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ELECTROPHORETIC AND ENZYMATIC IDENTIFICATION OF PROTEINS ASSOCIATED WITH THE DEVELOPMENT OF COMPETENCE IN BACILLUS SUBTILIS

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ELECTROPHORETIC AND ENZYMATIC IDENTIFICATION OF PROTEINS
ASSOCIATED WITH THE DEVELOPMENT OF COMPETENCE IN BACILLUS SUBTILIS

BY

Susan A. Coughlin
B.A., Dowling College, 1975

Dissertation

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To George
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................ iv
LIST OF FIGURES ............................................. vii
LIST OF TABLES ............................................... ix
ABSTRACT ..................................................... x

I. TWO DIMENSIONAL ZYM OGRAM ANALYSIS OF
NUCLEASES IN BACILLUS SUBTILIS ..................... 1

Introduction .................................................. 1

Materials and Methods ...................................... 3
Preparation of DNA .......................................... 3
Preparation of Cell Lysates ................................. 3
Isoelectric Focusing and Non-Equilibrium pH
Gradient Electrophoresis .................................... 4
SDS Gel Electrophoresis ..................................... 5
Preparation of Samples for One-dimensional
Electrophoresis ................................................ 5
Nuclease Detection .......................................... 6
Protein Staining .............................................. 7
Autoradiography ............................................. 7
Analysis of Bacillus subtilis ............................... 7
Sensitivity of the Assay ..................................... 7

Results ......................................................... 9
Analysis of Bacillus subtilis nuclease.................... 9
Sensitivity of the Assay ..................................... 16

Discussion .................................................... 18

References .................................................... 20

II. IDENTIFICATION OF COMPETENCE-ASSOCIATED NUCLEASES AND
POLYPEPTIDES IN BACILLUS SUBTILIS STRAIN SB25 AND SEVERAL
ISOGENIC TRANSFORMATION DEFICIENT MUTANTS ... 23

Introduction .................................................. 23

Materials and Methods .................................... 28
Bacterial Strains ............................................. 28
Media and Growth Regimes .............................. 28
Radioisotopic Labeling of Cellular Protein .......... 28
Measurement of Competence ............................. 29
Preparation of Cell Lysates and Determination of Acid Precipitable Radioactivity ............ 29
Electrophoretic Analysis of Cellular Protein and Nuclease......................... 29
Molecular Weight Analysis ...... 30
Measurement of the pH Gradient Following Isoelectric Focusing ... 30

Results and Discussion .............. 31
Two-dimensional Electrophoretic Analysis of Competent and Non-competitive Bacillus subtilis SB25 .......... 31
Two-dimensional Zymogram Analysis of Nuclease from Competent Bacillus subtilis SB25 .......... 39
Nuclease Activity Associated with Competence in Bacillus subtilis .............. 59
Analysis of Transformation Deficient Mutants .......... 65

References ......................... 86
LIST OF FIGURES

1. Comparison of nuclease and protein distributions obtained from lysates of competent *Bacillus subtilis* following two-dimensional, IEF-SDS, electrophoresis. 11

2. Distribution of competent *B. subtilis* nucleases following two-dimensional, NEPHGE-SDS electrophoresis. 12

3. Response of the ethidium bromide fluorescence pattern to protein denaturing conditions. 15

4. Limit of detection of DNase I. 17

5. Two-dimensional, IEF-SDS, electrophoretic analysis of radioisotopically labeled competent and physiologically non-competent cultures of *B. subtilis*. 32

6. Two-dimensional, NEPHGE-SDS, electrophoretic analysis of radioisotopically labeled competent and physiologically non-competent cultures of *B. subtilis*. 33

7. Divalent cation activation profile of renaturable nucleases active against native calf thymus DNA in competent cultures of *B. subtilis*. 40

8. Divalent cation activation profile of renaturable nucleases active against denatured calf thymus DNA in competent cultures of *B. subtilis*. 42

9. Cd$^{2+}$-activated renaturable nucleases in competent cultures of *B. subtilis* active against native calf thymus DNA. 44

10. Schematic presentation of renaturable nucleases in competent *B. subtilis* cultures detected in gels containing native calf thymus DNA. 48

11. Schematic presentation of renaturable nucleases in competent *B. subtilis* cultures detected in gels containing denatured calf thymus DNA. 49

12. Activity classes of renaturable nucleases in competent *B. subtilis* cultures. 50

13. Two-dimensional zymogram analysis of renaturable nucleases in competent and physiologically non-competent cultures of *B. subtilis*. 62
14. Comparison of protein pattern and distribution in a pair of tandem gels analyzed prior to or after nuclease analysis.

15. Location of nuclease activity relative to autoradiographic polypeptide patterns of radioisotopically labeled competent cultures of B. subtilis.

16. One-dimensional zymogram analysis of renaturable nucleases in competent cultures of transformation-deficient mutants of B. subtilis.

17. Two-dimensional zymogram analysis of renaturable nucleases active in competent cultures of transformation-deficient mutants of B. subtilis.

18. Two-dimensional, IEF-SDS, electrophoretic analysis of radioisotopically labeled competent cultures of transformation-defective mutants of B. subtilis.

19. Two-dimensional, NEPHGE-SDS, electrophoretic analysis of radioisotopically labeled competent cultures of transformation-defective mutants of B. subtilis.
LIST OF TABLES

I. Competence associated polypeptides in *B. subtilis* SB25, IEF-SDS. 35

II. Competence associated polypeptides in *B. subtilis* SB25, NEPHGE-SDS. 36

III. Activity classes and physical characteristics of renaturable nucleases in *B. subtilis* identified by two-dimensional zymogram analysis. 46

IV. A summary of reported *B. subtilis* nucleases. 51

V. Nucleases which may result from persistent subunit interactions. 56

VI. Identification of nuclease pairs differing only in mobility during NEPHGE. 57

VII. Characteristics of transformation-defective mutants examined in this study. 66

VIII. Competence-associated polypeptides in transformation defective mutants of *B. subtilis*, IEF-SDS. 79

IX. Competence-associated polypeptides in transformation defective mutants of *B. subtilis*, NEPHGE-SDS. 80
ABSTRACT

ELECTROPHORETIC AND ENZYMATIC IDENTIFICATION OF PROTEINS ASSOCIATED WITH THE DEVELOPMENT OF COMPETENCE IN BACILLUS SUBTILIS

by

Susan A. Coughlin

University of New Hampshire, September, 1983

A two-dimensional zymogram procedure for the analysis of nucleases is described. Using purified deoxyribonuclease I (bovine pancreas), as little as 10 pg of nuclease can be detected. Isoelectric focusing (IEF) and non-equilibrium pH gradient electrophoresis (NEPHGE) were compared as first dimensions, in combination with SDS electrophoresis as the second dimension, in analyzing nucleases in lysates of Bacillus subtilis. All renaturable nuclease activities detected following SDS electrophoresis alone were resolved in NEPHGE-SDS electrophoresis gels whereas, in IEF-SDS gels, most were either at the basic end or were not present in the second dimensional gel. This method of analysis has revealed a complexity in nuclease species in B. subtilis not previously recognized.

Eighty-three discrete nuclease activities have been detected in B. subtilis lysates. These nucleases have been characterized with respect to monomeric molecular weights, cation-activation requirements, and preference for single or double stranded substrate. Substrate and cation analysis reveals a minimum of 16 classes of activities. The nucleases identified in this study are compared to those previously
The total protein composition and nuclease composition of cellular lysates from competent and non-competent *B. subtilis* strain SB25 and several transformation-defective mutants of SB25 has been examined by two-dimensional electrophoretic and zymogram analysis. Thirty-six competence-associated polypeptides have been identified. Nuclease analysis has revealed that the development of competence is accompanied by the induction of 4 nucleases and the suppression of 6 nucleases. Three of the competence-associated nucleases (monomeric MW; 17,500, 18,000, and 18,500) are primarily activated by Mn$^{2+}$ and are most active on denatured DNA. The fourth (monomeric MW; 17,000) is also Mn$^{2+}$-activated but is distinguished by its preference for native DNA. The nuclease activities which are reduced in competent cells are all primarily Mn$^{2+}$-activated. Three of these nucleases (monomeric MW; 18,000, 21,000, and 24,000) show equal activity on native and denatured DNA. The remaining three (monomeric MW; 17,000, 18,500, and 19,250), show a preference for native DNA.

Transformation-defective mutants have been identified which have a reduced level of the 17,000 MW competence-associated nuclease. These mutants are deficient in uptake and the ability to inactivate exogenous DNA. In addition to the suppression of the 17,000 MW nuclease, these mutants fail to induce a large number of additional competence-associated polypeptides.
I. TWO DIMENSIONAL ZYMGRAM ANALYSIS OF NUCLEASES IN BACILLUS SUBTILIS

Introduction

A number of methods have been reported for the separation and analysis of nucleases following electrophoresis in polyacrylamide gels containing high molecular weight DNA or RNA (1-9). The resolution obtained with these procedures is not great enough to completely analyze a complex mixture of nucleases. Very active enzymes often obscure minor activities. In addition, enzymes which differ only slightly in charge or molecular weight may be indistinguishable. Improving resolution assumes importance since these are precisely the kinds of differences one expects to find in analyzing altered forms of enzymes resulting from either post-translational modification, partial degradation, or mutational changes.

We have combined the resolution afforded by the two dimensional procedures of O'Farrell (10) and O'Farrell et al. (11) with the sensitive method for nuclease detection described by Rosenthal and Lacks (5). The nuclease detection method of Rosenthal and Lacks (5) depends on the renaturability of nucleases following electrophoresis in the SDS-gel dimension and their ability to then hydrolyze nucleic acid which has been trapped in the gel. Among the substrates that have been successfully used to detect nucleases are, native and denatured DNA and ribosomal RNA. DNA hydrolysis is detected by the loss of fluorescence of the DNA-ethidium bromide intercalation complex (1,3). Both the disruption of DNA secondary structure due to extensive hydrolysis, and dif-
fusion of fragmented DNA from the gel during incubation under hydrolytic conditions lead to reduction in fluorescence. It is possible to determine substrate specificities, ion activation and co-factor requirements, pH optima, and response to inhibitors (5,12) of the renatured nucleases.
Materials and Methods

Ampholytes were purchased from LKB; urea, enzyme grade acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylenediamine and ammonium persulfate from Eastman-Kodak; electrophoresis grade sodium dodecyl sulfate (SDS) from BioRad; lysozyme (L-6876), deoxyribonuclease I (DN-CL grade), calf thymus DNA (D-1501), phenylmethylsulfonfyl fluoride (PMSF), 1,10-phenanthroline, and N-ethylmaleimide from Sigma; ribonuclease A (5679) from Worthington; Non-Idet P-40 (NP-40) from Bethesda Research Laboratories; $^{14}$C-labeled L-amino acid mixture (NEC-445) from New England Nuclear.

