Length of Deoxyribonucleic Acid of PBSX-like Particles of *Bacillus subtilis* Induced by 4-Nitroquinoline-1-Oxide

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The formation of particles resembling PBSX phages was induced by 4-nitroquinoline-1-oxide in a Marburg strain of *Bacillus subtilis*. All particles were homogeneous in their morphology. Physical and biological analyses revealed that the deoxyribonucleic acid (DNA) carried by these particles are fragments of host-cell DNA. The contour length of the DNA is 4.25 μ m, corresponding to a molecular weight of 8.1×10^6 daltons.

Bacillus subtilis releases defective phages by various phage-inducing treatments; e.g., ultraviolet (UV) light, mitomycin C, and hydrogen peroxide (5, 12, 14; F. A. Eiserling and W. R. Romig, *Bacteriol. Proc.*, p. 118, 1964). Although different names were given to these defective phages by each author, they may be identical, since they revealed somewhat similar morphology. It was also found, however, that mitomycin C induction produced simultaneously three different types of particles (PBSX lysate) from *B. subtilis* 168 (12). Recently, a single type of particle (PBSX) was further purified from PBSX lysate, and the properties of its deoxyribonucleic acid (DNA) were analyzed by Okamoto et al. (9).

We studied the relationship between UV sensitivity and 4-nitroquinoline-1-oxide (4NQO) sensitivity on *B. subtilis* Marburg strain (3). This drug, a water-soluble strong carcinogenic agent (8), can induce λ phage in *Escherichia coli* K-12(λ) (2). We also found that 4NQO treatment introduced cell lysis and produced a single type of PBSX-like particles from *B. subtilis* wild-type strain. In this paper, we report that DNA extracted from these particles is characterized as fragmented host-cell DNA of a homogeneous size, as determined by contour length measurement on electron micrographs.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* Marburg, a prototrophic strain (wild) supplied by S. Zamenhof to the Institute of Applied Microbiology, the University of Tokyo (10), was used for the induction of the PBSX-

like particles and for the isolation of wild-type DNA for transformation studies. An auxotrophic strain (3115) of *B. subtilis* (*arg-15*, *his-31*, *ind-168*) requiring arginine, histidine, and indole was used as the recipient in transformation (4).

Induction and purification of PBSX-like particles. A 10-ml amount of an overnight culture of the wild strain grown in nutrient broth [nutrient medium (Difco), 8 g; NaCl, 5 g; in 1 liter of water, pH 7.0] was added to 1 liter of nutrient broth and was incubated with vigorous shaking at 37 C. When the cell density reached approximately 10^8 cells per ml, 4NQO (Beacon Chemical Co., Boston, Mass.) was added to the culture at a final concentration of 10^{-3} M. After subsequent incubation of this culture for 10 min, 4NQO was removed by centrifugation ($13,000 \times g$ for 10 min at 4 C). Sedimented cells were suspended in 200 ml of nutrient broth and were further incubated with vigorous shaking at 37 C until the cell density decreased (usually in 7 hr). Initially, the cell density increased by a change in cell shape to long form, but colony-forming ability decreased drastically from 10^8 to 10^4 /ml within 30 min after the resuspension in nutrient broth. Alternatively, after 7 hr of incubation, muramidase (final concentration, 50 μ g per ml; Worthington Biochemical Corp., Freehold, N.J.) was added to the culture to complete cell lysis, which occurred within 10 min at 37 C. Cell debris was removed by low-speed centrifugation. PBSX-like particles were then harvested by high-speed centrifugation ($22,000 \times g$ for 2 hr in a Spinco R19 rotor at 4 C). The pellet was kept in a small volume of nutrient broth in the refrigerator.

The concentrated particle suspension (1.5 to 2 ml) was added to the top of two layers (1.5 ml each) of CsCl solutions with concentrations of 56% (w/w) and 38% (w/w) respectively, and was placed in a 5-ml nitrocellulose tube of the SW50 rotor (Spinco). After 2 hr of centrifugation at $130,000 \times g$, the blue-white band in the middle of the 38% layer of CsCl was collected and dialyzed for a few hours at room

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temperature against an excess volume of minimal medium without glucose (13).

An electron micrograph of this preparation is shown in Fig. 1. The picture reveals only one type of particle, although many separate components of it, heads, contracted sheaths, and tail needles, were observed in other fields. Most of the particles show empty heads and contracted sheaths. The head diameter is about 43 nm and the tail length is about 220 nm. The size and shape of the particles are similar to those of phage PBSX (9) and of phage SP α (F. A. Eiserling and W. R. Romig, *Bacterial Proc.*, p. 118, 1964).

DNA extraction. DNA was extracted from purified particles with an equal volume of freshly distilled phenol saturated with SSC (0.15 M NaCl, 0.015 M sodium citrate) by gentle rocking by hand for 15 min at room temperature. After centrifugation ($10,000 \times g$ for 5 min), the phenol phase was taken off with a pipette and discarded. This extraction was repeated once more, and finally the DNA fraction was dialyzed against an excess volume of SSC overnight at 4 C.

