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SHORT COMMUNICATION

EXPRESSION OF TOX+ GENE CARRIED BY AN EARLY MUTANT (DNA NEGATIVE MUTANT) OF NONLYSOGENIZING PHAGE β^{hv} IN NON-TOXINOGENIC DIPHTHERIA BACILLI

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In a previous paper (Matsuda et al., 1971a), we reported the isolation of various temperature-sensitive (=*ts*) mutants of a nonlysogenizing toxinogenic corynebacteriophage β^{hv} (Matsuda and Barksdale, 1966, 1967). One of these, *ts e*21, seemed to be a DNA negative mutant. Thus, under nonpermissive conditions, this particular *ts* mutant failed to synthesize DNA and development of the phage was arrested at an early stage of phage multiplication. The questions thus arose of whether expression of the tox+ gene carried by this phage mutant could occur under the nonpermissive conditions and if so of how it occurs. This is a brief report of studies on the kinetics of formation of toxin, phage and DNA during the cycle of infection of nontoxinogenic diphtheria bacilli by this phage mutant.

The system (Matsuda and Barksdale, 1967) used to investigate phage-directed synthesis of diphtheria toxin was applied in characterization of the phage mutant and in studies on the kinetics of toxin production, employing a nontoxinogenic, nonlysogenic C7 (—) strain (Barksdale and Pappenheimer, 1954) of *Corynebacterium diphtheriae*. The mutant *ts e*21 of nonlysogenizing phage β^{hv} used in this study was isolated from a stock of phage which had been treated with N-methyl-N′-nitro-N-nitroso guanidine as previously described (Matsuda et al., 1971a). It was isolated by the special selection procedures reported by Edgar and Lielausis (1964) as the temperature-sensitive mutant which forms plaques at 30 C but not at 38 C. The methods used for preparing the plating bacteria and for assay of plaque forming particles were as described previously (Matsuda et al., 1971a). The conditions for the experiments are given in the legends to the figures.

Fig. 1 shows DNA synthesis and the change in the optical density of C7 (—) cells infected with *ts e*21 mutant during incubations at 30 C and 38 C. As can be seen, at low temperature (30 C), the mutant phage-infected cells lysed at the time characteristic for the wild-type phage and thus produced plaque forming, mature progeny phage particles (1.7×10^{11} plaque forming units/ml). The total amount of DNA in the culture began to increase about 30 min after infection and increased linearly with time. At the end of the latent period it reached a value about 2 times that at the time of infection, as in the case of infection with the wild-type phage. In sharp contrast, at the elevated temperature (38 C), the *ts e*21 mutant

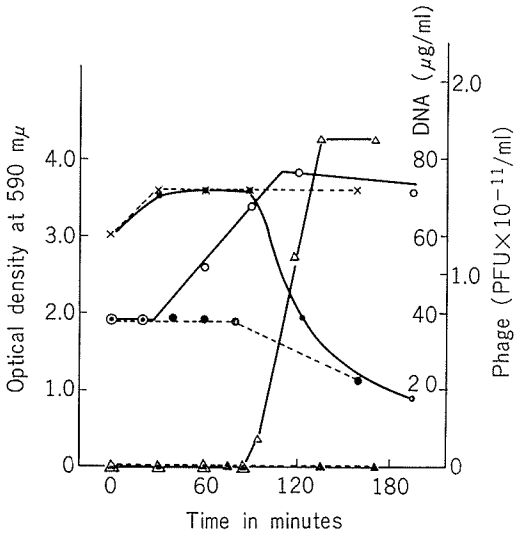

FIGURE 1. *DNA synthesis and phage production and lysis of C7(−) cells infected with β^{hv} phage mutant ts e21 at 30 C and 38 C.*

Nontoxinogenic, nonlysogenic C7(−) cells were grown to an optical density (OD) of 1.8 in deferrated PGT medium (Barksdale and Pappenheimer, 1954) containing 4% maltose and 0.04 μg iron/ml. Then they were infected with phage mutant ts e21 at a multiplicity of infection of about 6. The procedures used for synchronizing infection and for one step growth were as reported previously (Matsuda et al., 1971 a), except that, for adsorption, the temperature was 30 C and the period of incubation was 22 min. The washed, infected cells were resuspended to OD 3.0 in chilled fresh deferrated PGT medium containing 4% maltose without iron, and divided into two parts. One was incubated at 30 C and the other at 38 C, with gentle shaking. For measurement of DNA the method of Burton (1956) as modified by Epstein et al. (1963) was employed taking 1 ml samples of the experimental cultures. Lysis of the infected cultures was determined by measuring the optical density at 590 mμ of samples using a Bausch & Lomb spectrophotometer. Optical density at 30 C (•) and at 38 C (×); DNA at 30 C (○) and at 38 C (●); plaque forming phage particles at 30 C (△) and at 38 C (▲).

