several possibilities which are not mutually exclusive. (i) The physical barrier near the membrane may slow diffusion of wall precursors or exoenzymes involved in wall synthesis, so that a priming quantity of cell wall can accumulate. (ii) The physical barrier may adsorb wall precursors and/or exoenzymes and thereby allow priming. In these instances, priming is regarded as the production of a wall polymer sufficiently large to permit its own further extension by the organism's normal wall-building system (as in a quasi-spheroplast). (iii) The barrier may adsorb, and thereby block the action of, a wall-destroying enzyme or repressor of wall biosynthesis. (iv) The barrier may induce, by an unknown mechanism, renewed formation of wall precursors whose synthesis has been repressed by removal of wall. This last possibility is underlined by the finding (Bond, 1969) that diaminopimelic acid production is profoundly depressed in protoplasts and L forms of *B. subtilis*.

While the research reported in this paper has helped us to eliminate some hypotheses, particularly these involving chemical priming, we cannot decide which of the foregoing possibilities, if any, is correct. Whatever the molecular events underlying reversion, the characteristics of all known reversion-inducing systems (hard agar, gelatin, partly walled 'quasi-spheroplasts', membrane filters, exogenous wall or autoclaved bacteria) suggest that, for a period early in the process, the naked cells must be kept in close, undisturbed physical contact with a solid substratum.

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The Isolation and Characterization of Alkane-oxidizing Organisms and the Effect of Growth Substrate on Isocitric Lyase

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SUMMARY

A mycobacterium, two pseudomonads and a torula were isolated from soil using selective enrichment techniques in mineral salts-kerosene media. The properties of the organisms and their possible identity are outlined. Hydrocarbons, fatty acids, *n*-alcohols, dicarboxylic acids and acyl amides able to support growth were determined. Growth of the torula on hydrocarbons increased the ability of the organisms to oxidize other alkanes and fatty acids; the organism was, however, able to oxidize these substrates, at a slow rate, irrespective of the substrate of growth. When the torula was grown on an alkane with an even number of carbon atoms, there was no evidence that the induced enzymes favoured the oxidation of fatty acids with even rather than odd numbers of carbon atoms.

Growth on hydrocarbons increased the amount of isocitric lyase present in all four micro-organisms, suggesting that β-oxidation of fatty acids derived from alkanes gives rise to acetyl-CoA and that this pathway and the glyoxylate bypass are important in the metabolism of alkanes.

INTRODUCTION

The metabolism of straight-chain hydrocarbons by micro-organisms has been reviewed by Foster (1962), Johnson (1967), McKenna & Kallic (1965), and van der Linden & Thijsse (1965). It is generally agreed that the primary attack on *n*-alkanes is by oxidation of the terminal methyl group to a carboxyl group. There is evidence that diterminal oxidation also occurs (Kester & Foster, 1963), but it is probable that this is a minor pathway. In both cases, the major pathway for the subsequent metabolism of the fatty acid is thought to be by β-oxidation to yield acetyl-coenzyme A units. Since the organisms would thus be growing essentially on acetate, it might be expected that isocitric lyase (EC 4.1.3.1), a key enzyme of the glyoxylate bypass, would be induced by prior growth on *n*-alkanes. This paper reports assays for this enzyme in cells grown on alkane and non-alkane substrates.

It is still commonly said that prior growth on alkanes induces oxidation systems in the cells such that the fatty acids in the homologous series, each with two carbon atoms less than the alkane used for growth, are oxidized more rapidly than the fatty acids with one, three, five, etc. less carbon atoms. Azoulay & Senez (1960) claim such sequential induction in a pseudomonad grown on C_{10}, C_8, C_7 or C_6 alkanes. Although they obtained some data inconsistent with this interpretation, they attributed these results to impurities in their reagents. The results of Thijsse & van der Linden (1958,

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1963) with heptane or hexane-grown pseudomonads did not show sequential induction nor were Treccani, Canonica & de Girolamo (1955) able to demonstrate this phenomenon with Nocardia, Achromobacter or Mycobacterium grown on C_{12}, C_8, C_7, C_6, C_5 alkanes or C_6 or C_5 fatty acids.

This paper reports first the isolation and characterization of widely different organisms, pseudomonads, a mycobacterium and a torula, capable of growth on alkanes as the sole source of carbon. The organisms were then tested for their ability to grow on a number of alkanes and fatty acids. In addition, the effect of the growth substrate on the ability of the torula to oxidize likely metabolites was investigated. While the enzyme systems concerned in the oxidation of alkanes and fatty acids were induced by prior growth on alkanes, there was no evidence of sequential induction of the enzymes associated with the oxidation of the series of fatty acids with two less carbon atoms than the alkane used for growth.

METHODS

Media. The basal mineral salts medium contained (g./l.): $MgSO_4.7H_2O$, 0·04; $CaCl_2.6H_2O$, 0·02; $FeSO_4.6H_2O$, 0·002; NH_4Cl, 2·7; KH_2PO_4, 8·5; Na_2HPO_4, 8·9. The modified mineral salts medium used for the isolation of the torula was made in tap water and contained (g./l.): $MgSO_4.7H_2O$, 0·5; NH_4Cl_2, 1·0; KH_2PO_4, 1·0; Difco yeast extract, 0·25. This medium was adjusted to pH 5·5 by the addition of 0·25 M-NaOH. All media were autoclaved at 15 lb./sq. in. for 15 min. D-Glucose solution 25 % (w/v) was autoclaved at 5 lb./sq. in. for 25 min. and added to sterile basal mineral salts medium to give 0·05 M concentration. Sodium acetate was added to give 0·05 M in the mineral salts medium before the medium was autoclaved. Carboxylic acids were added to the basal mineral salts medium to give 0·005 M before the medium was autocalved. Seitz-filtered liquid alkane (5 ml.) was added to 1 l. of the sterile mineral salts medium. Solid media were prepared by the addition of 1·2 % (w/v) agar to liquid media. Pigment production was measured on King's media A and B (King, Ward & Raney, 1954).

Isolation and maintenance of organisms. A hydrocarbon-utilizing torula was isolated from soil using selective enrichment with kerosene modified mineral salts (pH 5·5) medium. Hydrocarbon-utilizing pseudomonads were isolated from soil using kerosene-basal mineral salts medium at pH 7·0 and a hydrocarbon-utilizing mycobacterium was isolated by selective enrichment on kerosene-mineral salts agar at pH 7·0.

Stock cultures were kept at 4° on acetate-mineral salts agar slopes and were subcultured monthly. Freeze-dried cultures of all strains were kept as reserve stock.

Characterization of the organisms. The tests for the properties of the isolates were those described by Skerman (1967).

Tests for growth on hydrocarbons and fatty acids. The ability of the organisms to use various hydrocarbons and fatty acids as sole source of carbon and energy for growth was tested in 100 ml. of medium in shaken flasks. The cultures were incubated at 30° and, after 7 days, the amount of growth in each flask was observed visually and recorded according to the following scale: 1+ = light but definite turbidity; 2+ = moderate turbidity; 3+ = heavy turbidity.

Growth of cells for respiration and enzyme studies. Cells were grown at 30° in 4 l. batches in a 6 l. glass fermenter. Air was passed into the fermenter at a rate of 4 l./min. and the agitator rotated from 600 to 1000 rev./min. After 36 hr, cultures were cooled

and the cells centrifuged at 10,000 *g* for 15 min. at 4°. The cells were resuspended and washed in 0·02 M-KH_2PO_4–Na_2HPO_4 buffer (pH 7). Cells grown on glucose or acetate were washed twice; for those grown on hydrocarbons, six washes were necessary to remove the alkane.

Respiration studies. Cells were resuspended in 0·05 M-KH_2PO_4–Na_2HPO_4 buffer (pH 7) and the turbidity of the cell suspension was standardized to contain 1 mg. dry weight/ml. of buffer. The Warburg flasks contained 0·2 ml. of 20 % (w/v) NaOH in the centre well, 1 ml. of organisms and 1 ml. of 0·05 M-KH_2PO_4–Na_2HPO_4 buffer (pH 7) in the main compartment and either 0·2 ml. of liquid hydrocarbon, 50 mg. of solid hydrocarbon or 10 μmoles of carboxylic acid (pH 7) in the side arm. To measure endogenous respiration, 0·2 ml. of distilled water replaced the substrate in the side arm. Measurements were made at 30° in air. The Q_{O_2} (μl. of oxygen taken up/mg. dry weight of cells/hr) was calculated.

Preparation of crude cell-free extracts. After washing, 1 g. of wet cell paste was resuspended in 4 ml. of 0·05 M-KH_2PO_4–Na_2HPO_4 buffer (pH 7) containing 1 mM-2-mercaptoethanol and 1 mM-$MgCl_2$. Cells were exposed to ultrasonic disintegration for five periods of 60 sec., care being taken not to overheat the cell suspension. Cell debris was removed by centrifugation at 13,000 *g* for 15 min. at 4°; the supernatant was assayed for isocitric lyase activity.

Protein estimation. Protein was estimated by the biuret method of Gornall, Bardawill & David (1949).

Assay of isocitric lyase. The method of Dixon & Kornberg (1959) was used. The assay mixture contained 100 μmoles KH_2PO_4–Na_2HPO_4 buffer (pH 7), 15 μmoles $MgCl_2$, 10 μmoles phenylhydrazine HCl, 10 μmoles reduced glutathione, 5 μmoles sodium (D,L)-isocitrate and cell-free extract in a final volume of 3 ml. The blank lacked substrate. Glyoxylate phenylhydrazone was estimated by measuring the increase in OD_{324} on incubation at 30° and comparing this with a standard curve of glyoxylate phenylhydrazone. One unit of isocitric-lyase activity is defined as mμmoles phenylhydrazone formed/5 min. and the specific activity is expressed as units/mg. protein.

Chemicals. Hexadecane was American Standard Testing Material. The other alkanes used for growth and oxidation studies were supplied by Newton Maine Rare Chemicals guaranteed not less than 99 % pure. The fatty acids were Sigma Grade supplied by Sigma Chemical Co. Mixed hydrocarbons were commercially available products of Mobil Oil Co., Australia. All other chemicals were of the highest purity commercially available and were not purified further.

RESULTS

Characteristics of Torulopsis Y8. The alkane-oxidizing torula, Torulopsis Y8 was a Gram-positive, non-capsulate oval organism (dimensions 2·5–4 μm.) which stained regularly and divided by budding. After 2 days aerobic growth at 30° on glucose-yeast extract agar, the matt colonies were circular, 1·5 mm. diameter with a regular edge and a low convex elevation. They had a white opaque colour, fruity odour and a butyrous consistency. Despite repeated attempts to induce sporulation, spores were never observed.

Peptone, yeast extract, ammonium ions but not nitrate were able to act as sole nitrogen source for growth. Glucose, sucrose, and galactose, but not lactose, maltose or arabinose acted as the sole source of carbon for growth. The organism was unable

to grow in ethanol-mineral salts medium. It produced acid from glucose and galactose after 1 day, a little acid from sucrose and raffinose after 2 days and a little acid from trehalose after 30 days. Fructose, maltose, xylose, arabinose, inositol, mannitol, glycerol and rhamnose were not fermented. The organism was able to metabolize glucose oxidatively and fermentatively. Litmus milk was made alkaline after 30 days.

Lodder & Kreger-van Rij's *The Yeasts, a Taxonomic Study* (1952) was used for the identification of the organism. Torulopsis Y8 appears to be similar to but not identical with *Torulopsis gropengiensseri* and *Torulopsis magnoliae.*

Characteristics of Pseudomonas 22 and Pseudomonas 14. The alkane-oxidizing pseudomonads 22 and 14 were Gram-negative, non-capsulated, evenly stained, non-sporing rods (dimensions $0{\cdot}4 \times 0{\cdot}8$ μm.). Both were motile by means of a single polar flagellum. They metabolized glucose oxidatively but no acid was produced fermentatively after 17 days incubation. After 2 days aerobic growth at 30° on nutrient agar, the smooth, creamy opalescent colonies were circular, 2 mm. diameter with a regular edge and a low convex elevation; they had a foul ammoniacal odour and a butyrous consistency. Aerobic growth in nutrient broth at 30° gave a dense uniform turbidity with a deposit and a slight ring pellicle; neither organism produced any pigment. Both organisms were able to grow at 12, 22, 30 and 37° but not at 45° with an optimum at 30°. Neither organism produced a pigment in King's A, but organism 22 produced a yellowish green, water-soluble pigment in King's B which was not pyocyanin. On Dorset egg medium, strain 22 gave orange yellow pigmented growth, and strain 14 gave light yellow growth. Both organisms hydrolysed urea and tributyrin, but not gelatin, gave negative indole, methyl-red and Voges-Proskauer tests, and strain 14 was able to reduce nitrate (no gas production). Neither was able to ferment maltose, inositol, sucrose, glycerol, mannitol, rhamnose, lactose, xylose, raffinose or trehalose. A small amount of acid was produced by both organisms from glucose after 3 days and by organism 14 from fructose and arabinose after 7 days.