Preparation of DNA

SP82 bacteriophage DNA was prepared as described (13,14) from purified phage isolated by centrifugation into preformed gradients of cesium chloride. Calf thymus DNA, 2 mg/ml in 15mM NaCl 0.15mM sodium citrate (0.1 x SSC) and 1% SDS was shaken overnight at room temperature. The solution was extracted with an equal volume of chloroform: isooamy l alcohol 20:1 (v/v) and centrifuged (10,000xg for 15 minutes). The upper viscous layer was removed and an equal volume of filtered 95% ethanol was overlaid. The DNA was wound onto a glass rod, transferred to 0.1x SSC. DNA preparations were dialyzed against 3 changes of 10 vol of 0.25x lower gel buffer. DNA was denatured by boiling for 10 min and chilled rapidly.
Preparation of Cell Lysates

*B. subtilis* strain SB25 was made competent by the method of Spizizen (15) as modified by Kohn and Green (16). In cell preparations which were to be radioisotopically labeled, casein hydrolysate was omitted from both stages of growth and a mixture of $^{14}$C-labeled L-amino acids was added to a final concentration of 10μCi/ml during the final 60 min of the second stage of growth. Typically, the volume of radioisotopically labeled preparation was 10 ml. Cells were concentrated by centrifugation and resuspended in 0.01 vol of 0.01 M Tris-HCl, pH 7.4, 1 mM disodium EDTA, 1 mM 1,10-phenanthroline, 5 mM N-ethylmaleimide, 250 μg/ml PMSF and subjected to 5 cycles of freezing and thawing in a dry ice and acetone bath. The cells were then treated with lysozyme at 10 mg/ml and RNase at 50 μg/ml for 15 min at 37° followed by an additional 5 min incubation in the presence of 2% (w/v) NP-40. Solid urea was added to 9 M at room temperature (this is accompanied by a 1.7x increase in volume). After the urea was dissolved, an equal volume of 9.5 M urea containing 2% (w/v) NP-40, 5% 2-mercaptoethanol, 2% (w/v) ampholytes (1.6% pH 5-7, 0.4% pH 3.5-10) was added. The lysates were maintained at -80° until use.

Isoelectric Focusing and Non-equilibrium pH Gradient Electrophoresis

The procedures of O'Farrell were used for isoelectric focusing (IEF) (10) and non-equilibrium pH gradient electrophoresis (NEPHGE) (11) with the exception that gels were not pre-run. The ampholyte composition was 1.6% pH 5-7 and 0.4% pH 3.5-10 for IEF. IEF gels were run for 15 h at 400 V followed by 1 h at 800 V. NEPHGE gels were composed of either 2% pH 3.5-10 ampholytes or 1.6% pH 5-7 and 0.4% pH 3.5-10 ampho-
lytes and were run at 500 V for 90 min or 100 min, respectively. The mobilities of proteins will be affected by the length of the gels and the particular electrophoresis equipment utilized. This is due to changes in the resistance in the system which alters the voltage drop across the gels (17).

Following IEF, the pH gradient was determined as follows: gel sections (1 cm) were transferred to tubes and 0.3 ml of a degassed solution of 10 mM NaCl was added. After flushing with nitrogen the tubes were sealed and shaken at room temperature for 10 min prior to measuring the pH.

**SDS Gel Electrophoresis**

IEF and NEPHGE gels were equilibrated as described (10) and run in a second dimension consisting of the discontinuous SDS gel system of Laemmli (18) as described by O'Farrell (10). The second dimension slab gel consisted of an 11-14% exponential gradient of acrylamide. The volume of the 14% solution was 35% of the total gel volume. Gels which were to be used for nuclease detection included DNA at 20 μg/ml and bovine serum albumin at 10 μg/ml (final concentrations) in the separating gel. A stock solution of bovine serum albumin (10 mg/ml) was boiled for 10 min to destroy contaminating nucleases. Gels were run at 30 mA constant current until the marker dye (bromphenol blue) front had reached the bottom of the gel.

**Preparation of Samples for One-dimensional SDS Electrophoresis**

Samples subjected to SDS electrophoresis alone were frequently run in slab gels alongside IEF and NEPHGE gels. Lysates for such samples
were prepared in the following manner; 0.1 ml of lysate was transferred to a test tube in a 47° heating block. Immediately, 0.5 ml of a liquidified (47°) agarose solution (1.5% agarose, 0.075 M Tris-HCl, pH 6.8, 2.8% SDS, 6% 2-mercaptoethanol, 12% glycerol) was added. The solution was mixed thoroughly, drawn into a 15 cm section of glass tubing (2.5 mm ID), allowed to solidify and then stored at -80°. Prior to electrophoresis a tube was brought to room temperature and a section was extruded and cut off. These samples were applied to SDS slab gels in the same manner as equilibrated IEF or NEPHGE gels.

**Nuclease Detection**

The procedure for nuclease detection is that described by Rosenthal and Lacks (5) with several modifications. SDS was removed from gels by incubating them at 37° with constant agitation in 3 consecutive changes of 500 ml (20 x the gel volume) of 0.04 M Tris-HCl, pH 9.0, 0.02% sodium azide, 2 mM disodium EDTA; twice for 30 min and finally for approximately 15-20 h. Gels were rinsed with distilled water at 37° between buffer changes. All buffer solutions were prewarmed to 37° prior to use.

The gels were rinsed again in distilled water and transferred to 500 ml activation buffer (0.04 M Tris-HCl, pH 7.6, 0.02% sodium azide) along with the cations and/or cofactors required for nuclease activity. 1 M stock solutions of CaCl₂, MgCl₂ and MnCl₂ were filtered through 0.45 μm nitrocellulose filters immediately prior to use. Final cation concentrations were 2 mM. The reaction was terminated by transferring the gel to 500 ml 0.04 M Tris-HCl, pH 7.6, 0.02% sodium azide, 2 mM disodium EDTA, 2-4 μg/ml ethidium bromide. In situ hydrolysis of the DNA trapped
In the gel results in a reduction in ethidium bromide fluorescence.

Gels were photographed on a Chromato-Vue Transilluminator Model C-62 (UV Products Inc.) using a Kodak #16 orange filter and Polaroid Type 55 film (typical exposure time: 4-7 min).

Protein Staining

SDS slab gels were stained in 10 vol of 0.1% Coomassie Blue R-250 in a solution consisting of 5:5:1 methanol: distilled water: glacial acetic acid and destained in 1:17:2 methanol: distilled water: glacial acetic acid.

IEF and NEPHGE gels were fixed in 3.5% perchloric acid, stained with 0.01% Coomassie Blue G-250 in 3.5% perchloric acid (19) and finally transferred to 7.5% acetic acid.

Autoradiography

Gels containing $10^6$ CPM $[^{14}C]$-labeled protein were dried onto filter paper (Whatman, 3 mM) on a slab gel dryer (Hoeffer Scientific) and placed in direct contact with Kodak SB-5 X-ray film at room temperature for autoradiography.

Analysis of B. subtilis Nucleases

Samples of B. subtilis lysates containing either 175 or 350 μg protein were analyzed for nuclease activity following SDS electrophoresis or two-dimensional electrophoresis, respectively.

Sensitivity of the Assay

Deoxyribonuclease I (bovine pancreas) was dissolved and diluted in 3 mM Tris-HCl, pH 7.4, 0.3 mM disodium EDTA, 0.3 mM 1,10-phenanthroline, 1.5 mM N-ethylmaleimide, 74 μg/ml PMSF, 9.25 M urea, 2.5% 2-mercapto-
ethanol, 1% (w/v) NP-40, 1% ampholytes (0.8% pH 5-7, 0.2% pH 3.5-10), 0.1% bovine serum albumin. A NEPHGE gel containing 10 µg of DNase I was stained according to Reisner et al (19). The section of gel corresponding to this position was cut from gels which had been loaded with varying amounts of DNase I and applied to a SDS slab gel containing native calf thymus DNA. Following electrophoresis, the gel was analyzed for nuclease activity as described.
Results

Analysis of B. subtilis Nucleases

A lysate from B. subtilis was analyzed for renaturable nuclease activities in a gel containing native calf thymus DNA following SDS electrophoresis and following the two-dimensional, IEF-SDS electrophoretic procedure of O'Farrell (10). Nuclease activity was terminated after five days in activation buffer containing MgCl$_2$ and CaCl$_2$. The results, presented in Fig. 1a, indicate that many of the proteins which demonstrate nuclease activity following SDS electrophoresis alone are not seen in the two-dimensional pattern.

Comparison of the two-dimensional nuclease pattern to the distribution of radioisotopically labeled polypeptides (Fig. 1b.) obtained with this procedure demonstrates that the isionic properties of the observed nucleases (number average pH$_1$=6.5) are more basic than those of the protein population in general (number average pH$_1$=6.1). It seemed probable, therefore, that nucleases which were lost following IEF-SDS two-dimensional electrophoresis had isionic points greater than the pH gradient employed. Therefore, the NEPHGE procedure was substituted for IEF in the first dimension. In this procedure proteins are subjected to electrophoresis toward the cathode through a rapidly forming pH gradient and are not allowed to reach equilibrium (11). This method permits the detection of basic proteins which migrate off the gel during IEF.

The number of B. subtilis nucleases obtained with this procedure (Fig. 2) is greatly increased. All major renaturable nuclease activities that are detected following SDS electrophoresis are represented in
Fig. 1. Comparison of nuclease and protein distributions obtained from lysates of competent *B. subtilis* following two-dimensional, IEF-SDS electrophoresis.

a. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing native calf thymus DNA. 350 µg of protein was subjected to two-dimensional electrophoresis. 175 µg of protein was subjected to SDS electrophoresis alone, along the right edge of the same gel. Activation buffer contained MgCl₂ and CaCl₂ at 2 mM.

b. Autoradiographic pattern obtained following electrophoresis of samples containing 10⁶ CPM of acid precipitable [³¹C]-labeled protein.
Fig. 2. Distribution of competent *B. subtilis* nuclease following two-dimensional, NEPHGE-SDS electrophoresis. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing native calf thymus DNA. 350 µg of protein was subjected to two dimensional electrophoresis. 175 µg of protein was subjected to SDS electrophoresis alone, along the right edge of the same gel. Activation buffer contained MgCl₂, CaCl₂, and MnCl₂ at 2 mM. The position of lysozyme is identified with an arrow.
the two-dimensional pattern. A total of 72 nucleases have been detected in gels containing native and denatured calf thymus DNA when these are activated by a combination of Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$. In a series of eight independent analyses using four different lysate samples the nuclease pattern was completely reproducible for all major activities. However, seven weak activities that are close to the limit of detection and a series of six Ca$^{2+}$-activated high molecular weight activities that are seen when denatured DNA is the substrate were often, but not reliably, detected.

