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Deoxyribonucleases expressed in competence-induced com and spo0 mutants of Bacillus subtilis

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Deoxyribonucleases expressed in competence-induced *com* and *spo*0 mutants of *Bacillus subtilis*   .

McGrew, Billy Robert, Ph.D.

University of New Hampshire, 1989

DEOXYRIBONUCLEASES EXPRESSED IN COMPETENCE-INDUCED

COM AND SPOO MUTANTS OF

BACILLUS SUBTILIS

BY

Billy R. McGrew
B.S., Fitchburg State College, 1981

Dissertation

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

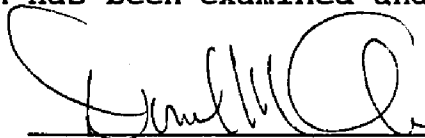Doctor of Philosophy

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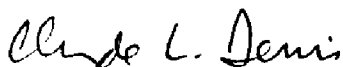
Biochemistry

May, 1989

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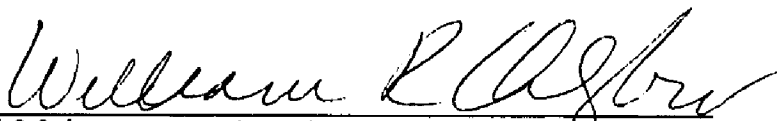
Dissertation director, Donald M. Green
Professor of Biochemistry

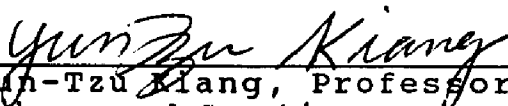Clyde L. Denis, Associate Professor of
Biochemistry

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William R. Chesbro, Professor of
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Science and Genetics

April 7, 1914
Date

ABSTRACT

DEOXYRIBONUCLEASES   EXPRESSED IN   COMPETENCE-INDUCED

com AND spo MUTANTS OF BACILLUS SUBTILIS

by

Billy R. McGrew

University of New Hampshire, May, 1989

The inclusion of 1% casein in buffer used to
reactivate enzymes subjected to SDS-polyacrylamide
electrophoresis resulted in faster and more complete
restoration of nuclease and B-galactosidase enzyme
activities.   Enzyme activities which were absent from
gels during longer wash procedures were detectable
with this technique. The threshold of detection of
two-dimensionally separated Deoxyribonuclease I was 1
picogram, tenfold lower than for previously reported
wash procedures.   Addition of BSA at concentrations
above 50 ug/ml to nuclease gels was found to result in
less effective detergent removal during wash
procedures and reduced recovery of enzyme activity.

General and specific nuclease expression patterns
were observed in two-dimensional sodium dodecyl
sulfate polyacrylamide gels of 64 competence-induced
com and spo mutants of Bacillus subtilis. General

patterns of nucleases expression were as follows:

1. Four different groups of nucleases called "clusters" were found to be co-expressed in greater than 95% of the mutants.

2. Some cluster members are probably produced by post-translational modification.

3. A 14 kd nuclease reported by Venema was detected in the gels after use of the casein wash procedure.

Specific patterns of expression are as follows:

1. Sigma factor H is not required for nuclease 2 expression.

2. spo0K is required for nuclease 2 production.

3. Nuclease 2 is regulated differently than nuclease clusters A, B, C, and D.

4. $crsa_{47}$ (sigma A) is required for competence.

To: Mom and Dad, Will and Lou, Robbie and Rick.

ACKNOWLEDGEMENTS

Thank you to my best friend, Lou McGrew, who has helped me get through the rough spots. She has always inspired me to persevere even though she doesn't know what I do for a living! It's probably best left that way. I hope that I have been as good an influence upon her life as she has been on mine.

Thanks also to Karen Rasmussen for her friendship and understanding. We have spent much time discussing the satisfying as well as the disappointing aspects of graduate school. I hope those conversations have made us stronger and have kept us focused on the important things in life.

Thanks Mom, your love and blind pride for me has been a great source of confidence.

I am grateful to the university for awarding me a dissertation year fellowship and a CURF grant.

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I. Restoration of Enzymatic Activity following Sodium

   Dodecyl Sulfate Polyacrylamide Gel Electrophoresis:

             Use of Casein in Gel Wash Buffer

     The ability to observe enzymatic activities
within SDS-PAGE gels is dependent on several
variables: i) overall purity of the SDS preparation
employed, ii) identity and quantity of a given SDS
preparation's contaminants iii) the presence in some
instances of proteins embedded in the gel matrix and
iv) the ability to lower the detergent after
electrophoresis to a concentration that will allow
renaturation (2,10,11,16,20,27,28).

     In agreement with others (2,16) we recommend the
use of high purity SDS to circumvent problems in
recovering enzymatic activities associated with major
quantities of SDS contaminants. However, a rapid
method for quantitative SDS removal is still required
to obtain optimum results, especially if the enzyme of
interest is unstable or capable of diffusing from the
gel prior to activation.

     The complete removal of SDS from proteins by
dialysis has met with varied success (3,6,22,23,24).
Since both the purity of the SDS and the nature of the
proteins involved are probable factors in achieving
complete removal, it may be unrealistic to expect

                         1

total recovery of enzymatic activity within polyacrylamide gels. Nevertheless, better gel wash procedures may extend the sensitivity of activity assays beyond those previously reported.

