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# FURTHER STUDIES ON COMPLEMENTATION BETWEEN MUTANTS OF *CLOSTRIDIUM PERFRINGENS*

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SUMMART 1. Mutants devoid of λ- and κ-toxin and hemagglutinin (HA), respectively, were isolated from *Cl. perfringens* PB6K. The $\lambda^-$ and $HA^-$ mutants could be classified into *a* and *b* groups by complementation but the $\kappa^-$ mutants were all of the *a* group.

2. All *b* group mutants isolated, irrespective of the marker used for isolation, were pleiotropically negative or leaky with respect to θ-, λ- and κ-toxin and HA production.

3. Lambda-toxin produced by complementation was proved to be a rennet-like protease.

4. The activities of 12 extracellular enzymes, including sialidase, of several *b* group strains and the parent PB6K were compared, but no definite differences were observed. From this finding, the productions of these enzymes were concluded not to be regulated by the same mechanism as θ-, λ- and κ-toxin and HA.

5. *Cl. perfringens* CN3870 was also studied. Findings were similar to those on PB6K except for very low activity of HA.

## INTRODUCTION

Higashi et al. (1973, 1976) of this laboratory described θ-toxin production by complementation between *a* and *b* group $\theta^-$ mutants of *Clostridium perfringens* PB6K when they were co-cultured in proteose-peptone broth or on a sheep blood agar plate. In the complementation, *b* group mutants produced θ-toxin and *a* group mutants produced a stimulating sub-

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stance, but genetic transfer did not play a role.

According to Hobbs et al. (1958), heat-resistant type A food poisoning strains of *Cl. perfringens* are devoid of $\theta$-toxin. We obtained 30 strains isolated during outbreaks of food poisoning in Japan and examined their hemolysis, finding that 28 were $\theta^-$ and belonged to *a* group and the other 2 were $\theta^+$, but were easily segregated $\theta^-$ colonies which also showed the *a* group character. As Hobbs et al. reported that heat-resistant food poisoning strains were also devoid of $\lambda$-toxin (protease), we examined the Japanese strains and found that they all produced $\lambda$-toxin when casein or gelatin was used as substrate as described in this paper. But, when we examined $\lambda$-toxin production of $\theta^-$ mutants, we found that all *b* group mutants of $\theta^-$ were $\lambda^-$ or $\lambda^\pm$ (leaky) and that $\lambda$-toxin production by *a* group mutants varied according to the strain. When, $\lambda^-$ strains from both groups were co-cultured on casein anaerobe agar they showed complementation (Higashi et al., 1976). In addition, collagenase (Higashi et al., 1976) and hemagglutinin (HA) production of $\theta^-$ mutants behaved in similar fashions.

We attempted to isolate $\lambda^-$, $\kappa^-$ and HA$^-$ mutants from PB6K and the present paper reports results of these studies.

## MATERIALS AND METHODS

### 1. *Bacterial strains*

*Cl. perfringens* PB6K, given by Dr. Ryosuke Murata of the National Institute for Preventive Hygiene, was used as the parent strain to isolate mutants. This strain is identical with BP6K, and with ATCC 10543 used in other countries. Strain CN3870 was obtained from the Welcome Laboratory, England and used for studying HA and sialidase.

### 2. *Medium used*

As liquid media for growth, anaerobe agar (Eiken) or TEP anaerobe agar (Eiken) was added to cold distilled water according to directions, filtered through filter paper to remove agar granules and autoclaved. Proteose peptone broth was also used in some experiments. It consisted of 50 g proteose peptone (Daigo), 2 g NaCl, 12.6 g $Na_2HPO_4 \cdot 12H_2O$, 0.1 g $KH_2PO_4$, 0.1 g thioglycolate and 10 g fructose per liter of distilled water. For assays of HA and sialidase, Fructose Trypticase Soy Broth containing 0.4% yeast extract was used. Trypticase Soy Broth consisted of 17 g Trypticase Peptone (BBL), 3 g Phytone Peptone (BBL), 5 g NaCl, 2.5 g $K_2HPO_4$ and 4 g yeast extract per liter. The mixture was autoclaved and then sterile fructose was added to 0.25%. For isolation of HA mutants, NG treated cells were spread on Trypticase Soy Agar prepared by adding 1.5% agar to Trypticase Soy Broth, and 0.25% sterile fructose was added after autoclaving.

