AN ABSTRACT OF THE THESIS OF

DARREL DEAN GWINN (name) for the M. S. (degree) in MICROBIOLOGY (major)

Date thesis is presented August 6, 1963

Title STUDIES ON TRANSFORMATION OF BACILLUS LICHENIFORMIS

Redacted for Privacy

Abstract approved (Major Professor)

The original purpose of this research was to study the metabolic pathway to the synthesis of glutamyl polypeptide in B. licheniformis. Colonies of B. licheniformis 9945A, when grown on an appropriate medium appear very smooth as a result of glutamyl polypeptide accumulation. The plan of approach was to obtain mutants blocked at various steps in the route of synthesis and to attempt to identify the blocked reactions by searching for accumulation of precursors by certain classes of mutants and utilization of the accumulated products by other classes of mutants. Two transducing phages for B. licheniformis were available and the plan of approach included the use of these phages to distinguish classes of mutants by transduction. However, with the low frequencies of transduction which were characteristic of this sytem and without a specific method for selecting cells that were transduced for the ability

to synthesize peptide, it soon became apparent that a different procedure would have to be used.

Since frequencies of transformation are, in general, much higher than frequencies of transduction, it seemed logical to use transformation as the system of genetic transfer. Transformation had not been reported in B. licheniformis, but it was expected that techniques which gave good results with B. subtilis would work with B. licheniformis as well. It was soon learned, however, that B. licheniformis behaved very differently from B. subtilis, and because of this, the problem became one of how to get transformation to occur in B. licheniformis.

Auxotrophic mutants and rough mutants of B. licheniformis 9945A were isolated and a number of experiments were run in unsuccessful attempts to transform them. The conditions for growth of the recipient cells, the media, and the mutants were manipulated in trying to obtain a transforming system. In most of the later experiments efforts were concentrated on attempts to transfer nutritional markers since selection methods for these markers presented no problem and the probability of detecting transformants occurring at low frequencies would be increased. All of these attempts to transform B. licheniformis failed.

The next approach was based on the assumption that it should be possible to isolate transformable mutants.

The plan was to isolate numerous mutants and to screen them for transformation. The screening method consisted of spreading 0.1 ml of a culture and 0.05 ml of DNA, prepared from wild type 9945A, on a minimal agar plate. Control plates included one with cells alone and one with cells and DNA plus DNase. The plates were incubated and observed daily for four or five days. This approach turned out to be very fuitful within a short period of time. Three auxotrophs, 9945A-M28 (glycine$^-$), -M30 (uncharacterized), and -M33 (purine$^-$), produced transformants. M28 transformed at a much higher frequency than M30 or M33 and for that reason, it was studied in greater detail.

The transformants of 9945A-M28 were of two colonial types on minimal agar; a few of them synthesized peptide and were smooth and a larger number did not synthesize peptide and were rough. DNA was prepared from the two types of transformants and from 9945A-M28. No transformants were produced with the DNA isolated from the mutant. Transformants were produced with the DNA isolated from the two types of transformants. The transformants that were produced with the DNA from the rough type were all of the rough colony type, whereas the transformants produced with the DNA from the smooth type included both rough and smooth colony types.

Cells of 9945A-M28$ when spread on minimal agar plates became competent after a period of incubation and transformed well when 9945A DNA was present. The most competent M28$ cells for transformation in liquid medium were from 22-hour cultures grown in minimal medium salts plus nutrient broth and glycerol and the highest frequency of transformation occurred when the DNA-cell mixture was incubated in a serum bottle on a rotary shaker for 1 hour.

Transformation was obtained with five doubly-marked auxotrophs of 9945A-M28$. Glycine^{+} transformants were obtained with each of the five mutants and transformants for three of the other markers, serine, histidine, and leucine, were also obtained. However, transformants for adenine or tryptophan were not detected.

From the above experiment, glycine^{+}serine^{-}, glycine^{+} leucine^{-}, and glycine^{+}histidine^{-} transformants were isolated and tested for transformability. Each of the three isolates, although glycine independent, was transformed to prototrophy. These results, which show that transformation was not specific for the glycine marker, suggested that M28$ carries, in addition to the mutation responsible for glycine dependence, a second unidentified mutation which renders it amenable to transformation.

STUDIES ON TRANSFORMATION OF *BACILLUS LICHENIFORMIS*

by

DARREL DEAN GWINN

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1964

APPROVED: Redacted for Privacy

Professor of Microbiology

In Charge of Major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented August 6, 1963

Typed by Ruth Baines

ACKNOWLEDGMENTS

The author wishes to express his appreciation:

To Dr. Curtis B. Thorne for his guidance and advice throughout this investigation.

To the members of the Microbiology Department for their assistance.

To his mother for her patience and understanding.

TABLE OF CONTENTS

Table of Contents - continued

LIST OF ILLUSTRATIONS

LIST OF TABLES

List of Tables - continued

STUDIES ON TRANSFORMATION OF *BACILLUS LICHENIFORMIS*

INTRODUCTION

Bacillus licheniformis strain 9945A synthesizes a large amount of glutamyl polypeptide during growth in a synthetic medium, with the peptide occurring free in the medium. *Bacillus anthracis* produces a capsule composed largely, if not entirely, of a similar glutamyl polypeptide. The capsule is thought to be one of the factors which makes *B. anthracis* virulent.

The original purpose of this research was to study the metabolic pathway to the synthesis of glutamyl polypeptide in *B. licheniformis*. The plan of approach was to obtain mutants blocked at various steps in the route of synthesis and to attempt to identify the blocked reactions by searching for accumulation of precursors by certain classes of mutants and utilization of the accumulated products by other classes of mutants. Two transducing phages for *B. licheniformis* were available (21, in press), and the plan of approach included the use of these phages to distinguish classes of mutants by transduction. However, with the low frequencies of transduction which were characteristic of this system and without a specific method for selecting cells that were transduced for the

ability to synthesize peptide, it soon became apparent, that a different procedure would have to be used.

Since frequencies of transformation are, in general, much higher than frequencies of transduction, it seemed logical to use transformation as the system of genetic transfer. Transformation of B. licheniformis had not been reported, but it was expected that techniques which gave good results with Bacillus subtilis would be applicable to B. licheniformis as well. It was learned, however, that B. licheniformis behaved very differently from B. subtilis, and because of this, the problem became one of how to get transformation to occur in B. licheniformis.

HISTORICAL

The first report of genetic exchange between bacteria (the researcher didn't know this was the phenomenon that was occurring) was made by the English bacteriologist, Griffith (9, 115-159) in 1928. Griffith injected viable avirulent and heat-killed virulent pneumococci subcutaneously into mice and found that the injected animals died of pneumonia. Virulent encapsulated pneumococci were recovered from the dead mice. The significant finding was that the type of virulent strain recovered did not correspond to the type of virulent strain from which the living, unencapsulated strain used in the experiment had been derived. It corresponded, however, to the type of virulent strain from which the heat-killed vaccine had been prepared. Avery and his co-workers (4, 137-158) in 1944 concluded after over 10 years of work that DNA was the agent which transmitted genetic information and which transformed the unencapsulated avirulent cells to encapsulated virulent cells.

Transformations have been reported in the following genera: Diplococcus, Streptococcus, Hemophilus, Neisseria, Escherichia, Agrobacterium, Xanthomonas, Bacillus, Rhizobium, Staphylococcus, (18, 65-69). Several other reports of genetic transformation

of bacteria have been made, but further data are needed before the phenomena reported can be related properly to other observations in the field of bacterial transformations (3, 31-50).

In the genus Bacillus, the species subtilis (19, 1072-1078), natto, and subtilis variety aterrimus (14, 461-467) have been transformed. It has been found that phage-mediated transduction of genetic characters occurs in Bacillus subtilis (22, 106-111) and in Bacillus licheniformis (21, in press). With the exception of the results in this thesis there have been no reports of transformation in B. licheniformis.

The variety of genetic characters capable of being transformed does not appear to be limited in any way to a certain class or category. Whatever be the species of bacterium in which transformation is studied, one generally finds that any character which can be conveniently investigated is susceptible to hereditary transformation by the appropriate DNA agent (18, 69-71). Examples of some nutritional markers that have been transformed in Bacillus subtilis are indole, anthranilic acid, nicotinic acid (18, 71), various amino acids (27, 559-566), uracil, purines, quinolinic acid and niacin (8, 407). Some characters other than nutritional characters are sporulation (18, 71), sulfanilamide resistance (6, 56-63), the ability to grow at 55°C (16, 218-220), mutation rates (28, 944-947),

streptomycin resistance (10, 327-328), and flagellation (17, 43).

The term competence is defined as the capacity of cells to take up DNA irreversibly and to undergo transformation. The transformable genera and species become competent at different points in their respective growth cycles and each has optimum growth conditions and growth medium for maximum competence. The physiological state of competence seems to be in some way connected with the mechanism of DNA penetration through the cell wall. Two hypotheses have been proposed to account for penetration: one, termed the "localized protoplast" hypothesis and the other, the "enzymic receptor" hypothesis (18, 100-111). The "localized protoplast" hypothesis regards the competent cell as one having at some period in its life cycle naked areas on the surface through which DNA can penetrate. According to the "enzymic receptor" hypothesis, competent cells produce on their surface an enzyme which catalyzes DNA uptake.

According to Fox (7, 1043-1048) the recombination act in transformation is completed in the absence of any DNA synthesis in the region of the newly introduced element of DNA. In addition, the segment that is integrated must be at least 900 nucleotide pairs in length and is perhaps

much longer. Following its integration, this DNA appears to replicate in synchrony with the host DNA.

The glutamyl polypeptide produced by B. anthracis is contained mostly in a capsule and is released into the medium upon autoclaving or upon aging and autolysis of the cells, while that produced by B. licheniformis occurs free in the medium (23, 68-80). The peptide from B. anthracis is composed mainly, if not entirely, of the D-isomer of glutamic acid. B. licheniformis, however, produces two glutamyl peptides, one containing only the D-isomer and the other containing only the L-isomer (26, 1109-1112). The proportion of D-glutamic acid in the total peptide increased from 39 to 84 per cent with increasing concentrations of Mn^{++} in the medium (11, 499-503). An enzyme, glutamyl transferase, present in culture filtrates of B. licheniformis, hydrolyzes the polypeptide and also catalyzes the synthesis of glutamyl peptides by a transfer reaction. When reaction mixtures containing enzyme, L-glutamine, and D-glutamic acid were allowed to incubate for several hours, peptides of a chain length greater than that of a dipeptide were synthesized (23, 68-80). However, the synthesis of high molecular weight peptides by the transferase could not be demonstrated, and it seems unlikely that this enzyme is involved in the in vivo synthesis of peptide.

