Clostridium botulinum

Ecology and Control in Foods

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Marcel Dekker, Inc. New York • Basel • Hong Kong

Library of Congress Cataloging-in-Publication Data

Clostridium botulinum : ecology and control in foods / edited by
Andreas H. W. Hauschild and Karen L. Dodds.
p. cm.—(Food science and technology ; 54)
Includes bibliographical references and index.
ISBN 0-8247-8748-X
1. Botulism. 2. *Clostridium botulinum*. 3. Food—Microbiology.
I. Hauschild, Andreas H. W. II. Dodds, Karen L.
III. Series: Food science and technology (Marcel Dekker, Inc.) :
54.
QR201.B7C56 1992
664'.07—dc20 92-29096
CIP

This book is printed on acid-free paper.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

Preface

When we were asked to write or edit a comprehensive book on *Clostridium botulinum*, we felt that a general review of the subject was not needed at the time. Smith and Sugiyama (1988) had only recently published such a review, Eklund and Dowell (1987) had edited a book entitled *Avian Botulism*, and Simpson's book *Botulinum Neurotoxin and Tetanus Toxin* (1989) was in press. However, while writing a chapter for Doyle's *Foodborne Bacterial Pathogens* (1989), it occurred to us that two aspects of the microorganism—its ecology and its control in foods—had been somewhat neglected. These are addressed in this book.

This book is intended to serve as a reference for professionals and graduate students in the fields of bacterial pathogens and microbial ecology, as well as for food microbiologists and food scientists. We are confident that the book will indeed be a useful tool, thanks to the wide-ranging expertise and enthusiasm of our coauthors. The chapters are arranged according to subjects, and the chapter formats have been left largely to the discretion of the authors.

In recent years, strains of recognized clostridial species other than *C. botulinum* have been found to be capable of producing neurotoxins and of causing botulism. These discoveries have fundamentally altered our concept of the group of microorganisms associated with botulism. Therefore, as an introduction to this book, and in particular to its ecological aspects, we asked Charles Hatheway to write on currently known clostridia producing botulinum neurotoxins.

A short chapter on infant botulism (Chapter 5) has been included because ecological aspects such as *C. botulinum* in the environment and in foods and its competitive development in the infant's intestine are significant factors in the etiology of infant botulism. Wound botulism and "unclassified" botulism have been dealt with briefly in Chapter 1.

In conclusion, we are much indebted to our coauthors for their excellent contributions, and we wish to thank them for doing their utmost to finish their work within the given time frame.

ANDREAS H. W. HAUSCHILD
KAREN L. DODDS

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Contents

I

ECOLOGY

1

Clostridium botulinum and Other Clostridia that Produce Botulinum Neurotoxin

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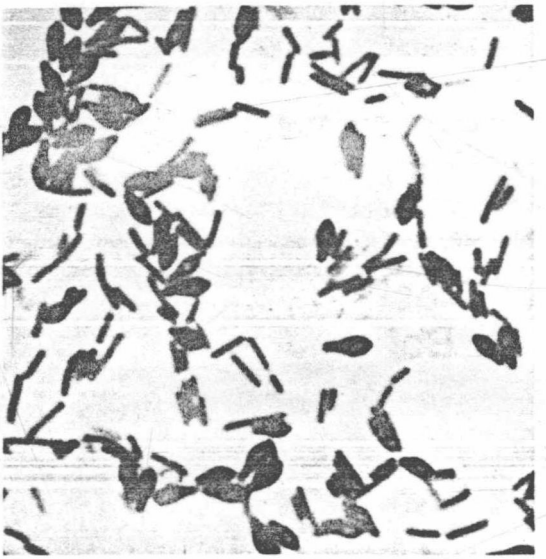
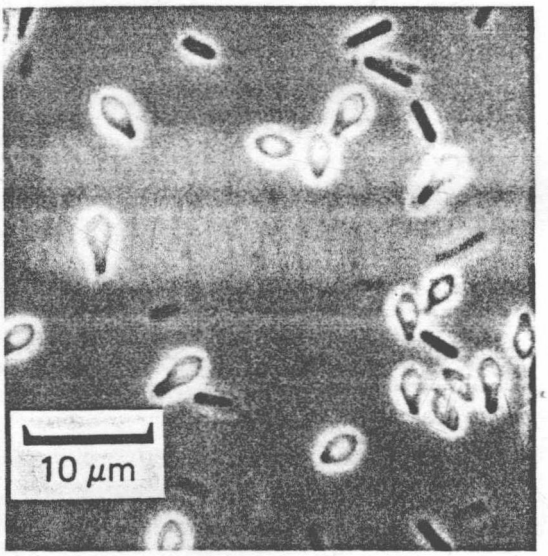
I. INTRODUCTION

