INFECTION AND IMMUNITY, Jan. 1974, p. 15-19
Vol. 9, No. 1

Isolation of *Clostridium absonum* and Its Cultural and Biochemical Properties

M. HAYASE, N. MITSUI, K. TAMAI, S. NAKAMURA, AND S. NISHIDA

Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Japan

Received for publication 5 September 1973

A new procedure for isolation of *Clostridium absonum* was devised. Sixty-seven strains of *C. absonum* were isolated from 135 soil samples, but no strain of *C. absonum* could be found from human fecal samples. The lecithinase, hemolysin, and lethal toxin in the culture filtrates of this species exhibited low avidity for *C. perfringens* type A antitoxin. The three activities were inseparable by the present method of purification. A reinvestigation of biochemical properties revealed that incomplete suppression of lecithinase reaction by *C. perfringens* type A antitoxin and no fermentation of raffinose, melibiose, and starch are useful criteria to differentiate *C. absonum* from *C. perfringens*, and that positive, although weak, gelatin liquefaction and fermentation of trehalose are useful to differentiate it from *C. paraperfringens*.

Nakamura et al. (5) have proposed a new species named *Clostridium absonum* for a group of *C. perfringens*-like strains which were isolated by one of us from soil. *C. absonum* strains produced toxin(s), hemolysin(s), and lecithinase with low avidities to *C. perfringens* type A antitoxin. Considering the possible clinical importance of this species, we carried out investigations on the method of isolation and the biochemical and immunological properties of toxins produced by *C. absonum* strains.

MATERIALS AND METHODS

Strains. A total of 54 strains were used: 23 strains of *C. absonum* consisting of three strains used by Nakamura et al. (5) and 20 strains isolated by a new method described in Results; 21 strains of *C. perfringens* consisting of a stock strain, BP6K, and 20 strains isolated from soil by us; and 10 strains of *C. paraperfringens*, the biological properties of which have already been described (5).

Biochemical properties. Routine biochemical tests were carried out by the method of Sterne and van Heyningen (8). Gelatin liquefaction, however, was examined also by a modified method of the Virginia Polytechnic Institute (VPI) (2), which gave a more decisive result than did the routine method. The modified VPI method used is as follows. A 5-ml portion of gelatin medium in a tube (16.5 by 165 mm), consisting of 10% (wt/vol) gelatin, 0.5% proteose peptone, 1.0% (wt/vol) yeast extract (Difco), 0.1% sodium thioglycollate, 0.1% glucose, and 0.5% NaCl (pH 7.2), was inoculated with a 0.5-ml portion of a 15-h liver broth culture. The results obtained were read as described in the VPI manual (2). Stormy fermentation of milk was examined in milk medium consisting of 20 g of skim milk and 180 ml of distilled water. A 10-ml portion of the medium in a tube (16.5 by 165 mm) was inoculated with a 15-h liver broth culture and was observed for 3 weeks.

DNA-DNA homology. Deoxyribonucleic acid (DNA)-DNA homology was carried out by the same procedure as described by Nakamura et al. (5).

Lecithinase activity. Cooked meat broth containing 1% (wt/vol) fructose (6) was used for production of lecithinase. The activity of lecithinase was estimated by the method of van Heyningen (9).

Hot-cold hemolysin activity. Estimation of hot-cold hemolysin activity was by the method of Florence and Pillemer (1).

Lethal toxin. Lethal toxicity of the above-mentioned cooked meat broth culture was assayed by injecting a 0.4-ml amount of a 7- to 9-h culture filtrate into two mice intravenously. The minimal lethal dose (MLD) was read after 2 days of observation.

Estimation of protein. Protein concentrations were estimated by the method of Lowry et al. (3).

