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COMPLEMENTATION CHARACTERISTICS OF NEWLY ISOLATED MUTANTS FROM TWO GROUPS OF STRAINS OF *CLOSTRIDIUM PERFRINGENS*

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SUMMARY 1. Mutants of *a* group were converted to *b* group by a second NG treatment. The resulting *b* group mutants could not produce the marker products that had been lost on the first NG treatment but could produce the others by complementation.

2. Mutants of *b* group were converted to constitutive mutants by a second NG treatment. No back mutation from *b* group was observed.

INTRODUCTION

Complementation in θ-toxin production was observed when two groups (*a* and *b*) of θ^- mutants of *Clostridium perfringens* PB6K were cocultured on blood agar plates or in proteose peptone broth in this laboratory (Higashi et al., 1973). It was found that the complementation took place without genetic transfer and that θ-toxin was produced by *b* group mutants by transfer of a stimulating substance from *a* group mutants (Higashi et al., 1973; Higashi et al., 1976). Further studies on this phenomenon revealed that respective negative mutants of λ-toxin (rennet-like protease), κ-toixn (collagenase) and hemagglutinin (HA) could also be classified into two groups (Higashi et al., 1976; Tatsuki et al., 1981). The *b* group mutants were pleiotropic and were always devoid of the four extracellular products irrespective of which marker was used for selection, except for κ^- because fewer plates were used. On the other hand, the *a* group mutants differed depending on the markers used for selection (Tatsuki et al., 1981).

To gain more insight into the genetic difference between *a* and *b* group mutants, attempts were made to obtain further mutants from these mutants. This paper describes the characters of the newly isolated mutants.

MATERIALS AND METHODS

1. *Bacterial strains*

Clostridium perfringens strain PB6K was used as

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a parent strain, and *a*M4–1 (θ^-, λ^-, κ^-, HA$^-$) and *b*300–11 ($\underline{\theta}^-$, $\underline{\lambda}^-$, $\underline{\kappa}^-$, $\underline{\text{HA}}^-$) isolated previously in our laboratory were used as standard strains of group *a* and *b*, respectively, for analysis of the complementation characters of the mutants. A bar " _ " under a marker of *b* group strains mean the ability to produce the extracellular products by complementation with *a* group strains. Strain *b*M2–11 ($\underline{\theta}^-$, $\underline{\lambda}^-$, $\underline{\kappa}^-$, $\underline{\text{HA}}^-$) was used together with *b*300–11 to isolate further mutants. The typical group *a* mutants strain *a*32 (θ^-, λ^+, κ^+, HA$^-$) and *a*F11 (θ^+, λ^-, κ^+, HA$^+$) were used for isolation of new type mutants.

2. *Agar plates*

Sheep blood agar, TEP anaerobe agar (Eiken Chem. Co., Ltd) containing 2% sodium caseinate and TEP agar containing 0.3% powder of native bovin Achilles tendon were used for qualitative examinations of θ-, λ- and κ-toxin activities, respectively.

3. *Liquid media*

The media used for productions of θ- and λ-toxins and HA were proteose peptone broth (Higashi et al,. 1973), filtrates of TEP anaerobe agar medium through filter paper to remove agar granules and Trypticase Soy Broth containing 1% frutose instead of glucose described in the previous report (Tatsuki et al., 1981), respectively.

4. *Quantitative assays for θ- and λ-toxins and HA*

Assays for θ-toxin and HA were made essentially by the methods of Akama et al., (1969) and Rood et al., (1974), respectively. The assay methods for rennet-like protease was improved for quantitative use in a liquid reaction mixture. The reaction mixtures (3 ml) contained final concentrations of 1 m*M* $CaCl_2$, 7.5 m*M* cysteine, 0.1 *M* imidazole-HCl buffer, pH 6.8, 0.3 vol. of an appropriate dilution of enzyme sample, and 0.2% sodium caseinate, previously boiled for 10 min. After incubation for 2 h at 37 C, the trubidity reaction mixtures was quickly measured at 550 nm.

