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INCORPORATION OF BACTERIOPHAGE GENOME BY SPORES OF *BACILLUS SUBTILIS*[1]

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ABSTRACT

TAKAHASHI, I. (Microbiology Research Institute, Ottawa, Ontario, Canada). Incorporation of bacteriophage genome by spores of *Bacillus subtilis*. J. Bacteriol. **87**:1499-1502. 1964—The buoyant density in a CsCl gradient of deoxyribonucleic acid (DNA) extracted from spores of *Bacillus subtilis* was found to be identical to that of DNA from vegetative cells. Density-gradient centrifugation of DNA of spores derived from cultures infected with phage PBS 1 revealed the presence of a minor band whose density corresponded to that of the phage DNA in addition to the spore DNA. No intermediate bands were present. The relative amount of the phage DNA present in the spores was estimated to be 11%, suggesting that spores of this organism may incorporate several copies of the phage genome. Although the possibility that true lysogeny may occur cannot be entirely eliminated, the results seem to indicate that the phage genomes incorporated into spores are not attached to the host chromosome in this system.

It has been shown that spores derived from cultures of *Bacillus subtilis* infected with the transducing phage PBS 1, even after heat treatment at 85 C for 15 min, form colonies which are immune to infection with homologous phages and produce free phage particles (Takahashi, 1961). Although similar observations have been made by a number of investigators with a variety of *Bacillus* species, the direct demonstration of phage genomes in spores has never been achieved.

Recently, phage PBS 1 for *B. subtilis* was reported to have deoxyribonucleic acid (DNA) which differs markedly from that of the host bacteria in its physical and chemical properties. The phage DNA contains glucose as an integral part of the molecule (Takahashi and Marmur, 1963*a*), and thymidylic acid is substituted by deoxyuridylic acid (Takahashi and Marmur, 1963*b*). The buoyant density of the phage DNA in a CsCl gradient is significantly higher than that of the host DNA, although the former has a guanine plus cytosine content much lower than the latter (Takahashi and Marmur, 1963*a*).

The present study was undertaken to determine whether the phage DNA in spores can be separated from the host DNA by density-gradient centrifugation and, if so, to estimate the relative amount of the phage genome present in these bodies.

MATERIALS AND METHODS

Bacterial cultures and techniques for the preparation of phage lysates and for assay of phages were as described previously (Takahashi, 1963). It was found earlier that vegetative cells of *B. subtilis* which carried the phage PBS 1 were unstable and reverted to the sensitive state at a fairly high frequency (Takahashi, 1963). Consequently, spores were used as the source of DNA thoughout the present study.

The DNA from infected spores was prepared as follows. Cells of *B. subtilis* SB 19 grown in Penassay Broth (Difco) for 4 hr were infected with phage PBS 1 at a multiplicity of infection of 4, plated on the sporulation agar of Schaeffer (1961), and incubated for 24 hr. The spore preparations were treated with lysozyme (100 μg/ml) in saline (0.85% NaCl) buffered with 0.01 M phosphate at pH 7.0 (PS) for 30 min to lyse vegetative cells still present at this stage. Deoxyribonuclease (5 μg/ml) and $MgSO_4$ (0.01 M) were then added to the suspension, followed by incubation for 20 min. Spores were collected by centrifugation (3,000 $\times$ *g* for 20 min) and

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washed twice in PS. The washed spores were resuspended in 10% 2-mercaptoethanol in 8 M urea (pH 3.0) and incubated for 60 min (Gould and Hitchins, 1963). Mercaptoethanol was removed by five cycles of washing in PS, and the spores were resuspended in PS and treated with lysozyme (100 μg/ml) for 60 min or longer. The resulting viscous suspension was centrifuged at 12,000 $\times$ g for 20 min, and crude DNA was precipitated from the supernatant liquid by the addition of 95% alcohol. All treatments described above were carried out at 37 C, except centrifugations which were at 4 C. It was found that DNA could be most readily extracted from spores obtained from cultures incubated for 24 hr; those incubated more than 2 days were not sensitized to lysozyme by the mercaptoethanol treatment. Purified DNA was obtained by the method of Marmur (1961). DNA from phage PBS 1 was prepared as outlined previously (Takahashi and Marmur, 1963*a*).

The technique for density-gradient centrifugation was similar to that reported by Meselson, Stahl, and Vinograd (1957). Details of the centrifugation technique and the method for the determination of the buoyant density were as described by Schildkraut, Marmur, and Doty (1962). The relative amount of DNA banded in the CsCl gradient was estimated from the area occupied in microdensitometer tracings of DNA bands.