In litmus milk, organism 22 produced an alkaline reaction after 3 days and weak reduction after 11 days. Organism 14 produced acid and clot formation with slight reduction at 7 days and partial liquefaction of the clot at 18 days.

Using *Bergey's Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957), Pseudomonas 22 was found to be similar to the *Pseudomonas putida, P. striata* and *P. ovalis* group; Pseudomonas 14 was similar to the *P. desmolytica, P. rattours* and *P. salopnia* group.

The cells of Mycobacterium B4 were irregularly Gram-positive, acid-fast, non-motile, non-capsulated, non-sporing rods ($0{\cdot}8 \times 2{\cdot}5$ μm.). Some cells were pleomorphic with bent axes. After aerobic growth on nutrient agar for 2 days, the colonies were circular, 1 to 3 mm. in diameter, with an irregular edge, flat, with a very wrinkled matt surface. The colonies had an orange-yellow pigment and were difficult to emulsify. No pigment was produced by growth on King A or King B medium, but yellow growth was produced on a potato slice after 2 days. The organism grew at 22, 30, 37 and 45° but not at 12 or 52°; 37° was the optimum temperature.

The organism hydrolysed urea and tributyrin but not gelatin. Cooked meat was not digested but a ring pellicle of growth did occur. The organism was indole-negative, methyl-red negative, Voges-Proskauer negative and nitrate negative, and produced weak acid from Hugh and Liefson medium aerobically after 14 days. Glycerol, inositol,

Table 1. *Growth of Torulopsis Y8, Pseudomonas 22, Pseudomonas 14 and Mycobacterium B4 on n-alkanes, mixed hydrocarbons, alcohols, monocarboxylic acids, dicarboxylic acids and acyl amides*

Substrate	Number of carbon atoms	Growth* after 6 days			
		Y8	22	14	B4
n-Alkanes					
Hexane	6	0	2+	0	0
Octane	8	0	2+	1+	0
Decane	10	0	2+	1+	1+
Dodecane	12	1+	2+	2+	1+
Tetradecane	14	2+	2+	2+	2+
Hexadecane	16	2+	2+	2+	2+
Octadecane	18	1+	1+	2+	1+
Eicosane	20	1+	1+	2+	1+
Monocarboxylic acids					
Formate	1	0	0	0	0
Acetate	2	2+	3+	3+	2+
Propionate	3	0	0	1+	0
Butyrate	4	0	2+	2+	0
Pentanoate	5	0	1+	1+	0
Hexanoate	6	0	1+	1+	0
Octanoate	8	0	2+	0	0
Decanoate	10	1+	3+	2+	1+
Dodecanoate	12	1+	3+	2+	2+
Tetradecanoate	14	2+	3+	2+	1+
Octadecanoate	18	2+	3+	2+	1+
Eicosanoate	20	2+	2+	2+	1+
n-Alcohols					
Methanol	1	0	0	0	0
Ethanol	2	0	0	0	0
Propanol	3	0	0	0	0
Butanol	4	0	0	0	0
Pentanol	5	0	0	0	0
Undecanol	11	1+	1+	1+	1+
Octadecanol	18	1+	1+	1+	1+
Dicarboxylic acids					
Ethanedioate	2	0	0	0	—
Propanedioate	3	0	2+	2+	—
Butanedioate	4	0	2+	2+	—
Hexanedioate	6	0	0	2+	—
Octanedioate	8	0	0	2+	—
Decanedioate	10	0	2+	2+	—
Acyl amides					
Acetamide	2	2+	2+	0	
Octanamide	8	0	0	0	
Dodecanamide	12	0	0	0	
Mixed hydrocarbons					
White lighting kerosene		1+	3+	3+	3+
Power kerosene		1+	3+	3+	3+
Industrial diesel oil		1+	3+	3+	3+
Mobiljet A kerosene		1+	3+	1+	3+
Mobiljet B kerosene		2+	2+	3+	2+
Thermally stable jet fuel		0	3+	3+	3+
Brake fluid		2+	1+	1+	1+
Automotive diesel oil		1+	3+	3+	3+

* 0 = No growth; 1+ = slight turbidity; 2+ = moderate turbidity; 3+ = dense turbidity; — = not tested.

lactose, maltose, mannitol, rhamnose, sucrose, xylose, arabinose, raffinose and trehalose were not fermented.

Using *Bergey's Manual of Determinative Bacteriology* (Breed, *et al.* 1957) Mycobacterium B4 is a new species similar to *M. smegmatis*.

Table 2. *Oxygen uptake (Q_{O_2}) from alkanes and fatty acids by resting cell suspensions of Torulopsis Y8 grown on different substrates*

Substrate	Number of carbon atoms	Substrate of growth Q_{O_2} (μl. O_2/mg. dry wt/hr)				
		Glucose	Acetate	ADO	Dodecane	Hexadecane
n-Alkanes						
Heptane	7	6	2	45	10	9
Octane	8	8	16	142	66	35
Nonane	9	27	30	120	60	55
Decane	10	45	38	170	95	35
Undecane	11	9	13	95	37	115
Dodecane	12	15	12	67	64	65
Tridecane	13	5	15	68	38	55
Tetradecane	14	7	—*	—	—	60
Pentadecane	15	7	15	60	26	79
Hexadecane	16	4	13	55	29	75
Heptadecane	17	22	29	60	32	68
Octadecane	18	0	13	40	26	39
Nonadecane	19	2	20	45	20	30
Eicosane	20	4	7	35	10	12
Docosane	22	8	12	15	10	15
Tetracosane	24	4	8	14	11	10
Octacosane	28	4	6	16	10	11
Dotriacontane	32	4	6	16	32	12
Monocarboxylic acids						
Formate	1	0	0	0	—	0
Acetate	2	30	43	27	—	50
Propionate	3	0	0	0	—	0
Butyrate	4	0	0	0	—	0
Hexanoate	6	0	0	0	—	75
Octanoate	8	0	0	22	—	95
Decanoate	10	0	0	0	—	165
Undecanoate	11	0	0	0	—	112
Dodecanoate	12	0	0	17	—	80
Tridecanoate	13	20	45	141	—	90
Tetradecanoate	14	120	115	156	—	190
Pentadecanoate	15	82	60	104	—	112
Hexadecanoate	16	29	50	37	—	135
Heptadecanoate	17	12	—	28	—	45
Octadecanoate	18	18	5	2	—	25
Eicosanoate	20	0	5	5	—	0
Docosanoate	22	0	0	0	—	0

* — = Not tested.

Substrates able to support growth. Table 1 shows the results of experiments in which either Torulopsis Y8, Pseudomonas 22, Pseudomonas 14, or Mycobacterium B4 was inoculated into media with different sources of carbon. It is clear that organisms isolated using kerosene are best able to grow on the *n*-alkanes containing 12 to 20 carbon atoms, although Pseudomonas 22 was able to grow on the alkanes from C_6 to C_{20}. All organisms were able to use a number of mixed hydrocarbons for growth; the growth obtained was much heavier than that obtained with single *n*-alkanes.

The ability of these organisms to grow on the C_1 to C_{20} monocarboxylic acids, the C_2 to C_{10} dicarboxylic acids, the C_2, C_8 and C_{12} acyl amides and several straight chain alcohols was also tested. The organisms were able to grow on a far greater range of monocarboxylic acids than *n*-alkanes and growth was much heavier with the monocarboxylic acids as substrates. The pseudomonads grew on most of the C_2 to C_{20} monocarboxylic acids tested, but Torulopsis Y8 and Mycobacterium B4 could not grow on the C_3 to C_8 monocarboxylic acids. Torulopsis Y8 was also unable to grow on the C_2 to C_{10} dicarboxylic acids. The C_8 and C_{12} acyl amides and the short chain alcohols did not support the growth of any of the organisms tested but both undecanol and octadecanol supported poor growth.

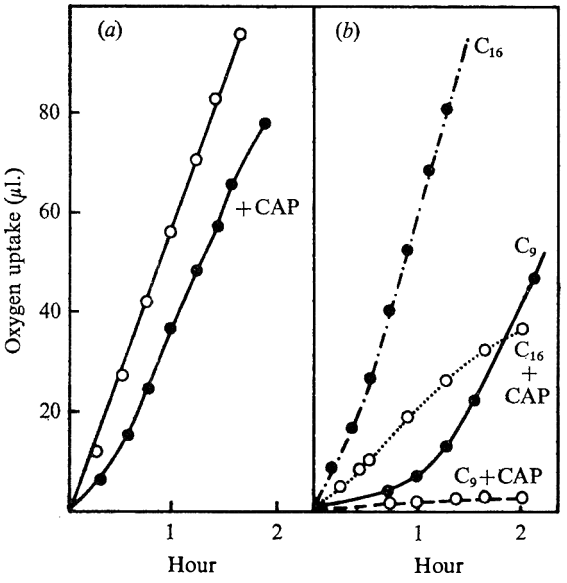

Fig. 1. Oxygen uptake (µl.) by cells of Mycobacterium B4 grown on glucose. (*a*) Cells exposed to glucose (○——○) and glucose+24 µg. chloramphenicol/ml. (●——●); (*b*) cells exposed to: hexadecane (●- - - -●); hexadecane+24 µg. chloramphenicol/ml. (○- - - -○); nonane (●——●) and nonane+24 µg. chloramphenicol/ml. (○- - - -○).

Substrates oxidized. The ability of washed, resting-cells of torula to take up oxygen on exposure to *n*-alkanes and to monocarboxylic acids was tested (Table 2). Torulopsis Y8 was able to oxidize the C_7 to C_{32} *n*-alkanes regardless of the substrate on which the cells had been grown. In glucose and acetate-grown cells, the ability to oxidize alkanes was present at a low basal level and oxygen uptake increased with time until a constant rate was achieved between 0·5 and 2·5 hr. Prior growth on Automotive Diesel Oil (ADO), dodecane or hexadecane and subsequent exposure of the cells to alkanes resulted in significantly higher Q_{O_2} values from zero time. Prior growth of the cells on hydrocarbons presumably induced enzyme systems in the cells.

The alkanes most readily oxidized were not analogous to the fatty acids most readily oxidized. Torulopsis Y8 oxidized C_8 to C_{13} alkanes fastest but fatty acids C_{13} to C_{16} were most readily oxidized. The C_{20} to C_{32} alkanes and acids were oxidized at a slow rate and then only after a long lag period. The C_1, C_3, C_4, C_6, C_8 and C_{10} fatty acids were often toxic to the cells. Oxygen uptake on exposure to these acids gradually

decreased with time and, in certain cases, even endogenous respiration was inhibited. The enzymes concerned in the oxidation of fatty acids by glucose-grown and acetate-grown cells of Torulopsis Y8 were presumably induced, since these cells initially oxidized the acids at only a low basal rate but this rate increased with time. Cells grown on ADO or hexadecane exhibited a higher rate of oxygen uptake initially and this did not increase with time.

Induction of n-alkane oxidation. To confirm that the oxidation of *n*-alkanes was inducible, the effect of chloramphenicol (CAP) (24 μg./ml.) on the oxidation of glucose, nonane and hexadecane by glucose-grown cells of Mycobacterium B4 was tested. The results are shown in Fig. 1. The lower rates of oxidation of alkanes observed in the presence of CAP suggests that the enzymes for alkane oxidation are inducible but that, in glucose-grown cells, these enzymes are present constitutively at a low concentration.

Induction of isocitric lyase by growth of organisms on hydrocarbons. Torulopsis Y8, Pseudomonas 22 and Mycobacterium B4 were grown on two non-hydrocarbon substrates (glucose and acetate), two mixed hydrocarbon substrates (Mobiljet A kerosene and Mobil ADO) and two pure *n*-alkanes (dodecane and hexadecane). Cell-free extracts were assayed for isocitric lyase and the results obtained are seen in Table 3. Growth on hydrocarbons induced isocitric lyase formation; however, the amount of induction varied both from organism to organism and from substrate to substrate.

Table 3. *Effect of the substrate of growth on the content of isocitric lyase in Torulopsis Y8, Pseudomonas 22 and Mycobacterium B4*

Growth substrate	Torulopsis Y8 S.A.*	Pseudomonas 22 S.A.	Mycobacterium B4 S.A.
Glucose	30	10	40
Acetate	550	60	600
Dodecane	120	70	130
Hexadecane	140	120	60
Mobiljet A	130	20	—
ADO	—†	40	—

* S.A. = Specific activity (units/mg. protein).
† — = Not tested.