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Materials and Methods

Sodium dodecyl sulfate preparations were the
following grades: Biorad electrophoretic purity (lot
#M2276), Fisher laboratory grade (lot # 710486), and
Baker (95%) practical grade. Sodium dodecyl sulfate
labeled with ^{35}S (4.66MBq/mg) was purchased from
Amersham. Urea and acrylamide were electrophoresis
grade from Biorad. Biorad gelatin was EIA grade;
Ampholytes were from LKB; N,N,N',N'-
tetramethylenediamine, N,N'-methylenebisacrlyamide,
and ammonium persulfate were from Eastman Kodak; Calf
thymus DNA (D-1501), Trizma base, phenylmethylsulfonyl
fluoride (PMSF), 1,10-phenanthroline, N-
ethylmaleimide, deoxyribonuclease I (DNase I) #DN-25
and purified casein (# C-5890) were from Sigma; Knox
brand gelatin was purchased at the supermarket; Brain-
Heart Infusion agar (BHI agar) was from Difco; Nonidet
NP-40 was from Bethesda Research Laboratories; DE-52
was from Whatman; Egg albumin and reagent grade
isopropanol was from Baker; bovine serum albumin (BSA)
was Pentax fraction V; ammonium sulfate ultra-pure was
from Schwartz-Mann; commercial beta-galactosidase, a
1.7M ammonium sulfate suspension (G-6512, lot#102F-
683, 600-900 units per mg protein was from Sigma. 1-
Chloro-2-Bromo galactopyranoside (X-gal) was from
Boerhinger Mannheim. All other chemicals were reagent

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grade.

DNA preparation

Calf thymus DNA used for embedding in SDS-PAGE gels was prepared as described (1). DNA prepared for use within BHI agar was prepared the same way but was dialyzed against Buffer C [0.01M Tris-HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA)].

Preparation of BHI agar containing calf thymus DNA

Calf thymus DNA in Buffer C was added to molten 48°C Brain/Heart Infusion Agar (BHI) to a final concentration of 3.5ug/ml and distributed into 21 x 21 x 5cm sterile pyrex baking dishes, 100 ml per dish. The dishes were covered with aluminum foil and the agar allowed to solidify.

Preparation of the secreted bacterial proteins

Pyrex dishes containing the BHI agar-plus DNA described above, were individually seeded with 100 ul of frozen late log phase stock cultures of Bacillus subtilis. Bacteria were spread over the entire surface using bent glass rods and incubated for 12.5 hours at 37°C.

The following steps were carried out at 4°C unless otherwise specified: Agar from two plates containing the strain of interest was combined into a

single centrifuge bottle and macerated. To the agar was added 100 ml of 50 mM Tris-HCl pH 8.1, 1 mM EDTA and 0.5 ml 100 mM PMSF in 2-propanol was added. The bottle was then capped and agitated on its side for 60 minutes. The sample was centrifuged at 5,000 rpm for 10 minutes and the supernate collected into several 50 ml polyallomer centrifuge tubes. The pooled supernates were recentrifuged in a SS-34 rotor at 10,000 rpm for 10 minutes. This supernate (120 ml) was collected and warmed to room temperature before adding ammonium sulfate gradually to a concentration of 3.8 M. The sample was cooled to 4°C and stirred for 7 hours. The precipitate was sedimented in a GSA rotor at 5,000 rpm for 25 minutes. The pellet was resuspended in 5 ml of 0.01M Tris-HCl pH 7.4, 1 mM EDTA, 250uG/ml PMSF, 5 mM N-ethyl maleimide, 1 mM 1,10-phenanthroline.

The suspension was placed into 6-8 k cut-off dialysis tubing and dialyzed against four 2 l changes of 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.5 gm solid phenylmethyl sulfonyl fluoride (PMSF) over a 24 hour period. After 12 hours the pellet had fully dissolved nd the dialysate was clear. The recovered volume was approximately 10 ml. This preparation was stored at -80°C after addition of 100 mM PMSF in 2-propanol to 3 mM.

Preparation for Electrophoretic Separations

Because of the increased sensitivity of the gel system, the following precautions were taken to avoid contaminating nucleases in the nuclease detection gels: i) All first dimension and second dimension gel solutions were prepared with autoclaved glass-distilled/deionized water unless specified. ii) NEPHGE reservoir solutions were prepared with autoclaved distilled/deionized water (ddH_2O). iii) All gel apparati were rinsed in ddH_2O followed by 95% ethanol after each use and stored in dust free cabinets. iv) SDS-PAGE running buffer and protein containing wash buffer were prepared with autoclaved ddH_2O and then autoclaved. v) Other gel wash and activation buffers were prepared with autoclaved ddH_2O. vi) All tools used to handle gels were boiled in ddH_2O water for 5 minutes prior to use. vii) Glass tubes used for the first dimension were acid washed and rinsed in sterile ddH_2O. viii) Glass plates used in the second dimension were soaked overnight in saturated potassium hydroxide, washed and final rinsed in ddH_2O. ix) If the SDS-PAGE apparatus is constructed so that one glass plate becomes one wall of the upper buffer reservoir, this plate was thoroughly washed with ddH_2O and 95% ethanol prior to the electrophoretic run.

Nonequilibrium pH gradient electrophoresis of DNase I

O'Farrell's procedure for nonequilibrium pH gradient electrophoresis (NEPHGE) (17) was used as described (1). DNase I was prepared for NEPHGE as described by Coughlin for assessing the sensitivity of the assay (1). To isolate DNase I, I removed the top 2.5 cm of the NEPHGE gel for equilibration in the O'Farrell second dimension equilibration buffer.

Preparation of samples for one dimensional electrophoresis

Samples were embedded as 160mm x 2.5mm i.d. tubes in 1% agarose sample buffer as described (1).