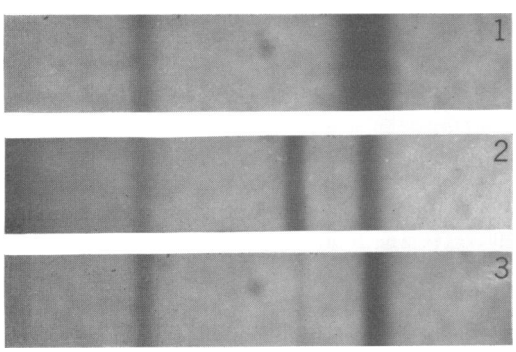

FIG. 1–3. *Ultraviolet absorption photographs taken after centrifuging DNA samples at 44,770 rev/min for 20 hr. The band at the far left was used as standard and was DNA from deuterium oxide-labeled Pseudomonas aeruginosa (ρ = 1.763 g/cc). The band at the far right was DNA from spores of Bacillus subtilis (ρ = 1.703 g/cc). The phage DNA banded at ρ = 1.722 g/cc, between the standard and the spore DNA (Fig. 2 and 3). In a separate experiment the density of DNA extracted from B. subtilis cells was found to be 1.703 g/cc. Figure 1 shows DNA from noninfected spores of B. subtilis banded in a CsCl gradient. Figure 2 shows separation of PBS 1 DNA from the spore DNA by density-gradient centrifugation. Figure 3 shows the banding pattern of DNA from spores of B. subtilis infected with PBS 1.*

RESULTS AND DISCUSSION

DNA extracted from noninfected *B. subtilis* spores banded at a position corresponding to the density (ρ = 1.703 g/cc) of DNA from vegetative cells (Fig. 1). This result is not surprising, since Mandel and Rowley (1963) reported that spore DNA of *B. subtilis* var. *niger* had the double-stranded structure, and its thermal transition profile was very similar to that of DNA extracted from vegetative cells. Also, Young and Fitz-James (1959) with *B. cereus* and Szulmajster and Canfield (*personal communication*) with *B. subtilis* demonstrated that spore DNA was derived directly from vegetative cells. Furthermore, it should be noted that the spore DNA used in this experiment had a specific transforming activity comparable with that of DNA from vegetative cells with respect to an indole marker and a histidine marker, as determined by the technique of Anagnostopoulos and Spizizen (1961).

When equal amounts of DNA prepared from noninfected *B. subtilis* spores and DNA from the phage PBS 1 were mixed and centrifuged in a CsCl gradient, the former banded at the position corresponding to a density of 1.703 g/cc and the latter at a density of 1.722 g/cc (Fig. 2). Thus, these two DNA species can be separated readily from each other by density-gradient centrifugation.

Figure 3 illustrates the banding pattern obtained with DNA extracted from infected spores. In addition to the band of spore DNA, a minor band with the density of the phage DNA was present. The density of this minor band and the fact that DNA of noninfected spores showed no additional band (Fig. 1), even though the concentration of the DNA centrifuged was about five times higher than that used in the standard technique, seem to indicate that the minor band represents the phage genome in the infected spores. However, no further attempts were made to identify the functional nature of the minor band.

The relative amount of DNA in the minor band was estimated to be 7.7% of the total DNA centrifuged. Since 70% of the spores used in this experiment could form colonies which are immune to superinfection with PBS 1 and carry free phages, the relative amount of the phage DNA actually present in these spores would be 11%. The amount of phage genome incorporated into organisms of other lysogenic systems is generally considered to be on the order of 1 to 2% (Jacob and Wollman, 1961) although no exact data are available at present. If this is also the case with phage PBS 1, the figure obtained here seems to be too high for a single phage genome. It is suggested that spores used in this experiment have incorporated several copies of the phage genome. It should be mentioned that, when these spores were prepared, a high multiplicity of infection was used, and phage antisera were not present on agar plates. It is thus probable that the multiple infection of cells took place during the sporulation process.

It is interesting that no intermediate bands were found between the spore DNA and the phage DNA (Fig. 3). The absence of the intermediate type of DNA seems to indicate that there is no material integration of the phage genome into the host chromosome. The possibility that a small number of PBS 1 genomes may also be integrated stably into the spore chromosome in addition to the presence of a relatively large amount of free phage DNA, however, cannot be excluded by this experiment. To detect any significant change in the density of the spore DNA, the presence of at least 10% of stably integrated phage DNA would be required. In lysogenic systems with enteric bacteria (for example, lambda or P2), phage DNA and bacterial DNA are very similar in chemical composition and physical properties. Prophages in these cases are usually firmly attached to the host chromosome at a specific site, and the loss of prophage occurs at a very low frequency. On the contrary, preliminary results with the phage PBS 1 indicate that in vegetative cells the phage DNA and the host DNA are not replicating synchronously (*unpublished data*) and during growth the phage-carrying cells segregate sensitive cells at a fairly high frequency (Takahashi, 1963). This rather unusual situation may be understood if one considers differences in the chemical composition of the phage DNA and the host DNA. Kahan (1963) reported that for the replication of the phage DNA at least two new enzymes, which were absent in noninfected cells, were required. It would be difficult to visualize the situation where bacterial cells can synthesize simultaneously these two types of DNA whose chemical compositions are entirely different. Moreover, attempts to induce phage development in this system by ultraviolet irradiation were not successful (Takahashi, 1963). The PBS 1 system thus differs in many respects from other systems, and presumably this is a case of pseudo-lysogeny.

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