As indicator plates for $\lambda$-toxin production, 3% gelatin (Difco) or 2% Na-caseinate (Wako Pure Chemicals) were added to anaerobe agar (Eiken) or TEP anaerobe agar (Eiken). In casein agar, white turbidity around and / or under colonies, due to rennet-like enzyme activity, and transparent haloes around and / or under colonies in the background of white turbidity developed by adding glacial acetic acid were taken as indications of $\lambda$-toxin production. In gelatin agar, very faint white turbidity around colonies and transparent haloes colonies in the background of white turbidity developed by adding saturated ammonium sulfate were taken as indication of $\lambda$-toxin production. Human fibrin agar plates were also used; 10 ml of anaerobe agar was poured into a Petri dish, and when it had solidified, another 10 ml of agar containing 0.5% sterile human fibrinogen (Midori-juji Co.) clotted by adding 50 units thrombin (Midori-juji Co.) was poured into the plates and solidified. Lambda positive colonies were surrounded by clear haloes in a background of white turbidity.

Bovine Achilles tendon agar was used for detecting $\kappa$-toxin. Native bovine Achilles tendon from a slaughter house was freed from connective tissue as far as possible and sliced with a knife in dry ice. The frozen slices were homogenized in a Waring blender with dry ice, and the fine frozen debris was filtered though a 30 mesh stainless wire net and stored at 4 C as a dense suspension in phosphate (M/75, pH 7.2) buffered saline with $CHC1_3$ to prevent contamination. Then 10 ml of anaerobe agar containing 20 $\mu$g/ml of streptomycin was poured into a Petri dish, and solidified. *Cl. perfringens* is completely resistant to streptomycin up to 40 $\mu$g/ml. Then a mixture of another 10 ml of anaerobe agar, 20 $\mu$g of antibiotics and an appropriate volume of bovine Achilles tendon suspension at about 45 C was poured onto the anaerobe agar. The disappearance

of the debris around colonies after incubation for 3 days was taken as an indication of $\kappa$-toxin production.

3. *Isolation of mutants*

A culture of PB6K or CN3870 in the log phase in Proteose peptone or anaerobe broth (Eiken) was treated with 0.02% NG for 1 h, washed and resuspended in the same medium in more than 10 tubes and incubated overnight. These cultures were spread on respective indicator plates to give 50–100 colonies per dish. After overnight incubation for $\lambda^-$ mutant isolation, colonies showing negative or questionable results were recultured on the indicator plates and negative or leaky mutants were kept in liver-liver broth. For $\kappa^-$ mutant isolation, incubation was continued for 3 days.

For isolation of HA$^-$ mutants, PB6K was grown in Tryptic Soy Broth. NG-treated cultures were grown in 20 tubes, and then each culture was spread on Tryptic Soy Agar to give about 100 colonies. These colonies were numbered, and 70 randomly selected colonies from each culture were inoculated onto blood agar plates by sticking to find $\theta^-$ colonies and also inoculated into small tubes of Tryptic Soy Broth. The broth cultures were each diluted 1 : 200 and a drop of chick erythrocyte suspension was added to each diluted culture. The presence of the bacterial cells did not disturb the hemagglutination. After this preliminary selection, mutants giving negative or questionable results were again grown in the same medium, the cultures were centrifuged and the supernatants were examined at serial 1 : 2 dilutions. Mutants giving 32 HAU/ml or less were scored as negative and those giving 64–256 HAU/ml were scored as $\pm$(leaky) mutants. PB6K gave 4000–8000 HAU/ml and CN3870 gave only 128–256 HAU/ml.

4. *Chromatography of $\lambda$-toxin*

Volumes of 200 ml of culture supernatants of PB6K or co-cultures of *a* and *b* group strains in TEP anaerobe broth were concentrated to 8 ml in vacuo in Visking cellophane tubing. The concentrated solution was applied to a CM-Sephadex C50 column (2$\times$20 cm), previously equilibrated with 0.02 *M* phosphate buffer, pH 6.0, material was eluted successively with 187 ml of the same buffer, and 437 ml of a linear gradient of saline formed with 0.5 *M* NaCl in the buffer and 1 *M* NaCl in the bufier.

The activity of $\lambda$-toxin was estimated in a reaction mixture consisting of 0.2 ml of enzyme preparation of appropriate dilution, 0.3 ml of 10 m*M* $CaCl_2$, 0.3 ml of 75 m*M* cysteine, 1.0 ml of 0.3 *M* imidazole (Wako Pure Chemicals)-HCl buffer at pH 6.8 and 1.2 ml of 0.25% Na-caseinate at 37 C for 2 h. The turbidity was read in a Hitachi Perkin-Elmer spectrophotometer at 550 m$\mu$. A linear relationship was obtained up to O.D.$=$1.0. Enzyme preparation giving higher O.D. values than 1.0 were diluted before measurement.