Van Ermengem (1897) first established that botulism is caused by a toxin consumed in a food and then isolated the organism responsible for the toxin from the food. He named the organism *Bacillus botulinus*. As subsequent incidents of botulism were investigated, it was found that sometimes the toxins had different serological properties and that the causative organisms had varying physiological characteristics. The organisms were always anaerobic and formed spores, and they were later placed into the genus *Clostridium* (Bengtson, 1924); the genus *Bacillus* was restricted to aerobic spore-forming rods.

The genus *Clostridium* includes all anaerobic spore-forming rods except for those classified as *Desulfotomaculum* (Cato et al., 1986). Clostridia are considered gram-positive, although many strains stain positive only in very young cultures and may appear as gram-negative cells in more mature cultures. Clostridia can be distinguished from non–spore-forming organisms by visualizing the spores on a stained smear or wet mount or by demonstrating heat resistance. The morphology of a typical strain of *Clostridium botulinum* is shown in the photomicrographs in Figure 1. Most species of *Clostridium* will not grow on agar surfaces in the presence of oxygen; some, however, are aerotolerant and could be judged to be aerobic organisms, i.e., *Bacillus* species, but they can be distinguished from these by their failure to produce catalase.



(A)



(B)

Figure 1 Photomicrographs of egg yolk agar culture of a typical strain of *Clostridium botulinum* type A (CDC strain 5356). (A) Gram-stained smear from 3-day culture. (B) Phase-contrast view of wet-mount suspension from 4-day culture. Scale for both photomicrographs is indicated by bar in B.

C. botulinum was defined in 1953 as the species designation for all organisms known to produce botulinum neurotoxin and thereby having the capability of causing botulism in humans or animals (Prévot, 1953). This nomenclature eliminated the use of the name *C. parabotulinum* to distinguish the proteolytic group of toxigenic organisms from the nonproteolytic group. There had been a problem of other ambiguous uses of that species name. The unifying feature of the organisms known as *C. botulinum*, then, has been their toxigenicity. It will be seen that *C. botulinum*, as presently constituted, can be separated into four different species. In the investigation of some recent botulism incidents, the causative organisms have been found to be other established species of *Clostridium* that possess the ability to produce botulinum neurotoxin and to cause botulism. This illustrates the difficulty inherent in a nomenclature rigidly based on the ability to produce a toxin (Hatheway and McCroskey, 1989).

Thus, botulinum toxin can be produced by a variety of clostridia. The neurotoxins produced by various organisms can be differentiated serologically by specific neutralization into seven toxin types: A, B, C, D, E, F, and G. A given toxin type is not necessarily restricted to one phenotypic group of organisms. Regardless of serological type, the toxins are structurally and pharmacologically similar, causing the same flaccid paralysis of botulism in susceptible animal species.

II. TOXIN TYPES

Nine years after van Ermengem (1897) established that botulism was caused by a bacterial toxin, a second outbreak due to bean salad was investigated and confirmed in a similar manner by Landmann (1904) in Darmstadt, Germany. Leuchs (1910) compared the organisms isolated from the Belgian and the German outbreaks and, using the method of Kempner (1897) for preparing antitoxins against botulinum toxin, found that the toxins were serologically distinct, each being neutralized only by its homologous antiserum. Letter designations were not assigned to toxin types until Burke (1919) reported the results of studies on the characteristics and toxins of strains recovered from botulism outbreaks in the United States. Toxins produced by these organisms were identified as either type A or type B on the basis of neutralization with rabbit antitoxins. Although the van Ermengem and the Landmann strains were no longer available for testing with the typing reagents, it was apparent from the descriptions of the cultural characteristics of the organisms in the earlier literature that the former would correspond to type B and the latter to type A. Additional types were established as other organisms were recovered from incidents of botulism in animals and humans that produced toxins that did not correspond to existing types.

