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DNA Replication in Bacteriophage-Infected *Staphylococcus aureus*

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The synthesis of viral and host DNA in phage-infected *Staphylococcus aureus* was examined. Three intracellular forms of phage 52HJD DNA were demonstrated: covalently closed circular, open circular, and linear DNA species. It was noted that infection of *S. aureus*-propagating strains 81 and 52 with phage 52HJD inhibited the replication of the bacterial chromosome and a stringently controlled penicillinase plasmid. A small tetracycline plasmid, normally under relaxed replication control, continued to replicate in the postinfection period. No breakdown of the host chromosome into small-molecular-weight fragments or utilization of bacterial DNA material for the synthesis of viral DNA was observed.

In recent years a great deal of information has become available concerning the developmental patterns of bacteriophages. Of particular interest have been studies concerning the replication of temperate phages such as λ (10, 20, 25, 30, 32, 34, 37) and *Salmonella* phage P22 (5-8, 36) and the effects of infection with such phages upon host synthetic patterns (14, 31). However, to date little is known concerning the intracellular events following infection with the staphylococcal phages. This study was undertaken to investigate the patterns of DNA synthesis in *Staphylococcus aureus* after infection with a temperate bacteriophage.

MATERIALS AND METHODS

Organisms. *S. aureus* phage-propagating strains 52 and 81 were obtained from our stock culture collection. PS 81 contains a penicillinase (pP_{81}) and a tetracycline (pT_{81}) plasmid (28). The pP_{81} plasmid has a molecular weight of 18×10^6 to 21×10^6 and possesses determinants conferring resistance to arsenate, cadmium, and mercury, in addition to the penicillinase structural gene and regulatory loci. Resistance to tetracycline is the only known determinant associated with the pT_{81} plasmid. The molecular weight of this plasmid is 2.7×10^6. Staphylococcal group B phage 52HJD of the International Typing Series (hereafter called phage 52), a variant of phage 52 which is lytic for PS 81 (2), was used. The latent period of this phage is 40 min and progeny viral particles reach a maximum titer at 60 min postinfection (unpublished data). The molecular weight of staphylococcal group B phage DNA ranges from 29×10^6 to 31×10^6 (26). Phage stocks were prepared by the soft agar overlay method (3, 35).

Media. Bacterial stock cultures were maintained on Tryptone soy agar (Oxoid) slants. Trypticase soy broth (TSB; Baltimore Biological Laboratory, Cockeysville, Md.) was used to harvest and maintain phage stocks, to harvest bacteria from slants, and as a diluent in phage and bacterial assays. CY broth (22) was used as the growth medium in labeling experiments.

Labeling of viral and bacterial DNA. Overnight cultures were diluted into CY broth and allowed to grow in a shaking water bath at 37°C to a cell density of 2.5×10^8 CFU/ml. Phage at a multiplicity of infection of 2 to 3 and $CaCl_2$ to a concentration of 4 mM were added. After 2.5 min of incubation, 5 μCi of [^{3}H]thymidine per ml (International Chemical and Nuclear Corp.) was added, and incubation was continued for up to 25 min. In some experiments, cells were grown before infection in the presence of 0.25 μCi of [^{14}C]thymidine per ml (International Chemical and Nuclear Corp.), and in the postinfection period, in the presence of 5 μCi of [^{3}H]thymidine per ml. In each experiment, uninfected control cells were labeled in the same manner as infected cells.

Isolation of DNA. Covalently closed circular (CCC) DNA was isolated and separated from open circular (OC) and linear (LIN) DNA by centrifugation in cesium chloride-ethidium bromide (CsCl-EB) dye-buoyant density gradients following the cleared lysate method as modified by Novick and Bouanchaud (23) in which the bulk of the chromosomal DNA is eliminated. Labeled cells were harvested, washed by filtration, and suspended to a final cell concentration of 10^{10} CFU/ml. The suspensions were treated with lysostaphin (Schwarz/Mann) at a level of 15 μg/ml for 15 min at 37°C and subsequently lysed as described by Novick and Bouanchaud (23). The lysate was centrifuged at 49,000 × *g* for 30 min at 0°C, and 3.4 ml of the supernatant, 4.4 ml of saturated CsCl (refractive index 1.4178), and 0.2 ml of an ethidium bromide (Calbiochem) solution (10 mg/ml) were mixed in a polyallomer centrifuge tube. The mixture was centrifuged at 43,000 rpm for 40 h at 20°C in a type 50 rotor of a Spinco preparative ultracentrifuge.