DISCUSSION

The bacteria isolated for this study are representative of very different genera in the Schizomycetes but we are unable to assign them to species. Dr R. R. Colwell of Georgetown University examined Pseudomonas 22 and Dr R. E. Gordon of Rutgers University examined Mycobacterium B4 and could not give them a species name in their respective genera. Similarly, the torula appears to be different from presently described species but we are unable to do more than place strain Y8 within the genus Torulopsis. As might have been expected, all four isolates had a similar general pattern of growth on pure alkanes and fatty acids; the pseudomonads, however, could use a rather wider range of substrates for growth than the representatives of other genera. All organisms grew better on crude mixed hydrocarbons than on pure alkanes and this is probably related to the presence of more readily available impurities (especially fatty acids) in the crude hydrocarbons; this is significant for the storage life of fuels.

Rapid oxidation of a particular alkane was not necessarily correlated with rapid oxidation of the corresponding saturated fatty acid. The results indicate that cells selected for their ability to utilize alkanes as sole source of carbon do have a basal level of enzymes capable of oxidizing hydrocarbons and cells grown on hexadecane (and other alkanes) had the same general pattern of oxidation of both alkanes and fatty acids as cells grown on non-hydrocarbon substrates. Prior growth of the organisms on alkanes did, however, induce the cells (over a wide range of chain lengths) to oxidize alkanes at a faster rate and several alkanes which were not oxidized by glucose-grown cells were oxidized by alkane-grown cells. The monocarboxylic acids were oxidized faster than the *n*-alkanes and this might be expected since the initial oxidation of the alkyl group of the alkane is already accomplished when the acid is supplied. In addition, the carboxyl group increases the polarity and solubility of the acid, thus the acid presents a far greater surface to the organism than does the corresponding alkane. The poor availability of insoluble substrates probably accounts for the long lag periods and the slow rates of oxygen uptake shown with C_{20} to C_{32} alkanes and acids.

The lack of growth on and oxidation of the short chain fatty acids is possibly due to their toxicity. The short chain acids have a detergent action on membranes and denature proteins. These effects could account for the observation that, when the C_1, C_3, C_4, C_6, C_8 and C_{10} fatty acids were oxidized at all, oxygen uptake was often inhibited soon after exposure to these fatty acids. In addition to toxicity, failure to metabolize certain fatty acids may be associated with lack of enzymes vital for their metabolism.

While growth of the torula on alkanes did increase its ability to oxidize fatty acids, the effect was far less pronounced with the acids than it was with the alkanes. Further, the oxidation of fatty acids by torula grown on alkanes showed no evidence that growth on the alkane had induced any particular pattern of enhanced oxidation of the fatty acids of smaller chain length than the growth-alkane (as was reported by Azoulay & Senez, 1960). This result is not totally unexpected since during β-oxidation the monocarboxylic acid intermediates are in the CoA-ester form and, in this form, they may not act as inducers of the enzymes associated with the oxidaton of the free fatty acids. The inability to demonstrate sequential induction cannot be interpreted as meaning that β-oxidation is unimportant in the oxidation of alkanes and acids in these organisms. On the contrary, the finding that the enzyme isocitric lyase was induced by growth of the organisms on alkanes is consistent with β-oxidation being important in the metabolism of these substrates. The fact that this induction was found in widely different organisms grown on very different hydrocarbon substrates indicates that the glyoxylate cycle has an important role in the metabolism of hydrocarbons by micro-organisms.

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Inhibition by D-Glutamate of Growth and Glutamate Dehydrogenase Activity of *Neurospora crassa*

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SUMMARY

D-Glutamic acid (D-glu) inhibited strongly the growth of two strains of *Neurospora crassa* in a minimal medium. The inhibition was completely annulled by equivalent concentrations of L-glutamic acid (L-glu) or L-glutamine. D-Glu also inhibited glutamate dehydrogenase (GDH) and was only antagonized completely by 10 equivalents of L-glu. D-Glu in media containing L-glu increased GDH-activity, presumably by de-repression. D-Glu showed no effect on glutamine synthetase and γ-glutamyl transferase activities. Inhibition of growth of *N. crassa* by D-glu thus seems due to the interference with glutamate synthesis by inhibition of GDH-activity.

INTRODUCTION

Neurospora crassa is a prototrophic fungus which grows well in a minimal medium consisting of ammonia, glucose and various inorganic salts (Beadle & Tatum, 1941). The α-ketoglutarate (α-kg) formed from the sugar in the tricarboxylic acid cycle (Strauss, 1955; Krebs & Lowenstein, 1960) is converted to glutamate in the presence of ammonia and the glutamate dehydrogenase enzyme (GDH). The glutamate then participates both directly in protein synthesis and as an intermediate in the production of many other amino acids by transamination and other reactions (Fincham, 1951*a*, *b*). The GDH enzyme is therefore of pivotal importance to this organism.

Certain D-amino acids inhibit growth of certain bacteria in the absence of the L-isomers (Fox, Fling & Bollenback, 1944; Prescott, Schweigert, Lyman & Kuiken, 1949). D-Amino acids also inhibit the enzymic activity of the corresponding natural amino acid. Thus, for example, D-asparagine inhibits L-asparaginase in extracts of *Mycobacterium phlei* (Grossowicz & Halpern, 1956; Grossowicz & Halpern, 1957), and D-glutamic acid causes partial inhibition of the L-glutamic decarboxylase of *Escherichia coli* (Roberts, 1953). Olson & Anfinsen (1953) reported that D-glutamic acid inhibits the activity of crystalline GDH from beef liver. The present paper deals with the inhibition of growth and of GDH-activity of *Neurospora crassa* by D-glutamic acid (D-glu) and with the mechanism of these inhibitions.

METHODS

Media. A minimal medium prepared according to Horowitz & Beadle (1943) at a 5:4 concentration was distributed in 4 ml. amounts into test-tubes (18 × 150 mm.). Water or substances to be tested were added to the medium and its final volume

brought to 5 ml. L-Glutamic acid (L-glu) and/or D-glu were added in suitable amounts to the medium before sterilization; sucrose and L-glutamine were sterilized separately (the last by filtration) and added aseptically to the sterile medium.

Strains. Most of the work was done with strain no. 1 from the collection of the Bacteriology Department which was sensitive to D-glu. Some experiments were done with the wild-type strain no. 262 (STA-4) obtained from Dr B. D. Sanwal (Department of Microbiology, The University of Manitoba, Winnipeg, Canada). Another strain sensitive to inhibition by D-glu was IMI-31288, a mutant which requires *p*-amino-benzoic acid for growth. Two other strains (LBS304.59 and a local isolate) proved much more resistant to D-glu.

Estimation of growth. Each tube was inoculated with 0·2 ml. of a barely turbid spore suspension of the no. 1 strain of *Neurospora crassa* prepared from a 48 hr culture grown on Bacto-Neurospora Culture Agar (Difco); the tubes were incubated at 30° for 2 to 3 days in a sloped position of 15° above horizontal to ensure adequate aeration.

After incubation, the mycelium was separated from the medium by filtration through filter paper on a small Büchner funnel. The mycelium was thoroughly washed with distilled water, air dried and then dried in an oven at 80° for 2 to 3 hr. The different mycelial mats were weighed separately on a Sauter spring balance of 0·2 mg. sensitivity. With the Horowitz-Beadle medium, without any additions, the dry weights of the mycelia were between 18 and 25 mg./tube.

Growth of Neurospora for enzyme preparations. To obtain enough enzyme, the fungus was grown in 2 l. Erlenmeyer flasks containing 500 ml. medium and shaken at 30° for 2 days.

Acetone dry powder preparation. After incubation, the mycelium was collected by filtration; 20 vol. cooled acetone/g. mycelium were added and homogenized for 2 to 3 min. at maximum speed in an M.S.E. Atomix-homogenizer. The acetone was removed by decantation and filtration through a Büchner funnel and the powdered mycelium was dried rapidly on filter sheets, yielding a fine flaky substance; 1 g. of the acetone dry powder was extracted with 20 ml. 0·05 M-K-potassium phosphate (pH 8·0) for 1 hr in the cold, centrifuged at 10,000 rev./min. for 15 min. The supernatant fluid was dialysed in the cold against a large volume of the same buffer and served as the GDH enzyme preparation.

Protein was measured by the biuret method modified according to Gornall, Bardawill & David (1949).

Assay of GDH-activity. The enzymic activity was assayed at 35° by measuring the changes in optical density (O.D.) at 340 mμ in a S.P. Unicam Spectrophotometer due to oxidation of the reduced nicotinamide dinucleotide phosphate (NADPH) or to the reduction of NADP. The reaction mixture for glutamate synthesis (forward reaction) contained the following components (unless otherwise stated) in a final volume of 3 ml.: α-ketoglutarate, 20 μmole; ammonium sulphate, 10 μmole; phosphate buffer (pH 7·5), 0·1 M; NADPH, 300 μg.; enzyme protein, 250 μg. For the reverse reaction the mixture was composed of L-glu, 80 μmole, phosphate buffer (pH 8·3), 0·1 M; NADP, 300 μg.; enzyme protein, 750 μg. The reaction was started by adding enzyme. The differences in extinction were examined every 15 to 30 sec. for 3 min.

RESULTS

Effect of D-glutamate on growth of Neurospora crassa. D-Glu in the minimal medium inhibited growth; at 0·5 to 1·0 μmole/ml. D-glu caused 50 % inhibition over 2 days but less inhibition after 3 days (Table 1). The strain no. 262 was slightly less sensitive than the departmental culture (strain no. 1).

Effect of L-glu and L-glutamine on growth inhibition caused by D-glu. As Table 2 shows, the growth inhibition by D-glu was overcome by either L-glu or glutamine at 2 to 3 times lower molar concentrations.

Table 1. *Effect of D-glutamate (D-glu) on growth of Neurospora crassa*

Tubes containing 5 ml. of minimal medium with increasing concentrations of D-glu were inoculated with 0·2 ml. of barely turbid spore suspensions of the respective *Neurospora crassa* strains. After 3 days at 30° the mycelia were removed by filtration through small Büchner funnels, washed with distilled water, dried to constant weight for 2 hr at 80°, and weighed.

	Strain no. 1		Strain no. 262	
D-glu added (μmole/ml.)	Growth (mg. dry wt)	Inhibition (%)	Growth (mg. dry wt)	Inhibition (%)
0	21	—	25	—
0·2	16	24	22	12
0·4	13	38	17	32
0·6	12	43	15	40
1·0	10	52	13	44
2·0	5	76	10	60
3·0	2·5	88	8	68
4·0	1·5	93	5	80
5·0	0·5	98	1	96

Table 2. *Effect of L-glu and glutamine on the growth inhibition caused by D-glu*

Strain no. 1. Growth in mg. dry wt after 3 days at 30°.

D-glu added		L-glu (μmole/ml.)				Glutamine (μmole/ml.)			
(μmole/ml.)	0·0	0·2	0·3	0·6	1·0	0·2	0·3	0·6	1·0
0	18	20	20	20	20	18	18·5	18·5	18
0·3	10·5	18	19	20	20	17	17·5	18	18
0·6	9	16	18	19	20	17	17·5	19	19
1·0	7	15	17·5	18	19	14	18	18	19

Effect of D-glu on enzymes involved in the metabolism of glutamic acid and glutamine. D-Glu had no effect on glutamine synthetase and γ-glutamyl transferase but glutamate dehydrogenase (GDH) activity, as preliminary experiments indicated, was inhibited.

Inhibition of the forward reaction: synthesis of glutamate. Enzymic activity decreased with the increase of the D-glu concentration; 50 % inhibition was caused at a molar D-glu: α-kg ratio of 1:1 (Fig. 1). Similar results were obtained with the strain no. 262.

Inhibition of the backward reaction: oxidative deamination of glutamate. The reversal of glutamate formation was inhibited by D-glu in a similar manner as the forward reaction (Fig. 2); 50 % inhibition was obtained at a ratio inhibitor:substrate of 1:2 to 1:4.

Effect of α-ketoglutarate (α-kg). In the absence of D-glu, 20 μmole α-kg saturated 0·25 mg. enzyme but with 20 μmole D-glu, 80 μmole α-kg were required to overcome the inhibition completely. When the results were plotted according to Lineweaver & Burk (1934), the two lines intersected on the ordinate.