SDS-PAGE gel electrophoresis

One or two-dimensional gel electrophoresis was performed using the discontinuous system of Laemmli as described by O'Farrell (17) and revised by Coughlin (1). During some experiments BSA concentrations were increased from 10ug/ml to 50 or 500ug/ml as described.

SDS Removal and Nuclease detection

The basic procedure of Rosenthal and Lacks (20,21) as modified by Coughlin (1) was used for SDS removal, nuclease activation and detection within the gels. However, I have added protein or chromatographic resins to the SDS removal buffer (Buffer A) to investigate their effect on nuclease

detection. All incubations were performed at 37°C with agitation on a New Brunswick Scientific gyrotory shaker rotating at 50-60 rpm.

To prepare a SDS removal buffer containing a specific protein, Buffer A (0.04M Tris-HCl pH 9.0, 0.02% sodium azide, 2 mM EDTA) was made 1% in protein and autoclaved (20psi., 121°C) for 30 minutes to denature contaminating enzymes.

Buffer A containing DE-52 was prepared as follows: 16 grams pre-swollen DE-52 per gel slice was equilibrated in 5X strength buffer A until the pH was 9.0. Then 1X strength buffer A was used to wash the resin until the conductivity of buffer A alone equaled that of the DE-52 slurry. The final volume of the DE-52 slurry was adjusted to 40 ml per 4 grams of DE-52.

Vertical 12mm x 160mm x 1mm gel strips were cut from one-dimensional SDS-gels and incubated in 20 gel volumes of either buffer A, buffer A containing 1% protein, or buffer A containing DE-52. Gel washing proceeded for 2 hours with 4 buffer changes unless otherwise stated. Gels were washed in either a multiwell plexiglass tray or by placing in Corning wide-mouth bottles (#1368) laid on their sides.

SDS removal by isopropanol was performed as described by Blank et. al. except that buffer A rather than 0.01M Tris-HCl-HCL pH 7.4 was made 25% in

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isopropanol. Gel strips were incubated twice for 15 minutes in 20 gel volumes of isopropanol containing buffer A, then twice for 15 minutes in buffer A without isopropanol, and then again in buffer A, twice for 30 minutes.

After washing to remove SDS, gels were placed in 20 volumes of activation Buffer B (0.04M Tris-HCl pH 7.6, 0.02% sodium azide) containing the cation concentrations required for nuclease activity. In situ DNA hydrolysis was terminated and the results photographed as previously described (1).

Protein determination

Protein determinations were performed using the BioRad Protein Assay kit based on the method of Bradford (29).

Preparation of Commercial Beta-galactosidase for SDS-PAGE

An initial stock solution of beta-galactosidase was prepared by diluting 3 ul of Sigma ammonium sulfate suspension into 47.5 ul of Lacks 1X sample buffer (20). Additional dilutions of the stock (1:5, 1:10, 1:100 and 1:1000) were also prepared. 10 ul of the initial stock and each of the dilutions was run on multiwelled SDS-PAGE vertical gels in duplicate. Each gel was washed and activated as described.

Wash procedure for recovery of Beta-galactosidase activity

Gels were washed in either isopropanol or casein as described. Following SDS removal, gels were equilibrated by two-45 minute incubations (500 ml at room temperature) in Z-buffer (0.06 M disodium phosphate, 0.04 M monosodium phosphate pH 7.0, 0.01 M KCl, 1 mM magnesium sulfate and 39 mM 2-mercaptoethanol). Activation of the gels was done in 100 ml of Z-buffer supplemented with 1 ml of 2% X-gal in dimethylformamide. Activated gels were incubated with agitation at 37°C. overnight or until color developed. Beta-galactosidase activity is represented by a blue colored band within the gel.

[35]S-labeled SDS Elution from Polyacrylamide Gels

Eleven percent polyacrylamide gels (8 x 12 x 0.1cm, 11.72 ml) were poured as described by Coughlin but contained 1uCi of ^{35}S-labeled SDS. Individual gels differed in make-up by the inclusion of various concentrations of: i) calf thymus DNA ii) BSA, or both. Control gels contained no additives. After polymerization, identical gel plugs were cut using a number 8 stopper hole cutter (1.5 cm diameter) and washed in 50 ml of one of the described wash buffers. Time course elution studies were performed by removing individual gel sections from the wash buffer at

prescribed intervals. Gel samples were allowed to equilibrate for 30 minutes with shaking in scintillation vials containing 5 ml of scintillation cocktail (1:2) (Triton-X 100:toluene), 0.56% omnifluor prior to counting in a Beckman LS-7000 scintillation counter.

Effect of protein addition to SDS removal buffer A on nuclease reactivation

Reactivation of B. subtilis nucleases was enhanced with increased washing time (Fig. 1). Detection of the nuclease activity of high molecular weight nucleases required three 30-minute wash periods when gels were washed in unsupplemented buffer A. In contrast, in gel strips washed in gelatin wash buffer, both high and low molecular weight nuclease activity bands were visible following two, 30-minute washes. After three 30-minute washes in the gelatin system, nuclease bands were more intense than after 6, 30-minute washes in buffer A alone. More importantly, small molecular weight bands which were missing in gel strips washed in unsupplemented buffer A (Lanes A-F) were present in gels washed in the gelatin containing buffer(Lanes I-L). Elution of the low molecular weight bands from the gel during extensive washing is a plausible cause of this effect. Two nucleases at approximately 17 kd gained activity throughout the wash procedure as do nuclease activities at 160, 150, 125, and 70 kd.

