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COMPLEMENTATION OF θ-TOXINOGENICITY BETWEEN MUTANTS OF TWO GROUPS OF *CLOSTRIDIUM PERFRINGENS*

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SUMMARY Eighty two θ^- or $\theta^\pm$ mutants were isolated from *Clostridium perfringens* by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants were classified into two groups (*a* and *b*) by the type of their complementation in θ-toxin production. Group *a* comprised 72 mutants which produce some factor which activates the 10 mutants in *b* group to form θ-toxin.

Of 30 food poisoning strains of type A *Clostridium perfringens* examined, 28 were θ^-, belonging to group *a*, and 2 mutants were θ^+, but easily segregated θ^- mutants belonging to group *a*.

INTRODUCTION

Results of kinetic studies on hemolysis by θ-toxin of *Clostridium perfringens*, suggested the possibility that hemolysis of red cells might be due to the successive attack of two or more components. If so, θ-toxin negative mutants may lack one of multi-components involved and some mutants may complement each other in the hemolysis. To test this, several θ-toxin and α-toxin negative mutants, which had been isolated for another purpose, were cross-streaked on sheep blood agar. Astonishingly enough, some mutants were complementary in θ-hemolysis. This paper describes results of studies on this complementation.

MATERIALS AND METHODS

1. *Bacterial strains*

Clostridium perfringens PB6K was a generous gift from Dr. Ryosuke Murata (National Institute of Health, Tokyo, Japan) and was used for isolation of mutants. Food poisoning strains were obtained by courtesy of Prof. Shoki Nishida (Kanazawa University, Kanazawa, Japan).

2. *Liquid medium*

Two g NaCl, 12.6 g $Na_2HPO_4 \cdot 12\,H_2O$, 0.1 g KH_2PO_4, 0.1 g thiogycolate and 10 g fructose were

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added to 1000 ml of 5% proteose peptone (DAIGO) which had previously been treated with acetone to remove cholesterol. For production of θ-toxin, an overnight culture of cells was inoculated into 15 volumes of fresh medium and incubated at 37 C for 7 hr, unless otherwise specified.

3. *Isolation of mutants*

1) Streptomycin resistant strains (sm^R) and rifampicin resistant strains (rf^R) were selected by the method of one step growth on Eiken Anaerobic Agar plates containing 1000 μg/ml streptomycin and 20 μg/ml rifampicin, respectively. None of the strains isolated were antibiotic-dependent.

2) For isolation of mutants of α-toxin a culture in the exponential growth phase was treated with 0.02% *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 37 C for 1 hr. Then cells were washed and grown in fresh liquid medium overnight. Then they were spread on egg yolk agar plates, and colonies giving a negative Nagler reaction were picked up.

3) For isolation of θ-negative mutants, cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosogunidine, as described above and then mutants were isolated by two procedures. In one, cells were grown overnight and then spread on sheep blood agar plates, and mutants were isolated from colonies which had no clear hemolytic halo inside of partial hemolytic zone due to α-toxin (Fig. 1). In the other procedure, cells were grown overnight and then spread on bovine serum agar plates and replica plates were made on the same medium. The original plates were overlayed with soft agar containing M/200 EDTA (α-toxin inhibitor) and bovine blood and incubated. Doubtful colonies were picked up from the replica plates and serially transferred on sheep blood agar plates for reconfirmation. Eighty-two θ-negative mutants were isolated. Their culture supernatants had less than 16 units of hemolytic activity, if any, whereas the parent produced 250–1000 units. Mutants not producing θ-toxin and leaky producers are expressed as θ^- and $\theta^\pm$, respectively. The characters of the representative mutants used in this study are listed in Table 1.