5. *Isolation of mutants*

A late-log culture in liquid TEP anaerobe medium was treated with N-methly-N'-nitro-N-nitrosoguanidine (NG) at a final concentration of 0.02% at 37 C for 1 h. The cells were washed and grown overnight in fresh liquid TEP anaerobe medium at 37 C. Then appropriate dilutions were spread on blood agar or 2% sodium caseinate-containing TEP agar, and cultured overnight anaerobic conditions at 37 C.

6. *Complementation of θ-toxin production through a cellophane membrane*

A small loopful of a culture of *b*300–11 was spotted onto a blood agar or a casein-TEP anaerobe agar plate and the spot was covered with a single sheet of sterile open Visking membrane 24/32 (M.W. cut off. 10,000–20,000) or Spectra / Por 6 membrane (M.W. cut off. 1,000–2,000). Proteose peptone soft agar containing the parent strain PB6K or a *a* group mutant was dropped on top of the membrane. After incubation under anaerobic conditions, complete θ-hemolysis of the blood agar or a turbid zone of casein digests on a casein-TEP anaerobe agar was found under the membrane sheet where the spotted strain grew.

RESULTS

1. *Further mutation in a group strains*

Strain *a*32 (θ^-, λ^+, κ^+, HA$^-$) was treated with NG and 25 λ^- mutants were isolated on casein-TEP anaerobe agar. Five of them were pleiotropic negative mutants (θ^-, $\underline{\lambda}^-$, $\underline{\kappa}^-$, HA$^-$), and in complementation tests they behaved as *b* group mutants with regard to λ and κ production, but did not produce θ-toxin or HA in mixed culture with *a* group strains devoid of θ and / or HA. Results on a representative strain, *b*32–C5, are shown in Table 1 and Fig. 1b. The other 20 λ^- mutants still retained the character of *a* group.

In another experiment, *a*F11 (θ^+, λ^-, κ^+, HA$^+$) was treated in the same way and 10 θ^- mutants were isolated on sheep blood agar. Two of them had lost the character of *a* group and gained the character of *b* group ($\underline{\theta}^-$, λ^-, $\underline{\kappa}^-$, $\underline{\text{HA}}^-$). One of the mutants was designated as *b*F11–T23; it could produce θ, κ and HA but not λ by complementation, as shown in Table 1 and Fig. 1c.

From these data, the stimulating substance produced by *a* group strains was named *a*-substance and *b* group strains were considered as a^-.

TABLE 1. *Phenotypic and complementation characters of representative mutants*

Strain	Phenotype				Complementation			
	HA	θ	λ	κ	HA	θ	λ	κ
PB6K	+	+	+	+				
*a*M4-1	−	−	−	−	a[a]	a	a	a
*b*300-11	−	−	−	−	b[b]	b	b	b
*a*32	−	+	+	−	a			a
*b*32-C5	−	−	−	−	−[c]	b	b	−
32-C5 R3	−	+	+	−	−			−
*a*F11	+	+	−	+	b	b	a	b
*b*F11-T23	−	−	−	−	b	b	−	b
F11-T23 R1	+	+	−	+			−	

[a] Complementation with *b*300-11.
[b] Complementation with *a*M4-1.
[c] No complementation with either strain *a*M4-1 or *b*300-11.

TABLE 2. *Characteristics of b mutants and their derivatives*

Mutant	Phenotype				Complementation			
	θ	λ	κ	HA	θ	λ	κ	HA
*b*300-11	−	−	−	−	b	b	b	b
C320	−	−	−	−	−[a]	−	(b)[b]	−
C350	−	−	−	−	(b)	−	−	−
C353	−	−	−	−	b	−	b	−
C365	−	−	−	−	b	−	b	b
*b*M2-11	−	−	−	−	b	b	b	(b)
C331	−	−	−	−	b	−	b	(b)
C340	−	−	−	−	−	−	−	−
R1	+	+	+	±				
R3	±	+	±	+	(b)		(b)	
R4	+	+	+	−				−

[a] No complementaion with either strain *a*M4-1 or *b*300-11.
[b] Leaky and weak nature of group *b* in the complementation test.

