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Relationship Between Toxigenicity and Sporulating Potency of *Clostridium novyi*

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ABSTRACT

NISHIDA, SHOKI (Kanazawa University, Kanazawa, Japan), AND GIZO NAKAGAWARA. Relationship between toxigenicity and sporulating potency of *Clostridium novyi*. J. Bacteriol. **89**:993-995. 1965.—The less toxigenic the strains, the stronger was the sporulating potency of *Clostridium novyi* strains isolated. This was confirmed by investigation of the toxigenicity of substrains of *C. novyi* 140 possessing different degrees of sporulating potency. Atoxic strains or type C strains could be obtained from the parent type A strain (no. 140) by heat selection. This phenomenon was also observed in the other five strains. Prolonged storage of *C. novyi* strains also resulted in selection of cells with stronger sporulating ability and lower toxigenicity.

We reported previously (Nishida and Nakagawara, 1964) that the longer the duration of sample incubation, the less toxigenic were the strains of *Clostridium novyi* isolated. We also reported that when higher temperatures were employed to preheat samples, strains isolated were less toxigenic. Since these procedures for isolation result in selection of strongly sporulating strains, an experiment was undertaken to elucidate the relationship between the sporulating potency and toxigenicity of these strains. Attention was given also to the relation of atoxic type C strains to toxigenic type A strains of *C. novyi*.

MATERIALS AND METHODS

Strains. C. novyi 140 is a stock strain kindly supplied by S. Miyasaki of the Institute for Infectious Disease, Tokyo, Japan. *C. novyi* 37101, 37102, 37103, 37104, and 37105 were isolated by us from soil specimens heated at 70 C for 10 min and incubated for 3 days. *C. novyi* 77101, 77102, 77103, 77104, and 77105 were obtained from soil specimens heated at 70 C for 10 min and incubated for 7 days. Five atoxic strains of *C. novyi*, namely 3-1101, 3-1102, 3-1103, 3-1104, and 3-1105, were arbitrarily selected from strains isolated from soil specimens heated at 100 C for 10 min and incubated for 3 weeks; these strains, and the exact procedure of their isolation, were described previously (Nishida and Nakagawara, 1964). All the toxic strains isolated were identified as type A and all the atoxic strains, as type C, according to the description of Oakley, Warrack, and Clarke (1947).

Toxin production. The medium and procedure for toxin production were described previously (Nishida and Nakagawara, 1964).

Estimation of sporulating potency. After the strains were cultivated in the medium for toxin production for 96 hr, Gram-stained smears of these cultures were prepared. The ratio of the number of spores to total cells per milliliter is designated as sporulating potency. Sporulating potency was calculated for 20 microscopic fields containing approximately 50 to 150 cells per field. Gram-positive forespores were also included in the number of spores. Although the most probable number method (Hoskins, 1934) was routinely employed in our laboratory for estimating the sporulating potency, we preferred the present method to the most probable number method, because spores of *C. novyi* can be more easily counted microscopically than those of other clostridia and because we believed that a different method to estimate sporulating potency is useful for analysis of the nature of this mechanism.

RESULTS

Sporulating potency of strains isolated. Toxigenicity and sporulating potency of the strains isolated were estimated (Table 1). The results imply that the longer the sample incubation and the higher the temperatures applied to the samples, the stronger was the sporulating potency and the weaker was the toxigenicity of the strains isolated. These findings indicate also that the type C strains of *C. novyi* are possibly the type A strains possessing strong sporulating potency. An experiment was, therefore, undertaken to determine whether or not a conversion from type A to type C could be obtained by selecting heat-resistant substrains from parent type A strains.

Toxigenicity and sporulating potency of C. novyi

TABLE 1. *Sporulating potency of strains with different degrees of toxigenicity*

Strain no.	Sporulating potency*	Toxigenicity (MLD/ml)	Type
37101	1/1,650 (0.06)	10^3	A
37102	2/2,302 (0.08)	10^3	A
37103	0/2,406 (0)	10^3	A
37104	0/1,232 (0)	10^3	A
37105	2/1,562 (0.1)	10^3	A
77101	231/1,622 (14.2)	10^2	A
77102	199/2,146 (9.2)	10^2	A
77103	245/2,232 (10.9)	10^2	A
77104	102/1,245 (8.1)	10^2	A
77105	92/1,741 (5.2)	10^2	A
3-1101	642/1,622 (39.5)	0	C
3-1102	321/932 (34.4)	0	C
3-1103	462/1,432 32.2)	0	C
3-1104	388/1,262 (31.2)	0	C
3-1105	762/1,633 (46.6)	0	C

* Sporulating potency (expressed as number of spores/number of total cells counted) is based on examination of 20 arbitrarily selected microscopic fields. Figures in parentheses indicate the numerical ratio (percentage) of spores to total cells.