TABLE 1. *Characteristics of representative mutants used in this study*

Mutant	Toxinogenicity		Drug resistance		Classified Group
MLH10[a]	α^-	$\theta^\pm$	sm^S	rf^S	*a*
32	α^+	θ^-	sm^R	rf^S	*a*
46	α^+	$\theta^\pm$	sm^R	rf^S	*b*
300-2	α^+	$\theta^\pm$	sm^S	rf^R	*b*
300-11	α^-	$\theta^\pm$	sm^S	rf^R	*b*

[a] Strains of *Cl. perfringens* usually resist up to 200 μg/ml of streptomycin. Therefore, resistance and sensitivity to streptomycin were tested using a concentration of 1000 μg/ml.

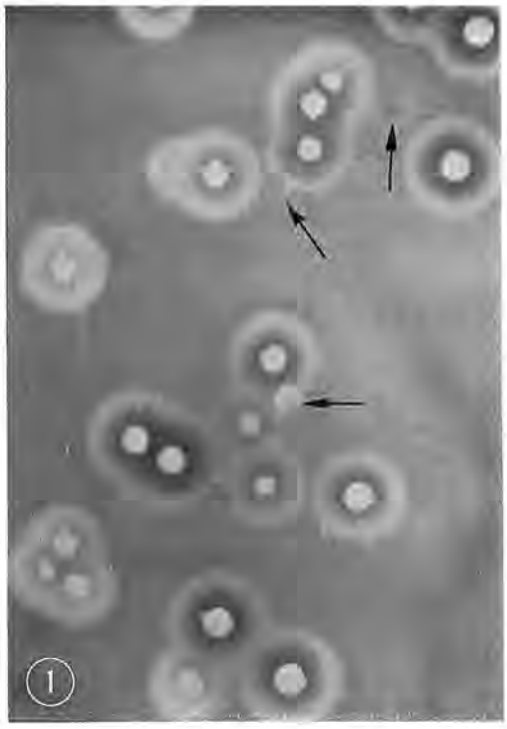

FIGURE 1. *Sheep blood agar plate showing parent and θ^- mutant colonies of Clostridium perfringens PB6K. The wide and hazy zones of incomplete hemolysis are due to α-toxin, and the narrow and clear haloes of complete hemolysis are due to θ-toxin. Arrows indicate θ^- mutant colonies.*

4. *Assay of hemolytic activity of θ-toxin*

The method used for assay of hemolytic activity of θ-toxin is a modification of that of Akama et al. (1969). Serial 1 : 2 diulutions of culture supernatants in PBS, pH 6.8 containing M/200 EDTA and M/200 cysteine were incubated for 15 minutes at 37 C, and then an equal volume of a suspension of sheep blood cells in PBS was added. The mixtures were incubated for 30 minutes more at 37 C. One

unit of θ-toxin was defined as the amount causing complete hemolysis of a 0.5% suspension of sheep blood cells. In some experiments the degree of hemolysis was graded as 0 to 4.

5. *Tests for bacteriocinogeny and sensitivity to bacteriocin*

Strains to be tested for bacteriocinogeny were stab-inoculated into Eiken Anaerobic Agar plates and incubated for 2 days. Then the agar surface was treated with chloroform vapor and covered with soft agar inoculated with the strains to be tested for sensitivity. Plates were incubated overnight, and inhibition zones around the stab-inoculated colonies were examined.

6. *Antitoxin to θ-toxin*

Partially purified anti-θ antitoxin (γ-globulin fraction) devoid of anti-α antitoxin was donated by Dr. Ryosuke Murata.

7. *Micellaneous*

Cysteine was a product of Wako Pure Chemical Industries, Ltd. Streptomycin and rifampicin were from Takeda Chemical Industries, Ltd. and Daiichi Pure Chemicals Co., respectively. Cholesterol was purchased from Nutritional Biochemicals Co. It was dissolved in a small volume of chloroform, diluted with ethanol and added to saline buffered at pH 7.2.