2. *Further mutations in b group strains*

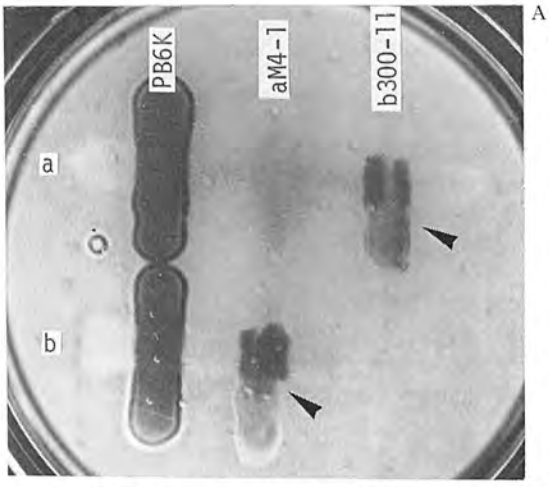
Since *b* group strains were considered to be in a repressed state in the absence of *a*-substance, a mutation in the regulation system should lead the mutants to be constitutive producers.

When NG-treated cultures of *b*300-11 and *b*M2–11 were spread on casein-TEP agar to give 10^3—10^4 colonies per plate and a total of 10^6 colonies were examined, 3 λ^+ colonies (R1, R3 and R4) were isolated from *b*M2–11, but none from *b*300–11. Examination of other markers revealed that R1 and R3 also recovered θ, κ and HA producing ability and R4 recovered θ and κ but not HA, as shown in Table 2. The results showed that none of the 3 mutants recovered the a^+ character when tested for complementation with *b* group strains through cellophane membranes on assay plates. These data indicate that all 3 mutants had a mutation in the regulator gene and hence that they had became constitutive. The HA^- character of R4 might have been due to an aditional mutation in the structural gene of HA.

Similarly *b*32–C5 and *b*F11–T23, described above, were treated again with NG and 10^6 colonies of each were examined, the former on casein-TEP agar plates and the latter on sheep blood agar plates. From the former 3 λ^+ mutants (R1, R2 and R3) were isolated and these had recovered κ-toxin producing ability. From the latter, only one θ^+ mutant (R1) was obtained and it had recovered κ-toxin and HA producing abilities as shown in Table 1 and Fig. 1. Tests for the a^+ character gave negative results. These data are quite consistent with those on M2–11 R1 and R3. The mutants 32–C5 R1, R2, R3 and F11–T23 R1 had a mutation in regulator genes and became constitutive except for the extracellular products, that had been lost on isolation of their *a* group parents.

Direct back mutation from *b*300–11 and *b*M2–11 to *a* group strains such as PB6K was tried, but without success, although as many as 10^6 colonies of each were examined for the λ^+ character on casein TEP agar.