The optimum medium for production of glutamyl polypeptide by growing cultures of B. licheniformis contains a large amount of L-glutamic acid, but the glutamic acid of the polypeptide contains a large proportion of the D-isomer. A glutamic acid racemase has not been demonstrated in this organism, but alanine racemase and D-amino acid transaminases have been demonstrated. The following reactions, which occur in the presence of extracts of B. licheniformis and B. anthracis could result in the indirect conversion of L-glutamic acid to the D-isomer.

(1) L-glutamic acid + pyruvic acid $\rightleftharpoons$ α-ketoglaturic acid + L-alanine,

(2) L-alanine $\longrightarrow$ DL-alanine,

(3) D-alanine + α-ketoglutaric acid $\rightleftharpoons$ pyruvic acid + D-glutamic acid (23, 68-80).

METHODS AND MATERIALS

Cultures

Bacillus licheniformis strains ATCC 9945A and ATCC 10716 and *Bacillus subtilis* strains 168 (indole$^-$) and W-23-S^r were obtained from Dr. Curtis B. Thorne. Rough mutants (unable to synthesize glutamyl polypeptide) and auxotrophic mutants derived from *B. licheniformis* 9945A were isolated following irradiation by ultraviolet light. The auxotrophic mutants of 9945A are listed in Tables 1 and 2. Mutants of strain 10716 requiring methionine and isoleucine, respectively, were also isolated following ultraviolet irradiation. Phages Sp-5, Sp-10, and Sp-15 were obtained from Dr. Thorne.

Media

NBY medium was composed of 8 g of Difco nutrient broth and 3 g of Difco yeast extract per liter. NBYG medium was NBY with 1 g of glucose per liter.

Potato medium (22, 106-111) was prepared as follows: Diced potatoes (200 g) were boiled 5 minutes in 1 liter of water and the material was filtered through Whatman No. 1 paper on a Buchner funnel. Two g of Difco yeast extract and 20 g of N-Z Case peptone were added. The pH was adjusted to 7.2 with HCl and/or NaOH and the solution was diluted to 2 liters. Potato agar was made by adding

Table 1

Auxotrophic mutants of 9945A with single markers

Mutant	Requirement
9945A-M1	Thiamine
M2	Lysine
M3	Lysine
M4	Arginine
M5	Undiagnosed
M6	Arginine
M8	Methionine
M9	Undiagnosed
M10	Hypoxanthine
M11	Undiagnosed
M12	Undiagnosed
M14	Methionine
M15	Undiagnosed
M16	Uracil
M17	Undiagnosed
M19	Adenine
M22	Undiagnosed
M24	Tryptophan
M26	Histidine
M28	Glycine
M29	Undiagnosed
M30	Undiagnosed
M32	Undiagnosed
M33	Purine
M34	Undiagnosed
M35	Arginine
M36	Undiagnosed
M37	Undiagnosed

Table 2

Auxotrophic mutants of 9945A with double markers

Mutant	Requirements
9945A-M28-D1	Glycine + Tryptophan
D2	" + Histidine
D3	" + Histidine
D4	" + Adenine
D5	" + Histidine
D6	" + Serine
D7	" + Serine
D8	" + Threonine
D9	" + Threonine
D10	" + Guanine
D11	" + Adenine
D12	" + Unknown
D13	" + Adenine
D14	" + Leucine
D15	" + Adenine
D16	" + Unknown
D17	" + Uracil
D18	" + Histidine
D19	" + Lysine
D20	" + Unknown
D21	" + Unknown
D22	" + Unknown
D23	" + Uracil
D24	" + Unknown
D25	" + Unknown
D26	" + Unknown
D27	" + Unknown
D28	" + Unknown
D29	" + Tryptophan
D30	" + Unknown
D31	" + Unknown
D32	" + Serine
D33	" + Unknown
D34	" + Unknown
D35	" + Unknown
D36	" + Unknown
D37	" + Unknown

20 g of agar per liter.

Medium E (11, 499-509) contained 20 g of L-glutamic acid, 12 g of citric acid, 80 g of glycerol, 7 g of NH_4Cl, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $FeCl_3 \cdot 5H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $MnSO_4 \cdot H_2O$ per liter. The pH of the medium was adjusted to 7.4 with HCl and/or NaOH.

Minimal medium (2, 741-746) was composed of 2 g of $(NH_4)_2SO_4$, 6 g of KH_2PO_4, 14 g of K_2HPO_4, 1 g of sodium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$ and 5 g of glucose (added aseptically after sterilization) per liter. For the initial growth of a competent culture of B. subtilis 168 (indole$^-$) the minimal medium was supplemented with 0.05 g of tryptophan and 0.2 g of casamino acids per liter. The final growth of the culture was in minimal supplemented with 0.005 g of tryptophan and 0.1 g of casamino acids per liter and an additional amount of $MgSO_4$ (5 μmoles per ml).

Minimal I medium (22, 106-111) was composed of 2 g of $(NH_4)_2SO_4$, 6 g of KH_2PO_4, 14 g of K_2HPO_4, 1 g of sodium citrate, 5 g of glucose (autoclaved separately), 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $FeCl_3 \cdot 6H_2O$, 0.00025 g of $MnSO_4 \cdot H_2O$, and 1 g of L-glutamic acid per liter. Three g of Difco yeast extract per liter was added to assay mutants which needed added growth factors. The pH of the medium

was adjusted to 6.9-7.0 with HCl and/or NaOH. Enriched minimal I had 2.5 ml of nutrient broth added per 100 ml.

Phage assay agar (22, 106-111) was the medium used for titrating phage suspensions by the soft agar overlay method described by Adams (1, 27-34). The technique is one of overlaying hard agar with soft agar. The bottom or hard layer was composed of 8 g of Difco nutrient broth, 5 g of Nacl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$ and 15 g of agar per liter. The pH was adjusted to 5.9-6.0 with HCl. The soft agar for overlaying contained the same constituents as the hard agar with the exception that it had only 7 g of agar per liter.

NBSG medium was prepared by adding 8 g of Difco nutrient broth and 5 g of glycerol per liter of minimal I medium with glucose omitted.

Tryptose blood agar base was composed of 40 g of Difco blood agar base per liter.

The standard diluent used for diluting cells and phage contained 5 g of Difco peptone per liter of distilled water.

Preparation of DNA

The following procedure is essentially that of Marmur (13, 208-218). Each of two 500-ml Erlenmeyer flasks containing 100 ml of NBY broth was inoculated with 0.1 ml (10^8 spores) of spore suspension and the flasks were incubated on the shaker at 37°C for 16 hours. The resulting

cultures were pooled and centrifuged at 4080 x g for 15 minutes. The supernatant fluid was discarded and the cells were resuspended in 5 ml of saline (0.15 M) - Versene (0.1M) at pH 8.0 in a screw cap tube. The suspension was mixed thoroughly with a Vortex Jr. mixer. One ml of lysozyme (40 mg/ml; 3 x crystalized preparation from egg white obtained from California Corporation for Biochemical Research) was added to the suspension and it was again mixed with the mixer. The tube was incubated in a 37°C water bath with periodic mixing with the Vortex Jr. mixer until the suspension became homogeneously viscous. Four-tenths ml of a saturated solution of Dupanol (sodium lauryl sulfate) in 45% ethanol was added and incubation was continued with occasional mixing until the contents of the tube became clear. One and three-tenths ml of 5 M $NaClO_4$ was added and mixed (with wrist action) and then 6 ml of chloroform and 0.3 ml of isoamyl alcohol were added and the tube was shaken (with wrist action) for 10 minutes. The mixture was centrifuged at 7710 x g and the top layer, aqueous phase, was removed (with a pipet and rubber bulb) and transferred into an eight-inch test tube. Two volumes of 95% ethanol were layered on top of the aqueous phase and a sterile glass rod (wet with saline) was inserted to the bottom of the tube. The tube was held at a 45° angle while the rod was wound slowly around the inside until no

more precipitate seemed to be forming on it. Then the DNA precipitate was dissolved in 5 ml of 2 M NaCl in a screw cap tube. The DNA preparation was sterilized by adding 2.2 ml of melted phenol (making the solution 30% v/v phenol) and incubating with periodic shaking in a 65°C water bath for 30 minutes. The entire DNA-phenol solution was taken up into a 10-ml pipet and blown into an eight-inch test tube containing 40 ml of 95% ethanol. The resulting DNA clump was removed with a sterile glass rod (wet with saline) and dissolved in 5 ml of 2M NaCl in a screw cap tube. The DNA preparation was checked for sterility by spreading 0.2 ml of it onto a nutrient agar plate which was then incubated at 37°C for 16 hours. Preparations were stored at 4°C.

Irradiation with ultraviolet light

The light source was two 15-watt General Electric germicidal tubes. Unless otherwise stated, materials were irradiated in a flat bottom petri dish placed on a rotating platform 16 inches from the light source.

Isolation of auxotrophic mutants of B. licheniformis

Five ml of spore suspension (10^9 spores/ml) was exposed to ultraviolet light for one minute. The 5 ml of irradiated spore suspension was added to a 250-ml flask containing 50 ml of minimal I medium and the flask was

incubated on a rotary shaker at 37°C for about 10 hours. The resulting culture was held at 65°C for 30 minutes to kill the germinated prototrophs. The culture was then plated on enriched minimal I agar at a dilution that gave two or three hundred colonies per plate. Any tiny colonies that came up among the large prototrophic colonies after an appropriate incubation time were picked and tested to see whether they were auxotrophs.

