AN ABSTRACT OF THE THESIS OF

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(Name of student) (Degree)

in Microbiology presented on 7-21-72
(Major) (Date)

Title: GENETIC MAPPING AND PHYSIOLOGICAL CHARACTERIZATION OF RNA POLYMERASE MUTANTS OF *BACILLUS SUBTILIS*

Abstract approved: Redacted for Privacy
Lyle R. Brown

DNA-dependent RNA polymerase mutants of *B. subtilis* strain Marburg resulting from EMS mutagenesis of spores were isolated and characterized. Genetic analysis by PBS1 phage mediated transduction and by transformation indicated that all of the four phenotypic classes studied (rifampin resistance, streptovaricin resistance, streptolydigin resistance and temperature sensitivity) were clustered close to the *cys* A14 locus. Three factor transformation analysis indicated the following marker order: $Rif^R (Stv^R)$ Std^R $Ts_{418} (Ts_{427})$. Physical separation of the Rif^R Std^R and Ts phenotypes by extensive mechanical shearing of transforming DNA indicated that these phenotypic classes were the result of independent, physically separated mutations. Centrifugation of sheared DNA samples on $Hg\text{-}Cs_2SO_4$ gradients indicated that the rifampin resistant mutations reside in an

area of higher A + T content than do the streptolydigin resistant mutations.

In vivo and *in vitro* measurements of RNA synthesis by isotope incorporation confirmed the genotype as RNA polymerase mutants. Measurements of growth and sporulating ability indicated that the rifampin resistant and streptovaricin resistant mutants studied were not affected by their altered RNA polymerases. However, the streptolydigin resistant mutants examined had varied generation times both in the absence and presence of streptolydigin and sporulated with reduced efficiency. *In vivo* studies indicated that the wild-type strain lost sensitivity to streptolydigin at the onset of sporulation. However, *in vitro* data with RNA polymerase from sporulating cells on poly-(dA-dT) template indicated that the sporulating polymerase was sensitive to streptolydigin. This paradox has not been resolved.

Genetic Mapping and Physiological Characterization of RNA Polymerase Mutants of *Bacillus subtilis*

by

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A Thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1973

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Date thesis is presented 7-21-72

Typed by Ilene Anderton for Stephen Randall Haworth

To my wife, Mary Ruth, without whose love, patience, and understanding this work could not have been completed

ACKNOWLEDGEMENT

I wish to express my sincere thanks to:

My major professor, Dr. Lyle Brown, for his guidance and for the many hours of stimulating discussions;

The members of my committee for their time and suggestions;

Dr. K. E. Van Holde and his associates for their assistance with the DNA molecular weight determinations;

Joe Hanus for his excellent technical assistance; and

My close friends here in Corvallis for their interest and encouragement.

This research was supported by Public Health Service Training Grant AI00186-10 from the Institute of Allergy and Infectious Diseases.

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GENETIC MAPPING AND PHYSIOLOGICAL CHARACTERIZATION OF RNA POLYMERASE MUTANTS OF *BACILLUS SUBTILIS*

INTRODUCTION

The primary function of the DNA-dependent RNA polymerase is the synthesis, using DNA as a template, of the various RNA molecules which enable an organism to utilize the information coded in its DNA (38, 56, 93).

Structure of DNA-Dependent RNA Polymerase of Bacteria

RNA polymerase isolated from several different genera of bacteria appear to be very similar. Those isolated from *Escherichia coli* (10, 113) *Pseudomonas putida* (41), *Azotobacter vinelandii* (46), and *Bacillus subtilis* (5, 51) each have two identical α subunits with a molecular weight of approximately 42,000, a β subunit with a molecular weight of approximately 150,000 daltons, and a β' subunit with a molecular weight of about 160,000 daltons. A transcription stimulating factor, sigma, which can be co-purified with the core enzyme (10) has a molecular weight of approximately 90,000 daltons in *E. coli* (10, 113), *P. putida* (41) and *A. vinelandii* (46), but only 56,000 daltons molecular weight for the enzyme isolated from *B. subtilis* (5, 51). However, RNA

polymerases isolated from T3-or T7-infected *E. coli* cells have only one sub-unit and are much smaller with a molecular weight of approximately 110,000 daltons (16, 23).

Inhibitors of RNA Polymerase

Antibiotics are available which specifically inhibit RNA polymerase activity. A group of antibiotics produced by *Streptomyces mediterranei*, the rifamycins, were first described in 1959 (77). Hartmann *et al*. (34) and Umezawa *et al*. (98) first demonstrated that the rifamycins specifically inhibited the DNA-dependent RNA polymerase of bacteria. Later workers showed that the antibiotics bound to the RNA polymerase and specifically blocked the initiation step of RNA synthesis (19, 50, 81, 86, 101). After the structure of the rifamycins was determined (69), a large number of semi-synthetic derivatives were prepared among which was the derivative of rifamcycin SV, rifampin (rifampicin) (54).

Another very similar antibiotic is streptovaricin. Its production by *Streptomyces spectabilis* was first described by Siminoff *et al*. (80) and further characterized by Sokolski *et al*. (88), and Yamazaki (106, 107).

Like rifampin, streptovaricin also inhibits the initiation step of RNA synthesis in bacteria by binding to RNA polymerase (59, 60, 111).

A third compound, streptolydigin, produced by *Streptomyces lydicus*, was first described by DeBoer *et al*. (18) and further characterized by Eble *et al*.(24), Lewis *et al*.(49), and Rinehart *et al*. (70). Its mode of action differs from that of rifampin and streptovaracin in that it blocks translocation of the enzyme along the DNA (13, 75, 79) by inhibiting phosphodiester bond formation and by altering the affinity of the enzyme for CTP and UTP (14, 99).

Role of RNA Polymerase in Regulation

That RNA polymerase can exert a positive control function on the transcription of DNA is suggested by several types of evidence. The transcription stimulating properties of the sigma factor have been described by Burgess *et al* (10) and a possible role for RNA polymerase in the activation of catabolite sensitive genes has been suggested by Zubay (114). During T4 phage infection of *E. coli*, the host polymerase is modified at several stages, each time followed by the appearance of new classes of phage specific mRNA (30, 76, 100). Evidence has been reported for the interaction of N gene product of λ bacteriophage with the RNA polymerase of *E. coli* to allow transcription to proceed past normally terminating signals (29). Finally, Losick *et al*. (51) have described an alteration in the β subunit of *B. subtilis* at the onset of sporulation. The altered form has a molecular weight of 110,000 daltons as compared to the

unaltered molecular weight of 145,000 daltons.

Genetic Analysis of RNA Polymerase Genes in *E. coli*

The RNA polymerase genes in *E. coli* have been genetically analyzed by conjugation and P1 phage mediated transduction. Mutants resistant to rifampin, streptovaricin or streptolydigin have been shown to map between *arg* H and *thi* which corresponds to about 77.5' and 78' on the *E. coli* chromosome (2, 42, 58, 67, 87, 111, 112). RNA polymerase sub-unit reconstitution studies (35) and studies with merodiploids (43) offer strong evidence that the mutations conferring rifampin resistance or streptolydigin resistance both reside in the β subunit. Several temperature sensitive mutations have also been shown to reside in the RNA polymerase molecule and to map between *arg* H and *thi* (42, 112). One such mutation simultaneously confers temperature sensitivity and resistance to streptovaricin (112).

Genetic Analysis of the *B. subtilis* Chromosome

PBS1 phage mediated transduction (95), transformation (91), density transfer analysis (65, 109), and marker frequency analysis (108) have been used to construct a genetic map of the *B. subtilis* chromosome (110). Although the *B. subtilis* genetic map is depicted

as circular, the evidence available is not sufficient to confirm this hypothesis. Terminal markers and origin markers have not been linked by co-transformation or co-transduction. The segment containing *pur* B, *pur* C, *pur* D, *tem*-1, *pig*, *tsi* and the germination marker Gsp II 115 has not been linked to any other markers although density transfer mapping (65, 109) marker frequency analysis (108) and transformation with DNA from germinating spores (6) indicate that this segment does replicate in a linear order with the rest of the chromosome. However, pending circularization of the *B. subtilis* chromosome there, at present, exist three linkage groups in *B. subtilis*. In the first linkage group, the region between *cys* A14 and the second linkage group, has been studied extensively. Genetic analysis has shown that the DNA between *cys* A14 and *lin* contains 80% of the genes coding for 5S, 16S, and 23S ribosomal RNA (22, 64, 84, 85) and also genes for t-RNA (63). A number of mutations conferring resistance to antibiotics known to affect ribosomal proteins also map in this region (31, 33, 83). Hoch *et al*. (37) Rogolsky (71) and Takahashi(96) have shown that twelve asporogenous mutants blocked at two different stages of sporulation map very close to the *cys* A14 locus.

The RNA polymerase genes of *B. subtilis* have not been mapped as extensively as in *E. coli*; however, recent studies indicate that rifampin resistant and streptovaricin resistant mutations are closely

linked to *cys* A14 by co-transformation and co-transduction. Harford and Sueoka(33) first demonstrated linkage of rifampin resistance to *cys* A14 by co-transformation. Brown and Doi (9) genetically analyzed over forty rifampin resistant and streptovaricin resistant mutants by PBSl mediated transduction. Both the rifampin resistant and the streptovaricin resistant phenotypes were closely linked by co-transduction to *cys* A14.

Physical Analysis of the *B. subtilis* Chromosome

The intramolecular heterogeneity of the *B. subtilis* chromosome has been demonstrated by absorption melting curves (68), comparison of relative sensitivites of several genetic markers to ultraviolet irradiation (61), comparison of relative elution profiles of various genetic markers from methylated albumin-kieselguhr columns (74), and by comparison of the bouyant densities of sheared pieces of DNA (28, 66, 94, 97, 103). The data indicate that the G + C content of the chromosome is not uniform throughout and that the origin proximal region of the chromosome is of higher G + C content than is the remainder of the chromosome (97).

DNA molecules can be broken into families of double stranded fragments of various sizes by subjection to appropriate levels of hydrodynamic shear (11, 12, 15, 36, 72). Shearing results in decreased total transforming activity and in decreased

co-transformation of linked markers (92, 97). The range of $S_{20,w}$ values which describes the family of DNA molecules present in a sheared preparation can be determined by sedimentation velocity centrifugation (78). The fragments can be separated on the basis of G + C content by centrifugation in Hg-Cs_2SO_4 gradients (62, 82, 103, 104). Preferential binding to A + T rich regions by Hg^{++} ions causes A + T regions to band at a position of higher bouyant density in a Hg-Cs_2SO_4 gradient (62).

Bacterial Sporulation as a Model for Cellular Differentiation

Bacterial sporulation has been viewed as a model system for cellular differentiation (32, 45, 56). The developmental processes occuring during the life cycle of a sporulating bacterium yield a differentiated cell with physical and physiological properties entirely different from those of the mother cell. *B. subtilis* offers a unique system in which to study this process in that it lends itself to both physiological and genetic analysis. *B. subtilis* is sensitive to rifampin, streptovaricin, and streptolydigin, and mutants resistant to these drugs can be isolated (9, 20, 89).

Several types of evidence suggest that the RNA polymerase of *B. subtilis* may be directly involved in the regulation of the sporulation process.

Recent studies have shown that the alteration in the β subunit of RNA polymerase described by Losick (51) is due to the specific cleavage by a catabolite repressible serine active protease (48).

B. subtilis vegetative cells support the growth of virulent phage φe but sporulating cells do not (52). *In vitro* analysis of RNA polymerase from vegetative and sporulating cells shows that the vegetative enzyme can use φe DNA as a template but that sporulating enyzme can not (52).

Hussey *et al.* (39) have shown that the percentage of radioactive uracil incorporated into ribosomal RNA or ribosomal subunits decreases greatly during early sporulation. This finding suggests that ribosomal RNA genes are turned off during sporulation.

In vitro analysis of ribosomal RNA synthesized from *B. subtilis* DNA template indicates that vegetative polymerase, even after extensive purification, can synthesize ribosomal RNA (40). However, if the sigma factor is missing or if polymerase from sporulating cells is used, little ribosomal RNA is sythesized *in vitro* (40).

Doi *et al.* (20) and Korch and Doi (44) have reported a rifampin resistant mutant which produces spores with greatly altered morphology. Altered spore morphology and rifampin resistance are

100% co-transformable. Another rifampin resistant mutant reported by Sonenshein and Losick (89) was shown to have simultaneously gained resistance to rifampin and the inability to sporulate. A revertant of this strain simultaneously regained sensitivity to rifampin and the ability to sporulate. This rifampin resistant strain maintains vegetative template specificity during stationary phase (89) and continues to synthesize ribosomal sub-units and ribosomal RNA after log growth ceases.

None of these observations are sufficient in themselves to show that RNA polymerase is acting in a positive way to control transcription. However, taken as a whole, the observations do provide substantial evidence that RNA polymerase does play a role in regulating the sporulation process.

In order to better understand the B. subtilis RNA polymerase, new classes of RNA polymerase mutants should be isolated and characterized.

Objectives

The primary goals of this investigation were: (1) to isolate mutants resistant to the RNA polymerase inhibitor, streptolydigin, 2) to isolate conditional RNA polymerase mutants, 3) to determine by both genetic and physical means the location on the chromosome of the RNA polymerase mutations, and 4) to compare their

physiological properties to those of the wild-type strain and to other classes of RNA polymerase mutants.

The knowledge gained in this study should contribute to a fuller understanding of the functions of DNA-dependent RNA polymerase.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophage

The bacterial strains and bacteriophage used in this study are listed in Table 1.[1/]

OSB 3 (Cys^-Rif^R) was constructed by bacteriophage PBS1 mediated transduction by the following genetic cross: OSB 2 (Rif^R) x OSB 154 (Cys^-).

OSB 11 (Cys^-Rif^R) was constructed by PBS1 mediated transduction by the following genetic cross: OSB 10 (Rif^R) x OSB 154 (Cys^-).

OSB 192 (Cys^-) was constructed by transformation by the following cross: OSB 154 (Cys^-) x OSB 418 ($Std^R Ts$).

OSB 431 ($Rif^R Std^R Ts$) was constructed by transformation by the following genetic cross: OSB 423 (Rif^R) x OSB 418 ($Std^R Ts$).

[1/] A genetic cross by transduction or transformation will be depicted in the following manner:

OSB x (Rif^R) x OSB y (Std^R)

The strain on the left is the donor and the strain on the right is the recipient. The phenotype of each strain is included in the parentheses.

The following abbreviations will be used when referring to the phenotype of a strain: rifampin resistant, Rif^R; rifampin sensitive Rif^S; Streptovaricin resistant, Stv^R; streptolydigin sensitive, Stv^S; streptolydigin resistant Std^R; streptolydigin sensitive, Std^S; temperature sensitive, Ts; thermal stable, +; unable to grow without cysteine supplement, Cys^-; able to grow without cysteine supplement, Cys^+.

Table 1. Bacterial strains and bacteriophage.

Strain Designation	Phenotype		Source
OSB 2	prototroph	Rif^R	L. R. Brown NTG Mutagenesis
OSB 3	Cys^-	Rif^R	Construction
OSB 10	prototroph	Rif^R	L. R. Brown NTG Mutagenesis
OSB 11	Cys^-	Rif^R	Construction
OSB 17	prototroph	Rif^R	L. R. Brown NTG Mutagenesis
OSB 114	prototroph	$Stv^R Rif^R$	L. R. Brown ^3H-5-cytidine decay
OSB 122	prototroph	Stv^R	L. R. Brown ^3H-5-cytidine decay
OSB 154	Cys^-		N. Sueoka
OSB 158	prototroph <u>B. subtilis</u> strain Marburg		N. Sueoka
OSB 159	prototroph <u>B. pumilus</u>		F. Young
OSB 192	Cys^-		Construction
OSB 400	prototroph	Std^R	EMS Mutagenesis
OSB 401	prototroph	Std^R	Spontaneous
OSB 402	prototroph	Std^R	Spontaneous
OSB 403	prototroph	Std^R	EMS Mutagenesis
OSB 404	prototroph	Std^R	EMS Mutagenesis
OSB 405	prototroph	Std^R	EMS Mutagenesis
OSB 406	prototroph	Std^R	EMS Mutagenesis
OSB 407	prototroph	Std^R	EMS Mutagenesis
OSB 408	prototroph	Std^R	EMS Mutagenesis
OSB 409	prototroph	Std^R	EMS Mutagenesis
OSB 410	prototroph	Std^R	EMS Mutagenesis
OSB 412	prototroph	Std^R	EMS Mutagenesis

Table 1. Continued.

Strain Designation	Phenotype		Source
OSB 413	prototroph	Std^R	EMS Mutagenesis
OSB 414	prototroph	Std^R	EMS Mutagenesis
OSB 415	prototroph	Std^R	EMS Mutagenesis
OSB 416	prototroph	Std^R	EMS Mutagenesis
OSB 417	prototroph	Std^R Ts	EMS Mutagenesis
OSB 418	prototroph	Std^R Ts	EMS Mutagenesis
OSB 419	prototroph	Std^R Ts	EMS Mutagenesis
OSB 420	prototroph	Rif^R	EMS Mutagenesis
OSB 421	prototroph	Rif^R	EMS Mutagenesis
OSB 422	prototroph	Rif^R	EMS Mutagenesis
OSB 423	prototroph	Rif^R	EMS Mutagenesis
OSB 424	prototroph	Rif^R	EMS Mutagenesis
OSB 425	prototroph	Rif^R	EMS Mutagenesis
OSB 426	prototroph	Rif^R	EMS Mutagenesis
OSB 427	prototroph	Rif^R Ts	EMS Mutagenesis
OSB 428	prototroph	Rif^R Ts	T. Leighton EMS Mutagenesis
OSB 429	prototroph	Rif^R Ts	T. Leighton EMS Mutagenesis
OSB 431	prototroph	$Rif^R Std^R$ Ts	Construction
OSB 432	prototroph	$Rif^R Std^R$ Ts	Construction
OSB 433	prototroph	$Rif^R Std^R$ Ts	Construction
OSB 439	prototroph	$Rif^R Std^R$	Construction
OSB 440	prototroph	$Rif^R Std^R$	Construction
PBS1 bacteriophage	*B. subtilis* generalized transducing phage		J. Hock

A Rif^{R}Std^{S}Ts recombinant was selected on rifampin and used as the recipient in the following cross: OSB 404 (Std^R) x Rif^{R}Std^{S}Ts. OSB 432 (Rif^{R}Std^{R}Ts) and OSB 433 (Rif^{R}Std^{R}Ts) were constructed in exactly the same way except that OSB 405 (Std^R) and OSB 415 (Std^R) respectively were used as donors in the cross with the Rif^R Std^{S}Ts recombinant.

OSB 439 (Rif^{R}Std^R) was constructed by PBS1 mediated transduction in the following cross: OSB 406 (Std^R) x OSB 3 (Rif^R).

OSB 404 was constructed by PBS1 mediated transduction in the following cross: OSB 405 (Std^R) x OSB 11 (Rif^R).

Culture Media

Unless otherwise stated, all culture media were sterilized by autoclaving. Glucose was sterilized separately and was added aseptically to a medium just prior to its use. Media which were filter sterilized were passed through a 0.22 nm Millipore membrane.

The commercially prepared dehydrated media used were Penassay Broth (PAB) or Antibiotic Medium Number 3, Difco (Detroit, Mich.) and Tryptose Blood Agar Base (TBAB), Difco. M medium was the minimal medium described by Anagnostopolous and Spizizen (4). MG medium was the same medium except D-glucose was supplemented to 0.5%. MG agar was MG medium solidified with 1.5 g per liter of Bacto-Agar, Difco. Double strength M medium

and double strength agar were sterilized separately and then combined aseptically before distribution into petri dishes. When this agar was used to select for *cys* A14 transformants, it was supplemented with the filter sterilized enhancing amino acid mixture described by Wilson and Bott (102). SG medium was 2x Schaffer's medium as modified by Maia (55). SSM medium was a filter sterilized, defined sporulation medium as described by Freese and Fortnagel (27) except that the tryptophan and methionine supplements were omitted.

Reagents

Liquified phenol (J. T. Baker Chem. Co., Phillipsburg, N.J.) was mixed with 10 percent(v/v)0.05 M Na_2HPO_4 and adjusted to neutral pH with NaOH before use in DNA extraction. Chloroform iso - amyl alcohol solution was a 1:24 mixture of iso-amyl alcohol to chloroform. Saline - EDTA contained 0.15 M NaCl plus 0.1 M EDTA with the pH adjusted to 8.0. Concentrated saline citrate solution (10 x SSC) contained 1.5 M NaCl plus 0.15 M trisodium citrate and was adjusted to pH 7.0. This solution was diluted 1:10 to achieve standard saline citrate concentration (1 x SSC) and was diluted 1:100 to achieve the dilute saline citrate concentration (0.1 x SSC). Acetate - EDTA contained 3.0 M sodium acetate plus 0.001 M EDTA and was adjusted to pH 7.0.

Scintillation fluid contained, in an appropriate volume of reagent grade toluene, 0.4% 2, 5-Diphenyloxazole (PPO), scintillation grade, (Packard Instrument Co., Downers Grove, Ill.) and 0.01% 1, 4-bis-[2-(5-phenyloxazolyl)]-Benzene (POPOP), scintillation grade (Packard).

Mutagens

Ethylmethane sulfonate (EMS) was purchased from Eastman Kodak Chemical Company, Rochester, New York.

Enzymes

DNase I and 5 x crystalline, salt free, A-grade Bovine Pancreatic RNase were purchased from Calbiochem, Los Angeles, California. RNase was dissolved in 0.15 M NaCl, pH 5.0 to a final concentration of 0.2% and was heated at 80 C for ten minutes to destroy any residual DNase activity.

Egg white lysozyme was purchased from Sigma Chemical Company, St. Louis, Missouri.

Heterologous DNA Templates

Salmon sperm DNA was purchased from Calbiochem. Highly polymerized poly (dA-dT) was purchased from Research Products Division, Miles Laboratories, Inc., Kankakee, Illinois.

Antibiotics

Chloramphenicol (CAP), Lot Rx291064A, was a gift from Parke-Davis Company, Detroit, Michigan.

Rifampin (Rif), lot D-1281, was a gift from Dr. Justus Gelzen, CIBA Pharmaceutical Company, Summit, New Jersey. Additional Rifampin, Lot OD1760, was purchased from Cal Biochem, Los Angeles.

Streptovaricin (Stv) U-7750 (complex) Lot 11560-5 and streptolydigin (Std) U-5482 (sodium), Lot 4969-WTP-54, were kindly donated by Dr. G. B. Whitfield, the Upjohn Company, Kalamazoo, Michigan.

Rifampin, streptovaricin, and streptolydigin stock solutions were prepared in M-medium. The final concentrations used in the growth media were Rif, 10 μg/ml; Stv, 30 μg/ml; Std, 100 μg/ml.

Radioisotopes

^{3}H-5-Uridine (25 Ci/mM) and uniformly labelled ^{14}C-L-Phenylalanine (384.5 Ci/mM) were purchased from Amersham/Searle Radio Chemical, Arlington Heights, Illinois. Unless otherwise stated, ^{3}H-5-Uridine was supplemented in the growth medium at a final concentration of 0.4 μCi/ml and uniformly labelled ^{14}C-Phenylalanine was used at 0.04 μCi/ml. Cold carrier phenylalanine

was present at 5 μg/ml.

^{3}H-UTP (8-22 Ci/mM) was purchased from Amersham/Searle Radiochemical. Each *in vitro* reaction mixture contained 2 μCi of ^{3}H-UTP.

Preparation of Spore Stocks

SG medium was inoculated with a five percent inoculum of log phase OSB 158 and incubated on a rotary shaker for 24-30 hr. The resulting spores were harvested by centrifugation at 13348 x g for 20 min at 4 C and the pellet was washed exhaustively with sterile distilled water. Successive centrifugations were at 3337 x g for 15 min at 4 C. The clean spores were stored at 4 C in sterile distilled water.

Selection of Drug Resistant or Thermal Resistant Mutants

Cells to which resistance to Rif, Stv, or Std had been conferred by mutagenesis, transduction, or transformation were allowed an expression time, during which new altered RNA polymerase could be synthesized, before they were challenged with the appropriate drug. The same technique was used for temperature sensitive strains that had been transformed to thermal stability.

Routinely, the spores or cells were first plated on an 86 mm

diameter 0.45 nm Millipore membrane and washed once with six ml of M medium. The membrane was transferred immediately to a TBAB plate, incubated for an appropriate time at 37 C, then transferred to another TBAB plate containing an appropriate drug and incubated at 37 C for 24 hr.

Temperature sensitive recipient cells transformed to thermal resistance were plated directly onto TBAB agar, incubated for an appropriate time at 37 C, and then shifted to 50 C for 24 hr.

Maximum recovery of drug resistant mutants from EMS mutagenized spores was obtained by a 3.5 hr delayed selection time. Drug resistant or thermal resistant transformants were delayed two hr before challenging with drug or shifting to 50 C.