Standard strains, *b*300–11 and *b*M2–11, of group *b* mutants were treated with NG in the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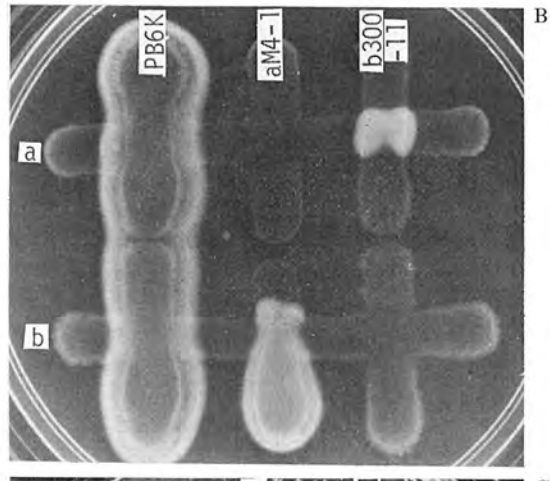
aM4-1
b300-11
a
b
A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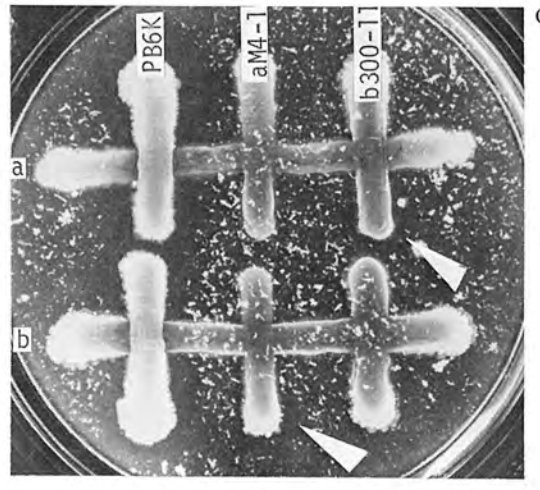
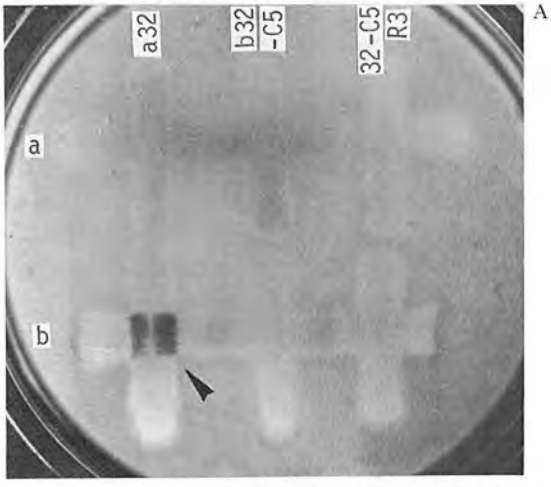
a
b
B