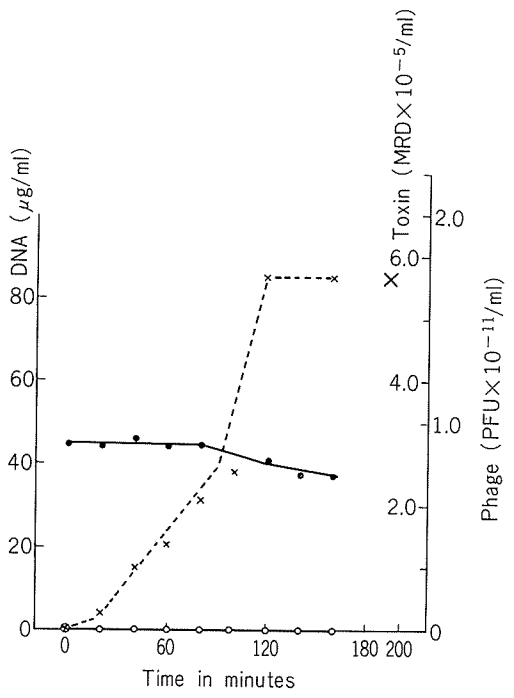

FIGURE 2. *Toxin, DNA and phage synthesis by C7(−) cells infected with β^{hv} phage mutant ts e21 at 38 C.*

Conditions were as described for Fig. 1. The toxin content of the infected culture was determined by injecting 0.1 ml of various dilutions of the culture filtrates (Millipore filter, 0.45 μ) intradermally into the depilated backs of rabbits, following the parallel line assay method of Kondo et al. (1960). Symbols: (•), DNA; (×), toxin; and (○), plaque forming phage particles at 38 C; (×), toxin yield on incubation at 30 C for 200 min.

did not lyse the cells or produce plaque forming particles and no appreciable increase in DNA in the cells could be observed after infection with this mutant phage. Previous work on *ts e*21 phage infected cells (Matsuda et al., 1971a) showed that the temperature-sensitive step for this phage mutant occurs 28 min after infection, early in the latent period of phage growth. Thus *ts e*21 is an early mutant which has a defect in DNA synthesis.

Fig. 2 shows the kinetics of the production of toxin and phage particles and of DNA synthesis by *ts e*21 phage-infected cells during incubation at the nonpermissive temperature (38 C) for the mutant. It is clear that toxin production occurred under the conditions; toxin began to increase about 20 min after phage infection and reached a plateau after about 120 min, as observed with the wild-type phage (Matsuda and Barksdale, 1966, 1967).

During the incubation, there was no increase in the amount of DNA or the number of plaque forming particles. The yield of toxin was 6×10^5 MRD/ml, 120 min after infection, and this is comparable with that observed on incubation of mutant phage-infected cells at the permissive temperature (30 C) for the mutant. The toxin content was also assayed by the semi-quantitative immunodiffusion method described previously (Matsuda et al., 1971a) and found to be 5~10 Lf eq. units per ml.

The results presented here clearly show that replication of phage DNA is not a prerequisite for expression of the tox$^+$ gene. Moreover, since the kinetics and yield of toxin by cells infected with the DNA negative mutant and with the wild-type phage are similar, only the parental DNA injected into the cell appears to function in toxin synthesis, as in the case of early functions for phage growth.

No mutations of phage genes essential for phage growth have so far been found to affect toxin production of cells infected with the mutant phage (Matsuda et al., 1971a) and phage growth does not appear to be affected by mutations in the tox$^+$ gene that result in production of fragments of the toxin protein molecule (Uchida et al., 1971; Matsuda et al. 1972). From these facts and results obtained with the DNA negative mutant, it appears very unlikely that the tox$^+$ gene is essential for phage growth, although it is possible that expression of the tox$^+$ gene may occur in some association with phage genes for early functions.

It is interesting that toxin production can proceed in the absence of phage DNA synthesis because this may imply that the number of tox$^+$ genes in cells remains constant. This provides a basis for calculating the rate of toxin production in this mutant phage-infected C7(--) cell system. Calculation revealed that the rate of toxin production in this system was about 1.0×10^5 MRD/10^9 cells·hr. This value is about three times that (3×10^4 MRD/10^9 cells·hr) estimated (Matsuda et al., 1971b) under growth-limiting conditions (Hirai et al., 1966) of the same host strain lysogenized with β phage (C7(β)). The high rate of toxin production observed in this system may be due to a dose effect of tox$^+$ gene injected with parental phage DNA (multiplicity of infection, about five), or to a dose effect of the tox$^+$ gene which may be copied and increase two-three fold in a very brief period after infection, or to liberation from some regulatory mechanism of the host operating in the lysogenic state. However, one must await further investigation to clarify this.

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