Avidity of C. absonum and C. perfringens culture filtrates to C. perfringens type A antitoxin. Avidity of *C. absonum* lecithinase to *C. perfringens* type A antitoxin was assayed as follows. Increasing amounts of the antitoxin contained in 0.05 ml were added to 0.4 ml of 7-h culture filtrates of *C. absonum* and of *C. perfringens*, and the mixtures were incubated for 20 min at 37 C. Then, 0.55 ml of 0.2 M borate buffer (pH 7.1) containing 0.01 M $CaCl_2$ and 2.0 ml of egg yolk solution (9) were added to make a total volume of 3 ml. The mixtures were incubated at 37 C for 60 min, and optical densities (470 nm) of increasing turbidity were read every 5 min during the ensuing 60 min.

The avidity of *C. absonum* lethal toxin for *C. perfringens* type A antitoxin was also examined in mice. Increasing amounts of the antitoxin contained in 0.05 ml were added to 0.4 ml of culture filtrates, and the mixture was incubated for 20 min at 37 C.

Then, 0.45 ml of the mixture was injected into two mice intravenously. The result was read after 2 days. The *C. perfringens* type A antitoxin serum (550 IU/ml) was purchased from Chiba Serum Institute, Chiba, Japan.

Oxygen-labile (OL-) hemolysin. PF medium consisting of 3% (wt/vol) proteose peptone (Difco), 0.25% fructose, 0.5% NaCl, and 0.1% sodium thioglycollate (pH 7.4) was used for the production of OL-hemolysin. The activity of OL-hemolysin in a 7-h culture filtrate was estimated against sheep blood by the method of Florence et al. (1). To examine avidities of the OL-hemolysins of *C. absonum* and *C. perfringens* to *C. perfringens* theta-antitoxin, the minimal dose of the antitoxin to neutralize 0.05 ml of the culture filtrates was assayed. The purified theta-antitoxin serum (618 U/ml) of *C. perfringens* was obtained from R. Murata, National Institute of Health, Tokyo, Japan.

Purification of toxin. A medium was devised for convenience of purification. It consisted of proteose peptone (Difco) (3 g), fructose (0.25 g), NaCl (0.5 g), sodium thioglycollate (0.1 g), $FeSO_4 \cdot 7H_2O$ (0.1 mg), $MnSO_4 \cdot 7H_2O$ (20 mg), $ZnSO_4 \cdot 7H_2O$ (0.6 mg), $MnCl_2 \cdot 4H_2O$ (0.4 mg), Na_2HPO_4 (0.57 g), KH_2PO_4 (0.14 g), thiamine-2 HCl (0.1 mg), pyridoxamine-2 HCl (0.1 mg), nicotinic acid (0.1 mg), calcium-pantothenate (0.1 mg), pimelic acid (0.1 mg), riboflavin (0.05 mg), biotin (0.0005 mg), and distilled water (100 ml) (pH 7.4). Six 1-liter Erlenmyer flasks containing a 900-ml volume of the above-mentioned medium were used for culture. Preculture with the same medium was repeated four times, and then a 9-ml amount of the fifth 5-h preculture was added to each 900 ml of the medium. The cultures were incubated for 7 h at 37 C. A total of 5,400-ml culture filtrates obtained were purified according to a slightly modified method of Mitsui et al. (4). The detailed procedure is also referred to in Results.

RESULTS AND DISCUSSION

A new procedure to isolate *C. absonum* was devised as follows. A pea-size sample was added into a tube (10 by 105 mm) containing 3.5 ml of a medium consisting of 2% (wt/vol) proteose peptone (Difco), 0.5% NaCl, 0.5% salicin, 0.1% agar, and 0.1% sodium thioglycollate (pH 7.2) and was incubated overnight. A loopful of inoculum from only those gas-producing cultures was plated on Nagler medium containing 0.1 mg of kanamycin per ml and 2 U of *C. perfringens* type A antitoxin per ml, which were demonstrated to be the minimal concentrations to suppress the lecithinase activities of *C. perfringens* and *C. paraperfringens* and to leave that of *C. absonum* uninfluenced (Table 1).

