

Isolation and Characterization of *Corynebacterium diphtheriae* Nontandem Double Lysogens Hyperproducing CRM197

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Phage $\beta 197^{tox-}$, which codes for CRM197, a nontoxic protein immunochemically indistinguishable from diphtheria toxin, was UV induced from a culture of the C7($\beta 197$) $^{tox-}$ strain. A total of 191 C7($\beta 197$) $^{tox-}$ lysogens were isolated and selected according to the halo produced on TYE agar containing antidiphtheria toxin serum and were further characterized by Southern blots of their chromosomal DNA. Most of the isolates turned out to be monolysogens, but some tandem and nontandem double lysogens were also found. The nontandem double lysogens were stable and capable of giving high yields of CRM197, up to threefold higher than monolysogens. They are, therefore, suitable for large-scale industrial production.

$\beta 197^{tox-}$ is a nontoxic phage obtained by nitrosoguanidine mutagenesis of toxigenic corynephage β^{tox+} (21). It directs the synthesis of a mutant protein (cross-reacting material 197, or CRM197) which has the same molecular weight as diphtheria toxin and is able to compete with it for toxin receptors on HeLa cells but is devoid of any toxic activity in vivo and in vitro (22-24). CRM197 is immunologically indistinguishable from diphtheria toxin, and after mild stabilization with formaldehyde, it is able to induce protective levels of antibodies in guinea pigs (11, 13).

The diphtheria vaccine presently in use is obtained by formaldehyde detoxification of crude supernatants of the highly toxigenic PW8 strain (12) followed by partial purification of the chemically modified toxin. The use of the nontoxic protein CRM197 as a starting material would offer some obvious advantages. In fact, the use of CRM197 would avoid the problem of handling large volumes of toxic supernatants, and the protein could be purified to homogeneity before formaldehyde stabilization and would never revert to toxicity. Furthermore, when chemically coupled to bacterial polysaccharides, CRM197 enhances their immunogenicity (1). However, the development of new CRM197-derived biologicals has been hampered by the low yield obtained, which is 15 to 20 flocculation limit (Lf)/ml, compared with 150 to 200 Lf of toxin per ml obtained with the PW8 strain.

We have recently shown that the high yield of diphtheria toxin by the PW8 strain is due in part to the facts that it contains two ω^{tox+} phages integrated in the chromosome (15) and that the yield of toxin in C7 lysogens can be

increased two or three times by selecting double or triple lysogens (16). In the present paper, we report the isolation and characterization of C7($\beta 197$) $^{tox-}$ monolysogens and double lysogens, and we show that by using nontandem double lysogens under optimized growth conditions, the yield of CRM197 can be increased up to 65 Lf/ml.

MATERIALS AND METHODS

Strains. The nonlysogenic, nontoxic strain C7_s(-) $^{tox-}$ has already been described (2). The lysogenic, nontoxic strain C7($\beta 197$) $^{tox-}$ (21) was obtained from A. M. Pappenheimer Jr. (Biological Laboratories, Harvard University, Cambridge, Mass.). Phage $\beta 197^{tox-}$ was obtained by UV induction (8) from the C7($\beta 197$) $^{tox-}$ lysogen.

Media. PT medium (8) was used as a standard medium for bacterial growth. For the production of CRM197, C7($\beta 197$) $^{tox-}$ lysogens were grown on CY medium (11) containing 20 g of yeast extract, 10 g of Casamino Acids, 5 g of KH_2PO_4 , 10 ml of 1% (wt/vol) L-tryptophan, and 2 ml of 50% CaCl_2 per liter. The pH was adjusted to 7.4, and the medium was boiled and filtered through Whatman no. 40 paper. Two ml of solution II (9) and 1 ml of solution III (9) were added before sterilization. Before use, 30 ml of 50% (wt/vol) maltose-0.5% (wt/vol) CaCl_2 solution and 0.5 μg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per ml (0.1 μg of Fe^{2+} per ml) were added. Lysogens were screened by the halo plate assay (6, 16) in TYE agar (11). This was prepared as follows: 10 g of tryptose, 5 g of yeast extract, 5 g of NaCl, and 5 g of KH_2PO_4 were dissolved in 1 liter of water and brought to pH 7.4. Next, 2 ml of 50% CaCl_2 was added. The medium was boiled and allowed to sediment for 24 h (or was centrifuged). Two ml of solution II (9), 1 ml of solution III (9), and 12.5 g of Noble agar were added for each liter of supernatant before autoclaving. After sterilization, the TYE agar

medium was cooled to 55°C, and 4 U of horse diphtheria antitoxin (Production Control Department, Istituto Sieroterapico e Vaccinogeno Toscano Sclavo, Siena, Italy) per ml was added.