Comparison of various SDS removal methods

To determine if the inclusion of protein in

Figure 1

Secreted nuclease activities from <u>Bacillus</u> <u>subtilis</u> visualized on a single one-dimensional SDS nuclease activity gel. Lanes A-F are identical gel slices washed for increasing lengths of time in Buffer A unsupplemented with protein. From left to right, the wash period lengths are 30, 60, 90, 120, 150, and 180 minutes respectively with buffer changes every 30 minutes. Slices G-L were washed as in A-F but in Buffer A containing 1% gelatin. After individual wash periods, each slice was immediately activated in Buffer B containing 5 mM $MnCl_2$ at 37^OC for 48 hours with gyration, and the activation terminated in 500 ml 0.04 M Tris-HCl, pH 7.6, 0.02% sodium azide, 2 mM EDTA, 2-4 ug/ml ethidium bromide. Gels were photographed as described (1).

Figure 2

One-dimensional SDS-PAGE separation of <u>B.</u> <u>subtilis</u> secreted nucleases as a function of different washing protocols. Lane A was washed for 2 hours with 30 minute buffer A changes prior to activation. Lanes B and C were washed for 1 and 2 hours respectively by the isopropanol procedure described in "methods". Lanes D, F, H, and J were washed for 1 hour in Buffer A containing BioRad gelatin, Knox gelatin, casein or egg albumin respectively. Lanes E, G, I, and K were washed for 2 hours in Buffer A containing Biorad gelatin, Knox gelatin, casein, or egg albumin respectively. Wash buffers were changed at 30 minute intervals prior to activation. Nucleases were activated in Buffer B containing 5 mM $MnCl_2$ at 37^OC with gyration for 50 hours. Enzymatic activity was terminated as described at the appropriate intervals to maintain equal times of activation for all slices.

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Figure 1   A B C D E F G H I J K L

Figure 2   A   B   C   D   E   F   G   H   I   J   K

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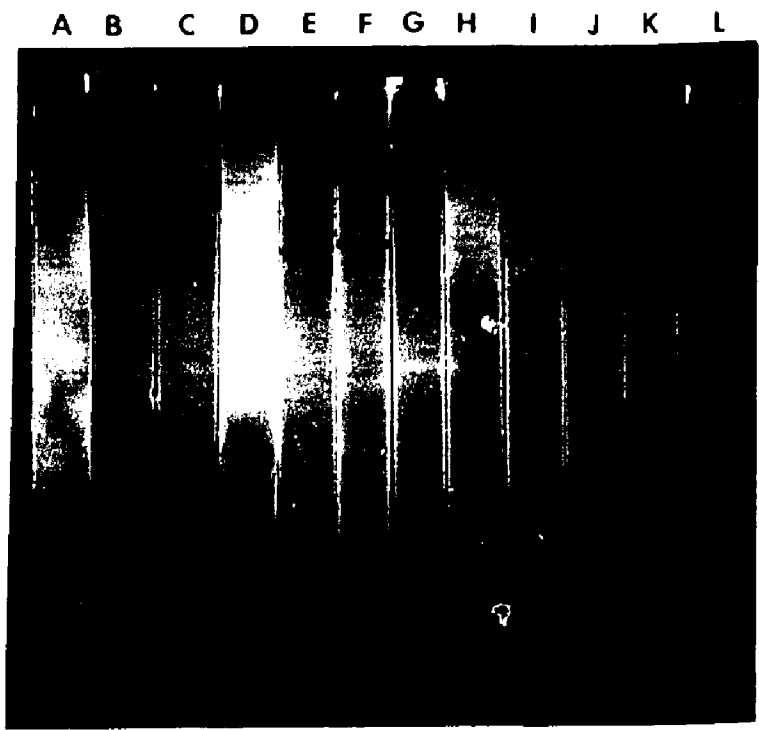
buffer A was as effective as other reported methods of reactivation of nucleases, I compared the reported isopropanol wash procedure (2) with Buffer A supplemented with other protein additives.

Maximal band reactivation was obtained in gel slices washed for 2 hr in either the isopropanol or casein-containing wash buffers (Fig. 2, Lanes C and I). The sensitivities of the two procedures were generally comparable. However, the casein wash procedure revealed nucleases in the 20-29 kd range which were not detected in the isopropanol washed gels. Both procedures showed greater sensitivity than the gels washed in other solutions. As result of these findings, additional comparisons centered on casein effects rather than gelatin.

Various proteins or chromatographic resins were again compared to casein in buffer A to determine their relative effects on reactivation. Proteins investigated were acetone liver powder, alpha-amylase, bovine serum albumin (BSA), fibrinogen, gelatin, lipase, trypsin, and urease (data not shown). Chromatographic resins chosen were DE-52, Dowex1-8A, BioRad Mixed bed resin , and Octyl agarose (data not shown). Of these additional substances only BSA and DE-52 showed enhancement of nuclease reactivation comparable to that of casein.

Gels containing BioRad SDS were tested using the isopropanol procedure and the Buffer A procedure with either BSA, casein, 1% BSA + 1% casein, or DE-52. All bands present in the isopropanol washed gel were present or enhanced by the protein containing wash buffers except for the smallest band approximately 14.6 kd (Fig. 3). This band was present in gels treated by isopropanol or DE-52, less obvious in gels treated with BSA or Casein, and not detected in the gel treated with both BSA and Casein. The combination of casein and BSA showed no greater sensitivity than casein alone.