A distinctly lecithinase-positive colony on Nagler plate containing 2 U of alpha-antitoxin per ml was fished and transferred into a tube (10 by 105 mm) containing 3.5 ml of medium consisting of 2% (wt/vol) proteose peptone, 1% (wt/vol) lactose, 0.1% agar, and 0.1% sodium thioglycollate. After incubation at 37 C for 24 h, an inoculum was obtained only from the lactose-fermenting culture and was plated on human blood agar containing 1% (wt/vol) glucose. A fairly round, button-like colony surrounded by a distinctly hemolytic zone was fished out, and the strain obtained was examined for cultural and biochemical properties according to routine biochemical tests. Tests for raffinose fermentation and 2% gelatin liquefaction were included in the routine biological test. Practically, however, identification was based on five main criteria, viz., fermentation of lactose, immediate fermentation of salicin, no fermentation of raffinose, lecithinase poorly neutralized by *C. perfringens* alpha-antitoxin, and about 0.8 to 1.0 μm in cell width. A total of 67 strains of *C. absonum* were isolated from 135 soil samples, but none of the strains could be obtained from human feces. Routine biological tests on all of these isolates disclosed that their properties were entirely in agreement with those of *C. absonum*, as shown by Nakamura et al. (5).

To confirm the identity of the new isolates obtained by the new method to the type strain of *C. absonum*, three new isolates, Amano-8, CPP-8, and Maruhashi-3, were tested by DNA-DNA homology procedures against *C. absonum* HA 7103 (ATCC no. 27555), *C. paraperfringens* 2227 and *C. perfringens* BP6K. The three

TABLE 1. *Suppression of lecithinase reaction on Nagler plate agar by C. perfringens type A antitoxin*

Strain	*C. perfringens* type antitoxin in Nagler plate (U/ml)[a]					
	0	1	2	3	6	9
C. paraperfringens						
2227	++	+	+	±	±	−
3-1	++	+	+	−	−	−
H	++	+	+	+	−	−
C. absonum						
HA 7103	++	++	++	++	++	++
HA 9103	++	++	++	++	++	++
C. perfringens						
BP6K	++	++	+	+	−	−
S 008-1	++	+	−	−	−	−
S 7142	++	+	±	−	−	−
S 7137	++	+	±	−	−	−
S 7132	++	+	±	−	−	−

[a] Symbols: ++, lecithinase reaction around colony was positive; +, lecithinase reaction was positive only underneath colony; ±, lecithinase reaction was positive in trace underneath colony; −, lecithinase reaction was suppressed completely.

strains, Amano-8, CPP-8, and Maruhashi-3, exhibited high homologies of 76, 90, and 80%, respectively, to *C. absonum* HA-7103; they were related with low homologies of 31, 28, and 29%, respectively, to *C. paraperfringens* 2227, and with much lower homologies of 12, 11, and 12%, respectively, to *C. perfringens* BP6K.

Properties of C. absonum culture filtrates. Culture filtrates of 26 *C. absonum* strains randomly selected from our collection were examined for toxicity for mice. Toxigenicity ranging from 2.5 to 5 MLD/ml was demonstrated in 18 of them, and the remaining strains were nontoxigenic. Lecithinase strengths of the 26 culture filtrates ranged between 0.3 and 6.9 egg units (EU)/ml. The ratios of toxicity to EU seemed to be much lower in the culture filtrates of *C. absonum* than in those of *C. perfringens*.