Sucrose gradient centrifugation. Fractions from the CsCl dye-buoyant density gradients containing the

desired DNA species were pooled, extracted with isopropanol to remove the ethidium bromide, and dialyzed overnight against dilute saline citrate (0.015 M NACl-0.0015 M sodium citrate) with 0.01 M EDTA (pH 7.0). Sucrose solutions were prepared in 0.05 M EDTA (pH 7.0). Gradients of 5 to 20% (wt/vol) sucrose formed over a 0.2-ml cushion of 50% sucrose were layered with 0.2 ml of the DNA preparation. The gradients were centrifuged in a type 50.1 rotor at 45,000 rpm for 114 min at 5°C.

Collection and assay of fractions. Both CsCl and sucrose gradients were fractionated by puncturing the bottom of the centrifuge tube. Fractions were prepared for assay of the trichloroacetic acid-precipitable counts by the method of Bollum (4). The samples were counted in a Nuclear-Chicago Unilux II scintillation counter.

RESULTS

Phage 52 DNA in CCC form. To examine the replication of phage 52 DNA, PS 52 was chosen as the host bacterium since it contains no extrachromosomal DNA (28). A CsCl-EB dye-buoyant density gradient of PS 52 labeled with [^{3}H]thymidine was undertaken (Fig. 1A). Only one peak of material, representing linear chromosomal DNA, was present. A similar gradient of PS 52 infected with phage 52 and labeled with [^{3}H]thymidine from 2.5 to 12.5 min into the infectious cycle is shown in Fig. 1B. Two peaks of DNA synthesized after phage infection were observed, one sedimenting in the region characteristic of chromosomal DNA, and a second peak of denser material not observed in DNA preparations isolated from uninfected cells. This second peak represents viral DNA found in a CCC form. To further investigate the molecular forms of DNA synthesized in the postinfection period, PS 52 was grown in a medium containing [^{14}C]thymidine, and the cells were washed and infected with phage 52 in a medium containing [^{3}H]thymidine. After 10 min, the CCC forms of DNA from these infected complexes were separated from LIN and/or OC forms by centrifugation in a CsCl-EB gradient. Fractions of each of the two peaks were pooled and prepared for centrifugation in sucrose gradients as described above. The sucrose gradient of the denser band of material, which is represented in Fig. 2, revealed a peak of DNA associated only with the postinfection tritium label. This peak sediments as would be expected for a CCC form of the viral DNA, as suggested by previous data.

OC forms of viral DNA. The sucrose profile of the less dense DNA from the CsCl-EB gradient is presented in Fig. 3. As in Fig. 2, one band of material containing only the postinfection label was noted. The DNA was presumably phage DNA in OC or LIN form. The homogeneity of the peak indicates that the bulk, if not all, of the postinfection DNA synthesis in this strain involves replication of the phage genome. Thus, infection with this phage apparently interrupts the replication of the bacterial chromosome.