Comparison of ^{35}S-SDS elution from polyacrylamide gels by different wash methods

The rate of S-35 labelled SDS elution from polyacrylamide gels containing both DNA and BSA was studied. (Fig. 4) From the slopes of the graphs between 20 and 40 minutes the elution rate was greatest for casein and decreased respectively for gels washed in isopropanol and buffer A. At equilibrium, the casein washed gels showed the lowest levels of residual SDS, closely followed by the isopropanol wash. Gels washed with unsupplemented Buffer A had significantly higher residual SDS levels.

Figure 3

    B. subtilis secreted nuclease pattern as demonstrated by optimal wash conditions. All lanes were washed for 2 hours as described. Lane A was washed using the isopropanol procedure. Lane B was washed in Buffer A containing 1% BSA. Lane C was washed in Buffer A that was 1% in BSA and casein. Lane D was washed in Buffer A containing 1% casein and lane E was washed in a slurry of Buffer A and DE-52 (4 gm/40 ml wash).
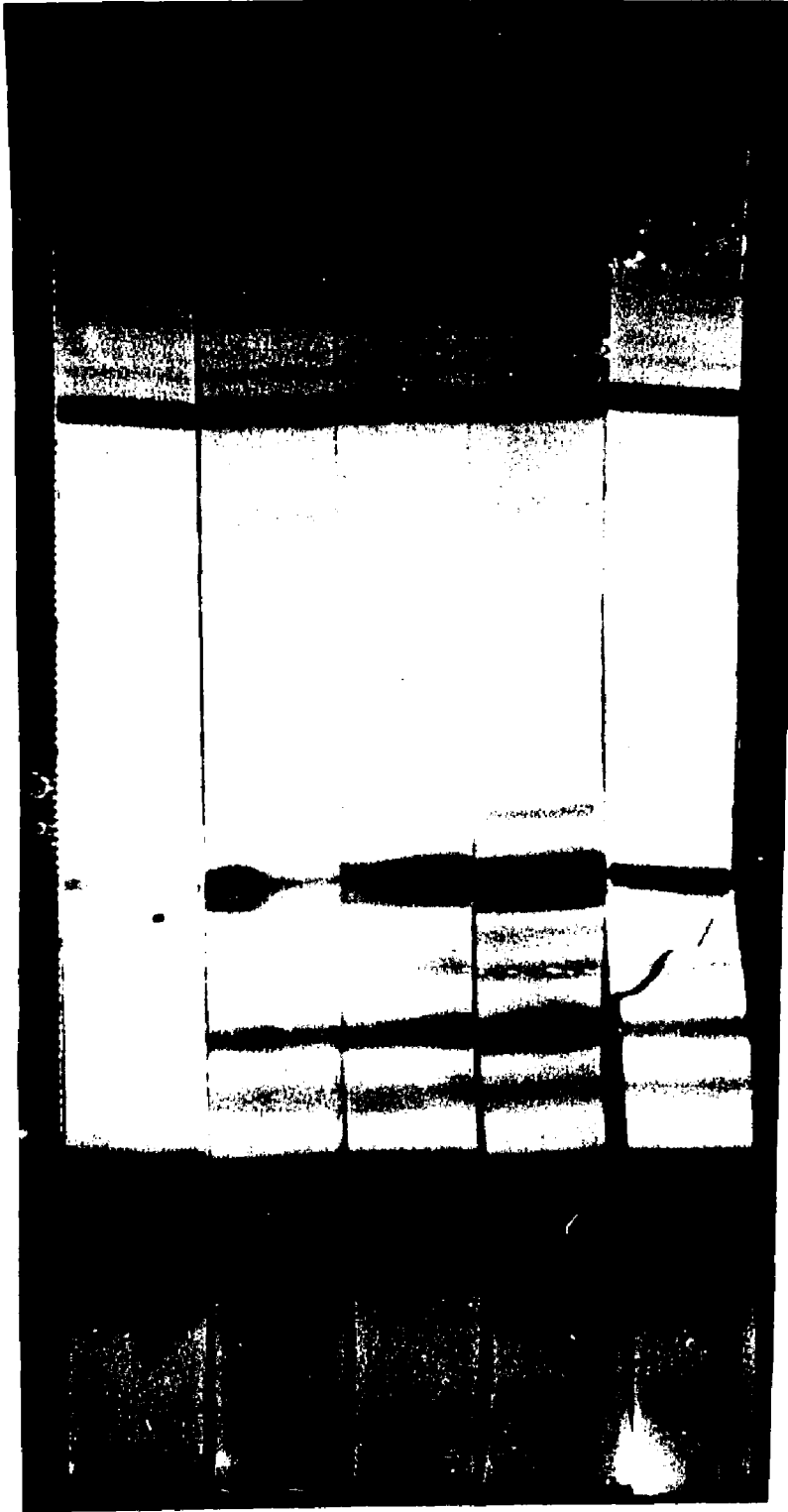
Figure 3