CRM197 production. Portions (0.1 ml) of overnight cultures of C7(β 197)^{tox-} lysogens were inoculated into 100-ml flasks containing 10 ml of deferrated CY medium with the addition of 0.1 μ g of Fe²⁺ per ml. Flasks were then incubated in a New Brunswick water bath rotary shaker at 35°C and 200 rpm for 24 to 48 h, and samples were taken at various intervals to monitor the optical density of the culture and the CRM197 released in the supernatant. The optical density at 590 nm was determined by a Perkin Elmer 35 spectrophotometer (light path of 1 cm).

Quantitative CRM197 determinations were performed by rocket immunoelectrophoresis (10) or by flocculation (14) or by both methods. Diphtheria toxin (Production Control Department, Istituto Sieroterapico e Vaccinogeno Sclavo) was used as standard antigen for rocket immunoelectrophoresis.

Isolation of new C7(β 197)^{tox-} lysogens. Phage β 197^{tox-} was obtained by UV induction of the original C7(β 197)^{tox-} lysogen as described by Miller et al. (8) and was plated on lawns of C7_s(-)^{tox-}. After incubation for 48 h at 35°C, lysogens growing in the middle of each turbid plaque were streaked for single colonies on CY agar and grown for 2 days at 35°C. Each colony was then tested for lysogeny by the phage release assay (8) on lawns of C7_s(-)^{tox-}. Positive colonies were then isolated twice more from a single colony, and the amount of CRM197 was evaluated by the halo assay (6, 16) on TYE agar containing 4 U of antitoxin per ml. Culture supernatants from new lysogens were tested for toxicity on CHO cells by the method of Murphy et al. (10).

DNA procedures. Chromosomal DNA was purified from C7(β 197)^{tox-} lysogens by a modification of the procedure of Shiller et al. (19). Bacteria grown overnight in PT medium were diluted in 30 ml of fresh medium to an optical density at 590 nm of 0.2 and were grown to an optical density of 0.6 to 0.8. Next, 1 μ g of penicillin G per ml was added, and the cultures were incubated for 2 h.

Bacteria were sedimented by centrifugation, suspended in 2 ml of 10 mM Tris (pH 8.2) containing 0.5 M sucrose and 5 mg of lysozyme per ml, and incubated at 37°C for 2 h. Cells were then harvested by centrifugation, suspended in 20 mM Tris (pH 8) containing 1 mM EDTA, lysed by the addition of sodium dodecyl sulfate to 1%, incubated at 68°C for 20 min, and digested for 2 h with pronase (5 mg/ml) which had been incubated for 1 h at 37°C to inactivate nucleases. The clear lysate was then extracted twice with phenol and twice with chloroform, and the DNA was precipitated with ethanol, suspended in distilled water, digested with 10 μ g of RNase A per ml for 30 min at 37°C, precipitated with ethanol again, and resuspended in distilled water. One drop of chloroform was then added to the DNA, which was stored at 4°C.

Purified DNA was digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's specifications. Enzyme-digested DNA was electrophoresed in vertical 1.3% slab gels in TEA buffer (50 mM Tris-acetate, 2 mM EDTA [pH 8]) and transferred to nitrocellulose filter paper (type BA85; Schleicher &

Schvell Co., Keene, N.H.) by the method of Southern (20). Southern blots were then processed by standard procedures (17, 20). Autoradiography was performed on Agfa-Gevaert Curix RPI film at -70°C with Curix MR800 intensifying screens.