Mutagenesis

Bacillus subtilis spores were diluted into sterile distilled water at 37 C and equilibrated for five min. EMS was added to a final concentration of 0.3 percent and after 20 min, 0.1 ml samples were removed and added to 9.9 ml of sterile distilled water. The entire diluted sample was filtered through an 86 mm 0.45 nm Millipore membrane and drug resistant mutants were then selected as described in the previous section.

The isolation of temperature sensitive mutants was identical except that mutants were first selected for drug resistance and then

tested for the ability to grow at 37 C and inability to grow at 50 C.

Stock and Donor Lysate Production

Twenty ml of PAB were inoculated to visible turbidity from an overnight plate of B. pumilus or appropriate donor strain and incubated with shaking at 37 C on a rotary shaker. When the culture reached peak motility, a sample was diluted 1:4 into fresh PAB. PBS1 bacteriophage were added to a multiplicity of infection (m. o. i.) of 1-2 and the cells were shaken for one hr at 37 C.

CAP was added to a final concentration of 5 μg/ml. The culture was shaken for two hr at 37 C and then placed in a 37 C stationary incubator overnight. DNase was added to a final concentration of 2 μg/ml. The culture was centrifuged at 2308 x g for 15 min, filtered through a 0. 45 nm Millipore membrane and then stored at 4 C. The lysate was checked for sterility on TBAB plates.

An alternate method of lysate production was achieved by plating approximately 1000 PBS1 bacteriophage with an appropriate bacterial strain using the standard overlay technique described by Adams (1). After 12 hr incubation at 37 C, 3-5 ml of PAB were pipetted onto the confluently lysed bacterial lawn and allowed to stand at room temperature for 15-20 min. The broth and overlay were removed and centrifuged at 2308 x g for 15 min. DNase was added to a final concentration of 2 μg/ml and the suspension was filtered

through a 0.45 nm Millipore membrane and stored at 4 C.

Bacteriophage Assay

PBS1 was assayed as described by Adams (1). Top agar was PAB plus 0.4% Bactoagar and bottom agar was TBAB. If fresh moist TBAB plates are not used, minute plaques will result which makes quantification difficult. Using this system, both OSB 158 and OSB 159 as hosts gave highly reproducible plaque counts.

Transduction Procedure

Ten ml of PAB were inoculated to visible turbidity with a recipient strain. When the culture reached peak motility, one ml of cells was mixed with one ml of phage of sufficient titer to give an m.o.i. of one and was shaken at 37 C for 30 min. The culture was then combined with five ml of M medium, centrifuged for 15 min at 1610 x g, and resuspended in two ml of M medium. Appropriate dilutions were plated on the required selection plates.

Preparation of Transforming DNA

Two hundred and fifty ml of log phase cells were centrifuged at 337 x g for 15 min and resuspended in five ml of Saline-EDTA. Lysozyme (10 mg/ml) was added to a final concentration of 900 μg/ml and the suspension was incubated at 37 C resulting in extensive

lysis in approximately one hr. Two percent sodium dodecyl sulfate was added to a final concentration of 0.07 % and the suspension was incubated at 37 C for five min with shaking. Five molar sodium perchlorate was added to the lysed suspension to a final concentration of 1 M. The mixture was then combined with an equal volume of chloroform iso-amyl alcohol, shaken for two minutes and centrifuged at 2308 x g for 10 min. The aqueous top layer was removed with a pipette and transferred to a 30 ml Corex centrifuge tube to which two volumes of cold 95% ethanol were pipetted slowly down the wall of the tube. The precipitate was wound onto a glass rod, rinsed with 70% and 100% ethanol and transferred to five ml of 0.1 M NaCl. The dissolved precipitate was pipetted into 20 ml of cold 95% ethanol and the resulting precipitate was collected with a sterile wire loop and transferred to five ml of 2 M NaCl. The DNA concentration was estimated by U.V. absorption at 260 nm.

Transformation Procedure

Preparation of competent cells was performed essentially as described by Anagnostopoulos and Spizizin (4). The recipient strain was incubated overnight on a rotary shaker at 37 C in PAB and transferred to MG medium ($\sim 1 \times 10^8$ cells/ml) containing 50 μg/ml L-tryptophan and 0.02% casein hydrolysate. For auxotrophic recipients, required amino acids were supplemented at 50 μg/ml.

Two and one half ml of this cell suspension were placed in a 19 x 150 mm screw-cap tube, shaken vigorously on a reciprocal shaker at 37 C for four hr, centrifuged at 3020 x g for five min and diluted 1:10 in MG medium containing 5 μg/ml L-tryptophan, 0.01% casein hydrolysate, an additional 5 μ moles/ml of $MgSO_4$, and 5 μg/ml of any other required amino acid. Nine tenths of a ml of this cell suspension and 0.1 ml of a 0.1 μg/ml DNA solution were placed in a 16 x 125 mm screw-cap tube which was placed at 37 C for 90 min on a reciprocal shaker. DNase I (100 μg/ml) was added to a final concentration of 33 μg/ml and the cells were plated on selective medium. Viable cell counts were done on TBAB.

Preparation of Purified DNA for Shearing Studies and for Hg-Cs_2SO_4 Gradients

Three grams of wet packed log phase cells grown in PAB were harvested, washed twice with saline-EDTA and frozen at 20 C overnight. The cells were resuspended in 30 ml of saline-EDTA and egg-white lysozyme (10 mg/ml) was added to a final concentration of 700 μg/ml. The suspension was incubated at 37 C for approximately 30 min until the suspension became viscous and microscopic observation showed extensive lysis. Twenty percent SDS was added to the suspension to give a final concentration of one percent and the mixture was agitated for ten min. Saline-EDTA was gradually added

with mixing to give a uniform suspension. An equal volume of phenol was added to the suspension which was shaken in a 250 ml glass stoppered Erlenmeyer flask for 10 min and centrifuged at 9230 x g for 15 min at 4 C. The aqueous top layer was removed and combined with an equal volume of chloroform iso-amyl alcohol. The mixture was shaken vigorously for 15 min and centrifuged at 9230 x g for 15 min at 4 C. Again, the aqueous top layer was removed and placed in a 30 ml Corex centrifuge tube. Two volumes of 95% ethanol were pipetted slowly down the side of the tube and the fibrous precipitate was wound onto a glass rod, washed in 70% ethanol and redissolved in six ml of 0. 1 x SSC. The dissolved precipitate was adjusted to approximately 1 x SSC concentration by the addition of 0. 6 ml of 10 x SSC. RNase solution was added to a final concentration of 40 μg/ml and the mixture was incubated for 30 min at 37 C. An equal volume of phenol was added and the mixture was shaken for 10 min in a 19 x 150 mm glass stoppered test tube. The resulting emulsion was centrifuged at 9230 x g for 15 min at 4 C and the aqueous top layer was removed and clarified by centrifugation at 9230 x g for 10 min at room temperature. The aqueous top layer was removed and placed in a 30 ml Corex centrifuge tube. Two volumes of 95% ethanol were pipetted slowly down the side of the tube and the precipitate was wound onto a glass rod, washed in 70% ethanol and resuspended in five ml of dilute saline citrate. The

dissolved precipitate was adjusted to 1 x SSC concentration by the addition of 0.5 ml of 10 x SSC. Acetate-EDTA was added to a final concentration of 10% (v/v) and, while rapidly stirring the solution with a glass rod, the DNA was precipitated by the dropwise addition of approximately 0.7 volumes of isopropanol. The precipitate was washed with 70% ethanol and resuspended in seven ml of 0.1 M Na_2SO_4. This solution was stored at 4 C in a screw cap test tube.

DNA Shearing

DNA extracted from OSB 431 was diluted to 50 µg/ml in 0.1 M Na_2SO_4. Using a Virtis "23" tissue homogenizer, ten ml of DNA solution were stirred at top speed for thirty minutes in a 50 ml homogenizer jar. Another ten ml sample was sheared in the same manner at a speed control setting of 60 on a rheostat scale of 0-100. The DNA solutions were held in an ice bath during the shearing process.

"100-1" sheared and "100-2" sheared refer to samples from two independently prepared DNA preparations which had each been separately sheared at top speed for 30 min. "60" sheared refers to a DNA sample that was sheared for 30 min at a rheostat setting of 60. The "100-1" sheared and the "60" sheared samples were both from the same DNA preparation.

DNA Molecular Weight Determination

The mean sedimentation coefficient, $S_{20,w}$ of the sheared DNA samples was determined by sedimentation velocity in a Spinco model E ultra-centrifuge at a concentration of 50 μg/ml in 0.1 M Na_2SO_4 at a speed of 40,000 RPM using U.V. scanner optics. The $S_{20,w}$ was corrected to infinite dilution by the relationship defined by Eigner, Schildkraut, and Doty (26).

The mean molecular weight of the sample was estimated using the equation described by Eigner and Doty (25).

Preparation of Hg-Cs_2SO_4 Gradients

Four ml Hg-Cs_2SO_4 gradients were prepared using stock solutions of the required components. Added to a 5/8" x 3" cellulose nitrate ultra-centrifuge tube in the order listed were: 1.92 ml of saturated Cs_2SO_4, 0.2 ml of 0.1 M $Na_2B_4O_7$ pH 9.2, 1.52 ml of DNA at 50 μg/ml, an appropriate volume of 1×10^{-3} $HgCl_2$ to achieve the proper Rf (molar ratio of Hg:Nucleotides) and an appropriate amount of distilled, deionized water. The contents were thoroughly mixed after each addition. Final concentrations of the gradient components were: Cs_2SO_4, 41.7% (w/w) $\rho = 1.4899$; $Na_2B_4O_7$, 0.005 M; DNA, 19 μg/ml; Rf of 0.19 or 0.22. The density of the gradient was measured by a refractometer and refractive index

was converted to density as described by Ludlum and Warner (53).

The solution was covered with mineral oil and centrifuged in a Spinco L2-65 ultracentrifuge at 36,000 RPM for 48 hr at 4 C using a #40 fixed angle rotor. Four drop fractions were collected into 0.3 ml of 1 M NaCl and the $OD_{260\ nm}$ was measured in a Ziess Spectrophotometer using 0.5 or 1.0 ml quartz cuvettes. The instrument was blanked against 1 M NaCl.

Fractions to be analyzed for transforming activity were dialyzed for 36 hr against three two-liter volumes of 1 M NaCl.

In vivo ^{3}H-5-Uridine and ^{14}C-L-Phenylalanine (U) Incorporation

In growing cultures of OSB 158, selected rifampin or streptolydigin resistant mutants, or selected temperature sensitive mutants, RNA and protein synthesis were monitored by incorporation of ^{3}H-5-Uridine and uniformly labelled ^{14}C-L-Phenylalanine. Routinely, an appropriate amount of SG medium plus cold carrier were inoculated to visible turbidity with log phase cells and incubated at 37 C on a rotary water bath shaker. When the culture turbidity reached 15 Klett units, the label was added. After 20 min, which was the empirically determined time required to achieve a constant incorporation rate, the culture was divided into other flasks which contained an appropriate drug or which were to be shaken at 50 C.

Duplicate one ml samples were withdrawn from the cultures at the indicated times and mixed with an equal volume of cold ten percent trichloroacetic acid (TCA). After at least four hr, the TCA precipitable material was collected on Millipore prefilters (AP 2002500) which had previously been soaked overnight in five percent TCA plus 500 μg/ml of cold carrier. The filtered material was washed exhaustively with cold five percent TCA and placed into glass scintillation vials. After the filters had been heated in a drying oven so that no residual TCA remained, ten ml of scintillation fluid was added to each vial and radioactivity was measured in a Nuclear-Chicago Liquid Scintillation Counter.

Preparation of Cell Free Extract

Five hundred ml of log phase cells were harvested by centrifugation at 7000 x g for six min and washed and resuspended in ten ml of buffer containing: $MgCl_2$, 0.01 M; KCl, 0.1 M; dithiothreotol, 10^{-4} M; Tris, 0.2 M pH 8.0; 10% glycerol. The resuspended cells were sonicated with a Bronwell III sonic-oscillator with the BP-111-12T probe. Twenty second bursts at 15 sec intervals for six min resulted in clearing of the cell suspension. The sonicated cells were centrifuged for three hr in a Spinco preparative ultra-centrifuge in a #30 rotor at 27,000 RPM at 4 C. RNA polymerase was assayed in the supernatant fraction.

A one-step partial purification of the crude extract was achieved by precipitation with 60% $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 9230 x g for 20 min at 4 C.

RNA Polymerase Assay

RNA polymerase was assayed according to the procedure of Harford and Sueoka (33) as modified by Leighton et al. (48). Each reaction mixture contained a total volume of 0.25 ml with the following ingredients in μ moles: KCl, 37.5; $MgCl_2$, 2.5; $MnCl_2$, 0.5; Tris-HCl, pH 7.9, 12.5; dithiothreotol, 0.1; UTP, 0.25; CTP, 0.25, GTP, 0.25; ATP, 0.25; UTP-^3H, 2 μc. Each reaction mixture also contained 10-40 μg of template DNA (105) which had either been dialyzed against 0.1 x SSC or dissolved in 0.1 x SSC before use. The mixture was incubated at the desired temperatures for ten min and precipitated with an equal volume of cold 10% TCA. Radioactivity was measured in a Packard Liquid Scintillation Counter. Specific activity is expressed as as nmoles of UMP incorporated/hour/mg of protein.

Growth Measurements of Bacterial Cultures

Growth measurements of bacterial cultures were made in SG medium contained in side-arm flasks by measuring culture turbidity with a Klett-Summerson colorimeter with a blue filter.

Measurement of Sporulation

Two fifty ml aliquots of SG or SSM sporulation medium were inoculated with one ml of late log phase cells. One of the aliquots contained rifampin, streptovaricin or streptolydigin and the other one did not. After 24 hr in SG medium or 48 hr in SSM medium, a three ml sample was withdrawn from each flask, and was heated at 80 C for 15 min, diluted and plated on TBAB to measure the number of heat resistant spores. At the same time, an unheated sample from each flask was diluted and plated on TBAB to measure the total number of viable cells.

At several times during the 24 or 48 hr growth and sporulation period, each culture was observed and evaluated both microscopically and macroscopically.

Measurement of the Sensitivity of Sporulating Cells to Rifampin and Streptolydigin

Three hundred ml of SG medium contained in a one liter side-arm flask were inoculated with a five percent inoculum of log phase OSB 158. The culture was placed at 37 C on a rotary shaker and at the indicated times, three nine ml samples and one, one ml sample were withdrawn. The nine ml samples were each placed into a separate 250 ml Erlenmeyer flask which contained either one ml of

spent SG medium or one ml of rifampin or streptolydigin dissolved in spent SG medium. The flasks were incubated at 37 C on a rotary shaker. The one ml sample was placed in a 16 x 125 mm tube and heated at 80 C for 15 min to kill vegetative cells. Dilutions were then plated on TBAB to measure the spores present at the times of sampling. Twenty-four hr after the first samples were taken, one ml samples from each of the flasks were heated at 80 C for 15 min and dilutions were plated on TBAB to measure the number of heat-resistant spores. Unheated samples from each flask were also diluted and plated on TBAB to measure the total number of viable cells.

The spent SG media used in this experiment were obtained from a 300 ml OSB 158 culture which had been inoculated in the same manner as was the above 300 ml culture. Spent medium samples were collected at times corresponding to the sampling times in the above experiment, e. g. cell samples collected one hr after inoculation were placed in flasks containing spent medium or drug plus spent medium that had been collected one hr after inoculation. Cells had been removed from the spent media by low speed centrifugation followed by filtration through a 0.22 nm Millipore membrane.

RESULTS

Mutagenesis

EMS mutagenesis of *B. subtilis* spores was used to isolate single point rifampin resistant, streptolydigin resistant, or conditional mutants in which a single step mutation conferred both drug resistance and temperature sensitivity. A number of rifampin resistant-temperature sensitive mutants had already been isolated by L. R.. Brown (8) and T. Leighton (47) using a similar technique, so only streptolydigin resistant mutants were tested for temperature sensitivity in this study. As shown in Table 2, large numbers of rifampin resistant and streptolydigin resistant mutants were isolated. The spontaneous mutation rate was 6.2×10^{-7} for streptolydigin resistance and 7.7×10^{-7} for rifampin resistance. The induced mutation rate by 20 min exposure to EMS was 5.9×10^{-6} for streptolydigin resistance and 8.7×10^{-6} for rifampin resistance, and there was no loss in viability after 20 min exposure to EMS.

In another experiment, attempts to recover mutants resistant to both Rif and Std at spore concentrations on the membrane up to 1×10^{11} were not successful. Increased concentrations of EMS or longer exposure to EMS yielded lower survival rates without appreciably increasing the recovery of drug resistant mutants.

L. R. Brown (8). A representative sample of mutants was picked for further study.

Table 2. Induction of rifampin resistant and streptolydigin resistant mutants by EMS mutagenesis.

Length of EMS Exposure (min)	Mutants per ml		
	Std^R	Rif^R	Std^R Rif^R
0	80	100	0
3	140	160	0
5	160	190	0
10	220	240	0
13	310	430	0
16	350	940	0
20	840	1200	0

Viable Count: Control 1.3×10^8
20 min EMS 1.3×10^8

Transductional and Transformational Analysis

As described earlier, resistance to rifampin and streptovaricin has been shown to be linked by co-transformation and co-transduction to the *cys* A14 locus in *B. subtilis* (9, 33). Resistance to streptolydigin in *E. coli* has been linked by co-transduction to rifampin resistance (87), and evidence was available that the loci for rifampin resistance and streptolydigin resistance were located in the DNA segment coding for the β subunit of RNA polymerase in *E. coli* (35

43). Thus, rifampin resistance and streptolydigin resistance might be linked by co-transduction in _B. subtilis_. To examine this possibility, two and three factor crosses were conducted using PBS1 mediated transduction. The data in Tables 3 and 4 confirm that streptolydigin resistance is linked to both rifampin resistance and the _cys_ A14 locus by co-transduction. Three factor crosses indicated that _cys_ A14 is an outside marker but could not differentiate between the _cys_ A14 Rif^R Std^R or _cys_ A14 Std^R Rif^R marker orders. It is apparent that within the genetic distances involved in these experiments, there is a bias towards incorporation of all the donor markers. Due to its greater resolving power, transformation was used to analyze further this small region of the _B. subtilis_ chromosome.

The primary reference marker used in these experiments, _cys_ A14, behaved as a complex locus in transformation experiments. Harford and Sueoka (33) reported two classes of Cys^+ transformants when selection was made on MG agar. The first class formed large, readily observable colonies. Upon close inspection, however, a second class of very minute colonies was also observed and represented 60-80% of the total transformants. Normal development of the minute colonies was achieved by supplementing the MG agar with a mixture of nine enhancing amino acids described by Wilson and Bott (102). DNA from the minute colony class yielded only minute

Table 3. Two-factor transductional analysis.

Donor		Recipient				Recombinant Classes		
Strain Designation	phenotype	Strain Designation	phenotype	Selected Marker	Total Colonies Tested	11[a]	10	% Co-transduction
OSB 17	Cys^+ Rif^R	OSB 154	Cys^- Rif^s	Cys^+	208	186	22	89.4
OSB 2	Cys^+ Rif^R	OSB 154	Cys^- Rif^s	Cys^+	175	162	13	92.5
OSB 404	Cys^+ Std^R	OSB 154	Cys^- Std^s	Cys^+	632	584	48	91.8

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

Table 4. Three-factor transductional analysis.

Donor		Recipient				Recombinant Classes						
Strain Designation	phenotype	Strain Designation	phenotype	Selected Marker	Total Colonies Tested	111[a]	110	100	101	010	011	001
OSB 439	$Cys^+Rif^RStd^R$	OSB 154	$Cys^-Rif^SStd^S$	Cys^+	512	475	6	38	1	-	-	-
				Rif^R	457	375	1	-	-	7	74	-
				Std^R	486	406	-	-	1	-	51	28
OSB 440	$Cys^+Rif^RStd^R$	OSB 154	$Cys^-Rif^SStd^S$	Cys^+	520	469	3	47	1	-	-	-
				Rif^R	472	434	1	-	-	2	35	-
				Std^R	521	474	-	-	0	-	30	17
OSB 406	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	798	421	343	29	5	-	-	-
				Std^R	208	199	-	-	1	-	4	4
OSB 400	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	658	454	165	33	5	-	-	-
				Std^R	510	486	-	-	17	-	14	3
OSB 401	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	785	392	358	30	5	-	-	-
				Std^R	260	254	-	-	1	-	2	3

Table 4. Continued.

Donor Strain Designation	Donor phenotype	Recipient Strain Designation	Recipient phenotype	Selected Marker	Total Colonies Tested	Recombinant Classes 111[a]	110	100	101	010	011	001
OSB 402	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	510	328	147	30	5	-	-	-
				Std^R	161	145	-	-	4	-	5	7
OSB 403	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	850	404	414	30	2	-	-	-
				Std^R	260	253	-	-	1	-	4	2
OSB 404	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	1029	606	373	47	5	-	-	-
				Std^R	506	400	-	-	4	-	5	7
OSB 405	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	521	336	159	23	3	-	-	-
				Std^R	156	153	-	-	0	-	1	2
OSB 407	$Cys^+Rif^SStd^R$	OSB 3	$Cys^-Rif^RStd^S$	Cys^+	846	261	548	36	1	-	-	-
				Std^R	208	207	-	-	1	-	0	0

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

transformants while DNA from the large colony class yielded both large and minute transformants. They also reported that no spontaneous Cys^+ revertants were ever observed from the _cys_ $A14^-$ carrying strain.

In our system, the _cys_ A14 locus, carried by OSB 154, behaved in a similar manner. The minute colony class comprised over 90% of the total yield of transformants recovered on MG agar and did not require the amino acid supplement for normal development. On the amino acid supplemented plates the two classes of transformants were still readily distinguishable in that the large colony class produced larger and more opaque colonies than did the minute colony class. Of 178 large colony Cys^+ transformants examined from various Rif^R x _cys_ $A14^-$ genetic crosses, 39 were rifampin resistant for a co-transformation frequency of 21.9%. Of 164 large colony Cys^+ transformants examined from various Std^R x _cys_ $A14^-$ genetic crosses, 17 were streptolydigin resistant for a co-transformation frequency of 10.4%. No spontaneous Cys^+ revertants were ever observed from OSB 154. In addition, we report some other interesting behavior concerning the OSB 154 strain.

Mapping studies with OSB 427, a rifampin resistant-temperature sensitive strain, indicated that rifampin resistance and temperature sensitivity in that strain were linked by 18% co-transformation when OSB 158 rifampin resistant recombinants were scored for

temperature sensitivity. However, when OSB 154 was the recipient strain and OSB 427 was again the donor strain, no temperature sensitive recombinants were recovered. Instead, when either Cys^+ or Rif^R transformants were scored at 50 C for temperature sensitivity, two easily distinguishable colony types were observed. The most numerous class displayed normal colony morphology. However, the second recombinant class yielded much smaller colonies whose phenotype will be referred to as pseudo-temperature sensitive (pTs). Rifampin resistance-pseudo-temperature sensitivity linkage by co-transformation was 19.4% which was consistant with the rifampin resistance-temperature sensitivity co-transformation frequency observed when OSB 158 was the recipient.

When the _cys_ A14 locus was transferred into a _B. subtilis_ strain Marburg chromosome, the new strain, OSB 192, exhibited some new properties. Cys^+ transformants of this strain were no longer of two classes and the amino acid supplement in the MG agar was no longer required for normal development of Cys^+ transformants. The colony morphology of this strain was very much like that of the recipient parent and spontaneous Cys^+ revertants could now be recovered from minimal agar plates. In addition, when OSB 192 was the recipient in a transformation experiment with OSB 427 as donor, the rifampin resistant-temperature sensitive recombinants no longer exhibited the pseudo-temperature sensitive phenotype.

Rifampin resistance linkage of various strains by co-transformation with the cys locus of OSB 192 was essentially the same as that found for the cys A14 locus of OSB 154 when it was the recipient strain (Table 5).

Transformation experiments were conducted to determine the relative location on the B. subtilis chromosome of the mutation(s) from which the Rif^R, Stv^R, Std^R, and Ts phenotypes result. As indicated in Table 6, when Cys^+ transformants were selected, co-transformation frequencies of rifampin resistance with the cys A14 locus varied from 21.7 to 36.1%. However, when recombinants were selected on rifampin, the Cys^+ Rif^R co-transformation frequencies varied from 10.6 to 19.8%. The one streptovaricin resistant strain tested behaved in a similar manner. The data in Table 7 show that co-transformation frequencies of streptolydigin resistance with the cys A14 locus vary from 8.9 to 23.6% when Cys^+ transformants were selected. However, when recombinants were selected on streptolydigin, the co-transformation frequencies dropped to a range of 5.4 to 14.3%.