RESULTS

1. *Complementation of θ-toxinogenicity between two groups of θ-negative mutants*

Complementation in θ-toxin production was detected around mixed colonies on cross streaking some combinations of θ-negative mutants on sheep blood agar plates: *i.e.* on cross-streaking mutants 32 and 46, 32 and 300–2, and 46 and MLH 10, respectively, complete hemolysis due to θ-toxin was found around mixed colonies. However, no complementation was observed between mutants 46 and 300–2, or 32 and MLH 10. The complementation of all the θ-negative mutants (82 strains) isolated was tested using strains 32 and 46 as references. From the results the mutants were classified into two groups (*a* and *b*). Mutants with the same character as strain 32 were classified as group *a* and the others, including strain 46, as group *b*. Any combination of a mutant from group *a* and one from group *b* showed complementation of θ-toxin production, while combinations of two mutants from the same group did not. Thus, 72 of the 82 θ-negative mutants of the strain PB6K were classified as group *a* and 10 as group *b*.

The complementation of the two groups was examined using the supernatants of cultures grown in proteose peptone borth. Serial 1 : 2 dilutions of culture supernatants of *a* 32 θ^- sm^R and *b* 300–2 $\theta^\pm$ rf^R were made with saline containing M/200 EDTA and M/200 cysteine at pH 7.2. Checker board mixtures were made from them and hemolysis of sheep red cells by θ-toxin in them was examined. Strain *a* 32 produced no θ-toxin and strain *b* 300–2 gave a very low titer (8～16 units), and the titers of none of the tubes exceeded that of θ-toxin of strain *b* 300–2. The same negative results were obtained using several other mutants of the two groups. Complementation was only detected with a mixed culture of one strain of each group in proteose peptone

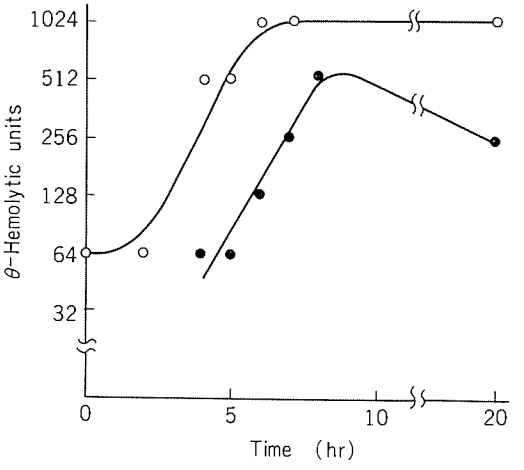

FIGURE 2. *Time course of production of θ-toxin in a culture of the parent PB6K and a mixed culture of a 32 θ^- and b 300–2 $\theta^\pm$.*

○———○, *Parent PB6K*; ●———●, Mixed *culture of a 32 θ^- and b 300–2 $\theta^\pm$.*

broth. Figure 2 shows the time course of θ-toxin production in a mixed culture of *a* 32 θ^- sm^R and *b* 300–2 $\theta^\pm$ rf^R when 0.3 and 0.7 ml of the respective overnight cultures were inoculated into 15 ml of fresh medium. As can be seen, the time course of θ-toxin production was quite similar to that of the parent strain PB6K. The above ratio of inocula gave the maximal titer of θ-toxin in the mixed culture. However, it was later found that *a* 32 and *b* 300–2 are bacteriocinogenic and each is partially sensitive to the bacteriocin of the other. When more resistant clones were selected from the partial inhibition zones produced by the bacteriocin of the other strain and examined for complementation in the mixed culture, inocula of the same size gave maximal θ-toxin production.