DNA concentration was measured in a Zeiss spectrophotometer. The extinction ratios (260 to 230, 260 to 250, and 260 to 280 μm) were 2.67, 1.14, and 2.00, respectively.

Bacterial DNA was extracted and purified by the method of Saito and Miura (10).

Length measurement of DNA. DNA samples for electron microscopy were prepared by the diffusion method of Lang et al. (6). Contour lengths of DNA molecules were measured accordingly.

Buoyant-density analysis. Analytical runs were performed according to the method of Schildkraut, Marmur, and Doty (11) in a Beckman model E analytical ultracentrifuge equipped with a monochromator. As a reference, *B. subtilis* phage SPO1 DNA was used.

Transformation. A culture of strain 3115 in MY1 medium (10) was added to glucose (6% of final concentration) at 4.5 hr from spore germination, then frozen in liquid nitrogen and kept at -70°C . These cells became competent after 60 min of incubation in MY2 medium (10) at 37°C . They were then exposed to either phage DNA or bacterial DNA for 30 min at 37°C , and plated in Spizizen minimal agar (13) supplemented with (i) arginine (50 $\mu\text{g}/\text{ml}$), (ii) arginine and tryptophan (30 $\mu\text{g}/\text{ml}$), or (iii) arginine and histidine (4), in order to identify different classes of transformants.

RESULTS

Contour length of the DNA of PBSX-like particles. Individual DNA molecules were measured in two ways. On random fields, all DNA mole-

FIG. 1. Electron micrograph of PBSX-like particles. Negatively stained with phosphotungstate. $\times 230,000$.

cules were measured regardless of length and then were plotted as per cent frequency against length (Fig. 2a). There were many small DNA fragments present, distributed about exponentially. The other method consisted of selection for reasonably long DNA molecules on the fluorescent screen of the electron microscope before taking a micrograph (Fig. 2b). In both cases, a peak in the distribution of DNA lengths appeared between 3.9 and 4.6 μm and the mean length was $4.25 \pm 0.19 \mu\text{m}$ (standard deviation). This length corresponds to a molecular weight of 8.1×10^6 daltons, since 0.1 nm of the B configuration of DNA is calculated as 191.3 daltons (7).

Density of DNA. The buoyant density of DNA of PBSX-like particles in CsCl solution was determined by analytic ultracentrifugation. Phage SPO1 DNA, with a density of 1.735 g/cm^3 (15),

FIG. 2. Length distribution of DNA molecules of a PBSX-like particle. (a) All molecules (518) found on random fields were measured. (b) Molecules longer than 3 μm , from fields which were selected for long molecules on the fluorescent screen of the electron microscope.

FIG. 3. Buoyant density of DNA of PBSX-like particles centrifuged at 44,000 rev/min and 25 C. A UV photograph taken after 21 hr and traced by a Joyce-Loebel recording microdensitometer. DNA from *Bacillus phage* SPO1 (kindly supplied by D. W. Romig), with a density of 1.736 g/cm^3 , was used as reference; it was measured versus *E. coli* DNA, which has a density of 1.7035 g/cm^3 (15).

was used as a reference. Figure 3 shows that the buoyant density of the DNA is 1.696 g/cm^3 . This is identical to *B. subtilis* DNA. All DNA density measurements were made versus *E. coli* DNA, which has a density of 1.7035 (15).

Transformation. To test whether the physical similarities between PBSX-like particles and host-cell DNA had a biological counterpart, the biological activity of the particle DNA was assayed by bacterial transformation (Table 1). DNA of PBSX-like particles possessed low activity for single genetic markers, as compared with that of host-cell DNA, and simultaneous transformation of the linked markers *ind-168*⁺ and *his-31*⁺ was not observed.

DISCUSSION

Particles induced by 4NQO are similar in size to those of purified PBSX (9, 12) and SP α (F. A. Eiserling and W. R. Romig, *Bacteriol. Proc.*, 1964, p. 118). Although all these particles may be identical phages, it seems that 4NQO-induced particles are more fragile than these other types. However, since the particles were purified in CsCl solution and dialyzed against minimal medium, their morphology might be damaged by osmotic and ionic changes,

TABLE 1. Transforming activity of DNA of PBSX-like particles^a

Source of donor DNA	Concn (final)	No. of transformants per ml in the classes (frequency of transformation)			
		<i>arg</i> ⁺	<i>ind</i> ⁺	<i>his</i> ⁺	<i>ind</i> ⁺ <i>his</i> ⁺
	$\mu\text{g/ml}$				
PBSX-like particle	1.2	3.5×10^2 (5.5×10^{-6})	1.1×10^3 (1.7×10^{-5})	1.7×10^3 (2.7×10^{-5})	0 (0)
<i>B. subtilis</i>	1.0	7.1×10^4 (1.1×10^{-3})	1.0×10^4 (1.6×10^{-4})	1.0×10^4 (1.6×10^{-4})	4.5×10^4 (7.1×10^{-4})

^a *B. subtilis* strain 3115 (*arg*-15⁻, *ind*-168⁻, and *his*-31⁻) was used as the recipient. The *ind*-168 and *his*-31 are linked (cotransfer index, 0.6) on DNA extracted from the wild strain by the phenol method (4, 10). Frequency of transformation = no. of transformants in each class per 6.4×10^7 recipient cells/ml.

hence the predomination of degenerated particles in the electron micrographs.

