COO-2084-19

5

STUDIES WITH INFECTIOUS FRAGMENTS OF PHAGE DNA

Final Report

January 1, 1970 - June 30, 1976

Charles F. Schachtele, Ph.D.

University of Minnesota

Minneapolis, Minnesota 55455

August 1976

PREPARED FOR THE U. S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION UNDER CONTRACT E(11-1)-2084

OT E(11-1)-2084

DISCLAIMER

NOTICE

ABSTRACT

The minute, virulent and structurally intricate Bacillus subtilis bacteriophage Ø29 was utilized to study in vivo viral development. Purified strands of Ø29 DNA were used to analyze transciption of the viral genome. Early mRNA hybridizes to the light DNA strand which controls DNA replication and other early functions. Late mRNA hybridizes to the heavy DNA strand which codes for phage structural proteins. The temporal sequence of specific viral protein synthesis was analyzed by gel electrophoresis and was shown to directly correlate with the RNA transcription pattern. The genes carried by Ø29 have been marked with ts and sus mutations and mapped by appropriate crosses yielding a linear map of 17 cistrons. Fragments of the Ø29 DNA were shown to retain their biological activity and marker rescue studies indicated that gene transfer could be performed with pieces having a molecular weight of less than 1 million daltons. Mutant infection under nonpermissive conditions and the analysis of precursor structures has allowed the formation of a tentative morphogenetic pathway leading to the formation of infectious particles. Work with Ø29 has established this virus as an advantageous model system for studying a variety of problems in molecular biology and approximately a dozen laboratories in the country and abroad are working with this phage.

TABLE OF CONTENTS

– reprints removed

I. INTRODUCTION

We are studying the minute, virulent and structurally intricate Bacillus subtilis bacteriophage Ø29. Data has been obtained which indicates that this virus will be extremely useful in studies aimed at several basic biological problems.

II. SCOPE OF THE PROJECT

The scope of the work under the contract consists of studies on B. subtilis phage Ø29 with the following objectives:

1. To study in detail the function of the 17 to 20 genes carried by the virulent B. subtilis phage Ø29.
2. To analyze the temporal control and orientation of expression of the phage Ø29 genome.
3. To use transfection and superinfection marker rescue to study the effects of various physical and chemical treatments on the infectivity and expression of fractionated Ø29 DNA containing specific genes. Fractionation will involve not only isolated duplex fragments but individual strands of phage Ø29 DNA.
4. To isolate by physical and enzymatic methods individual Ø29 genes and to use such DNA in studies on recombination and DNA repair.
5. To use a polymerase complex from B. subtilis to synthesize in vitro infectious duplex phage Ø29 DNA free from template and to use this system to investigate replication of the viral DNA molecule.
6. To lay the foundation for future studies projected at analyzing in detail the in vitro assembly of phage structural components and to clarigy the mechanism whereby large pieces of DNA can be condensed and packaged to make complete virions.

IV. PUBLICATIONS

COO-2084-1 Technical Progress Report Contract No. AT(11-1)-2084 1/1/70 to 8/1/70. Studies with Infectious Fragments of Phage DNA.

COO-2084-2 Schachtele, C. F., Bader, L. A., Hagen, E. W., Oman, R. W. & Anderson, D. L. (1970). Conditional Lethal Mutants of Bacteriophage Ø29. Bact. Proc. 1970, 202.

COO-2084-3 Schachtele, C. F. & Anderson, D. L. (1970). Effect of Elevated Temperature on the Development of Bacillus Bacteriophage Ø29. Bact. Proc. 1970, 176.

COO-2084-4 Mosharrafa, E. T., Schachtele, C. F. & Anderson, D. L. (1970). Isolation of the Complementary Strands of Bacteriophage Ø29 DNA. Bact. Proc. 1970, 175.

COO-2084-5 Mosharrafa, E. T., Schachtele, C. F. & Anderson, D. L. (1970). Isolation of the Complementary Strands of Bacteriophage Ø29 DNA. NATO Symposium on Uptake of Informative Molecules by Living Cells. Mol. Belgium. p. 16.

COO-2084-6 Schachtele, C. F., Oman, R. W. & Anderson, D. L. (1970). Effect of Elevated Temperature on DNA Synthesis in Phage Ø29-infected Bacillus amyloliquefaciens. J. Virol. 6, 430-437.

COO-2084-7 Mosharrafa, E. T., Schachtele, C. F., Reilly, B. E. & Anderson, D. L. (1970). The Complementary Strands of Bacteriophage Ø29 DNA: Preparative Separation and Transcription Studies. J. Virol. 6, 855-864.