By analysis of substrate specificity and response to individual ions a total of 83 nucleases have been detected. The two-dimensional clustering of some activities of similar substrate and ion-activation characteristics suggests that the same enzyme may occur in multiple forms (data not shown).

In spite of the large number of activities, only a small number of the nucleases detected can be attributed to aggregated states that migrated at different rates in the first dimension. Seventy-two different nucleases were detected in gels activated simultaneously by Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$. Of these, only 7 were observed to reside at molecular weight positions with nucleases of similar ion-activation and substrate specificities. Six of these pairs of activities with similar properties have been observed on at least three different gels in which different samples were run.

Most nuclease activities show discrete positions although they may have idiosyncratic shapes. However, an intense activity with a molecular weight of 17,000 shows a major activity at a low isionic point (PHI
= 4.8) and in NEPHGE preparations a pronounced tail of activity extending in both directions so that the band runs across the whole SDS gel. Similar anomalous electrophoretic behavior has been reported by Akriigg (20) for an extracellular endonuclease produced by \textit{B. subtilis} during sporulation. The two enzymes have a number of additional features in common including the isionic point of the major activity, ion-activation and substrate specificities, but apparently differ in molecular weight.

The possibility exists that, in some cases, the reduction in ethidium bromide fluorescence is due to the presence of a DNA binding protein rather than a nuclease activity. Lysozyme, which can bind to DNA, does reduce ethidium bromide fluorescence in this assay and is indistinguishable from nuclease activities (see Fig. 2). The following experiment was performed to differentiate between nuclease activity and DNA binding proteins. A lysate of \textit{B. subtilis} was subjected to SDS electrophoresis across the whole length of a gel containing native SP82 bacteriophage DNA. The gel was washed, as described, cut into strips and incubated in activation buffer containing either Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), or EDTA. After the nuclease pattern was recorded the gels were incubated for two days at 37\(^\circ\) in 0.06 M Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, to dissociate DNA binding proteins. The strips were washed again and re-photographed in the presence of EDTA and ethidium bromide. The results (Fig. 3) demonstrate that the band corresponding to lysozyme is the only band which disappears under these conditions. The changes in ethidium bromide fluorescence we have observed either represent proteins with nuclease activity, or binding proteins that do not denature
Fig. 3. Response of the ethidium bromide fluorescence pattern to protein denaturing conditions. A sample of *B. subtilis* protein was subjected to SDS electrophoresis in a slab gel containing native SP82 phage DNA. Following renaturation, gel strips were incubated in activation buffer containing (a) MgCl$_2$, (b) CaCl$_2$, (c) MnCl$_2$, or (d) EDTA at 2 mM for 5 days. Solid line; tracings of Polaroid type 55 film negatives made on a Joyce Loebel scanning densitometer. Broken line; tracings of the same strips after they were incubated for 2 days at 37°C in 0.06 M Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol, to dissociate DNA binding proteins, re-washed, and re-photographed. The position of lysozyme is indicated with an arrow.
under these conditions.

**Sensitivity of the Assay**

A reduction in ethidium bromide fluorescence resulting from the nuclease activity of DNase I at 10 pg was detectable (Fig. 4). This level of sensitivity is comparable to, or greater than, previously reported methods entailing *in situ* hydrolysis of DNA trapped in polyacrylamide gels (1,3,5-9,12).

Some nucleases are inhibited by ethidium bromide (21). In addition, ultraviolet irradiation in the presence of ethidium bromide introduces single strand breaks into DNA (22). Our approach, therefore, has been to record the pattern of ethidium bromide fluorescence only after exhaustive incubation (5-7 days) in activation buffer in the absence of ethidium bromide. This is to ensure that slow acting nucleases (e.g. exonucleases) and those in very low concentrations have sufficient time to act. Undoubtedly, shorter incubation times in activation buffer can be employed in many instances. In a separate experiment the nuclease activity of 300 pg of DNase I was detected within 2 hours (data not shown).
Fig. 4. Limit of detection of DNase I. Sections of NEPHGE gels containing (a) 0.1 pg, (b) 10 pg, (c) 1 ng, and (d) 0.1 μg DNase I (bovine pancreas) were subjected to SDS electrophoresis in a gel containing native calf thymus DNA and analyzed for nuclease activity. Activation buffer contained MgCl₂, CaCl₂ and MnCl₂ at 2 mM. Nuclease activity is identified by a reduction in ethidium bromide fluorescence.
A wide range of both monomeric enzymes and multimeric enzymes composed of identical subunits have been renatured and detected enzymatically in gels following SDS electrophoresis. These include proteases, amylases (23,24), dehydrogenases (23), lipases (24), nucleases (1-7,9,12), DNA polymerases (25), creatine kinase, alkaline phosphatase, uridine diphosphoglucose pyrophosphorylase (26), and fructosyl transferase (27).

Failure to restore enzymatic activity may be an inherent property of certain enzymes. However, at least in some instances, failure may result from inadequate removal of SDS and/or contaminants in different SDS preparations (7,28). The addition of isopropanol (7), and neutral detergent (26,27) have been shown to aid in the renaturation of certain enzymes.

We have detected increased enzymatic recovery in gels washed in pH 9.0 Tris buffer rather than pH 7.6 and have incorporated this modification of the Rosenthal & Lacks (5) procedure into our protocol. This increased activity may be attributed to the low ionic strength of this buffer. It is also possible that the polar nature of the unprotonated Tris molecule behaves in a manner similar to urea (29) in aiding renaturation. The critical micelle temperature (CMT) of SDS, while also a function of the physical conditions of its environment is very close to "room temperature" (30). For this reason it seems advisable to effect SDS removal at a controlled, elevated, temperature (37°), as described in our procedure.
Analysis of \textit{in situ} enzyme activity following two-dimensional electrophoresis has been applied to nucleases using zonal electrophoresis in the first dimension and SDS electrophoresis in the second dimension (9). Scheele, Pash, and Bieger (24) and Manrow and Dottin (26) have recently reported the application of IEF-SDS electrophoretic procedures, similar to that we have independently developed, to the study of a number of other enzymes. Thus, it is likely that the two-dimensional zymogram (2DZ) analysis is of broad utility. Of particular importance is the ability of the 2DZ analysis to distinguish multiple forms of enzymes in preparations previously thought to be homogeneous (25,30).

The 2DZ nuclease procedure was specifically developed for the purpose of identifying nuclease changes associated with the development of competence in \textit{B. subtilis}. We have found that the combination of NEPHGE and SDS electrophoresis is superior to IEF-SDS electrophoresis for the study of nucleases in \textit{B. subtilis}. The procedure may be applicable to the study of a wide range of enzymes with basic isionic points and enzymes associated with DNA management. This analysis has revealed a greater number of nucleases in \textit{B. subtilis} than previously described. More detailed characterization is under way in our laboratory. Preliminary results suggest that in some cases multiple forms of the same enzyme are being visualized. On the basis of cation activation and substrate specificity it is clear that a number of the activities represent unique nucleases previously undetected in \textit{B. subtilis}.

This approach offers a number of advantages. The procedure is rapid since no purification of sample is necessary. Conditions for enzymatic activity, the molecular weight and the ionic properties of each nuclease, in a whole class of nucleases, can be studied simultaneously.
References


Bacillus subtilis Bacteriophage SP82G, J. Virol. 9, 1033-1046.


INTRODUCTION

Competent *Bacillus subtilis* cells are capable of exchanging genetic information by releasing and absorbing DNA to and from the extracellular environment. DNA which has been taken up may be permanently fixed and expressed by the cell following recombination with a resident DNA molecule at a point of partial homology. Transformation occurs when the newly acquired DNA contains genetic information which is new to the recipient cell. This may result in a discernible phenotypic alteration in the transformed cell.

The identification of biochemical events essential for transformation in *Bacillus subtilis* has been complicated by the fact that the spontaneous development of competence, in this bacterium, is predominantly a feature of cultures which are near the stationary phase of growth (1,2). Many complex physiological changes are associated with the differentiation of *B. subtilis* to competence (for reviews on all aspects of competence see ref. 3-15). However, it remains unclear if these changes reflect the vitality and stage of growth of the culture or whether they are related to the biochemical mechanism necessary for transformation. More recent studies, therefore, have been directed toward following the physicochemical fate of transforming DNA and toward identifying competence-specific changes, both in transformation-defective mutants and in enzymes and proteins which interact with DNA.

The action of cell surface nucleases on transforming DNA has been
suggested by the observation that a large proportion of high molecular weight DNA bound to cells is converted endonucleolytically to double stranded fragments of approximately 1-2×10^7 MW prior to internalization (9,16,17). Consistent with this is the fact that there are unique nucleolytic activities associated with competent cells (18,19). Evidence has been presented (20) which indicates that nuclease activity on the cell surface may be essential for transformation in *B. subtilis*. Two competence-associated Mn^{2+}-activated nucleases are not found or if present are found at very reduced levels in spheroplast supernatants from a number of mutants (20) which are deficient in the binding and entry of DNA (21) and are nontransformable.