To examine whether or not the lethal toxin, lecithinase, and hot-cold hemolysin in the culture filtrate of *C. absonum* strains are due to an identical toxin, as in *C. perfringens* alpha-toxin, a culture filtrate of *C. absonum* HA-7103 was preliminarily purified according to the procedure as shown in Fig. 1 and then further purified by Sephadex G-100 gel filtration. Lecithinase, hemolysin, and lethal toxin activities at each step of the purification and their individual purification ratios are shown in Table 2. The

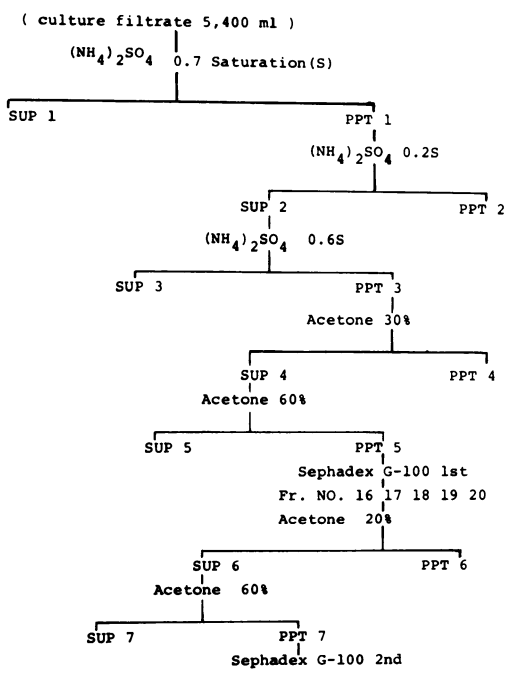

FIG. 1. *Procedure for purification of C. absonum HA 7103 culture filtrate. Abbreviations: SUP, supernatant; PPT, precipitate.*

three activities run in parallel at any fraction of purification. The greater part of OL-hemolysin was lost by the first 30% (vol/vol) acetone treatment. The precipitate (PPT) 5 fraction, when committed to Sephadex G-100 gel filtration, exhibited a peak of the remaining OL-hemolysin in the 13th fraction distinctly separate from the peaks of lecithinase, hot-cold hemolysin, and lethal toxin in the 18th fraction (Figure abbreviated). The final product (PPT 7), when committed to Sephadex G-100 gel filtration again, showed individual highest peaks of lecithinase, hot-cold hemolysin, and lethal toxin in the 16th fraction, whereas OL-hemolysin could not be detected anywhere (Fig. 2). The peaks of the three activities were not separable. Purification ratios of the lecithinase, hot-cold hemolysin, and lethal toxin in the 16th fraction were 3,333, 3,733, and 3,423, respectively. These findings imply that the three activities constitute an inseparable entity, at least by the present method.

Avidity of C. absonum lecithinase and OL-hemolysin to C. perfringens type A antitoxin. Since lecithinase production by *C. absonum* on Nagler plate was suppressed to some extent, although slightly, by the *C. perfringens* type A antitoxin, we carried out an experiment to examine whether or not much higher concentrations of *C. perfringens* antitoxin can neutralize the *C. absonum* lecithinase. Seven *C. absonum* strains and a reference strain of *C. perfringens*, BP6K, were used to obtain the lecithinase preparations. The lecithinase of *C. perfringens* BP6K with a strength of 2.1 EU/0.4 ml was completely inhibited in the presence of 1 U of *C. perfringens* type A antitoxin, whereas lecithinase activities ranging from 1.2 to 2.8 EU/0.4 ml of the seven *C. absonum* strains could not completely be inhibited even in the presence of 25 U of the antitoxin.

The avidity of the lethal toxin of *C. perfringens* type A antitoxin could neutralize 30 MLD of *C. perfringens* BP6K culture filtrate, whereas 1 U of the antitoxin could not neutralize 1 MLD of the culture filtrate of *C. absonum* (Table 3).

The OL-hemolysin of *C. absonum* HA 7103 was also demonstrated to exhibit the low avidity to *C. perfringens* theta-antitoxin (Table 4).

Reinvestigation of the biochemical properties of C. absonum and the related species. Twenty-one strains of *C. absonum*, 20 strains of *C. perfringens*, and 10 strains of *C. paraperfringens* were used. The main characters differentiating *C. absonum* from *C. perfringens* and *C. paraperfringens* are shown in Table 5.