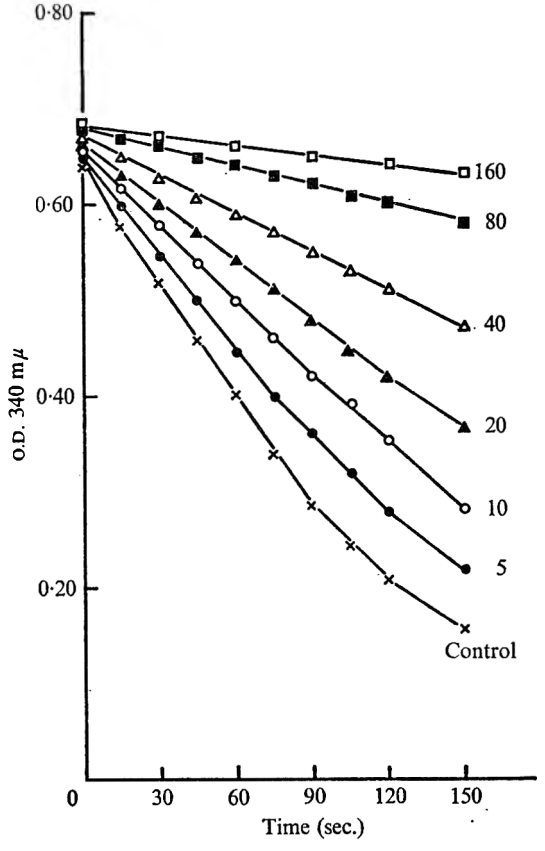

Fig. 1

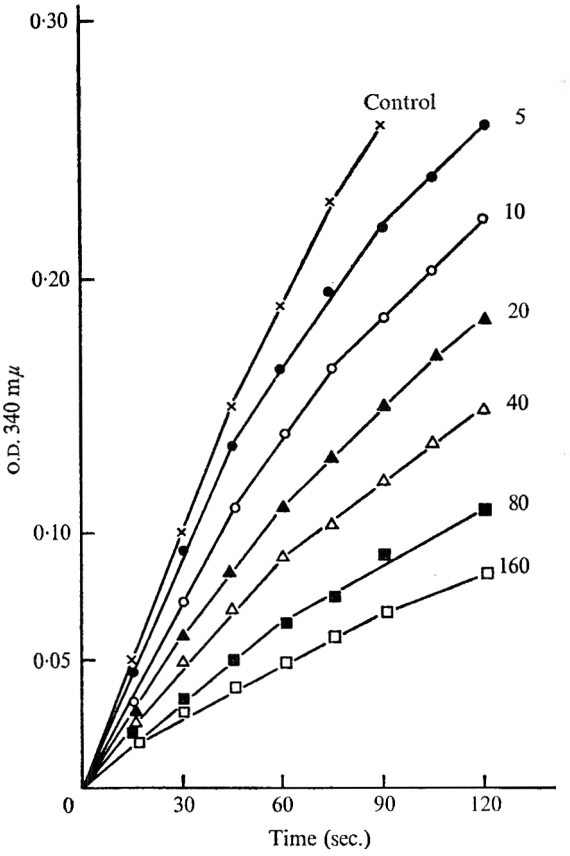

Fig. 2

Fig. 1. Glutamic dehydrogenase activity (forward reaction) as a function of the D-glu concentration. The reaction mixture contained: α-kg, 20 μmole; ammonium sulphate, 10 μmole; NADPH, 300 μg.; enzyme protein, 0·25 mg.; K-phosphate buffer (pH 7·5), 0·1 M; total volume, 3 ml. D-Glu μmole as indicated in the figure.

Fig. 2. Inhibition of the oxidative deamination of glutamate (backward reaction) by D-glu. The reaction mixture contained: L-glu, 80 μmole; NADP, 300 μg.; enzyme protein, 0·75 mg.; phosphate buffer (pH 8·3), 0·1 M; total vol., 3 ml. D-Glu μmole as indicated in figure.

Effect of ammonia. Increasing the concentration of ammonia hardly affected the inhibition of glutamate synthesis by D-glu: a fivefold increase in the ammonia concentration decreased the inhibition by 10 % only.

Effect of L-glu. In the absence of D-glu, 80 μmole L-glu were almost enough to saturate the enzyme (750 μg. protein): considerably higher concentrations of L-glu caused only a slight increase in activity. Addition of 20 μmole D-glu caused 50 % inhibition, but inhibition was not completely annulled until the L-glu concentration was increased sixfold, to a molar ratio of 24:1 of L-glu:D-glu (Table 3).

Effect of D-*glu and* L-*glu on GDH-formation.* The formation of many enzymes is repressed by the presence, during growth, of the product of the enzymic reaction. The NADPH-dependent GDH-activity of *Neurospora crassa* is repressed by L-glutamate and related amino acids (Sanwal & Lata, 1962*a*,*b*). To find out about the regulatory control of this biosynthetic enzyme the effect of D-glu on GDH-formation was investigated.

Table 3. *Rate of oxidative deamination of* L-*glutamic acid in the presence and absence of* D-*glu*

L-glu (μmole)	O.D. at 340 mμ after 60 sec. Without D-glu	D-glu added (20 μmole)
10	0·055	—
20	0·095	0·040
40	0·175	0·065
80	0·195	0·105
120	0·20	0·12
160	0·22	—
320	0·225	0·16
480	—	0·22

Table 4. *Glutamate dehydrogenase formation by Neurospora crassa grown in different media with or without* D-*glutamic acid*

Neurospora grown in	D-glu added (μmole/ml.)	GDH formed specific activity (mμmole/min./mg. protein)
Expt. 1		
Minimal medium	0	250
Minimal medium + L-glu, 0·5 M	0	50
Rich medium	0	40
Expt. 2		
Minimal medium	0	320
Minimal medium	0·5	600
Expt. 3		
Minimal medium	0	280
Minimal medium	0·7	360
Minimal medium + L-glu, 0·1 M	0	60
Minimal medium + L-glu, 0·1 M	50·0	80
Minimal medium + L-glu, 0·1 M	100·0	160
Expt. 4		
Rich medium	0	40
Rich medium	75·0	100
Rich medium	100·0	170

Neurospora crassa no. 1 was grown in the minimal medium in Erlenmeyer flasks with and without 0·5 mM-D-glu, which caused a 50 % inhibition of growth. After 2 days at 30° the mycelial mats were harvested and extracts prepared as described under Methods. The enzyme preparation from the D-glu-grown culture was almost twice as active as that obtained from the control culture (see below).

To test the effect of L-glutamate and metabolically related substances on GDH-formation, *Neurospora crassa* was grown for 2 days in various media without and with D-glu: (*a*) minimal medium; (*b*) minimal medium + 0·05 M-L-glu; (*c*) yeast-peptone

medium (rich medium). Preparations were obtained from the cultures and tested for GDH-activity. As Table 4 shows, addition of L-glu to the growth medium yielded extracts with considerably lower GDH-activity (25 %) than the controls. The extracts from cultures grown in the rich medium were even less active (15 %) than that of the L-glu-grown culture. On the other hand, extracts from cultures grown on the same medium supplemented with D-glu possessed considerable GDH-activity (Table 4, Expt. 4).

DISCUSSION

D-Glutamic acid (D-glu), the unnatural stereoisomer of L-glutamic acid (L-glu), was a potent growth inhibitor for the two *Neurospora crassa* strains studied, and its inhibition was easily annulled by L-glu or glutamine at 2·5 times smaller molar concentrations. Although cell-free preparations showed no glutaminase activity, it is not certain that the glutamine itself, and not some enzymic degradation product, was responsible for the annullment of the growth inhibition since glutamine did not annul inhibition of GDH-activity (see below).

Much less D-glu was required to inhibit growth than GDH-activity; growth inhibition was annulled by less than the equimolar L-glu. In contrast, inhibition of the GDH-activity was overcome only by 10-fold or higher molar concentrations of the natural amino acid. The inhibition of growth seems therefore to be non-competitive, due to the production of limiting concentrations of L-glu by the inhibited enzyme; indeed, addition of L-glu, the product of the enzymic reaction, restored growth to its full extent.

In accordance with the findings of Sanwal & Lata (1962*a*, *b*), formation of NADPH-dependent GDH enzyme was repressed by L-glu. Of interest is the finding that D-glu increased GDH-formation. L-Glu, the product of GDH-catalysed reaction, serves as a substrate in the biosynthesis of various amino acids and therefore should be used up rapidly, rather than accumulate in the cell. Thus, repression of GDH-activity can be expected to be pronounced only in a glutamate-containing medium, as was shown in our experiments (Table 4). D-Glu lowers the availability of L-glu to the fungus and so de-represses the formation of enzyme. D-Glu stimulation of enzyme formation resembles anthranilic acid and 3-methyl anthranilic acid induction of tryptophan synthetase formation (Lester & Yanofsky, 1961).

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The Occurrence and Genetics of Some CO_2 Mutants in *Streptomyces coelicolor*

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SUMMARY

We have isolated auxotrophic mutants of *Streptomyces coelicolor* which can grow on a minimal medium without growth factors when the gas phase is supplemented with CO_2. Usually they have an alternative requirement for a specific growth factor such as arginine, purines or vitamins. Some of the CO_2 mutants resemble those already known in *Neurospora crassa* and *Escherichia coli* but others present novel phenotypes.

INTRODUCTION

Carbon dioxide is present in ordinary air at a concentration of at least 0·03 % (v/v). It is essential for the growth, or initiation of growth, of bacteria and other micro-organisms (Valley & Rettger, 1927; Rockwell & Highberger, 1927). Recently it was shown that high concentrations of CO_2 in air stimulated the growth of some auxotrophic mutants of the fungus *Neurospora crassa* (Charles, 1962; Reissig & Nazario, 1962). Mutants stimulated by CO_2 were called CO_2 mutants (Charles & Broadbent, 1964). The purpose of the present study, and a related investigation on *Escherichia coli* (Charles & Roberts, 1968), was to see whether CO_2 mutants occurred in protokaryotic organisms. *Streptomyces coelicolor* was chosen because refined genetic analysis is possible (Hopwood, 1967). CO_2 mutants were obtained in this investigation; these are discussed in relation to mutants obtained in other micro-organisms.

METHODS

Media. Media used were the minimal (MM) and complete (CM) media of Hopwood (1967). Glucose was autoclaved separately as a 50 % (w/v) solution and added to each medium just before pouring into Petri dishes.

Culture methods. Incubation was at 30°, unless stated otherwise. When it was necessary to incubate the cultures in a gas phase other than air, 5 l. vacuum desiccators were used according to the procedure described by Broadbent & Charles (1965).

Strains. The strain of *Streptomyces coelicolor* in which mutants were induced and the multiply-marked mutant stocks used in linkage analysis were generously provided by Professor D. A. Hopwood. The terminology and mutant designations, and the procedures for making and analysing crosses, are those of Hopwood (1967). Mutants isolated during this study are identified by a *v* as a prefix to the isolation number.

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Isolation of CO_2 mutants. Mutants were induced by ultraviolet (u.v.) irradiation or by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Spore suspensions were made by suspending spores, from the surface of 5 day slope cultures on CM, in liquid MM and filtering through non-absorbent cotton wool to remove large mycelial fragments. For u.v. mutagenesis the spore suspension was irradiated with a Camag universal u.v. lamp, to a survival of about 0·5 %. For treatment with NTG the spore suspension was centrifuged and the spores suspended in 5 ml. liquid MM enriched with 2·5 % (v/v) liquid CM; NTG was added (4 mg./ml.) and the suspension incubated at 37° for 45 min. Liquid CM (5 ml.) was then added and incubation continued for 90 min.; this treatment killed about 99 % of the spores. At the time of this investigation no enrichment procedure for the isolation of auxotrophic mutants of Streptomyces was known, nor were the recently discovered optimal conditions for mutagenesis by NTG (Delić, Hopwood & Friend, 1970).

Before CO_2 mutants could be sought the CO_2 requirement of the wild-type strain, A 3(2), had to be determined. Growth was slightly inhibited in desiccators containing potassium hydroxide to remove CO_2 from the gas phase (Charles, 1964); for this reason CO_2 mutants were isolated on the basis of growth stimulation by CO_2 at concentrations greater than that in ordinary air.

Following mutagenic treatment, suitable dilutions of the spore suspensions were plated on either CM and incubated in air, or MM and incubated in air enriched with 10 % (v/v) CO_2. After 5 days the colonies from each Petri dish were replicated to MM with velvet pads and the replicas incubated in air. Those colonies that failed to grow on the replicas were picked from the original plates and purified by streaking on CM. They were then each replicated to two plates of MM, one of which was incubated in air and the other in air + 10 % (v/v) CO_2. In this way CO_2-requiring mutants were distinguished from other auxotrophs. Cultures which grew better in the presence of CO_2 were isolated for further study. Many of the colonies that initially appeared to respond to CO_2 did not respond when tested again.