Characterization of auxotrophic mutants

It is possible to diagnose the growth requirements of auxotrophic mutants by replica plating them onto plates of minimal I medium supplemented with appropriate nutrients. Stock solutions of the nutrients were made up in distilled water at a concentration of 2 mg of each constituent per ml and used in a final concentration of 20 μg per ml (thiamine was used at a final concentration of 4 μg per ml). Pools of these substances were set up as in Table 3. If an auxotroph grows on pools 3 and 8 only, for example, then it probably requires tryptophan. If an auxotroph won't grow on any of the pools, it probably requires more than one substance, or it may require other growth factors which are not included in the pools. A diagnosis can be confirmed by spreading cells of the auxotroph on a minimal agar plate and placing on the agar a filter paper disc saturated with a solution of the suspected compound. If

Table 3

Nutrient pools for characterization of auxotrophic mutants

Pool Number	1	2	3	4	5
6	adenine	guanine	cysteine	methionine	thiamine
7	histidine	leucine	isoleucine	valine	lysine
8	phenylalanine	tyrosine	tryptophan	threonine	proline
9	hypoxanthine	serine	uracil	glycine	arginine

the diagnosis is correct, the cells will grow near the disc but not elsewhere on the plate.

Isolation of rough mutants

Spores of 9945A were spread on plates of medium E agar which were then incubated for 14 hours. Six plates, each having about 100 colonies, were irradiated with ultraviolet light for one minute and incubation was continued for 20 hours more. Only 5 colonies survived the irradiation and each of them appeared to be rough. The colonies were isolated and spore suspensions were prepared. They were designated 9945A-R_1, R_2, R_3, R_4, and R_5.

Determination of DNA

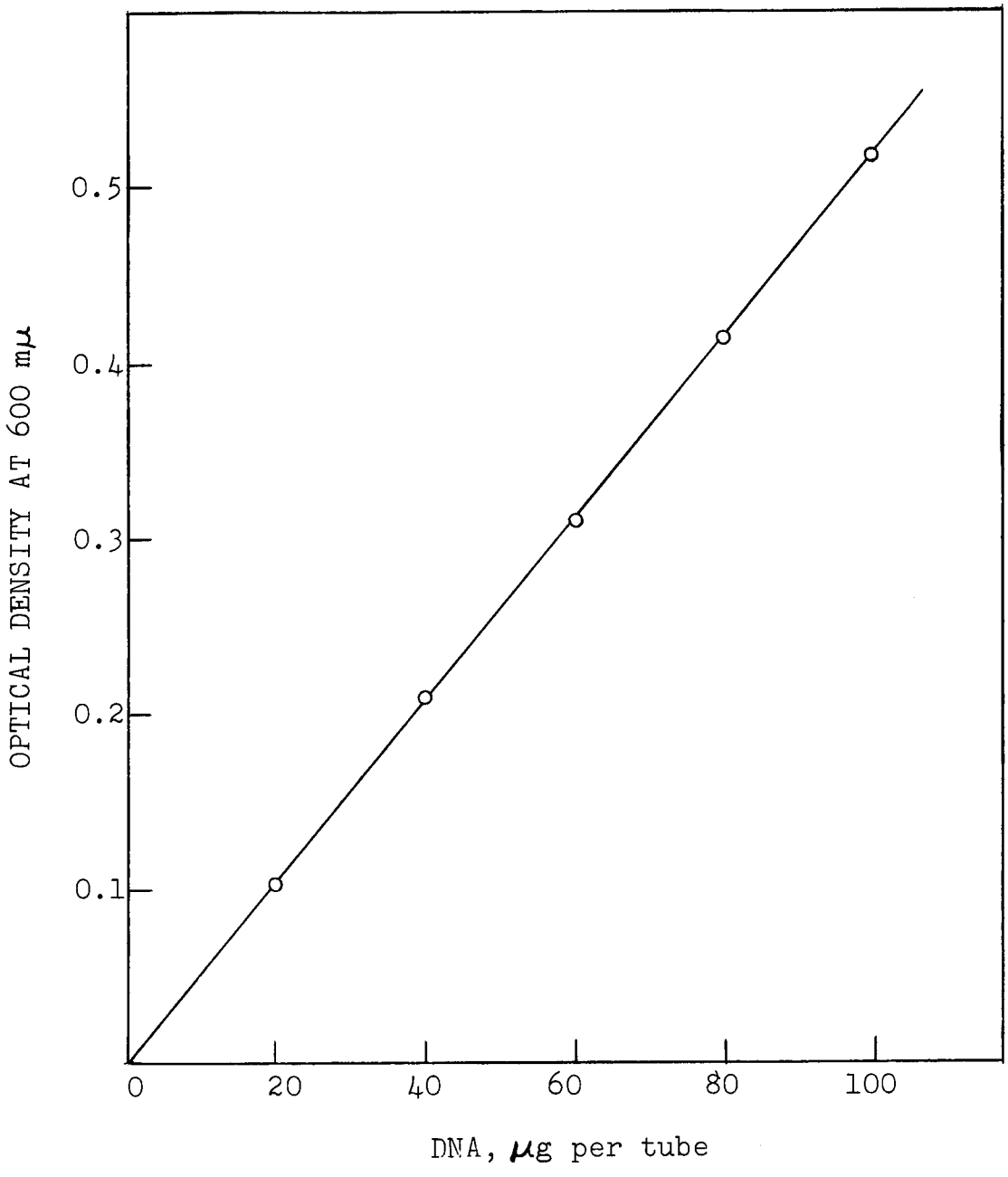
The quantity of DNA was determined by the method of Burton (5, 315-322). A typical standard reference curve from which the DNA content of unknown samples was determined is shown in Figure 1.

Preparation of spore suspensions

Stock spore suspensions were prepared by two methods.

(a) Potato agar slopes in screw cap tubes (6-inch) were inoculated with the appropriate culture and incubated at 37°C for about seven days or until most of the cells had sporulated. Four ml of sterile distilled water was added to each tube and the growth was suspended in it. The suspensions from the tubes were pooled, mixed thoroughly,

Figure 1



Standard reference curve for determining DNA concentration.

and heated at 65°C for 30 minutes.

(b) The appropriate culture was inoculated into a 250-ml flask containing 50 ml of phage assay medium (the broth without any agar) and the flask was incubated on a rotary shaker at 37°C for about 5 days or until most of the cells had sporulated. The culture was centrifuged at 3090 x g for 15 minutes. The pellet was suspended in 50 ml of sterile distilled water and the suspension was centrifuged at 3090 x g for 15 minutes. The pellet was finally suspended in 5 ml of sterile distilled water and the suspension was heated at 65°C for 30 minutes.

The number of viable spores in a suspension was determined by plating at appropriate dilutions on nutrient agar.

Propagation of phage

Phage was propagated in soft phage assay agar or in phage assay broth.

For propagation in soft agar, the phage was inoculated into 5 ml of soft phage assay agar which had been seeded with the host organism. The soft agar was poured onto 25 ml of hard phage assay agar in a petri dish which was then incubated upright at 37°C for 16 hours. Five ml of peptone diluent was pipetted onto the plate and the soft agar layer was macerated with a glass spreader. The macerated agar and peptone were transferred to a sterile tube and centrifuged at 3090 x g for 20 minutes. The supernatant fluid

containing the phage was stored frozen after 10% (v/v) of sterile glycerol was added. The phage suspensions were assayed with B. subtilis W-23-S^r as the indicator.

For propagation in broth, a 250-ml Erlenmeyer flask containing 50 ml of phage assay broth was inoculated with 0.1 ml (10^8 spores) of a lysogenic spore suspension or with 0.1 ml (10^8 spores) of host spore suspension and 0.1 ml (10^8 phage particles) of the phage suspension. The flask was incubated on the rotary shaker at 37°C for 16 hours. The resulting culture was centrifuged at 3090 x g for 20 minutes and the supernatant fluid was stored frozen with 10% (v/v) of glycerol.

Phage suspensions free of bacteria were prepared by filtering through Millipore DA membranes (0.65μ pore size).

Glutamyl polypeptide

The isolation of glutamyl polypeptide and the determination of the glutamic acid present in it were accomplished by the methods outlined by Thorne (25, 307-315).

Growth conditions

Unless otherwise stated, cultures were grown on a rotary shaker in 250-ml Frlenmeyer flasks containing 50 ml of medium.

All cultures were incubated at 37°C.

Screening of auxotrophs for transforming ability

The screening method consisted of spreading 0.1 ml of a 16-hour shaken culture grown in NBY or NBSG and 0.05 ml of DNA, prepared from wild type 9945A, on an agar plate containing 25 ml of minimal I medium. Control plates included one with cells alone and one with cells and DNA plus DNase. The plates were incubated at 37°C and observed daily for four or five days. This process is sometimes referred to in this thesis as transformation on the plate or the plate method of transformation.

Routine procedure for transformation in liquid medium

Routinely, experiments on transformation in liquid medium were set up in a manner similar to the following procotol. Sterile, 6-inch test tubes were set up as follows.

Tube	Recipient culture	DNA	DNase	Minimal 1 broth
1	1.0 ml	0.1 ml		0.1 ml
2	1.0 ml	0.1 ml	0.1 ml	
3	1.0 ml			0.2 ml

The DNA, DNase (200 μg), and diluent were added to the tubes and incubated in a 37°C water bath for 30 minutes. The recipient culture was added and the incubation was continued for 30 minutes. Samples, usually 0.1 ml, were spread onto appropriate agar plates for scoring transformants.

RESULTS AND DISCUSSION

Colonies of B. licheniformis 9945A, when grown on an appropriate medium, e.g. minimal 1 or medium E, appear very smooth as a result of glutamyl polypeptide accumulation. However, on media that are not conducive to peptide synthesis, e.g. nutrient agar, the colonies appear rough since peptide is absent. Thus, cells in a colony may be genotypically smooth although the colony, itself, appears phenotypically rough. As used in this thesis, the terms smooth and rough indicate the presence and absence, respectively, of glytamyl polypeptide. To avoid confusion, care has been taken where necessary to indicate whether the terms refer to genotype or phenotype.