The two-factor transformation data indicate that the mutation(s) that confer resistance to rifampin reside much closer to the cys A14 locus than do the mutation(s) conferring resistance to streptolydigin. The one streptovaricin resistant strain tested by two-factor transformation mapped as closely to the cys A14 locus as did the

Table 5. Genetic crosses comparing OSB 154 and OSB 192.

	Total Tested	Selected Marker	% Co-transformation	
			$Cys^{+}Rif^{R}$	$Rif^{R}Ts$
OSB 427 x OSB 154	856	Rif^{R}	12.7	21.3
OSB 427 x OSB 192	208	Rif^{R}	9.6	20.7
OSB 420 x OSB 154	416	Rif^{R}	17.6	-
OSB 420 x OSB 192	104	Rif^{R}	11.5	-
OSB 423 x OSB 154	416	Rif^{R}	13.9	-
OSB 423 x OSB 192	104	Rif^{R}	19.2	-
OSB 424 x OSB 154	406	Rif^{R}	18.2	-
OSB 424 x OSB 192	104	Rif^{R}	14.4	-
OSB 429 x OSB 192	416	Cys^{+}	31.3	-
OSB 429 x OSB 192	416	Rif^{R}	14.2	-

Table 6. Two-factor transformational analysis of rifampin resistant mutants.

Donor		Recipient		Selected Marker	Total Colonies Tested	Recombinant Classes			% Co-transformation
Strain Designation	phenotype	Strain Designation	phenotype			11[a]	10	01	
OSB 10	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	780	234	546	-	30.0
				Rif^{R}	416	64	-	348	15.4
OSB 420	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	435	157	278	-	36.1
				Rif^{R}	416	74	-	342	17.8
OSB 421	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	364	91	273	-	25.0
				Rif^{R}	416	48	-	368	11.5
OSB 422	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	364	86	278	-	23.6
				Rif^{R}	416	44	-	372	10.6
OSB 423	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	447	110	337	-	24.6
				Rif^{R}	416	58	-	358	13.9
OSB 424	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	412	104	308	-	25.2
				Rif^{R}	406	74	-	328	18.2
OSB 425	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Rif^{R}	364	70	-	294	19.3
OSB 426	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	717	156	561	-	21.7
				Rif^{R}	780	154	-	626	19.8
OSB 427	$Rif^{R}Ts$	OSB 158	$Rif^{S}Ts$	Rif^{R}	572	105	-	467	18.4
OSB 428	$Cys^{+}Rif^{R}$	OSB 154	$Cys^{-}Rif^{S}$	Cys^{+}	416	88	328	-	21.1
				Rif^{R}	416	46	-	370	11.1

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

Table 7. Two-factor transformational analysis of streptolydigin resistant mutants.

Donor		Recipient				Recombinant Classes			
Strain Designation	phenotype	Strain Designation	phenotype	Selected Marker	Total Colonies Tested	11[a/]	10	01	% Co-transformation
OSB 400	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	98	318	-	23.6
OSB 401	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	83	333	-	20.0
OSB 402	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	76	340	-	18.3
OSB 403	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	91	325	-	21.9
OSB 404	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	832	152	680	-	18.3
				Std^{R}	654	74	-	580	11.2
OSB 405	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	389	79	310	-	16.9
				Std^{R}	501	55	-	446	9.9
OSB 406	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	85	331	-	20.4
OSB 407	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	91	325	-	21.9
OSB 408	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	312	38	274	-	12.1
				Std^{R}	260	14	-	246	5.4
OSB 409	$Cys^{+}Std^{R}$	OSB 154	$Cys^{-}Std^{S}$	Cys^{+}	416	52	364	-	12.5
				Std^{R}	416	33	-	383	7.9

Table 7. Continued.

Donor Strain Designation	Donor phenotype	Recipient Strain Designation	Recipient phenotype	Selected Marker	Total Colonies Tested	Recombinant Classes 11[a]	10	01	% Co-transformation
OSB 410	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	292	27	265	-	9.3
				Std^R	346	32	-	314	9.2
OSB 412	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	404	80	324	-	19.8
				Std^R	398	32	-	366	8.0
OSB 413	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	398	46	352	-	11.9
				Std^R	416	46	-	370	11.1
OSB 414	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	416	63	353	-	15.1
				Std^R	416	49	-	367	11.8
OSB 415	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	416	37	379	-	8.9
				Std^R	364	22	-	342	6.0
OSB 416	Cys^+Std^R	OSB 154	Cys^-Std^S	Cys^+	416	55	361	-	13.2
				Std^R	416	46	-	370	11.1

Table 7. Continued

Donor		Recipient				Recombinant Classes			
Strain Designation	phenotype	Strain Designation	phenotype	Selected Marker	Total Colonies Tested	11[a]	10	01	% Co-transformation
OSB 417	$Cys^+ Std^R Ts$	OSB 154	$Cys^- Std^S$	Cys^+	416	54	362	-	12.9
				Std^R	416	56	-	360	13.5
OSB 419	$Cys^+ Std^R Ts$	OSB 154	$Cys^- Std^S$	Cys^+	859	142	717	-	16.5
				Std^R	562	75	487	-	13.3

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

rifampin resistant strains. Among the rifampin resistant strains tested, the co-transformation frequency was twice as great when Cys^+ transformants were selected as when rifampin resistant transformants were selected. This non-reciprocality was also present but was not nearly so pronounced among the streptolydigin resistant strains.

Drug resistant- temperature sensitive strains were genetically analyzed by transformation to determine if a single step mutation had conferred both drug resistance and temperature sensitivity. If the phenotype was the result of a single mutation, then drug resistance and temperature sensitivity should be co-transformed 100% of the time. Of the six strains examined, only two strains displayed any linkage of drug resistance and temperature sensitivity by co-transformation. OSB 427 showed an 18% rifampin resistance-temperature sensitivity co-transformation frequency and OSB 418 demonstrated a co-transformation frequency of 51.9% for streptolydigin resistance and temperature sensitivity. These data indicate that the drug resistant phenotype and the temperature sensitive phenotype are separable by genetic manipulation.

Even though the two temperature sensitive mutations co-transform with the *cys* locus at almost identical frequencies, they are probably two different mutations since one mutant yields pseudo-temperature sensitive recombinants from OSB 154 and the other one

yields true temperature sensitive recombinants. If the two mutations are identical, then no thermal stable recombinants should result at 50 C from an OSB 427 x OSB 418 genetic cross. However, if they are separate mutations, then thermal stable recombinants should be recovered at 50 C. As the data in Table 8 indicate, thermal stable recombinants were recovered when selected at 50 C. When transformants from this cross were selected on rifampin at 37 C, the rifampin resistant-thermal stable classes were diminished in number compared to the usual recovery of these classes. Since OSB 427 had never attained the competent state, the reciprocal of the above cross was not conducted.

Appropriate three factor crosses were performed to determine the probable sequence of markers which yield the Cys^-, Rif^R, Stv^R, Std^R, and Ts phenotypes. In Table 9 the markers are listed in the most probable order as determined by two factor crosses. The double recombinant class is always represented in the "101" column. In all cases, the data clearly confirm the suggested order. The marker order indicated by the three factor data is cys A14 Rif^R $(Stv)^R$ Std^R Ts_{418} (Ts_{427}). Rifampin resistance and streptovaricin resistance could not be ordered by three factor crosses because all rifampin resistant strains selected on rifampin were also resistant to streptovaricin. However, two thirds of the streptovaracin resistant strains that were originally selected on streptovaricin were

Table 8. Comparison of recombinant classes resulting from an OSB427 x OSB418 genetic cross and an OSB420 x OSB418 genetic cross.

Recombinant Classes Selected at 50 C	OSB427 x OSB418	OSB420 x OSB418
$Rif^R Std^R +$	0	10
$Rif^R Std^S +$	2	66
$Rif^S Std^R +$	47	139
$Rif^S Std^S +$	3	39

Recombinant Classes Selected on Rifampin	OSB427 x OSB418	OSB420 x OSB418
$Rif^R Std^R +$	7	3
$Rif^R Std^R Ts$	214	176
$Rif^R Std^S +$	0	34
$Rif^R Std^S Ts$	83	47

Table 9. Three-factor transformational analysis.

	Selected Marker	Total Recombinants Tested	Recombinant Classes						
			111[a]	110	101	100	010	011	001
Cross Ordering: Cys^{-}-Rif^{R}-Std^{R}									
OSB 440 x OSB 154	Cys^{+}	406	51	30	9	316	-	-	-
	Std^{R}	306	51	-	8	-	-	149	98
Cross Ordering: Cys^{-}-Rif^{R}-Ts_{427}									
OSB 427 x OSB 154	Cys^{+}	558	26	124	11	396	-	-	-
	Rif^{R}	852	19	78	-	-	564	147	-
Cross Ordering: Cys^{-}-Std^{R}-Ts_{418}									
OSB 418 x OSB 154	Cys^{+}	406	16	32	6	352	-	-	-
	Std^{R}	416	20	21	-	-	162	213	-
OSB 154 x OSB 418	50 C	416	28	-	5	-	-	193	190

Table 9. Continued.

	Selected Marker	Total Recombinants Tested	Recombinant Classes						
			111[a]	110	101	100	010	011	001
Cross Ordering:									
Stv-Std-Ts_{418}									
OSB 114 x OSB 418	Stv^R	260	37	178	3	42	-	-	-
	50 C	312	100	-	5	-	-	65	142
OSB 122 x OSB 418	Stv^R	312	90	81	2	139	-	-	-
	50 C	234	56	-	9	-	-	50	119
Cross Ordering:									
Rif^R-Std^R Ts_{418}									
OSB 420 x OSB 418	Rif^R	260	34	47	3	176	-	-	-
	50 C	254	66	-	10	-	-	39	139
OSB 423 x OSB 418	Rif^R	207	46	32	7	122	-	-	-
	50 C	208	95	-	5	-	-	33	75

[a] "1" and "0" refer to donor and recipient phenotypes, respectively.

resistant to rifampin (9). Although the Ts mutations cannot be definitely ordered with the existing mapping data, analysis of the recombinant classes that resulted from the OSB 427 x OSB 418 cross did suggest that Ts_{427} was the outside marker.

DNA Shearing

Genetic analysis has shown that the rifampin resistant, streptolydigin resistant and temperature sensitive phenotypes are the result of different classes of mutations. If the phenotypes are, in fact, the result of physically separated mutations on the chromosome, then it should be possible to separate the markers by shearing the DNA. Separation of the markers should be evident by a drop in co-transforming activity (28, 92). It can also be predicted that the percent co-transformation of the more closely linked markers will be decreased less by shearing than will that of the more widely separated markers. Since the mapping data indicate that the markers producing the rifampin resistant and the temperature sensitive phenotypes are more widely separated than are those producing the streptolydigin resistant and the temperature sensitive phenotypes or the rifampin resistant and streptolydigin resistant phenotypes, then rifampin resistance-temperature-sensitivity linkage by co-transformation should show the greatest decrease.

To test these predictions, co-transformation of the rifampin

resistant, streptolydigin resistant and temperature sensitive phenotypes was measured in DNA preparations of OSB 431 that had been extensively sheared.

The data in Table 10 indicate that with a decrease in molecular weight, there is a decrease in the co-transformation of the phenotypes and that the rifampin resistance-temperature sensitivity linkage by co-transformation is decreased more than that of rifampin resistance-streptolydigin resistance or streptolydigin resistance-temperature sensitivity.

Although the mapping data indicate that the mutations resulting in the rifampin resistant and the streptolydigin resistant phenotypes are more widely separated than those resulting in the streptolydigin resistant and temperature sensitive phenotypes, this prediction was not confirmed by the shearing data.

The total transforming activity of the DNA preparations, as measured by streptolydigin resistance transforming ability, was decreased by over 99% in both the "60" and "100" sheared preparations.

Molecular weight determinations were made on the sheared DNA preparations. $S_{20,w}$ values were calculated at various points along the boundary of the sedimenting material from a U V absorbtion scan taken early in the centrifuge run and from a U V absorbtion scan taken late in the centrifuge run. These $S_{20,w}$ values were

Table 10. Effect of shearing on co-transformation frequencies of rifampin resistance, streptolydigin resistance, and temperature sensitivity.

Unsheared OSB 431 DNA	% Decrease in Total Transforming Activity	% Co-transformation	% Decrease in Co-transformation	Mean $S_{20,w}$
$Rif^R Std^R$		31.9	-	
$Rif^R Ts$	-	16.5	-	26.0*
$Std^R Ts$		24.7	-	
"60" Sheared OSB 431 DNA				
$Rif^R Std^R$		8.5	73.4	
$Rif^R Ts$	99.91	3.1	81.9	11.1
$Std^R Ts$		9.6	61.1	
"100-2" Sheared OSB 431 DNA				
$Rif^R Std^R$		1.7	94.7	
$Rif^R Ts$	99.98	0.22	98.7	10.1
$Std^R Ts$		0.99	96.0	

*Estimation from Marmur (59).

then plotted against the percent of the total OD present in the cell at the point at which the $S_{20,w}$ value was calculated. In this way, the range of molecular weights of the molecular species present can be estimated and the extent of diffusion of the boundary can be measured (78).

As shown in Figures 1, 2 and 3, each sheared preparation represents a polydisperse system. Comparison of early and late U.V. absorption scans from each DNA preparation showed that there was only minimal diffusion indicating that the calculated $S_{20,w}$ values weregood estimates of the size of the molecules present in the DNA preparations.

About 80% of the U.V. absorbing material in the most highly sheared preparations could be described by $S_{20,w}$ values between 6 and 12. Ten percent of the U.V. absorbing material had $S_{20,w}$ values less than 6 and 10% had values over 12. In the "60" sheared preparation, 80% of the U. V. absorbing material had $S_{20,w}$ values between 8 and 13, 10% had $S_{20,w}$ values below 8, and 10% were greater than 13. Therefore, 80% of the U.V. absorbing material in the most highly sheared preparations had a molecular weight between 1.9×10^5 and 4.0×10^6 daltons. Eighty percent of the U.V. absorbing material in the "60" sheared preparation had a molecular weight between 4.6×10^5 and 5.8×10^6 daltons.

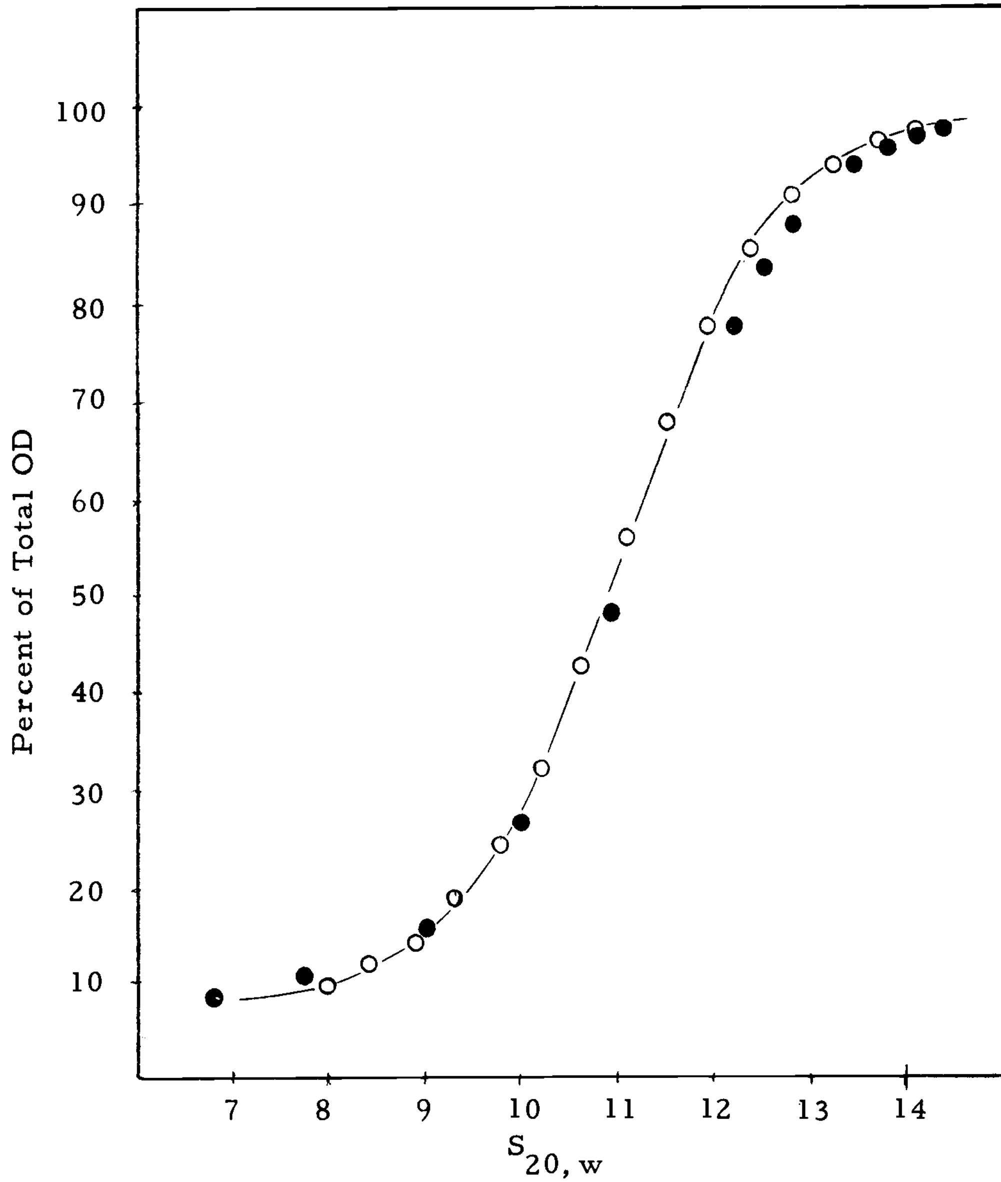

Figure 1. Distribution curves of sedimenting DNA from the "60" sheared preparation. UV scans were taken at 16 min ●--● , and at 76 min o--o .

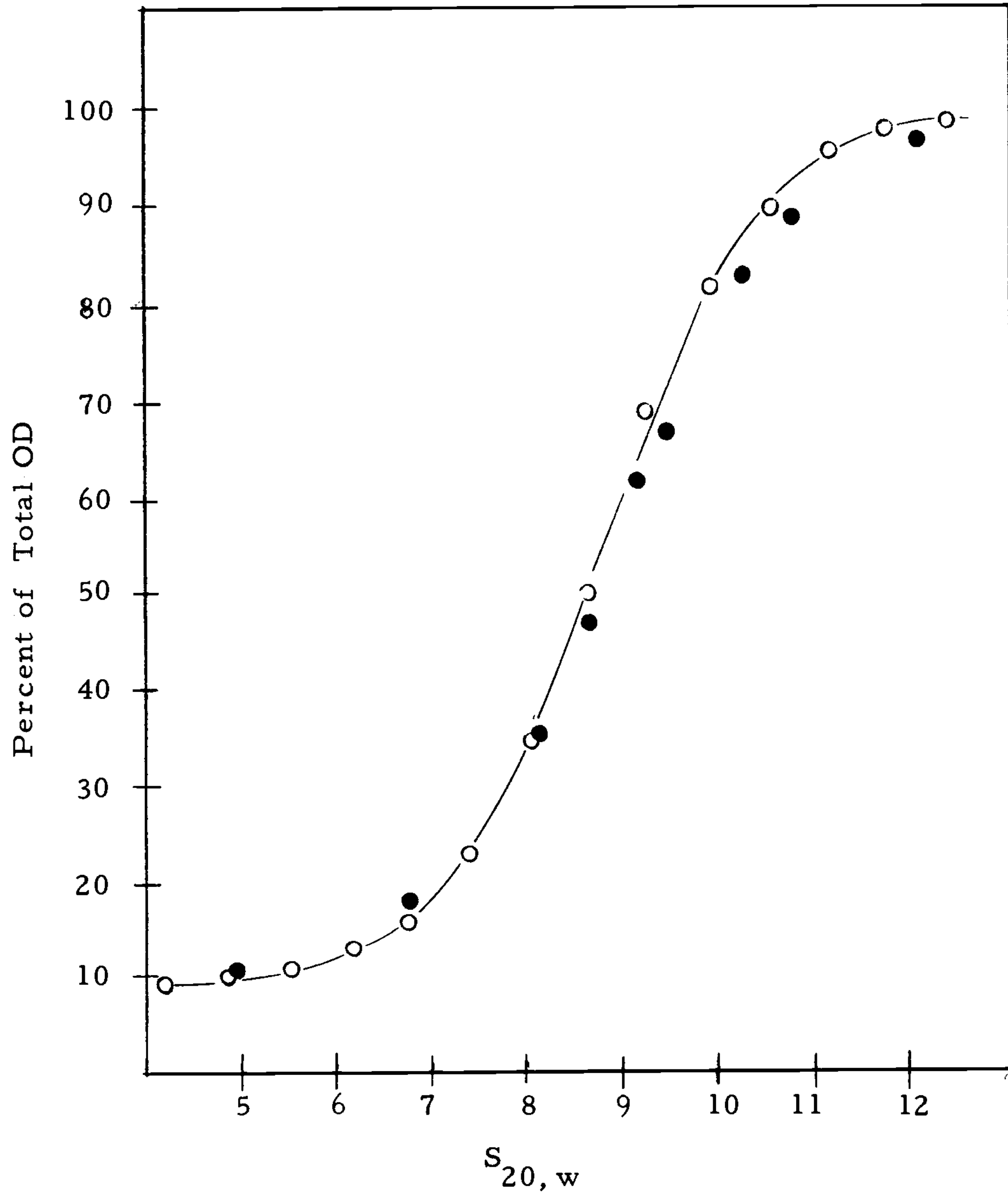

Figure 2. Distribution curves of sedimenting DNA from the "100-1" sheared preparation. UV scans were taken at 20 min ●--● , and at 52 min ○--○ .

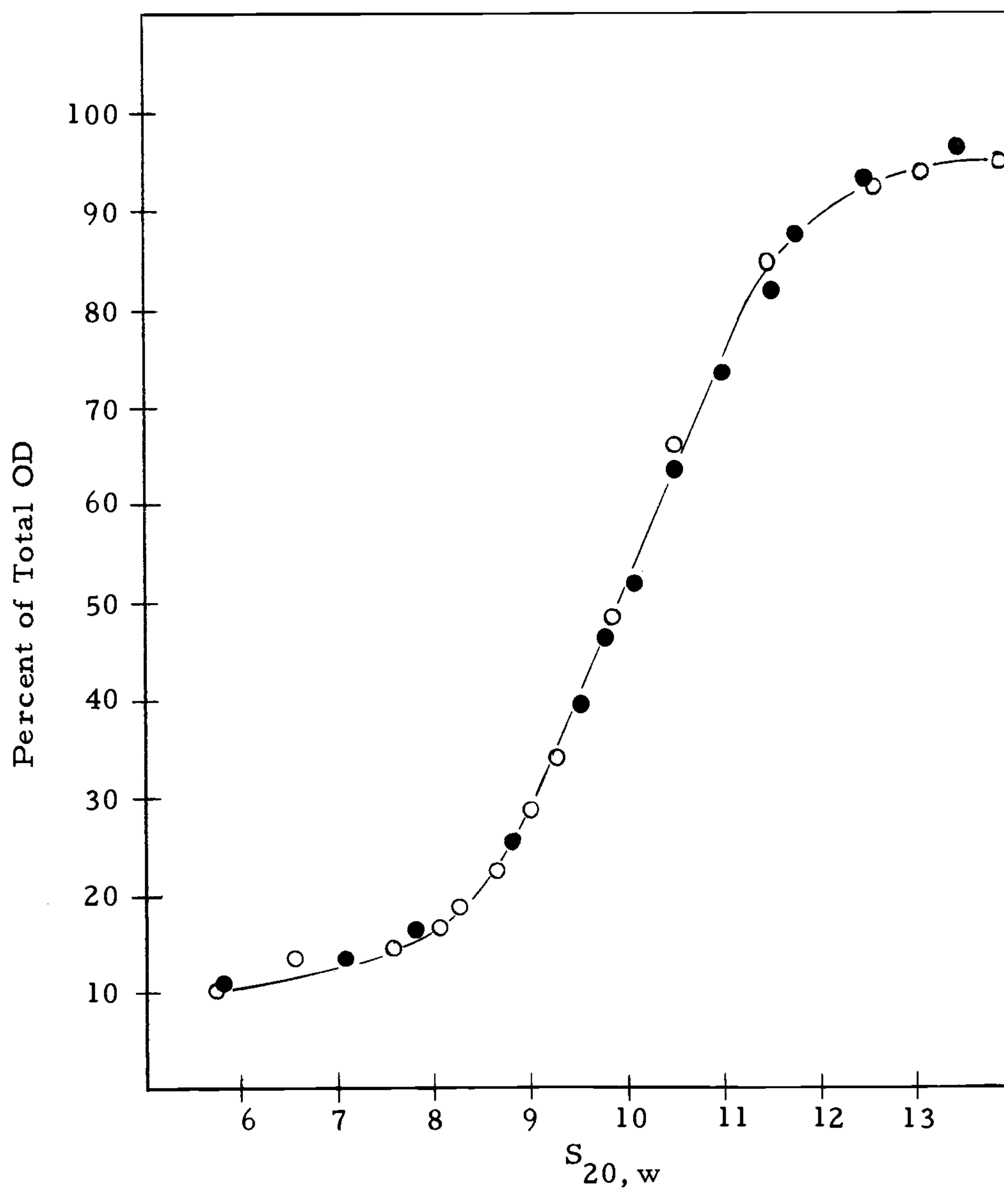

Figure 3. Distribution curves of sedimenting DNA from the "100-2" sheared preparation. UV scans were taken at 22 min ●--● , and at 56 min ○--○ .

