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PRELIMINARY REPORT

DEGREE OF EXPRESSION OF THE TOX$^+$ GENE INTRODUCED INTO CORYNEBACTERIA BY PHAGES DERIVED FROM DIPHTHERIA BACILLI HAVING DIFFERENT CAPACITIES TO PRODUCE TOXIN

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The toxinogenicity of *Corynebacterium diphtheriae* that is determined by the genetic information (tox$^+$ gene) carried by specific corynebacteriophages (Freeman, 1951; Groman, 1953, 1955; Barksdale and Pappenheimer, 1954; Barksdale, 1955), is known to vary widely in different strains. However, the mechanism causing this inherent difference between strains is unknown. In studies on this problem, we recently obtained evidence that the degree of ability to produce diphtheria toxin in corynebacteria depends on the host bacterial strain, not on the tox$^+$ phage tested with which the host bacteria are lysogenized.

This study was to examine to what extent the tox$^+$ gene carried by phages derived from diphtheria bacilli having different capacities to produce toxin, can be expressed when a single host strain is lysogenized with different tox$^+$ phages and when different host strains are lysogenized with a single tox$^+$ phage. The host bacterial strains employed were the nontoxinogenic nonlysogenic C7 (—) strain (Barksdale and Pappenheimer, 1954) of *C. diphtheriae* and the 9304 strain of *C. ulcerans* (Maximescu, 1968), kindly provided by Drs. Saragea and Maximescu ('Dr. Cantacuzino' Institute, Bucharest), while the phage strains were the phages ω and ω_7 (Matsuda et al., 1971a) derived from a highly toxinogenic substrain, Biken, of the Park Williams No. 8 (PW8) strain (Park and Williams, 1896) and classic corynebacteriophage β obtained from a low toxinogenic C7 (β) strain (Barksdale and Pappenheimer, 1954) of *C. diphtheriae*. The remarkable difference in the capacities to produce toxin between the PW8 and C7 (β) strains used as sources of phages is shown in the accompanying tables. Lysogens were prepared by combination of host bacterial strains and the phage preparations which had been purified by successive single plaque isolations on *C. ulcerans* for ω and C7 (—) for ω_7 and *C. ulcerans* or C7 (—) for β. They were of the following five kinds: C7 (ω_7), C7 (β), *C. ulcerans* (ω), *C. ulcerans* (ω_7) and *C. ulcerans* (β). Incidentally, no C7 (ω) could be obtained because the C7 (—) strain was insensitive to phage ω. These lysogens were each isolated from resistant outgrowths in giant plaques formed on the lawn of sensitive bacteria by Millipore filtrates of each phage lysate and subsequently a pure clone of each lysogen was obtained after several successive single colony isolations. The lysogens were then tested for their stability in terms of phage

producing ability, immunity against homologous phage and of toxinogenicity by examining these properties of the subclones from at least ten colonies of each culture. They were also tested to see whether these properties could be eliminated by incubating the lysogens in medium containing anti β^{hv} phage serum (Matsuda et al., 1971a). The test showed that these lysogens were all stable.

The toxinogenicities of the lysogens thus obtained were then compared both in terms of the rate of toxin production (amount of toxin produced per unit of bacteria per unit of time) under growth-limiting conditions (Hirai et al., 1966) and of the final toxin yields obtained under growing conditions which were optimal for toxin production. Since only traces of toxin were detectable in cell-extracts of the lysogens tested, the toxin content was assayed of the culture filtrates. It was determined as minimal reaction dose (MRD) on rabbit skin and as the limit of flocculation (Lf) or Lf equivalence (Lf eq.) by the methods described previously (Matsuda et al., 1971b). Growth of the cultures was determined by measuring the optical density (OD) of samples at 590 mμ using a Bausch and Lomb spectrophotometer. The experimental conditions are given in the legends to the tables.

The results summarized in tables 1 and 2, show the final toxin yields (Lf/ml) and the rates of toxin production (Lf or Lf eq. or MRD/OD·hr) of the various lysogens described above. Several independently isolated strains were used for each lysogen and they gave similar results. From the tables, it is apparent that the degree in toxinogenicity of the lysogens varies with the kind of host bacteria, irrespective of the source of the tox$^+$ phages with which the hosts were lysogenized. Thus a marked difference was seen between the lysogens derived from C7 (−) and those from *C. ulcerans*. This is particularly noticeable with respect to their rates of toxin production, the toxinogenicity of the latter lysogens being about one tenth of that of the former. In sharp contrast, almost no differences were seen between the lysogens derived from the same host with different tox$^+$ phages, either in their rates of toxin production or in their toxin yields.