Additional nucleolytic processing of transforming DNA on the cell surface has been suggested by the fact that a large portion of bound DNA is converted to single strands concomitant with DNA uptake (16,22). Other reports suggest that duplex transforming DNA also occurs intracellularly (23-25). Much of the bound DNA is released to the medium as either acid soluble oligonucleotides (16,19) or as a biologically inactivated limit duplex product (16,17). Several reports suggest the participation of additional DNA repair and management enzymes. Among these, a high level of single-strandedness, introduced into recipient cellular DNA (26,27) is thought to be protected by the simultaneous induction of a DNA-binding protein during competence (28,29). Mazza and Riva (30) have reported that one of four *B. subtilis* DNA-dependent ATPases is increased six fold in competent cells. Yasbin's (31,32) results suggest that competence development activates an error-prone DNA repair system resulting in induction of prophage and the expression of a previously silent DNA modification system.
A potential role for the ATP-dependent nuclease activity of *B. subtilis* in transformation was suggested following the demonstration that two poorly transformable mutants are deficient in this enzyme (33,34). A number of unpublished observations, cited recently by Venema (12) and Dubnau (15), conflict with this interpretation, however. In addition Doly et al. (35) have indicated that the failure of one of these mutants (33) to transform and transduce may be explained by a reduced cell viability. These reports, in combination with the diversity of phenotypes in the transformation-deficient mutants which have been isolated, give an indication of the potential complexity of the biochemical mechanism of transformation.

Dubnau has suggested (15), that rather than identifying the specific enzymatic changes in individual transformation-deficient strains, a more fruitful approach would be to isolate mutants altered in specific enzymes which one might suspect play an essential role in transformation. This approach has at least one shortcoming. Heretofore unidentified enzymes, perhaps those uniquely associated with the biochemical mechanism of transformation would be the last to be discovered and in fact could overlooked indefinitely.

The identification of specific alterations in transformation-defective strains is complicated by the large number of potential enzymatic activities which appear to be involved. This investigation was undertaken, in part, to provide a simple basis for screening transformation-defective mutants. Initially, our approach was as broad as possible. A two-dimensional electrophoretic map of *B. subtilis* polypeptides synthesized during the development of competence was constructed. Secondly, those polypeptides uniquely associated with competence were identified.
by comparison to polypeptides present in physiologically non-competent cells. This approach led to the identification of 14 novel polypeptides in cultures of competent cells. In a series of transformation-defective mutants that were analyzed, an alteration in one or more of 7 of these peptides was detected (36). A number of these competence-associated polypeptides have since been shown to be present predominantly in the competent fraction of cells isolated by renografin gradient centrifugation (37).

The demonstration that the majority of renaturable *B. subtilis* nucleases are too basic to be well resolved by isoelectric focusing (IEF) in pH 5-7 gradients (38), along with the possibility that many DNA-interacting proteins may be similarly charged, led to a search for competence-associated polypeptides with increased emphasis on basic polypeptides. This was accomplished by employing non-equilibrium pH gradient electrophoresis (NEPHGE) in addition to IEF as the first dimension of electrophoresis. A number of additional competence-associated polypeptides have been detected and are reported in this dissertation.

Considerable evidence suggests nucleases play some role at many steps in the biochemical pathway of transformation. However, very little is known about the nuclease content of competent cells. Using a recently developed technique for the identification of the renaturable activities of nucleases following two-dimensional electrophoresis (38), we have begun the characterization of 83 renaturable nucleases in competent cultures of *B. subtilis*.

To identify competence-associated nucleases we have compared nuclease activities derived from competent cells, physiologically non-competent cells, and four transformation-defective mutants of *B. sub-
Ills SB25. In addition, the two-dimensional nuclease pattern was compared directly to the two-dimensional autoradiographic polypeptide pattern.
MATERIALS AND METHODS

Bacterial Strains

*Bacillus subtilis* strain SB25 (hisB₂ trpC₂) and a number of isogenic transformation-deficient mutants (36,39) were used.

Media and Growth Regimes

Competent cells were made by the Spizizen (40) technique as modified by Kohn and Green (41). Physiologically non-competent cells were grown at 37°C in a Tris-salt solution (42) supplemented with 0.5% glucose, 2.5 mM MgCl₂, 0.1% yeast extract, 0.05 mg/ml D,L-tryptophan, 0.4% arginine, 0.02% L-histidine and 0.2% casein hydrolysate. Cells were inoculated at approximately 10⁵ cells/ml, grown to 5 X 10⁷ cells/ml, diluted ten-fold and grown for an additional 90 min. After the first stage of growth the cultures were stored at -80°C following the addition of dimethyl sulfoxide to 7.5%.

Radioisotopic Labeling of Cellular Protein

**Media.** Radioisotopically labeled competent cells were grown in the absence of casein hydrolysate throughout both stages of growth. The first and second stage media for radioisotopically labeled non-competent cellular preparations were identical to unlabeled preparations with the exception that the amino acid composition was adjusted to match the media used for competent cell preparations as follows; casein hydrolysate was eliminated from media for both stages of growth; arginine and histidine concentrations were 0.8% and 0.4%, respectively, in the first stage of growth, and were eliminated from the second stage media.
Labeling. Protein was radioisotopically labeled during the final 60 min of the second stage of growth of competent and non-competent cells by the addition of a mixture of \[^{14}C\]labeled amino acids (NEN) to 10μCi/ml. The uptake and incorporation of radioisotopically labeled amino acids was stopped by adding cold, vitamin free, casein hydrolysate to 5%, chloramphenicol to 200 μg/ml, and rapidly chilling the cells.

Measurement of Competence

The level of competence was determined in a marker rescue assay as previously described (43) by adsorbing cells that had been preinfected with bacteriophage H167-A4 (44), a double temperature-sensitive mutant of SP82, to a saturating level of wild type SP82 bacteriophage DNA (45).

Preparation of Cell Lysates and Determination of Acid Precipitable Radioactivity

The procedure for the preparation of cell lysates has been described (38). The amount of acid precipitable radioactivity was measured as follows; A 10 μl sample of each lysate was added to 2 ml of cold 5% trichloroacetic acid. An aliquot of this suspension and an equal aliquot of the supernatant following centrifugation (10,000 X g for 10 min) was counted in a Nuclear Chicago liquid scintillation counter. The liquid scintillation cocktail contained 0.56% omnifluor in 1:2 (vol/vol) Triton X 100: toluene.

Electrophoretic Analysis of Cellular Protein and Nucleases

The procedures for electrophoresis, nuclease detection, staining and autoradiography have been described (38). Fluorography was done at -80° following impregnation of gels with New England Nuclear Enhance solution. X-ray film was preflashed according to the procedure des-
Molecular Weight Analysis

Molecular weight was determined by comparison to the mobility of a series of methylated $[^{14}C]$-labeled proteins purchased from New England Nuclear. The protein standards and their molecular weights were as follows; phosphorylase B, 92,500; albumin (bovine serum) 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lactoglobulin A, 18,367; cytochrome C, 12,300.

Measurement of the pH Gradient Following Isoelectric Focusing

The procedure used for determining the pH gradient following isoelectric focusing has been described (38, Chapter 1).
RESULTS AND DISCUSSION

Two-dimensional Electrophoretic Analysis of Competent and Non-competent B. subtilis SB25

B. subtilis, strain SB25, grown in the competence regime, was radioisotopically labeled during the final 60 min of the second stage of growth. During this time interval a typical culture increases 10-50 X in the level of competence. At the completion of the labeling period these cultures contained 3-5% competent cells as determined in a marker rescue assay. Physiologically non-competent cultures of SB25 were labeled for 60 min during logarithmic growth and contained, at the termination of the labeling period, 0.16% the level of competence exhibited by cultures grown in the competence regime.

Lysates from competent and non-competent cellular preparations containing 1.7 x 10^6 CPM of acid precipitable counts were analyzed by two dimensional electrophoresis with either IEF or NEPHGE the first dimension and SDS electrophoresis in the second dimension. The IEF-SDS and NEPHGE-SDS electrophoretic patterns from cells grown under the two culture conditions are presented in Fig 5 and Fig 6, respectively. We have focused our attention on those polypeptides which were either markedly increased or were novel in competent cell preparations.

Twenty-two new competence-associated polypeptides were detected in this study in addition to the 14 previously reported (36). The addition of lactoglobulin (18,400 MW) as a molecular weight standard for SDS electrophoresis permitted more accurate estimation of the molecular weights of the competence-associated polypeptides than in the original
Fig 5. Two-dimensional, IEF-SDS, electrophoretic analysis of radioisotopically labeled competent and physiologically non-competent cultures of *B. subtilis*. Autoradiographic pattern obtained following electrophoresis of samples containing $1.7 \times 10^6$ CPM of acid precipitable [14C]-labeled protein prepared from A, competent and B, physiologically non-competent cultures.
Fig 6. Two-dimensional, NEPHGE-SDS, electrophoretic analysis of radiolabeled competent and physiologically non-competent cultures of B. subtilis. Autoradiographic pattern obtained following electrophoresis of samples containing $1.7 \times 10^6$ CPM of acid precipitable $[^{14}C]$-labeled protein prepared from A, competent and B, physiologically non-competent cultures.
study (36). A summary of the thirty seven competence-associated polypeptides is presented in Table I and II.

The results indicate that *B. subtilis* is similar to a number of transformable bacteria in which are induced a unique set of polypeptides while undergoing differentiation to the competent state. In *Streptococcus pneumoniae* the development of competence is accompanied by the synthesis of 16 new polypeptides (47). Similarly, 10 competence-associated polypeptides have been detected in *Streptococcus sanguis* (48) and in *Haemophilus influenzae*, 6 polypeptides, synthesized specifically during competence development were detected in purified cell envelope preparations (49).

The induction of competence-associated polypeptides in *S. pneumoniae* (47,50) and *S. sanguis* (48) is accompanied by the overall suppression of non-competence-associated protein synthesis. Shortly following the competent phase, competence-associated protein synthesis is again turned off and precompetent protein synthesis is resumed. This evidence suggests that the abrupt transient appearance of competence, which in these bacteria occurs in the midst of logarithmic growth (48,51), involves a complete reorganization of gene expression. The competent state in these two bacteria is elicited by the synthesis of a small protein which triggers the rapid and synchronous development of competence in nearly the entire population of cells (52,53). Competence, which normally develops spontaneously at a specific cell density (48,51), can be induced by the addition of competence factor, isolated from the supernatant of a competent culture. Morrison and Baker (50) and Raina and Ravin (48) demonstrated that the precocious induction of competence following the addition of purified competence factor, was
Table I. *B. subtilis* Competence Associated Polypeptides

<table>
<thead>
<tr>
<th>Molecular wt (X10^-3)</th>
<th>IsoIonic pH</th>
<th>Type</th>
<th>Intensity</th>
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<td>17</td>
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<td>17.5</td>
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<td>20.5</td>
<td>5.32</td>
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<td>weak</td>
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<td>25</td>
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<td>moderate</td>
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<tr>
<td>31</td>
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<td>strong</td>
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<td>33</td>
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<td>increased</td>
<td>moderate</td>
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<td>moderate</td>
</tr>
<tr>
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<td>increased</td>
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<tr>
<td>43</td>
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<td>50</td>
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<td>56</td>
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<tr>
<td>69</td>
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<tr>
<td>86</td>
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</tr>
<tr>
<td>100</td>
<td>5.46</td>
<td>increased</td>
<td>moderate</td>
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</table>
Table II. *B. subtilis* Competence Associated Polypeptides

**NEPHGE-SDS**

<table>
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<tr>
<th>Molecular wt. (X10^-3)</th>
<th>Mobility NEPHGE/Lysozyme</th>
<th>Type</th>
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<td>24.5</td>
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<td>27.5</td>
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<tr>
<td>36</td>
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<td>moderate</td>
</tr>
<tr>
<td>46</td>
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</tr>
<tr>
<td>48</td>
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<td>51</td>
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<td>58</td>
<td>0.30</td>
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</tr>
<tr>
<td>104</td>
<td>0.16</td>
<td>Increased</td>
<td>strong</td>
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</table>
accompanied by the same abrupt change-over to the selective synthesis of competence-associated polypeptides, as that observed when competence developed spontaneously. It can be concluded, therefore, that in *S. pneumoniae* and *S. sanguis*, these events are not dependent on a specific cell density.