PB6K
aM4-1
b300-11
a
b
C

1a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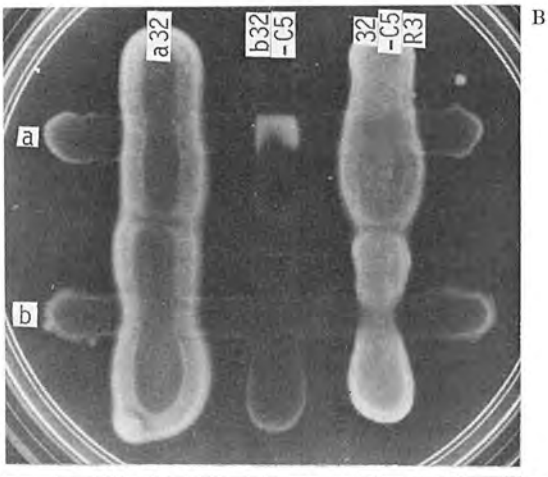
b32
-C5
32-C5
R3
a
b
A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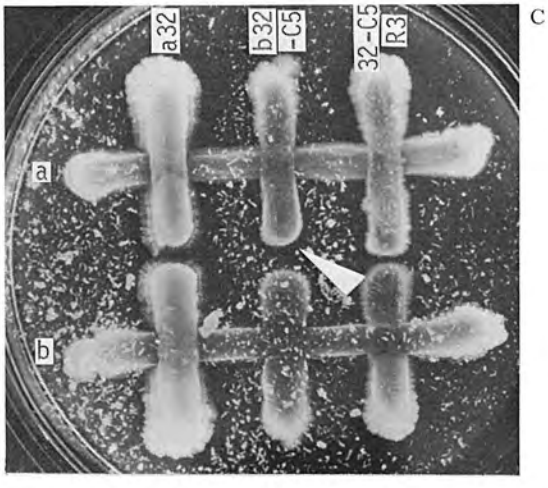
b32
-C5
32
-C5
R3
a
b
B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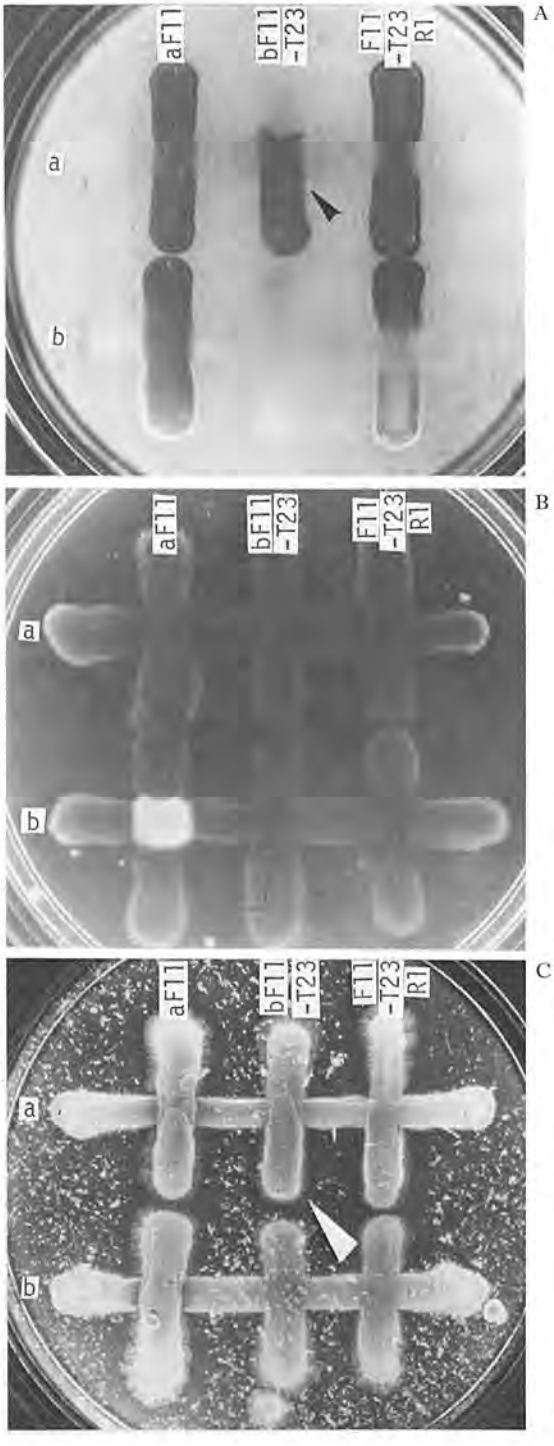
b32
-C5
32-C5
R3
a
b
C

1b



1c

◀

FIGURE 1. Complementation of *θ*-toxin production on blood agar (A), *λ*-toxin (rennet-like protease) on casein-TEP anaerobe agar (B), and *κ*-toxin (collagenase) on bovine Achilles tendon-TEP agar (C) after cross-streak culture against the standard strains *a*M4–1 and *b*300–11. Arrows indicate that the complementation has occurred.

FIGURE 1a. The parent strain PB6K, streaked as a control, and the standard strains *a*M4–1 and *b*300–11 were cross-streaked against *a*M4–1 and *b*300–11, respectively, on three kinds of agar plates cultured under anaerobic conditions, for three days on bovine Achilles tendon-TEP agar for *κ*-toxin production and overnight in other cases.

FIGURE 1b. Strain *a*32 and its derivatives *b*32-C5 and 32-C5 R3 were cross-streaked against *a*M4–1 and *b*300–11, respectively, on three kinds of agar plates. Other procedures were as for Fig. 1a.

FIGURE 1c. Strain *a*F11 and its derivatives, *b*F11–T23 and F11–T23 R1, were cross-streaked against *a*M4–1 and *b*300–11, respectively. Conditions were as for Fig. 1a.

usual manner then plated at appropriate dilutions on casein-TEP agar plates to give about 100 colonies, and incubated at 37 C overnight. Then replica plates were made on casein-TEP agar plates on which about 10^6 cells of *a*M4–1 had been dispersed. After overnight-culture, six *λ*-toxin negative colonies from *b*300–11 and three from *b*M2–11 were isolated in an approximate frequency of 10^{-2} from the original plates. Complementation characters of the representative mutants on other markers of toxin production were further characterized as shown qualitatively in Table 2. They did not recover the a^+ character and are λ^- even on conplementation with *a* group strains. With regard to other markers they differ from strain to strain and they all show *b* group characters on complementation with *a* group strains in some of the four markers except for one mutant (C340 from *b*M2–11) which gave negative results for all markers on complementation as shown in Table 2.