Failure of rough mutants to produce glutamyl polypeptide. When the five presumed rough mutants, 9945A-R_1 through R_5, were grown on medium E agar, the resulting colonies were rough. Each of the mutants was grown in medium E broth for 48 hours and the culture supernatant fluids were tested for the presence of glutamyl polypeptide. No peptide could be detected although a similar supernatant fluid prepared from a culture of 9945A contained an abundance of peptide. From these tests it was concluded that these mutants are probably genotypically rough. The fact that they grew well on medium E indicates they

retained the prototrophic characteristic of 9945A.

Attempts to detect smooth cells in a predominantly rough population. The following experiments were run to test whether it would be possible to detect a small number of transduced smooth cells in a large population of rough cells. Six medium E agar plates were inoculated by the spreading technique with 10^8 cells of 9945A-R_2 and another set of six were inoculated with 10^7 cells. Duplicate plates from each of the two sets were then spread with 10^1, 10^2, or 10^3 9945A cells. After 48 hours of incubation the plates had confluent rough growth; no smooth growth could be detected. It would seem from these results that the large amount of rough growth masked the small amount of smooth growth.

Flasks of medium E broth were inoculated with 10^8, 10^3, 10^2, and 10^1 spores of 9945A respectively, and 10^8 spores of 9945A-R_2 were added to each of the flasks. The cultures were tested for peptide production after 48 hours of incubation. Peptide was present in the flask inoculated with 10^8 rough spores and 10^8 smooth spores but it was not detected in any of the three other flasks. The rough cells may have inhibited peptide production or they may have inhibited growth of the smooth cells. Another possibility is that the rough cells utilized any peptide produced by the smooth cells.

When grown under conditions favorable for peptide synthesis, rough cells of B. anthracis are lysed by virulent phage while smooth (capsulated) cells are apparently resistant to the phage (15, 114-125). This difference in susceptibility of the two types to lysis by phage is the basis of a method for detecting small numbers of genotypically smooth cells in a population predominantly genotypically rough (24, 290-291). It seemed worthwhile to test whether smooth and rough cells of 9945A would behave in an analogous manner when exposed to a virulent phage.

Phage SP-5, which was known to lyse phenotypically rough cells, was chosen for the test. Plates of medium E and nutrient agar were spread with $1x10^8$ phage particles and $5x10^8$ spores of 9945A-R_2. Other plates were spread with phage and spores of wild type 9945A in the same manner. Control plates were spread with 9945A or 9945A-R_2 and phage was omitted. After 20 hours of incubation there was good growth on all the plates without phage, but no growth occurred on any of the plates with phage added. Thus, both rough and smooth cells were lysed by the SP-5 phage.

Egg white lysozyme and glutamyl polypeptide react in aqueous solution to form an insoluble complex which

precipitates (12, 175-188). It had already been determined that lysozyme lyses rough cells of B. licheniformis but its action on smooth cells had not been determined. It seemed possible that the coat of glutamyl polypeptide around smooth cells might combine with lysozyme and prevent it from lysing the cell. Such an effect might be used as a basis for selecting smooth cells.

To test this, cells of 9945A-R_2 and 9945A were exposed to lysozyme in the following manner. Cultures of 9945A-R_2 and 9945A were grown in medium E broth and in nutrient broth for 24 hours. One-tenth ml samples of these cultures were spread onto plates of medium E agar and nutrient agar which had been previously spread with 0.15 ml (8 mg/ml) of lysozyme. After 16 hours of incubation there was no growth of either 9945A-R_2 or 9945A cells on the nutrient agar plates treated with lysozyme. However, neither culture seemed to be affected by the lysozyme on the medium E plates. Possibly the metal ions in medium E inhibited lysozyme activity. Since rough cells were not lysed under conditions which allowed expression of the smooth genotype, no further tests were made with lysozyme.

The results of the preceeding experiments indicated that it would not be possible with any of the techniques tested, to detect a small number of smooth transductants among a large number of rough cells. We therefore turned to transformation as a means of genetic transfer since frequencies of transformation are, in general, much higher than those of transduction. High frequencies of genetic exchange would minimize the problem of detection. Transformation had not been reported in B. licheniformis but it was expected that techniques which gave good results with B. subtilis would work with B. licheniformis as well and that the rough mutants could be transformed to the smooth type. It was soon learned, however, that B. licheniformis behaved very differently from B. subtilis, and because of this, the problem became one of how to get transformation to occur in B. licheniformis.

Sterilization of DNA. DNA preparations obtained from B. licheniformis 9945A were invariably contaminated with spores which were carried through the DNA isolation process. Several different procedures were tested in efforts to obtain a sterile DNA preparation. This is not usually a problem with DNA from B. subtilis; spores of this species seldom survive the DNA isolation procedure.

The first approach to the problem was an attempt to grow a culture of B. licheniformis 9945A which had no spores or at least very few spores in it. Since liquid medium E is a poor medium for spore production, it was thought that by transferring a culture into a fresh medium and incubating subsequently for a short period, a crop of cells with very few spores would be obtained. Ten ml of a 16-hour culture of 9945A-R_4 grown in medium E plus yeast extract (0.3%) was inoculated into a flask of the same medium. After 5 hours of incubation the procedure was repeated. The final culture was incubated for 3 hours. The cultures were all assayed for spore content and it was found that the 16-hour culture had fewer spores than the 5-hour culture and the 5-hour culture had fewer spores than the 3-hour culture. This approach to the problem of getting rid of spores was obviously of no value.

An attempt was made to obtain cells free of spores by adding alanine to cause the spores to germinate. Alanine was added to a 16-hour culture to bring the concentration to 10 mM and the culture was incubated for 90 minutes more. DNA was harvested from the culture and the precipitated DNA was allowed to stand in 95% ethanol for three days before it was dissolved in 2 M NaCl. The resulting DNA preparation had 9×10^2 spores per ml.

The next approach to the problem centered around

methods of killing the spores present in DNA preparations without losing transforming activity. A DNA preparation was precipitated in 95% ethanol and the DNA clump was transferred to 70% ethanol and stored in the refrigerator. At the end of 16 days when the DNA clump was dissolved in 2 M NaCl, viable spores were present.

A 9945A spore suspension was diluted in 70% ethanol to give 10^3 spores per ml. The diluted spore suspension was held in a 65°C water bath and 0.1 ml of it was spread on duplicate nutrient agar plates after 30, 60, 90 and 120 minutes. After 48 hours no growth was present on any of the plates, indicating that the 70% ethanol at 65°C was lethal to spores of 9945A. The next step was to see whether the hot 70% ethanol would kill the spores in a contaminated DNA preparation. A DNA preparation contaminated with 9×10^2 spores per ml was precipitated in 95% ethanol and the DNA clump was transferred to 70% ethanol. The preparation was heated at 65°C for 30 minutes after which it was cooled and precipitated again in 95% ethanol. After the precipitated DNA was dissolved in the original volume of 2 M NaCl, it was found to have 1.6×10^2 spores per ml. The clump of DNA didn't completely dissolve in the hot ethanol and this precipitated DNA must in some way have protected the contaminating spores.

Phenol (5% v/v, final concentration) was added to a DNA preparation contaminated with spores and the mixture

was heated in a 65°C water bath for 30 minutes. After the DNA was precipitated in 95% ethanol and dissolved in 2 M NaCl, viable spores were still present. The same procedure was repeated on another contaminated preparation except that the final phenol concentration was 30% (v/v). After this treatment no viable spores could be detected. The results were reproducible and the method was adopted as a routine procedure for sterilizing DNA preparations.

Since a transforming system with B. licheniformis was not yet available, I could not test the biological activity of B. licheniformis DNA following sterilization by the phenol treatment. However, the effect of the phenol treatment was tested with the B. subtilis transforming system, and the assumption was made that the biological activity of the two types of DNA would have similar responses to the phenol treatment. This assumption proved to be correct in a later test after the isolation of a transformable mutant of B. licheniformis made such a test possible. To test whether the transforming ability of a B. subtilis DNA preparation would be impaired by treatment with 30% phenol, a sample of a sterile DNA preparation from W-23-S^r was treated as described above. A transformation experiment was performed with B. subtilis 168 to compare the treated and untreated DNA samples. The results obtained from the experiment are given in Table 4. The phenol-

Table 4

Test of the effect of sterilizing DNA with phenol on its activity for transforming *B. subtilis* 168

Recipient cells		Treated DNA	Untreated DNA	Minimal I broth	Transformants
ml	number	ml	ml	ml	per ml
0.1	5×10^7	0.1		0.8	1480
0.1	5×10^7		0.1	0.8	2220
0.1	5×10^7			0.9	0

The recipient cells were from a 16-hour NBY culture. The mixtures were incubated in 6-inch test tubes in a water bath for 30 minutes. Duplicate plates of minimal I plus casamino acids (0.3%) were spread with 0.1 ml from each mixture. The donor DNA (465 μg/ml) was obtained from *B. subtilis* W-23-S^r. The number of transformants per ml is an average derived from duplicate plates.

treated DNA had essentially the same activity as the untreated DNA. This sterilization method then made it possible to perform transformation experiments on B. licheniformis 9945A mutants with sterile DNA preparations.

Unsuccessful attempts to transform B. licheniformis mutants. A number of experiments were run in unsuccessful attempts to transform mutants of 9945A. The conditions for growth of the recipient cells, the media, and the mutants were manipulated in trying to obtain a transforming system. Some of these experiments are summarized below.

Generally these experiments were carried out by incubating recipient cells with DNA in test tubes according to the protocol given under Methods and Materials. Samples of the transformation mixture were plated on appropriate media devised for selection of transformants. In one of the early experiments an attempt was made to transform rough cells to the smooth type. Recipient cells of 9945A-R_4 were from a 4-hour culture (5×10^8 cells per ml) prepared by inoculating medium E broth plus yeast extract (0.3%) with 5 ml of an overnight culture grown in the same medium. The DNA (approximately 600 µg per ml) was from smooth 9945A cells. Samples of transformation

and control mixtures were plated at dilutions of 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6} on plates of medium E agar with yeast extract. After 48 hours no smooth colonies could be detected on any of the plates indicating that transformation of cells from rough to smooth had not occurred.