The toxin produced by organisms causing botulism in chickens in the United States (Bengtson, 1922) and in cattle in Australia (Seddon, 1922) was designated

as type C. Upon observing that antitoxin produced against the Bengtson strain neutralized the toxin of either strain, while antitoxin against the Seddon strain neutralized only toxin from the homologous strain, the former was designated C_α, and the latter strain, C_β (Gunninson and Meyer, 1929). Another outbreak in cattle in South Africa was caused by an organism subsequently designated type D because its toxin was serologically distinct from types A, B, and C (Meyer and Gunnison, 1928–29). Type E was established to designate the toxin type of organisms causing botulism in the United States and in the Soviet Union caused by consumption of fish (Gunnison et al., 1936–37). An outbreak of botulism caused by a liver paste in Denmark involved a sixth type of toxin, known as F (Moller and Scheibel, 1960). Finally, the toxin produced by an organism isolated from soil in Argentina was designated type G (Giménez and Ciccarelli, 1970b).

The distinction between the C_α and the C_β toxin types is a source of confusion. C_α strains produce C_1, a neurotoxin, and C_2, which is not a neurotoxin but is lethal in mice, ducks, and geese; congestion and hemorrhage in the lung and dripping of the nares are observed in birds injected with C_2 toxin, but paralytic signs of botulism are absent (Jensen and Duncan, 1980; Ohishi and DasGupta, 1987). C_β strains produce only C_2 toxin.

Thus, there are seven serologically differentiable types of botulinum neurotoxin. Although, in general, each type is serologically distinct, slight partial cross-neutralizations of the neurotoxins produced by types C and D strains occur because type C strains produce a minor amount of type D neurotoxin, and type D strains produce a trace of type C neurotoxin (Jansen, 1971a, b, 1987). Oguma et al. (1984) have also found evidence for common antigenic sites on types C and D toxin molecules. The initial studies of *C. botulinum* type F showed that its toxin was neutralized by a large excess of type E antitoxin (Moller and Scheibel, 1960; Dolman and Murakami, 1961). A later study indicates that the cross-neutralization between type E and type F is reciprocal (Yang and Sugiyama, 1975). There are also subtle differences between toxins produced by strains of the same toxin type (Ciccarelli and Giménez, 1971; Giménez and Ciccarelli, 1972; Hatheway et al., 1981). Strains have been found that produce mixtures of two types of toxin, A + F (Giménez and Ciccarelli, 1970a), A + B (Poumeyrol et al., 1983), and B + F (Hatheway and McCroskey, 1989).

III. PHYSIOLOGICAL AND GENETIC DIFFERENCES AMONG ORGANISMS PRODUCING BOTULINUM NEUROTOXIN

A. Physiological Groups

Since botulism had always been associated with meat products, as denoted by its name (derived from *botulus*, the Latin word for sausage), it was surprising that the illness in Darmstadt (Landmann, 1904) due to consumption of bean salad was also confirmed as botulism. Leuchs (1910) noted that the Ellezelles strain

(van Ermengem, 1897) and the Darmstadt strain were similar in their morphological and cultural characteristics but differed in their optimal temperatures for growth and toxin production; for the former it was 18–25°C, and for the latter, 35–37°C. Burke (1919) found that the strains of both toxin variants (type A and type B) of organisms isolated from botulism incidents in the United States resembled the Darmstadt strain, with 37°C as their optimum temperature for growth and toxin production. Bengtson (1924) found that all the American isolates of either toxin type were strongly proteolytic, i.e., they digested meat particles, casein, and coagulated egg albumin, while a European isolate that produced type B toxin clearly was not proteolytic. The latter corresponded very closely to the organism isolated and described by van Ermengem (1897) in all of its cultural characteristics.