Figure 4

Comparison of three different wash methods on the elution rate of labeled SDS from gels containing both DNA (20 ug/ml) and BSA (10 ug/ml). Gel sections (1.5 cm diameter) were placed into scintillation vials and counted after being washed in buffer for 20, 40, 60, 80, 100, 120, 140, and 160 minutes. Wash buffers contained either no additives (triangles), 25% isopropanol (circles), or 1% casein (squares).
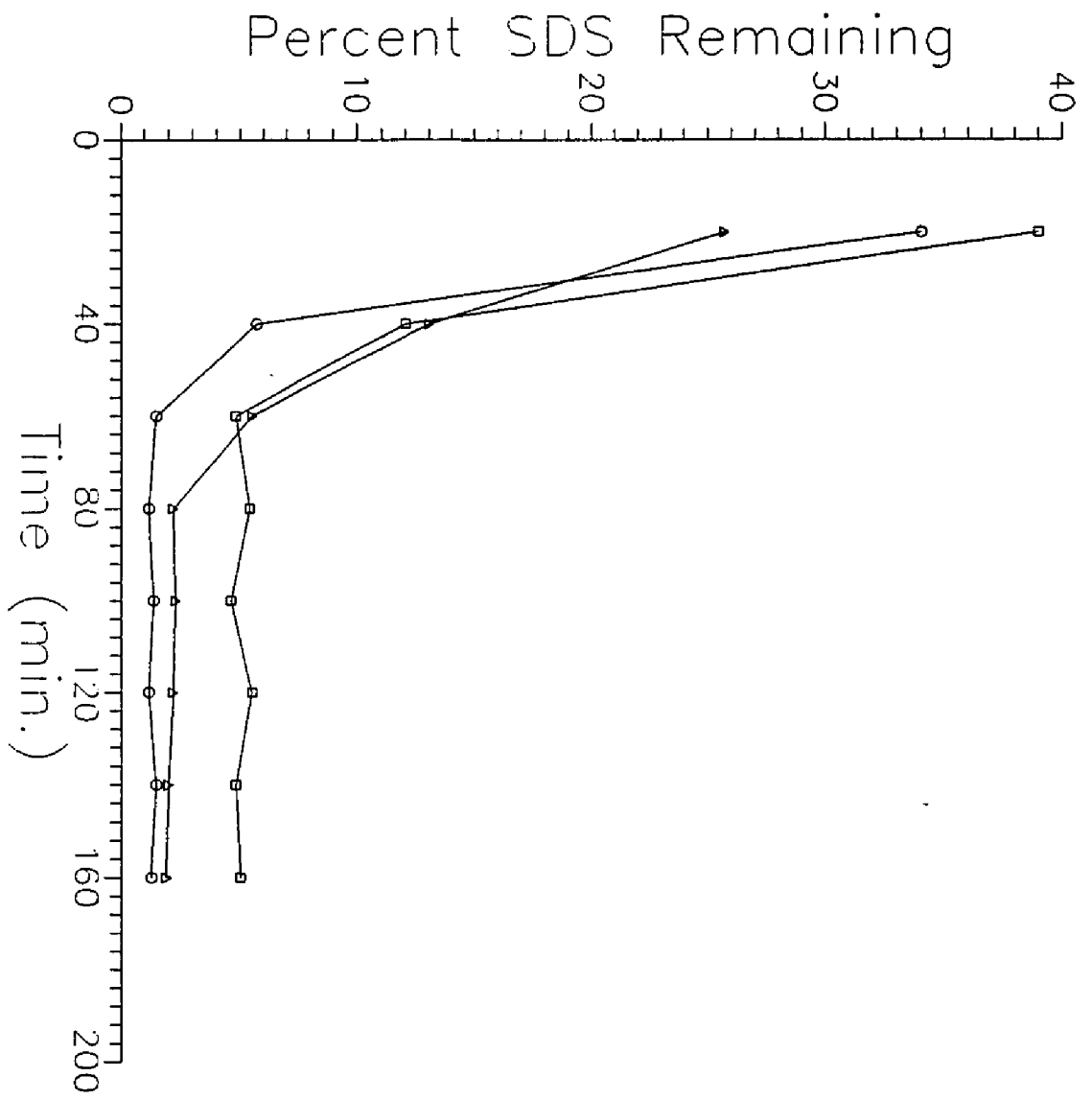
Figure 4

Effect of wash procedure on nuclease reactivation in SDS gels containing low purity SDS

The quality of the SDS used in electrophoresis has been implicated as a major factor in the renaturation of enzyme activity (16). The ability of different wash procedures to overcome the reported inhibitory effects of SDS contaminants on enzyme reactivation were examined. Fisher "laboratory grade" SDS is described by the manufacturer as a mixture of SDS and other alkyl sulfates. Gels prepared and run in this grade of SDS were washed with different buffers as described in Figure 5. The gel washed in buffer containing casein showed the presence of more nucleases than any other procedure including the gel washed in isopropanol.

Gels prepared and run with Baker (95%) "practical" grade SDS revealed more nuclease activities than the Fisher SDS preparation. (See Fig. 6). In this case both casein and BSA as well as the combination of the two (Fig. 6, lanes C, D, and E) showed improved reactivation over other wash methods. In this experiment, BSA was more effective than casein in reactivating several nuclease bands. The combination of casein and BSA (Fig. 6, lane D) did not result in an additive improvement but showed reactivation which appeared to be intermediate

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<u>Figure 5</u>

    <u>B</u>. <u>subtilis</u> secreted nuclease pattern observed in one-dimensional SDS-PAGE gels that were prepared and run with Fisher "laboratory grade" SDS. Lanes A-F were washed in Buffer A, isopropanol, Biorad gelatin, Knox gelatin, casein, or egg albumin respectively. All washes were for 2 hours as described. Gel slices were activated in Buffer B containing 5 mM $MnCl_2$ at $37^{\circ}C$ for 50 hours with gyration.