Table 1. *CO_2 mutants of Streptomyces coelicolor*

Mutation	Locus	Characteristics
ade-v9	—	Requirement for purines or CO_2
arg-v7	—	Requirement for arginine, partially satisfied by CO_2
cdx-v1	*cdxA*	Requirement for CO_2
nic-v1	*nicB*	Requirement for nicotinamide partially satisfied by CO_2
pdx-v1	*pdxA*	Requirement for pyridoxine, partially satisfied by CO_2

RESULTS

Classes of CO_2 mutants. Four CO_2 mutants came from platings on MM in air + 10 % (v/v) CO_2 and one (*ade-v9*) from CM in air (Table 1). Three of these mutants defined new loci (*cdx*A, *nicB*, *pdxA*); their positions and those of mutants that have not yet been assigned to a locus are shown in Fig. 1.

It was found that a gas phase of air + 10 % (v/v) CO_2 stimulated the best growth of the CO_2 mutants. All the mutants responded moderately to CO_2; none grew as quickly

with CO_2 as did the parental strain without it. Mutant *cdxAv1* showed the strongest stimulation by CO_2 and was of special interest because no other substance has so far been found to replace CO_2 as a growth factor for it. The remaining four mutants grew well without supplementary CO_2 when supplied with other growth factors; growth stimulations by these other substances were generally better than by CO_2.

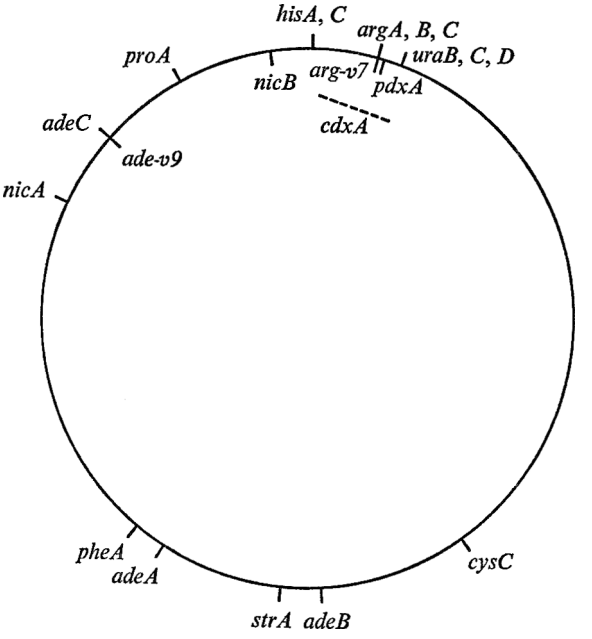

Fig. 1. *Streptomyces coelicolor.* Linkage map showing on the outside of the circle the location of previously known markers mentioned in text, and on the inside of the circle, the location of the CO_2 mutants. The marker designations are given by Hopwood (1967) with the exception of *adeC* which has a requirement for purines.

Adenine/CO_2 mutant ade-v9. This mutant grew well when supplied with adenine or supplementary CO_2, and to a lesser extent with guanine, hypoxanthine or xanthine. The mutation was mapped by the method of haploid recombinant selection (Hopwood, 1967) as follows. The mutant, *ade-v9*, was crossed with strain 876 carrying a series of well-separated markers, and spores from the mixed culture were plated on a medium selecting distant markers, *hisC9*$^+$ and *strA1*. Fig. 2 shows the marker arrangements in the parental strains and gives the results. The allele ratios for non-selected markers amongst the 142 selected recombinants indicated a position for *ade-v9* either between *proA* and *pheA* (as shown) or between *argA* and *cysC*. The former location was indicated by a consideration of the frequencies of individual segregant classes: with this location only one recombinant (*ade*, *pro*), required more than the minimum number of one crossover in each arc between the selected markers, whereas with the alternative location 16 recombinants (the *pro phe* and *phe* classes) required complex crossover patterns.

A sample of 27 adenine segregants all responded well to air + 10 % (v/v) CO_2 in the absence of adenine, suggesting that the adenine/CO_2 phenotype resulted from a single mutation.

An adenine locus had not been defined in the region of *ade-v9*, but another adenine mutant isolated during this study, *ade-v1*, not a CO_2 mutant, also mapped in this region. Recently a new locus, *adeC*, has been defined between *cysA* and *proA* (E. J. Friend, personal communication) and it seems probable that *ade-v9* is either at this locus or near it.

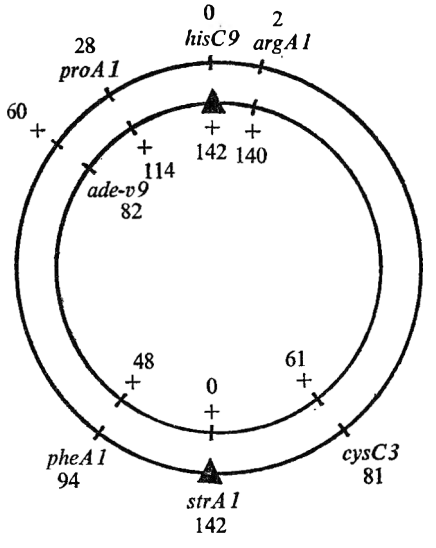

Fig. 2

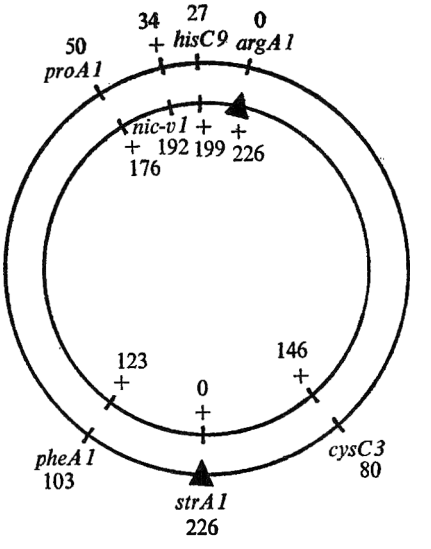

Fig. 3

Fig. 2. Location of *S. coelicolor* mutant *ade-v9*. Results of a cross between CO_2 mutant *ade-v9* (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele frequencies in a sample of recombinants containing the selected markers indicated by triangles.

Segregant phenotype	Number	Segregant phenotype	Number
arg, pro, phe, cys	2	*ade, phe, cys*	20
pro, phe, cys	19	*ade, phe*	14
pro, phe	6	*ade, cys*	17
phe, cys	23	*ade*	30
phe	10	*ade, pro*	1
		Total	142

Fig. 3. Location of *S. coelicolor* mutant *nic-v1*. Results of a cross between CO_2 mutant *nic-v1* (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele frequencies in a sample of recombinants containing the selected markers indicated by triangles.

Segregant phenotype	Number	Segregant phenotype	Number
nic	97	*nic, pro, phe, cys*	10
nic, cys	26	*pro, phe*	7
nic, phe, cys	31	*pro, phe, his*	14
nic, phe	22	*pro,phe, his, cys*	13
nic, pro, phe	6	Total	226

Arginine/CO_2 mutant arg-v7. This mutant was slightly leaky, in that some growth occurred on MM without supplementary CO_2 or arginine. Arginine allowed good growth and CO_2 gave a weaker stimulation. Ornithine and citrulline were not stimulatory. Arginine/CO_2 mutants are known in *Neurospora crassa* (Charles, 1962) and

Escherichia coli (Charles & Roberts, 1968). The *Streptomyces coelicolor* mutant differed from the mutants in *N. crassa* and *E. coli* in not responding to citrulline, not having its response to CO_2 inhibited by pyrimidines, and not having its response to arginine further enhanced by pyrimidines. In *N. crassa* and *E. coli* the CO_2 stimulation is explained in terms of a defect in carbamyl phosphate (CAP) synthesis which is overcome, directly or indirectly, by CO_2. This explanation requires that the mutants should respond to citrulline. It is probable that the *S. coelicolor* mutant *arg-v7* has the same defect: failure to respond to citrulline is expected because arginine mutants of *S. coelicolor* that grow on ornithine respond very poorly to citrulline (Hopwood, 1967), suggesting a possible impermeability to citrulline. CAP is also required for pyrimidine synthesis, and the failure of mutant *arg-v7* to be inhibited or stimulated by pyrimidines suggests differences in the regulatory effect of pyrimidines on CAP utilization in *S. coelicolor*.

A preliminary cross indicated a location for *arg-v7* rather close to *hisA1*, either clockwise or anticlockwise. Three previously recorded loci, *argA*, *B*, *C* (Hopwood, 1967), lie clockwise of *hisA*, and an attempt was made to see if *arg-v7* was closely linked with this cluster of *arg* loci. A strain carrying the *arg-v7* mutation was crossed with multiply-marked strains carrying either *argB2* or *argC4*, and spores were plated in parallel on media selecting for recombination between *arg-v7*$^+$ and *argB2*$^+$ or *argC4*$^+$ on the one hand, and between the distant markers *hisA1*$^+$ and *pheA*$^+$ on the other. Colony counts for the first selection were some 7 to 40 times lower than for the second, indicating close linkage between *arg-v7* and the *argB, C* cluster. In both crosses *arg-v7* was quite leaky in air, so that background growth on the Petri dish made it impossible to decide whether heteroclones (Sermonti, Mancinelli & Spada-Sermonti, 1960), which would have indicated complementation between the *arg* mutations (Hopwood, 1967), were present on the medium selecting for recombination between pairs of *arg* loci. Consequently it was not possible to decide whether *arg-v7* was an allele of either *argB* or *argC*. Mutant *arg-v7* resembles mutants at *argB* in responding to arginine but not to ornithine or citrulline; mutants at *argB* have not been tested for a CO_2 response.

Nicotinamide/CO_2 mutant nic-v1. This mutant responded moderately to CO_2 on MM, and nicotinamide caused growth stimulation which was not further increased by CO_2. Some other *nic* mutants (*nicA1*, *nicA3*, *nic-v2*, *nic-v3*, *nic-v4*) were tested for stimulation by CO_2 on MM but did not show it. The *nic-v1* mutant was crossed with strain 876 and *argA1*$^+$, *strA1* recombinants were selected (Fig. 3). The results indicated a location for the *nic-v1* mutation between *proA* and *hisC*, that is in a clearly different region from the previously known *nicA* locus; *nic-v1* therefore defined a new locus, *nicB*.

Pyridoxine/CO_2 mutant pdx-v1. This mutant showed a weak to moderate response to supplementary CO_2; pyridoxal, pyridoxine or pyridoxamine caused better growth stimulations. This was the first pyridoxine mutant of *Streptomyces coelicolor* to be studied. The results of a cross between strain 876 and *pdx-v1* (Fig. 4) indicated a location for the *pdx-v1* mutation clockwise of *argA* at a locus designated *pdxA*.

CO_2 mutant cdx-v1. This mutant did not grow on MM except when the air was supplemented with 10% (v/v) CO_2. Slow growth occurred on CM without supplementary CO_2, and yeast extract was implicated as a source of an alternative growth factor; auxanographic experiments did not reveal its identity. Yeast extract was a

less effective growth factor than CO_2 in two ways: it caused only a small proportion (about 10 %) of the plating units in a suspension to give rise to colonies, as compared with CO_2, and many of the resulting colonies were very small. These defects of growth on MM + yeast extract, as compared with CO_2, may perhaps be attributed to delayed germination.

Because mutant *cdx-v1* required CO_2 for uniform colony initiation and growth it was necessary, in mapping experiments, to incubate the cultures in a gas-phase containing 10 % (v/v) CO_2; so far as could be determined this did not adversely affect the growth of any of the segregants. A cross (Fig. 5) located *cdx-v1* close to *argA*, and

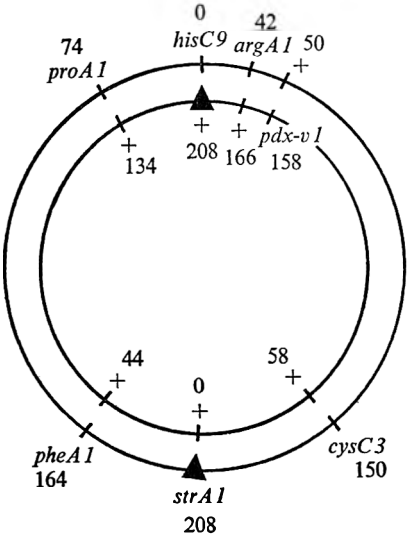

Fig. 4

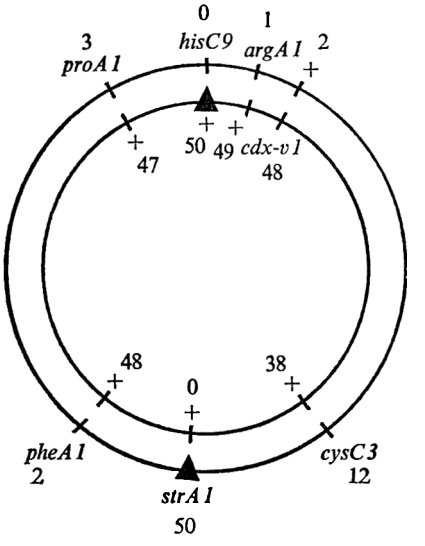

Fig. 5

Fig. 4. Location of *S. coelicolor* mutant *pdx-v1*. Results of a cross between CO_2 mutant *pdx-v1* (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele frequencies in a sample of recombinants containing the selected markers indicated by triangles.

