METHODS in MICROBIOLOGY

Edited by

T. BERGAN

J. R. NORRIS

Volume 13

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T. BERGAN

Department of Microbiology,
Institute of Pharmacy and Department of Microbiology,
Aker Hospital, University of Oslo,
Oslo, Norway

J. R. NORRIS

Agricultural Research Council,
Meat Research Institute,
Bristol, England

Volume 13

1979

ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

London · New York · Toronto · Sydney · San Francisco

ACADEMIC PRESS INC. (LONDON) LTD
24–28 Oval Road
London NW1

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

British Library Cataloguing in Publication Data

Methods in Microbiology.
Vol. 13
1. Microbiology–Technique
I. Bergan, T
II. Norris, John Robert
576′.028 QR65 68–57745

ISBN: 0–12–521513–4

PRINTED IN GREAT BRITAIN BY
ADLARD AND SON LIMITED
DORKING, SURREY

PREFACE

Volume 13 of "Methods in Microbiology" is a continuation of the series of Volumes describing the methods available for typing major pathogens by serology, bacteriophage typing, and bacteriocin typing.

This Volume has a Chapter dealing with bacteriophage typing and bacteriocin typing of clostridia. Chapters II and III detail the typing of *Listeria monocytogenes* and *Erysipelothrix insidiosa*. In Chapter IV, the bacteriology and epidemiology of diphtheria bacilli are discussed. Chapter V presents bacteriophage typing of the *Shigella* species. Then follow serology and phage typing of *Actinomyces*, *Arachnia*, and *Mycobacterium*. Chapter IX describes the characterisation of *Mycoplasma* and Chapter X deals with *Campylobacter*.

We have been fortunate in having had the good collaboration of internationally recognised authorities. The Chapter on phage typing of *Shigella* originally had a contributor who unfortunately had to withdraw because of other commitments. Since this happened two years after the deadline and we could not proceed without this important topic, we prepared it ourselves in consultation with experts in the field. We are grateful for this support.

We hope that Volumes 10–13 will serve as useful references for microbiologists who need to know most methods available to type bacteria in the context of their epidemiological relationships. The Volumes were originally planned five years ago. Since then there have been significant developments in the characterisation of several groups of bacteria. When the time is ripe for it, we hope to collect the methodology for typing further microbes in a later volume of "Methods in Microbiology".

T. Bergan
J. R. Norris

August, 1979

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CHAPTER I

Bacteriocin, Bacteriophage and other Epidemiological Typing Methods for the Genus *Clostridium*

D. E. MAHONY

Department of Microbiology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada

I. INTRODUCTION

Many of the bacteria classified as clostridia are harmless. Their habitat is in the soil and intestinal tract of man and animals; yet some of these

same species, under appropriate conditions, are capable of causing destructive and sometimes fatal disease such as botulism, gas gangrene, clostridial cellulitis, tetanus and the non-fatal but frequently encountered *Clostridium perfringens* food poisoning. Because such organisms are ubiquitous, the possible typing of these bacteria presents an intriguing problem. Just what is the origin of a hospital acquired case of gas gangrene—spore-laden dust in an operating theatre, inadequately sterilised instruments or materials, or the patient's own flora? What is the source of an enterotoxin-producing strain of *C. perfringens* in an outbreak of food poisoning? Similar questions might be asked of botulism and tetanus. Means are available for answering a few of these questions.

Historically, typing of only pathogenic bacteria has been considered and this Chapter will be restricted to the pathogenic species of *Clostridium* associated with gas gangrene, botulism, tetanus and food poisoning. Unfortunately, little work has been done on bacteriocin or bacteriophage typing of clostridia and perhaps one of the major objectives of this contribution is to review much of our knowledge on clostridial bacteriocins and bacteriophages with a view to suggesting potential typing schemes for various species. An appreciation of some of the difficulties which might impede the development of typing schemes should also be considered. A bacteriocin typing scheme for *C. perfringens* developed in our laboratory is presented as one possible model for typing the clostridia.

II. APPROACHES TO BACTERIOCIN TYPING

There are two approaches to bacteriocin typing. The first involves examining the ability of isolates to produce bacteriocins active against a standard set of indicator bacteria. The host range of any bacteriocin produced by the test strains then defines the typing pattern. One method of performing this test is to inoculate a plate of an appropriate medium with a single wide diametrical streak of the test organism. After growth (18 or more hours) the bacteria are scraped off the plate and the plate exposed to chloroform vapours. Subsequently the plate is aired to remove the chloroform before the standard indicator bacteria are streaked across the plate at right angles to the original streak. The plates are incubated to allow growth of the indicator bacteria. If bacteriocin has been produced by the test strain, the growth of one or more of the indicator strains should be inhibited where the streakings intersect.