RESULTS

Isolation of new C7(β 197)^{tox-} lysogens. Serial dilutions of sterile filtered supernatants from UV-induced C7(β 197)^{tox-} cultures, containing about 10⁸ phage per ml, were plated on C7_s(-)^{tox-} and were incubated at 35°C for 48 h. Lysogens growing in the middle of each turbid plaque were streaked for single colonies on CY agar, and the colonies obtained were tested for lysogeny by the phage release assay. Positive colonies were plated individually with a sterile toothpick on TYE agar containing 4 U of horse antidiphtheria serum per ml. After 24 to 48 h at 35°C, several types of colonies were observed. Most of the colonies had small halos, some had medium halos, and a few showed large halos (see Fig. 1). Southern blot analysis of chromosomal DNA from the above lysogens showed that the colonies with small halos were monolyso- gens, those with the medium halos were tandem double lysogens, and those with large halos were nontandem double lysogens. Out of 191 lysogens tested, 178 isolates were monolyso- gens, 8 were tandem double lysogens, and 4 were nontandem double lysogens. When tested for toxicity on cultures of CHO cells, all the new lysogens turned out to be negative.

Chromosomal arrangement. It has been previously shown that corynephages β ^{tox+}, ω ^{tox+}, and γ ^{tox-} are able to form monolyso- gens and double and triple lysogens in the C7_s(-)^{tox-} strain (16). This analysis was based on probing *Bam*HI digested chromosomal DNA with a DNA fragment from the vegetative phage genome containing the *attP* site and labeled with ³²P. In the present study, the same strategies were adopted with the β 197 *Bam*HI fragment 4 as probe.

This gave a single band (*attP*) of 3.9 kilobases (kb) when hybridized to the vegetative phage DNA (Fig. 2, lane A). Since the *attP* fragment was split during the lysogenization process, monolyso- gens gave two bands of different sizes: 4.6 and 2.7 kb if the phage was integrated in bacterial attachment site 1 (*attB1*), 3.4 and 2.2 kb if the phage was integrated in bacterial attachment site 2 (*attB2*) (Fig. 2, lanes B and C, respectively). Tandem double lysogens gave a third band in addition to the two bands characteristic of monolyso- gens. This corresponded to the reconstituted *attP* fragment of 3.9 kb (Fig. 2, lanes D and E).

Nontandem double lysogens showed four bands which were the sum of a monolyso- gen in *attB1* and a monolyso- gen in *attB2* (Fig. 2, lane

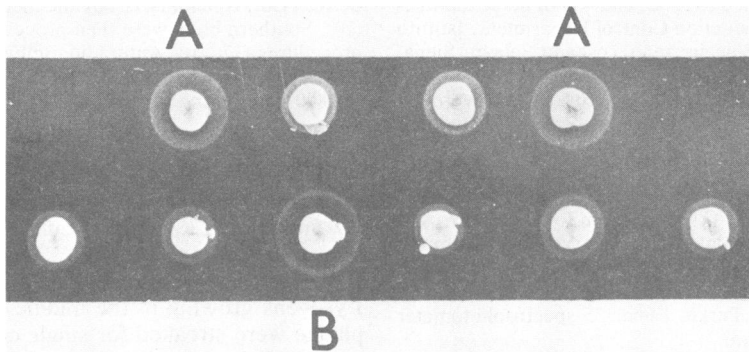


FIG. 1. Halo production of $C7(\beta 197)^{tox-}$ lysogens on TYE agar containing 4 U of anti-diphtheria toxin serum per ml. Colonies giving small halos are monolysogens; colonies with medium halos (A) are tandem double lysogens; the colony with the large halo is a nontandem double lysogen (B).

F). When analyzed by the above method, 10 out of 10 lysogens giving small halos in plates with 4 U/ml turned out to contain one phage, either in *attB1* or in *attB2*. All those giving medium halos (Fig. 1, A colonies) contained two tandem phages, either in *attB1* or in *attB2*, and all those giving large halos (Fig. 1, B colony) contained two nontandem phages, one in *attB1* and one in *attB2*.

Stability of lysogens. The stability of different lysogens was determined by plating dilutions of 48-h-old cultures on CY agar for single colonies and then transferring each colony with a sterile toothpick onto TYE plates containing 4 U of serum per ml. About 350 colonies were tested for each lysogen, and the number of phages in each colony was determined from the size of the halo (16). No segregants were found out of seven monolysogens and four nontandem double lysogens tested. On the contrary, all the tandem double lysogens always produced monolysogenic segregants, with a frequency ranging from 20 to 80%, depending on the age of the original isolate.