Segregant phenotype	Number	Segregant phenotype	Number
pdx, phe, pro, cys	40	*pdx, pro, phe*	10
pdx, phe, cys	49	*arg, pro, cys*	2
pdx, phe	23	*arg, cys*	5
pdx, cys	13	*phe*	2
pdx	22	+	1
arg, phe, pro, cys	21	*arg, pdx, pro, phe, cys*	1
arg, phe, cys	13	*cys*	1
phe, cys	5	Total	208

Fig. 5. Location of *S. coelicolor* mutant *cdx-v1*. Results of a cross between CO_2 mutant *cdx-v1* (inner circle) and multiple-marked strain 876 (outer circle). Numbers are the allele frequencies in a sample of recombinants containing the selected markers indicated by triangles.

Segregant phenotype	Number	Segregant phenotype	Number
cys, cdx	10	*pro, phe, cys, cdx*	1
cdx	34	*arg*	1
pro, cdx	2	*cys*	1
phe, cdx	1	Total	50

further analysis suggested a location clockwise of *argA*, that is in the region of *uraB,C,D*. The mutant is provisionally accepted as representative of a new locus, designated *cdxA*. Mutations at *uraD* result in a requirement for arginine+uracil (Hopwood, 1967) probably due to defective CAP synthesis. In *Escherichia coli* some mutations at the equivalent locus cause a CO_2 requirement (Charles & Roberts, 1968; M. Mergeay, personal communication) and this should also be true of *uraD* in *Streptomyces coelicolor*, in which case there may be a functional relationship between *cdxA* and *uraD*. A possible alternative explanation of the CO_2 response in *cdx-v1* is considered in the Discussion.

DISCUSSION

CO_2 mutants have previously been investigated in the mould *Neurospora crassa* (Broadbent & Charles, 1965; Broadbent, 1965; de Serres, 1966), and in the eubacterium *Escherichia coli* (Charles & Roberts, 1968). The present study has shown that they can also be isolated in *Streptomyces coelicolor*, a protokaryote not closely related to *E. coli*, suggesting that CO_2 mutants are probably of general occurrence in micro-organisms.

So far the CO_2 mutants that are most easily understood are those defective in the incorporation of CO_2 into a biosynthetic sequence, for example that of adenine (Broadbent, 1965; de Serres, 1966) or arginine (Charles, 1962, 1964; Charles & Broadbent, 1964; Charles & Roberts, 1968). The adenine/CO_2 mutant of *Streptomyces coelicolor* may be of this type. Many other CO_2 mutants (Broadbent, 1965; Roberts, 1968; Vivian, 1968; H. P. Charles, unpublished results) offer no obvious explanations for their response to CO_2. The vitamin/CO_2 mutants of *S. coelicolor* are examples: no CO_2 incorporation reaction has been shown to be involved in the biosynthesis of either nicotinamide or pyridoxine. Possibly CO_2 causes growth without being incorporated into the final product of the defective biosynthetic sequence, for example by a regulatory function. CO_2 has recently been shown to have a regulatory function in an inducible anaerobic enzyme system in *Escherichia coli* by Swanson & Ogg (1969), who concluded that, under appropriate conditions, CO_2 could function either as a metabolic stimulator or as an inhibitor. Alternatively, growth might be possible because of a partial restoration of activity to a mutant enzyme by an effect of CO_2.

Some answers to the more fundamental aspects of CO_2 metabolism, particularly the dependence of heterotrophic organisms upon CO_2 for growth (Valley & Rettger, 1927; Rockwell & Highberger, 1927) might come from a study of some of the CO_2 mutants poorly understood at present. The work of Lwoff & Monod (1947) and Ajl & Werkman (1949) indicated that the CO_2 requirement of heterotrophs was partly due to the incorporation of CO_2 in biosynthetic reactions, although CO_2 could not be entirely replaced as a growth factor under all conditions of inoculum size. If wild-type *Streptomyces coelicolor* requires small quantities of CO_2 in essential reactions for which no other growth factor will substitute, the CO_2 requirement of *cdx-v1* might result from an enzyme involved in the utilization of CO_2 having a decreased affinity for CO_2, or because some defect of permeability hinders the uptake of CO_2. This is an interesting possibility because CO_2, usually in low concentration, is required for uniform germination of many micro-organisms, and this is probably true of *S. coelicolor* spores. The mutant *cdx-v1* may have a defect in the germination process causing an increased requirement for CO_2. The problem of germination of spores of *S. coelicolor*

is complicated because a spore suspension contains not only separate spores, but also clumps of spores and mycelial fragments. Bacteria in clumps require less exogenously supplied CO_2 for initiation of growth (Gladstone, Fildes & Richardson, 1935) presumably because the CO_2 they themselves generate may reach a higher concentration within a clump. The colonies which arise on yeast-extract medium may arise from clumps which may generate a sufficiently high concentration of CO_2 from the yeast extract to initiate growth.

CO_2 has been found to have some effect on morphogenesis in various organisms, from moulds (Bartnicki-Garcia & Nickerson, 1962) to coelenterates (Loomis, 1961). Hopwood (1967) has pointed out the suitability of *Streptomyces coelicolor* for the study of morphogenesis in protokaryote systems. It could be worth future investigation to see whether CO_2 does have any significance as a morphogenetic agent in *S. coelicolor*. A start has been made towards unravelling the development of the aerial stages of growth in *S. coelicolor* (Wildermuth, 1970; Wildermuth & Hopwood, 1970) by the study of mutants defective in sporulation (Hopwood, Wildermuth & Palmer, 1969); it would be interesting to see if the defects in some of these mutants could be overcome by supplementary CO_2.

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Analysis of an R$^+$ Strain Carrying Two *fi*$^-$ Sex Factors

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SUMMARY

An *Escherichia coli* K12 strain, J5-3 (R313), was presumed to carry an *fi*$^-$ R factor, R313, which determined resistance to the drugs tetracycline (Tc), streptomycin (Sm) and Sulphonamide (Su), and also determined a host specificity, hsII. When this strain was used as a donor of drug resistance, separation of R factor-carried determinants was observed in ex-conjugants. Resistance to Tc and hsII character were not separated, nor was resistance to Sm separated from resistance to Su, but separation of these two pairs of characters was observed. Tetracycline-sensitive segregants, obtained by penicillin selection, were resistant to Sm and Su, but had lost the hsII character. Similarly, segregants for selected sensitivity to Su were sensitive to Sm, but resistant to Tc and hsII$^+$. The same pattern of separation of characters was also observed when drug resistance was transduced with phage P1, with the additional finding that the Sm and Su resistant transductants lacked sex factor activity. The resistance to Sm carried by these transductants could be mobilized by an *fi*$^-$ R factor, R143. Explanations of this behaviour are considered, including the possibility that the strain J5-3 (R313) had carried two *fi*$^-$ R factors. This explanation would also require that the transduction of an R factor by phage P1 is not always complete.

INTRODUCTION

Resistance transfer factors (R factors) of the Enterobacteriaceae determine transmissible resistance to antibiotics and have also been shown to be associated with genetic determinants for other characters (Meynell, Meynell & Datta, 1968). These include reduction of the efficiency of plating (e.o.p.) of bacteriophages which in several cases has been shown to be due to the operation of host specificity determinants (Yoshikawa & Akiba, 1962; Molina, 1964; Watanabe, Takano, Arai, Nishida & Sato, 1966; Bannister & Glover, 1968). Host specificity determinants control two bacterial functions: restriction of foreign DNA and modification of DNA synthesized in the bacterial cell (Luria, 1953; Arber & Dussoix, 1962). At least two different host specificities are associated with R factors (Bannister & Glover, 1968), one of which, termed hsII (identical with the R factor-associated host specificity observed by Watanabe *et al.* 1966), proved to be convenient marker in the investigation of an unusual R$^+$ strain of *Escherichia coli* K12. The e.o.p. of bacteriophage λ grown on *E. coli* K (λ.K) is 1·0 on *E. coli* K, and 1×10^{-2} on a strain carrying hsII (Bannister, 1969), a difference which can be detected by use of a single critical dilution of bacteriophage λ.

The R$^+$ strain J5-3 (R313) is resistant to streptomycin (Sm), sulphonamide (Su),

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and tetracycline (Tc), and is hsII$^+$. This strain acts as a donor for all three drug resistances and for the hsII determinants, but segregation of these four characters was observed in exconjugants obtained after mating. The R factor R313 behaved on testing as *fi*$^-$, i.e. it did not repress the expression of F (Watanabe, Nishida, Ogato, Arai & Sato, 1964). Generally, two R factors of the same *fi* type cannot co-exist stably in the same cell (Harada *et al.* 1961; Mitsuhashi *et al.* 1962; Watanabe *et al.* 1964), and since the strain clearly did not carry an *fi*$^+$ R factor, it was investigated to determine the number and type of sex factors and plasmids it carried. This paper presents the results of that investigation.

METHODS

Bacterial strains. Two strains of *Escherichia coli* K12 were used: C600 *thr leu thi lac* (Appleyard, 1954) and J5-3 *pro met* (Clowes & Rowley, 1954). J5-3 (R313) was the gift of Drs Elinor Meynell and Naomi Datta. The R factor, R313, is *fi*$^-$ and determines I-like sex pili (Lawn, Meynell, Meynell & Datta, 1967).

Bacteriophages. Phage λ *vir*, a virulent mutant of phage λ (Jacob & Wollman, 1954). Phage P1 (Lennox, 1955).

Media. Oxoid nutrient broth: Oxoid no. 2 broth powder, 25 g.; distilled water, 1·0 l. L-broth: Difco tryptone, 10 g.; yeast extract, 5 g.; NaCl, 10 g.; distilled water, 1·0 l.; pH 7.2. Nutrient agar: Oxoid nutrient broth solidified with 12·5 g./l. of Davis New Zealand agar. Difco agar: Oxoid tryptone, 10 g.; NaCl, 8 g.; glucose, 1 g.; Difco Bacto agar, 10 g.; distilled water, 1·0 l. VB agar: VB salts (Vogel & Bonner, 1956) solidified with 15 g./l. of Davis New Zealand agar. Soft agar for overlays: Difco Bacto agar, 6 g.; distilled water, 1·0 l. M9 medium: Na_2HPO_4, 7 g.; KH_2PO_4, 4 g.; NaCl, 0·5 g.; NH_4Cl, 1 g.; distilled water, 1·0 l. with addition after sterilization of $MgSO_4$ to 0·001 M and $CaCl_2$ to 0·0001 M. Amino acids used at a final concentration of 20 μg./ml.; sugars used at final concentration of 0·2 %; thiamine hydrochloride used at final concentration of 1 μg./ml. Antibiotics: Tetracycline (achromycin hydrochloride, Cyanamid) used at a final concentration of 50 μg./ml. Streptomycin (streptomycin sulphate BP, Glaxo) used at final concentration of 20 μg./ml.) Sulphonamide (sulphamezathine, ICI) used at a final concentration of 20 μg/ml. Penicillin (benzyl penicillin, Glaxo) used at a final concentration of 20 μg./ml.

Phage P1 transductions. Overnight cultures of an R$^+$ strain were diluted 1 in 10 into L-broth, grown at 37° for 90 min. and harvested by centrifugation. The pellet was resuspended in L-broth containing 0·01 M-$CaCl_2$, to give a titre of 2 to 5 × 10^8 bacteria/ml. Phage P1 was added at a multiplicity of 0·1, and was allowed to adsorb for 20 min. at 37°. Unadsorbed phage were removed by centrifugation, and samples of the resuspended bacteria, and suitable dilutions, were added to 3 ml. of soft agar and poured over supplemented VB agar plates. Since the VB agar contains citrate, no further precaution against readsorption of phage P1 was taken. Colonies appearing after 24 to 36 hr incubation were picked for purification.