COO-2084-8 Schachtele, C. F., Anderson, D. L. & Liljemark, W. R. (1970). Formation and Structure of Canavanyl-protein-DNA-membrane Complexes in Excherichia coli. Electron Microscopy 1, 619-620.

COO-2084-9 Tsien, H. C., Mosharrafa, E. T., Hickman, D. D., Hagen, E. W., Schachtele, C. F. & Anderson, D. L. (1971). Studies with Bacteriophage Ø29 and its Infectious DNA. Chapter 24. In "Informative Molecules in Biological Systems: (L. Ledoux, Ed.), North-Holland Publishing Company, The Netherlands. p. 431-444.

Coo-2084-10 Schachtele, C. F., Hagen, E. W. & Anderson, D. L. (1971). Temperature-shift Analysis of Bacteriophage Ø29 Gene Expression in Bacillus amyloliquefaciens. J. Virol. 8, 352-354.

COO-2084-11 Technical Progress Report Contract No. AT(11-1)-2084 1/1/71 to 9/15/71. Studies with Infectious Fragments of Phage DNA.

COO-2084-12 Schachtele, C. F., De Sain, C. V., Hawley, L. A. & Anderson, D. L. (1972). Transcription During the Development of Bacteriophage Ø29: Production of Host and Ø29-specific Ribonucleic Acid. J. Virol. 10, 1170-1178.

COO-2084-13 Schachtele, C. F., De Sain, C. V. & Anderson, D. L. (1972). Transcription During the Development of Bacteriophage Ø29: Definition of "Early" and "Late" Ø29 Ribonucleic Acid. J. Virol. 11, 9-16.

COO-2084-14 Schachtele, C. F., Reilly, B. E., De Sain, C. V., Whittington, M. O. & Anderson, D. L. (1972). Selective Replication of Bacteriophage Ø29 Deoxyribonucleic Acid in 6-(p-hydroxyphenylazo)-Uracil Treated Bacillus subtilis. J. Virol. 11, 153-155.

COO-2084-15 Technical Progress Report Contract No. AT(11-1)-2084 9/16/71 to 9/15/72. Studies with Infectious Fragments of Phage DNA.

COO-2084-16 Anderson, D., Hagen, E., Peterson, C., Reilly, B., Schachtele, C. & Tosi, M. (1974). Studies on Phage Ø29 Morphogenesis. Squaw Valley-ICN Phage Assembly Meeting.

COO-2084-17 Technical Progress Report Contract No. AT(11-1)-2084 1/1/73 to 3/30/74. Studies with Infectious Fragments of Phage DNA.

COO-2084-18 Technical Progress Report Contract No. AT(11-1)-2084 4/1/74 to 3/30/75. Studies with Infectious Fragments of Phage DNA.

V. EVALUATION OF PROGRESS IN THE AREA OF RESEARCH SUPPORTED BY THE CONTRACT

As listed in the Scope of the Project (Page 1) the objectives of our research was to define the genetic system of B. subtilis bacteriophage Ø29 and to analyze the interaction of the host and viral genomes during viral replication and morphogenesis. Phage Ø29 was selected because it was small but morphologically complex, because it had a genome of DNA with a molecular weight of about 11 X 10^6 daltons and because the genetic system of one host, B. subtilis strain 168 was under intensive investigation (3,51).

Phage Ø29 was one of a series of viruses isolated from soil (B. E. Reilly, Ph.D. Thesis, Case-Western Reserve Univ., 1965). Bacteriophage of this series have been shown to have a wide host range (12). Reilly and Spizizen (32) reported that phage Ø29 was active in transfection and could infect competent bacterial cells unable to adsorb the virus. The cell population competent for transformation was infected by the viral DNA. The relationship of Ø29 DNA infectivity to DNA concentration and the kinetics of DNA infection were similar to those observed in bacterial transformation and suggested that a single native DNA molecule was necessary if not sufficient to initiate an infection. We (COO-2084-8) have been able to rescue genetic markers on half- and quarter-length Ø29 DNA fragments using competent B. subtilis cells infected by ts mutant phage and superinfected with fragments of viral DNA. However, Hirokawa (17) demonstrated that Ø29 DNA transfection was protease sensitive. Ortin et al. (29) reported a protein molecule bound to the circular form of DNA released from the virion. Hirokawa suggests that this protein may function at an early stage of transfection and be protease sensitive. On the other hand, DNA annealed from denatures molecules is also active in transformation (4). Perhaps there is only an apparent conflict in this data. The absolute efficiency of transfection is low in terms of viral genome equivalents of DNA added to the cells (47). It is possible that there is more than one form infections agent present in the phenol extracts of Ø29 particles.