Enrichment of Genetic Regions Responsible for Rifampin Resistant and Streptolydigin Resistant Phenotypes by Hg-Cs_2SO_4 Centrifugation

The region of the B. subtilis chromosome from which at least some of the RNA polymerase mutant phenotypes arise is of higher G + C content than is the bulk of the chromosome (97). Since Hg^{++} ions preferentially bind to A + T rich regions of the chromosome (62), a significant enrichment of the RNA polymerase genes should occur if a sheared preparation of B. subtilis DNA is centrifuged to equilibrium in a Hg-Cs_2SO_4 gradient. Since the shearing data in the previous section clearly indicate that the markers resulting in the Rif^R, Std^R and Ts phenotypes can be physically separated, the peaks of transforming activity for each of these markers should be contained in different fractions of a Hg-Cs_2SO_4 gradient if the DNA fragments on which they reside differ significantly in G + C content. The location of the rifampin resistance and streptolydigin resistance transforming activities should be easily monitored by measuring the transforming activity in selected fractions from the gradient. The fractions containing temperature sensitivity transforming activity cannot be measured due to the absence of a method with which to select for temperature sensitive transformants.

To test these predictions, sheared OSB 431 DNA was

centrifuged to equilibrium in a Hg-Cs_2SO_4 gradient and the rifampin resistance and the streptolydigin resistance transforming activity was measured in selected fractions. Figures 4 and 5 indicate that significant enrichment for rifampin resistance transforming activity was obtained by raising the Rf from 0.19 to 0.22 without changing the Cs_2SO_4 content of the gradients. Rifampin resistance and streptolydigin resistance transforming peaks were shifted to the heavier side of the A_{260} profile, and were separated slightly under the conditions employed (Figure 6 and 7).

Cys^+ transforming activity was also analyzed in both gradients and in each, two widely separated peaks were observed. Only 1 out of 308 Cys^+ transformants was also resistant to rifampin. No Cys^+ transformants were resistant to streptolydigin or displayed any temperature sensitivity.

In the R_f 0.22 gradient (Figure 7) one fraction which coincided with the peak of the A_{260} profile was observed which contained extremely high transforming activity for all three markers. The reason for this peak, which was not present in the R_f 0.19 gradient is unknown.

<u>In vivo</u> and <u>In vitro</u> Analysis of RNA and Protein Synthesis

To confirm that the phenotypes of the drug resistant mutants weredue to resistant RNA polymerases, synthesis of RNA and protein

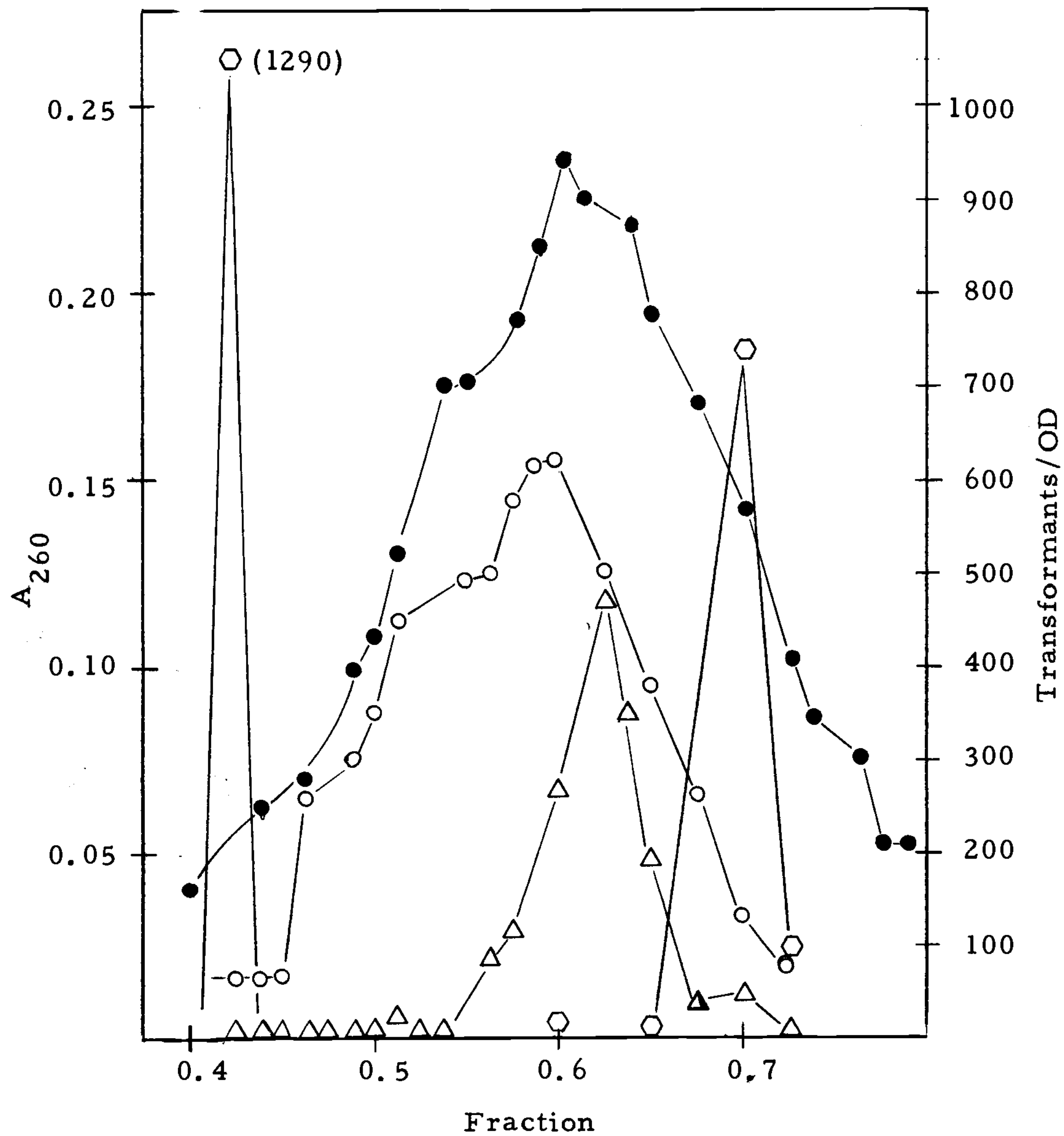

Figure 4. Transformants/OD contained in selected fractions of a Hg-Cs_2SO_4 gradient (R_f = 0.19). DNA was from the "100-2" sheared preparation. ●--●, A_{260}; ○--○, rifampin resistant; △--△, streptolydigin resistant; ⬡--⬡, Cys^+.

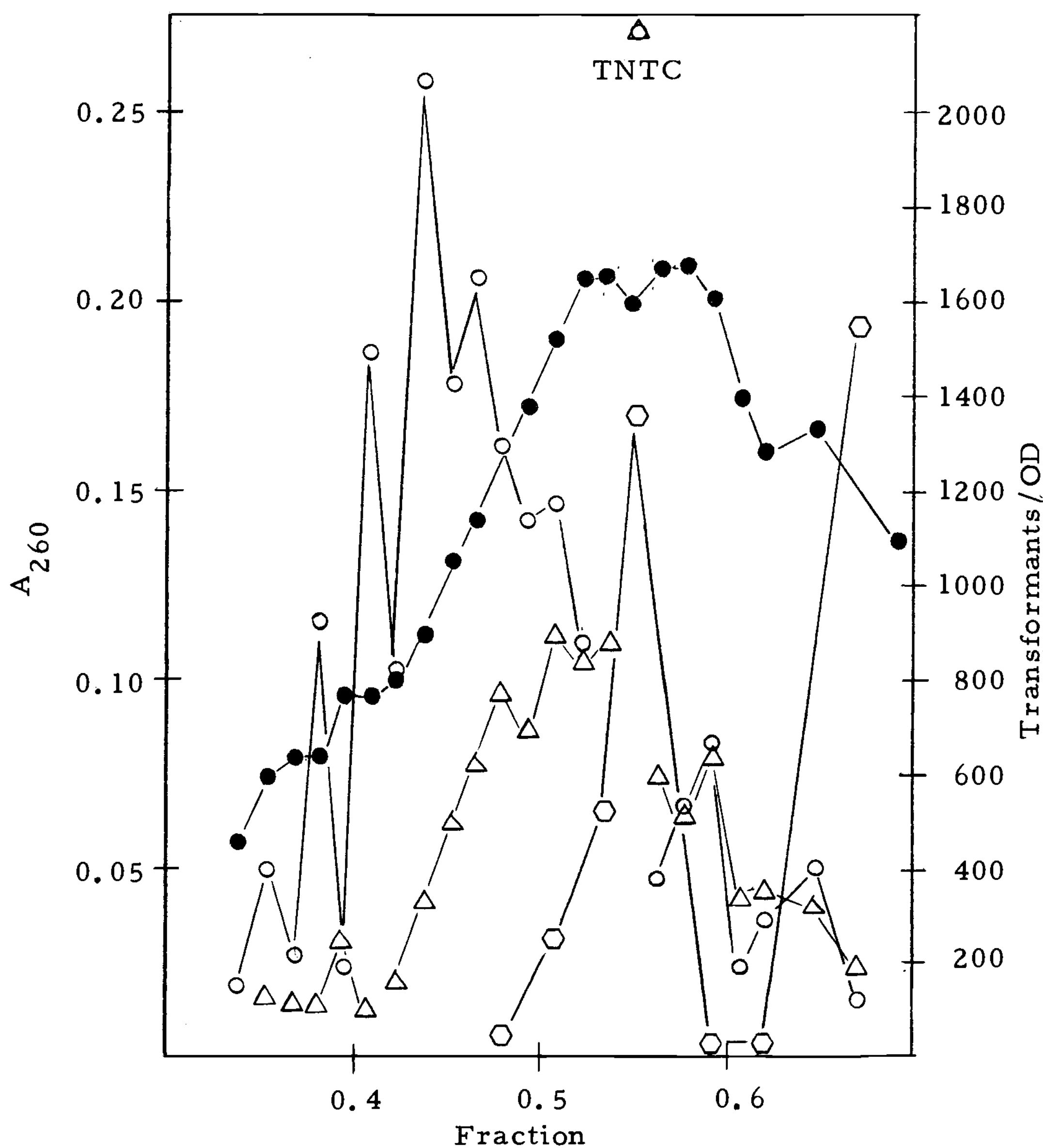

Figure 5. Transformants/OD contained in selected fractions of a Hg-Cs_2SO_4 gradient (R_f = 0.22). DNA was from the "100-2" sheared preparation. ●--●, A_{260}; ○--○, rifampin resistant; △--△, streptolydigin resistant; ⬡--⬡, Cys^+.

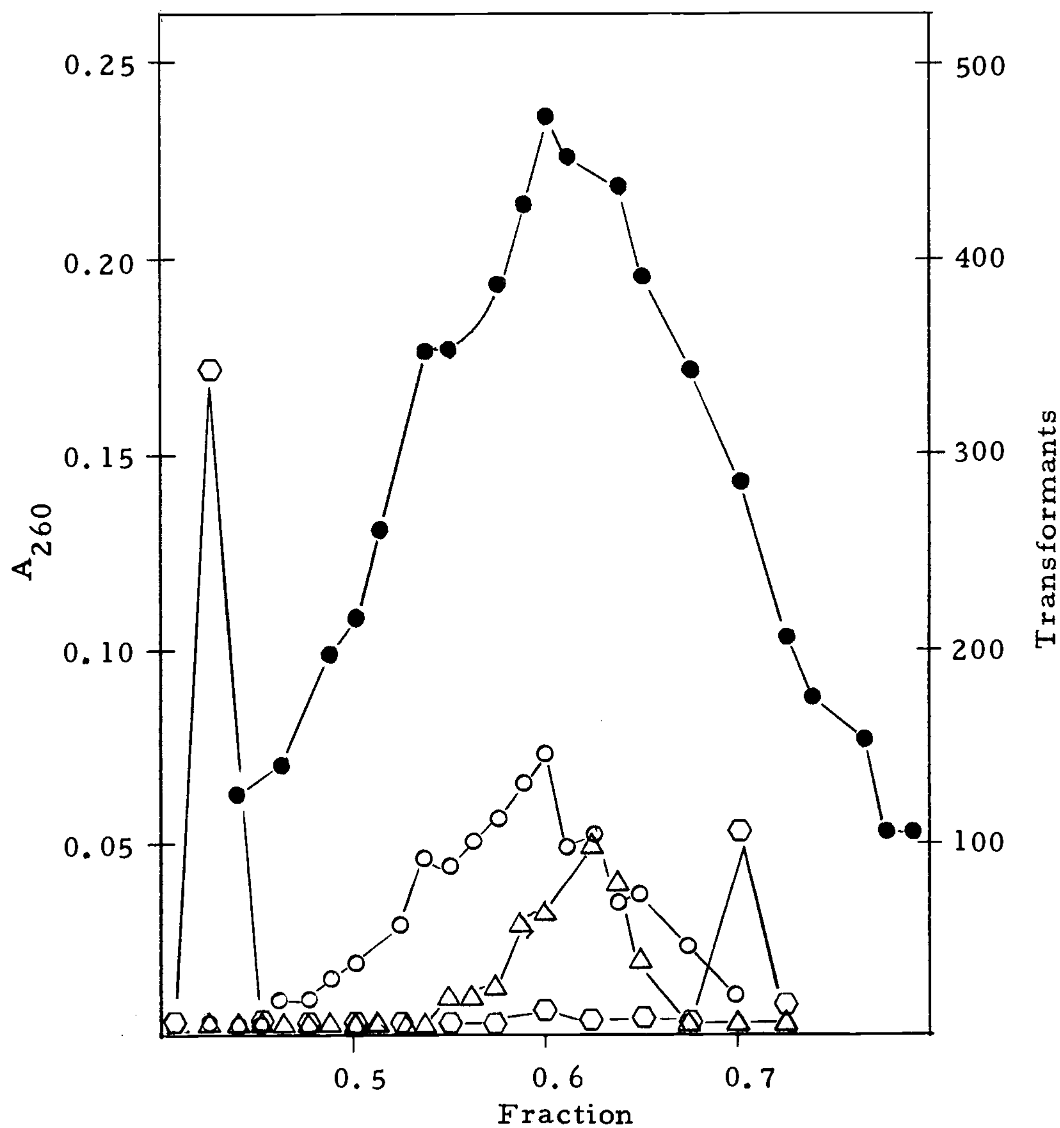

Figure 6. Transforming activity distribution in a Hg-Cs_2SO_4 gradient (R_f = 0.19). DNA was from the "100-2" sheared preparation. ●--●, A_{260}; ○--○, rifampin resistance; △--△, streptolydigin resistance; ⬡--⬡, Cys^+.

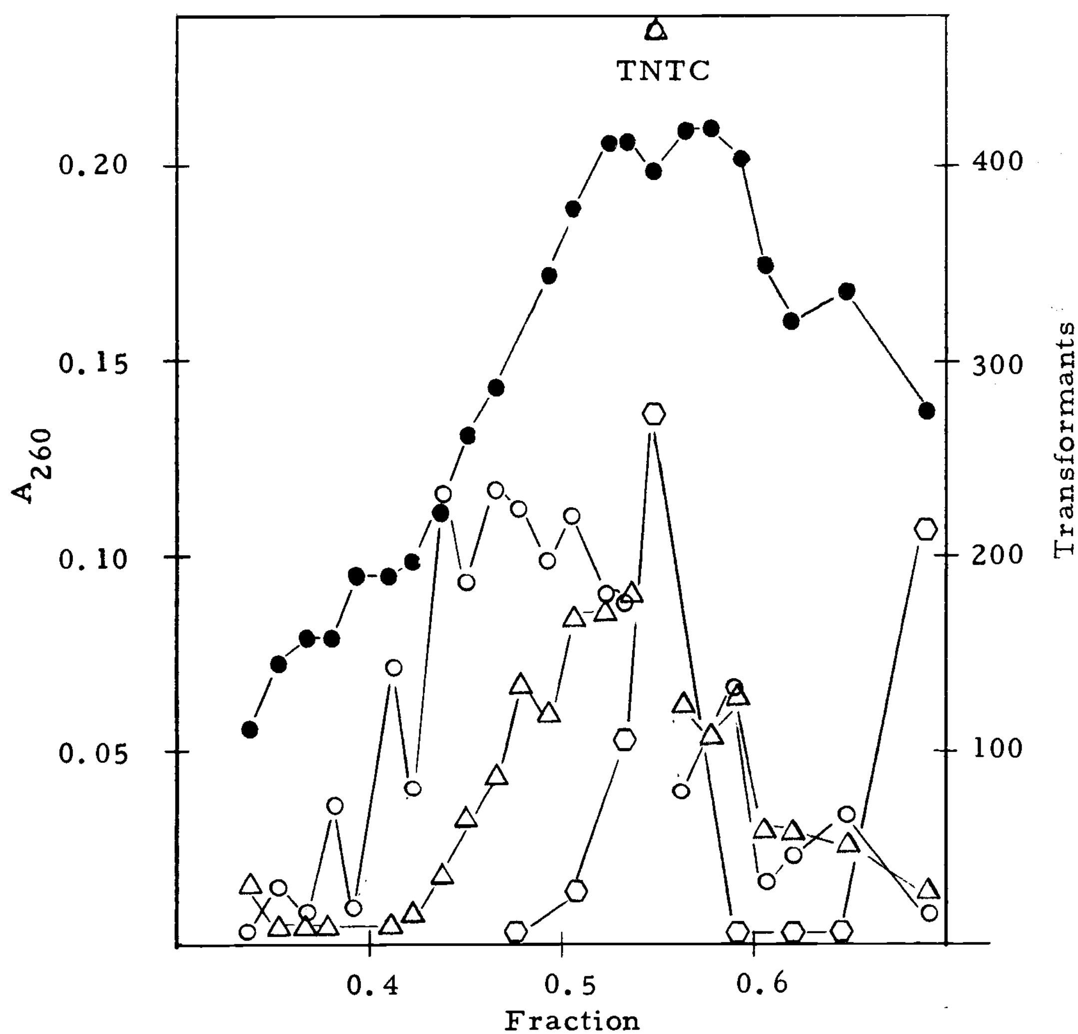

Figure 7. Transforming activity distribution in a Hg-Cs_2SO_4 gradient (R_f = 0.22). DNA was from the "100-2" sheared preparation. ●--●, A_{260}; ○--○, rifampin resistance; △--△, streptolydigin resistance; ⬡--⬡, Cys^+.

was measured _in vivo_ by incorporation of uniformly labelled ^{14}C-L-phenylalanine and ^{3}H-5-uridine. RNA systhesis was measured _in vitro_ by incorporation of ^{3}H-UTP. In addition, by using the same technique, we wanted to determine if OSB 418 and OSB 427 contained temperature sensitive RNA polymerases.

The data in Figure 8 and 9 indicate that _in vivo_ RNA and protein synthesis in OSB 158 ceased after addition of Rif, Stv, or Std into the growth medium. Upon shifting the culture from 37 C to 50 C, RNA synthesis first slowed and then assumed a faster rate. Protein synthesis did not lag.

Synthesis of RNA and protein was measured _in vivo_ in several rifampin resistant, streptolydigin resistant or temperature sensitive strains. As shown in Figures 10 and 11, in the presence of rifampin, neither RNA nor protein synthesis was inhibited in the rifampin resistant mutants. In the presence of Std, on the other hand, the streptolydigin resistant mutants displayed either an immediate shift to a reduced rate of synthesis, or a lag and then a shift to a reduced rate of RNA synthesis (Figures 12 and 13). Protein synthesis did not lag before assuming the reduced rate of synthesis. In both OSB 418 and OSB 427, RNA and protein synthesis ceased soon after shifting to 50 C. Another temperature sensitive mutant, OSB 419, in which the Ts phenotype is not linked by co-transformation to _cys_ A14, rifampin resistance or streptolydigin resistance, did not

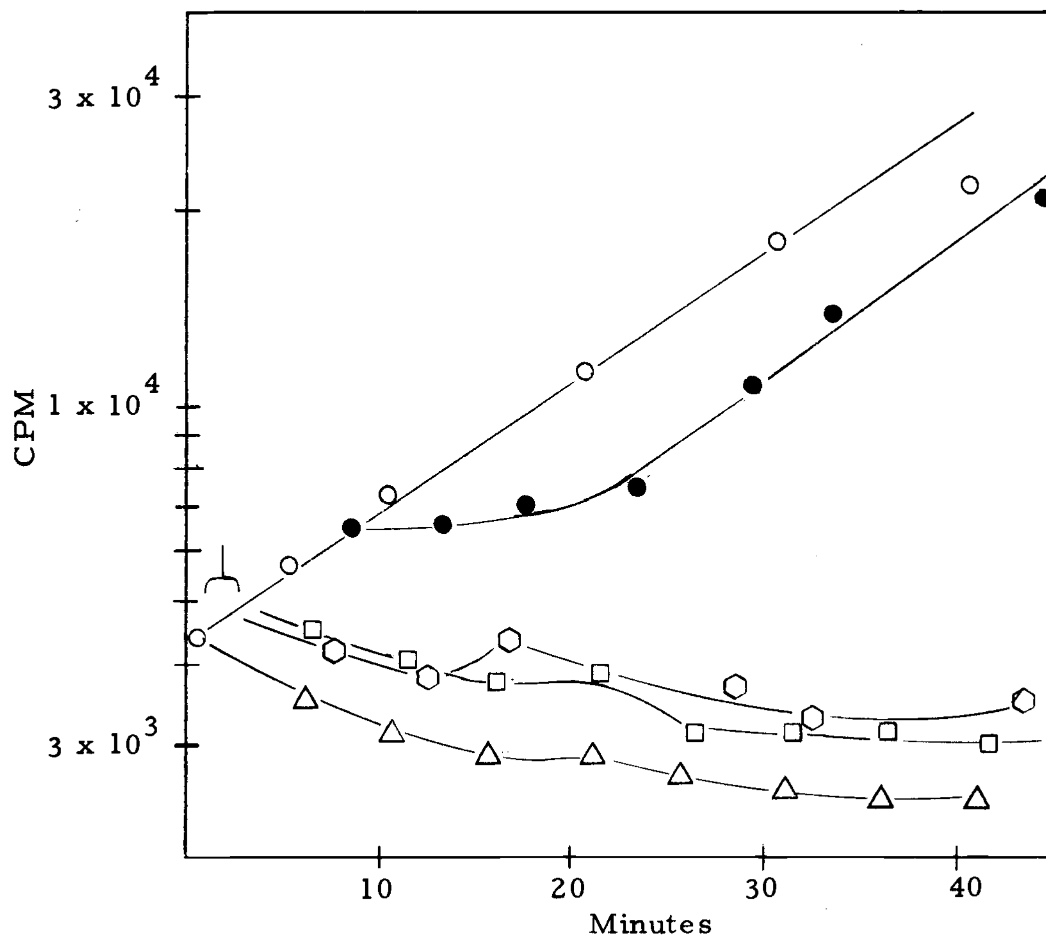

Figure 8. ^{3}H-5-Uridine incorporation by OSB 158. ○--○, control; ⬡--⬡, plus rifampin; □--□, plus streptovaricin; △--△, plus streptolydigin; ●--●, at 50 C.