To distinguish which molecular species of

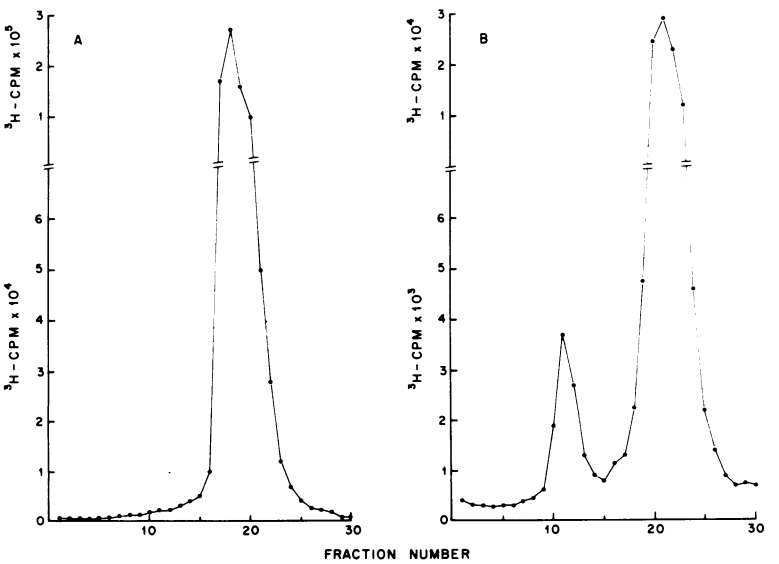

FIG. 1. *CsCl-EB dye-buoyant density gradients of DNA isolated from uninfected and phage-infected PS 52. DNA labeled with [^{3}H]thymidine was isolated from PS 52 and separated on a CsCl-EB dye-buoyant density gradient. (A) PS 52; (B) PS 52 infected with bacteriophage 52.*

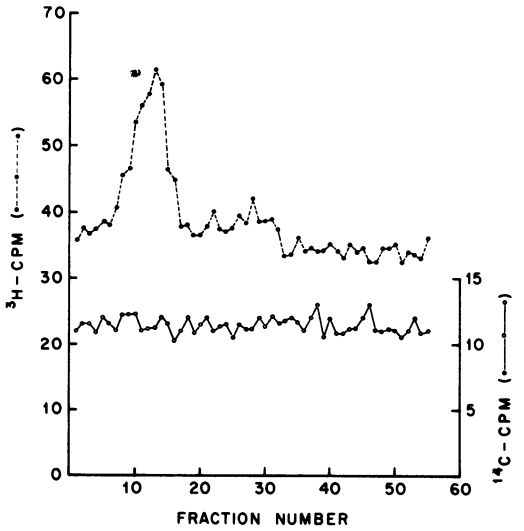

FIG. 2. *Neutral sucrose gradient profile of CCC DNA isolated from phage-infected PS 52. PS 52, incubated before infection with [^{14}C]thymidine, was infected with phage 52 in the presence of [^{3}H]thymidine, and the DNA was isolated 12.5 min after infection and separated on a CsCl-EB dye-buoyant density gradient. Fractions comprising the CCC DNA were pooled, extracted with isopropanol, dialyzed against dilute saline citrate in 0.01 M EDTA (pH 7.0), and sedimented through a 5 to 20% neutral sucrose gradient.*

phage DNA was represented, the material pooled from the two peaks seen in the CsCl gradient was cosedimented through a neutral 5 to 20% sucrose gradient. The ratio of the distance traveled into the gradient by the more rapidly sedimenting CCC material to the slower moving material is indicative of the molecular nature of this latter DNA species (9). The expected ratios of the distances traveled by various forms of viral DNA of molecular weight 3×10^7 are as follows: CCC to OC = 1.50; and CCC to LIN = 1.74. The ratio observed was 1.58, compatible with the conclusion that the second peak represents an OC form of viral DNA.

LIN form of viral DNA. The sucrose profiles demonstrating the CCC and OC species of viral DNA (Fig. 2 and 3) were derived from cells labeled during a period of 12.5 min after infection with phage. When the infected cells were labeled for a longer period into the infectious cycle, there was an indication of the appearance of linear viral genomes. Sucrose gradients of the less dense CsCl-EB peaks of PS 52 (Fig. 4A) and PS 81 (Fig. 4B) infected with phage 52 and labeled for 25 min with [^{3}H]thymidine are presented. In contrast to the single peak of viral DNA seen in Fig. 3, these profiles contained two peaks of DNA, representing OC (the more rapidly sedimenting bands) and LIN viral molecules. The ratios of the distances traveled by the two peaks into the gradients, 1.18 for both Fig. 4A and B, were compatible with this conclusion since the expected ratio of OC to LIN was 1.17.

These data suggest that staphylococcal phage DNA is found in three distinct forms during the infectious cycle, i.e., as CCC, OC, and LIN molecules. The late appearance of the LIN forms may indicate that this species of DNA is the final replicated product, which is packaged into phage protein coats. Infection with this phage represses replication of the bacterial chromosome but does not cause a measurable breakdown of host DNA into phage size or smaller fragments. It was noted in the double-label experiment represented in Fig. 2 and 3 that viral DNA contained only the postinfection tritium label. This observation strongly suggests that little or no conversion of bacterial DNA material into viral DNA (less than 1%) occurs in the course of a lytic infection.