The hemolysin produced by the complementation appeared to be θ-toxin judging from the nature of the hemolytic zones around mixed colonies and from the fact that it was not inhibited in a test tube by EDTA. Further experiments were carried out to prove that it was θ-toxin: 1) As shown in Table 2, when the supernatant of a mixed culture was kept

TABLE 2. *Reactivation of θ-hemolytic activity in culture supernatant by cysteine*

Culture supernatant	Hemolytic unit	
	--	with cysteine
PB6K (after 1 day)	1024	1024
PB6K (after 2 months)	4	512
Mixed culture of *a* 32 θ^- and *b* 46 $\theta^\pm$ (after 1 day)	128	128
Mixed culture of *a* 32 θ^- and *b* 46 $\theta^\pm$ (after 2 months)	2	128

TABLE 3. *Inhibition of θ-hemolysis by cholesterol*

		Dilution of cholesterol solution						
		2	4	8	16	32	64	—
Dilution of culture sup. of parent PB6K	16	0	1	4	4	4	4	4
	32	0	0	1	4	4	4	4
	64	0	0	0	1	4	4	4
	128	0	0	0	0	3	4	4
	256	0	0	0	0	0	2	4
	512	0	0	0	0	0	0	4
	1024	0	0	0	0	0	0	4

		Dilution of cholesterol solution						
		2	4	8	16	32	64	—
Dilution of culture sup. of mixed culture of *a* 32 and *b* 300–2	16	0	0	4	4	4	4	4
	32	0	0	1	4	4	4	4
	64	0	0	0	0	3	4	4
	128	0	0	0	0	0	3	4
	256	0	0	0	0	0	0	3
	512	0	0	0	0	0	0	1
	1024	0	0	0	0	0	0	0

0.02% cholesterol solution was diluted serially and 0.1 ml of each dilution was added to 1.0 ml of diluted culture supernatant. After 30 minutes at 37 C 1.0 ml of 1% sheep blood cell suspension was added. Values show the degrees of hemolysis after incubation at 37 C for 30 minutes, graded as 0 to 4.

TABLE 4. *Neutralization of θ-hemolysis by anti-θ antitoxin*

		Dilution of antitoxin					
		400	800	1600	3200	6400	—
Dilution of culture sup. of parent PB6K	8	0	0	2	4	4	4
	16	0	0	0	3	4	4
	32	0	0	0	0	4	4
	64	0	0	0	0	1	4
	128	0	0	0	0	0	4
	256	0	0	0	0	0	2

		Dilution of antitoxin					
		400	800	1600	3200	6400	—
Dilution of culture sup. of mixed culture of *a* 32 and *b* 300–2	8	0	0	2	4	4	4
	16	0	0	0	3	4	4
	32	0	0	0	0	3	4
	64	0	0	0	0	0	4
	128	0	0	0	0	0	2
	256	0	0	0	0	0	0

Volume of 0.5 ml of serially diluted antitoxin were added to 0.5 ml of diluted culture supernatant. After 30 minutes at 37 C, 1.0 ml of 1% sheep blood cell suspension was added. Results are expressed as in Table 3.

in a Petri dish in a sealed vessel for 2 months in a cold room its hemolytic activity decreased from 128 to 2 units. The activity was restored by addition of M/200 cysteine. 2) The hemolytic activity was completely inhibited by cholesterol, as shown in Table 3. 3) As shown in Table 4, the γ-globulin fraction of anti-θ serum, devoid of anti-α antitoxin, neutralized the hemolytic activity completely. These results indicate that the hemolytic activity produced by the complementation was due to θ-toxin.

2. *No genetic transfer in the complementation*

To see whether the complementation was caused by genetic transfer, a mixed culture of *a* 32 θ^- sm^R and *b* 300–2 $\theta^\pm$ rf^R was spread on a blood agar plate to give about 1000 colonies. Many of these colonies were surrounded by clear haloes of θ-hemolysis. These colonies were printed on two velveteens and each was printed again on blood agar plates containing streptomycin (1000 μg/ml) or rifampicin (20 μg/ml). After incubation, no colony on either type of plate showed a clear halo of θ-hemolysis. Colonies on these plates were replicated on blood agar plates without antibiotics, and again no clear halo of θ-hemolysis was found around the colonies.

3. *No requirement of direct contact between mutants for complementation*

No genetic transfer was shown in the complementation, so next the problem of whether direct contact between the mutants was necessary for complementation was investigated, as follows.