Young (49) has demonstrated that glucosylated teichoic acid is a requirement for adsorption of Ø29 to B. subtilis. However, Ø29 can adsorb to and replicate in protoplasts of mutants that cannot glucosylate teichoic

acid (21). The absolute efficiency of plating of Ø29 approaches one on B. amyloliquefaciens H (3). We (COO-2084-12) have shown that host DNA synthesis continues during infection and that transcription continues at the preinfection rate throughout the latent period. Ribosomal RNA production is not inhibited. RNA-DNA hybridization and amino acid-labeling indicate that functional host messenger synthesis continues late into the viral lytic cycle and that this RNA is used to produce protein.

Anderson et al. (3) first described the complex morphology of Ø29. The virion has a number of protein fibers attached to both ends of its prolate head. The neck assembly consists of an upper collar that is attached twelve appendages. An elongate tail axis, enlarged at the distal portion, projects from the collar complex. Tosi and Anderson (45) have shown that antibodies to Ø29 bind to head fibers, the appendages and the head surface but apparently not to the tail axis and that the appendages are the organelles of adsorption. Tosi et al. (46) demonstrated that each of the twelve appendages consists of a single protein molecule.

Our molecular weight determination for Ø29 DNA, 11 X 10^6 daltons, was derived by electron microscopy and band sedimentation (3,4). This DNA molecule is a linear nonpermated duplex with intact single strands (4). D. Lang has redetermined the molecular weight of Ø29 DNA of electron microscopy and reports a value of 12.3 $\pm$ 0.4 megadaltons (personal communication). Rubio et al. (36) calculated the molecular mass of Ø29 to be 18 $\pm$ 1 X 10^6 daltons from sedimentation and diffusion coefficients of Ø29, the partial specific volume obtained by pycnometry, and the Svedberg equation. An independent estimate of the molecular weight of 19 $\pm$ 1 X 10^6 daltons was derived from the phosphorous and nitrogen content, and the amino acid and nucleotide composition, assuming a DNA molecular weight of 11 X 10^6 daltons.

The structural proteins of Ø29 were examined in detail by the Vinuela group of Madrid. Mendez et al. (25) demonstrated three head proteins and showed that the morphological entities named upper and lower collar, appendages and tail each seemed to contain a single type polypeptide resolvable by electrophoresis. Alvarez et al. (2) also resolved seven structural proteins. Analysis of peptides generated by tryptic digestion by Salas et al. (37) suggested nonidentity of the head fiber and the major capsid protein. The removal of the head fibers from the mature virion did not alter infectivity. The protein of the DNA-protein complex reported

by Ortin et al. (29) could be an eighth structural protein.

Using profiles generated by tracing the proteins revealed by sodium dodecylsulfate polyacrylamide gel electrophoresis followed by autoradiography we have been unable to revolve the third or minor head protein from among the proteins of infected cells (5,15) but have observed a minor band occasionally when we have examined virions purified in CsCl gradients (unpublished observations). We favor the hypothesis that the minor head protein might be generated by cleavage of the major head protein, perhaps nonspecifically. The protein of the DNA-protein complex (29) may be hidden by the major head protein band, be the third or minor head protein, or represent a second function of the major head protein.

By using 6-(p-hydroxyphenylazo)-uracil to inhibit host DNA biosynthesis, viral DNA synthesis was detected at about 12-15 minutes after infection and continued at a high rate until lysis (24, 44, COO-2084-14). Mutation in at least two ts cistrons blocks DNA synthesis at restrictive temperature (44). In the presence of the azopyrimidine inhibitor, sus mutants of two cistrons were unable to synthesize viral DNA (COO-2084-14). Mutants of two of the eight cistrons identified by McGuire et al. (26) did not synthesize Ø29 DNA. There is no evidence for a Ø29-specific DNA polymerase.