Isolation of plasmid DNA. To determine

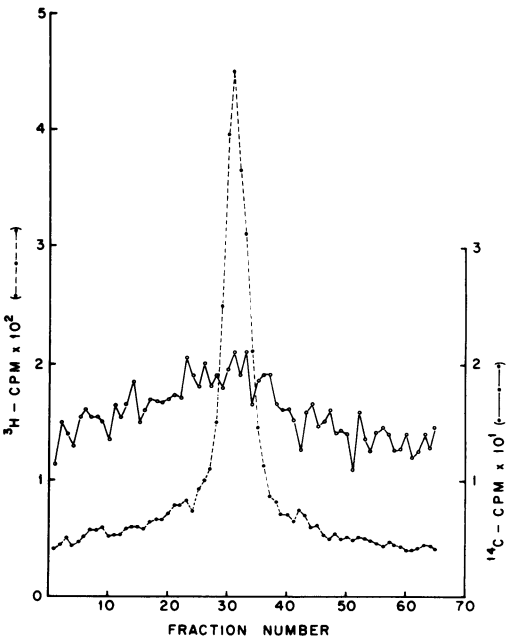

FIG. 3. *Neutral sucrose gradient profile of OC DNA isolated from phage-infected PS 52. PS 52, incubated before infection with [^{14}C]thymidine, was infected with phage 52 in the presence of [^{3}H]thymidine, and incubation was continued for 10 min. The DNA was isolated and separated on a CsCl-EB dye-buoyant density gradient. Pooled fractions of the less dense band of material from the resulting gradient were sedimented through a 5 to 20% neutral sucrose gradient.*

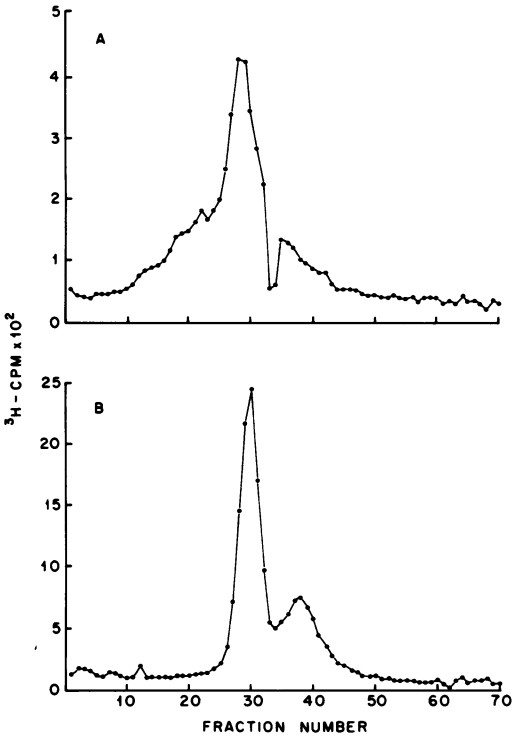

FIG. 4. *Neutral sucrose gradient profiles of OC and LIN DNA from phage-infected PS 52 and PS 81. The DNA of PS 52 (A) and PS 81 (B) infected with phage 52 and labeled with [^{3}H]thymidine for 25 min after infection was separated on CsCl-EB dye-buoyant density gradients. The pooled fractions of the less dense peaks of material from the resulting gradients were sedimented on 5 to 20% neutral sucrose gradients.*

the effects of phage infection upon replication of plasmid DNA, PS 81, which contains a penicillinase and a tetracycline plasmid, was chosen for study. A CsCl-EB gradient of PS 81 labeled with [^{3}H]thymidine is presented in Fig. 5. As was expected, two peaks of DNA were observed, the denser band representing CCC plasmid DNA. Sucrose gradient analysis of the denser material observed in Fig. 5 is shown in Fig. 6. The material from the "plasmid" band separated into two very distinct peaks, representing the larger, more rapidly sedimenting penicillinase plasmid (47S) and the smaller, tetracycline plasmid (20S). No distinctive peak of DNA was observed in the sucrose profile of the less dense peak of DNA from the CsCl gradient (data not shown).