Two blood agar plates were streaked with lines of *a* 32θ^- sm^R and *b* 300–2 $\theta^\pm$ rf^R, respectively. Then, each was covered with a membrane filter (Fuji-film microfilter No. 45, bacteria tight) which does not hemolyze sheep red cells on the agar plate. The other mutants (*b* 300–2 $\theta^\pm$ rf^R and *a* 32 θ^- sm^R, respectively) were streaked on top of the membrane in a line at right angles to the first streak on the

agar, but separated from it by the membrane. After incubation overnight, the membranes were carefully removed, and hemolysis was found at the intersection of the streaks on each plate. Each streaked culture on the plate was replicated on blood agar containing rifampicin or streptomycin to allow the cells inoculated on the membrane to grow and to kill the cells inoculated on the agar. After incubation, no colony was found on either type of replica plate. Thus the hemolysis observed on the plate was evoked by θ-toxin produced by complementation through the membrane.

A mixture of cultures of *a* 32 θ^- sm^R α^+ and *b* 300–11 $\theta^\pm$ rf^R α^- (the latter is an α-toxin negative derivative of *b* 300–2 $\theta^\pm$ rf^R) was spread on blood agar plates to give about 100 colonies. After incubation, some θ-hemolytic colonies formed were fused colonies of mutants

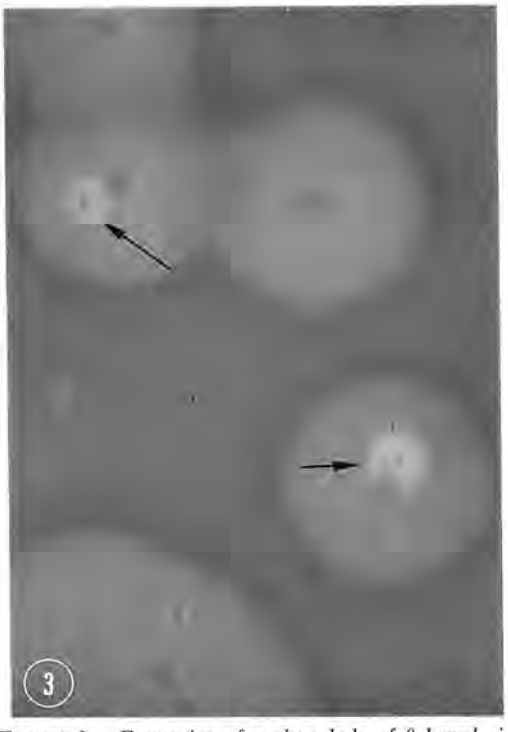

FIGURE 3. *Formation of a clear halo of θ-hemolysis by complementation between two mutants. Arrows indicate colonies of b 300–11 $\theta^\pm$ rf^R α^- surrounded by complete hmolysis where there is a very close colony of a 32 θ^- sm^R α^+.*

of both groups, because they showed discoloration due to α-toxin and because they gave θ-negative colonies after replication on blood agar containing rifampicin (group *b*) or streptomycin (group *a*). However, there were some α-toxin negative colonies (group *b*) surrounded by θ-hemolysis, and these were always found very close to α-toxin positive colonies (group *a*), as shown in Fig. 3. The distance between two such colonies was up to 1 mm. A sterile glass capillary was inserted into the agar in the region where the colonies were closest together, then the capillary was sticked into another sterile blood agar plate using a micromanupilator under the low power of a microscope. This plate was then incubated. No colonies grew. The same experiment was carried out using *a* MLH 10 $\theta^\pm$ α^- and *b* 46 $\theta^\pm$ α^+ rf^R. No α-toxin negative colonies (group *a*) gave θ-hemolysis on blood agar. Haloes due to θ-hemolysin were always found around α-toxin positive colonies (showing discoloration of red cells by α-toxin). Some of them were mixed colonies, which gave colonies of group *a* and *b* after restreaking on another plate. Others gave only group *b* colonies after restreaking, and these were always found very close to group *a* colonies. These results indicate that the complementation dose not require the direct contact of cells of the two groups and that cells of group *b* seem to produce θ-toxin after activation by some product diffusing from group *a* cells.