A B C D E F

Figure 5

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Figure 6

Gel slices containing Baker SDS were washed (A-F) with Buffer A, isopropanol, casein, casein + BSA, BSA, and DE-52 respectively. All washes were for 2 hours as described. Gel slices were activated in Buffer B containing 5 mM $MnCl_2$ at $37^{\circ}C$ for 50 hours with gyration.

A   B   C   D   E   F

Figure 6

25

between the casein wash (lane C) and BSA wash (lane E). DE-52 reactivation buffer (lane F) showed less reactivation than either of the other additives used in the experiment. Casein wash buffer allowed the detection of one nuclease band at approximately 29 kd that only the BSA + casein buffer detected as well. (Lanes C and D). Conversely, the isopropanol (lane B) and BSA (lane E) wash procedures demonstrated the presence of a nuclease at approximately 70 kd that was less pronounced in the casein washed gel.

Sensitivity of the Nuclease Assay

Coughlin and Green (1) reported 10 pg as the lower limit for detection of DNase I after 5-7 days of nuclease activation. We repeated the experiment using the same commercial DNase I preparation and compared gel wash procedures as described in Figure 7. Using the casein wash procedure on gels containing high quality SDS we detected 1.0 pg of DNAse I after 24 hr of enzyme reactivation (Fig. 7A). With the isopropanol wash procedure, 1 pg of DNase I activity was weakly detected after 24 hr of activation (Fig. 7B).

Simplificaton of the Wash Procedure

In place of 4 30 minute 500 ml buffer changes, a single wash for 120 minutes in one liter of casein

Figure 7

Two-dimensional separation of increasing amounts of a "chromatographically purified" commercial DNase I preparation in the presence of BioRad SDS. Four NEPHGE gel tops containing decreasing amounts of DNase I were embedded in 1% agarose sample buffer (1) across the top of both SDS-PAGE gels from left to right. The gels in Figures 7A, B and C are identical except for the wash procedure employed. 7A was washed in Casein wash buffer for 2 hours as described while 7B was washed by the isopropanol method. 7C was washed in casein wash buffer by the following simplified protocol: The gel was placed for two hours in 1 liter of 1% casein buffer with no replacement with fresh buffer. Amounts of DNase I applied to the NEPHGE gels from left to right on all gels were 10.0 pg, 1.0 pg, 100 fg, and 0g. All gels were activated in Buffer B that was 2 mM in $CaCl_2$, $MgCl_2$, and $MnCl_2$ at 37°C for 24 hours.

Figure 7

28
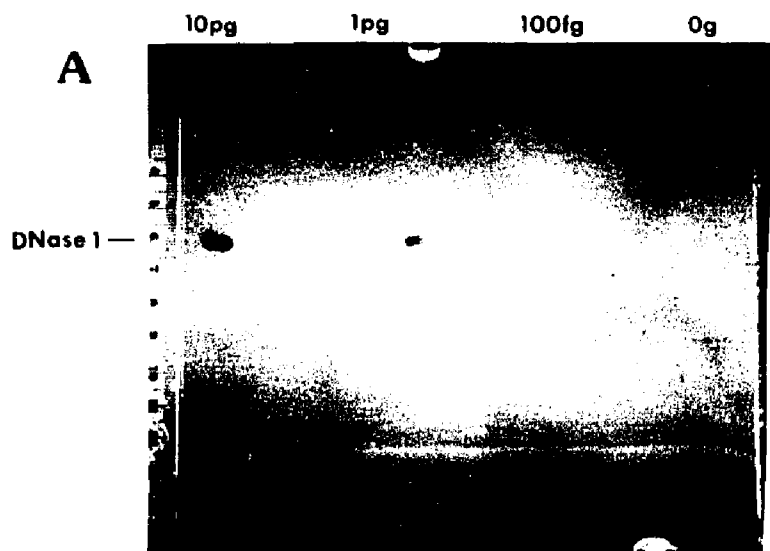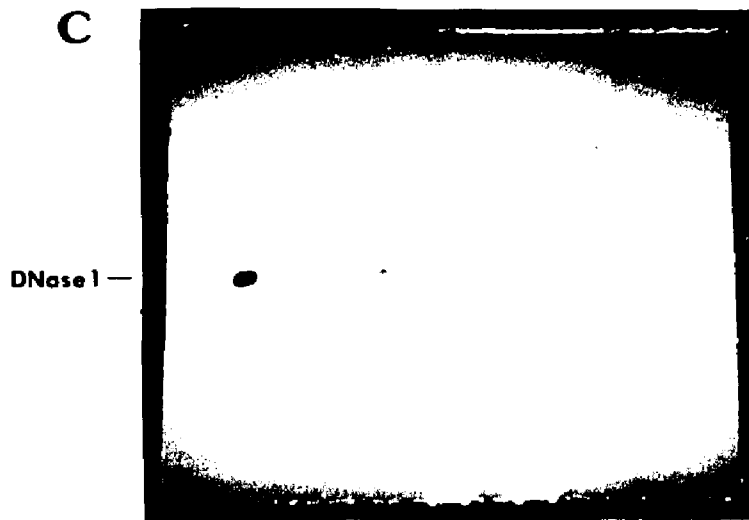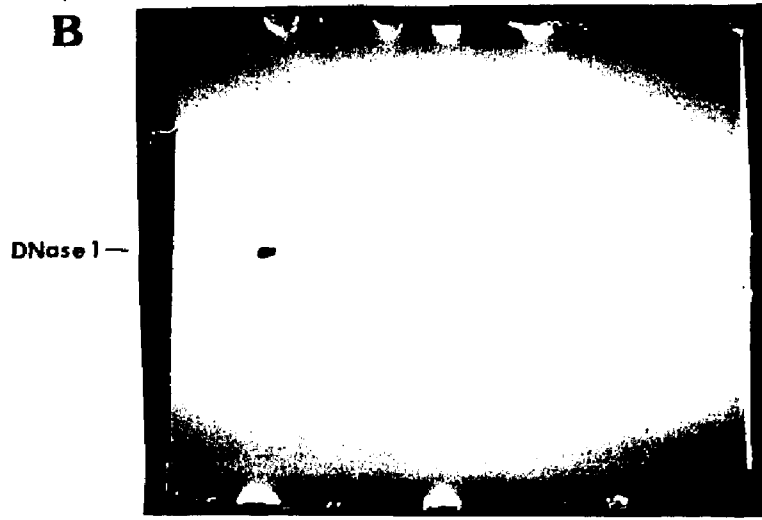
wash buffer was found to be sufficient for maximum reactivation (Fig. 7C). Nuclease gel sensitivity was identical to that of gels washed as described in Figures 7A and 7B.