CRM197 production on liquid culture. Figure 3 shows the production of CRM197 as a function of time by typical representatives of monolysogens and tandem and nontandem double lysogens of $C7(\beta 197)^{tox-}$ during a 45-h culture. As shown, no CRM197 production was detected before the optical density of the culture reached 4.5 to 5 (the end of the log phase). On entering the late log phase, massive CRM197 production started, and it continued through the beginning of the stationary phase (about 20 h), when maximal optical density and maximal CRM197 levels were reached. Entering the late stationary phase, cells started to lyse and release proteolytic enzymes which digested the CRM197, the recovery of which in the supernatant decreased by 25% after 30 h and by up to 57% after 45 h.

The growth pattern and the CRM197 release

pattern of $\beta 197^{tox-}$ monolysogens and tandem and nontandem double lysogens did not show any significant difference. However, the

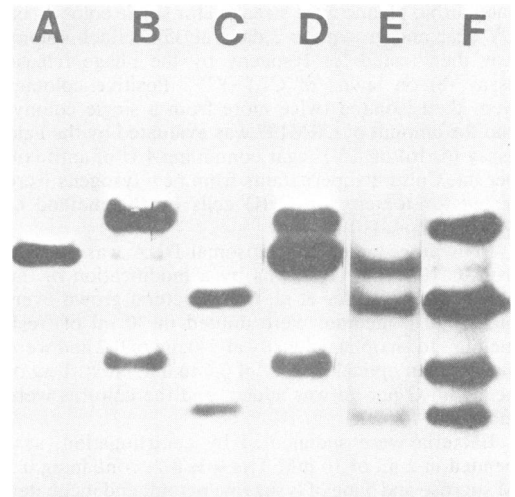


FIG. 2. Southern blot showing the chromosomal arrangement of different lysogens. Purified DNAs from phage $\beta 197^{tox-}$ (A) and from different $C7(\beta 197)^{tox-}$ lysogens (B through F) were digested with the restriction enzyme *Bam*HI, run on 1.3% agarose gels, transferred to nitrocellulose filters, and probed with ^{32}P -labeled $\beta 197^{tox-}$ *Bam*HI fragment 4, which contains the phage attachment site (*attP*). This probe gave a 3.9-kb band when hybridized to the vegetative phage DNA (A). Upon lysogenization, the *attP* fragment was split into two fragments of different sizes: 4.6 and 2.7 kb if the phage was integrated in bacterial attachment site 1 (*attB1*) (B), and 3.4 and 2.2 kb if the phage was integrated in bacterial attachment site 2 (*attB2*) (C). Tandem double lysogens in *attB1* (D) or in *attB2* (E) had, in addition to the monolysogen pattern, a third band corresponding to the reconstituted *attP* fragment. Nontandem double lysogens (F) gave four bands, two for the phage in *attB1* and two for the phage in *attB2*.

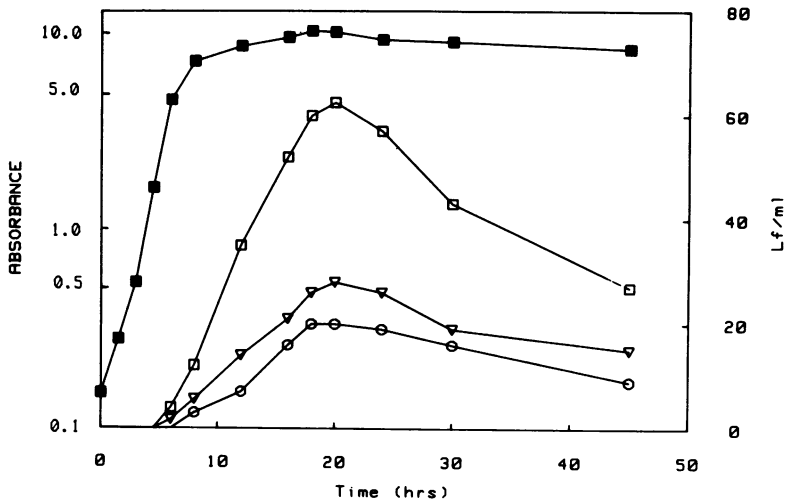


FIG. 3. CRM197 production on CY medium by a monolysogen (O), a tandem double lysogen (∇), and a nontandem double lysogen (□). ■, Optical densities of the cultures.

amounts of CRM197 produced were completely different. Monolysogens reached maximal levels of 15 to 20 Lf/ml, tandem double lysogens produced 25 to 30 Lf/ml, and nontandem double lysogens produced about 55 to 65 Lf/ml.