3. *Quantitative estimation of four extracellular products of newly isolated mutants*

Results on quantitative estimation of θ, λ and HA of the newly isolated mutants are shown in Table 3 together with those on κ-toxin with their titers on complementation.

TABLE 3. *Quantitative data on four extracellular products of representative mutants*

Strain	HA[a]	θ[b]	λ[c]	κ[d]
Single culture				
PB6K	1024	512	2.71	+
*a*M4-1	0	4	0	−
*b*300-11	0	4	0	−
*a*32	0	0	2.56	+
*b*32-C5	0	0	0	−
32-C5 R3	0	0	1.66	+
*a*F11	1024	512	0	+
*b*F11-T23	0	4	0	−
F11-T23 R1	1024	256	0	+
Mixed culture with *a*M4-1				
*b*300-11	256	128	0.84	+
*a*32	0	2		
*b*32-C5	0	2	0.23	+
32-C5 R3	0	4		
*a*F11			0	
*b*F11-T23	512	128	0	+
F11-T23 R1			0	
Mixed culture with *b*300-11				
*a*32	256	256		
*b*32-C5	0	2	0	−
32-C5 R3	0	4		
*a*F11			2.60	
*b*F11-T23	0	8	0	−
F11-T23 R1			0	

[a] HA: units/ml.
[b] θ: units.
[c] λ: O.D. at 550 nm (37 C, 2 h).
[d] 37 C for 2 days on bovine Achilles tendon-TEP agar.

4. *Complementation through cellophane membranes*

Complementation in production of θ-, λ- and κ-toxin occurred through cellophane membranes (open Visking 20/32 tubing) when *b* group strains were spotted on indicator plates under the membranes and *a* group strains or PB6K were dropped on the membranes in nutrient soft agar (Higashi et al., 1976; Tatsuki et al., 1981).

A similar experiment on complementation was carried out with a Spectra / Por 6 membrane, which permits penetration of substances with molecular weight of 2000 or less. Surprisingly, complementation occurred between *a* and *b* group strains as shown in Fig. 2. Thus the gene *a* product has a very small molecular weight of about 2,000 or less.

DISCUSSION

In our previous studies on complementation between *a* and *b* group strains through cellophane membranes, we found that production of an extracellular product synthesized by *b* group strains was stimulated by *a* substance diffusing through the membrane from *a* group colonies. This finding led us to the following hypothesis: 1) the structural genes of four extracellular product are intact in *b* group strains, but are mutated in *a* group strains, 2) *a* group strains can produce a stimulating substance, *a*-substance, 3) *b* group strains have lost the capacity to produce *a*-substance and they are in a repressed state for production of extracellular products, 4) when *b* group strains obtain *a*-substance from *a* group strains, they were freed from repression and switched on synthesis of extracellular products.

To examine this hypothesis, we attempted to convert *a* group strains to the *b* group by a second NG treatment. We obtained *b* group mutants in this way but these *b* group mutants still retained the mutations present in their *a* group parents. Therefore, on complementation they could produce all the markers except for those lost previoulsy but they had lost the capacity to produce *a*-substance as shown by complementation through cellophane membranes. From these results we concluded that