5. *Activity of $\lambda$-toxin of $\kappa$-casein and whole casein*

Whole Na-caseinate or $\kappa$-casein, the most sensitive substrate among the components of casein to rennet enzyme, were treated with enzyme fractions. The reaction mixture consisted of 50 $\mu$l of 4 mg/ml of Na-caseinate or $\kappa$-casein, 30 $\mu$l of 0.3 *M* imidazole-HCl buffer of pH 6.8, 10 $\mu$l of 255 m*M* cysteine, 10 $\mu$l of 300 m*M* EDTA to prevent precipitation of split peptidase and 0.2 ml of the test enzyme preparation. After incubation at 37 C for 2 h 25 $\mu$l of a mixture of 50% glycerol and 0.05% bromophenol blue was added to 0.1 ml of the reaction mixture. The resultant mixture was subjected to polyacrylamide gel electorophoresis in gel consisting of 5% separation gel and 3% concentrating gel buffered at pH 8.3. A current of 4 mA was applied to the gel column. Columns were stained with Coomassie brilliant blue.

6. *Estimation of HA titer*

HA was estimated as decribed by Rood and Wilkinson (1974). The sample was diluted serially 1 : 2 in 0.25 ml of 1 m*M* $NiCl_2$ in saline containing borate buffer (5 m*M*, pH 7.2) in a perspex tray and 0.25 ml of 1% chicken erythrocyte suspension was added to each sample. The end point was read after 3 h at room temperature. The HA titer is shown as the reciprocal of the endpoint dilution multiplied by 4 to express the titer per ml the original sample.

7. *Estimation of sialidase activity*

The substrate used, Collocaria sialomucoid, was prepared by digesting finely ground nests, obtained from a Chinese food shop, with ficine as described by Aminoff (1961) and Lawton, McLoughlin & Morgan (1956). Insoluble material was removed by centrifugation and the solution was neutralized and heated in a boiling water bath for 10 min. Then it was dialyzed against distilled water and stored at 4 C with added $CHCl_3$.

The estimation was carried out as described by

Rood & Wilkinson (1974). The reaction mixture consisted of 0.1 ml of 1 : 5 diluted substrate solution, 0.1 ml of 1 *M* potassium acetate buffer (pH 5.5) containing 1.5 mg per ml of bovine serum albumin and appropriately diluted culture supernatant previously dialyzed against saline. The total volume was adjusted to 0.5 ml with distilled water. Incubation was carried out for 20 min at 37 C and then the mixture was heated at 100 C for 2 min to stop the reaction and promptly cooled in an ice-water bath. Enzyme or substrate was omitted from control mixtures. Free sialic acid was estimated with thiobarbituric acid as described by Aminoff (1961). One unit of enzyme was defined as the amount necessary to release 1 $\mu$mole of sialic acid per min from the substrate under the assay condition.

8. *Micellaneous enzyme assays*

DNase, RNase and mucinase, respectively, were assayed on DNase test agar (BBL), TEP agar containing 0.2% RNA (Merck) and TEP agar containing 1% hog gastric mucin (Wilson Lab.). DNA and RNA plates were stained with 1.5% toluidine blue after growth. The widths of the pink zones around colonies were compared. On mucin plates the clear zones around colonies were measured. $\alpha$-Amylase was assyed on TEP agar containing 1% filter-sterilized soluble starch; after growth a solution of iodine-potassium iodide was poured onto the plates. End-$\beta$-N-acetylglucosaminidase was assayed in culture supernatants using a suspension of *M. lysodeikticus* cell walls. $\mu$-Toxin (hyaluronidase) was assayed by the ACRA test described by Evans et al. (1951) using normal human synovial fluid as substrate. The following enzymes were assayed as described in references: acid and alkaline phosphatase (Shibco et al., 1965), $\alpha$-glucosidase (Halvorson et al., 1958), arylsulfatase (Breslow et al., 1972; Roy. 1953), $\beta$-glucuronidase (Shinoyama et al., 1969), $\beta$-glucosidase (Beck et al., 1968), $\beta$-galactosidase (Furth et al. 1965) and elastase (Sachar et al., 1955). Enzymes destroying blood group A substance were assayed as described by Morgan & King (1943) with hog gastric mucin as substrate and inhibitor for type A erythrocyte agglutination.

## RESULTS

1. $\lambda^-$ *mutants*

On casein agar plates, 24 cas$^+$ and 13 cas$^\pm$ (leaky) mutants were obtained. One of the 24 cas$^-$ mutants was a *b* group mutant and the other 36 were *a* group. Group determination carried out using cas$^-$ mutants isolated as $\theta^-$ as reference strains of the *a* and *b* group. Group *a* mutants of cas$^-$ were identified by positive $\lambda$-toxin production with the reference *b* group strain, and group *b* mutants were identified with the reference *a* group strain. The culture supernatants of cas$^-$ mutants had no activity on casein, but they had 1/2—1/100 of the activity of PB6K on gelatin.

On gelatin agar plates, 53 mutants were isolated: 47 were of the *a* group, and 6 of the *b* group. Of the *a* group mutants, 44 were cas$^-$ and 3 were cas$^\pm$, but all of them showed reduced activity on gelatin. The enzymic activities of these *a* group mutants against gelatin were 1/4—1/100 of that of the parent. Therefore, they were quite similar to cas$^-$ or cas$^\pm$ mutants.