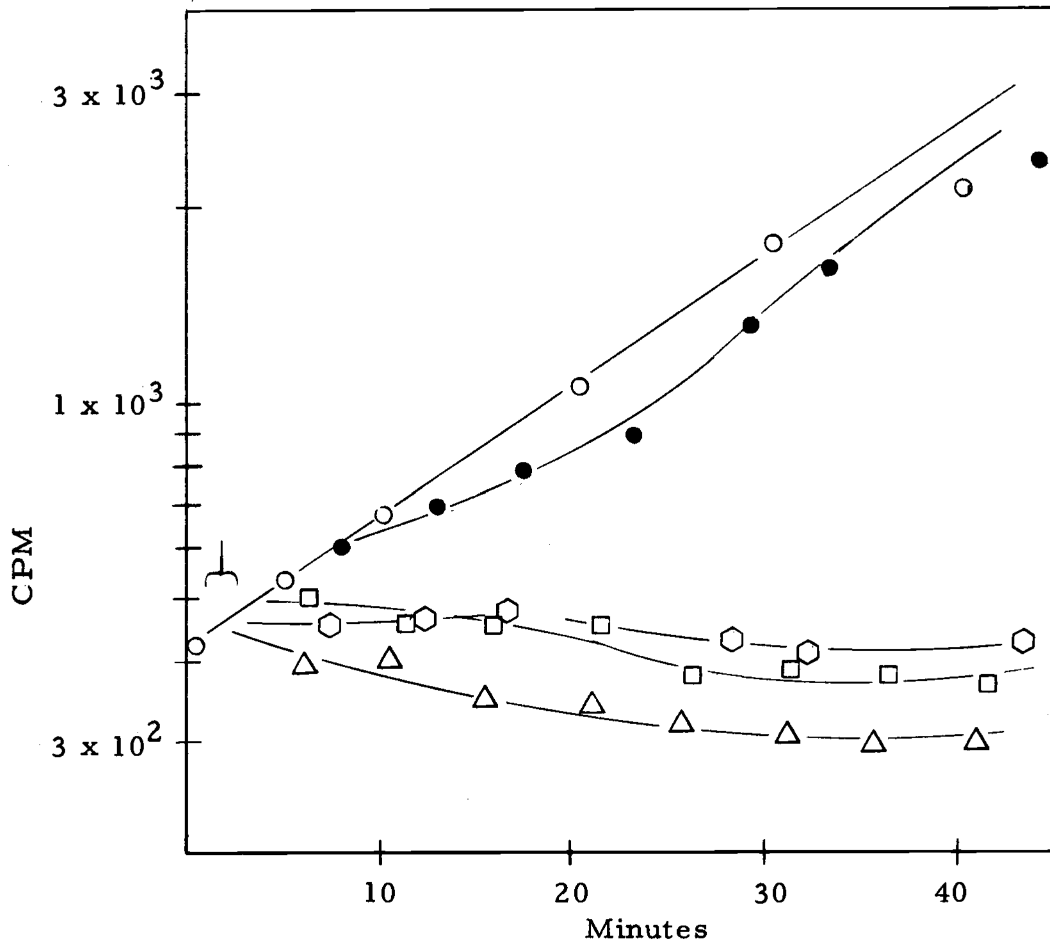

Figure 9. ^{14}C-Phenylalanine incorporation by OSB 158. ○--○, control; ⬡--⬡, plus rifampin; □--□, plus streptovaricin; △--△, plus streptolydigin; ●--●, at 50 C.

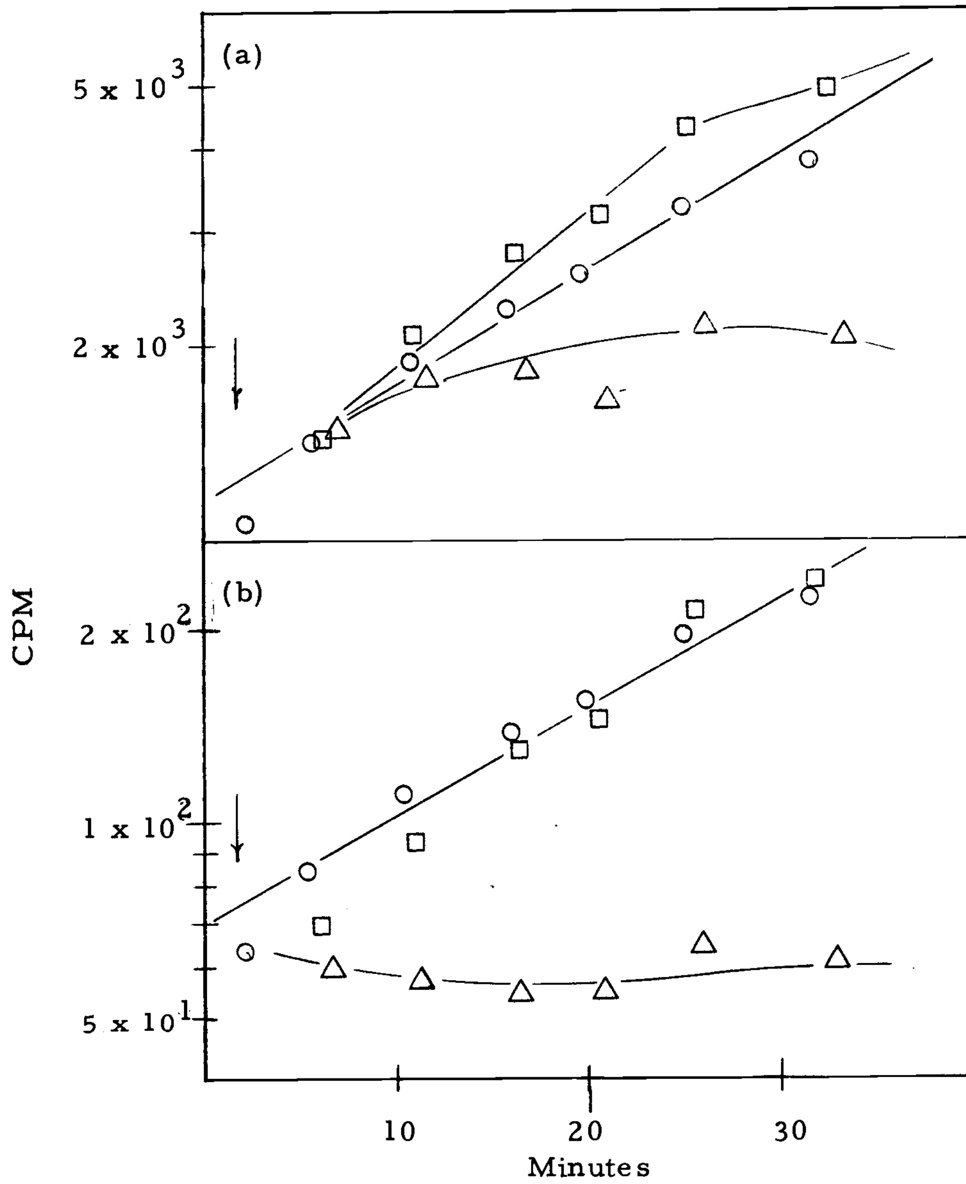

Figure 10. (a) ^{3}H-5-Uridine incorporation by OSB 427.
(b) ^{14}C-Phenylalanine incorporation by OSB 427.
○--○, control; □--□ plus rifampin; △--△, at 50 C.

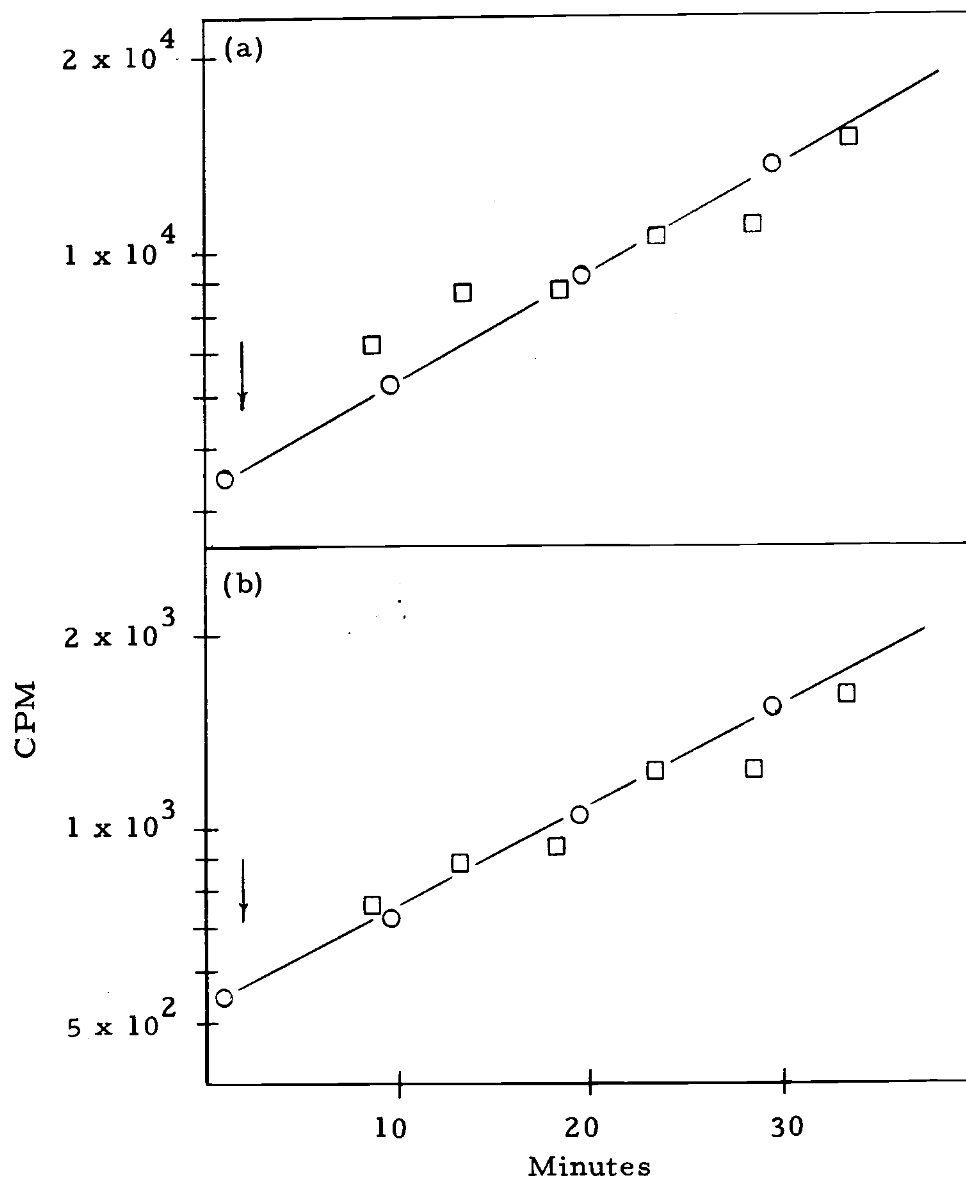

Figure 11. (a) ^{3}H-5-Uridine incorporation by OSB 423.
(b) ^{14}C-Phenylalanine incorporation by OSB 423.
○--○, control; □--□, plus rifampin.

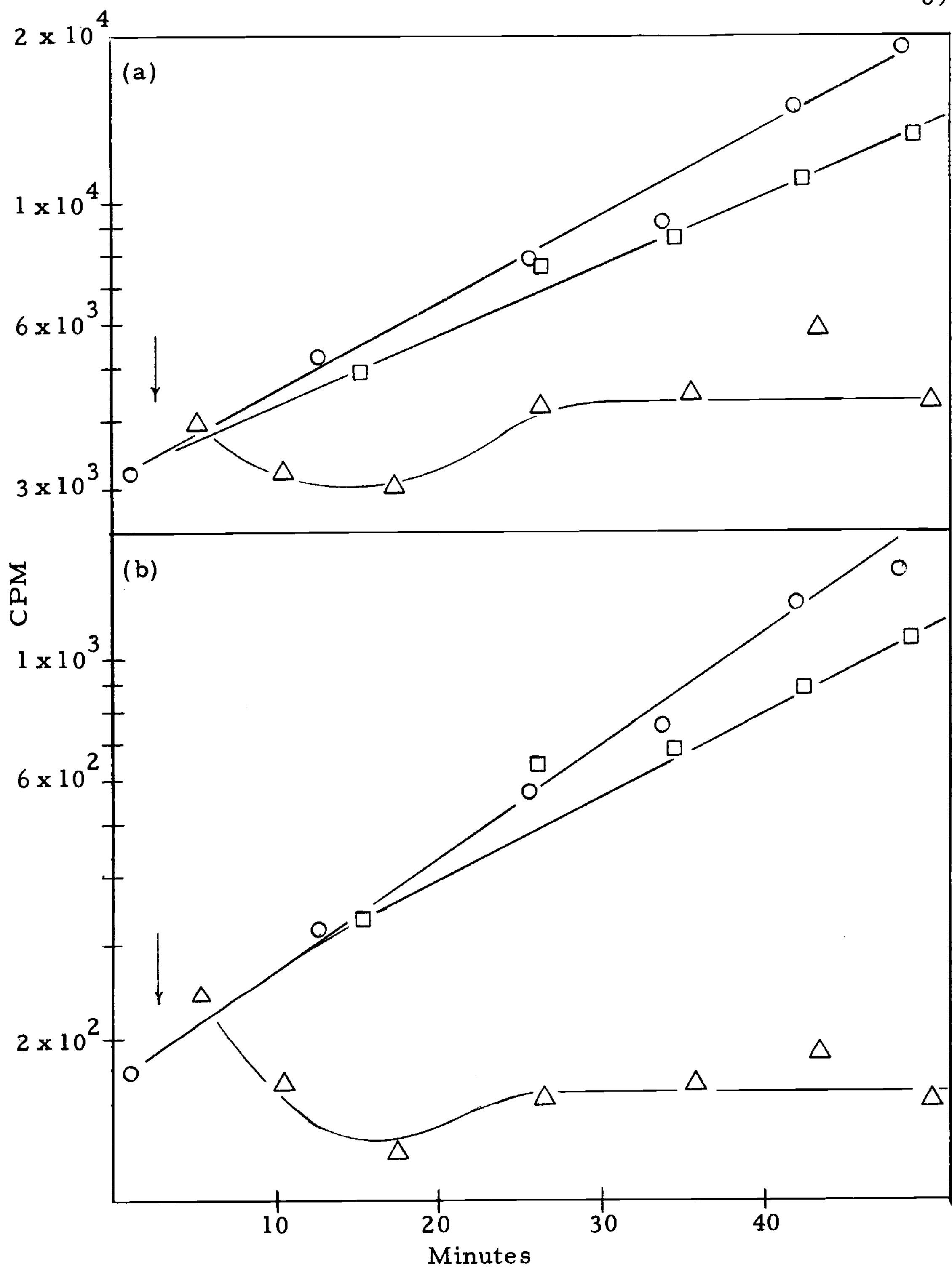

Figure 12. (a) Incorporation of ^{3}H-5-Uridine by OSB 418.
(b) Incorporation of ^{14}C-Phenylalanine by OSB 418.
○--○, control; □--□, plus streptolydigin;
△--△, at 50 C.

display the Ts phenotype until the onset of sporulation. The data indicate that during logrithmic growth, RNA synthesis assumed a slower rate at 50 C but protein synthesis appeared to be unaffected. At the end of logrithmic growth as the culture started into sporulation, however, the total incorporation of labelled RNA and protein was much less at 50 C than at 37 C (Figure 14).

Synthesis of RNA and protein in OSB 431 was unaffected by rifampin but assumed a reduced rate in the presence of strepto-lydigin (Figure 15). RNA synthesis' response to the simultaneous presence of Rif and Std was essentially the same as its response to Std alone. At 50 C, both RNA synthesis and protein synthesis were shut off.

In vitro ^{3}H-UTP incorporation was measured in cell-free extracts of OSB 158, OSB 418, and OSB 427 to show that the Rif^{R} and Std^{R} phenotypes are the result of drug resistant RNA polymerases and that the Ts phenotype is the result of a temperature sensitive RNA polymerase.

As indicated in Tables 11 and 12, RNA polymerase from OSB 427 lost over 85% of its specific activity at 47.5 C while OSB 158 lost only 50%. In the partially purified extract, OSB 427 lost over 70% of its activity while the wild type enzyme lost only 39% of its specific activity. On poly (dA-dT), the crude enzymes from both OSB 158 and OSB 427 showed increased activity at 37 C with very

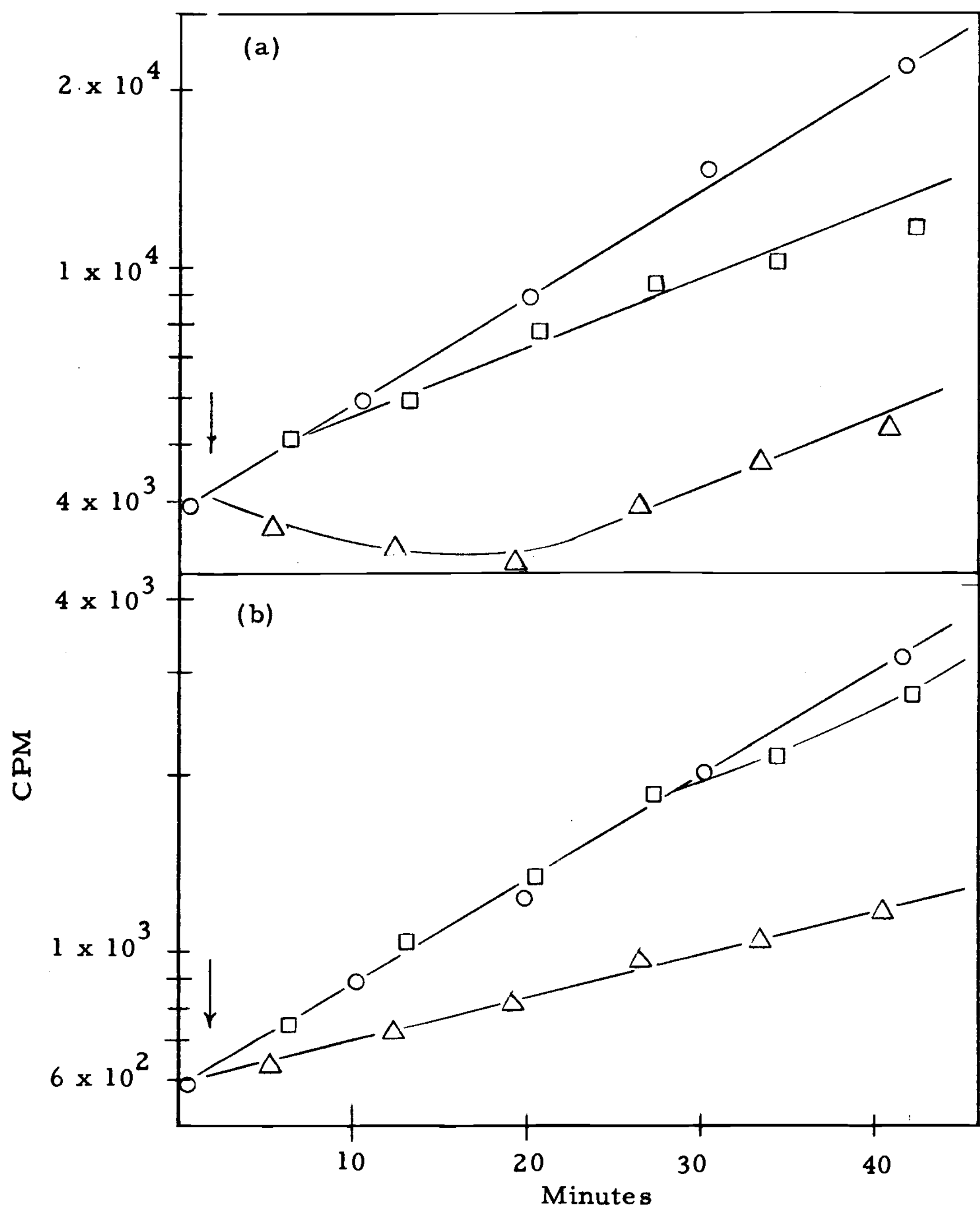

Figure 13. (a) Incorporation of ^{3}H-5-Uridine by OSB 419.
(b) Incorporation of ^{14}C-Phenylalanine by OSB 419.
O--O, control; □--□, plus streptolydigin;
△--△, at 50 C.

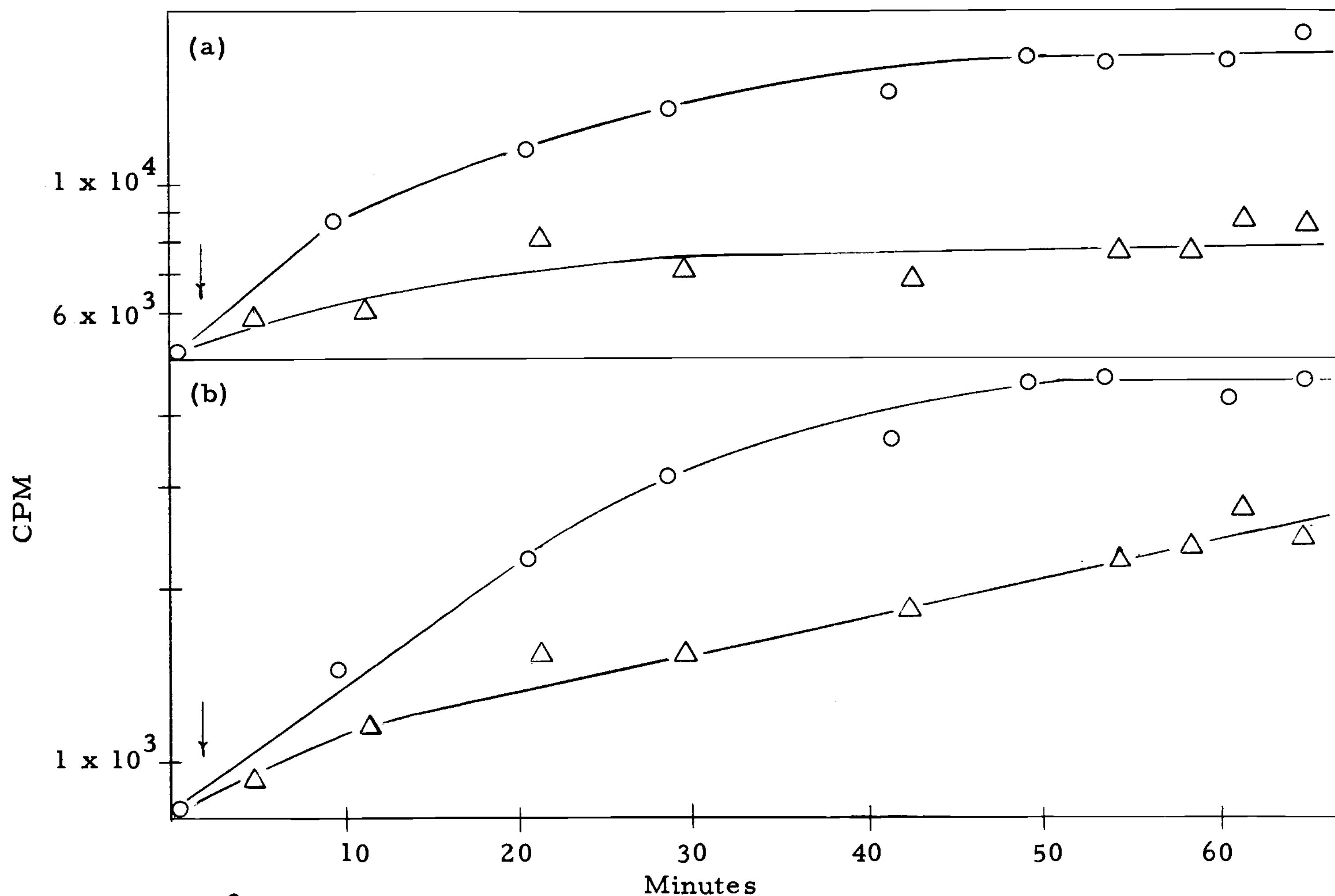

Figure 14. (a) ^{3}H-5-Uridine incorporation by OSB 419 as logrithmic growth ceases and sporulation begins. (b) ^{14}C-Phenylalanine incorporation by OSB 419 as logrithmic growth ceases and sporulation begins. o--o, control; Δ--Δ, at 50 C. Cold carrier uridine and phenyl-alanine were supplemented at 80 μg/ml and 10 μg/ml respectively.