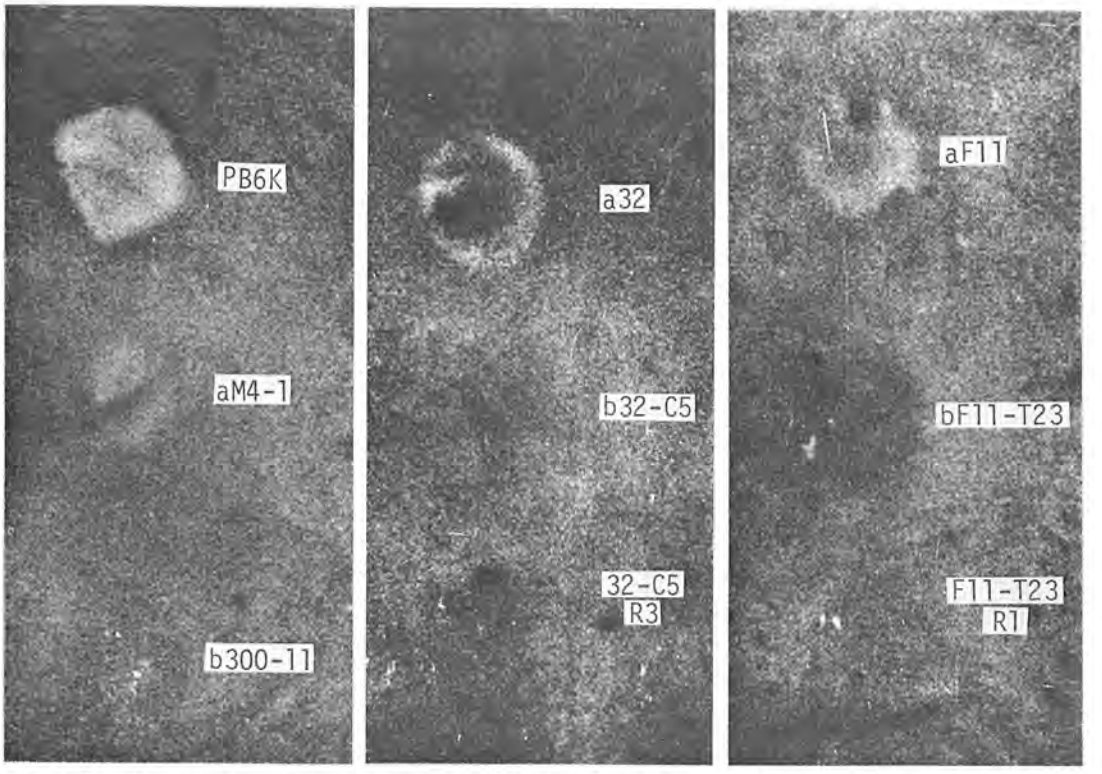


FIGURE 2. The complementation of θ-toxin production through a cellophane membrane. A blood agar plate was streaked with *b*300–11 and covered with a sheet of sterile Spectra/Por 6 membrane over which nutrient soft agar inoculated with the test derivative mutant was dropped. After incubation at 37 C overnight, complete hemolysis was found under the membrane on which PB6K, *a*M4–1, *a*32 and *a*F11 were growing, as described previously, but no hemolysis was found with other mutants even when they were growing on the membrane. These results indicate that *a*M4–1, *a*32, *a*F11 and PB6K produce *a* substance but *b*300–11, *b*32-C5, *b*32–C5, R3, *b*F11–T23 and F11–23 R1 do not.

b group strains bear mutations in the gene of *a*-substance, which operates as a derepressor. This would account for the pleiotropic loss of four marker products in *b* group strains isolated in the first NG treatment.

Since we explain the pleiotropic loss of four marker products in *b* group strains as due to repression, it should be possible to introduce a mutation in a repressor gene that enables the strains to self-produce the marker products without recovery of *a*-substance. After the second NG treatment of *b*M2–11, and after the third NG treatment of *b*32–C5 and *b*F11–T23, constitutive mutants were isolated and they were all shown not to produce *a*-substance by complementation through cellophane membranes. However, similar treatment of *b*300–11 did not give a constitutive, for some unknown reason. Of the three constitutive mutants obtained from *b*M2–11, two (R1 and R3) were θ^+, κ^+, HA^+ in addition to λ^+ and the third (R4) was θ^+, κ^+, HA^- and λ^-. The loss of HA in R4 can be explained by considering that it had another mutation in the structural gene of HA.