Penicillin selection. Selection for loss of tetracycline resistance was carried out in Oxoid nutrient broth, but M9 medium was used for selection of sulphonamide sensitive segregants. Overnight cultures were diluted to about 10^4 cells/ml. and were grown with aeration by bubbling at 37° for 2 hr. when 50 μg./ml. of tetracycline, or 20 μg./ml. of sulphonamide, were added. Aeration was continued at 37° for a further 60 min. and while the number of bacteria was still less than 10^7 cells/ml., 20 μg./ml. of benzyl

penicillin were added. Aeration at 37° was continued for 4 hr, the culture was centrifuged and samples spread on supplemented VB agar plates. Colonies appearing after 24 hr incubation were purified by two serial single colony isolations and re-tested.

Transfer of drug resistance. Overnight cultures of an R^+ donor strain in Oxoid nutrient broth were diluted 1 in 10 fresh broth and grown for 90 min., then diluted 1 in 10 into an overnight broth culture of a recipient strain. The mating mixture was grown at 37° for 30 min. and then centrifuged and resuspended in M9 medium to reduce the amount of broth added to the plates at the next stage—an important consideration whenever sulphonamide resistance is to be selected. Samples of the mating mixture, or suitable dilutions, were added to 3 ml. of soft agar and poured over appropriately supplemented VB agar plates. Contraselection against the growth of the donor relied on omission of its growth requirements. Each cross was controlled by plating donor and recipient separately on the various selective media, and the donor bacterial concentration was assayed on Oxoid nutrient plates. The drug resistances of donor cultures were checked by use of an Oxoid multodisk code no. 30-19N. After 24 to 36 hr incubation at 37°, R^+ recipient colonies were picked and stabbed onto selective VB agar plates, grown for a further 24 hr at 37°, and their drug resistances scored by replica plating.

Detection of the hsII determinant. Detection of the presence of hsII was based on the reduction in e.o.p. of bacteriophage λ.K on hs_{II}^+ strains. A confluent streak was made on Difco agar plates of each colony under test and the streaks were spotted with critical dilutions of phage λ *vir* grown on R^- *Escherichia coli* K12, followed by overnight incubation at 37°. The dilution of bacteriophage λ *vir* was so chosen that $hsII^+$ strains showed no plaques and $hsII^-$ strains showed confluent lysis.

RESULTS

Separation of two linkages groups during conjugal transfer. In preliminary experiments, the *Escherichia coli* strain J5-3 (R313) acted as a donor of resistance to each of the drugs tetracycline (Tc), streptomycin (Sm) and sulphonamide (Su), but it was observed that not all exconjugants selected for resistance to Sm were resistant to all three drugs, nor were all of them $hsII^+$. Using J5-3 (R313) as the donor, and C600 as the recipient, a more detailed analysis was carried out by selecting exconjugants resistant to Tc, Sm and Su separately, and scoring their resistance to each of the other two drugs and for the presence of hsII. The results of two such experiments are presented in Table 1. Separation of R factor-associated characters occurred on conjugal transfer according to a pattern giving only three types of exconjugant. Selection for resistance to tetracycline produced exconjugants resistant to Tc and $hsII^+$ as well as exconjugants resistant to Tc, Sm, Su and also $hsII^+$, Selection for either Sm resistance or for Su resistance also resulted in the production of two types of exconjugant. One type (also observed when Tc resistance was selected) was resistant to all three drugs Tc, Sm and Su and $hsII^+$, the second type was resistant to Sm and Su. The R factor-associated characters of each type of exconjugant remained associated with one another and were transmissible in subsequent matings.

When a purified exconjugant, resistant to Tc, Sm and Su, and $hsII^+$, was used as a donor and J5-3 as the recipient the same pattern of segregation was observed, as was found when J5-3 (R313) was the donor (Table 2).

Spontaneous segregation of drug resistance. The separation of donor characters on transfer could have reflected a high rate of segregation of these characters in the donor strain itself. To test this, single colonies of J5-3 (R313) were surveyed by replica plating for loss of drug resistance, but after screening several hundred colonies no colony sensitive to any of the drugs Tc, Sm or Su was found.

Table 1. *Properties of exconjugants from the cross J5-3 (R313) × C600*

Overnight cultures of J5–3 (R313) were diluted 1/10 in broth, grown for 90 min., diluted 1/10 into an overnight culture of C600. After 30 min. the mixture was centrifuged, resuspended in minimal M9 medium and samples added to tubes containing 3 ml. of water soft agar which was then poured on to supplemented VB agar plates. The colonies appearing after 24 to 36 hr were purified and tested for drug resistances. At least 20 colonies (if available) were tested for presence of hsII by spotting streaks on Difco agar plates with critical dilutions of phage λ.K (see Methods). Presence of a sex factor was determined by using at least two purified single colonies as donors, following the procedure for transfer of drug resistance outlined in methods.

Drug used for selection	Drug resistances present	No. of colonies Expt. 1	Expt. 2	hsII	Presence of sex factor
Sm	Sm Su	71	98	−	+
	Tc Sm Su	48	30	+	+
Su	Sm Su	42	104	−	+
	Tc Sm Su	65	17	+	+
Tc	Tc	75	68	+	+
	Tc Sm Su	−	3	+	+

Table 2. *Properties of exconjugants from the cross C600 (R313) × J5-3*

Drug used for selection	Drug resistances present	No. of colonies	hsII	Presence of sex factor
Tc	Tc	109	+	+
	Tc Sm Su	3	+	+
Sm	Sm (Su)*	67	−	+
	Tc Sm (Su)*	67	+	+

* 10 colonies scored for Su resistance. Experimental procedure as in Table 1.

Table 3. *Properties of drug sensitive segregants of J5-3 (R313)*

The drug sensitive segregants were obtained after penicillin selection (see Methods). Presence of hsII and of sex factor were determined as in Methods.

Drug sensitivity selected	Drug resistances still present	No. of colonies	hsII	Presence of sex factor
Tc	Sm Su	2	−	+
Su	Tc	5	+	+

Isolation of drug sensitive colonies after penicillin selection. Following the failure to find drug-sensitive colonies by simple replica plating, a small number of segregants was obtained after applying penicillin selection to enrich the culture for rare cells sensitive to one or other of the bacteriostatic drugs Tc and Su. The drug-sensitive segregants found are listed in Table 3, which shows that loss of resistance to Tc was invariably accompanied by loss of the hsII determinants, while resistance to Sm and

Su was retained. On the other hand, loss of resistance to Su was accompanied by loss of resistance to Sm, but retention of resistance to Tc and of the hsII determinants. Both classes of segregants acted as donors of the characters which had been retained.

Transduction of drug resistance by phage P1. The results of conjugal transfer and penicillin selection suggest the presence of two sets of linked characters, Tc resistance associated with hsII, and Sm resistance associated with Su resistance. The strain must also carry at least one sex factor, and transduction with phage P1 was used to investigate the relationship of the sex factor with the other transmissible determinants. Phage P1 generally transduces the entire R factor, in fact co-transduction of drug resistance determinants and sex factor activity is generally regarded as evidence of a single structural unit for the R factor (Mitsuhashi *et al.* 1962). The results of two transductions using phage P1 grown on J5-3 (R313) are shown in Table 4. In agreement with the

Table 4. *P1 transduction of drug resistance*

Phage P1 transduction was carried out as in Methods. The presence of hsII and sex factor activity were determined as outlined in Methods.

Drug used for selection	Drug resistances present	No. of colonies		hsII	Presence of sex factor
		Expt. 1	Expt. 2		
Tc	Tc	123	20	+	+
Sm	Sm Su	25	19	−	−
Su	Sm Su	6	28	−	−

Table 5. *Transfer frequencies from strains carrying phage P1 transduced Sm resistance and a sex factor*

Transfer was measured by the procedure outlined in Table 1. The first row of figures for each sex factor refers to a transductant originally selected for Sm resistance, the second row to transductant selected for Su resistance. Both types are resistant to both Sm and Su.

Sex factor	Average frequency of transfer/donor of resistance to		
	Tc	Sm	Km
F	—	1×10^{-6}	—
	—	2×10^{-6}	—
R124, *fi*⁺	5×10^{-4}	1×10^{-7}	—
	1×10^{-4}	1×10^{-7}	—
R143, *fi*⁻	—	1×10^{-5}	1×10^{-3}
	—	4×10^{-5}	2×10^{-3}
R313-T-1, *fi*⁻	2×10^{-3}	1×10^{-6}	—
	3×10^{-3}	1×10^{-6}	—

results presented above there was separation of the R factor-associated characters; selection for resistance to Tc yielded colonies which were also hsII⁺, and selection for either Sm or Su resistance produced colonies resistant to both these drugs. Colonies carrying more than two of these characters, or any other combination of them, were not observed. One major difference was observed between the two separated groups of characters after phage P1 transduction: although the Tc-resistant hsII⁺ colonies were donors of Tc resistance and hsII determinants, the transductants resistant to Sm and Su did not act as donors of resistance to either of these drugs.

Mobilization of non-transmissible characters introduced by transduction. Transductants carrying the non-transmissible Sm an Su resistance determinants were infected with the following sex factors: F; R124, an *fi*$^+$ R factor; R143, an *fi*$^-$ R factor; R313-T-1, an *fi*$^-$ segregant of R313, produced during conjugal transfer. The sex factor-infected transductants were used as donors to the recipient strain J5-3, and the frequency of transfer of Sm resistance, and any other resistance carried by the strain, was measured. No transfer of Sm resistance was observed when the introduced sex factor was R124 or R313-T-1, although resistance to Tc was transferred at frequencies of 10^{-4} and 10^{-3} donor bacterium respectively. A very low transfer of resistance to Sm, about 1×10^{-6}/donor, was observed when the sex factor was F, although transfer of F itself would be expected to be 50 to 100 % under the same conditions. The rare F$^+$ bacterium acting as a donor of the Sm resistance did not appear to be a recombinant of F and the Sm and Su resistance determinants, since exconjugants which were F$^+$ and resistant to Sm and Su did not transfer Sm resistance at an increased frequency. In the presence of R143, resistance to Sm was transferred at a frequency of about 1×10^{-4}/donor bacterium, a figure not very much less than the transfer frequency of the kanamycin (Km) resistance carried by R143 itself (10^{-3}). About 1 to 10 % of exconjugants resistant to Km were also resistant to Sm.

DISCUSSION

Although co-existence of an *fi*$^+$ and an *fi*$^-$ R factor can occur (Romero & Meynell, 1969), there has been only one previous report of the stable co-existence of two or more *fi*$^-$ R factors in *Escherichia coli* (Bouanchaud & Chabbert, 1969). Two R factors of the same *fi* type can be forced to co-exist in the presence of a combination of drugs selective for the presence of both R factors, but segregation occurs if the strain is subcultured on media lacking the drugs, and recombinant R factors may arise if forced maintenance is prolonged (Mitsuhashi *et al.* 1962; Watanabe *et al.* 1964). The strain J5-3 (R313) was originally assumed to carry a single *fi*$^-$ R factor until separation of the transmissible characters into two groups was observed after conjugal transfer of drug resistance. Exconjugants of three types were observed: donor type, that is resistant to Tc, Sm and Su and carrying hsII; resistant to Sm and Su; and resistant to Tc and hsII$^+$. All three types of exconjugant acted as donors of the characters carried, indicating the presence of a sex factor in all types of exconjugant. Although the frequent separation of donor characters during transfer was not due to a high rate of segregation in the parent strain, since no spontaneous segregant types were observed among several hundred donor colonies tested, the two segregant types can occasionally arise without conjugal transfer as demonstrated by the observation that the types of segregant found after penicillin selection are indistinguishable from those found after conjugal transfer. The same two types of segregant are again found after phage P1 transduction, with the important difference that the Sm and Su resistant transductants do not have detectable sex factor activity.

The conjugal separation of drug resistance determinants carried in association with an *fi*$^-$ R factor, and the successful isolation of an *fi*$^-$ sex factor not associated with any observed drug resistance determinants, has been reported previously, and the existence of variable degrees of attachment of the determinants to the sex factor postulated to explain it (Anderson, 1966; Anderson & Lewis, 1965). If such an hypothesis was

adopted here, the phage P1 transduction showed clearly that the determinants for Tc resistance and hsII were very closely associated with the sex factor, while the determinants for resistance to Sm and Su were not. Such a conclusion is untenable since both exconjugants and segregants which had lost Tc resistance and hsII, and may thus be assumed to have lost the sex factor associated with them in transduction, are still capable of transferring Sm and Su resistance. Hence it seems preferable to regard the evidence of the phage P1 transductions as demonstrating the presence of an R factor, characterized by its association with resistance to Tc and being hsII$^+$. A second sex factor could be present in J5-3 (R313) not associated with any other detected determinants, in which case the Sm and Su resistance determinants must be on a third plasmid. Alternatively, this sex factor may be attached to the determinants for resistance to Sm and Su, which would mean that a second *fi*$^-$ sex factor was present in the strain. Although two *fi*$^-$ R factors do not usually co-exist stably, there is one report of a strain simultaneously carrying three *fi*$^-$ R factors (Bouanchaud & Chabbert, 1969). In view of their results and ours, it is therefore possible that the *fi*$^-$ class of R factors comprises more than one group. Until recently the *fi*$^-$ class could only be negatively defined by failure to repress the expression of F. The recent isolation of a phage which specifically adsorbs to the pilus produced by *col* I and the majority of *fi*$^-$ R factors, the I-specific phage, has revealed a relationship between *col* I and some R factors (Meynell & Lawn, 1968), but there is no information as to the nature of the remaining factors.