On fibrin agar plates, 11 *a* and 2 *b* group mutants were also isolated, The 11 *a* group mutants were also similar to cas$^-$ or cas$^\pm$ mutants.

These data indicate that mutants of the *a* group were very similar irrespective of the substrate used for their isolation, and hence they were named $\lambda^-$ mutants. As gelatin and collagen are common substrate for $\kappa$-toxin (collagenase), these $\lambda^-$ mutants were examined for activity against debris of bovine Achilles tendon. Many *a* group mutants showed collagenase activity and a few showed leaky activity. This would account for the weak activity of $\lambda^-$ *a* group mutants on gelatin. A remarkble finding was that *b* group mutants were either non-producers / or leaky producers of $\kappa$-toxin, $\theta$-toxin and HA. The results of examinations of other markers are shown in Table 1.

Complementation of $\lambda$-toxin production was attained through a cellophane membrane on casein agar; *b* group strains produced $\lambda$ in the complementation.

TABLE 1. *Properties of $\lambda^-$ mutants isolated with various protein substrates*

| Phenotype[a] | | | | Selecting marker | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | casein | | gelatin | | fibrin | |
| | | | | no. of mutants | | no. of mutants | | no. of mutants | |
| $\lambda$ | $\kappa$ | $\theta$ | HA | *a* group | *b* group | *a* group | *b* group | *a* group | *b* group |
| − | − | − | − | 7 | 1 | 5 | 6 | 4 | 2 |
| − | − | − | + | 0(1)[b] | | 0 | | 0 | |
| − | − | + | − | 0 | | 0 | | 0 | |
| − | − | + | + | 0 | | 0 | | 0 | |
| − | + | − | − | 13 | | 39 | | 1 | |
| − | + | − | + | 2(5)[b] | | 1(2)[b] | | 0(1)[b] | |
| − | + | + | − | 0 | | 0 | | 0 | |
| − | + | + | + | 7(1)[b] | | 0 | | 5 | |
| total | | | | 36 | 1 | 47 | 6 | 11 | 2 |

[a] Negative and leaky (±) mutants indicated as −.
[b] These strains were dead when HA was examined.

2. *Enzymic nature of protease produced by complementation*

The proteases produced by complementation between *a*M4–1 and *b*300–11 and by PB6K were compared. Previous studies (Ohashi et al., 1975; Sato et al., 1978) showed that PB6K produces two or three proteases.

The concentrated culture supernatants were applied to a CM-Sephadex column and chromatographed as described in the "Methods". The results are shown in Fig. 1. With PB6K, three casein-hydrolyzing activities were found: one in the unabsorbed fraction (P1) and one each in the fractions eluted with 0.25 *M* (P2) and 0.4 *M* (P3) NaCl. With the mixed culture supernatants, the activity was found in a single fraction (M) corresponding to P2 (or P3 in some experiments) of PB6K. The optimal pH of P1 was 6.6–6.8 and those of P2 and P3 were 6.2–6.4. Cysteine was essential for casein hydrolyzing activity and sodium tetrathionate and iodoacetamied were inhibitory. $Ca^{++}$ was necessary for turbidometric determination but not for proteolysis, and phosphate, maleate and EDTA were inhibitory to turbidometric determination but not to proteolysis.

The sensitivities of $\kappa$-casein, the most sensitive of the components of casein to rennet (chymosin, rennin), and whole casein to P2, P3 and M were compared as described in the "Methods". As shown in Fig. 2, $\kappa$-casein was very sensitive to these proteolytic enzymes and it disappeared preferentially among the casein components. Though no figure is shown, other casein components also disappeared when the incubation period was prolonged or the amount of enzyme added was increased. These data indicate that P2, P3 and M are rennet-like enzymes.

3. *$\kappa^-$ mutants*

Only 13 $\kappa^-$ mutants were isolated, because of the paucity of substrate; 12 were $\theta^+$, $\lambda^-$ and $HA^+$ and one was $\theta^\pm$, $\lambda^\pm$ and $HA^-$.