Other attempts to obtain *a* group mutants from *b* group strains after NG treatment were made by two procedures: 1) Direct back mu-

tation of the a^+ character selecting λ^+ colonies on casein-TEP agar, this was unsuccessful. 2) Selection of back mutation in the a^+ character as complementation negative colonies on casein-TEP agar, on which about 10^6 λ^- *a* group strain cells had been seeded. All the mutants obtained in this way still retained the *b* group character on complementation of other markers except for one mutant, which did not produce any of the four markers on complementation and was still a^-.

The relatively higher incidence of isolation of *a* (θ^-, λ^-, κ^-, HA^-) was a remarkable result, as described in the provious report (Tatsuki et al., 1981). To see whether the structural genes of *a* mutants are intact, we isolated these mutants as complementation negative colonies with a *b* group strain on casein-TEP agar. These a^- mutants were isolated from 6 *a* group strains, at an insidence of 10^{-3}. Although they should be *b* group strains, they were still non-producers of the four markers in complementation with other *a* group strains. As they were all produced α-toxin and were agglutinated by antiserum to the parent PB6K, they were not contaminants. In addition, no λ^+ revertants were obtained from NG-treated cultures of as many as 10^6 colonies each of 6 *a* (θ^-, λ^-, κ^-, HA^-) strains. From these data, we suppose that these *a* group mutants bear mutations in each of the respective 4 marker genes.

The possibility of genetic transfer in complementation was examined by spreading *a*32 $\theta^-\alpha^+sm^Rrf^S$ together with *b*300–11 $\theta^-\alpha^-sm^Srf^R$ on blood agar plates, and θ^+ colonies among θ^- colonies were replicated onto blood agar containing streptomycin or rifampicin. All $rf^R\alpha^-$ or $sm^R\alpha^+$ colonies from θ^+ mixed colonies were θ^- and no recombinant was detected. This results excludes the possibility of genetic transfer in complementation (Higashi et al., 1973). However, there is another possibility of genetic transfer, *i.e.* complementation might be evoked by transfer of a plasmid DNA that could not replicate effectively, as in abortive transdution, and hence the actual transformant would not be isolated. This possibility seemed to be excluded by the fact that complementation was achieved through a cellophane membrane (open Visking tubing 20/32) as described previously (Higashi et al., 1976). But, here again, there is a possibility that the parent strain PB6K might be a cellobiose fermenter and hence enlarge pores of the cellophane membrane by hydrolysis of cellulose fibers. This possibility, was examined by autoclaving the cellophane membrane in TEP liquid medium and growing PB6K in it for 3 days. Then the membranes were washed, sterilized by autoclaving in a wet state, and put on EMB Gal plates prespotted *E. coli* K12 594 Gal^- sm^R. Soft agar containing *E. coli* K12 W3101 sm^S/F′Gal^+ was then dropped on top of the membranes. When the plates were then incubated for 3 days, no Gal^+ points appeared under the membranes. This result excludes the possibility that PB6K enlarged pores in the cellophane membrane.

The complementation also occurred through a Spectra / Por 6 membrane (M.W. cut off: 1,000–2,000) indicateing that *a*-substance has a very small molecular weight. However, *a*-substance could not be detected in the supernatant of a liquid culture of *a* group strains after its filtration through Millipore HA membranes or cellophane membrane even in anaerobic conditions. We are attempting to find the reason for this discrepancy. In relation to the fact that *a* substance is very small in molecular weight, another possibility has been offered to explain the complementation phenomenon. It is the cyclic AMP system that may act the production of four kinds of extracellular products. The *b* group strains would be negative mutants of adenylate cyclase which catalizes the formation of cAMP from ATP, but could be induced to produce these products by recieving cAMP from the parent or the *a* group strains. Therefore, we have tried the experiments of the addition of cAMP or cGMP in doses of 5 to 500 μg/ml to the cultures of the *b* group strains, but failed to show any evidence of the production of these extracellular products in

both agar plates and liquid media.

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