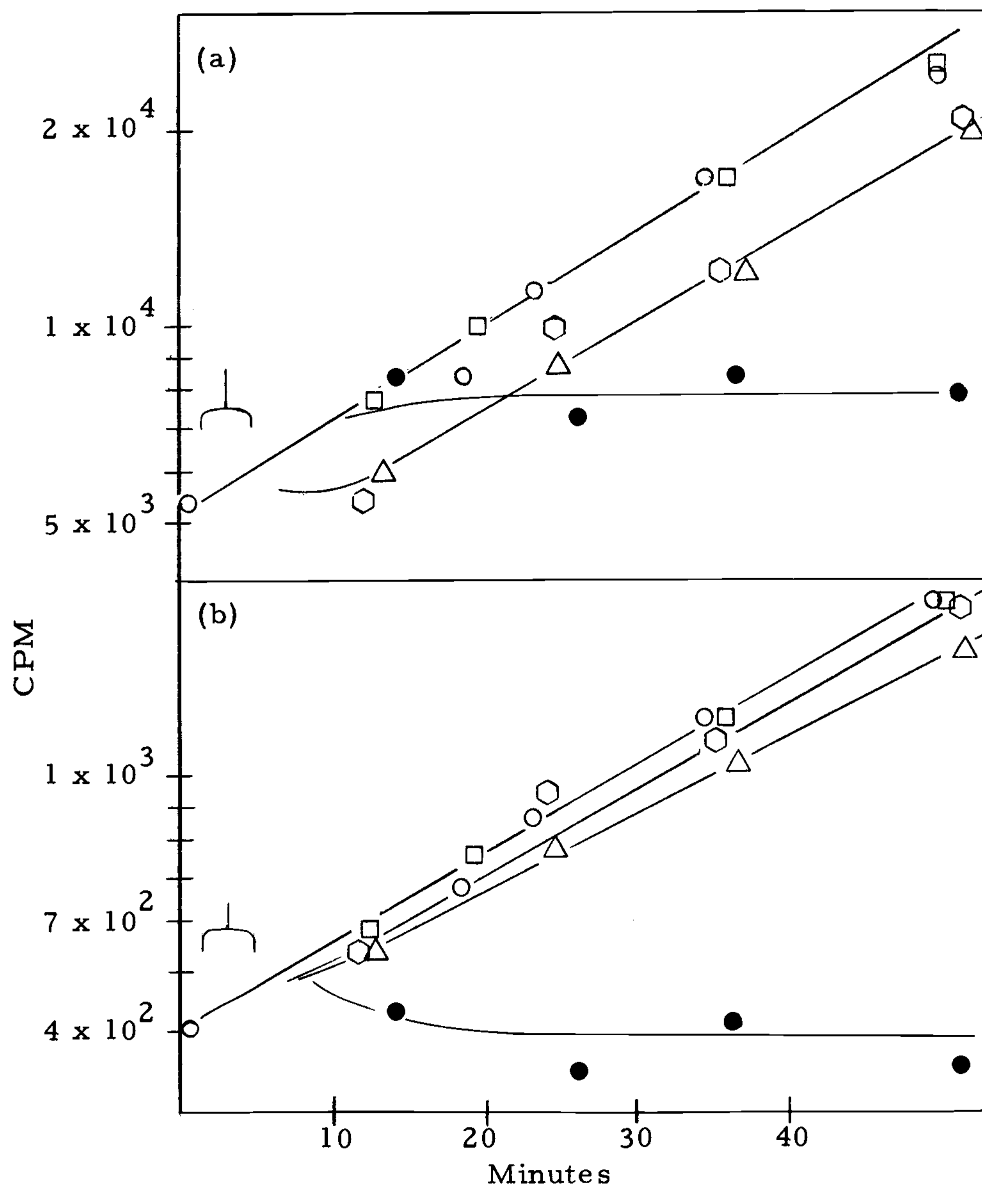

Figure 15. (a) ^{3}H-5-Uridine incorporation by OSB 431.
(b) ^{14}C-Phenylalanine incorporation by OSB 431. ○--○, control; □--□, plus rifampin; △--△, plus streptolydigin, ⬡--⬡, plus rifampin and streptolydigin; ●--●, at 50 C.

little loss of specific activity at 47.5 C. On salmon sperm DNA template, the ammonium sulfate fractions of both OSB 158 and OSB 427 extracts showed equal reductions in RNA polymerase specific activity at 47.5 C. The wild type polymerase was sensitive to rifampin at both 35.5 C and 47.5 C while the OSB 427 polymerase was resistant at both temperatures. The data in Table 12 show that OSB 158 RNA polymerase was sensitive to Std at all concentrations of drug tested. The resistant polymerase, however, maintained high levels of resistance up to the concentration of drug normally used *in vivo*. A substantial reduction in specific activity was observed at a Std concentration equivalent to 200 μg/ml which is twice the concentration used in *in vivo* experiments.

Table 11. *In vitro* RNA polymerase specific activity in a crude extract.

Source of Enzyme	Template Activity: *B. subtilis* DNA 35.5C	*B. subtilis* DNA 47.5C	poly (dA-dT) 35.5 C	poly (dA-dT) 47.5C	poly(dA-dT)+ Rifampin (7 μg/assay) 35.5C	poly(dA-dT)+ Rifampin (7 μg/assay) 47.5C
OSB 158	3.8	1.9	45.0	34.0	0.1	0.2
OSB 427	4.3	0.6	55.0	39.0	41.0	35.5

Table 12. *In vitro* RNA polymerase specific activity in an ammonium sulfate fraction.

Source of Enzyme	Template Activity: *B. subtilis* DNA		Salmon Sperm DNA	
	35.5C	47.5C	35.5C	47.5C
OSB 158	6.5	4.0	3.8	1.7
OSB 427	6.9	2.0	4.3	1.9

Table 13. *In vitro* RNA polymerase sensitivity to streptolydigin in a crude extract.

Source of Enzyme	Template poly (dA-dT): No Std	12.5 μg Std	25 μg Std	50 μg Std
OSB 158	58	18	13	7.2
OSB 418	50	49	48	28

The *in vitro* data confirm that the RNA polymerase from OSB 158 is sensitive to Rif and Std but does continue to function at 47.5 C. The RNA polymerase from the rifampin resistant OSB 427 strain is resistant to Rif and is temperature sensitive at 47.5 C. The RNA polymerase from the streptolydigin resistant OSB 418 strain is resistant to increasing concentrations of Std while the wild type enzyme is inhibited at all concentrations of drug tested.

Growth and Sporulation Studies

To evaluate the ability of drug resistant strains to carry out

normal cellular functions, measurements were made of growth and sporulation in the presence and absence of drug. For temperature sensitive strains, measurements were made at 50 C.

Figure 16 indicates that OSB 158 ceased to grow in the presence of Rif, Stv, or Std but did continue to grow normally when shifted to 50 C. The data presented in Figures 17 and 18 and in Table 14 can be summarized in the following manner. The rifampin resistant strains had similar generation times to that of the wild type strain and were not affected by the presence of rifampin. The strepto-varicin resistant strains also had generation times like the wild type strain. OSB 122 was sensitive to Rif and OSB 114 was not affected by its presence. The streptolydigin resistant strains were widely varied in generation time and drug tolerance. Strains carrying two drug resistant markers plus the Ts_{418} marker had longer generation times than did the parent strains, although each marker still exerted essentially the same effect as it did in the parent strain, e.g. Rif in the growth medium did not alter the generation time in OSB 432 just as it did not in OSB 423 (Figure 19). Strains carrying either the Ts_{418} mutation or the Ts_{427} mutation did not exhibit any unique characteristics at 37 C. However, OSB 418 did have a greatly increased generation time in the presence of Std. Whether or not the Ts mutation contributed to this increase is unknown. OSB 419, which carries an unlinked Ts marker, did not display any temperature

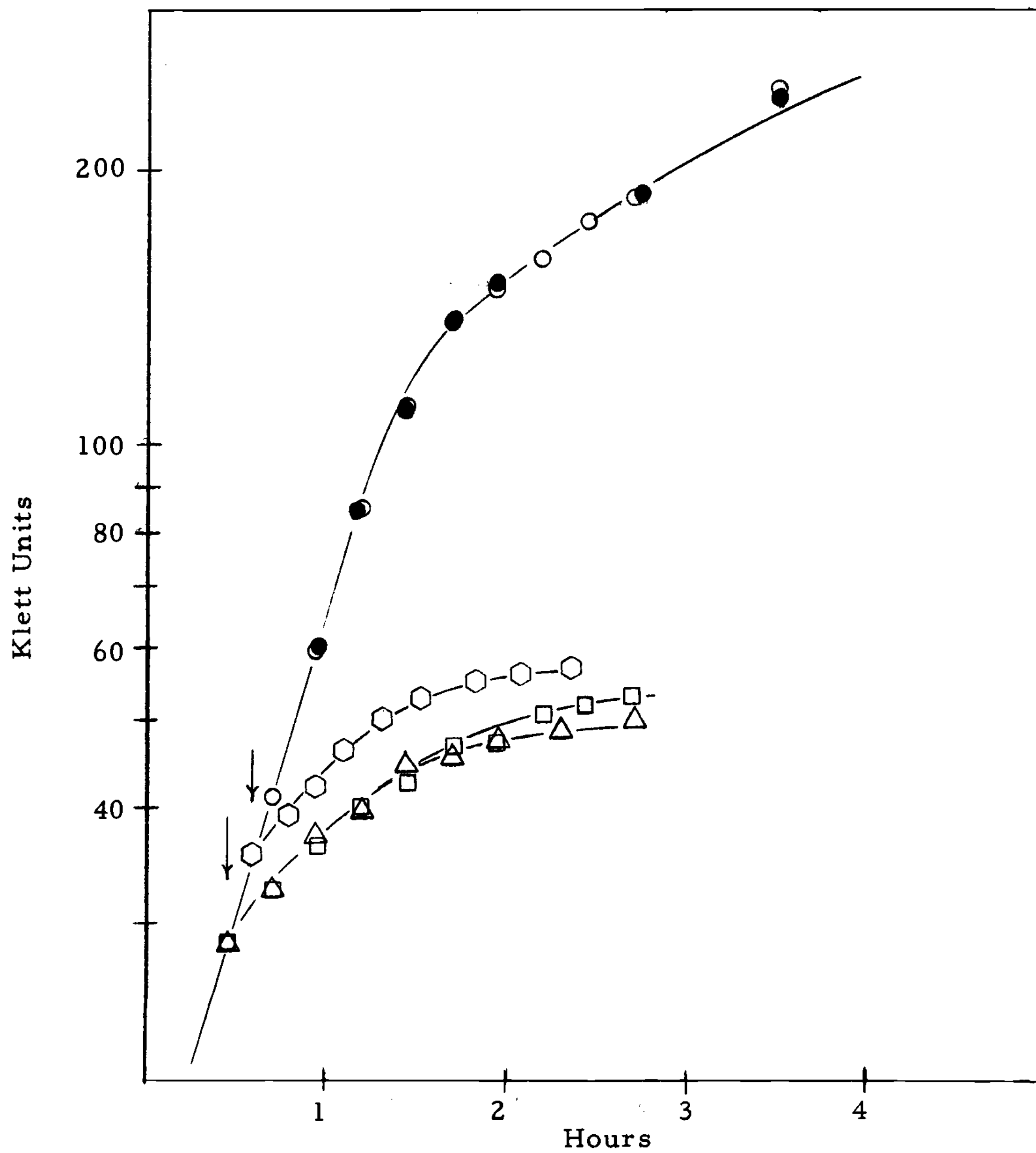

Figure 16. OSB 158 growth curves. ○--○, control; △--△, plus rifampin; □--□, plus streptovaricin; ⬡--⬡, plus streptolydigin; ●--●, at 50 C.

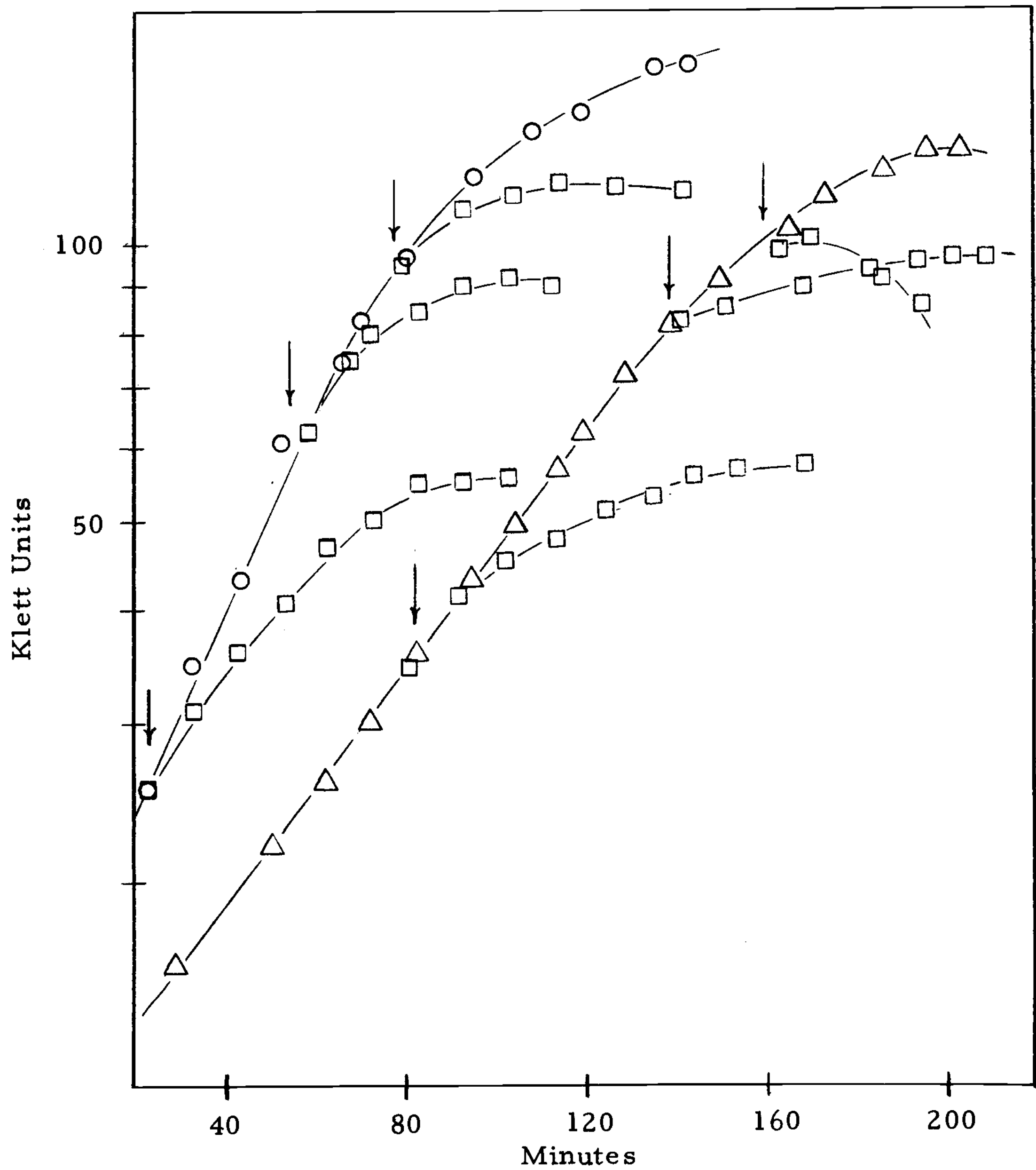

Figure 17. OSB 418 growth curves.
○--○, at 37 C without streptolydigin.
△--△, at 37 C with streptolydigin.
□--□, at 50 C.

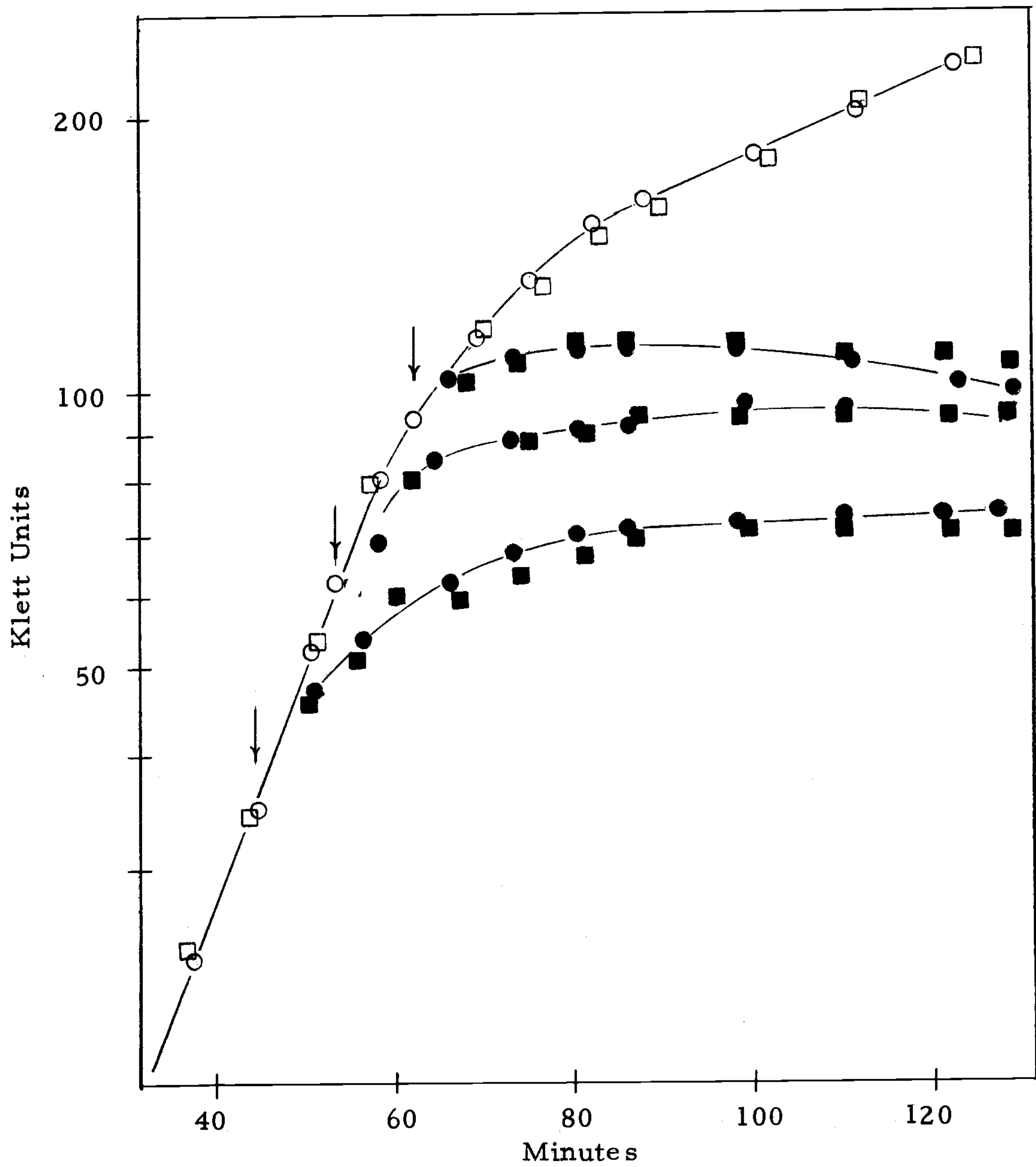

Figure 18. OSB 427 growth curves.
○--○, at 37 C without rifampin; □--□, at 37 C with rifampin; ●--●, at 50 C without rifampin; ■--■, at 50 C with rifampin.

Table 14. Summary of growth measurements.

Strain	Phenotype	Generation Time (min) w/o Drug	Generation Time (min) with Drug or at 50 C
OSB 158	wt	25	lysed under all conditions
OSB 420	Rif^R	26	26 + Rif
OSB 423	Rif^R	26	26 + Rif
OSB 424	Rif^R	28	29 + Rif
OSB 427	$Rif^R Ts$	25	25 + Rif, lysed at 50 C
OSB 114	$Rif^R Stv^R$	25	25 + Rif
OSB 122	Stv^R	25	lysed with Rif
OSB 404	Std^R	25	29 + Std
OSB 405	Std^R	40	40 + Std
OSB 415	Std^R	35	35 + Std
OSB 418	$Std^R Ts$	28	46 + Std, lysed at 50 C
OSB 419	$Std^R Ts$	29	29 50 C log phase lysed at 50 C post log
OSB 431	$Rif^R Std^R Ts$	37	37 + Rif 42 + Std 42 + Rif + Std lysed at 50 C
OSB 432	$Rif^R Std^R Ts$	42	42 + Rif 67 + Std 67 + Rif + Std lysed at 50 C
OSB 433	$Rif^R Std^R Ts$	41	41 + Rif 44 + Std 44 + Rif + Std lysed at 50 C

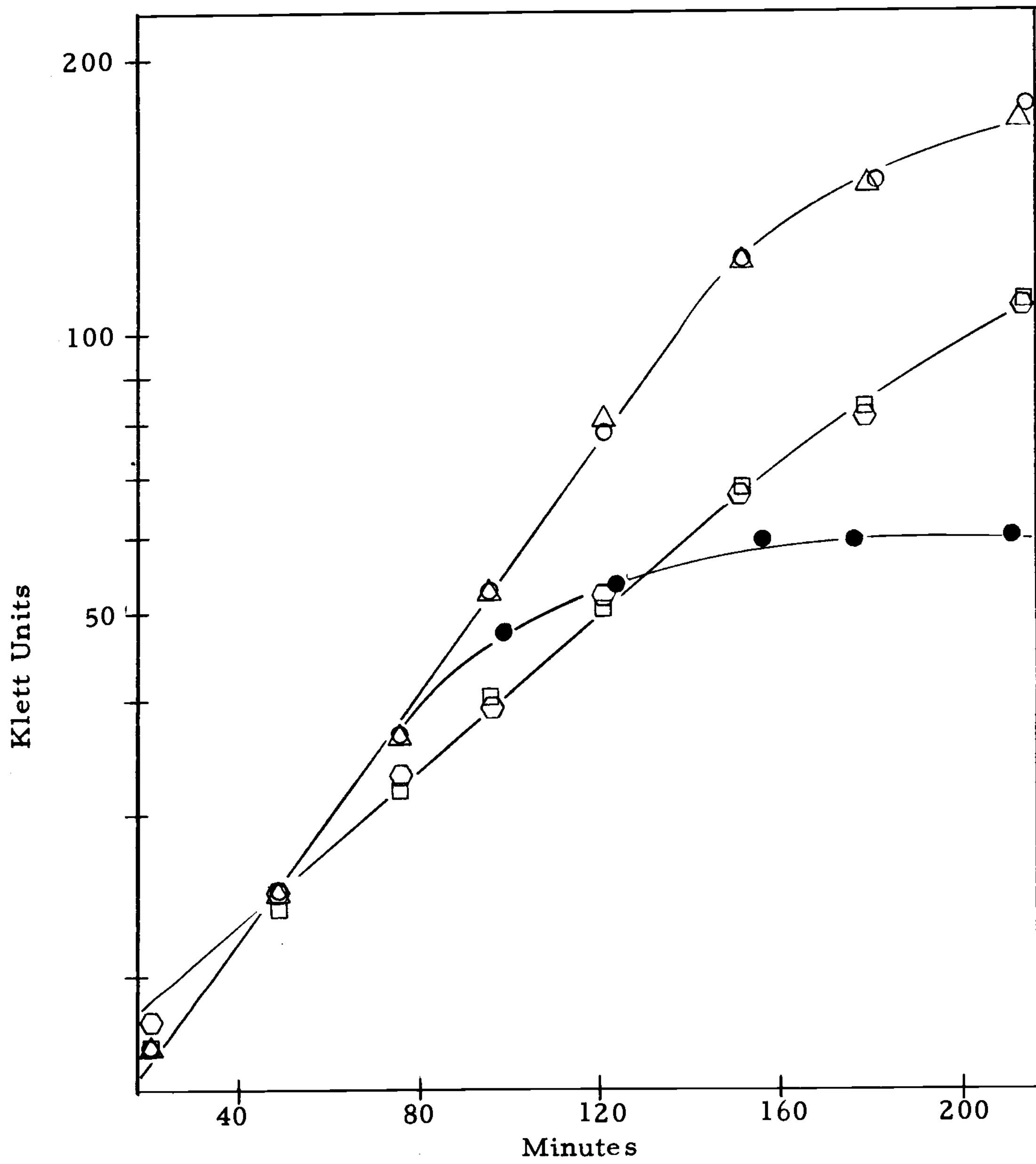

Figure 19. OSB 432 growth curves.
○--○, control; △--△, plus rifampin; □--□, plus streptolydigin; ⬡--⬡, plus rifampin and streptolydigin; ●--●, at 50 C.

sensitivity until logrithmic growth had ceased (Figure 20).

When attempts were made to measure the ability of selected mutants to sporulate, extensive aggregation of the cells and spores was encountered, making quantitation studies impossible. General observations on these sporulating cultures will be reported, however (Table 15).

Table 15. Strains observed in sporulation studies.

	SG Medium	Minimal Medium
OSB 158	x[a]	x
OSB 423	-	x
OSB 427	x	x
OSB 114	x	-
OSB 122	x	-
OSB 404	x	x
OSB 405	x	-
OSB 418	x	x
OSB 431	x	x

[a] x denotes if tested.