TABLE 2. *Purification of C. absonum HA 7103 culture filtrate*

Fraction[a]	Hot-cold hemolysin activity		Lecithinase activity		Lethal activity	
	Hemolytic U/mg	Purification ratio	EU/mg	Purification ratio	MLD/mg	Purification ratio
Starting material	6.0	1	0.24	1	0.16	1
PPT 1	260.0	63	11.7	48	6.3	40
PPT 3	545.5	90	24.0	100	11.4	73
PPT 5	1,290.0	215	60.0	250	30.8	200
PPT 7	5,208.0	766	231.6	965	122.6	786
Sephadex G-100 fraction no. 16	22,400.0	3,733	800.0	3,333	534.0	3,423

[a] Refer to precipitate (PPT) fraction numbers in Fig. 1.

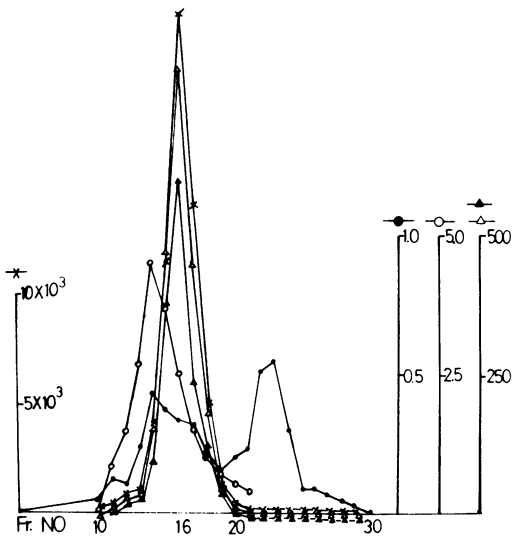

FIG. 2. *Sephadex G-100 gel filtration of C. absonum HA 7103 culture filtrate precipitate-7 fraction. A sample (4 ml) was passed over a column (40 by 2.2 cm) with 0.02 M borate buffer in 0.003 M $CaCl_2$ (pH 7.1). Flow rate was 10 ml/h; fraction volume was 5 ml. Symbols: O, total protein (mg); ▲, toxicity (MLD/mg); Δ, lecithinase activity (EU/mg); ×, hot-cold hemolysin activity (hemolytic U/mg); ●, optical density at 280 nm.*

TABLE 4. *Neutralization of OL-hemolysins of C. absonum and C. perfringens by C. perfringens theta-antitoxin*

No. of *C. perfringens* theta-antitoxin units added	OL-hemolysin[a]	
	C. absonum HA-7103 (3.7 OL-HU)[b]	*C. perfringens* BP6K (79.8 OL-HU)
61	±	
12	+	−
6	+	−
3	+	±
1		+

[a] Symbols: +, hemolysin was not neutralized; ±, hemolysin was partially neutralized; −, hemolysin was neutralized.

[b] OL-HU, oxygen-labile hemolysin unit.

The useful criteria to differentiate *C. absonum* from *C. perfringens* are as follows: *C. absonum* ferments salicin and cellobiose and does not ferment raffinose, inositol, melibiose, and starch. The main criteria to differentiate *C. absonum* from *C. paraperfringens* are as follows: *C. absonum* ferments trehalose, gives rise to stormy fermentation of milk, and digests gelatin.

All strains used of the three species fermented

TABLE 3. *Lethal toxin, lecithinase, and antitoxin equivalents of culture filtrates of C. absonum and C. perfringens*

Activities/0.4 ml	*C. perfringens* BP6K	*C. absonum*				
		Amano-8	Maruhashi-3	Kawai	Amano-5	Amano-6
Lethal toxin (MLD)	30	2	1	1	0	0
Lecithinase (EU)	ND[a]	1.8	1.3	ND	0.07	0.01
Antitoxin equivalents[b]	1	4	4	2	ND	ND

[a] ND, not done.