Our analysis of Ø29 DNA transcription was based on preferential binding of the polynucleotide, polyuridylic-guanylic acid to the intact complementary strands of denatured Ø29 DNA (COO-2084-7). This permitted the separation of the DNA strands in neutral CsCl gradients. We (COO-2084-13) showed that early mRNA is transcribed from the light Ø29 DNA strand, and that infection in the presence of chloramphenicol yields mRNA that forms hybrids only with the light strand of Ø29 DNA. After the onset of viral DNA synthesis, late mRNA is made that will hybridize with the heavy Ø29 DNA strand. DNA-RNA hybridization-competition experiments indicate the continued synthesis of early mRNA throughout the cycle. Loskutoff et al. (24) confirmed these results and demonstrated that late mRNA synthesis was not dependent upon viral DNA synthesis. Loskutoff and Pene (23) used gel electrophoresis to revolve at least six species of early mRNA, and these mRNAs reflect about 35% of the coding capacity of the Ø29 genome. Only one species of early mRNA was absent in chloramphenicol treated cells. In the typical infection, as viral DNA was being synthesized,

three species of late mRNA were formed in addition to most species of early mRNA.

The existence of a Ø29 soded DNA-dependent RNA polymerase remains in doubt. Since only early mRNA is formed when cells are infected in the presence of chloramphenicol, one might expect to find a polymerase similar to the enzyme reported for T7 (11,24, COO-2084-13). However, all Ø29 mRNA synthesis is sensitive to the initiation inhibitor rifamycin, and Ø29 message is formed in rifamycin resistant host cells in the presence of the antibiotic (COO-2084-13). The host RNA polymerase of both B. subtilis and B. amyloliquefaciens has been purified by students of Ø29 (6, 16). Holland and Whiteley (18) have observed differences in specific activity, template specificities, stability and sedimentation properties of the polymerase isolated from Ø29 infected cells. A 30,000 molecular weight polypeptide appeared in association with the polymerase early in infection, and this observation may account for the chloramphenicol sensitivity of the late mRNA synthesis.

Since Ø29 infection does not stop host protein synthesis (COO-2084-12), viral-induced protein synthesis has been studied in ultraviolet-irradiated cells (9,15, 26, 30). Hawley et al. (15) resolved 23 virus-induced proteins by SDS polyacrylamide gel electrophoresis and autoradiography. Although the results of the three groups are not in complete agreement, there is a consensus that an early and a late set of proteins are synthesized and that the late proteins consist of the viral structural proteins and at least three nonstructural proteins. Carrascosa et al. (8) have reported the in vitro synthesis of Ø29 early proteins with an Escherichia coli cell-free system.

The initial genetic study of Ø29 employed ts mutants and two-factor crosses for amp construction; 13 cistrons were identified (14). Reilly et al. (34) placed 133 suppressor-sensitive (sus) mutants in 13 cistrons and employed three-factor crosses to assign an unambiguous order for ten cistrons. McGuire et al. (26) and Reilly et al. (33) have provided additional genetic data as an adjunct to other studies. Moreno et al. (27) have employed the ts mutants ot Talavera et al. (44) and a collection of sus mutants to construct a linear genetic map containing mutants in 17 complementation groups. Ito et al. (19) have generated five fragments of the Ø29 genome with the restriction endonuclease EcoR1. Inciarte et al. have used EcoR1 fragments, marker rescue by transfection and cell-free

protein synthesis to construct a physical map of Ø29 (personal communication).

Although our analysis of both the Ø29 genetic system and the pattern of Ø29-specific protein synthesis is not completed we can provide an estimate of the number of Ø29 cistrons and the number of viral specified polypeptides that we can detect.

We have examined the patterns of Ø29-specific protein synthesis after infection by sus mutants under restrictive conditions. The ^{14}C-labeled proteins were resolved and identified by SDS gel electrophoresis and autoradiography (5, 33). This approach was also employed by McGuire et al. (26).

We have summarized our image of the time course of protein synthesis (15) and have preliminary evidence of a prevursor for the early protein AF and BF (unpublished observation), and since point mutation in cistron 3 results in the loss of both proteins during infection of nonpermissive cells, we could consider the cistron 3 product a single protein, P3 (5). We do not know if this cleavage is specific or if the proteins we have labeled AF and BF can function.

The proteins A1 of the supernatant and A2 of the cell pellet (15) migrate at different rates during electrophoresis. The amount of A2 is variable but it is always present in the cell pellet, probably associated with the cell membrane. Both proteins are absent following point mutation (5). We have observed fragments of these proteins in the infected nonpermissive host. The fragments of the proteins A1 and A2 always partition between the supernatant and the cell pellet and also migrate at the characteristic differential rates relative to one another (unpublished observations). Revertants of cistron 17 mutants synthesize both the A1 and A2 proteins. Therefore we could also consider the product of cistron 17 to be a single polypeptide P17. The migration of protein A2 may reflect modification of the protein during function.

