JOURNAL OF BACTERIOLOGY, Apr. 1971, p. 289-291

Vol. 106, No. 1
Printed in U.S.A.

Induction of Mutants of *Staphylococcus aureus* 100 with Increased Ability to Produce Enterotoxin A

MISCHA E. FRIEDMAN[1] AND MARY B. HOWARD

U.S. Army Biological Defense Research Laboratories, Fort Detrick, Frederick, Maryland 21701

Received for publication 11 November 1970

As a result of serial exposures to a mutagenic agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the yield of enterotoxin A produced by the last mutant in the series was increased nearly 20-fold over the amount produced by the parent *Staphylococcus aureus* 100.

The identification and quantitation of staphylococcal enterotoxin A are complicated by the fact that only small amounts are elaborated by enterotoxigenic strains either in contaminated food or in laboratory cultures. The sensitivity of the serological tests for these purposes (1, 7, 9) necessitates the use of antiserum with a high degree of specificity, the preparation of which requires large volumes of culture to provide sufficient crude toxin for purification (4). Increased amounts of enterotoxins A, B, and C have been reported (1-3) by growth in or on dialysis membranes which prevented dilution by the medium. However, the volumes recovered were small. Despite medium modifications (4, 5), the yield still did not exceed 3 to 8 μg/ml in normal cultures.

This report describes experiments which enabled the collection of large amounts of crude material containing an approximately 20-fold increase in toxin. This was accomplished through the selection of high toxin-producing mutants of *Staphylococcus aureus* 100 after a series of exposures to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG).

S. aureus 100 was obtained from M. S. Bergdoll. In a preliminary study on the effects of mutagenic agents, this strain was exposed to ultraviolet light (15-w G. E. Germicidal Lamp, 60 cm for 60 sec). A two-log decrease in viable cells resulted. One of the survivors was designated isolate 64-11A-5 and seemed to produce slightly greater amounts of enterotoxin A than did its parent. Isolate 64-11A-5 was exposed to NTG to begin the serial selection of mutants designated 1N through 13N. The culture to be treated was adjusted to an optical density (OD) of 0.26 (Coleman model 9 colorimeter, red filter, with distilled water as the blank). A 1-ml amount of this was inoculated to 10 ml of medium consisting of 4% N-Z-Amine type NAK (Sheffield Chemical Company) supplemented with 0.2% glucose and 0.2% yeast extract (Difco), *p*H 6.7, and incubated at 37 C on a reciprocal shaker. When the OD reached 0.24, the cells were centrifuged from the medium and resuspended in the same volume of freshly prepared NTG solution (1 mg/ml in 0.85% NaCl). The suspension was held at room temperature for 30 min and centrifuged, and the cells were resuspended in the original volume of fresh, pre-warmed broth and incubated at 37 C on the shaker for a 4-hr expression period. The centrifuged cells then were suspended in 10 ml of sterile saline and stored at 4 C. Samples from the dilution shown to provide approximately 50 to 100 well-isolated colonies were spread on a large number of 4% NAK agar plates containing rabbit antiserum to highly purified enterotoxin A. After 24 to 72 hr of incubation, enterotoxigenic colonies were surrounded by a zone or halo of immune precipitate (Fig. 1). From 2,000 to 10,000 colonies were observed after each NTG treatment, and those with the highest ratio of halo width to colony size were selected for quantitative assay. The highest producer was exposed to NTG to provide the next series of mutants, and the 1N through 12N series was selected in this manner. Isolate 12N-8 was exposed to theee successive cycles of NTG treatment with intervening 6-hr expression periods and a final 22.5-hr expression time. The treated cells were plated on approximately 300 4% N-Z-Amine NAK agar plates without added antiserum. Over 3,000 large colonies were picked and

[1] Present address: Division of Research Grants, National Institutes of Health, Bethesda, Md. 20014.

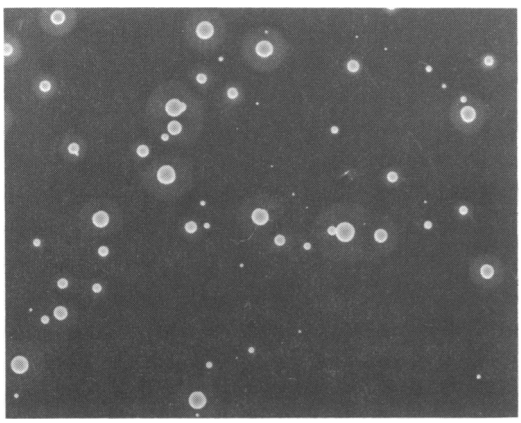

FIG. 1. *Isolate 10N-34 plated on antiserum agar after exposure to N-methyl-N'-nitro-N-nitrosoguanidine. Incubation was for 41 hr at 37 C. × 2.*

subsequently assayed by the Oudin immunodiffusion technique as described by Silverman (6).