[b] Expressed as minimal units of *C. perfringens* type A antitoxin needed to neutralize lethal toxin in 0.4 ml of culture filtrate.

TABLE 5. *Criteria of biochemical properties for differentiation*

Criteria for differentiation	Number of test-positive strains		
	C. paraperfringens (10 strains)	*C. perfringens* (20 strains)	*C. absonum* (21 strains)
Salicin	10	4[a]	21
Cellobiose	10	3[a]	21
Raffinose	0	20	0
Inositol	0	20	0
Melibiose	7	19	0
Starch	7	20	0
Trehalose	0	16	20
Gelatin liquefaction			
2%	0	20	21
10%	0	20	5
10% (VPI)	0	20	21
Stormy fermentation	0	19	16

[a] Delayed fermentation.

glucose, maltose, lactose, sucrose, fructose, galactose, and mannose. None of the strains fermented sorbitol, mannitol, and inulin, produced indole, or digested coagulated albumen. A few strains of *C. perfringen* and *C. paraperfringens* fermented arabinose, xylose, and rhamnose.

Some criteria of the biochemical properties to differentiate *C. perfringens*, *C. absonum*, and *C. paraperfringens* from each other were added in this investigation. Nishida et al. (7), however, demonstrated that the biochemical properties of *C. perfringens* differed according to the conditions of heating used for their isolation. Recent investigation in our laboratory revealed also that fermentability of salicin, cellobiose, and inositol of *C. perfringens* differed in accordance with the conditions of preheating (S. Nakamura and S. Nishida, unpublished data); all of 10 *C. perfringens* isolates obtained from unheated soil samples could ferment inositol, whereas none of 14 isolates obtained from soil samples heated at 100 C for 1 h could ferment the sugar. The longer the preheating at 100 C, the greater was the number of strains fermenting cellobiose and salicin.

It seems reasonable, therefore, to remove the variable characters from the present criteria for differentiation.

ACKNOWLEDGMENTS

We thank R. Murata for a supply of purified *C. perfringens* theta-antitoxin and C. T. Huang for helpful comments.

LITERATURE CITED

1. Florence, B. R., and L. Pillemer. 1953. The separation of alpha toxin (lecithinase) from filtrates of *Clostridium welchii*. J. Immunol. **70**:533-537.
2. Holdeman, L. V., and W. E. C. Moore (ed.). 1972. Anaerobe laboratory manual. 2nd ed. Virginia Polytechic Institute, Blacksburg.
3. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
4. Mitsui, K., N. Mitsui, and J. Hase. 1973. *Clostridium perfringens* exotoxin. I. Purification and properties of α-toxin. Jap. J. Exp. Med. **43**:65-80.
5. Nakamura, S., T. Shimamura, M. Hayase, and S. Nishida. 1973. Numerical taxonomy of saccharolytic clostridia, particularly *Clostridium perfringens*-like strains: description of *Clostridium absonum* sp. n. and *Clostridium paraperfringens*. Int. J. Syst. Bacteriol. **23**:419-429.
6. Nishida, S., M. Murakami, and T. Yamagishi. 1962. Chopped meat broth as a medium for the production of toxins by clostridia. I. Production of toxins by *Clostridium welchii*. Jap. J. Microbiol. **6**:33-40.
7. Nishida, S., N. Seo, and M. Nakagawa. 1969. Sporulation, heat resistance, and biological properties of *Clostridium perfringens*. Appl. Microbiol. **6**:303-309.
8. Sterne, M., and W. E. van Heyningen. 1965. Bacterial and mycotic infections of man, 4th. ed. J. B. Lippincott Co., Philadelphia.
9. Van Heyningen, W. E. 1945. The biochemistry of the gas gangene toxins. I. Estimation of α-toxin of *Cl. welchii*, type A. Biochem. J. **35**:1246-1256.