We have not identified the polypeptides coded for by cistrons 2, 13 or 14 (mutant sus14(1241)). If we assume that each cistron codes for a unique polypeptide, and also assume that none of the low-molecular-weight proteins with the exception of LM2 represent cleavage products, we can estimate the number of Ø29-specified proteins. If the protein of Ortin et al. (29) is unique, there may be 25 of these proteins.

To facilitate our genetic analysis we have recently exchanged mutants with the Madrid group and have examined the mutants by quantitative complementation (joint unpublished observations). One ts mutant, ts5(219) (COO-2084-10), represents a cistron that maps between cistrons 4 and 6. We have defined 17 cistrons by quantitative complementation and/or electrophoresis (5,33).

Mutants of cistrons 2 and 3 are unable to synthesize viral DNA in the nonpermissive host in the presence of 6-(p-hydroxyphenylazo)-uracil (COO-2084-16). Under these conditions, sus mutants of cistrons 1, 15 and 17 synthesize substantial but variable amounts of DNA on what appears to be a random basis. With mutant sus6(626) viral DNA synthesis is delayed, and the DNA is not synthesized at the wild-type rate or extent. Under restrictive conditions, these mutants also give small bursts, but again the burst size is quite variable. This may relfect the need of Ø29 to modulate the host biosynthesis that continues throughout the latent period as demonstrated previously (COO-2084-10). Talavera et al. (43) showed that ts mutants in cistrons 2 and 3 were unable to synthesize DNA.

Ivarie and Pene (20) present evidence that the mutation in ts2(35) (14) appears to affect a protein that serves to associate viral DNA with the host cell membrane in the replicating complex. Mutant sus2(628) of the sus collection does not complement mutant ts2(35).

Point mutation in a series of sus mutant of cistron 6 results in a protein profile that lacks proteins LM3 and LM8 (5). Revertants of sus6(626) synthesize both proteins. We have isolated mutants that cannot synthesize LM3 and other independent isolates that cannot make LM8. These mutants can form plaques on the restrictive host. We conclude that the cistron 6 product may have a regulatory function.

The mutant sus1(629) is our only isolate that cannot synthesize the proteins LM7 and LM9. Yet we have identified mutants that cannot synthesize LM7 and others that cannot make LM9. Both types of mutant make plaques on the restrictive host.

Our current hypothesis regarding the phenotype of the mutants that cannot synthesize low molecular weight proteins is as follow. Some of these proteins modulate biosynthesis in the host bacteria. In the absence of certain proteins, the burst size is reduced. When these mutations are present in certain combinations the burst size is reduced to the extent that plaques are not visible. We are sure that mutants that cannot synthesize

the proteins LM3, LM5, LM7, LM8 and LM9, respectively, can form plaques on the nonpermissive host.

In our initial analysis, mutants of cistron 10 failed to synthesize the neck collar proteins C1 (P19) and C2 (P11) (5). The genetic results of Moreno et al. (27) with their mutants of cistrons 10 and 11 were interpreted to indicate a strong polarity effect of the nonsense supressor translation of the mRNA transcribed from the collar region of the genome.

We have since isolated a series of mutants that can synthesize the neck protein P10 but not P11. These mutants are in cistron 11. We have confirmed the results of the quantitative complementation experiments of Moreno et al. (27) using mutants of both collections. This leads us to the hypothesis that the product of cistron 8 could play a role analogous to that postulated for the cistron 6 product, or that the cistron 8 phenotype reflects the situation described for cistron 10.

We have reported a precursor-product relationship between the product of cistron 12, P12, and both the appendage protein Ap and a low-molecular-weight product, LM2 (5). Infection with a mutant having a clear plaque (c) phenotype, Ø29c, permits the uncleaved P12 to accumulate (5). We believe this cleavage is specific because we have demonstrated by in vitro complementation that the cleaved form of the appendage is functional and that cleavage of the appendage precursor protein does not occur in situ on the maturing virus (46). Carrascosa et al. (7) have comfirmed the relationship of P12 to the appendage by pulse-chase experiments and by comparison of peptides obtained from tryptic digests.

If we assume that a separate cistron codes for each low molecular weight protein with the exception of LM2 and that the major head protein and the head fiber are also the products of separate cistrons, then our estimate of total cistrons would be at least 25.

Our data suggest that there may be some functional clustering of genes in Ø29. The mutants that cannot synthesize DNA are in the cistron 2 and 3 at the left of our genetic map. The cistrons that are known to code for structural proteins are centrally located. The cistrons IV, V and VI defined by McGuire et al. (26) are in the central region of their genetic map and correspond to our cistrons 9, 10 and 12, respectively, in the same order. We have presented evidence that

transcription in this region probably proceeds from cistron 10 itno cistron 12 (33).