In most of the later experiments efforts were concentrated on attempts to transfer nutritional markers or, in some instances, resistance to streptomycin, since selection methods for these markers presented no problem and the probability of detecting transformants occurring at low frequencies would be increased. Many experiments were done with 9945A-M2 (lysine$^-$) and 9945A-M14 (methionine$^-$) but all results were negative. Methods for producing competent cells of B. subtilis (2, 741-746) and Hemophilus influenzae (20, 537-549) were not effective for these B. licheniformis auxotrophs. Cells were grown in the presence of DNA in NBY broth and on potato agar to test whether they would become competent and be transformed during some step of the cycle of germination, growth, and sporulation. However, no evidence of transformation was found in these experiments. Other experiments which gave negative results with 9945A-M2 and/or M14 included a test over a broad range of pH values (pH 5.0, 6.0, 7.0, and 8.0); the addition of lysozyme (1.0 mg and 0.1 mg per ml) to transformation mixtures as a possible way of increasing the permeability of the cell walls; attempts to

transform cells in the presence of a temperate phage, SP-10, on the assumption that phage might aid in penetration of DNA into the cell; attempts to transform freshly germinated spores by adding spores and DNA to a solution of alanine and adenosine at concentrations effective for rapid germination; impinging cells from transformation mixtures onto Millipore membranes and incubating them on complete medium (NBY agar) for a period of three hours to allow expression of transformed characters and then transferring them to minimal agar; and attempts to transform cells by incubating them in a culture filtrate prepared from wild-type 9945A. This last experiment seemed worthwhile in view of the fact that culture filtrates of B. subtilis W-23-S^r contain DNA effective in transforming B. subtilis auxotrophs.

Since all attempts thus far to transform B. licheniformis had failed, it seemed possible that the cells were destroying the DNA before penetration could occur. Evidence that this was not true is found in the results of an experiment presented in Table 5. In this experiment B. subtilis 168 was transformed in the presence of cells of B. licheniformis 9945A-M14. It seemed likely that if B. licheniformis cells were inactivating homologous DNA they would also inactivate B. subtilis DNA. Since transformants of B. subtilis were isolated from the mixture,

Table 5

The transformation of mixed recipient cells with mixed donor DNAs

Recipient culture (ml)			DNA (ml)		Minimal I broth (ml)	Transformants* per ml
9945A-M14	168	9945A-M14+168	W-23-S^r	9945A		
0.8			0.1		0.1	0
0.8				0.1	0.1	0
0.8			0.1	0.1		0
0.8					0.2	0
	0.8		0.1		0.1	33,440
	0.8			0.1	0.1	0
	0.8		0.1	0.1		16,780
	0.8				0.2	0
		0.8	0.1		0.1	1,720
		0.8		0.1	0.1	0
		0.8	0.1	0.1		880
		0.8			0.2	0
0.4	0.4		0.1		0.1	10,960
0.4	0.4			0.1	0.1	0
0.4	0.4		0.1	0.1		4,800
0.4	0.4				0.2	0
			0.1		0.9	0
				0.1	0.9	0

The recipient cells were from a 5-hour culture grown in minimal I broth plus yeast extract (0.3%). The mixtures were incubated in 6-inch test tubes in a water bath for 90 minutes. Duplicate minimal I agar plates were spread with 0.1 ml from each mixture.

*The transformants were identified as B. subtilis by their distinctive colonial morphology and no B. licheniformis colonies were observed.

this hypothesis was abandoned. In the same experiment, B. subtilis was transformed when the DNA was a mixture of preparations from B. subtilis and B. licheniformis, indicating that DNA from B. licheniformis did not contain nuclease or other inactivating substances. Another bit of information to be derived from the results in Table 5 is that the presence of competent cells of B. subtilis 168 in a mixture with cells of B. licheniformis did not effect competence in the latter cells. In this regard the results were the same when the two organisms were grown together as when they were mixed after growth. If competence in B. subtilis is effected by an enzyme which aids in penetration of DNA, such enzyme is probably specific for B. subtilis or perhaps it is not liberated into the medium, since B. licheniformis cells did not become competent in the presence of competent cells of B. subtilis.

A few experiments were done in efforts to transform auxotrophs of another strain of B. licheniformis, 10716. Cells of mutants requiring methionine (10716-M1 and M75) and isoleucine (10716-M4, M5, and M6) failed to yield detectable transformants when tested with DNA isolated from wild-type 10716.

Transformable mutants. The next approach was based on the assumption that it should be possible to isolate transformable mutants. The plan was to isolate numerous

mutants and to test them for transformation by the screening procedure given under Methods and Materials. The technique used in the screening test had been found to produce transformants of B. subtilis 168 and it was assumed, therefore, that it would suffice for detecting transformable mutants of B. licheniformis. Fortunately this approach turned out to be very fruitful within a short period of time. When the 28 auxotrophs listed in Table 1 were subjected to the screening test, three of them, 9945A-M28 (glycine$^-$), -M30 (uncharacterized), and -M33 (purine$^-$), produced transformants. M28 transformed at a much higher frequency than M30 or M33 and for that reason, it was studied in greater detail.

To confirm that the results obtained with M28 in the screening test were produced by transformation, the experiment was repeated and DNA from the mutant itself was included as a control. The results of such an experiment are shown in Table 6. Transformants were produced with the DNA isolated from wild-type 9945A but not with the DNA isolated from the mutant. Spontaneous reversion to prototrophy did not occur and the addition of DNase prevented the occurrence of prototrophs. These results provide strong evidence that M28 is a transformable mutant.

Colonies of M28 grow very slowly on NBY agar as well as on minimal I. On NBY agar colonies of M28 are

Table 6

Transformation of 9945A-M28ˢ with homologous and heterologous DNA

Recipient cells ml	Recipient cells number	9945A-M28ˢ DNA (300μg/ml)	9945A DNA (925μg/ml)	DNase (2mg/ml)	Transformants per ml
0.1	$1x10^8$	0.05			0
0.1	$1x10^8$	0.05		0.05	0
0.1	$1x10^8$		0.05		5800
0.1	$1x10^8$		0.05	0.05	0
0.1	$1x10^8$				0

The recipient cells were from an 18-hour NBY broth culture. Duplicate minimal I agar plates were spread with 0.1 ml of cells and 0.05 ml of DNA with or without 0.05 ml of DNase, as given in the table.

circular, with entire margins, and grow only to a diameter of about 2 mm; colonies of wild type 9945A on NBY agar are circular, with erose margins, and grow much larger to a diameter of about 6 mm. On minimal I medium supplemented with glycine, colonies of M28 are about 2 mm in diameter after 72 hours and appear rough. Hence, it is referred to as a rough mutant. In contrast, after only about 16 hours, wild type 9945A colonies are large on minimal I medium and very smooth as a result of glutamyl polypeptide accumulation. Upon prolonged incubation for 96 to 120 hours, occasionally colonies of M28 on minimal I medium exude small amounts of viscous material which is probably peptide. On medium E, supplemented with glycine or yeast extract (0.15%), M28 colonies are generally smooth although they appear not to produce nearly as much peptide as the wild type does. Therefore, although M28 appears rough on minimal I medium, it is probably a leaky mutant and is thus able to produce peptide on medium E which has much higher concentrations of peptide precursors than has minimal I. Another possibility is that small amounts of peptide are produced as a result of a suppressor mutation. In the remainder of this thesis M28 is referred to as a rough mutant which is descriptive of its characteristics on the minimal I medium used routinely.

Smooth and rough transformants. The transformants

of 9945A-M28ˢ were of two colonial types on minimal I plates; a few of them were smooth and a large number were rough. Cultures from each of the two types of colonies were purified and spore suspensions were prepared from single colony isolates. They were designated 9945A-M28ˢ-ST for the smooth transformant and 9945A-M28ˢ-RT for the rough transformant. Colonies of 9945A-M28ˢ-RT on minimal I agar cannot be distinguished from colonies of 9945A-M28ˢ on minimal I agar supplemented with glycine. Likewise, the cultures have similar colonial morphology on NBY agar. However, colonies of 9945A-M28ˢ-ST on minimal I agar or on NBY agar cannot be distinguished from colonies of 9945A on the same respective media. A test was made to determine whether the viscous material produced by M28ˢ-ST was in fact glutamyl polypeptide. The organism was grown in medium E and peptide was isolated (but not purified extensively) by the methods outlined by Thorne (25, 307-315). A paper chromatogram of the acid hydrolyzed material showed that it was predominantly glutamic acid.

DNA was prepared from cells of 9945A-M28ˢ-ST and 9945A-M28ˢ-RT and the two preparations were tested in a transformation experiment with M28ˢ. DNA from 9945A was also included in the experiment. The results are given in Table 7. The transformants that were produced with the

Table 7

Transformation of 9945A-M28 cells with DNA prepared from 9945A-M28 transformants

Recipient cells		DNA Source				
ml	number	9945A-M28-RT (395μg/ml)	9945A-M28-ST (473μg/ml)	9945A (925μg/ml)	DNase (2mg/ml)	Transformants per ml
0.1	1.2×10^8	0.05				1030*
0.1	1.2×10^8	0.05			0.05	0
0.1	1.2×10^8		0.05			420
0.1	1.2×10^8		0.05		0.05	0
0.1	1.2×10^8			0.05		8000
0.1	1.2×10^8			0.05	0.05	0
0.1	1.2×10^8					0

The recipient cells were from an 18-hour NBY broth culture. Duplicate minimal I agar plates were spread with 0.1 ml of cells and 0.05 ml of DNA with or without 0.05 ml of DNase, as given in the table.

*All these colonies were rough but about 10% of the transformants produced with DNA from 9945A or 9945A-M28 were smooth.

DNA from 9945A-M28-RT were all of the rough colony type, whereas the transformants produced with the DNA from 9945A-M28-ST or 9945A included both rough and smooth colony types.

Many transformation experiments were done with 9945A-M28. When 10^7 recipient cells grown in NBY were spread together with DNA on minimal I agar plates, there would be about 600 transformant colonies after 72 hours of incubation. When the recipient cells were grown in NBY, the first colonies appeared after about 48 hours of incubation and these were the smooth transformants. The rough transformants would begin to appear on the plates a few hours later. When the recipient cells were grown in NBSG the first colonies, which were smooth transformants, appeared in about 28 hours and the final number of transformant colonies was much greater than when the recipient cells were grown in NBY. The greater number of transformants from cells grown in NBSG might be related to the fact that fewer spores were produced in NBSG than in NBY.