To observe the development of the hemolytic zone between the colonies of mutants of group *a* and *b*, a blood agar plate was stab-inoculated with a loop of a heavy inculum. Group *a* cells were inoculated at the center and four stabs of group *b* cells were made round the central inoculum, very close to it. After incubation for 5 hr in an anaerobic jar, the plate was examined for initial signs of hemolysis. The group *b* colony separated by about 1 mm showed the beginning of θ-hemolysis at the edge closest to the group *a* colony. The hemolytic zone was lentiform

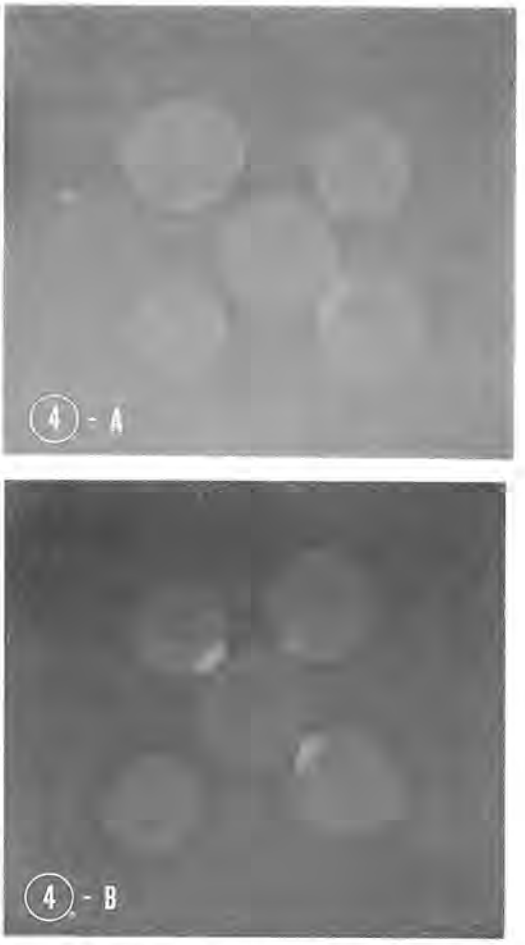

FIGURE 4. *Clear hemolytic zone between colonies of a MLH* 10 $\theta^{\pm}$ and *b 300–11* $\theta^{\pm}$ α^{-}. *A, 5 hr incubation*; *B, 10 hr incubation.*

after 5 hr incubation as shown in Fig. 4A, and another plate, similarily prepared but incubated for 10 hr, showed more developed hemolytic zones as can be seen in Fig. 4B.

4. *Theta-toxin negative character of food poisoning stains of type A Clostridium perfringens*

According to Hobbs et al. (1953) most type A food poisoning strains are devoid of θ-toxin. To clarify whether they belong to group *a* or *b* or whether they are completely negative and do not belong to either group, thirty strains isolated during outbreaks of food poisoning in the Tokyo, Osaka and Shizuoka districts were examined. The complementation in θ-toxin production was examined by cross streaking using *a* 32 θ^{-} sm^{R} and *b* 46 $\theta^{\pm}$ sm^{R} as references.

Fifteen of the 30 strains were found to belong to group *a*.