OSB 158 sporulated in about 24 hr in SG medium but required about 48 hr in SSM medium. In both cases, only minor aggregation was observed. Rifampin resistant and streptovaricin resistant strains sporulated in SG medium in the presence and absence of drug to about the same extent but were several hours slower in completing sporulation than was OSB 158. The rifampin resistant strains

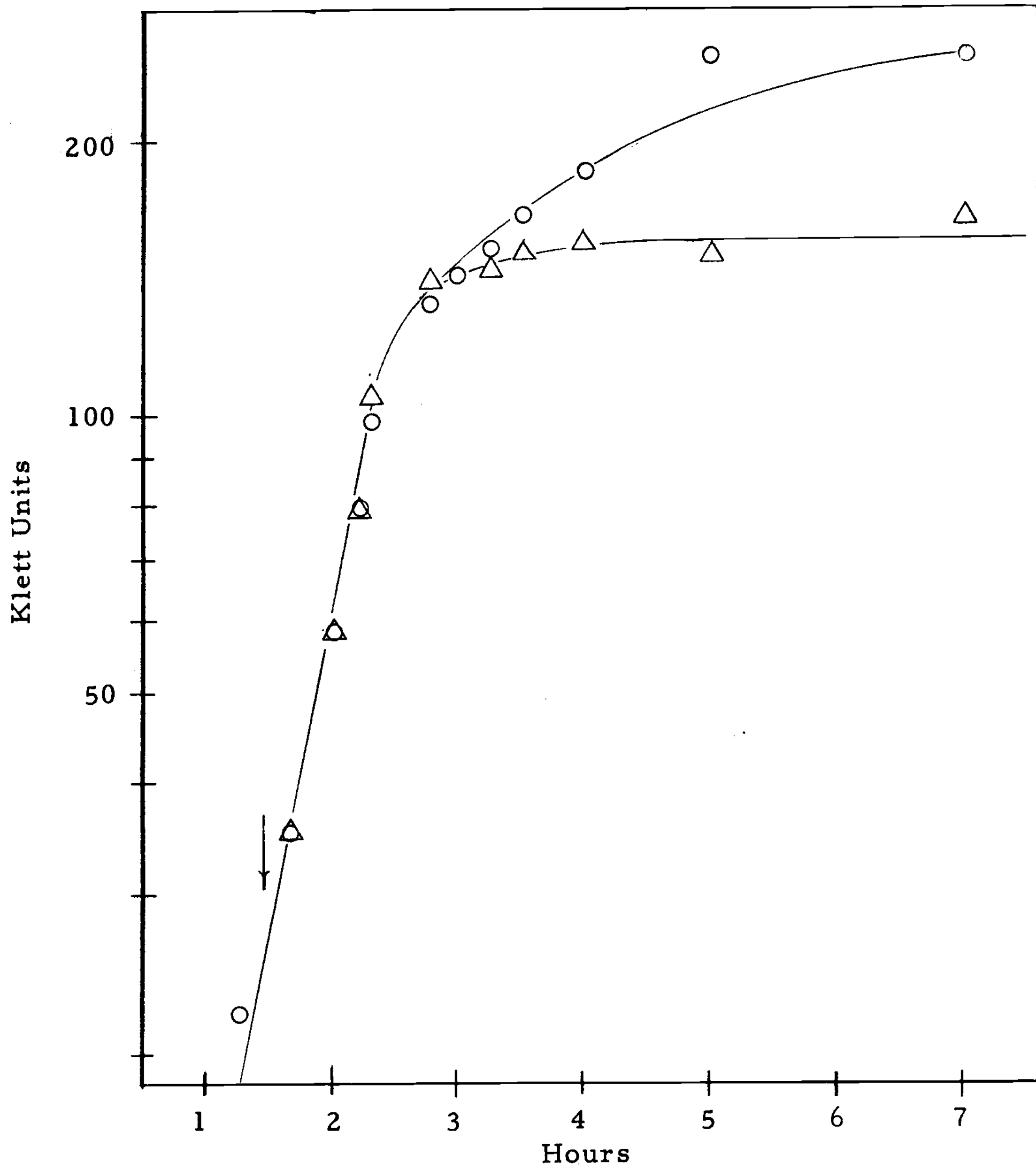

Figure 20. OSB 419 growth curves.
○--○, control; △--△, at 50 C.

behaved similarly in SSM as they did in SG medium except that aggregation was not as extensive.

The streptolydigin resistant strains required over twice as long to sporulate in SG medium and considerable cellular debris was observed in the cultures. In SSM medium, the streptolydigin resistant strains were again far behind the wild type strain and the rifampin resistant strains. The presence of Std in the growth medium significantly depressed sporulation, especially in the case of OSB 418 where extensive lysis was observed. OSB 431 sporulated very poorly under all conditions. The strain grew extremely poorly in the presence of Rif and Std in the SSM medium.

In summary, the rifampin resistant and the streptovaricin resistant strains grew and sporulated very well in the presence and absence of drug. The streptolydigin resistant strains showed rather variable growth rates and produced fewer spores than did the rifampin resistant and the streptovaricin resistant strains. OSB 431 grew and sporulated poorly under all conditions.

Sensitivity of Sporulating OSB 158 Cells to Rifampin and Streptolydigin

In the *E. coli* RNA polymerase, strong evidence (35, 43) indicates that the Rif and Std binding sites both reside in the β subunit of the RNA polymerase. If one or both of the loci resides in the

portion of the β sub-unit which is cleaved at the onset of sporulation, then the normally sensitive culture may become resistant to one or both of the drugs. This possibility was tested by in vivo and in vitro analysis as described in the Materials and Methods.

Figure 21 indicates that although OSB 158 remained sensitive to Rif throughout sporulation, the culture was no longer sensitive to Std after the onset of sporulation and was able to produce spores.

In vitro studies comparing specific activity of OSB 158 RNA polymerase from vegetative and sporulating cells indicated, however, that the sporulating polymerase did not lose its sensitivity to streptolydigin (Table 16).

Table 16. Comparison of vegetative RNA polymerase and sporulating RNA polymerase sensitivity to streptolydigin on poly (dA-dT) template.

Source of Enzyme	No Std	12.5 μg Std	25 μg Std	50 μg Std
OSB 158 (vegetative)	50	18	13	7.2
OSB 158 (Stage II-III)	200	72	56	38

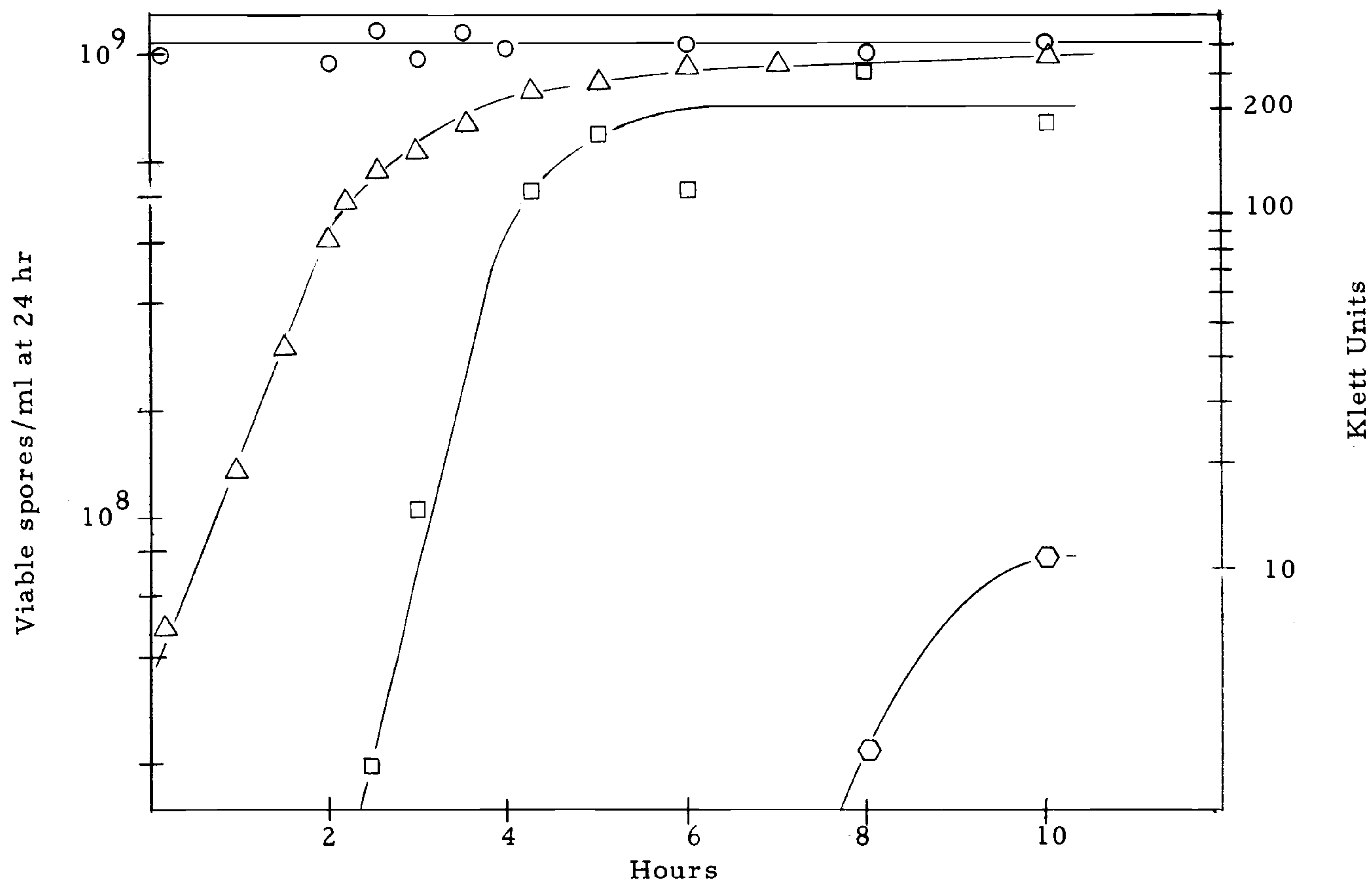

Figure 21. Sensitivity of OSB 158 to rifampin and streptolydigin during sporulation. ○--○, viable spores in control flasks after 24 hr. □--□, viable spores in the presence of streptolydigin; ⬡--⬡, viable spores in the presence of rifampin. △--△, Klett units.

DISCUSSION

A primary objective of this study was to isolate and genetically map new classes of *B. subtilis* RNA polymerase mutants. Harford and Sueoka (33) genetically mapped three rifampin resistant mutants of *B. subtilis* by transformation and Brown (9) genetically mapped over 40 rifampin resistant and streptovaricin resistant mutants of *B. subtilis* by PBS1 mediated transduction.

We obtained large numbers of streptolydigin resistant mutants by EMS mutagenesis and genetically mapped 18 of them by transduction or transformation. Two temperature sensitive mutants which we believe are also RNA polymerase mutants were also isolated by EMS mutagenesis and genetically mapped by transformation. A genetic map of these mutations has been constructed from two and three factor transformation data.

Mutagenesis

That controlled mutagenic conditions resulted in the isolation of single point mutants of RNA polymerase rather than large numbers of double mutants can be inferred from several observations. Of the mutants examined, all of the rifampin resistant and the streptolydigin resistant mutants were prototrophic, none were cross resistant, and

only three streptolydigin resistant mutants were also temperature sensitive.

Selection of Temperature Sensitive Mutants

Temperature sensitive RNA polymerase mutants could undoubtedly be isolated in the same manner as were the rifampin resistant and streptolydigin resistant mutants. However, no selection pressure was available for specifically isolating temperature sensitive RNA polymerase mutants. Yura *et al.* (112) had shown that a class of RNA polymerase mutants in *E. coli* could be isolated that had obtained temperature sensitivity and resistance to streptovaricin by a single step mutation. Therefore, in our isolation procedure, mutants were first selected for streptolydigin resistance and then tested for temperature sensitivity.

Genetic Analysis of Temperature Sensitive Mutants

Genetic analysis of three Std^R Ts mutants and three Rif^R Ts mutants which had been obtained by the above selection procedure indicated that in only two of the strains, (OSB 418, OSB 427) were drug resistance and temperature sensitivity genetically linked by co-transformation. In these strains, a single step mutation did not result in both drug resistance and temperature sensitivity since the two phenotypes could be separated by genetic manipulation and by

shearing of transforming DNA.

Genetic analysis also indicated that the temperature sensitive phenotype of OSB 418 and OSB 427 is the result of at least two different mutations.

Location of RNA Polymerase Genes on the *B. subtilis* Chromosome

Genetic mapping by transduction and transformation indicates that all of the Rif^R, Stv^R , Std^R and two of the Ts phenotypes are the result of tightly clustered mutations that co-transduce or co-transform with the *cys* A14 locus. With the exception of the Rif^R Stv^R phenotype all of the different phenotypes appear to be the result of different classes of mutations that can be genetically and physically separated, e.g. a $Rif^R Std^S$ strain can be transformed to a $Rif^R Std^S$ or a $Rif^S Std^R$ phenotype as well as to a $Rif^R Std^R$ phenotype. Mutants within each phenotypic class display a range of co-transformation frequencies with the *cys* A14 locus but the actual number of different mutations represented by these mutants cannot be determined from existing data. Fine structure mapping by the recombination index method should answer this question, however.

A genetic map has been constructed from two and three factor transformation data (Figure 22). Map distances were calculated from data in which *cys* was the selected marker. Co-transformation

frequencies were converted to map units by the following relationship:

100 --percent co-transformation = map units

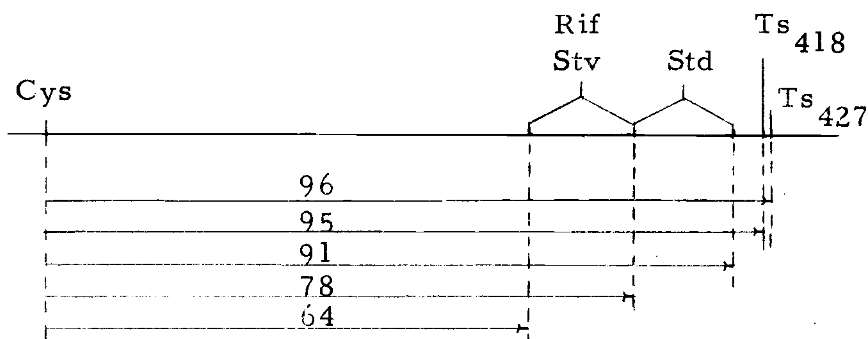

Figure 22. Genetic map of mutations which result in resistance to Rif, Stv, or Std or which result in temperature sensitivity.

Confirmation of Genotypes

In vivo and *in vitro* isotope incorporation data indicate that the rifampin resistant and streptolydigin resistant mutants that were isolated and genetically mapped actually contain a rifampin resistant or streptolydigin resistant RNA polymerase. *In vivo* incorporation of ^{3}H-5-uridine and uniformly labelled ^{14}C-L-phenylalanine showed that RNA and protein synthesis were blocked in OSB 158 by addition of Rif or Std. However, rifampin resistant and streptolydigin resistant strains continued to synthesize RNA and protein in the presence of Rif or Std. *In vitro* analysis of a rifampin resistant

strain, OSB 427, and a streptolydigin resistant strain, OSB 418, confirmed that these strains contained drug resistant polymerases. This finding inferred that the other rifampin resistant, and streptolydigin resistant mutants analyzed in this study also contained drug resistant RNA polymerases. This assumption is also an extension of the genetic mapping data which indicated that all of the drug resistant mutants examined were very tightly clustered.

That OSB 418 and OSB 427 contain temperature sensitive RNA polymerases is supported by _in vivo_ and _in vitro_ isotope incorporation data. _In vivo_ incorporation of ^{3}H-5-Uridine and ^{14}C-Phenylalanine into RNA and protein ceases upon shifting growing cultures of OSB 418 and OSB 427 to 50 C. This response is consistent with a temperature sensitive RNA polymerase genotype.

In vitro incorporation of ^{3}H-UTP was measured in a crude extract and in a partially purified extract of OSB 427. In both cases, the specific activity was greatly reduced at 47.5 C indicating that the RNA polymerase is temperature sensitive.

Crude and partially purified extracts of OSB 158 also showed reduced specific activity at 47.5 C. However the reduction was much less than in the OSB 427 extracts.

Growth Characteristics and Sporulating Ability of Rifampin Resistant, Streptovaricin Resistant, Streptolydigin Resistant and Temperature Sensitive Mutants

In order to evaluate the effect of an altered polymerase on a cell's ability to perform normal physiological functions, generation times and sporulating ability of selected mutants were measured.

In both the presence and absence of drug, the rifampin resistant and streptovaricin resistant mutants exhibited essentially the same growth characteristics and sporulating ability as those of OSB 158. However, rifampin resistant mutants have been isolated in which the ability to sporulate is greatly diminished (89) or in which spores with altered morphology are produced (20).

The streptolydigin resistant mutants examined, however, exhibited diverse generation times in the absence of Std and varied responses to the presence of Std.

In the absence of Std, the streptolydigin resistant strains produced fewer spores than did the rifampin or streptovaricin resistant strains. In the presence of Std, this effect was much more exaggerated with some strains showing large amounts of cellular debris within the culture.

Several strains were constructed by transformation whose phenotype included rifampin resistance, streptolydigin resistance and temperature sensitivity. The effect of these additional alterations in the RNA polymerase were manifested in longer generation times and in decreased sporulating ability. The effect of the presence of Rif or of shifting to 50 C was the same on the multiply marked strains as it was on the strain that originally carried the

given marker, e.g. the constructed strains had the same generation time in the presence and absence of Rif as had OSB 423, and the temperature sensitive phenotype was conserved. However, the multiply marked strains' responses to Std were not always the same as those of the streptolydigin resistant strains from which they were constructed. In the presence of streptolydigin, OSB 432 showed a 15 min increase in generation time as opposed to no increase by OSB 405 in the presence of Std. However, the responses of OSB 431 and OSB 433 to Std were essentially the same as those found for their respective streptolydigin resistant donor strains.

The location of the rifampin resistant mutation is apparently not affected by the presence of either the streptolydigin resistant mutations or the temperature sensitive mutation. The same observation is true for the temperature sensitive mutation in regard to the rifampin resistant mutation and the streptolydigin resistant mutation. However, depending on the streptolydigin mutation, the presence of the rifampin resistant and temperature sensitive mutations appears to influence a strain's response to the presence of Std. In lieu of fine structure mapping data, this kind of anlysis may be useful in classifying different strains of the same phenotype.

The effects of the mutations conferring resistance to Rif, Stv, or Std are presumably due to an altered binding site for a given drug. An altered binding site could either completely block

inhibitor binding or result in varying degrees of inhibitor binding. At the same time, the altered drug binding site may also affect the enzymatic properties of the RNA polymerase by affecting the conformation of the enzyme molecule. An increased generation time in the absence of drug would suggest that the enzymatic properties were affected. An increased generation time in the presence of drug would indicate greater binding of inhibitor.

It appears that in the rifampin and streptovaricin resistant mutants examined in this study, the alterations in the enzyme were such that they completely blocked rifampin binding to the RNA polymerase without disturbing its enzymatic properties. This was evidenced by the fact that the generation times were affected little in the presence or absence of Rif. However, with the streptolydigin resistant mutants, the enzymatic properties of the RNA polymerase were apparently altered in some strains as reflected by the increased generation times in the absence of Std. However, the extent to which Std binding is blocked varied among the strains examined as evidenced by the various responses of the streptolydigin resistant strains to the presence of Std.

Physical Analysis of RNA Polymerase Genes

Sedimentation velocity experiments with sheared preparations of OSB 431 DNA indicate that the sheared DNA molecules present

are not of uniform molecular weight. The sheared preparations have lost over 99% of their total transforming activity, and linkage by co-transformation of the Rif^R, Std^R and Ts phenotypes was greatly reduced. The physical separation of the phenotypes by shearing, as evidenced by decreased co-transformation, compliments the genetic evidence that the phenotypes are the result of separated independent mutations. The mapping data indicated that the mutations resulting in rifampin resistance and temperature sensitivity are more widely separated on the chromosome than are those resulting in a rifampin resistant-streptolydigin resistant or a streptolydigin resistant-temperature sensitive phenotype. The shearing data agree with this prediction in that the rifampin resistance-temperature sensitivity linkage by co-transformation is decreased more than the rifampin resistance -- streptolydigin resistance linkage or the streptolydigin resistance-temperature sensitivity linkage. Although the mapping data indicate that the mutations causing the $Rif^R Std^R$ phenotype are more closely linked by co-transformation than are those which result in the Std^R Ts phenotype, the shearing data could not confirm this prediction.

By centrifuging the sheared DNA preparations on Hg-Cs_2SO_4 gradients, the peak transforming activities for rifampin resistance and streptolydigin resistance were shown to reside in different fractions. The data indicate that the mutations resulting in rifampin resistance and streptolydigin resistance reside in areas of the chromosome which differ in G + C content. The extent of this

difference can be calculated by base composition analysis of each DNA containing fraction (82).

Significant enrichment for rifampin resistance transforming activity was accomplished by varying the Hg^{++} ion content in the Hg-Cs_2SO_4 gradient. If the RNA polymerase genes are contiguous, then centrifugation of DNA in Hg-Cs_2SO_4 gradients may be a useful tool for enriching for the intact polymerase genes.

Cys^+ transforming activity was also measured in the Hg-Cs_2SO_4 gradients. In both the R_f 0.19 and the R_f 0.22 gradients, two peaks of Cys^+ transforming activity were observed. The colony morphology of the Cys^+ transformants from the two separated peaks were identical and only one drug resistant recombinant was observed in the 308 transformants examined. The reason for two peaks of transforming activity is unknown.

Sporulating Cells' Sensitivity to Streptolydigin

In vivo and *in vitro* experiments to determine whether or not OSB 158 retains its sensitivity to Rif or Std during sporulation yield conflicting results. The *in vivo* experiments clearly indicate that sporulating wild type cells escape the effects of Std soon after the onset of sporulation but remain sensitive to Rif. Whether or not this apparent resistance to Std is a permeability phenomenon has not been determined. The *in vitro* data indicate that the vegetative

polymerase and the sporulating polymerase are both sensitive to Std. It is possible that the resistance of Std cannot be manifested on poly (dA-dT) template. Further _in vitro_ studies using _B. subtilis_ DNA template may clarify these conflicting data.

The _cys_ A14 Locus

In our transformation system, the _cys_ A14 locus behaves in a manner similar to that described by Harford and Sueoka (33). However, instead of the reported 60-80%, we find in our system that over 90% of the Cys^{+} transformants are of the minute colony class. Harford and Sueoka also reported, for three spontaneous rifampin resistant mutants, an average co-transformation frequency with _cys_ A14 of 63% when _cys_ was the selected marker. This frequency is over twice as great as that found for our rifampin resistant strains. The reason for this discrepancy in unknown.

As calculated from two-factor transformation data, when _cys_ is the selected marker, the average co-transformation frequency of the Cys^{+} Rif^{R} phenotype is 28% and that for the Cys^{+} Std^{R} phenotype is 15.5%. However, when only the large colony class of Cys^{+} transformants is considered, the Cys^{+} Rif^{R} phenotypes are co-transformed only 21.9% of the time and the Cys^{+} Std^{R} phenotypes are co-transformed only 10.4% of the time. These data suggest that the _cys_ A14 locus is composed of two separate mutations which will

be referred to as x^- and y^-. The genotype of the large colony Cys^+ transformant class is x^+y^+ and that of the small colony class is x^-y^+. The mapping data are consistant with the x mutation being located to the left of y.[2/] In the genetic cross OSB 154 x OSB 418 where the Cys^- phenotype was transferred into essentially an OSB 158 chromosome (OSB 418 is a mutant derived from OSB 158), the method of selection of transformants made it probable that only the y^- mutation was integrated. Until a detailed study of the complexities of the cys A14 locus are conducted, the reasons for its behavior can only be surmised.

Non-Reciprocality of Genetic Crosses

The non-reciprocality of genetic crosses in *B. subtilis* has been described by other workers (3, 31). Generally, when the marker on the right in a given pair is the selected marker, the co-transformation frequency is lower than when the marker on the left is the selected marker. Although this discrepancy makes it difficult to assign recombinational distances to the intervals between mutations, it does not affect the ordering of the mutant sites. In three-factor crosses, although the apparent linkage by co-transformation is different for

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[2/] With respect to a given marker, left refers to the chromosome origin direction and right refers to the chromosome terminus direction.

the markers depending on whether the right hand or the left hand marker is the selected marker, both sets of data are consistent with only one marker order. Spatz and Trautner (90) and Bresler *et al*. (7) have presented evidence for the existence of a heteroduplex correction system in *B. subtilis*, but this kind of mechanism does not explain the polarity of the recombination events.

Behavior of OSB 427 Temperature Sensitive Mutation in OSB 154 Recombinants

When Cys^{+} or rifampin resistant recombinants from an OSB 427 x OSB 154 genetic cross were tested at 50 C for temperature sensitivity, no temperature sensitive recombinants were observed. Instead, the pseudo temperature sensitive phenotype resulted. This behavior suggests that the temperature sensitive mutation is being suppressed by OSB 154, but it seems unlikely that this is a case of nonsense suppression.

A similar situation has been described in *E. coli* where a suppressor Q can suppress a Ts mutation in the *phe* S gene (73). The suppressor did not suppress several known amber or ochre mutations and was not gene specific.

Further analysis will be required to determine by what mechanism the OSB 427 temperature sensitive phenotype is being suppressed.

Behavior of OSB 419

OSB 419, a streptolydigin resistant, temperature sensitive mutant, does not reveal its temperature sensitive phenotype until after logrithmic growth ceases. Although the temperature sensitive phenotype does not co-transform with streptolydigin resistance, the possibility that the temperature sensitive phenotype is due to an altered polymerase has not been excluded.