The second approach tests the susceptibility of unknowns to a standard set of bacteriocins. This approach is less time consuming than the former since only one incubation period is required. The test organism is simply inoculated confluently over the surface of an agar plate as is done for anti-

biotic sensitivity testing, and defined volumes of the different bacteriocins are dropped on to sectors of the seeded plate. The pattern of inhibition which develops after subsequent growth of the lawn defines the bacteriocin type of the organism.

There are factors in favour of each method and further detail may be found in the literature (Shannon, 1957; MacPherson and Gillies, 1969).

III. GROWTH AND IDENTIFICATION OF CLOSTRIDIA

Many excellent references may be found on the growth and identification of the genus *Clostridium* (Dowell and Hawkins, 1974; Holdeman and Moore, 1972; Smith, 1975; Smith and Holdeman, 1968; Willis, 1969). In most laboratories, cooked meat or thioglycollate-containing broths and the Gaspak anaerobic jar (Bioquest, B.B.L.) are used in cultivation. It is beyond the scope of this Chapter to discuss the many variations in growth techniques and identification procedures.

The clostridia are usually Gram-positive, spore-forming, catalase-negative anaerobic bacteria. Some species are much more aerotolerant than others, but anaerobic techniques must be applied to assure the isolation of any member of the genus. *C. perfringens* is often reluctant to produce spores and is also non-motile. In addition, a double zone of haemolysis surrounds the smooth, entire-edged colonies of this organism on human or sheep blood agar plates; the inner zone of beta haemolysis is produced by the theta toxin while the outer weaker zone is caused by the alpha toxin. Some isolates, however, may lack the theta toxin or produce rough, irregular colonies which will confuse identification. The organism can be specifically identified by its saccharolytic activity on various sugars and neutralisation of its toxins with specific antisera. Some clostridia may only be specifically identified by toxin neutralisation tests, e.g. *C. botulism* and *C. tetani*. Newer analytical tools such as gas–liquid chromatography have enhanced the identification of many anaerobic bacteria by detecting specific organic acids produced by the fermentation of defined carbohydrates. This technology is described in detail by Holdeman and Moore (1972).

IV. NATURE AND MODE OF ACTION OF CLOSTRIDIAL BACTERIOCINS

A. Bacteriocins of *C. perfringens*

1. *Incidence of bacteriocinogenicity in* C. perfringens

Although the first observation of bacteriocin-like activity in this species was made by Smith in 1959 while studying lysogeny, the first major screen-

ing of large numbers of *C. perfringens* strains for bacteriocins was carried out by Sasarman and Antohi (1963). From 24 strains tested against one another, four bacteriocin-producing strains were identified: strain 1241 (toxin type E), 2077 (type F) and P24 and 353 (non-typed strains). Undoubtedly more bacteriocins would have been detected in supernatant fluids of cultures had these investigators not heated the supernatant fluid at 70°C for 1 h before spotting on to the plates. Using these bacteriocins, 237 strains of *C. perfringens* were tested for susceptibility. Sasarman and Antohi concluded that 81·4% of their strains were typable and 12 different typing patterns were obtained. Sixty-seven per cent of the strains fell within the first five types while the remaining seven types represented small numbers of strains. There was no apparent association between either the production of, or susceptibility to, bacteriocins and the classical toxin typing of these organisms.

This latter fact was also confirmed by Uchiyama (1966a) when 74 strains of *C. perfringens* were divided into four groups based upon their ability to produce bacteriocins. Twelve bacteriocin-producing strains were detected. In testing 14 strains of toxin types B, C, D, E and F, there was no type specificity in the action of bacteriocin. Only *C. perfringens* was sensitive to these bacteriocins during a survey of a number of Gram-positive and Gram-negative organisms. Also Tubylewicz (1966a) reported that 5 of 35 strains of *C. perfringens* Type A were bacteriocinogenic. A further study by Sasarman and Antohi (1968) showed that of 251 strains of *C. perfringens* examined, 12 elaborated bacteriocins (no heat treatment of the supernatant fluids). Hirano *et al.* (1972) showed that 14 of 176 strains produced bacteriocin while Mahony and Butler (1971) detected four bacteriocinogenic strains amongst 33 tested. In 1974, Mahony used ten bacteriocins to type 274 cultures of *C. perfringens* and observed 50 different bacteriocin typing patterns. Currently, 65 typing patterns have been observed (Mahony and Swantee, 1978).

The naming of bacteriocins of *C. perfringens* is still unresolved. Some authors call such bacteriocins "perfringocins", while others have chosen the term "welchicins".

2. *Production and detection of bacteriocins*

The methods used to produce and detect bacteriocins of *C. perfringens* are similar to those employed for bacteriocins of other bacteria with the exception that growth of the organisms is carried out under anaerobic conditions.

Spontaneous production of bacteriocins has been reported by Uchiyama (1966a), Sasarman and Antohi (1968), Mahony and Butler (1971), and Hirano and Imamura (1971). Tubylewicz (1966a) used ultraviolet light

induction to get an increased production of the active principle. Mahony and Butler (1971) were unable to induce one strain (strain 28), although one other strain was later shown to produce a higher titre after treatment with UV light (Mahony, 1973). In 1977, Mahony reported the induction of six of ten bacteriocinogenic strains of *C. perfringens* using Mitomycin C. The response to this antibiotic was peculiar in that induction occurred only after removal of Mitomycin C from the treated culture. Cell death was associated with induction of bacteriocin.