Mutants of cistron 4 synthesize neither protein LM3B nor any protein placed in the late category (5, 15). Our data on heavy DNA strand transcription leads us to the hypothesis that one function of cistron 4 might be to regulate late transcription.

One of the most striking observations is that two cistrons specifying proteins of the early I class, cistrons 3 and 17, map at opposite ends of the genome.

We have estimated that the genome of Ø29 contains at least 25 cistrons and that there are probably about 25 viral-specific proteins. These estimates are reasonable but may be low.

Our analysis of the temporal sequence of protein synthesis, although incomplete, is in good agreement with the published transcription data (23, 24, 28, COO-2084-13). Heavy DNA strand transcription begins late in infection, and mutants in cistron 4 fail to synthesize protein LM3B and all known proteins of the late class (Table 1). Loskutoff and Pene (23) report the existence of an early mRNA, not synthesized late in infection, that has an expected coding capacity of about 95,000 daltons of protein. If we place protein LM3B in the class early I (Table 1) with AF, BF, A1 and LM6B, the combined molecular weights would approximate 95,000 daltons.

Loskutoff and Pene (24) estimate that the 6 species of early mRNA account for about 35% of the Ø29 transcription. We estimate that the early proteins we detect reflect about 32% of transcription and that the assigned molecular weights of the Ø29-specified proteins we resolve is about 550,000 daltons. This is in excess of 90% of the expected coding capacity of the genome.

We have demonstrated the presence of a prohead that functions as a precursor in Ø29 assembly. In the absence of protein P16, a similar particle accumulates. Cells infected by mutants that cannot synthesize protein P7 contain a fragile, unstable, membrane-associated structure. In the prohead the protein P7 is present. We believe we have presented unequivocal evidence that al least two nonstructural proteins, P16 and P7, play a role in morphogenesis.

Structural proteins can also have a dual function. For example, the neck upper collar protein, P10, plays a role in form determination because only isometric heads are formed in its absence. There may be other examples, e.g., the protein of cistron 9.

Because of the relatively extensive commitment of the coding capacity of the Ø29 genome to the synthesis of structural proteins and morphopoietic factors, we have assumed that Ø29 must have evolved some compensatory mechanism to increase the complexity of its phenotype.

Proteins may play multiple roles as a result of specific cleavage or other types of modification during function. We believe we have detected cleavage of the proteins P3 and P12 as well as a potential modification of protein A1. We can only speculate as to the functional significance of these observations in this context.

Since Ø29 infection was not disruptive of host biosynthesis (COO-2084-12) and we could detect at least 12 proteins ranging in molecular weight from 17,500 to less than 4,000 daltons following infection, it may be significant that so few of these proteins were proven to be essential for the completion of the viral infectious cycle. It will be of interest to determine just where and how these proteins function and the affect they have on the phage burst size.

LITERATURE CITED

1. Ackermann, H.-W. 1974. La classification des bacteriophages de Bacillus et Clostridium. Path. Biol. 22:909-917.
2. Alvarez, G., E. Salas, N. Perez, and J. E. Celis. 1972. Ø29 bacteriophage structural proteins. J. Gen. Virol. 14:243-250.
3. Anderson, D. L., D. D. Hickman, and B. E. Reilly. 1966. Structure of Bacillus subtilis bacteriophage Ø29 and the length of Ø29 deoxyribonucleic acid. J. Bacteriol. 91:2081-2089.
4. Anderson, D. L., and E. T. Mosharrafa. 1968. Physical and biological properties of phage Ø29 deoxyribonucleic acid. J. Virol. 2:1185-1190.
5. Anderson, D. L., and B. E. Reilly. 1974. Analysis of bacteriophage ø29 gene function: protein synthesis in suppressor-sensitive mutant infection of Bacillus subtilis. J. Virol. 13:211-221.
6. Avila, J., J. M. Hermoso, E. Vinuela, and M. Salas. 1970. Subunit composition of B. subtilis RNA polymerase. Nature 226:1244-1245.
7. Carrascosa, J. L., A. Camacho, E. Vinuela, and M. Salas. 1974. A precursor of the neck appendage protein of B. subtilis phage Ø29. FEBS Lett. 44:317-321.
8. Carrascosa, J. L., F. Jimenez, E. Vinuela, and M. Salas. 1975. Synthesis in vitro of Ø29-specific early proteins directed by phage DNA. Eur. J. Biochem. 51:587-591.
9. Carrascosa, J. L., E. Vinuela, and M. Salas. 1973. Proteins induced in Bacillus subtilis infected with bacteriophage Ø29. Virology 56:291-299.
10. Casjens, S., and J. King. 1974. P22 morphogenesis I: catalytic scaffolding protein in capsid assembly. J. Supramol. Structure 2:202-224.