The *b* group mutants, obtained during isolation of $\theta^-$ or $\lambda^-$, were all $\kappa^-$ or $\kappa^\pm$. Co-cultures of any one of the $\kappa^-$ *b* group mutants with any of the 13 $\kappa^-$ mutants in complementation tests resulted in production of $\kappa$-toxin on agar plates. No $\kappa$-toxin was produced on co-culture of any of the 13 $\kappa^-$ mutants with an *a* group mutant, obtained during $\theta^-$ or $\lambda^-$ isolation, which are

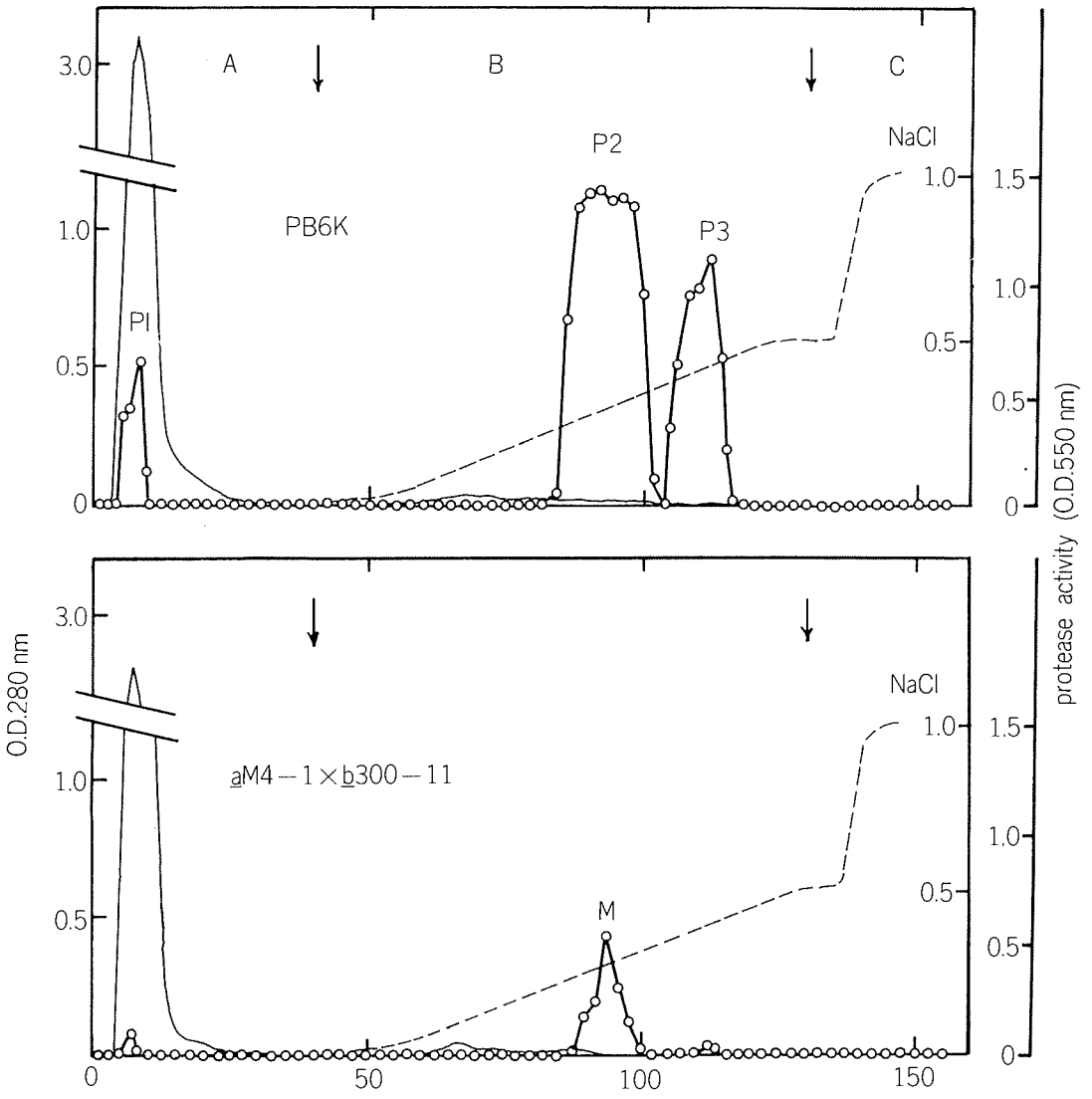


FIGURE 1. CM Sephadex G-50 chromatograms of proteases produced by PB6K parent strain and by complementation between *a*M4–1 and *b*300–11.

coincidently $\kappa^-$. Therefore, the 13 $\kappa^-$ mutants were *a* group.

The $\kappa$-toxin produced by complementation was neutralized by specific $\kappa$-antitoxin obtained from Wellcome Research Laboratories as described by Higashi et al. (1976).