It is more difficult to demonstrate that competence is independent from cell titre in *B. subtilis*. The growth regimens which have been designed to produce maximal competence in *B. subtilis* (40,54) demonstrate that the development of competence occurs near the stationary phase of growth. It is probable that early sporulation events are also induced in these nutrient depleted cultures. Based on the observation that some mutants deficient in sporulation are also poorly transformed, Young and Spizizen (55) postulated in 1961, that competence in *B. subtilis* is associated with the early events of spore formation. The extent of interdependence between these two pathways of differentiation has not been determined.

This question might be partially resolved by monitoring the synthesis of competence-associated polypeptides following the precocious induction of competence by a competence factor. The existence of a *B. subtilis* competence factor, with the ability to accelerate development of competence, has been suggested by the observation that competence appears earlier in cultures grown in supernatants removed from competent cultures (56). However, these cultures also enter stationary phase prematurely. Attempts to supplement the culture medium and prolong growth were partially successful in separating the development of competence from stationary growth, but resulted in the severe depression of transformability. It would appear, therefore, that the *B. subtilis*
competence factor in its present state of understanding will not be useful in this respect.

The study of *B. subtilis* competence-associated protein synthesis is complicated by the fact that, in maximally competent cultures, only 10-20% of the cells are competent (57). It has not been determined if, protein synthesis of non-competence-associated proteins is severely depressed in *B. subtilis* as it is in *S. pneumoniae* (47,50) and *S. sanguis* (48). A higher percentage of competent cells can be obtained by fractionating the culture on gradients of Renograffin (58,59). This method is based on the fact that competent cells are less dense than most of the non-competent population. An analysis of competence-associated polypeptides in cells fractionated in Renograffin gradients indicated that, a number of competence-associated polypeptides were synthesized predominantly in the competent fraction. Many radiolabelled polypeptides not associated with competence, were present as well (37). However, this should not be taken as evidence that during the development of competence in *B. subtilis* a generalized suppression of background protein synthesis does not occur since the development of competence in *B. subtilis* cultures is asynchronous. Smith et al. (37) employed a 1.5 h labelling period in their investigation. Undoubtedly, many cells incorporated radiolabelled into protein before the initiation of differentiation to competence.

The development of competence in *B. subtilis* results in the spontaneous induction of lysogenic bacteriophage (60). Since non-infectious prophage are ubiquitous in *B. subtilis* (61), it is probable that a number of the competence-associated polypeptides detected in this study resulted from the concomitant activation of genes specified by these
defective lysogens. In at least one instance, this is known to occur. The genetic information controlling the expression of a DNA modification system which is induced during the development of competence has been located within the genome of the defective prophage SPβ (62). However, *B. subtilis* strains which do not carry the defective prophages PBSX and SPβ, develop competence normally (62). Therefore, the proteins specified by these phage are not of primary importance in this study. The identification of these proteins, however, will aid in categorizing transformation-deficient strains which are altered in the ability to induce prophage.

Competent cultures contain an elevated level of one *B. subtilis* DNA-dependent ATPase (30). The physical properties of this enzyme (monomeric MW = 108,000; pH₁ = 5) resemble those of a competence-associated polypeptide identified in this study (monomeric MW = 100,000; pH₁ = 5.46) (see Table 1 and Fig. 5). It has been suggested that this enzyme may function during the recombination of donor transforming DNA and the host chromosome. The enzyme increases the formation of stable D-loops between single stranded DNA and homologous circular DNA (30) acting similarly to the *E. coli* recA protein (63-65).

**Two-Dimensional Zymogram Analysis of Nucleases from Competent *B. subtilis* SB25**

*Comparison of 2DZ nucleases to previously characterized *B. subtilis* nucleases.* Lysates prepared from competent SB25 cultures were analyzed for renaturable nuclease activity by two-dimensional zymogram (2DZ) analysis (38). Nuclease activity was compared using native (Fig 7) and denatured (Fig 8) calf thymus DNA following activation by A, Mg²⁺; B, Ca²⁺; C, Mn²⁺; or D, a combination of all three divalent cations. A
Fig 7. Divalent cation activation profile of renaturable nucleases active against native calf thymus DNA in competent cultures of B. subtilis. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing native calf thymus DNA. Approximately 350 μg of
protein prepared from a competent culture of *B. subtilis* SB25 was subjected to two-dimensional, NEPHGE-SDS, electrophoresis and analyzed for nuclease activity. Activation buffer contained A, Mg^{2+} at 2mM. Approximately 175 μg of protein was subjected to SDS-electrophoresis alone, along the right edge of gels A, B, and C.
Fig 8. Divalent cation activation profile of renaturable nucleases active against denatured calf thymus DNA in competent cultures of B. subtilis. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing denatured calf thymus DNA. Approximately 350 µg
of protein prepared from a competent culture of **B. subtilis** SB25 was subjected to two-dimensional, NEPHGE-SDS, electrophoresis and analyzed for nuclease activity. Activation buffer contained A, Mg$^{2+}$; B, Ca$^{2+}$; C, Mn$^{2+}$; and D, Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ at 2 mM. Approximately 175 µg of protein was subjected to SDS-electrophoresis alone, along the right edge of gels A, G, and C.
Fig 9. Cd$^{2+}$-activated renaturable nucleases in competent cultures of *B. subtilis* active against native calf thymus DNA. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing native calf thymus DNA. Approximately 350 µg of protein prepared from a competent culture of *B. subtilis* SB25 was subjected to two-dimensional, NEPHGE-SDS, electrophoresis and analyzed for nuclease activity supported by Cd$^{2+}$.
A total of 83 discrete nucleases were detected. A numerical assignment is given to each nuclease in Figs 10 and 11. The distribution of nucleases belonging to individual activity classes (described in Table III) is presented in Fig 12. The characteristics of the nucleases are summarized in Table III. A literature survey of *B. subtilis* nucleases is presented in Table IV. Subunit molecular weight and substrate preference were the primary characteristics considered in attempting to identify those *B. subtilis* nucleases which have been previously reported within the 2DZ pattern. The cation activation properties are a less reliable means of comparison as this may vary with reaction conditions and can be markedly influenced by minor contamination in a preparation.

2DZ nucleases 55, 53 and 51 closely match the Ca$^{2+}$-activated, single-stranded-DNA-specific endonuclease described by Ciarrochi et al. (66) and Cobianchi et al. (67). A Ca$^{2+}$-activated nuclease with subunit molecular weight and substrate specificity identical to that of 2DZ nuclease 62 has been described (78). This nuclease, reportedly, (78) retains 40% of its activity in the presence of 10 mM EDTA. There is presently no evidence for Ca$^{2+}$-independent activity for 2DZ nuclease 62. However, partial cation independence has not been ruled out, since the level at which this enzyme was observed was close to the limit of detection in the 2DZ gel.

2DZ nuclease 2 behaves, in many respects, like the sporulation-associated extracellular nuclease characterized by Akrigg (84). The isionic pH ($pH_i$) of nuclease 2 is 4.8, identical to that reported (84) for the extracellular enzyme. In addition, when electrophoresis is conducted in pH gradients, neither enzyme forms a discrete sharp band. This tendency to streak is very pronounced during non-equilibrium pH
### TABLE III. Activity Classes and Physical Characteristics of Renaturel Nucleases in E. coli

Identified by Two-dimensional Zymogram Analysis

<table>
<thead>
<tr>
<th>Primary Cation</th>
<th>Substrate Preference</th>
<th>Activity Class</th>
<th>Secondary Cation</th>
<th>Nuclease Classification</th>
<th>Mobility (kD)</th>
<th>Inhibition on Mixed Cation Gel</th>
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<tbody>
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<td>Ca</td>
<td>A</td>
<td>Mn</td>
<td>71</td>
<td>75.0</td>
<td>0.37</td>
<td>+</td>
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<tr>
<td></td>
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a. Na, native DNA; D, denatured DNA
b. Estimated to nearest 500
c. * indicates reduced activity in prescence of Ca, Mg + Na in comparison to activity in presence of primary cation
Fig 10. Schematic presentation of renaturable nucleases in competent *B. subtilis* cultures detected in gels containing native calf thymus DNA.
Fig 11. Schematic presentation of renaturable nucleases in competent *B. subtilis* cultures detected in gels containing denatured calf thymus DNA.
Fig 12. Activity classes of renaturable nucleases in competent *B. subtilis* cultures. Specific characteristics of activity classes, based on cation activation profiles and substrate preferences are given in Table III.
Table IV. A Summary of Reported *Bacillus subtilis* Nuclease

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<td></td>
</tr>
<tr>
<td>I</td>
<td>Exo</td>
<td>R</td>
<td>Mg, Mn, Ca</td>
<td>7.0-8.5</td>
<td></td>
<td>105</td>
</tr>
</tbody>
</table>

a. I, intracellular; E, extracellular; P, periplasmic

b. Endo, endonuclease; exon, exonuclease

c. R, RNA; D, denatured DNA; N, native DNA; Uv/0, ultraviolet irradiated; AP, adenosine or adenosine DNA

References:

1. Endo, exonuclease, endonuclease
2. R, RNA; D, denatured DNA; N, native DNA; Uv/0, ultraviolet irradiated; AP, adenosine or adenosine DNA DNA
gradient electrophoresis (see Fig. 7 and 8). It is the only enzyme which migrates in this manner. The results of a recent study on the membrane bound form of *B. subtilis* α-amylase (104) is of interest in this context. Highly purified membrane-derived α-amylase contains phospholipid. The phospholipid-α-amylase complex is not dissociated by Triton X-100 and is partially insensitive to SDS. However, the presence or absence of the phospholipid component does not affect the mobility of the protein during SDS electrophoresis (104). A complex with a highly charged component would explain the unusual electrophoretic behavior of 2DZ nuclease 2 during NEPHGE.