The Sm and Su resistant phage P1 transductants were not transmissible so that if these resistance determinants are normally linked to a sex factor, a second unusual situation has to be postulated, namely incomplete transduction of the plasmid. There has been one previous suggestion that incomplete transduction might occur with phage P1 (Romero & Meynell, 1969) and in Salmonella incomplete transductions with the generalized transducing phages P22, ϵ 15 and ϵ 34 are the rule (Watanabe & Fukasawa, 1961; Harada, Kameda, Suzuki & Mitsuhashi, 1963; Dubnau & Stocker, 1964). The only evidence supporting the possibility that the Sm and Su resistant transductants are the result of incomplete transduction of an R factor is the mobilisation of the Sm resistance by the *fi*$^-$ R factor, R143. R143 permits the adsorption of the I-specific phage (Meynell & Lawn, 1968). No mobilization of Sm resistance was observed with the *fi*$^+$ R factor R124, nor with the Tc resistance R factor obtained from R313, R313-T-1, and the frequency of mobilization with F was very low. It is possible that restoration of transmissibility to the Sm resistant transductants by R143 represents complementation of the defective sex factor produced by incomplete transduction. Since the mechanism by which sex factors mobilize non-transmissible plasmids is still unknown, the evidence derived from mobilization studies cannot be conclusive.

In summary, J5-3 (R313) could carry an *fi*$^-$ R factor (with Tc resistance and the hsII determinants), a plasmid (carrying resistance to Sm and Su, but having no sex factor activity), and an *fi*$^-$ sex factor (not associated with any detectable determinants other than those for sex factor activity). However, there is no direct evidence for the existence of a second sex factor and an alternative is that there are two *fi*$^-$ R factors, one with Tc resistance and hsII determinants, the other with Sm and Su resistance determinants. Both alternatives involve combinations of plasmids not previously reported and while the evidence is not conclusively in favour of either, the recent findings of Bouanchaud & Chabbert (1969) make it more probable that two *fi*$^-$ R factors do co-exist stably in J5-3 (R313).

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Explanation of the Apparent Association of Host Specificity Determinants with *fi*$^+$ R Factors

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SUMMARY

Two strains of *Escherichia coli* K12 carried R factors associated with the hsII host specificity determinants although they were apparently *fi*$^+$, while all other R factors carrying this host specificity have been found to be *fi*$^-$. When these two strains were used as donors of drug resistance, separation of two R factors from each strain was observed. The separated R factors were tested for *fi* character, and it was found that in both cases the original strains carried an *fi*$^+$ and an *fi*$^-$ R factor, and it was the *fi*$^-$ R factor which carried the hsII determinants.

INTRODUCTION

Transmissible drug resistance in the Enterobacteriaceae is determined by drug resistance factors (R factors) which are divisible into two classes, *fi*$^+$ and *fi*$^-$, on the basis of the repression of the expression of the sex factor F (see Watanabe, 1963), as well as by the structure of their sex pili (see Meynell, Meynell & Datta, 1968). An apparent third, distinguishing feature between the *fi*$^-$ and *fi*$^+$ R factors, was that the presence of certain *fi*$^-$ R factors led to a reduction in efficiency of plating (e.o.p.) of a number of bacteriophages such as λ and T1 (Watanabe, Takano, Arai, Nishida & Sato, 1966).

In a survey of 153 R$^+$ *Escherichia coli* K12 strains this affect was observed with both *fi*$^-$ and *fi*$^+$ R factors (Bannister & Glover, 1968). In some instances, a reduction in e.o.p. may be due to the operation of a host-controlled modification, the host range of a bacteriophage being altered by a single cycle of growth in a new host strain (Luria, 1953; Arber & Dussoix, 1962). In 12 R$^+$ strains, the reduction in e.o.p. of a number of bacteriophages was found to be due to R factor-associated host controlled modification, and two different host specificities were observed. One of these, termed hsI, was present only in one R$^+$ strain, carrying the *fi*$^+$ R factor R124, while the second, hsII, was found in the remaining 11 strains (Bannister & Glover, 1968), and was also shown to be identical to that found in two strains with *fi*$^-$ R factors by Watanabe *et al.* (1966). Careful examination of the *fi* character of the 11 hsII$^+$ strains showed that two of them were apparently exceptional in that they carried *fi*$^+$ R factors. This paper presents the results of an investigation of these two strains.

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METHODS

Media. The media have been described previously (Bannister, 1970; Bannister & Glover, 1970).

Antibiotics. Tetracycline: (achromycin hydrochloride from Cyanamid of Great Britain, Bush House, London, W.C.2) used at a final concentration of 50 μg./ml. Streptomycin: (streptomycin sulphate BP from Glaxo Laboratories Ltd., Greenford, Middlesex) used at a final concentration of 20 μg./ml. Sulphonamide: (sulphamezathine from ICI Pharmaceuticals Division, Macclesfield, Cheshire) used at a final concentration of 20 μg./ml. Chloramphenicol: (chloromycetin from Parke Davis, Hounslow, Middlesex) used at a final concentration of 20 μg./ml. Kanamycin: (kanamycin sulphate from Bayer Products Co., Surbiton, Surrey) used at a final concentration of 20 μg./ml.

Bacterial strains: The following *Escherichia coli* strains were used: C600 *thr leu thi lac* (Appleyard, 1954) from the collection of Dr S. W. Glover, and J5-3 *pro met* (Clowes & Rowley, 1954), J5-3 F^+ and J5-3 R^+ strains kindly provided by Dr Elinor Meynell and Dr Naomi Datta.

Bacteriophages. Phage λvir (Jacob & Wollman, 1954); MS2 (Davis, Strauss & Sinsheimer, 1961); T3.

Techniques. These are described elsewhere (Bannister, 1970; Bannister & Glover, 1970).

RESULTS AND DISCUSSION

Association of hsII with the fi⁻ character. Two strains apparently carrying *fi*$^+$ R factors associated with hsII were examined J5-3 (R269), which was resistant to the following antibiotics; tetracycline (Tc), streptomycin (Sm), sulphonamide (Su), and kanamycin (Km); and J5-3 (R348), resistant to Tc, Sm, Km and chloramphenicol (Cm). These two strains were used as donors of drug resistance to C600, and exconjugants selected for one resistance were scored for the others, and for the presence of the hsII determinants. The results of two such experiments are presented in Table 1. With R269, three classes of exconjugants were observed: those resistant to Tc, Sm, Su and hsII$^+$; those resistant to Tc, Sm, Su, Km and hsII$^+$, and those resistant to Sm, Su, Km and not carrying the hsII determinants. The most likely explanation of these findings is that two R factors were present in the original strain, one carrying resistance to Tc, Sm, Su and hsII$^+$ determinants, and the other carrying resistance to Sm, Su and Km. Those exconjugants carrying all the determinants of the parent strain would then arise by transfer of both R factors to the recipient. Similarly, three types of exconjugant were found for R348: those resistant to Tc, Sm and hsII$^+$; those resistant to Tc, Km and Cm, and those resistant to Tc, Sm, Km and Cm, and also carrying hsII$^+$. Again, the result can be explained in terms of the presence of two R factors in the original strain, one carrying resistance to Tc and to Sm, and also carrying the hsII determinant, the other carrying resistance to Tc, Km and Cm.

The fi character of the separated R factors from J5-3 (R269) and J5-3 (R348). A number of R^+ strains have been shown to carry both an *fi*$^+$ and an *fi*$^-$ R factor, though the presence of the *fi*$^-$ R factor was masked by the *fi*$^+$ R factor when the strains were tested originally for their ability to plate the F-specific phage MS2 (Romero & Meynell, 1969). Since two *fi*$^+$ R factors do not co-exist stably (Watanabe *et al.* 1964), it was pre-

Table 1. *Properties of exconjugants obtained after mating R^+ strains with strain C600.*

An overnight culture of the J5-3 R^+ donor was diluted 1 in 10 into fresh broth, grown on an inclined rotor at 37° for 90 min., then diluted 1 in 10 into an overnight culture of the recipient and kept at 37° for 30 min. This mating mixture was diluted and samples plated in soft agar overlays on supplemented VB agar plates, which were incubated at 37° for 36 to 48 hr. Colonies were purified by stabbing on to selective VB agar and grown overnight at 37° before replica-plating to determine drug resistance. The hsII character was determined by streaking colonies on Difco agar plates which were spotted with dilutions of phage *λvir* grown on K, and incubated overnight at 37°.

R factor and determinants carried	Drug resistance selected	Determinants found*†	Nos. observed Expt. 1	Nos. observed Expt. 2
R269	Tc	Tc Sm hsII	97	35
Tc Sm Su Km $hsII^+$		Tc Sm Km hsII	30	117
	Sm	Tc Sm hsII	15	2
		Tc Sm Km hsII	40	46
		Sm Km	15	96
	Su	Tc Sm Su hsII	0	2
		Tc Sm Su Km hsII	2	39
		Sm Su Km	128	102
	Km	Tc Sm Km hsII	25	36
		Sm Km	120	99
R348	Tc	Tc Sm hsII	16	112
Tc Sm Km Cm $hsII^+$		Tc Km Cm	97	27
		Tc Sm Km Cm hsII	8	2
	Sm	Tc Sm	75	68
	Km	Tc Sm Km Cm hsII	19	4
		Tc Km Cm	109	33
	Cm	Tc Km Cm	123	51
		Tc Sm Km Cm hsII	33	3

* At least 20 colonies of each tested for hsII where available.

† It is not possible to score Su resistance by replica-plating, hence Su resistance is not recorded for R 269, except where Su resistance was selected directly.

Table 2. *Phage MS2 plating and presumed fi character of F^+R^+ strains*

Parent R factor	Drug resistances present	hsII	MS2 plating*	*fi* character deduced	No. of colonies
R269	Tc Sm Su	+	+	−	3
R269	Sm Su Km	−	−	+	6
R348	Tc Sm	+	+	−	3
R348	Tc Cm Km	−	−	+	7

* MS2 plating was determined by spotting 0·01 ml. of an MS2 lysate on to the F^+ R^+ strain in soft agar on Difco agar plates.

sumed that the two strains J5-3 (R269) and J5-3 (R348), had both harboured an fi^+ and an fi^- R factor. To test this assumption the presumed separated R factors were transferred by conjugation to J5-3 F^+ strain, which was then tested for lysis with the F-specific phage MS2. The results are presented in Table 2. Lysis was abolished by the segregant of R269 carrying resistance to Sm, Su and Km, and similarly by the segregant of R348 carrying resistance to Tc, Km and Cm. Tests which showed a re-

duced efficiency of plating of phage T3 (Schell *et al.* 1963) confirmed that F was still present and thus that these R factors were acting by preventing its expression, and were therefore *fi*$^+$. The remaining two components of the original R269 and R348 factors, which carried the hsII$^+$ character, were both *fi*$^-$.

There have been three previous reports of R factor associated host specificities. For two *fi*$^-$ R factors described by Watanabe *et al.* (1966), it had been possible to identify the host specificity since one of the R factors, R15, was made available for this purpose, and the host specificity found was hsII. Yoshikawa & Akiba (1962) also found a host specificity associated with *fi*$^-$ R factors, while the third report (Molina, 1964) did not specify the *fi* character nor give details of the host specificity. There remains, therefore, only one instance of an *fi*$^+$ R factor carrying host specificity determinants, this being the association of R124 with the determinants for hsI, a host specificity distinct from hsII (Bannister, 1969). It is possible that once again the *fi*$^+$ R124 masks the presence of a second *fi*$^-$ R factor which carries the hsI determinants, since it is not unusual for an *fi*$^-$ R factor to be present in an apparently *fi*$^+$ strain (Romero & Meynell, 1969). However, it has not been possible to separate hsI from the *fi*$^+$ character and resistance to Tc, the only drug resistance carried by this R factor (Bannister, 1969).

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