Studies on competence of 9945A-M28. In experiments with transformation in liquid medium, conditions which produced high yields of transformants of B. subtilis mutants failed to produce transformants of 99445A-M28. Many unsuccessful experiments were done with cells and DNA incubated together in liquid medium for various periods of

time. Samples that were treated with DNase at the time of plating yielded no transformants, although similar samples without DNase treatment yielded transformants. Since it appeared as though the recipient cells became competent after they had been spread on minimal I agar plates, several experiments were run in attempts to learn what changes were occurring in the cells on the agar plates.

The following experiment was done to learn whether the mutant cells divided and/or sporulated after being spread on minimal I agar. Each of 12 plates was spread with 0.1 ml of a 16-hour NBY culture of M28. At various times corresponding to 0, 2, 5, 8, 13, and 25 hours of incubation the cells were washed from duplicate plates with 10 ml of peptone diluent and the resulting cell suspensions were assayed for total count and spore count. The results of this experiment are shown in Table 8. In 25 hours the total count increased to more than 30 times the initial value while the spore count remained nearly constant. The increase in the number of auxotrophic cells on the minimal I agar was probably a reflection of endogenous growth of the cells and, in part, of growth supported by small amounts of nutrients transferred to the plates with the 0.1 ml of culture.

To ascertain the time necessary for 9945A-M28 cells to become competent on minimal I agar, 40 plates were

Table 8

Increase in numbers of cells of 9945A-M28 on minimal I agar

Incubation time (hours)	Total count (cells/plate)	Spore count (spores/plate)
0	2.8×10^8	1.5×10^7
2	1.6×10^8	3.4×10^6
5	8.4×10^8	3.8×10^7
8	1.8×10^9	5.0×10^7
13	3.5×10^9	3.5×10^7
25	9.6×10^9	3.4×10^7

Each of 12 plates was spread with 0.1 ml of a 16-hour NBY culture. At the designated incubation times the cells were washed from duplicate plates with 10 ml of peptone diluent and the resulting cell suspensions were assayed for total count and spore count. The cell count and the spore count are averages derived from duplicate NBY agar plates.

spread with 0.05 ml of 9945A-DNA and 0.1 ml of an 18-hour NBSG culture of 9945A-M28. The plates were incubated and at 0, 1, 2, 3, 4, 5, 6, 7, 9, and 11 hours, two plates from a set of four were sprayed with DNase. The results given in Table 9 were obtained after 90 hours of incubation. The cells, as evidenced by the appearance of transformants, began to become competent after about 5 hours. There was a 10-fold increase in the number of transformants during the period between 5 and 11 hours. The number of transformants was not maximum at 11 hours as was evidenced by the larger number of transformants on the control plates that were not sprayed with DNase.

Since it appeared that the M28 cells became competent after a period of incubation on minimal I agar, the following experiment was designed to test whether they would become competent in minimal I broth. An 18-hour NBSG culture of 9945A-M28 was centrifuged at 5860 x g for 15 minutes to deposit the cells. The cells were washed once with an equal volume of minimal I broth, centrifuged again, and resuspended in 100 ml of minimal I broth. Fifty ml of the suspension was put into each of two sterile 250-ml flasks. Glycine was added to one of the flasks to give a final concentration of 25 μg per ml. The cell suspensions were incubated on a rotary shaker and samples were removed at 0, 1, 2, 3, 6, 9, 11 and 22 hours. The samples were

Table 9

Competence of 9945A-M28 cells incubated on minimal I agar for various periods of time

Incubation time (hours)	Transformants per ml	
	DNase treated	no DNase
0	0	>10,000
1	0	>10,000
2	0	>10,000
3	20	>10,000
4	0	>10,000
5	570	>10,000
6	400	>10,000
7	640	>10,000
9	950	>10,000
11	5700	>10,000

Minimal I agar plates were spread with 0.05 ml of 9945A-DNA and 0.1 ml of an 18-hour NBSG broth culture of 9945A-M28. At the designated incubation times, 2 plates from a set of 4 were sprayed with DNase (2 mg/ml). The number of transformants per ml is an average derived from duplicate minimal I agar plates.

assayed for total count and spore count, and the cells were tested for competence by exposing them to 9945A-DNA for 30 minutes. Each of four minimal I agar plates was spread with 0.1 ml of the mixture of DNA and cells, and 0.05 ml of a DNase solution containing 2 mg/ml was then spread on two of the plates. As a check for spontaneous revertants two additional plates were spread with 0.1 ml of a control sample from which DNA was omitted. Tables 10 and 11 give the results of this experiment. Some of the cells became competent and were transformed in liquid suspension with maximum competence occurring after two to three hours of incubation. The cells in minimal I broth supplemented with glycine appeared to be slightly more suceptible to transformation than those in the unsupplemented medium. The results from this experiment indicate that cells of 9945A-M28^{s} became competent in liquid medium after a long incubation period. The original culture was incubated for 18 hours and after the cells were resuspended they reached their maximum competence after 2 to 3 hours, a total of about 20-21 hours. Although transformation did occur in liquid medium, the frequencies in this experiment were very low.

Since the results from the previous experiment indicated that 9945A-M28^{s} became competent in liquid medium after a long incubation time, the following experiment was

Table 10

The competence, growth, and sporulation of 9945A-M28 cells when incubated in minimal I broth

Incubation time (hours)	Total count (cells/ml)	Spore count (spores/ml)	Transformants per ml		Revertants per ml
			DNase-treated	no DNase	
0	3.6×10^9	1×10^5	20	>10,000	0
1	3.4×10^9	1×10^5	190	>10,000	0
2	3.9×10^9	1×10^5	1150	>10,000	0
3	3.0×10^9	1×10^5	1250	>10,000	0
6	3.2×10^9	1×10^5	210	>10,000	0
9	3.2×10^9	1×10^5	30	>10,000	0
11	2.8×10^9	$>1 \times 10^6$	0	>10,000	0
22	3.4×10^9	$>1 \times 10^6$	0	>10,000	0

The recipient cells were from an 18-hour NBSG broth culture, centrifuged and resuspended in minimal I broth. Samples of the suspension were tested for spore count, total count, and competence at the designated incubation times. Six-inch test tubes were inoculated with 0.5 ml of the sample and 0.2 ml of DNA (9945A) and incubated in a water bath for 30 minutes. Four minimal I agar plates were spread with 0.1 ml of the DNA-cell mixture and 0.1 ml of DNase (2 mg/ml) was spread onto two of the four plates. To test for spontaneous reversion tubes were inoculated with 0.5 ml of the sample and 0.2 ml of minimal I broth and incubated as above. Duplicate minimal I agar plates were spread with 0.1 ml of the mixture.

Table 11

The competence, growth, and sporulation of 9945A-M28 cells when incubated in minimal I broth supplemented with 25 μg glycine per ml

Incubation time (hours)	Total count (cells/ml)	Spore count (spores/ml)	Transformants per ml		Revertants per ml
			DNase-treated	No DNase	
0	2.8×10^9	8.6×10^4	20	>10,000	0
1	2.6×10^9	1.0×10^5	210	>10,000	0
2	2.5×10^9	1.0×10^5	1620	>10,000	0
3	4.0×10^9	8.6×10^4	1770	>10,000	0
6	5.2×10^9	1.3×10^5	460	>10,000	0
9	3.8×10^9	7.3×10^5	660	>10,000	0
11	4.2×10^9	$>1.0 \times 10^6$	280	>10,000	0
12	3.8×10^9	$>1.0 \times 10^6$	30	>10,000	0

The recipient cells were from an 18-hour NBSG broth culture, centrifuged and resuspended in minimal I broth containing 25 μg of glycine per ml. Samples of the culture were tested for spore count, total count, and competence at the designated incubation times. Six-inch test tubes were inoculated with 0.5 ml of sample and 0.2 ml of DNA (9945A) and incubated in a water bath for 30 minutes. Four minimal I agar plates were spread with 0.1 ml of the DNA-cell mixture and 0.1 ml of DNase (2 mg/ml) was spread onto two of the four plates. To test for spontaneous reversion tubes were inoculated with 0.5 ml of the sample and 0.2 ml of minimal I broth and incubated as above. Duplicate minimal I agar plates were spread with 0.1 ml of the mixture.

done to determine whether cells became competent at some point during growth in NBSG broth. Samples of a culture growing in NBSG were removed at 18, 20, 22, 24, 26 and 28 hours and the cells were tested for transformation. Table 12 gives the results of this experiment. The culture seemed to be most competent at 22 hours when 1.2×10^{-4} per cent of the cells were transformed. The total counts indicated the culture was in the stationary phase of the growth cycle during the period between 18 and 28 hours.

The previous experiments on transformation in liquid medium were done by incubating cells with DNA under static conditions in test tubes. An experiment was designed to compare the results obtained under these conditions with those obtained when the mixture of cells and DNA was incubated on a shaker. The results of this experiment are shown in Table 13. More than ten times as many transformants were produced in the shaken mixture as in the static mixture. The advantage of shaking the transformation mixture was confirmed in another experiment, also shown in Table 13, in which an incubation time of one hour was used. This procedure for transformation in liquid medium gave better results than any other method tested.

It was thought that perhaps the transformation frequency would be increased if there were fewer cells in the DNA-cell mixtures and an experiment was designed to

Table 12

The competence, growth, and sporulation of 9945A-M28 cells in NBSG broth

Incubation Time (hours)	Total count (cells/ml)	Spore count (spores/ml)	Transformants per ml		Revertants per ml
			DNase-treated	No DNase	
18	3.5×10^9	1.1×10^5	720	>10,000	0
20	3.7×10^9	1.4×10^5	940	>10,000	0
22	3.6×10^9	1.9×10^5	2200	>10,000	0
24	4.2×10^9	3.2×10^5	1640	>10,000	0
26	2.7×10^9	7.8×10^5	1700	>10,000	0
28	2.0×10^9	$>1.0 \times 10^6$	830	>10,000	0

Samples of the culture were tested for spore count, total count, and competence at the designated incubation times. Six-inch test tubes were inoculated with 0.5 ml of the sample and 0.2 ml of DNA and incubated in a water bath for 30 minutes. Four minimal I agar plates were spread with 0.1 ml of the DNA-cell mixture and 0.1 ml of DNase (2 mg/ml) was spread onto two of the four plates. To test for spontaneous reversion tubes were inoculated with 0.5 ml of the sample and 0.2 ml of minimal I broth and incubated as above. Duplicate minimal I agar plates were spread with 0.1 ml of the mixture.