Since Cd$^{2+}$ has been reported (84) to activate the extracellular enzyme, renaturable *B. subtilis* nuclease were analyzed for activation by Cd$^{2+}$. The nuclease pattern (Fig 9) indicates that nuclease 2, along with at least 5 additional nucleases (2DZ nucleases 18, 19, 30, 34 and 54), are activated by Cd$^{2+}$.

Although similar in many characteristics, the extracellular enzyme and 2DZ nuclease 2 appear to differ in molecular weight. The extracellular nuclease is reported to have a native molecular weight of 12,000 (determined by gel filtration) while the subunit molecular weight of 2DZ nuclease 2 (determined by SDS electrophoresis) is 17,000. It is possible that the apparent difference in molecular weight between 2DZ nuclease 2 and the Mn$^{2+}$-activated, extracellular enzyme described by Akridge (84) reflects the methods used to estimate molecular weight.

2DZ nuclease 24 resembles a Mg$^{2+}$-activated, apurinic site-specific nuclease recently identified in *B. subtilis* (93,94). The activity of 2DZ nuclease 24 was only detected in gels containing denatured DNA. This is consistent with the possibility that they are the same enzyme
since a limited number of apurinic sites were introduced into DNA samples which underwent denaturation by boiling (105).

The competence-associated nuclease described by Burke and Spizizen (79) was not observed in standard gels nor in gels preincubated with 3M guanidine HCl (data not shown), a treatment which reportedly (79) activates this enzyme. This is not entirely surprising since the enzyme appears to act only once and results in a limiting product of $10^5$ MW. In addition, no nuclease with characteristics like those described for the exonuclease activity of *B. subtilis* polymerase III (pol III) (87,88) was detected. Since pol III is a monomeric protein, failure to detect it was probably due to one or more of the following reasons. The enzyme is sensitive to N-ethylmaleimide and, therefore, may have been partially or totally inactivated during sample preparation. Secondly, it hydrolyzes single stranded DNA exonucleolytically, from the 3′ end only, and is maximally active on small molecular weight DNA fragments (e.g. sonicated DNA). Lastly, it does not appear to hydrolyze DNA in a processive manner. Instead, it complexes briefly with its substrate, hydrolyzing short stretches of DNA with each interaction (87, 88). For these reasons it is likely that the exonuclease activity of *B. subtilis* Pol III, under the general 2DZ conditions employed, would be limited. However, this enzyme is capable of regaining activity following SDS electrophoresis since Spanos *et al.* (106) have detected *B. subtilis* Pol III exonucleolytic activity in SDS gels containing $[^{32}\text{P}]-3'$end-labeled substrate by monitoring release of the label. The remaining reports of *B. subtilis* nucleases which do not require a specialized substrate or ATP as a cofactor have not included subunit structure or molecular weight determination making further comparison impossible.
Analysis of the 2DZ nuclease pattern. The 2DZ nuclease pattern reveals a greater number and variety of nucleases in *B. subtilis* than previously identified by standard fractionation and isolation procedures. Categorization of the nucleases on the basis of cation activation and substrate preference (see Fig 10-12 and Table III) leads to a likely explanation for this complexity. The clustering of nucleases with similar or identical catalytic requirements in the two-dimensional pattern, suggests that a number of enzymes may occur in multiple forms. This has not been confirmed by direct isolation and examination of the nature of these potential heterogeneous forms. However, it is useful as a working hypothesis in the following discussion.

Multiple nuclease forms which differ in molecular weight were the most frequently encountered. This was sometimes accompanied by an alteration which influenced mobility during NEPHGE. A predominant form of post-translational modification which results in a change in molecular weight in bacteria results from proteolytic processing. Post-exponential growth phase cultures of *B. subtilis* synthesize a diverse group of proteases in copious amounts (107-114). To reduce the likelihood that different enzyme forms are being generated during sample preparation, the samples were lysed in the presence of a number of protease inhibitors which, in combination, act on all the types of proteases reported in *B. subtilis* to date (107-114). The relative proportions of multiple nuclease forms showed little variation in an analysis of 4 separate samples of competent cells (see mutant analysis). This suggests that either the different forms pre-exist in cells or that changes due to proteolytic cleavage during sample preparation are limited and controlled. The induction of at least one intracellular
protease during sporulation in *R. subtilis* results in an increased rate of protein turnover and site-specific proteolytic cleavage of a precursor form of a spore coat protein (111-114). Competent *R. subtilis* cells resemble, in some properties, early sporulating cells (115). It is possible that sporulation-associated proteases are responsible for the multiple enzyme forms observed. However, an examination of the heterogeneity of nucleases in logarithmically growing cultures, while incomplete, has not supported this conclusion (see Fig. 13).

In some instances, partial subunit association may persist if protein samples are not boiled in the presence of SDS (20,116). This possibility has not been examined because heating samples, which contain residual urea from the first dimensional electrophoretic analysis, would produce isocyanate resulting in carbamylation of free amino groups. The two-dimensional pattern was examined for nucleases with apparent molecular weights that were some multiple of a nuclease with similar catalytic requirements and mobility during NEPHGE. The 11 nuclease pairs which show such potential relationships are summarized in Table V.

Covalent modification of amino acid side chains may explain multiple enzyme forms for which only a charge difference can be detected. A wide range of covalently modified amino acids have been identified in bacteria (see for example ref. 117-120). The number of nucleases which potentially exhibit this type of microheterogeneity is limited (Table VI). The fact that it is not widespread reduces the likelihood that they are due to random artifactual modification of proteins.

A number of nuclease activities, detected in gels activated with Mn$^{2+}$ alone, were not observed in gels activated by Mn$^{2+}$ in combination with Mg$^{2+}$ and Ca$^{2+}$ (see in particular the intense activities correspond-
Table V. Nucleases which may result from persistent subunit interactions

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<tr>
<th>Monomer</th>
<th>Dimer</th>
<th>Tetramer</th>
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<tr>
<td>43 (29)</td>
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</tr>
<tr>
<td>2 (17)</td>
<td></td>
<td>60 (48)</td>
</tr>
<tr>
<td>52 (37)</td>
<td>57 (39.5)</td>
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</tr>
<tr>
<td>36 (27)</td>
<td>44 (30)</td>
<td>46 (31)</td>
</tr>
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<td>Activity Class</td>
<td>Nuclease#</td>
<td>MW x 10^{-3}</td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>B'</td>
<td>38</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>37</td>
<td>27.0</td>
</tr>
</tbody>
</table>
ing to the Mn$^{2+}$-activated nuclease cluster including 2DZ nucleases 18,19,20,23, and 27). The competitive inhibition of nucleases by incompatible divalent cations is not unusual, however, an interesting alternative explanation exists.

Mn$^{2+}$ can have a profound influence on the reaction mechanisms of a heterogeneous group of enzymes which interact with DNA. These include DNA and RNA polymerases where Mn$^{2+}$ promotes the misincorporation of ribonucleotides (121) and deoxyribonucleotides (122), respectively. In addition, Mg$^{2+}$-activated DNase I typically proceeds by a "double hit" mechanism in which only one strand of the double helix is cleaved at a time. In the presence of Mn$^{2+}$, Ca$^{2+}$ or Co$^{2+}$, DNase I simultaneously cleaves both strands (123). Several restriction endonucleases alter or lose their specificity when Mn$^{2+}$ is substituted for Mg$^{2+}$ (124-125). This Mn$^{2+}$ effect is not an absolute feature of all site-specific restriction enzymes. Nevertheless, it is not known how widespread a phenomenon it is. It might be imagined that these Mn$^{2+}$-activated nucleases were not detectable in mixed cation gels as a result of an altered reaction mechanism. The end product of Mg$^{2+}$-activated, sequence-specific hydrolysis might be too large to diffuse from the gel, whereas relaxed specificity in the presence of Mn$^{2+}$ might result in a diffusible product. A number of restriction enzymes have been detected in B. subtilis (95-97) but have not been physically characterized. One of these, Bsu, decreases its substrate specificity at low ionic strength and high pH and high glycerol concentration (126). Under these conditions it cleaves at the dinucleotide sequence NGCN rather than the tetranucleotide G GCC recognized under standard conditions. Bsu activity has not been examined in the presence of Mn$^{2+}$. However, EcoRI site-
specificity is relaxed in the presence of Mn\(^{2+}\) (124) or under conditions very similar to those affecting Bsu specificity (127).

A similar class of Ca\(^{2+}\)-activated nucleases were detected in the presence of Ca\(^{2+}\) alone, but were inactive in gels incubated with Ca\(^{2+}\), Mn\(^{2+}\) and Mg\(^{2+}\). The possibility exists that these enzymes reflect a similar cation-specific effect. However, since these activities are all near the limit of detection, it would seem that competitive cationic inhibition is an equally plausible explanation.

**Nuclease Activity Associated with Competence in Bacillus subtilis.**

The Involvement of Mn\(^{2+}\) nucleases. Scher and Dubnau (85,86) were the first to identify and partially characterize a periplasmic, Mn\(^{2+}\)-activated endonuclease from *B. subtilis*. This nuclease was shown to preferentially hydrolyze native DNA to a non-discrete duplex product of approximately 2 x 10\(^6\) MW. Scher and Dubnau postulated that this enzyme might be responsible for the cell surface endonucleaseolytic fragmentation of transforming DNA which is known to occur prior to uptake. The enzyme was isolated from late log phase cultures in which the level of competence was not determined. However, it may have been appreciable.