4. *HA⁻ mutants*

Next the culture supernatants of 110 *a* group mutants, isolated as $\theta^-$, $\lambda^-$ or $\kappa^-$, respectively, were tested for HA. A high correlaiton was found between $\theta^-$ and HA$^-$ and between $\theta^+$ and HA$^+$, as shown in Table. 2. All *b* mutants were HA$^-$ or HA$^\pm$. In addition, co-

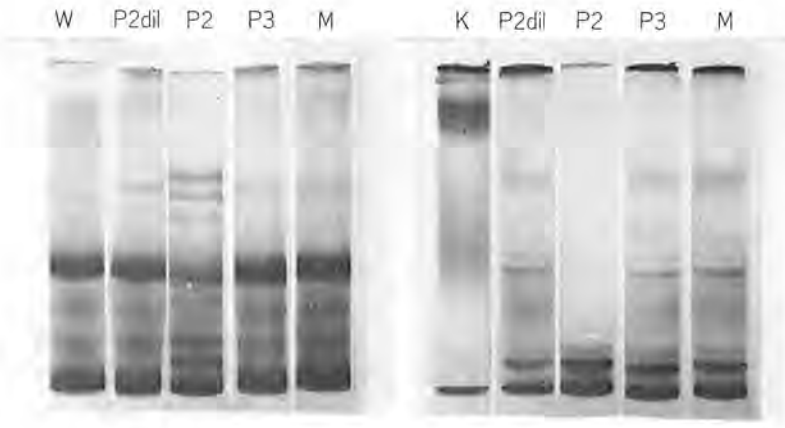


FIGURE 2. Polyacrylamide gel electrophoresis of hydrolytic products of whole casein and $\kappa$-casein by protease.
w: whole casein control.
k: $\kappa$-casein control.
P2 dil: hydrolytic products by 10-fold diluted P2.
P2, P3 and M: hydrolytic products by each protease.

TABLE 2. *Relationship between $\theta$ and HA activities of $\theta^-$, $\lambda^-$ and $\kappa^-$ mutants*

| phenotype[a] | | mutants isolated as | | |
|---|---|---|---|---|
| $\theta$ | HA | $\theta^-$ | $\lambda^-$ | $\kappa^-$ |
| − | − | 13 | 69 | 1 |
| − | + | 0 | 3 | 0 |
| + | − | 0 | 0 | 0 |
| + | + | 0 | 12 | 12 |

[a] Negative and leaky (±) mutant indicated as −.

TABLE 3. *Properties of mutants isolated as HA negative*

| Phenotype | | | | No. of mutants | | No. of clones | |
|---|---|---|---|---|---|---|---|
| HA | $\theta$ | $\kappa$ | $\lambda$ | *a* group | *b* group | *a* group | *b* group |
| − | − | − | − | 17 | 3 | 15 | 3 |
| − | − | − | + | 3 | | 3 | |
| − | − | + | + | 6 | | 4 | |
| − | + | − | + | 7 | | 6 | |
| − | + | + | − | 1 | | 1 | |
| − | + | + | + | 13 | | 8 | |
| total | | | | 47 | 3 | 37 | 3 |

[a] Negative and leaky (±) mutants indicated as −.

cultures of an HA$^-$ *a* group strain and a *b* group strain showed HA production in liquid medium. On the basis of these findings, we attempted to isolate HA$^-$ mutants as described in the " Methods ".

Fifty mutants were isolated. Among them, 23 were HA$^-$ and 27 were HA$^\pm$, and 47 were *a* group and 3 were *b* group.

The *b* group mutants were $\theta^-$, $\lambda^-$ and $\kappa^-$ or $\kappa^\pm$. The *a* group mutants were also tested for $\theta$, $\lambda$ and $\kappa$ activities. Mutants with the same pattern of $\theta$, $\lambda$ and $\kappa$ activities which were isolated from the same tube among the 20 tubes set up after NG treatment, were regarded as belonging to the same clone. The results are shown in Table 3. The activities of these 3 other markers than HA differed from clone to clone. Unexpectedly a high correlation between $\theta^-$ and HA$^-$ could not be seen, as shown in Table 3. In addition, the number of 16 HA$^+$ and $\theta^-$ mutants obtained in selection of HA$^-$ should also be considered.

A remarkable finding was that clones of mutants with no activity or leaky activity of all four markers showed the highest incidence among *a* group mutants. They showed *a* group character of $\theta$, $\lambda$ or $\kappa$ production in complementation tests through a cellophane membrane. Solid medium could not be used for HA production in complementation tests, and so complementation was assayed in mixed cultures in liquid medium. No complementation in production of HA or other markers could be attained through a cellophane membrane in liquid medium. These results show that mixed

culture supernant of HA$^-$ *a* and *b* group mutants contained HA, but results did not show which group strain produced HA and which group strain was stimulatory.

5. *Common nature of b group mutants irrespective of the marker used for isolation*

The *b* group strains were pleiotropic negative or leaky mutants devoid of the four marker products. These characters were common among *b* group strains irrespective of which marker was used in the isolation, as shown in Tables 1 and 3. This means that when the *b* group strains were compared with the parent strain, if some marker product was not detected or was markedly reduced this marker product could be considered to be regulated in the same way as $\theta$, $\lambda$, $\kappa$ and HA.