Replication of plasmid DNA in phage-infected *S. aureus*. A sucrose profile of the CCC DNA isolated from PS 81 infected with phage 52 is presented in Fig. 7. The DNA was derived from cells labeled in the preinfection period with [^{14}C]thymidine and, during the postinfection period, with [^{3}H]thymidine and harvested 12.5 min after infection. The preinfection label again demonstrates the two plasmids present in this strain. The profile of the postinfection label reveals that the penicillinase plasmid, like the host chromosome, underwent little or no replication with phage 52. The tetracycline plasmid did, however, continue to replicate after infection with this phage. Furthermore, the tetracycline plasmid DNA synthesized in the postinfection period is identical in size to that plasmid DNA labeled in the preinfection period, as indicated by the co-sedimentation of the [^{3}H]- and [^{14}C]-thymidine labels in this gradient. A new peak of material at fraction 13 (60S), associated only with the postinfection tritium label, was observed. This material represents the CCC form of phage DNA described previously.

DISCUSSION

It is becoming increasingly evident that bacteriophages with linear duplex chromosomes

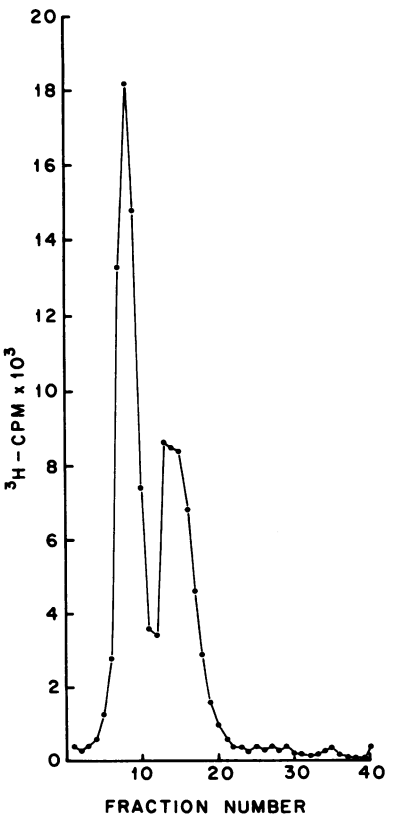

FIG. 5. *CsCl-EB dye-buoyant density gradient of [^{3}H]thymidine-labeled DNA from PS 81. DNA isolated from [^{3}H]thymidine-labeled PS 81 was centrifuged on a CsCl-EB dye-buoyant density gradient.*

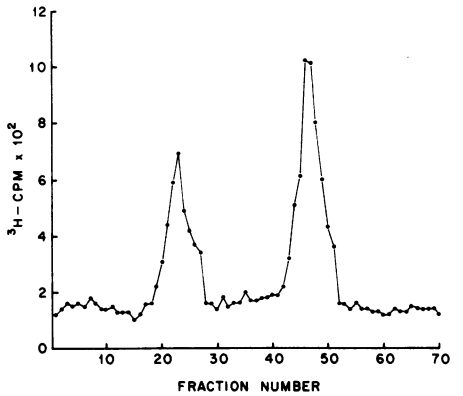

FIG. 6. *Neutral sucrose gradient profile of PS 81 CCC DNA from a CsCl-EB dye-buoyant density gradient. Fractions 6 through 10 from the CsCl-EB dye-buoyant density gradient shown in Fig. 5 were pooled and sedimented through a 5 to 20% neutral sucrose gradient.*

may share essentially similar replicative pathways. Circular and concatenated DNA replicative intermediates have been described from several systems. Circularization of the infecting linear parental DNA occurs very early in the infectious cycle and has been reported for phage λ (38), P22 (7), and T4 (1).