Table 13

A comparison of the effects of static and shaken conditions on transformation of 9945A-M28 cells in liquid medium

Recipient cells ml	Recipient cells number	9945A DNA (262μg/ml) ml	Minimal I broth ml	Incubation conditions	Transformants per ml
Experiment I					
0.5	1.5×10^9	0.2		static	760
0.5	1.5×10^9		0.2	static	0
1.0	3.0×10^9	0.4		shaken	8,290
1.0	3.0×10^9		0.4	shaken	0
Experiment II					
1.0	1.8×10^9	0.4		shaken	>10,000
1.0	1.8×10^9		0.4	shaken	0

The recipient cells were from a 22 hour NBSG broth culture. Six-inch test tubes were used for static incubation and 30 ml serum bottles on a rotary shaker were used for the aerated mixtures. The mixtures were incubated for 30 minutes in Experiment I and for 60 minutes in Experiment II. Duplicate minimal I agar plates were spread with 0.1 ml from each mixture and 0.1 ml of DNase (2 mg/ml) was spread with the mixtures containing DNA. The number of transformants per ml is an average derived from duplicate minimal I agar plates.

test this. The results are shown in Table 14. Transformability of cells was tested at several dilutions both in liquid suspensions and on agar plates. As the number of recipient cells decreased, the per cent transformation increased in the liquid suspensions but on the agar plates the per cent transformation was the same at the different cell concentrations.

To see whether different concentrations of DNA in the DNA-cell mixture would change the frequency of transformation of 9945A-M28, an experiment was done by the agar plate technique in which the cell concentration was held constant and the DNA concentration was varied. Table 15 gives the results of this experiment. As the concentration of DNA decreased below 462 μg/ml the numbers of transformants also decreased.

The phenol treatment didn't appear to affect the ability of the B. subtilis W-23-S^r DNA to transform B. subtilis 168, but it was not known whether phenol had any effect on DNA from B. licheniformis. It was important to test this since it seemed possible that the low frequencies of transformation might be a result of partial inactivation of DNA. To test whether the activity of the 9945A DNA was affected by the phenol treatment, a sterile DNA preparation that had not been treated with phenol was needed. In 16-hour NBY broth cultures of 9945A there

Table 14

The effect of decreasing the number of 9945A-M28 recipient cells on the number of transformants

Recipient cells ml	Recipient cells number	9945A DNA (262 μg/ml) ml	Transformation conditions	Transformants per ml	Per cent transformation
0.5	1.5×10^9	0.2	liquid	760	5.1×10^{-5}
0.5	1.5×10^8	0.2	liquid	190	1.3×10^{-4}
0.5	1.5×10^7	0.2	liquid	150	1.0×10^{-3}
0.5	1.5×10^6	0.2	liquid	50	3.3×10^{-3}
0.1	3.0×10^8	0.05	agar plate	6180	2.1×10^{-3}
0.1	3.0×10^7	0.05	agar "	1610	5.4×10^{-3}
0.1	3.0×10^6	0.05	agar "	100	3.3×10^{-3}
0.1	3.0×10^5	0.05	agar "	0	0

The recipient cells were from a 22-hour NBSG broth culture. For transformations in liquid medium the mixtures were incubated in 6-inch test tubes in a water bath for 30 minutes. Duplicate minimal I agar plates were spread with 0.1 ml from each mixture and 0.1 ml of DNase (2 mg/ml) was spread with the mixtures containing DNA. For transformations on agar plates, duplicate minimal I agar plates were spread with cells and DNA as given in the table. No spontaneous reversion occurred in the experiment.

Table 15

The transformation of 9945A-M28 with different amounts of DNA

Recipient cells ml	Recipient cells number	9945A-DNA μg/ml	DNase (2mg/ml) ml	Transformants per ml
0.1	1.5×10^8	925	0.05	0
0.1	1.5×10^8	0		0
0.1	1.5×10^8	925		7900
0.1	1.5×10^8	462		8310
0.1	1.5×10^8	46		5230
0.1	1.5×10^8	4.6		1240
0.1	1.5×10^7	925		260
0.1	1.5×10^7	462		440
0.1	1.5×10^7	46		160
0.1	1.5×10^7	4.6		15

The recipient cells were from a 16-hour culture grown in minimal I broth supplemented with 50 μg of glycine per ml. The transformation was done by the plate technique as given under Methods and Materials. The number of transformants per ml is an average derived from duplicate minimal I agar plates.

were usually 10^8 spores per ml, whereas in a 16-hour NBSG broth culture there were usually only about 10^5 spores per ml. Thus it seemed that a DNA preparation free of spores might be obtainable from cells grown in NBSG. DNA was harvested from 16-hour cells grown in NBSG broth and a test for sterility proved it to be free of viable spores and cells. The DNA preparation was tested for transformation of 9945A-M28 cells by the agar plate technique described under methods. The frequency of transformation was no greater than that usually obtained with phenol-treated DNA and therefore it was concluded that sterilization of 9945A-DNA with phenol doesn't appear to affect its biological activity.

Transformation of double mutants. It was of interest to determine whether 9945A-M28 was a transformable mutant because it had peculiar physiological characteristics or whether the glycine marker itself was for some reason particularly amenable to transformation. It seemed that this could be tested by introducing other markers into M28 and testing whether they, too, could be transformed. For this purpose other mutations were induced in M28 and the doubly-marked auxotrophs listed in Table 2 were isolated. Five of these mutants, M28-D1 (glycine$^-$, tryptophan$^-$), M28-D2 (glycine$^-$, histidine$^-$), M28-D4 (glycine$^-$, adenine$^-$), M28-D7(glycine$^-$, serine$^-$), and M28-D14 (glycine$^-$, leucine$^-$) were tested for transformation of both markers,

singly, by the plate technique. The results are shown in Table 16. Glycine$^+$ transformants were obtained with each of the five mutants and transformants for three of the other markers, serine, histidine, and leucine, were also obtained. However, transformants for adenine or tryptophan were not detected.

From the above experiment, glycine$^+$serine$^-$, glycine$^+$ leucine$^-$, and glycine$^+$histidine$^-$ transformants were isolated and tested for transformability. These results are shown in Table 17. Each of the three isolates, although glycine independent, was transformed to prototrophy. These results, which show that transformation was not specific for the glycine marker, suggested that M28^s carries, in addition to the mutation responsible for glycine dependence, a second unidentified mutation which renders it amenable to transformation.

Transformation of a smooth transformant of 9945A-M28^s. A smooth, glycine$^-$ culture was isolated from the transformation experiments with the doubly marked auxotrophs. Cells of this mutant, which had apparently been transformed for the ability to produce glutamyl polypeptide on minimal I agar, were tested for transformation with DNA from both wild type 9945A and 9945A-M28^s-RT (a rough glycine$^+$ transformant of M28^s). Results are shown in Table 18. Both of the DNA preparations were active in transforming the mutant

Table 16

The transformation of doubly-marked auxotrophs of 9945A-M28

Recipient cells			9945A-DNA (925μg/ml)	DNase (2mg/ml)	Transformants per ml	
Mutant	ml	number	ml	ml	glycine marker	other marker
M28-D1	0.1	1.8×10^8	0.05		3850	0
D1	0.1	1.8×10^8	0.05	0.05	0	0
D1	0.1	1.8×10^8			0	0
D2	0.1	3×10^8	0.05		6340	10
D2	0.1	3×10^8	0.05	0.05	0	0
D2	0.1	3×10^8			0	0
D4	0.1	3×10^7	0.05		110	0
D4	0.1	3×10^7	0.05	0.05	0	0
D4	0.1	3×10^7			0	0
D7	0.1	1.8×10^8	0.05		600	500
D7	0.1	1.8×10^8	0.05	0.05	0	0
D7	0.1	1.8×10^8			0	0
D14	0.1	3.5×10^8	0.05		>10,000	>10,000
D14	0.1	3.5×10^8	0.05	0.05	0	0
D14	0.1	3.5×10^8			0	0

The recipient cells were from 16-hour NBSG broth cultures. The transformation was done by the plate technique as given in Methods and Materials. The 5 mutants were, M28-D1 (glycine$^-$, tryptophan$^-$), M28-D2(glycine$^-$, histidine$^-$), M28-D4(glycine$^-$, adenine$^-$), M28-D7(glycine$^-$, serine$^-$), and M28-D14(glycine$^-$, leucine$^-$). The glycine transformants were scored on minimal I agar supplemented with the other required amino acids and the transformants of the second marker were scored on minimal I agar supplemented with glycine. The number of transformants per ml is an average derived from duplicate minimal I agar plates supplemented with the appropriate amino acids.

Table 17

Transformability of 9945A-M28(glycine$^+$, serine$^-$), M28 (glycine$^+$, histidine$^-$), and M28(glycine$^+$, leucine$^-$)

Recipient cells			9945A-DNA (925μg/ml)	DNase (2mg/ml)	Transformants
Marker	ml	Number	ml	ml	per ml
Serine$^-$	0.1	2.8×10^8	0.1		>10,000
Serine$^-$	0.1	2.8×10^8	0.1	0.1	0
Serine$^-$	0.1	2.8×10^8			0
Leucine$^-$	0.1	1.2×10^8	0.1		>10,000
Leucine$^-$	0.1	1.2×10^8	0.1	0.1	0
Leucine$^-$	0.1	1.2×10^8			0
Histidine$^-$	0.1	1.9×10^8	0.1		35
Histidine$^-$	0.1	1.9×10^8	0.1	0.1	0
Histidine$^-$	0.1	1.9×10^8			0

The recipient cells were from 17-hour NBSG broth cultures. The transformation was done by the plate technique as given under Methods and Materials. The number of transformants per ml is an average derived from duplicate minimal I agar plates.