6. *Other markers tested*

The productions by three randomly selected *b* group strains and the parent PB6K of the following extracellular products were compared: $\nu$-toxin (DNase), RNase, $\mu$-toxin (hyaluronidase), sialidase, lysozyme-like enzyme (end-$\beta$-N-acetylglucosaminidase), acid phosphatase, alkaline phosphatase, $\alpha$-amylase, mucinase, $\beta$-galactosidase, blood group A destroying enzyme. PB6K and *b* group strains showed no significant differences in these enzyme activities. However, in some experiments the activities could not be detected in one of the three *b* group strains. Therefore, two additional *b* group strains were examined. The four strains tested showed similar activities to those of the parent. Thus mutation resulted in loss of activity in only one strain. Arylsulfatase, $\beta$-glucuronidase, $\beta$-glucosidase, and elastase were also assayed, but even the parent strain PB6K did not have these activities.

As sialidase may be related to HA, its activity was assayed in randomly selected 6 *b* group strains. One strain had low activity, but the 5 others had similar activity to that of PB6K, as shown in Table 4.

The data show that these extracellular products are not included in the group of makers for which *a* and *b* group mutants show complementation.

TABLE 4. *Sialidase activity of b group mutants isolated from PB6K*

| | Sialidase Activity | |
|---|---|---|
| | $\mu$ mole/min/ml | % of PB6K |
| *b* groub strains | | |
| 300-11 | 0.016 | 100 |
| M2-11 | 0.015 | 94 |
| F-21 | 0.001 | 6 |
| F-22 | 0.015 | 94 |
| G-562 | 0.016 | 100 |
| MLH-14 | 0.011 | 69 |
| parent strain | | |
| PB6K | 0.016 | 100 |

7. *Mutants of Cl. perfringens CN3870*

Rood and Wilkinson (1975, 1976a, b) studied sialidase of *Cl. perfringens* CN3870 and found that this organism produced 1) three kinds of sialidase, *i.e.*, sialidase I, II and III, 2) sialidase II showed hemagglutin activity in the same molecule, 3) sialidase I was a trimer of sialidase II. They also found that sialidase I together with II showed about 10 times higher activity than sialidase III, that sialidase III was stable at pH 4.5 in potassium acetate buffer in the presence of 0.3 mg per ml of BSA in contrast to sialidase I and II, and that sialiase III and hemagglutinin III were distinct entities. Furthermore they found that *Cl. perfringens* ATCC 10543, which is identical to PB6K, produced only hemagglutinin III and sialidase III.

To know more about the relationship of sialidase to hemagglutinin of CN3870, we isolated 65 $\lambda^-$ mutants on casein TEP agar. They were classified into two groups by cross streaking on casein TEP agar. In complementation tests through cellophane membranes on casein TEP agar, strains that produced $\lambda$-toxin under the membrane on stimulation by a strain of the other group on the top of the membrane, were identified as *b* group, while stimulatory strains

on the top of the membrane were identified as *a* group. These *a* and *b* groups were confirmed by complementation through cellophane membranes using *a* and *b* group reference strains of PB6K. In this way 6 strains were classified as *b* group and 59 as *a* group. Again, *b* group strains were pleiotropically negative as regard $\kappa$ and $\theta$. As regards HA activity, even CN 3870 produced quite a low titer (128–256 HAU per ml) while *b* group strains produced 0–8 HAU per ml.

The sialidase activity of the culture supernatants of the 6 *b* group strains were assayed in potassium acetate buffer, pH 5.5, and results are shown in Table 5. The activity of CN3870 was weak and was comparable to that of PB6K. The activity of CN3870 reported by Rood & Wilkinson was 10 times that observed in our experiments. Pretreatment in potassium acetate buffer, pH 4.5, in the presence of BSA (0.3 mg per ml) did not cause marked decrease in activity assayed at pH 5.5 in our experiment, and from our results we concluded that our CN3870 does not produce much sialidase I or II, and thus that its production is similar to that of PB6K.

The *b* group strains also showed very similar sialidase activity except for 2 mutants, which showed only 1/10—1/5 as much activity. These data show that sialidase production is not included in the group of makers for which *a* and *b* group mutants exhibit complementation.

TABLE 5. *Sialidase activity of b group mutants isolated from CN3870*

| | Sialidase Activity | |
|---|---|---|
| | $\mu$ mole/min/ml | % of CN3870 |
| *b* group strains | | |
| 152 | 0.015 | 88 |
| 243 | 0.015 | 88 |
| 521 | 0.002 | 12 |
| 552 | 0.015 | 88 |
| 622 | 0.024 | 141 |
| C33 | 0.0007 | 4 |
| parent strain | | |
| CN3870 | 0.017 | 100 |