These results clearly indicate that the host must be involved in controlling the degree of diphtheria toxinogenicity in these lysogenic corynebacteria and that an important factor directly associated with such control by the host is regulation of the rate of toxin production. Careful inspection of the present data also suggests that another factor involved in control of toxin yield by the host could be regulation of the duration of toxin production under growing conditions. Incidentally, in case of the PW8 strain, Miller et al. (1966) suggested a possible importance of the metabolism of the host in determining the yields of toxin. They considered that, presumably because of its unique, abnormal respiratory system (Pappenheimer et al., 1962), the PW8 strain is able to continue its growth for a long

TABLE 1. *Toxin production by various strains of corynebacteria lysogenized with phages derived from diphtheria bacilli having different capacities to produce toxin (under growing conditions*[a])

Strain	Toxin yield (Lf/m*l* culture)	Final growth (OD at 590mμ)
C7 (ω_7)[b]	11	9.9
C7 (β)[c]	12	11.1
C. ulcerans (ω)	2~3	12.6
C. ulcerans (ω_7)	2~3	15.9
C. ulcerans (β)	2~3	12.0
PW8[d]	140	12.0

a Cells were grown in modified Pope's medium (Yoneda and Matsuda, 1961) containing 0.1 μg iron/m*l* with shaking at 35C for 52 hr.

b Phage ω could not lysogenize nor lyse C7(−).

c Similar results were obtained irrespective of the sources of β phage (C7 (β), *C. ulcerans* (β)) and of the bacterial strains (C7 (−), *C. ulcerans*) used for the preparation of the phage.

d Source of phages ω and ω_7 (Matsuda et al., 1971a).

TABLE 2. *Rates of toxin production by various strains of corynebacteria lysogenized with phage derived from diphtheria bacilli having different capacities to produce toxin (under growth-limiting conditions*[a])

Strain	Rate of toxin production	
	(Lf or Lf eq[b]./OD·hr	(MRD/OD·hr)
C7 (ω_7)[c]	0.25	3.1×10^4
C7 (β)[d]	0.25	3.1×10^4
C. ulcerans (ω)	0.01~0.03[b]	3.4×10^3
C. ulcerans (ω_7)	0.01~0.03[b]	—
C. ulcerans (β)	0.01~0.03[b]	3.1×10^3
PW8[e]	1.25	1.3×10^5

a Toxin production under growth-limiting conditions was performed as described by Hirai et al. (1966) as follows. Cells were precultured in deferrated PGT medium (Barksdale and Pappenheimer, 1954) containing 4% maltose and 0.04μg iron/m*l*. When the OD of the culture reached 1.7, the culture was chilled and centrifuged and the packed cells were resuspended in deferrated CST-6 medium (Uchida and Yoneda, 1967; 0.05M Tris-HCl buffer (pH 7.4) containing 0.6% casamino acid (Difco, certified), 0.003% DL-tryptophane, 0.01% cystine, 0.3% sodium succinate, 0.45% NaCl and 0.1% $MgSO_4\cdot7H_2O$) to give an OD of 9.0 (about 1×10^{10} cells/ml). Aliquots of 9 ml of the heavy cell suspension were put into 100 ml Erlenmeyer flasks and incubated at 35C in a rotary shaker at 140 rotations/min. Flasks were taken out after 60 min and 120 min and the cultures were chilled and centrifuged and the supernatants were assayed for toxin. Rates of toxin production (amounts of toxin produced/OD·hr) were calculated from data in the linear range of toxin production.

b Toxin content (Lf eq.—unit equivalent to the limit of flocculation (Lf)) of the concentrated culture filtrate was determined by the method described previously (Matsuda et al., 1971 b) using the immunodiffusion technique.

c Phage ω could not lysogenize nor lyse C7 (−).

d Similar results were obtained irrespective of the sources of β phage (C7 (β), *C. ulcerans* (β)) and of the bacterial strains (C7 (−), *C. ulcerans*) used for the preparation of the phage.

e Source of phages ω and ω_7 (Matsuda et al., 1971 a).

period until the cellular iron content falls to a very low level resulting in unusually high yields of toxin, However, this may not be the case in the present experiments where the yields of toxin were compared in fast-growing lysogenic strains, such as C7 (β) with a normal respiratory system (Pappenheimer et al., 1962). The intrinsic nature of the host factors is still unknown and requires further study.

Investigations are now in progress employing more strains and isolating host and phage mutants. The results will be published elsewhere.

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