11. Chamberlin, M., J. McGrath, and L. Waskell. 1970. Isolation and characterization of a new RNA polymerase from T7 infected E. coli. Nature (London) 228:227-231.

12. Gordon, R. E., W. C. Haynes, and C. H.-N. Pang. 1973. The Genus Bacillus. Agriculture Handbook No. 427. Agricultural Research Service, U. S. Dept. of Agriculture, Washington, D.C.

13. Hagen, E. W., and D. L. Anderson. 1975. In situ lysis of Ø29- and SPO1-infected Bacillus subtilis. J. Virol. 15:217-220.

14. Hagen, E. W., V. M. Zeece, and D. L. Anderson. 1971. A genetic study of temperature-sensitive mutants of the Bacillus subtilis bacteriophage Ø29. Virology 43:561-568.

15. Hawley, L. A., B. E. Reilly, E. W. Hagen, and D. L. Anderson. 1973. Viral protein synthesis in bacteriophage Ø29 infected Bacillus subtilis. J. Virol. 12:1149-1159.

16. Hermoso, J. M., J. Avila, F. Jimenez, and M. Salas. 1972. RNA polymerase from Bacillus amyloliquefaciens. Biochim. Biophys. Acta 277:280-283.

17. Hirokawa, H. 1972. Transfecting deoxyribonucleic acid of Bacillus bacteriophage Ø29 that is protease sensitive. Proc. Natl. Acad. Sci. U.S.A. 69:1555-1559.

18. Holland, M., and H. R. Whiteley. 1973. RNA polymerase from Bacillus amyloliquefaciens infected with Ø29 bacteriophage. Proc. Natl. Acad. Sci. U.S.A. 70:2234-2237.

19. Ito, J., F. J. Kawamura, and S. Yanofsky. 1975. Analysis of ø29 and Ø15 genomes by bacterial restriction endonucleases, EcoR1 and HpA1. Virology

20. Ivarie, R. D., and J. J. Pene. 1973. DNA replication in bacteriophage Ø29: the requirement of a viral-specific product for association of Ø29 DNA with the cell membrane of Bacillus amyloliquefaciens. Virology 52:351-362.

21. Jacobson, E. D., and O. E. Landman. 1975. Interaction of protoplasts, L forms, and Bacilli of Bacillus subtilis with 12 strains of bacteriophage. J. Bacteriol. 124:445-458.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

23. Loskutoff, D. J., and J. J. Pene. 1973. Gene expression during the development of Bacillus subtilis bacteriophage Ø29. II. Resolution of viral-specific ribonucleic acid molecules. J. Virol. 11:87-97.

24. Loskutoff, D. J., J. J. Pene, and D. P. Andrews. 1973. Gene expression during the development of Bacillus subtilis bacteriophage Ø29. I. Analysis of viral-specific transcription by deoxyribonucleic acid-ribonucleic acid competition hybridization. J. Virol. 11:78-86.

25. Mendez, E., G. Ramirez, M. Salas, and E. Vinuela. 1971. Structural proteins of bacteriophage Ø29. Virology 45:567-576.

26. McGuire, J. C., J. J. Pene, and J. Barrow-Carraway. 1974. Gene expression during the development of bacteriophage Ø29. III. Analysis of viral-specific protein synthesis with suppressible mutants. J. Virol. 13:690-698.

27. Moreno, F., A. Camacho, E. Vinuela, and M. Salas. 1974. Suppressor-sensitive mutants and genetic map of Bacillus subtilis bacteriophage Ø29. Virology 62:1-16.

28. Mosharrafa, E. T., C. F. Schachtele, B. E. Reilly, and D. L. Anderson. 1970. Complementary strands of bacteriophage Ø29 deoxyribonucleic acid: preparative seapration and transcription studies. J. Virol. 6:855-864.

29. Ortin, J., C. Vasquez, E. Vinuela, and M. Salas. 1971. DNA-protein complex in circular DNA from phage Ø29. Nature (London) New Biol. 234:275-277.

30. Pene, J. J., P. C. Murr, and J. Barrow-Carraway. 1973. Synthesis of bacteriophage Ø29 proteins in Bacillus subtilis. J. Virol. 12:61-67.