After circularization, phage DNA follows either of two modes of replication. In the sigma type of pathway, demonstrated for λ (30, 34), P22 (5, 6), T2 and T4 (11), T5 (34), and T7 (36), linear concatenated intermediates, generated by a rolling circle mechanism (12, 13), are formed. Mature phage DNA is cut from these oversize intermediates, and the chromosome maturation appears to be intimately associated with head assembly and the encapsidation process (8, 12, 30, 37). Alternatively, the theta type of replicative pathway, characteristic of SV40 (18), mitochondrial (27), and plasmid (15, 33) DNA, involves the generation of OC and CCC DNA species and has been observed for phage λ when it is perpetuated in a plasmid state (10, 20, 25, 32) and for a *Haemophilus influenzae* phage (21).

In this study, CCC, OC, and LIN forms of staphylococcal phage DNA were demonstrated, indicating that this phage may undergo replication via a theta-type pathway. However, the techniques used to examine viral DNA ensure the isolation of small supercoiled molecules and specifically exclude from the cleared lysate large linear molecules and membrane-bound DNA fractions (hence, concatenated forms). It is impossible therefore to determine conclusively which mode of replication is characteristic of this staphylococcal phage. A clear elucidation of the replicative pathway for this phage awaits development of more suitable techniques for examining staphylococcal DNA.

Infection of *S. aureus* strains with phage 52 caused a repression of chromosomal replication, and little, if any, conversion of bacterial DNA material into phage DNA was observed. In addition, infection with this phage did not cause a measurable breakdown of the host chromosome into phage-size or smaller pieces. Similar observations have been made with P22 infection of *Salmonella* (31). This conservation of bacterial DNA during a lytic infection is in contrast to the degradation of host material observed in infections with virulent bacteriophages such as T4 (19) and with virulent mutants of the temperate P1*kc* phage (14, 16).

The control of plasmid replication in the staphylococci is not entirely understood. Small plasmids, usually found in multiple copies, appear to function as autonomous replicons. In contrast, stringently controlled penicillinase plasmids code for a replicon-specific initiation factor, but most or all of the other replication functions are host coded, and, as a result, the replication of such plasmids is closely coordinated with that of the bacterial chromosome (24). However, Ruby and Novick (29) have demonstrated, by studying six *S. aureus* plasmids, that the intracellular pools of compatible plas-

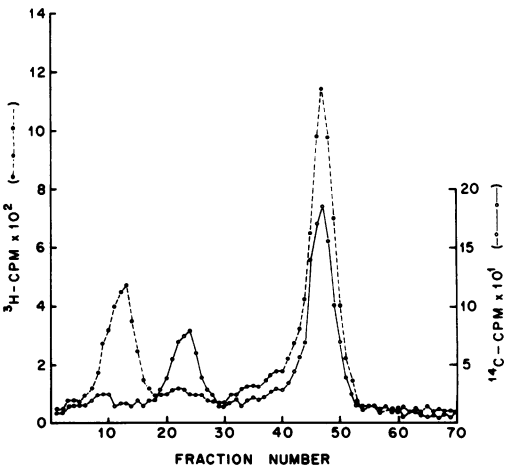

FIG. 7. *Neutral sucrose gradient profile of CCC DNA isolated from phage-infected PS 81. PS 81 incubated with [^{14}C]thymidine prior to infection was infected with phage 52 in the presence of [^{3}H]thymidine, the incubation was continued for 20 min, and the DNA was isolated and separated on a CsCl-EB dye-buoyant density gradient. Fractions comprising the denser peak of material from the resulting gradient were pooled and sedimented through a 5 to 20% neutral sucrose gradient.*

mid DNA are not additive. They suggested that there may be a cellular maximum for extrachromosomal DNA and, therefore, the replication of plasmids ultimately may be controlled not by replicon number or initiation events, as predicted by the classical replicon-maintainance site model (17), but by the availability of space for plasmid DNA in the bacterial host.

In this study, it was observed that infection with phage 52 inhibited the replication of the penicillinase plasmid but not of the tetracycline plasmid, which is normally under relaxed replication control. This differential effect upon plasmid DNA synthesis by phage infection does suggest that the concept of availability of DNA space as a major control mechanism for plasmid replication may be an oversimplification. The inhibition observed could result from the phage-directed repression of chromosomal DNA synthesis described previously. It is not clear, however, whether the postinfection replication of the small tetracycline plasmid is due to the normal synthesis of this plasmid DNA as an autonomous replicon or to a specific phage-directed stimulation of replication.

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