## DISCUSSION

Mutants of *Cl. perfringens* PB6K devoid of $\lambda$, $\kappa$ or HA were isolated and were classified into *a* and *b* groups as in the case of $\theta^-$ (Higashi et al., 1973). But, in case of $\kappa^-$ only 13 mutants were obtained and they all belonged to the *a* group. The *b* group mutants all showed the same characters, irrespective of which marker was used for their isolation (*i.e.*, $\theta$, $\lambda$ and HA). They were pleiotropically negative or leaky mutants with regard to $\theta$, $\lambda$, $\kappa$, and HA, and they were real producers of marked substances in complementation. But, in the case of HA production, this could not be proved, because HA production could only be detected in mixed culture in liquid medium and we have not yet succeeded in demonstrating complementation through a cellophane membrane separating strains of different group in liquid medium. However, although there is still some uncertainty as regards HA production, we conclude that *b* group mutants have a mutation in a regulatory gene not in structural genes of the four marker products. Therefore, *b* group mutants seem to be in a repressed state as regards production of the four markers. On the other hand, *a* group mutants have mutations in the respective structural genes and still retain the ability to produce a *b* group cell stimulating subtance that enables (derepresses) *b* group cells to synthesize the four marker products.

From the pleiotropic nature of *b* group mutants it was assumed that if another marker product was lost or markedly decreased in several *b* group strains examined, its production should be controlled by the same regulatory mechanism. On the basis of this assumption we compared the productions of 12 extracellular enzymes by several *b* group strains and the parent PB6K. However, none of them, including sialidase, showed any marked difference in activity in the mutants. This means that only $\theta$, $\lambda$, $\kappa$ and HA among the extracellular

products were controlled by a common regulatory mechanism.

Negative producers or leaky producers of the four markers were detected in highest incidence, even as numbers of clones, in HA$^-$ *a* group mutants. Since *a* group mutants are thought to have mutations in respective structural genes, this high incidence is hard to explain.

According to Rood & Wilkinson (1976a), *Cl. perfringens* ATCC 10543, which is identical to PB6K or BP6K, produces only HA III and sialidase III stable against previous treatment with potassium acetate buffer at pH 4.5 containing 0.3 mg per ml of BSA; they reported that HA III and sialidase III are distinct entities. We found that the sialidase of PB6K measured at pH 5.5 was also stable against previous treatment with potassium acetate buffer, pH 4.5. In addition HA of PB6K was controlled by the same regulatory mechanism as $\theta$-, $\lambda$- and $\kappa$-toxins, but its sialidase was not. These data are not necessarily incompatible with those of Rood & Wilkinson, because HA and sialidase of ATCC 10543 are distinct entities. However, our data on sialidase of *Cl. perfrigens* CN3870, are incompatible with those of Rood & Wilkinson, although the identity of their CN3870 with our CN3870 has not yet been confirmed. Their CN3870 produced more than 10 times stronger sialidase than their ATCC 10543 or our PB6K. They found that it produced HA-sialidase I, II and also sialidase III; HA-sialidase I and II were very labile in potassium acetate buffer, pH 4.5. In contrast, our data on sialidase of CN3870, sent from the Welcome Research Laboratory, did not indicate the presence of HA-sialidase I and II, because our CN3870 produced sialidase of similar activity and stability as that of PB6K, although we used *Collocalia* sialomucoid as substrate whereas Rood and Wilkinson used $\alpha$-acid glycoprotein. According to them, HA of CN3870 had 2/3 of the HA titer of ATCC 10543, whereas the HA titer of our CN3870 was about 1/20—1/30 of that of PB6K, which we regarded as HA$^\pm$. These discrepancies must be clarified by comparing our CN3870 with CN3870 of Rood and Wilkinson. Our CN3870 produced $\theta$-, $\lambda$-, and $\kappa$-toxins, and the $\lambda^-$ mutants could be classified into *a* and *b* groups. In addition, *b* group mutants were also pleiotropically negative or leaky producers and behaved in a similar manner in complementation with *a* group mutants in $\theta$-, $\lambda$- and $\kappa$-toxin production. From these data, our CN-3870 is regarded as $\theta^+$, $\lambda^+$, $\kappa^+$, HA$^\pm$. No complementation studies of mutants from CN3870 with reference strains of *a* and *b* groups from PB6K were performed, because CN3870 and PB6K bear different bacteriocinogenies which are very effective against each other. Although we had to isolate perfringocin-resistant strains from all these mutants for co-cultures, we avoided this by testing complementation through cellophane membranes, as described in the " Results ".

The enzymic nature of protease (M) produced by complementation between *a*M4–1 and *b*300–11 and proteases (P2 & P3) of PB6K, which were eluted in the same fractions on choromatography, were assumed to be rennet-like enzymes, because $\kappa$-casein, which is the most sensitive substrate for rennet among the components of casein, was hydrolyzed most rapidly. Rennet hydrolyses two or three definite peptide bonds of $\kappa$-casein most rapidly. However, we did not examine which peptide bonds were hydrolyzed perferentially. and so we use the term " rennet-like " enzyme.

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