31. Ravin, A. W. 1963. Experimental approaches to the study of bacterial phylogeny. Amer. Natur. 97:307-318.

32. Reilly, B. E., and J. Spizizen. 1965. Bacteriophage deoxyribonucleate infection of competent Bacillus subtilis. J. Bacteriol. 89:782-790.

33. Reilly, B. E., M. E. Tosi, and D. L. Anderson. 1975. Genetic Analysis of Bacteriophage Ø29 of Bacillus subtilis: mapping of the cistrons coding for structural proteins. J. Virol. 16:1010-1016.

34. Reilly, B. E., V. M. Zeece, and D. L. Anderson. 1973. A genetic study of suppressor-sensitive mutants of the Bacillus subtilis bacteriophage Ø29. J. Virol. 11:756-760.

35. Rima, B. K., and H. Y. Steensma. 1971. Bacteriophage of Bacillus subtilis: comparison of different isolation techniques and possible use for classification of Bacillus subtilis strains. Antonie van Leeuwenhoek J. Microbiol. Serol. 37:425-434.

36. Rubio, V., M. Salas, E. Vinuela, P. Usobiaga, J. L. Saiz, and J. F. Llopis. 1974. Biophysical properties of bacteriophage Ø29. Virology 57:112-121.

37. Salas, M., C. Vasquez, E. Mendez, and E. Vinuela. 1972. Head fibers of bacteriophage Ø29. Virology 50:180-188.

38. Schachtele, C. F., C. V. DeSain, and D. L. Anderson. 1973. Transcription during the development of bacteriophage Ø29: definition of "early" and "late" Ø29 ribonucleic acid. J. Virol. 11:9-16.

39. Schachtele, C. F., C. V. DeSain, L. A. Hawley, and D. L. Anderson. 1972. Transcription during the development of bacteriophage Ø29: production of host and Ø29-specific ribonucleic acid. J. Virol. 10:1170-1178.

40. Schachtele, C. F., E. W. Hagen, and D. L. Anderson. 1971. Temperature-shift analysis of bacteriophage Ø29 gene expression in Bacillus amyloliquefaciens. J. Virol. 8:352-354.

41. Schachtele, C. F., B. E. Reilly, C. V. DeSain, M. O. Whittington, and D. L. Anderson. 1973. Selective replication of bacteriophage Ø29 deoxyribonucleic acid in 6-(p-hydroxyphenylazo)-uracil treated Bacillus subtilis. J. Virol. 11:153-155.

42. Shimizu, N., K. Mivra, and H. Aoki. 1970. Characterization of B. subtilis bacteriophage II: isolation and morphology of phage Nf and properties of its DNA. J. Biochem. 68:277-286.

43. Talavera, A., F. Jimenez, M. Salas, and E. Vinuela. 1971. Temperature-sensitive mutants of bacteriophage Ø29. Virology 46:586-595.

44. Talavera, A., M. Salas, and E. Vinuela. 1972. Temperature-sensitive mutants affected in DNA synthesis in phage Ø29 of Bacillus subtilis. Eur. J. Biochem. 31:367-371.

45. Tosi, M., and D. L. Anderson. 1973. Antigenic properties of bacteriophage Ø29 structural proteins. J. Virology 12:1548-1559.

46. Tosi, M. E., B. E. Reilly, and D. L. Anderson. 1975. Morphogenesis of bacteriophage Ø29 of Bacillus subtilis: cleavage and assembly of the neck appendage protein. J. Virol. 16:1282-1295.

47. Trautner, T. A. 1970. An introduction to Bacillus subtilis transfection, p. 150-162. In L. G. Ledoux (ed.), International Symposium on Uptake of Informative Molecules by Living Cells. North Holland Publishing Company, Mol. Belgium.

48. Tsien, H. C., B. E. Reilly, and D. L. Anderson. 1970. Rescue of genetic markers from bacteriophage Ø29 DNA fragments. Path. Microbiol. 36:288-296.

49. Young, F. E. 1967. Requirement of glucosylated teichoic acid for adsorption of phage in Bacillus subtilis 168. Proc. Natl. Acad. Sci. U.S.A. 58:2377-2384.

50. Young, F. E., and G. A. Wilson. 1972. Genetics of Bacillus subtilis and other gram-positive sporulating bacillus, p. 77-106. In H. O. Halvorson, R. Handon and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.

51. Young, F. E., and G. A. Wilson. 1974. Chromosomal map of Bacillus subtilis, pp. 597-610. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.

52. Wilson, G. A., and F. E. Young. 1972. Intergenotic transformation of the Bacillus subtilis. J. Bacteriol. 111:705-716.