DNA isolated from 4NQO-induced particles had the same buoyant density as host-cell DNA and also introduced cell genetic markers into transformable cells. These findings indicate that the particles carry a part of host-cell DNA, and data are similar to those reported for PBSX by Seaman et al. (12) and Okamoto et al. (9). Furthermore, in these reports, PBSX DNA was shown to be of homogeneous size by sucrose density gradient centrifugation, and the molecular weight was calculated to be 1.2×10^7 daltons in the former paper and 8.35×10^6 daltons in the latter. The question of whether the DNA in defective phages is of homogeneous size becomes vital in the consideration of the mechanism of size determination of phage DNA. In 4NQO-induced particles, we were able to establish this homogeneity of particle DNA by contour length measurements of the individual DNA molecules, showing a mean length of 4.25 μm and a corresponding molecular weight of 8.1×10^6 daltons.

There are many small sizes of DNA in the contour length measurement (Fig. 2a). It could not be determined whether this small size of DNA exists intrinsically in PBSX-like particles or whether the particles are fragmented by DNA extraction. However, the latter possibility seems more likely, since Okamoto found that a small size of single strand of PBSX DNA appeared in alkaline-sucrose density gradient centrifugation (9). These breakages might easily cause a fragmentation of DNA by shearing.

DNA extracted from 4NQO-induced particles transformed genetic markers with less efficiency than cell DNA. This decreased efficiency might be caused by the small size and single-strand breakage of the particle DNA, since it was clearly shown that relative transforming activity is dependent upon the size of a single strand of

DNA in *B. subtilis* (1). It is worthwhile to note that the particle DNA never transforms the linked genetic markers indole and histidine. Therefore, the mean length of the particle DNA, 4.25 μm , may be a maximum size for these single genetic markers on *B. subtilis* transformation.

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LITERATURE CITED

1. Bodmer, W. F. 1966. Integration of deoxyribonuclease-treated DNA in *Bacillus subtilis* transformation. *J. Gen. Physiol.* 49:233-258.
2. Endo, H., M. Ishizawa, and T. Kamiya. 1963. Induction of bacteriophage formation in lysogenic bacteria by a potent carcinogen, 4-nitroquinoline-1-oxide, and its derivatives. *Nature* 198:195-196.
3. Felkner, I. C., and F. Kadlubar. 1968. Parallel between ultraviolet light and 4-nitroquinoline-1-oxide sensitivity in *Bacillus subtilis*. *J. Bacteriol.* 96:1448-1449.
4. Hirokawa, H., and Y. Ikeda. 1966. Genetic recombination of transforming deoxyribonucleic acid molecules with the recipient genome and among themselves in protoplasts of *Bacillus subtilis*. *J. Bacteriol.* 92:455-463.
5. Ionesco, H., A. Ryter, and P. Schaeffer. 1964. Sur un bactériophage hébergé par la souche Marburg de *Bacillus subtilis*. *Ann. Inst. Pasteur* 107:764-776.
6. Lang, D., H. Bujard, B. Wolff, and D. Russell. 1967. Electron microscopy of size and shape of viral DNA in solutions of different ionic strengths. *J. Mol. Biol.* 23:163-181.
7. Lang, D. and P. Coates. 1968. Diffusion coefficient of DNA in solution at "zero" concentration as measured by electron microscopy. *J. Mol. Biol.* 36:137-151.
8. Nakahara, W., F. Fukuoaka, and T. Sugimura. 1957. Carcinogenic action of 4-nitroquinoline-N-oxide. *Gann* 48:129-137.

9. Okamoto, K., J. A. Mudd, J. Mangan, W. H. Huang, T. V. Subbaiah, and J. Marmur. 1968. Properties of defective phage of *Bacillus subtilis*. *J. Mol. Biol.* 34:413-428.
10. Saito, H., and K. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* 72:619-629.
11. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* 4:430-443.
12. Seaman, E., E. Tarmy, and J. Marmur. 1964. Inducible phages of *Bacillus subtilis*. *Biochemistry* 3:607-613.
13. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U.S.* 44:1072-1078.
14. Stickler, D. J., and R. G. Tucker. 1965. Bacteriophage-like particles released from *Bacillus subtilis* after induction with hydrogen peroxide. *Virology* 26:142-145.
15. Szybalski, W. 1968. Use of cesium sulfate for equilibrium density gradient centrifugation, p. 330-360. *In* L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 12, part B. Academic Press Inc., New York.