Two of the 30 were θ-toxin positive, but the clear haloes around the mixed colonies after cross-streaking with *b* 46 $\theta^{\pm}$ sm^{R} were slightly wider than those formed with each strain alone. Therefore, the pure cultures were each spread on blood agar plates to give about 100 colonies. Among the colonies grown on the plates a few θ-negative colonies were found. One of them had been isolated and serotyped in the Public Health Laboratory of the Osaka City according to the method of Hobbs et al. (1953). So the θ-positive and θ-negative clones were also serotyped by courtesy of Dr. Akira Yasukawa at the Public Health Laboratory of Osaka City, and both were found to belong to type 6, like other strains isolated in the same outbreak of food poisoning. The hemolytic colonies were picked up, and spread on blood agar plates, and again a few θ-negative colonies were found on each plate. These θ-negative clones were also found to belong to group *a*.

Four of the remaining 13 strains showed no complementation. Before they could be regarded as completely θ-negative, their bacteriocinogeny and sensitivity to the bacteriocin of PB6K had to be tested. No bacteriocinogeny could be detected with PB6K as indicator, but they were sensitive to the bacteriocin of PB6K. The plates were incubated until the resistant mutants formed colonies in the inhibition zones. These resistant mutants were picked up, purified, and tested for complementation. All these mutants showed complementation with *b* 46 $\theta^{\pm}$ sm^{R}. Thus they also belong to group *a*.

When the remaining 9 strains were cross-streaked with *b* 46 $\theta^{\pm}$ sm^{R}, the mixed colonies did not show clear haloes, but the colonies of

b 46 $\theta^{\pm}$ sm^R were very thin at the portion very close to the intersection and these thin colonies showed hemolysis. Two ramdomly selected strains were tested for bacteriocinogeny for *b* 46 $\theta^{\pm}$ sm^R and for sensitivity to the bacteriocin of the latter. Both strains and *b* 46 $\theta^{\pm}$ sm^R were partially sensitive to bacteriocin of the other strains. Pure cultures were prepared from the partial inhibition zones of each strain and complementation was retested. Results showed that both strains also belonged to group *a*. Thus, 30 food poisoning strains were shown either to belong to the θ-negative group *a* or if θ-hemolytic, to segregate θ-negative mutants belonging to group *a* in a very high incidence.

DISCUSSION

Strains of *Clostridium perfringens* type A are well-known to produce two kinds of hemolysins active against sheep red cells, α-toxin and θ-toxin. Theta toxin causes complete hemolysis, even in presence of EDTA, an inhibitor of α-toxin, and it gives narrow clear haloes around colonies on sheep blood agar. In contrast α-toxin causes incomplete hemolysis and gives wide hazy zone at 37 C. These criteria were routinely used to identify hemolysis due to θ-toxin produced by the complementation. The hemolysins also satisfied other criteria necessary for identifying θ-toxin: inactivation by oxygen reactivation with cysteine, complete inhibition by a low concentration of cholesterol and complete neutralization by antitoxin. These results indicate that the hemolysin produced by the complementation was actually θ-toxin.

In the complementation, no genetic transfer took place and θ-toxin could be produced without direct contact between mutants. Group *b* cells produced θ-toxin after receiving some diffusible product from group *a* cells. However, the exact mechanism involved has not yet been clarified.

The hemolysin of *Streptococcus zymogenes* was demonstrated to be complemented by two appropriate negative mutants (Granato and Jackson, 1969) and the complementation was performed by merely mixing the culture supernatants of the two mutants. The authors postulated a similar mechanism to that observed with θ-toxin but results so far have not supported this.

The food poisoning strains of type A *Cl. perfringens* are mostly devoid of θ-toxin, as described by Hobbs et al. (1953). Most of the strains tested in this study were isolated after at 100 C for 1 hr. Some were isolated directly and serologically identical strains to these were also isolated after the heating procedure. Therefore, the food poisoning strains used in this study should be characterized to be heat resistant spore-formers. On the other hand, Hall et al. (1963) and Sutton and Hobbs (1965) reported food poisoning due to strains which were not heat resistant and these strains were mostly θ-toxin producers. From these results, it seems that loss of θ-toxinogenicity in heat resistant food poisoning strains has no relation to enterotoxin production but may have some causal relation to heat resistant spore formation.

ACKNOWLEDGEMENTS

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