type A strains and their heat-resistant substrains. *C. novyi* 140 was cultivated for 48 hr in cooked-meat broth containing 1% maltose; 1-ml portions of the suspension were distributed into five small test tubes (13 by 100 mm) and heated at 80, 90, or 100 C for 10 min, or at 100 C for 100 min; the fifth test tube was left unheated as a control. A 0.5-ml amount of each of the heated culture suspensions was transferred into liver broth and cultivated for 48 hr to obtain the heat-resistant substrains. The sporulating potency and toxigenicity of the heat-resistant substrains, as well as of the parent strains, were estimated (Table 2). The parent strain exhibited a high toxigenicity but an extremely low sporulating potency, whereas the heat-resistant substrains showed a decrease in toxigenicity and a reciprocally marked increase in their sporulating potency. The atoxic substrain obtained was identified as type C. A similar result obtained with the 10 substrains of *C. novyi* 140 established from 10 colonies developing on agar plates revealed that the conversion was not due to contamination by type C strain. Furthermore, the same findings were observed with all of five other strains examined (37101, 37102, 37103, 37104, and 37105).

Effect of storage on toxigenicity and sporulating potency. The sporulating potency and toxigenicity of five strains were compared immediately after isolation and after these strains had been stored in liver broth for 6 months (Table 3). The sporulating potency of these strains was heightened reciprocally with attenuation of toxigenicity. The sporulating potency of strain 37102 was particularly heightened, and its toxigenicity was markedly lowered.

TABLE 2. *Toxigenicity and sporulating potency of Clostridium novyi 140 and its heat-resistant substrains*

Strain used	Sporulating potency*	Toxigenicity (MLD/ml)	Type
Parent strain....	2/6,406 (0.03)	10^5	A
Substrain resistant to 80 C for 10 min.....	1,038/4,921 (21.09)	10^4	A
Substrain resistant to 90 C for 10 min.....	770/1,994 (38.61)	10^2	A
Substrain resistant to 100 C for 10 min.....	545/1,326 (41.1)	10^1	A
Substrain resistant to 100 C for 100 min.....	735/1,650 (44.5)	0	C

* See footnote to Table 1.

TABLE 3. *Changes in sporulating potency and toxigenicity during 6-month storage*

Strain	First* estimation		Second† estimation	
	Toxigenicity (MLD/ml)	Sporulating potency	Toxigenicity (MLD/ml)	Sporulating potency
37101	10^3	1/1,650	10^2	75/1,505
37102	10^3	2/2,302	10^1	533/2,221
37103	10^3	0/2,406	10^2	116/2,697
37104	10^3	0/1,232	10^2	264/1,922
37105	10^3	2/1,562	10^2	46/1,108

* The first estimation was performed immediately after the strains were isolated.

† The second estimation was performed 6 months later than the first estimation.

Definition of sporulating potency. Because the present method for estimating sporulating potency differs from the method employed by our colleagues (Yamagishi, Ishida, and Nishida, 1964; Nishida, Tamai, and Yamagishi, 1964; Sanada, 1963), we did experiments to confirm our definition of sporulating potency. Three strains of *C. novyi* (37101, 77101, and 3-1101) which were distinctive in sporulating potency and toxigenicity were plated on modified Weinberg's blood-agar (Nagler, 1944), and 10 substrains of each of the three strains were estab-

lished. Examination of their sporulating potency verified the general rule of the definition that strain A consists of cells possessing approximately the same sporulating potency as that of the parent strain A and strain B consists mainly of cells possessing approximately the same sporulating potency as that of parent strain B.

DISCUSSION

The intimate correlation between sporulating potency and toxigenicity was recently demonstrated in *C. perfringens* (Yamagishi, Ishida, and Nishida, 1964), *C. sordellii* (Nishida, Tamai, and Yamagishi, 1964; Tamai and Nishida, 1964), and *C. tetani* (Sanada, 1963). Since toxigenesis in *C. novyi* is also closely associated with its sporulating potency, we believe that the close correlation is a rather common phenomenon prevailing among clostridia. Manifestly, sporulating potency is the greatest agent controlling the mechanism of toxigenesis in *C. novyi*. However, we cannot deny the presence of some other agents which influence toxigenesis. For instance, a heat-resistant substrain of *C. novyi* 140 with a sporulating potency of 21.09% still possessed a toxigenicity of 10^2 MLD per ml (Table 2), whereas most of the wild strains or their heat-resistant substrains exhibiting approximately similar sporulating potency showed toxigenicity of only 10^0 MLD per ml.

Knaysi (1948) and Grelet (1957) reported that sporulation is an integrated biological process in spore-bearing organisms and that the asporogenous state will be brought about by inhibiting the integral process of metabolism. We have assumed that heating cultures results in selection of cells with integral physiology from a mixture with auxotrophic cells which were abortive in sporulation. We cannot be sure, however, that a heat-resistant strain is selected without dependence on any mutagenic effect (Curran and Evans, 1937; Nelson, 1943).

With regard to type differentiation, we believe that the designation of type C should be abolished, because type C strains are only strongly sporulating type A strains. However, this change should be delayed until further investigations along this line elucidate the relationships among the other types of *C. novyi*.

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