There is some evidence in *E. coli* that mutations not linked with rifampin, streptovaricin, or streptolydigin resistance can still alter the expression of an RNA polymerase mutation (67). This finding is unexplained but could suggest that the RNA polymerase genes are not all clustered.

SUMMARY

EMS mutagenesis of *B. subtilis* spores results in large numbers of single point streptolydigin resistant and rifampin resistant mutants. Two temperature sensitive RNA polymerase mutants were also isolated. *In vivo* and *in vitro* measurements of RNA synthesis by isotope incorporation confirmed the genotype of the RNA polymerase mutants.

Genetic analysis by PBSl phage mediated transduction and transformation indicates that all of the phenotypic classes examined (rifampin resistant, streptovaricin resistant, streptolydigin resistant, and temperature sensitive) are clustered close to the *cys* A14 locus. Three factor genetic analysis by transformation indicates the following marker order: *cys* A14 $Rif^R (Stv^R)$ Std^R $Ts_{418} (Ts_{427})$. Extensive shearing of transforming DNA preparations indicates that the Rif^R, Std^R, and Ts phenotypes are the result of independent, physically separated mutations. Centrifugation of sheared DNA samples in Hg-Cs_2SO_4 gradients shows that the rifampin resistant mutations reside in a region of higher A + T content than do the streptolydigin resistant mutations, and that a significant enrichment of the rifampin resistance transforming activity can be achieved.

Measurements of generation times and sporulating ability indicate that the rifampin resistant and streptovaricin resistant

mutants examined are not affected by their altered RNA polymerases. The streptolydigin resistant mutants display a wide range of generation times in the presence and absence of Std and sporulate with less efficiency than do the rifampin resistant or streptovaricin resistant mutants.

In vivo sporulation studies show that sporulating wild-type cells are no longer sensitive to Std. However, *in vitro* studies indicate that the RNA polymerase from sporulating cells is sensitive to Std. This conflict in data has not been resolved.

BIBLIOGRAPHY

1. Adams, M. H. Bacteriophages. New York, Interscience Publishers, 1959. 592 p.

2. Alikhanian, S. E., V. T. Debabov, C. N. Tovmasian, S. T. Kopeickina, I. D. Nickiforova, T. A. Severina, E. S. Piruzian and V. Z. Pogosov. Temperature dependent-rifampicin resistant mutations of DNA-dependent RNA-polymerase of E. coli. In: Proceedings of the First International Lepetit Colloquium on RNA Polymerase and Transcription, Florence, 1969, ed. by L. Silvestri. Amsterdam, North-Holland Publishing Company, 1970. p. 28-36.

3. Anagnostopoulos, C. and I. P. Crawford. Transformation Studies on the linkage of markers in the tryptophan pathway in B. subtilis. Proceedings of the National Academy of Science. 47:378-390. 1961.

4. Anagnostopoulos, C. and J. Spizizen. Requirements for transformation in B. subtilis. Journal of Bacteriology 81:741-746. 1961.

5. Avila, J., J. M. Hermoso, E. Vinuela and M. Salas. Purification and properties of DNA-dependent RNA polymerase from Bacillus subtilis vegetative cells. European Journal of Biochemistry 21:526-535. 1971.

6. Borenstein, S. and E. Ephrati-Elizur. Spontaneous release of DNA in sequential genetic order by B. subtilis. Journal of Molecular Biology 45:137-152. 1969.

7. Bresler, S. E., R. A. Kreneva and V. V. Kushev. Molecular heterozygotes in B. subtilis and their correction. Molecular and General Genetics 113:204-213. 1971.

8. Brown, L. R. Unpublished research on mutagenesis of B. subtilis spores with EMS. Corvallis, Oregon State University, Department of Microbiology, 1971.

9. Brown, L. R. and R. H. Doi. Rifampin and streptovaricin resistant mutants of *Bacillus subtilis* (Abstract) Bacteriological Proceedings, 1970, p. 131.

10. Burgess, R. R., A. A. Travers, J. J. Dunn and E. K. F. Bautz. Factor stimulating transcription by RNA polymerase. Nature 221: 43-46. 1969.

11. Burgi, E. and A. D. Hershey. A relative molecular weight series derived from the nucleic acid of bacteriophage T2. Journal of Molecular Biology 3:458-472. 1961.

12. Burgi, E. and A. D. Hershey. Sedimentation rate as a measure of molecular weight of DNA. Biophysical Journal 3:309-315. 1963.

13. Cassani, R., R. Burgess and H. M. Goodman. Streptolydigin inhibition of RNA polymerase. Cold Spring Harbor Symposia on Quantitative Biology. 35:59-63. 1970.

14. Cassani, G., R. Burgess, H. Goodman and L. Gold. Inhibition of RNA polymerase by streptolydigin. Nature New Biology 230:197-200. 1971.

15. Cavalieri, L. F. and B. H. Rosenberg. Shear degradation of deoxyribonucleic acid. Journal of the American Chemical Society 81:5136-5139. 1959.

16. Chamberlin, M., J. McGrath and L. Waskell. New RNA polymerase from *E. coli* infected with bacteriophage T7. Nature 228:227-231. 1970.

17. Colli, W. and M. Oishi. Ribosomal RNA genes in bacteria: Evidence for the nature of the physical linkage between 16S and 23S RNA genes in *Bacillus subtilis*. Proceedings of the National Academy of Science. 64:642-649. 1969.

18. DeBoer, C., A. Dietz, W. S. Silver and G. M. Savage. Streptolydigin, a new antimicrobial antibiotic. I. Biologic studies of streptolydigin. In: Antibiotics Annual, ed. by F. Marti-Ibanez. 1955-56, New York, Medical Encyclopedia, 1956. p. 886-892.

19. di Mauro, E., L. Snyder, P. Marino, A. Lamberti, A. Coppo and G. P. Tocchini-Valentini. Rifampin sensitivity of the components of DNA-dependent RNA polymerase. Nature 222:535-537. 1969.

20. Doi, R. H., L. R. Brown, G. Rodgers and Y. Hsu. Bacillus subtilis mutants altered in spore morphology and in RNA polymerase activity. Proceedings of the National Academy of Science 66:404-410. 1970.

21. Dubnau, D., C. Goldthwaite, I. Smith and J. Marmur. Genetic mapping in B. subtilis. Journal of Molecular Biology 27:163-185. 1967.

22. Dubnau, D., I. Smith and J. Marmur. Gene conservation in Bacillus species, II. The location of genes concerned with the synthesis of ribosomal components and soluble RNA. Proceedings of the National Academy of Science 54:724-730. 1965.

23. Dunn, J., F. Bautz and E. Bautz. Different template specities of phage T3 and T7 RNA polymerases. Nature New Biology 230:94-96. 1971.

24. Eble, T. E., C. M. Large, W. H. DeVries, G. F. Crum and J. W. Shell. Streptolydigin, a new antimicrobial antibiotic. II. Isolation and Characterization. In: Antiobiotics Annual, ed. by F. Marti-Ibanez. 1955-56. New York, Medical Encyclopedia, 1956. p. 893-896.

25. Eigner, J. and P. Doty. The native, denatured and renatured states of deoxyribonucleic acid. Journal of Molecular Biology 12:549-580. 1965.

26. Eigner, J., C. Schildkraut and P. Doty. Concentration effects in the hydrodynamic properties of deoxyribonucleic acid. Biochimica et Biophysica Acta 55:13-21. 1962.

27. Freese, E. and P. Fortnagel. Analysis of sporulation mutants. I. Response of uracil incorporation to carbon sources, and other mutant properties. Journal of Bacteriology 94:1957-1969. 1967.

28. Ganesan, A. T. and J. Lederberg. Physical and biological studies on transforming DNA. Journal of Molecular Biology 9:683-695. 1964.

29. Georgopoulos, C. P. Bacterial mutants in which the gene N function of bacteriophage lambda is blocked have an altered RNA polymerase. Proceedings of the National Academy of Science 68:2977-2981. 1971.

30. Goff, C., and K. Weber. A T4-induced RNA polymerase α subunit modification. Cold Spring Harbor Symposia on Quantitative Biology 35:101-108. 1970.

31. Goldthwaite, C., D. Dubnau and I. Smith. Genetic mapping of antibiotic resistance in markers Bacillus subtilis. Proceedings of the National Academy of Science 65:96-103. 1970.

32. Hansen, R. S., J. A. Peterson and A. A. Yousten. Unique biochemical events in bacterial sporulation. Annual Review of Microbiology 24:53-90. 1970.

33. Harford, N. and N. Sueoka. Chromosomal location of antibiotic resistance markers in Bacillus subtilis. Journal of Molecular Biology 51:267-286. 1970.

34. Hartmann, G., K. O. Honikel, F. Knusel and J. Nuesch. The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochimica et Biophysica Acta 145:843-844. 1967.

35. Heil, Alfred and Wolfram Zillig. Reconstitution of bacterial DNA-dependent RNA-polymerase from isolated subunits as a tool for the elucidation of the role of subunits in transcription. FEBS Letters 11:165-168. 1970.

36. Hershey, A. D., E. Burgi and L. Ingraham. Sedimentation coefficient and fragility under hydrodynamic shear as measures of molecular weight of the DNA of phage T5. Biophysical Journal 2:423-431. 1962.

37. Hoch, J. A. and J. Spizizen. Genetic control of some early events in sporulation of Bacillus subtilis 168. In: Spores IV, ed. by L. L. Campbell. Bethesda, American Society for Microbiology p. 112-120. 1969.

38. Hurwitz, J., A. Bresler and R. Diringer. The enzymic incorporation of ribonucleotides into polyribonucleotides and the effect of DNA. Biochemistry and Biophysics Research Communications 3:15-19. 1960.

39. Hussey, C., R. Losick. and A. L. Sonenshein. Ribosomal RNA synthesis is turned off during sporulation of Bacillus subtilis. Journal of Molecular Biology 57:59-70. 1971.

40. Hussey, C., J. Pero, R. Shorenstein and R. Losick. In vitro synthesis of ribosomal RNA by Bacillus subtilis RNA polymerase (sporulation/hybridization/heavy stand of DNA).Proceedings of the National Academy of Science 69:407-411. 1972.

41. Johnson, J. C., M. DeBacker and J. A. Boezi. Deoxyribonucleic acid-dependent ribonucleic acid polymerase of Pseudomonas putida. Journal of Biological Chemistry 246:1222-1232. 1971.

42. Khesin, R. B., Z. M. Gorlenko, M. F. Shemyakin, S. L. Stvolinsky, S. Z. Mindlin and T. S. Ilyina. Studies on the functions of the RNA polymerase components by means of mutations. Molecular and General Genetics 105:243-261. 1969.

43. Khesin, R. B., S. Z. Mindlin, T. S. Ilyina, M. I. Ovadis and Zh. M. Gorlenko. Mechanism of dominance of RNA-polymerase structural gene alleles causing sensitivity to antibiotics. Molecular Biology 5:862-877. 1971. (Translated from Molekulyarnaya Biologiya)

44. Korch, C. T. and R. H. Doi. Electron microscopy of the altered spore morphology of a ribonucleic acid polymerase mutant of Bacillus subtilis. Journal of Bacteriology 105: 1110-1118. 1971.

45. Kornberg, A., J. A. Spudich, D. L. Nelson and M. P. Deutscher. Origin of proteins in sporulation. Annual Review of Biochemistry 37:51-78. 1968.

46. Krakow, J. S. and K. von der Helm. Azotobacter RNA polymerase transitions an the release of sigma. Cold Spring Harbor Symposia on Quantitative Biology 35:73-83. 1970.

47. Leighton, T. J. Unpublished research on mutagenesis of B. subtilis spores with EMS. Davis, University of California, Department of Biochemistry, 1971.

48. Leighton, T. J., P. K. Freese, R. H. Doi, R. A. J. Warren and R. A. Kelln. Initiation of sporulation in Bacillus subtilis: Requirement for serine protease activity and ribonucleic acid polymerase modification. In: Spores V, ed. by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington, D. C., American Society for Microbiology, 1972, p. 238-246.

49. Lewis, C., J. R. Wilkins, D. F. Schwartz and C. T. Nikitas. Streptolydigin, a new antimicrobial antibiotic. III. In vitro and in vivo laboratory studies. In: Antibiotics Annual, ed. by F. Marti-Ibanez. 1955-56. New York, Medical Encyclopedia, 1956. p. 897-902.

50. Lill, H., U. Lill, A. Sippel and G. Hartmann. The inhibition of the RNA polymerase reaction by rifampicin. In: Proceedings of the First International Lepetit Colloquium on RNA Polymerase and Transcription, Florence, 1969, ed. by L. Silvestri. Amsterdam, North-Holland Publishing Company, 1969. p. 55-70.

51. Losick, R., R. G. Shorenstien and A. L. Sonenshein. . Structural alteration of RNA polymerase during sporulation. Nature 227:910-193.

52. Losick, R. and A. L. Sonenshein. Change in template specificity of RNA polymerase during sporulation of Bacillus subtilis. Nature 244:35-37. 1969

53. Ludlum, D. B. and R. C. Warner. Equilibrium centrifugation in cesium sulfate solutions. Journal of Biological Chemistry 240:2961-2965. 1965.

54. Maggi, N., C. R. Pasqualucci, R. Ballotta and P. Sensi. Rifampicin: A new orally active rifamycin. Chemotherapia 11:285-292. 1966.

55. Maia, J. C. C., P. Kerjan and J. Szulmajster. DNA dependent RNA polymerase from vegetative cells and spores of Bacillus subtilis. FEBS Letters 13:269-274. 1971.

56. Mandelstam, J. Regulation of bacterial spore formation. Symposium of the Society for General Microbiology 19:377-401. 1969.

57. Marmur, J. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. Journal of Molecular Biology 3:208-218. 1961.

58. Mindlin, S. Z., T. S. Ilyina, T. A. Voeykova and V. V. Velkov. Genetical analysis of rifampicin resistant mutants of E. coli K12. Molecular and General Genetics 115: 115-121. 1972.

59. Mizuno, S., H. Yamazaki, K. Nitta and H. Umezawa. Inhibition of initiation of DNA-dependent RNA synthesis. Biochemistry and Biophysics Research Communications 30:379-385. 1968.

60. Mizuno, S., H. Yamazaki, K. Nitta and H. Umezawa. Inhibition of DNA-dependent RNA polymerase reaction of E. coli by streptovaricin. Biochimica et Biophysica Acta 157:322-332. 1968.

61. Munakata, H., H. Saito and Y. Ikeda. Inactivation of transforming DNA by ultraviolet light. Mutation Research 3:93-100. 1966.

62. Nandi, U. S., J. C. Wang and N. Davidson. Separation of deoxyribonucleic acids by Hg(II) binding and Cs_2SO_4 density-gradient centrifugation. Biochemistry 4:1687-1696. 1965.

63. Oishi, M., A. Oishi and N. Sueoka. Location of genetic loci of soluble RNA on B. subtilis chromosome. Proceedings of the National Academy of Science 55:1095-1103. 1966.

64. Oishi, M. and N. Sueoka. Location of genetic loci of ribosomal RNA on B. subtilis chromosome. Proceedings of the National Academy of Science 54:483-491. 1965.

65. O'Sullivan, A. and N. Sueoka. Sequential replication of the B. subtilis chromosome, IV. Genetic mapping by density transfer experiment. Journal of Molecular Biology 27:349-368. 1967.

66. O'Sullivan, A., H. Yoshikawa and N. Sueoka. Sequential replication of the Bacillus subtilis chromosome, III. Regulation of initiation. Proceedings of the National Academy of Science 52:973-980. 1964.

67. Patterson, D., M. Weinstein, S. Marshall and D. Gillespie. A new RNA synthesis mutant of E. coli. Biochemical Genetics 5:563-578. 1971.

68. Pivec, L., H. Pivcova and Z. Sormova. Plurimodal heterogeneity of base composition of DNA isolated from Bacillus subtilis. Biochimica et Biophysica Acta 213: 343-351. 1970.

69. V. Prelog. Constitution of rifamycins. Pure and Applied Chemistry 7:551-564. 1963.

70. Rinehart, K. L., J. R. Beck, D. B. Borders, T. H. Kinstle and D. Krauss. Streptolydigin, III. Chromophore and structure. Journal of the American Chemical Society 85:4038-4039. 1963.

71. Rogolsky, M. Chromosomal regions which control sporulation in Bacillus subtilis. Canadian Journal of Microbiology 15:787-790. 1969.

72. Rosenkranz, H. S. and A. Bendich. Physiochemical effects of high-speed mixing on deoxyribonucleic acid. Journal of the American Chemical Society 82:3198-3201. 1960.

73. Russell, R. R. B. and A. J. Pittard. New suppressor in Escherichia coli. Journal of Bacteriology 107:736-740. 1971.

74. Saito, H. and Y. Mosamune. Fractionation of transforming deoxyribonucleic acid of Bacillus subtilis with the methylated-albumin column. Biochimica et Biophysica Acta 91: 344-347. 1964.

75. Schleif, Robert. Isolation and characterization of a streptolydigin resistant RNA polymerase. Nature 223:1068-1069. 1969.

76. Seifert, W., P. Qasba, G. Walter, P. Palm, M. Schachner and W. Zillig. Kinetics of the alteration and modification of DNA-dependent RNA polymerase in T4-infected E. coli cells. European Journal of Biochemistry 9:319-324. 1969.

77. Sensi, P., A. M. Greco and R. Ballotta. Rifomycin I. Isolation and properties of rifomycin B and rifomycin complex. In: Antibiotics Annual, ed. by F. Marti-Ibanez. 1959-60. New York, Antibiotica, 1960. p. 262-271.

78. Shooter, K. V. and J. A. V. Butler. Sedimentation of deoxyribonucleic acid at low concentrations. Transactions of the Faraday Society 52:734-742. 1956.

79. Siddhikol, C., J. Erbstoeszer and B. Weisblum. Mode of action of streptolydigin. Journal of Bacteriology 99: 151-155. 1969.

80. Siminoff, P., R. M. Smith, W. T. Sokolski and G. M. Savage. Streptovaricin: I. Discovery and biologic activity. American Review of Tuberculosis 75:576-583. 1957.

81. Sippel, A. and G. Hartmann. Mode of action of rifamycin on the RNA polymerase reaction. Biochimica et Biophysica Acta 157:218-219. 1968.

82. Skalka, A. A method for the breakage of DNA and resolution of the fragments. In: Methods of Enzymology, ed. by L. Grossman and K. Moldave. Vol. 12. New York, Academic Press, 1970. p. 315-320.

83. Smith, I., W. Colli and M. Oishi. Studies on the physical linkage of antibiotic resistance markers to ribosonal RNA genes in Bacillus subtilis. Journal of Molecular Biology 62:111-119. 1971.

84. Smith, I., D. Dubnau, P. Morell and J. Marmur. Chromomal location of DNA base sequences complementary to transfer RNA and to 5S, 16S, 23S ribosomal RNA in Bacillus subtilis. Journal of Molecular Biology 33:123-140. 1968.

85. Smith, I., C. Goldthwaite and D. Dubnau. The genetics of ribosomes in Bacillus subtilis. Cold Spring Harbor Symposia on Quantitative Biology 34:85-89. 1969.

86. So, A. G. and K. M. Downey. Studies on the mechanism of ribonucleic acid synthesis. II. Stabilization of the deoxyribonucleic acid-ribonucleic acid polymerase complex by the formation of a single phosphodiester bond. Biochemistry 9:4788-4793. 1970.

87. Sokolova, E. V., M. I. Ovodis, Zh. M. Gorlenko and R. B. Khesin. Localization of streptolydigin resistant mutations. Biochemical and Biophysical Research Communications 41:870-876. 1970.

88. Sokolski, W. T., N. J. Eilers and P. Siminoff. Paper chromatography and assay of components in streptovaricin. In: Antibiotics Annual, ed. by F. Marti-Ibanez. 1957-58. New York, Medical Encyclopedia, 1958. p. 119-125.

89. Sonenshein, A. L. and R. Losick. RNA polymerase mutants blocked in sporulation. Nature 227:906-909. 1970.

90. Spatz, H. Ch. and T. A. Trautner. One way to do experiments on gene conversion? Transfection with heteroduplex SPP1 DNA. Molecular and General Genetics 109:84-106. 1970.

91. Spizizen, John. Transformation of biochemically deficient strains of B. subtilis by deoxyribonucleate. Proceedings of the National Academy of Science 44:1072-1078. 1958.

92. Spizizen, J., B. E. Reilly and A. H. Evans. Microbial transformation and transfection. Annual Review of Microbiology 20:371-400. 1966.

93. Stevens, A. Incorporation of the adenine ribonucleotide into RNA by cell fractions from E. coli B. Biochemistry and Biophysics Research Communications 3:92-96. 1960.

94. Stewart, C. R. Physical heterogeneity among Bacillus subtilis deoxyribonucleic acid molecules carrying particular genetic markers. Journal of Bacteriology 98:1239-1247. 1969.

95. Takahashi, I. Transducing phages for *B. subtilis*. Journal of General Microbiology 31:211-217. 1963.

96. Takahashi, I. Localization of spore markers on the chromosome of *B. subtilis*. Journal of Bacteriology 89: 1065-1067. 1965.

97. Takahashi, H. and Y. Ikeda. The intramolecular heterogeneity of *Bacillus subtilis* DNA studied by isopycnic CsCl gradient. Biochimica et Biophysica Acta 213:523-525. 1970.

98. Umezawa, H., S. Mizuno, H. Yamazaki and K. Nitta. Inhibition of DNA-dependent RNA synthesis by rifamycins. Journal of Antibiotics 21:234-236. 1968.

99. Von Der Helm, K. and J. Krakow. Inhibition of RNA polymerase by streptolydigin. Nature New Biology 235: 82-83. 1972.

100. Walter, G., W. Seifert and W. Zillig. Modified DNA-dependent RNA polymerase from *E. coli* infected with bacteriophage T4. Biochemistry and Biophysics Research Communications 30:240-247. 1968.

101. Wehrli, W., J. Nuesch, F. Knusel and M. Staehelin. Action of rifamycins on RNA polymerase. Biochimica et Biophysica Acta 157:215-217. 1968.

102. Wilson, G. and K. Bott. Nutritional factors influencing the development of competence in the *B. subtilis* transformation system. Journal of Bacteriology 95:1439-1449. 1968.

103. Yamagishi, H. Nucleotide distribution in the DNA of *Escherichia coli*. Journal of Molecular Biology 49:603-608. 1970.

104. Yamagishi, H. and I. Takahashi. Heterogeneity in nucleotide composition of *B. subtilis* DNA. Journal of Molecular Biology 57:369-371. 1971.

105. Yamakawa, T. and R. H. Doi. Preferential transcription of *Bacillus subtilis* light deoxyribonucleic acid strands during sporulation. Journal of Molecular Biology 305-310. 1971.

106. Yamazaki, H. Studies of antimicrobial substance B44P (streptovaricin) produced by a strain of _Actinomycetes_. I. Production extraction and characteristics of substance B44P and the identity of the substance with streptovaricin. Journal of Antibiotics 21:204-208. 1968.

107. Yamazaki, H. Studies on antimicrobial stubstance B44P (streptovaricin) produced by a strain of _Actinomycetes_. II. Microbiológical and pharmacological studies. Journal of Antibiotics 21:209-221. 1968.

108. Yoshikawa, H. and S. Noboru. Sequential replication of _B. subtilis_ chromosome. I. Comparison of marker frequencies in exponential and stationary growth phases. Proceedings of the National Academy of Science 49:559-566. 1963.

109. Yoshikawa, H. and N. Sueoka. Sequential replication of the _B. subtilis_ chromosome. II. Isotopic transfer experiments. Proceedings of the National Academy of Science 49:806-813. 1963.

110. Young, F. E. and G. A. Wilson. Genetics of _Bacillus subtilis_ and other gram-positive sporulating bacilli. In: Spores V, ed. by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington, D. C., American Society for Microbiology, 1972. p. 77-106.

111. Yura, T. and K. Igarasahi. RNA polymerase mutants of _E. coli_. I. Mutants resistant to streptovaricin. Proceedings of the National Academy of Science 61:1313-1319. 1968.

112. Yura, T., K. Igarashi and K. Masukata. Temperature-sensitive RNA polymerase mutants of _Escherichia coli_. in: Proceedings of the First International Lepetit Colloquium on RNA Polymerase and Transcription, Florence, 1969, ed. by L. Silvestri. Amsterdam, North-Holland Publishing Company, 1970. p. 71-89.

113. Zillig, W., K. Zechel, D. Rabussay, M. Schachner, V. S. Sethi, P. Palm, A. Heil and W. Seifert. On the role of different subunits of DNA-dependent RNA polymerase from _E. coli_ in the transcription process. Cold Spring Harbor Symposia on Quantitative Biology 35:47-58. 1970.

114. Zubay, G., D. Schwartz and J. Beckwith. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proceedings of the National Academy of Science 66:104-110. 1970.