Sasarman and Antohi (1971) showed that the majority of their welchicin-producing strains gave the highest yield of bacteriocin when grown at the organism's optimum growth temperature (37°C). At 50°C bacteriocin was not produced, and at room temperature production was reduced. Other investigators have reported a temperature of 37°C for bacteriocin production. Mahony and Butler (1971) studied the production of bacteriocin 28, and found that optimum production was obtained in the late log phase between 2 and 3 h after initiation of growth. Uchiyama (1966b) found that maximum titre was achieved after 3 h of growth. In contrast, a bacteriocin of *C. perfringens* type A described by Clarke *et al.* (1975) was released into the culture fluid in the stationary phase of bacterial growth. With the method used by Tubylewicz (1966a) bacteriocin production was assayed after overnight incubation.

Ionesco *et al.* (1974) have described independent synthesis of bacteriocin and bacteriophage in one strain of *C. perfringens*. Both factors could be induced by UV light. Bacteriocin production demonstrated a lag period of 70 min while the maximum titre was achieved by 180 min after induction.

3. *Host range*

All the bacteriocins obtained by Tubylewicz (1966a) were active against *C. perfringens* and, under aerobic conditions, did not inhibit the growth of 37 bacterial strains belonging to ten other genera. Similar results have been published by Uchiyama (1966a). Sasarman and Antohi (1968) reported a wider range of activity for a bacteriocin (welchicin A) produced by *C. perfringens* type E (strain 1241). In addition to activity against 100% of *C. perfringens* tested including the producing strain, they found activity against *Clostridium oedematiens*, *Clostridium bifermentans*, and *Clostridium fallax*. Sensitivity to this bacteriocin (welchicin A) was also demonstrated by various species of the genus *Bacillus* and subsequently (1971) these workers also reported activity against *Corynebacterium diphtheriae*, staphylococci and streptococci. Sensitivity to a similar host range was later reported by the same authors (1970), where they showed that welchicin B (produced by *C. perfringens* type D, strain 366) was also active on Gram-positive organisms outside the family *Bacillaceae*. Sasarman and Antohi (1971), described the

existence of a new third class of welchicin which was active on *C. perfringens* and *Streptococcus*, but not on many strains of the other genera listed above.

A perfringocin active on *C. pasteurianum* has been described by Clarke *et al.* (1975).

Resistance of the producing strain to its own bacteriocin is a usual characteristic of bacteriocinogenic organisms, although one of the five bacteriocins studied by Tubylewicz (1966a), bacteriocin d, was found to be weakly active against the strain 496 from which it was derived, and one of the strains reported by Sasarman and Antohi (1968), strain 1241, type E, produced a welchicin A active against all the strains tested, including the producer organism.

4. *Chemical nature and antigenicity*

Most bacteriocins have been thermolabile (Tubylewicz and Uchiyama, 1966b; Mahony and Butler, 1971). Temperature sensitivity of the welchicins has been variable (Sasarman and Antohi, 1971). The bacteriocins active only against *C. perfringens* and *Streptococcus* showed reduced activity or complete inactivation above 50°C, while the activity of those inhibitory only to *C. perfringens* or to *Clostridium*, *Bacillus*, and *Streptococcus* was not reduced by temperatures up to and including 90°C for a period of 2 h. Hirano and Imamura (1972c) reported two types of *C. perfringens* bacteriocin—one heat stable (type S) and the other thermolabile (type L). The perfringocin described by Clarke *et al.* (1975) is heat stable (100°C, 10 min). We currently have two bacteriocins which are similarly heat stable (Mahony, unpublished data).

The sensitivity of bacteriocin to proteolytic enzymes was tested by Tubylewicz (1966b). Both trypsin and papain completely inactivated perfringocin. Sasarman and Antohi (1971), Mahony and Butler (1971) and Clarke (1975) reported similar results. The L type of Hirano and Imamura (1972a, b, c) was trypsin sensitive, but the S type was not affected by trypsin. Recently, we have observed two trypsin-resistant bacteriocins of *C. perfringens* (Mahony, unpublished data).

Tubylewicz (1966b) studied the influence of UV rays on bacteriocins. He found that, within a range of 537–12,888 erg/mm^2, UV light did not bring about any detectable change in the activity of bacteriocins *a* and *b*. Similar findings were reported by Hirano and Imamura (1972) for both their types of bacteriocins.

The influence of pH on bacteriocin activity was studied by Tubylewicz (1966b) who noted stability over a pH range of 4–10. Uchiyama (1966b) reported that their bacteriocins were inactivated below pH 3 and over pH 9. The S type bacteriocin of Hirano and Imamura (1972a, b, c) was