Recently, evidence has been presented that supports the possibility that periplasmic, Mn\(^{2+}\)-activated, nuclease activity is involved in the processing of DNA at the cell surface and the uptake of transforming DNA. The supernatants from spheroplasts of competent *B. subtilis* contain two Mn\(^{2+}\)-activated nucleases. The monomeric molecular weights determined by SDS electrophoresis are 13,000 and 15,000. The 13,000 MW enzyme predominates. Several transformation-deficient mutants of *B. subtilis*, which are reduced in their ability to bind and take up DNA, release less of these enzymes, as do physiologically non-competent cells.
We have studied a similar, if not identical, nuclease. 2DZ nuclease 2 requires Mn$^{2+}$ and is most active against native DNA. The location of this enzyme on the extracellular cell surface is implied by the fact that transformation-deficient mutants with a reduced level of this enzyme (see below) are phenotypically less able to inactivate extracellular transfecting DNA. The ability of intact *B. subtilis* cells to reduce the biological activity of exogenous DNA was first described by Haseltine and Fox (18). Inactivation was not observed in control experiments where DNA was exposed to cell-free culture fluids. It is possible, however, that at some stage following competence, this cell surface nuclease is released to the medium. As mentioned previously, 2DZ nuclease 2 closely resembles a Mn$^{2+}$-activated endonuclease which has been detected extracellularly during the onset of early sporulation events (84).

Comparison of nuclease levels in competent and physiologically non-competent SB25. To determine whether 2DZ nuclease 2 is associated with competence, the nuclease composition of competent and physiologically non-competent SB25 were compared. The level of competence in the two preparations differed by a factor of 1700. In these samples the concentration of lysozyme used to disrupt the cells exceeds that of the total cellular protein. Since a useful determination would be difficult to obtain, a protein sample derived from an equal number of cells was analyzed. Because 2DZ nuclease 2 is such an active nuclease in the competent preparations, the sample size was reduced to approximately 25% of that which was typically analyzed. Triplicate samples, containing $1.4 \times 10^8$ cellular equivalents of protein from either competent or
physiologically non-competent cultures of SB25, were subjected to 2DZ analysis. Following SDS removal, the renatured enzymes were activated with a combination of Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$. Nuclease activity was terminated periodically by transferring a set of gels to fresh activation buffer (20 x the gel vol) in which the divalent cations had been replaced by an equimolar concentration of disodium EDTA. All the gels were photographed at the completion of the experiment to ensure that diffusion of the hydrolyzed DNA out of the gel was complete. The results, presented in Fig 13, show the nuclease patterns following the shortest (24.5h) and the longest (163h) activation times. The activity of 2DZ nuclease 2, 6, 7 and 9 are clearly increased in the competent preparation. In addition, 6 very basic nuclease (2DZ nuclease 3, 5, 10, 12, 16, and 26), present as strong activities in the log phase sample are greatly reduced, or not detectable in the corresponding competent preparation. These 6 nucleases were detected in competent preparations in gels in which approximately four times as many cell equivalents of protein was analyzed (see Fig. 7 and 8). However, the level of activity is still not as high as in the log phase preparation.

Identification of nuclease within the two-dimensional polypeptide pattern. The location of nucleases within the two-dimensional polypeptide pattern was determined in two ways. A radioactively labeled cell lysate from a competent cell culture was analyzed for nuclease activity. The polypeptide pattern was then recorded fluorographically. Protein diffusion prior to acid fixation makes comparison of these patterns to the two-dimensional autoradiographic patterns difficult. Therefore, a second approach was taken in which a single first dimension sample was simultaneously electrophoresed into two identical slab gels. The de-
tails of this technique will be described elsewhere. One gel was analyzed first for nuclease activity and secondly for proteins by fluorography. The tandem gel was acid fixed and the proteins analyzed fluorographically immediately following electrophoresis. Fluorograms from a pair of tandem gels, analyzed in this manner, are presented in Fig 14. The extent of entrapment or diffusion from the polyacrylamide matrix prior to acid fixation is a reproducible characteristic of particular protein species. The near identity of the protein distributions in tandem gels is demonstrated in Fig 14C. Therefore, nuclease activity in these gels could be compared directly to both fluorographic patterns. By a combination of these two approaches the zymogram pattern was superimposed upon the autoradiographic pattern. Nuclease activity was tentatively ascribed to specific polypeptides as shown in Fig 15.

2DZ nuclease 2 coincides electrophoretically with a weak acidic competence-associated polypeptide (monomeric MW=17,000 pH\textsubscript{I} = 4.8). Many nucleases were not detected in the autoradiographic patterns. Included in this category are the remaining competence-associated nucleases and the nucleases which were strongly elevated in log phase cultures. It is possible that these nucleases are either present at very low levels or are not being actively synthesized since cultures which are developing competence are not rapidly dividing. Some of these nucleases may represent proteins which were synthesized prior to the labeling period. This, however, seems less likely in the case of those nucleases which were found to be elevated in log phase cultures. Proteins which are relatively acid soluble can diffuse out of polyacrylamide gels during acid fixation, staining and destaining. This could explain the failure to detect some or all of these nucleases in the radioactive polypeptide
Fig 13. Two-dimensional zymogram analysis of renaturable nucleases in competent and physiologically non-competent cultures of *B. subtilis*. Pattern of ethidium bromide fluorescence following renaturation and activation of nucleases in a gel containing native calf thymus DNA. $1.4 \times 10^8$ cellular equivalents of protein prepared from A, C; competent, and B, D; physiologically non-competent cultures. Activation of nucleases was terminated with EDTA following A, B; 24.5 h and C, D; 163 h in activation buffer containing Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ at 2 mM.
Fig 14. Comparison of protein pattern and distribution in a pair of tandem gels analyzed prior to or after nuclease analysis. Fluorographic pattern obtained following two-dimensional, NEPHGE-SDS, electrophoresis in a tandem gel, of a sample containing $1.7 \times 10^6$ CPM of acid precipitable $[^{14}C]$-labeled protein prepared from a competent culture of *B. subtilis*. One half of the tandem gel, A, was acid fixed and stained and prepared for fluorography immediately following electrophoresis. The other half of the tandem gel, B, was analyzed for nuclease activity prior to acid fixation, staining, and fluorographic analysis. C, superimposition of fluorograms from A and B.
Fig 15. Location of nuclease activity relative to the autoradiographic polypeptide pattern of a radiolosotopically labeled culture of B. subtilis.
Three additional nucleases coincide electrophoretically with competence-associated polypeptides. In the study in which the nuclease composition of competent and non-competent cultures was compared (Fig 13), one of these nucleases (2DZ nuclease 35) was not detected and the remaining two (2DZ nucleases 25 and 28) were slightly elevated in the physiologically non-competent cultures. Additional information will be required before the relationship of these nucleases to competence can be determined.

Analysis of Transformation Deficient Mutants

Characteristics of mutants. The transformation-deficient mutants analyzed in this study, NH03, NH46, NH4603 and NH9103, have been described previously (36,39), along with the manner in which they were isolated (36). A brief summary of their characteristics is presented in Table VII. Phenotypically, these strains represent two classes of mutations affecting competence. The sen (surface endonuclease\(^{-}\)) phenotype is expressed by mutants NH46, NH4603 and NH9103 which possess a reduced ability to biologically inactivate extracellular DNA. Sen mutants are characteristically deficient in transformation and transfection. These mutations block the conversion of DNA, which does bind to the cells, to a state which is insensitive to deoxyribonuclease. This state is presumed to be intracellular. Binding deficient and proficient strains are represented in this class. The ten (constitutive transfection enhancement) mutation present in NH03 and NH4603 results in lowered transformation. Susceptibility to infection by bacteriophage DNA, however, is elevated. An identical increase in transfection can be induced in the
TABLE VII. General Properties of Transformation Defective Mutants

<table>
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<tr>
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<th>Binding</th>
<th>Uptake</th>
<th>Transformation</th>
<th>Tranfection</th>
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</thead>
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<td>NH46 (sen, ten⁺)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH03 (sen⁺, ten)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>NH9103 (sen, ten⁺)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH4603 (sen, ten)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
parental strain, SB25, by pre-exposing the bacterium to ultraviolet irradiated DNA (128,129). Ten mutants, unaffected by this treatment, constitutively express the properties which result in enhanced transfection. This mutation affects neither binding nor uptake of DNA. The transformation-deficient strains NH46 (sen ten+) and NH03 (sen+ ten) were obtained by transformation of SB25 with DNA isolated from NH4603 (sen ten). NH9103 (sen ten+) is an independently isolated sen mutant.

Nuclease analysis of transformation-deficient mutants. These transformation-deficient strains were grown in the competence regime along with the transformation-proficient parental strain, SB25. Samples of cellular lysates were prepared and subjected to SDS electrophoresis as described (38) in gels containing either denatured calf thymus DNA or denatured SP82 bacteriophage DNA. The results of the nuclease analysis, presented in Fig 16, demonstrate that all three sen mutants, NH9103, NH46, and NH4603, are reduced in the 17,000 MW, competence-associated nuclease as compared to the levels seen in the parental strain SB25 and the sen+ transformation-deficient mutant, NH03. The nuclease compositions of NH46, NH4603, and NH03 were also examined following two dimensional electrophoresis. The results of this analysis are shown in Fig. 17. The sen-associated reduction in the competence-associated nuclease 17,000 MW, pH 4.8 does not appear to be as great as that observed in the one dimensional analysis. A larger amount of protein was analyzed in the two dimensional experiment. It may be that the nuclease concentration in both the deficient and proficient strains is sufficient to saturate the nucleic acid substrate. An alternative explanation may be that some component which modifies the activity of this nuclease co-migrates with it during SDS electrophoresis but is separated from it
Fig 16. One dimensional zymogram analysis of renaturable nucleases in competent cultures of transformation deficient mutants of *B. subtilis*. Samples of *B. subtilis* strains, from left to right, SB25, NH4603, NH03, NH46, and NH9103, were subjected to SDS-electrophoresis in gels containing A, B; denatured calf thymus DNA, and C, D; denatured SP82 bacteriophage DNA. Ethidium bromide fluorescence pattern following activation of renatured nucleases with A, C; Mg$^{2+}$ and Ca$^{2+}$, and B, D; Mn$^{2+}$ at 2 mM.
Fig 17. Two-dimensional zymogram analysis of renaturable nucleases in competent cultures of transformation defective mutants of *B. subtilis*. Approximately 350 μg of protein prepared from competent cultures of A, NH403; B, NH46; and C, NH4603; were subjected to electrophoretic analysis in gels containing, top, native, and bottom, denatured, calf thymus DNA. Pattern of ethidium bromide fluorescence following renaturation and activation by Mg²⁺, Ca²⁺, and Mn²⁺ at 2 mM.
Fig 17 B.
Fig. 17 C.
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during two dimensional electrophoresis. The actual quantitative difference represented by these results cannot be approximated in this type of assay. In one investigation in which the quantitation of enzyme activity in substrate containing gels was studied, the investigators concluded that "the data give the best straightline fit when the cube root of the peak area (referring to a densitometer recording of the area in which the substrate has been hydrolyzed) is plotted against the logarithm of the enzyme concentration" (130).