To test the effect of iron on the CRM197 production by these new lysogens, each strain was grown on CY medium containing no iron or 0.01, 0.1, or 1 μg of Fe^{2+} per ml. The concentration of 0.01 $\mu\text{g}/\text{ml}$ had no effect; 0.1 $\mu\text{g}/\text{ml}$ increased the CRM197 production (up to 40%), whereas 1 $\mu\text{g}/\text{ml}$ inhibited its synthesis completely.

DISCUSSION

The diphtheria toxoid presently used for human immunization is obtained by partial purification of crude culture supernatants from the highly toxigenic strain PW8 which have been detoxified by 0.4 to 0.7% formaldehyde for a period of 30 to 40 days. The process of formaldehyde detoxification involves intramolecular and intermolecular cross-linking (3-5), and the product obtained is extremely heterogeneous and difficult to purify.

Purification of the toxin before formaldehyde treatment would obviously be preferable, but toxoids obtained in this way show some reversion to toxicity (18). When detoxification of pure toxin was carried out in 0.05 M lysine, the toxoid obtained was stable (7); however, to our knowledge lysine has not been used as yet.

The use of a naturally nontoxic protein such as CRM197 for the production of diphtheria vaccines would eliminate most of the above problems. However, up to now, CRM197 has not been developed as an immunizing agent for

human use, mainly because of the poor yield of CRM197 obtained on liquid culture by the $C7(\beta 197)^{tox-}$ lysogen isolated by Uchida et al. (21).

It has been recently shown that the chromosome of *Corynebacterium diphtheriae* contains two phage attachment sites (*attB1* and *attB2*) and that the amount of toxin produced by $C7(\beta)^{tox+}$ and $C7(\omega)^{tox+}$ lysogens can be increased two or three times by selecting lysogens containing two or three prophages. In the present paper, we report the isolation of a series of $C7(\beta 197)^{tox-}$ monolysogens, tandem double lysogens (in *attB1* and *attB2*), and some nontandem double lysogens, and we show that the CRM197 production by a given $C7(\beta 197)^{tox-}$ lysogen depends on the number of phages integrated in the bacterial chromosome and on their stability. These results confirm and extend the data obtained for $C7(\beta)^{tox+}$ and $C7(\omega)^{tox+}$ lysogens, although some differences were found: $C7(\beta 197)^{tox-}$ monolysogens produced 15 to 20 Lf of CRM197 per ml, whereas under the same conditions $C7(\beta)^{tox+}$ and $C7(\omega)^{tox+}$ produced 25 to 35 Lf of toxin per ml. Furthermore, we would expect that nontandem double lysogens would produce 30 to 40 Lf of CRM197 per ml (i.e., twice that produced by monolysogens), but we routinely obtained 55 to 65 Lf/ml.

This is probably due to the fact that CRM197 in culture supernatants is degraded more rapidly than diphtheria toxin (data not shown). For example, if a monolysogen were to synthesize 35 Lf/ml and 15 Lf/ml were degraded, we would find 20 Lf/ml in the supernatant. On the other hand, a nontandem double lysogen would synthesize 70 Lf/ml, 15 Lf/ml would be degraded,

and we would recover 55 Lf/ml.

The results obtained show that CRM197 production can be raised to levels suitable for large-scale industrial production when cultures of nontandem double lysogens are used (tandem double lysogens are unstable, and their CRM197 production is low). It is to be hoped that this will allow the development of CRM197 as an immunizing agent for human use.

The methods used in the present study can be adopted for the isolation of nontandem double lysogens of other mutant corynephages and facilitate the production of other interesting CRM proteins, such as those codified by phages $\beta 45$, $\beta 30$, $\beta 228$, and $\beta 176$ (22).

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