A gradual but steady increase in the amount of enterotoxin A produced by the successively derived mutants was observed (Table 1). Other effects of the NTG exposure were a prolonged lag phase and a slower increase in turbidity of these mutant cultures. At no time during a 24-hr growth period did the OD of a mutant culture exceed that of the parent strain. At the end of the incubation, the OD of cultures of NTG-exposed isolates remained lower than for the parent cultures although the amount of enterotoxin was considerably greater (Table 2).

The isolates maintained on agar slants at 4 C were quite variable with regard to enterotoxigenicity and viability. However, storage of early log-phase liquid cultures at −20 C provided stable stocks of desirable growth and toxin-producing characteristics. A large inoculum was necessary to obtain good growth and optimum yields of enterotoxin in liquid media and significantly greater yields were not obtained when incubation was extended beyond 24 hr. The slow growth rate was particularly noticeable when the mutants were cultured on agar plates.

That the enterotoxin produced by the mutants was antigenically identical with that produced by the parent was evidenced by formation of a line of identity between these on gel diffusion plates when reacted with highly specific immune serum. Also, highly purified enterotoxin A prepared from a number of these isolates caused emesis in monkeys at the same dose levels as did enterotoxin A prepared from the parent strain (Table 3).

The stepwise increase in enterotoxin yield by the mutants resulting from serial exposure of *S.*

TABLE 1. *Enterotoxin A production by Staphylococcus aureus strain 100 and serial mutants derived from it.*

Isolate	Enterotoxin A (μg/ml)[a]
100-8-4	3
64-11A-5	4
1N-186	4
2N-64	5
3N-13	9
4N-45	9
5N-5	9
6N-15	11
7N-103	12
8N-63	12
9N-68	14
10N-34	38
11N-165	39
12N-8	41
13N-2909	57

[a] Medium: 4% N-Z-Amine type NAK, 0.2% glucose, 0.2% yeast extract, *p*H 6.7. Incubation: 37 C, 100 rev/min on reciprocal shaker, 24 hr.

TABLE 2. *Optical density and enterotoxin A content of cultures of the parent and some of the serially selected mutants*[a]

Organism	Optical[b] density	Enterotoxin A (μg/ml)
100	0.58	3
64-11A-5	0.50	4
3N-13	0.44	9
6N-15	0.42	11
10N-34	0.33	35
12N-8	0.32	41
13N-2909	0.32	57

[a] Incubation and medium as in Table 1.

[b] Optical density of a 10-fold diluted culture after 24 hr of growth.

TABLE 3. *Response of rhesus monkeys to enterotoxin A derived from Staphylococcus aureus 100 and some serially selected mutants of this strain*

Source of purified enterotoxin A	Dose (μg/kg)	Route	No. responding/ no. challenged
Strain 100	0.2	iv[a]	4/8
64-11A-5	0.2	iv	7/9
3N-13	0.1	iv	5/6
6N-15	0.2	iv	5/6
3N-13	50[b]	Oral	5/6
13N-2909[c]	60[b]	Oral	4/4

[a] Intravenous.

[b] Micrograms per monkey.

[c] Filtered culture supernatant was fed as no highly purified material from this isolate was available.

aureus 100 to NTG strongly suggests that an alteration in the ability of individual cells to synthesize or release enterotoxin A, or both, was induced. Unlike the S-6 strain of *S. aureus* (8), no spontaneous variability in toxin formation was observed with the enterotoxin A producer, strain 100 (4). Repeated exposures to NTG resulted in a continued increase in the amount of enterotoxin found in culture supernatants, despite negative effects on the growth and stability of the mutants. Preliminary results have indicated that some other extracellular products (coagulase, nuclease, hemolysins) elaborated by strain 100 occur in progressively decreasing amounts in the serially selected mutants, in contrast to the increase in enterotoxin A. Since mutants were selected on the basis of their ability to provide larger yields of enterotoxin A, a deleterious effect on the production of other substances is understandable as a consequence of the serial exposures to NTG.

We thank Robert A. Altenbern and Ivan D. Goldberg for helpful advice and review during the course of this work. We thank Guy Dayhoff, Steve Reid, Carl Valentine, and Allen Knott for valuable technical assistance.

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