Competence associated polypeptides in transformation deficient mutants. The transformation deficient strains NH46 (sen ten⁺), NH03 (sen⁺ ten), NH4603 (sen ten) and NH9103 (sen ten⁺) were grown and radiolabelled in the competence regime as described for the parental strain SB25. Samples of cellular lysates were analyzed by two-dimensional electrophoresis consisting of either IEF or NEPHGE in the first dimension and SDS electrophoresis in the second dimension. The IEF-SDS and NEPHGE-SDS electrophoretic patterns are presented in Fig 18 and Fig 19, respectively. The results of this analysis are presented in Table VIII and IX. Peptide changes observed in all three sen mutants remain potentially responsible for this phenotype. It is probable that the additional individual changes are not. The individual differences between these strains will need to be confirmed since the competence-associated polypeptide composition of these mutants has only been examined once under conditions resulting in sufficient incorporation of radioactive label into proteins to enable the detection of the complete inventory of competence-associated polypeptides.

The same competence-associated polypeptide (pHj=4.8, 17,000 MW) which co-migrated with 2DZ nuclease 2 during two-dimensional electro-
Fig 18. Two-dimensional, IEF-SDS, electrophoretic analysis of radiolabeled competent cultures of transformation defective mutants of *B. subtilis*. Autoradiographic pattern obtained following electrophoresis of samples of A, NH403; B, NH9103; C, NH4603; and D, NH46; containing A, B, C: 2 x 10^6 and D: 1,6 x 10^6 CPM of acid precipitable [14C]-labeled protein.
Fig 19. Two-dimensional, NEPHGE-SDS, electrophoretic analysis of radiolosotopically labeled competent cultures of transformation defective mutants of *B. subtilis*. Autoradiographic pattern obtained following electrophoresis of samples of A, NH03; B, NH9103; C, NH46Q3; and D, NH46; containing $2 \times 10^6$ CPM of acid precipitable $^{14}$C-labeled protein.
### Table VIII. Competence-Associated Polypeptides in Transformation Defective Mutants of *B. subtilis*

(IEF-SDS Polypeptides)

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<thead>
<tr>
<th>Molecular wt. (X10⁻³)</th>
<th>Isotonic pH</th>
<th>NH₀³</th>
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<tr>
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<td>6.85</td>
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<tr>
<td>56.0</td>
<td>5.83</td>
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<td>+</td>
</tr>
<tr>
<td>69.0</td>
<td>5.98</td>
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<tr>
<td>86.0</td>
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<td>+/-</td>
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<td>+</td>
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<td>5.46</td>
<td>+</td>
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</tr>
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</table>

ᵃ. ND - Not determined
Table IX. Competence Associated Polypeptides in Transformation Defective Mutants of *B. subtilis*

NEPHGE-SDS Polypeptides

<table>
<thead>
<tr>
<th>Molecular wt. (X10^-3)</th>
<th>Mobility NEPHGE/Lysozyme</th>
<th>NH03</th>
<th>NH46</th>
<th>NH4603</th>
<th>NH9103</th>
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<td>ND</td>
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<td>PNI</td>
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<td>+</td>
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</tbody>
</table>

a. ND - Not determined
b. PNI - Present, not increased
phoresis was not detected in all three of the sen mutants examined. It is likely, therefore, that the polypeptide and the nuclease are the same protein. A large number of additional competence-associated polypeptides are reduced or absent in these strains as well. This suggests that the primary mutation may reside somewhere other than in the gene that actually codes for the sen product. The primary mutation could affect some regulatory element or cause premature termination within a multi-gene transcriptional unit some distance upstream from the gene coding for the sen protein. The coordinate expression of a number of competence associated polypeptides in sen mutants has been reported (36) and is consistent with the latter possibility. In addition, Raina and Ravin (48) have detected a large competence-specific RNA which they estimated to have sufficient coding capacity to account for all the competence-associated polypeptides detected in Streptococcus sanguis. Alternatively, the induction of one or more competence-associated polypeptides may control the subsequent expression of additional competence-associated polypeptides. Consistent with this possibility is the fact that Raina and Ravin (48) have identified both early and late competence-associated polypeptides in S. sanguis.

The coordinate suppression of a large number of competence-associated polypeptides including 2DZ nuclease 2, in these sen mutants, raises the question of whether the deficiency in this nuclease is responsible for the low level of competence exhibited by these strains. Two alternative possibilities are that either the deficiency in this nuclease in combination with the suppression of additional specific competence-associated polypeptides results in a loss of transformability, or that the nuclease in itself is unrelated to transformability but
is suppressed simultaneously in these strains with proteins essential to transformation.

In *Streptococcus pneumoniae* mutants deficient in a membrane-bound (131) endonuclease are transformation-defective (132). In DNA-agar containing methyl green, wild type colonies are surrounded by a zone of hydrolyzed DNA whereas Noz mutants (for no zone) are not. The identity of the nuclease or nucleases responsible for similar zones of hydrolytic activity surrounding *B. subtilis* colonies in DNA-agar plates is not known. The physical and catalytic similarity between 2DZ nuclease 2 and an extracellular Mn$^{2+}$-activated endonuclease suggests that it may contribute to this activity. Therefore, if 2DZ nuclease 2 were essential for transformation we would predict that Noz mutants of *B. subtilis* would be similarly transformation-defective. However, those reported (21) are normal in transformation. It is possible that these mutants still retain sufficient nuclease activity to effect transformation. *S. pneumoniae* end mutants with as little as 1.4% of the wild-type level of the membrane-bound endonuclease are transformation proficient (130).

Joene and Venema (19) have examined the cell surface for nucleolytic activity in competent and non-competent cells fractionated on renograffin gradients. They detected a nuclease activity uniquely associated with the competent fraction of cells which was released from the competent cells following protoplast formation and catalyzed the release of acid soluble oligonucleotides from DNA. This nuclease activity did not appear to be involved in the cell surface inactivation phenomenon. This periplasmic nuclease activity was recently examined (20) in SDS gels containing native DNA and two competence-associated nucleases were identified. These nucleases were activated by Mn$^{2+}$
primarily, and by Mg$^{2+}$ secondarily. The monomeric molecular weights of these nucleases were reported to be 13,000 MW and 15,000 MW.

We have shown that the level of 2DZ nuclease 2 is elevated in competent cell cultures in comparison with physiologically non-competent cell cultures. However, we have not yet determined its relative distribution between the competent and non-competent cells existing within a competent culture. The phenomenon of cell surface inactivation of extracellular DNA was first examined by Haseltine and Fox (18). They found, by fractionating competent and non-competent cells on gradients of renograffin (58,59) that this activity was predominantly associated with the non-competent fraction of the population. This was apparently due to the fact that, although both cell types introduced double strand scissions into the DNA, the non-competent cells altered the DNA in some unique manner such that it could no longer compete in transformation. This suggests that a deficiency in sen activity would not result in lowered transformation capability. However, it might be imagined that the same nuclease which is responsible for the biological inactivation of DNA by non-competent cells, is modified, complexed, or located in such a manner in competent cells that it no longer results in the biological inactivation of DNA.

In summary, this study was undertaken to identify protein and enzymatic changes which accompany the development of competence. We have successfully identified thirty-six polypeptides and 4 nucleases which are increased in physiologically competent cell cultures when compared to physiologically non-competent cell cultures. Transformation-deficient mutants which fail to inactivate exogenous DNA (18) were shown to be deficient in a 17,000 MW competence-associated nuclease
and in 8-15 competence associated polypeptides. The combined analysis of transformation-deficient mutants for specific nuclease deficiencies and competence-associated polypeptides was useful in establishing the pleiotropic effect of the sen mutation in these strains. The nature of these mutations precludes the conclusion that the 17,000 MW competence-associated nuclease is essential for competence. The evidence presented indicates that it is probably the same enzyme as has been detected extracellularly in association with early sporulation events (84). Our laboratory is currently isolating the extracellular enzyme to confirm this relationship. The two periplasmic competence-associated nucleases identified by Mulder and Venema (20) are also similar to the extracellular enzyme in size and cation activation characteristics. We have not observed competence-associated nuclease activity with the low molecular weights reported for these nucleases. However, much less periplasmic protein was examined in our unfractionated lysates than in the study by Mulder and Venema. It will be necessary to determine what relationship exists between these enzymes and 2DZ nuclease 2.

Three additional Mn$^{2+}$-activated competence associated nucleases were identified in this study (2DZ nucleases 6, 7 and 9). They appear to be related to each other but are distinct from 2DZ nuclease 2 in that they exhibit a modest preference for denatured DNA and are not affected by the sen mutation. The relationship of these nucleases to competence remains to be established.

This approach has been a valuable one in studying the differentiation of B. subtilis to the competent state. It should be equally valuable in examining sporulation-associated events and in determining the extent of the relationship between these two pathways.
This method of analysis may be readily extended to the examination of many enzymatic activities (Chapter 1, ref 22-26) including proteases (Chapter 1, ref 23,23). A comparison between both protease and nuclease changes associated with competence development and sporulation would be useful in comparing the two pathways of differentiation since the temporal induction of both of these enzymatic activities is well documented in sporulation (133).

Sporulation appears to be controlled, in part, by the induction of new sigma factors which modify the affinity of RNA polymerase for particular promotor sites (133). It has not been determined if the changes in sigma factors which have been associated with sporulation direct the development of competence or if any novel sigma factors are involved. This should be a very interesting and exciting area of investigation.
REFERENCES


16) Dubnau, D., and Cirigliano. (1972) Fate of Transforming DNA Following Uptake by Competent *Bacillus subtilis*. Formation and Properties of Products Isolated from Transformed Cells which are Derived Entirely from Donor DNA. J. Mol. Biol. 64, 9-29.


54) Wilson, G.A. and Bott, K.F. (1968) Nutritional Factors Influencing the Development of Competence In the Bacillus subtilis Transforma-


Cahn, F.H., and Fox, M.S. (1968) Fractionation of Transformable Bacteria from Competent Cultures of *Bacillus subtilis* on Renografin Gradients. J. Bacteriol. 95, 867-875.