In examining an international collection of strains, Gunnison and Meyer (1929) confirmed the findings of Bengtson (1924). They found that two of three European toxin type B strains were nonproteolytic, as well as all strains of toxin type C and one strain (the only one tested) of toxin type D. Although the type C and D strains are nonproteolytic (or weakly so according to some researchers), they are distinguishable from the nonproteolytic type B strains by their high optimum growth temperature of 40°C (Smith and Sugiyama, 1988).

Type E strains resemble European strains of toxin type B in that they are clearly nonproteolytic (Gunnison et al., 1936–37). Although they may grow well at 37°C, toxigenicity is more constant at room temperature, and they are capable of growing and producing toxin at lower temperatures than the proteolytic strains. Nonproteolytic type B strains have been shown to grow and produce toxin at temperatures as low as 3.3°C (Eklund et al., 1967).

The initial isolate of *C. botulinum* type F (Moller and Scheibel, 1960) was proteolytic and resembled the American strains of toxin types A and B (Burke, 1919; Bengtson, 1924). A strain isolated from soil in Argentina also belongs to the proteolytic group (Giménez and Ciccarelli, 1968). Toxin type F strains isolated from marine sediments from the northwest coast of the United States (Eklund et al., 1967) and from the second outbreak of human type F botulism (Midura et al., 1972) are nonproteolytic, resembling the European type B strains (van Ermengem, 1897; Bengtson, 1924; Gunnison and Meyer, 1929) and the type E strains (Gunnison et al., 1936–37).

The type G organism isolated from soil in Argentina (Giménez and Ciccarelli, 1970b) is considerably different from the organisms discovered earlier that produce toxins of types A, B, C, D, E, and F. The latter all ferment glucose and may ferment several other carbohydrates; they also produce lipase, which is detectable on egg yolk agar (Cato et al., 1986). The type G organism is asaccharolytic and shows no reaction on egg yolk agar.

The various neurotoxigenic organisms described above are designated as *C. botulinum* and are divided, according to *Bergey's Manual* (Cato et al., 1986), into four groups. The distinguishing features are listed in Table 1.

Table 1 Characteristics of Organisms Capable of Producing Botulinum Neurotoxin[a]

	Groups					
	I	II	III	IV[b]	*C. butyricum*	*C. baratii*
Toxin types	A,B,F	B,E,F	C,D	G	(E)	(F)
Proteolysis	+	−	−	+	−	−
Liquefaction of gelatin	+	+	+	+	−	−
Fermentation of						
Glucose	+	+	+	−	+	+
Fructose	±	+	±	−	+	+
Mannose	−	+	+	−	+	+
Maltose	±	+	±	−	+	+
Sucrose	−	+	−	−	+	+
Trehalose	−	+	−	−	+	−
Lipase	+	+	+	−	−	−
Lecithinase	−	−	−	−	−	+
Metabolic acids[c]	A,iB,B,iV PP	A,B	A,P,B	A,iB,B,iV PA	A,B	A,B
Optimal growth temperature	35–40°C	18–25°C	40°C	37°C	30–37°C	30–45°C
Minimum growth temperature	10+°C	3.3°C	15°C		10°C	
Spore heat resistance (temp./D-value)	112°C/ 1.23	80°C/ 0.6–1.25	104°C/ 0.1–0.9	104°C 0.8–1.12		
Similar atoxic organism	*C. sporogenes*	(no species name assigned)	*C. novyi*	*C. subterminale*	(all typical strains)	(all typical strains)

[a] For biochemical reactions: +, all strains are positive; −, all strains are negative; ±, some strains are positive and some are negative. For temperature values, none are listed if not readily available in the literature.

[b] The name *Clostridium argentinense* has been proposed for group IV (Suen et al., 1988a).

[c] Metabolic acids: A, acetic; P, propionic; B, butyric; iB, isobutyric; iV, isovaleric; PP, phenylpropionic (hydrocinnamic); PA, phenylacetic.

Group I. The organisms typified by toxin type A strains are proteolytic, produce isobutyric, isovaleric, and beta-phenylpropionic (hydrocinnamic) acids, have an optimal growth temperature of 37°C and spores with a high heat resistance, and produce toxin of either type A, B, or F. Types A and B strains commonly produce 10^6 mouse lethal doses (LD) of toxin per ml in cultures;