Table 18

The transformation of a smooth, glycine$^-$ culture derived from 9945A-M28

Recipient cells		DNA source			Transformants per ml	
		9945A (262μg/ml)	9945A-M28-RT (395μg/ml)	Minimal I broth		
ml	number	ml	ml	ml	smooth	rough
0.5	1.3×10^9	0.2			210	0
0.5	1.3×10^9		0.2		270	20
0.5	1.3×10^9			0.2	0	0

The recipient cells were from a 22-hour NBSG broth culture. Six-inch test tubes containing the mixtures were incubated in a water bath for 30 minutes. Duplicate minimal I agar plates were spread with 0.1 ml of the mixtures.

to glycine independence, although the frequencies were considerably lower than those usually obtained with M28. The transformants produced with wild type DNA were all smooth although two rough colonies occurred among the prototrophic transformants produced with DNA from the rough transformant of M28 (9945A-M28-RT). At least two important bits of information can be deduced from these results: (1) Genotypically smooth cells can be susceptible to transformation and (2) the inability to produce glutamyl polypeptide on minimal I agar is a transformable characteristic.

Nature of the "rough" character in 9945A-M28. As reported earlier, when M28 was transformed with DNA from a rough prototrophic transformant all the transformants were rough in minimal I agar; when DNA from wild-type 9945A or a smooth prototrophic transformant was used, both smooth and rough transformants were obtained. It became of interest to see what types of transformants would be produced with the DNA from the rough prototrophic mutants, R_1 through R_5, described above, and with DNA from strain 10716. Apparently mutants R_1 through R_5, as well as strain 10716, are truly genotypically rough since they appear to produce no trace of glutamyl polypeptide on any medium tested. When DNA from each of these six cultures was tested with M28, both rough and smooth

transformants (on minimal I agar) were obtained. Such results should probably not be unexpected since, undoubtedly, there are at least several genes involved in peptide synthesis, and the lesion in M28δ could very well be at a site which is normal in the other rough cultures. However, with the available evidence, one cannot be certain that M28δ is a truly genotypically rough mutant which is leaky or suppressed. There remains the possibility that M28δ is genotypically smooth and that some other physiological characteristic interferes with glutamyl polypeptide synthesis.

Transduction of 9945A-M28δ. Phage SP-15, a transducing phage for both B. subtilis and B. licheniformis, transduced 9945A-M28δ to prototrophy. Results of an experiment on transduction are shown in Table 19. Although only a few transduction experiments were done, no smooth transductants were observed. The frequency of transduction was about 6×10^{-9} per plaque-forming unit, a value quite low compared to the frequency obtained with 9945A-M2 (lysine$^-$) which was $>1 \times 10^{-7}$ (21, in press).

Table 19

Transduction of 9945A-M28 cells with phage SP-15

Recipient cells		SP-15		DNase (2 mg/ml)	Phage assay	Transductants
ml	number	ml	number	ml	broth (ml)	per ml
0.5	1.3×10^9				0.6	0
0.5	1.3×10^9	0.5	2.5×10^{10}		0.1	135
0.5	1.3×10^9	0.5	2.5×10^{10}*		0.1	0
0.5	1.3×10^9	0.5	2.5×10^{10}	0.1		205
		0.5	2.5×10^{10}		0.6	0

*The SP-15 suspension was held for 1 hour in a 65°C water bath to inactivate phage particles.

The recipient cells were from a 6-hour NBSG broth culture. The mixtures were incubated in 30 ml serum bottles on a rotary shaker for 45 minutes. Duplicate minimal I agar plates were spread with 0.1 ml from each mixture. The number of transductants per ml is an average derived from duplicate minimal I agar plates. The phage was propagated on cells of wild-type 9945A.

SUMMARY

The original purpose of this research was to study the metabolic pathway to the synthesis of glutamyl polypeptide in B. licheniformis. The plan was to isolate mutants blocked at various steps in the route of synthesis and to study accumulation and utilization of intermediates by various classes of mutants. Two transducing phages for B. liceniformis were available, and the plan of approach included the use of these phages to distingish classes of mutants by transduction. However, with the low frequencies of transduction which were characteristic of this system and without a specific method for selecting cells that were transduced for the ability to synthesize peptide, it soon became apparent that a different procedure would have to be used.

Since frequencies of transformation are, in general, much higher than frequencies of transduction, it seemed logical to use transformation as the system of genetic transfer.

A number of experiments were run in efforts to transform mutants of B. licheniformis 9945A (auxotrophic mutants were included). The conditions for growth of the recipient cells, the media, and the mutants were manipulated in unsuccessful attempts to obtain a transforming system.

The next approach to the problem was to try to isolate transformable mutants. This approach turned out to be very fruitful within a short period of time. Three transformable auxotrophs were found, M28(glycine$^-$), M30(uncharacterized), and M33(purine$^-$). The auxotroph 9945A-M28 transformed at a much higher frequency than M30 or M33 and it was studied in greater detail.

Cells of 9945A-M28 spread on minimal I agar plates became competent after a period of incubation and transformed well when 9945A DNA was present. The most competent M28 cells for transformation in liquid were from 22-hour cultures grown in minimal medium salts plus nutrient broth and glycerol and the best transformation occurred when the DNA-cell mixture was incubated in a serum bottle on a rotary shaker for 1 hour.

BIBLIOGRAPHY

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

2. Anagnostopoulos, C. and John Spizizen. Requirements for transformation in Bacillus subtilis. Journal of Bacteriology 81:741-746. 1961.

3. Austrian, R. Bacterial transformation reactions. Bacteriological Reviews 16:31-50. 1952.

4. Avery, Oswald T., Colin M. MacLeod and Maclyn McCarty. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. Journal of Experimental Medicine 79:137-158. 1944.

5. Burton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochemical Journal 62:315-322. 1956.

6. Ephrati-Elizur, Erela, P. R. Srinivasan, and Stephen Zamenhof. Genetic analysis by means of transformation of histidine linkage groups in Bacillus subtilis. Proceedings of the National Academy of Sciences 47:56-63. 1961.

7. Fox, Maurice S. The fate of transforming deoxyribonucleate following fixation by transformable bacteria. Proceedings of the National Academy of Sciences 48:1043-1048. 1962.

8. Greer, Sheldon. Genetic studies of in vitro-induced auxotrophic mutational sites in Bacillus subtilis. Federation Proceedings 22:407. 1963.

9. Griffith, F. Significance of pneumococcal types. Journal of Hygiene 27:115-159. 1928.

10. Jannes, L. Deoxyribonucleic acid (DNA) transformation experiments with Escherichia coli and Bacillus subtilis. Acta Pathologica et Microbiologica Scandinavica, Supplementum 154:327-328. 1962.

11. Leonard, C. Gomez, Riley D. Housewright and Curtis B. Thorne. Effects of some metallic ions on glutamyl polypeptide synthesis by Bacillus subtilis. Journal of Bacteriology 76:499-503. 1958.

12. Leonard, C. Gomez and Curtis B. Thorne. Studies on the nonspecific precipitation of basic serum proteins with γ-glutamyl polypeptides. The Journal of Immunology 87:175-188. 1961.

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

14. Marmur, Julius, Edna Seaman, and James Levine. Interspecific transformation in Bacillus. Journal of Bacteriology 85:461-467. 1963.

15. McCloy, Elinor W. Studies on a lysogenic Bacillus strain. I. A bacteriophage specific for Bacillus anthracis. The Journal of Hygiene 49:114-125. 1951.

16. McDonald, William C. and Thomas S. Matney. Genetic transfer of the ability to grow at 55 C in Bacillus subtilis. Journal of Bacteriology 85:218-220. 1963.

17. Nasser, Delill S., and Henry Koffler. Transformation of the property of flagellation by deoxyribonucleic acid. Bacteriological Proceedings, 1962, p. 43.

18. Ravin, Arnold W. The genetics of transformation. In: Advances in Genetics, edited by E. W. Caspari and J. M. Thoday, vol 10. New York, Academic Press, 1961. p. 61-163.

19. Spizizen, John. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proceedings of the National Academy of Sciences 44:1072-1078. 1958.

20. Stuy, J. H. Transformability of Haemophilus influenzae. Journal of General Microbiology 29: 537-549. 1962.

21. Taylor, M. J. and C. B. Thorne. Transduction of Bacillus licheniformis and Bacillus subtilis by each of two phages. Journal of Bacteriology (in press). 1963.

22. Thorne, C. B. Transduction in Bacillus subtilis. Journal of Bacteriology 82:106-111. 1962.

23. Thorne, C. B. Capsule formation and glutamyl polypeptide synthesis by Bacillus anthracis and Bacillus subtilis. In: Bacterial anatomy, edited by E.T.C. Spooner and B.A.D. Stocker. Cambridge, Fngland, The University Press, 1956. p. 68-80.

24. Thorne, Curtis B. Bacteriophage in Bacillus cereus T. Discussion. In: Spores II, a Symposium held at Allerton Park, Illinois, 1960 under the auspices of the University of Illinois, edited by H. Orin Halvorson. Minneapolis, Minnesota, Burgess Publishing Company, 1961. p. 290-296.

25. Thorne, Curtis B., Carmen G. Gomez, Howard F. Noyes, and Riley D. Housewright. Production of glutamyl polypeptide by Bacillus subtilis. Journal of Bacteriology 68:307-315. 1954.

26. Thorne, Curtis B. and C. Gomez Leonard. Isolation of D- and L-glutamyl polypeptides from culture filtrates of Bacillus subtilis. The Journal of Biological Chemistry 233:1109-1112. 1958.

27. Yoshikawa, Hiroshi and Noboru Sueoka. Sequential replication of Bacillus subtilis chromosome, I. Comparison of marker frequencies in exponential and stationary growth phases. Proceedings of the National Academy of Sciences 49:559-566. 1963.

28. Zamenhof, Stephen, Rosalie de Giovanni-Donnelly and Lila H. Heldenmuth. Transfer, by transformation, of information determining mutation rates in Bacillus subtilis. Proceedings of the National Academy of Sciences 48:944-947. 1962.