Sensitivity of Nuclease Assay in Low Purity SDS Gels

The effect of low purity SDS on the sensitivity of detection of DNase I was examined in gels prepared with Fisher SDS (Fig. 8). Under these conditions the 1.0 pg activity was virtually absent from the gels while the 10 pg activity was markedly reduced.

Detection of Beta-Galactosidase Activity in SDS-PAGE gels

To determine if the casein wash procedure can be used to detect other enzyme activity stains, a SDS-PAGE separation of beta-galactosidase was performed followed by SDS removal in either buffer A, isopropanol, or casein wash buffer. Gels washed in buffer A failed to detect beta-galactosidase activity in the amounts applied to isopropanol or casein washed gels (data not shown).

Gels prepared with BioRad SDS revealed nearly identical beta-galactosidase sensitivities when comparing the isopropanol with the casein wash methods (Fig. 9A, lanes 4 and 5 and 9B, lanes 4 and 5). But in contrast, the isopropanol washed gel often

Figure 8

DNase I sensitivity gels prepared, loaded and run as described in Figure 7 but in the presence of Fisher SDS. Gel 8A was washed in isopropanol and 8B in Casein wash buffer. Both gels were activated as in Figure 7 for 24 hours.

Figure 8

A

B

31
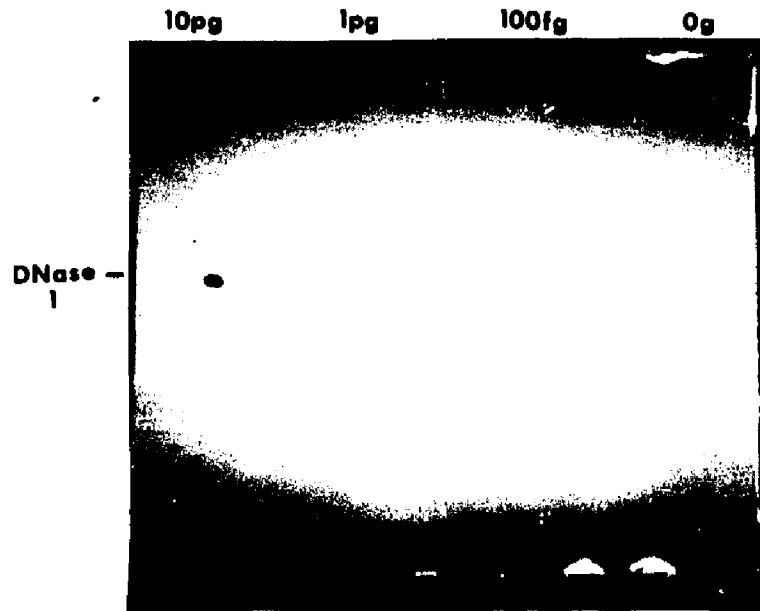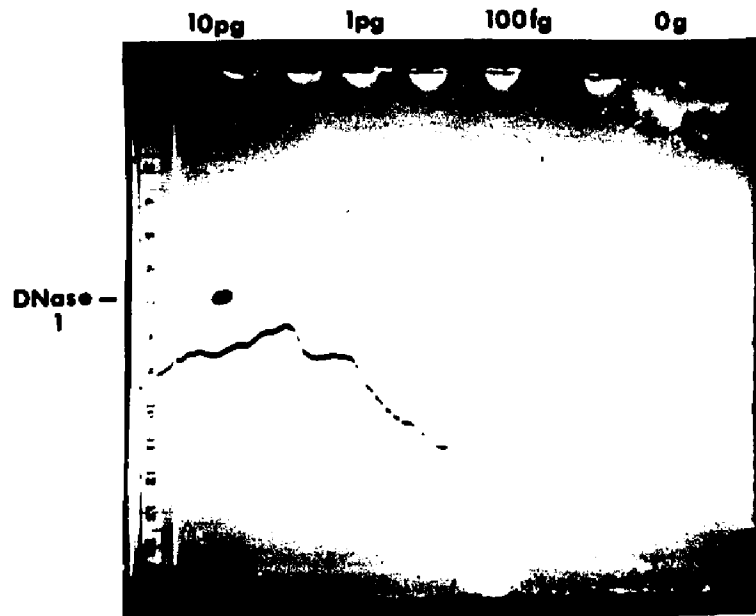
Figure 9

7% acrylamide gels containing either Biorad SDS (Fig. 9A and 9B) or Fisher SDS (Fig. 9C and 9D) run to separate duplicate samples of commercially prepared beta-galactosidase. In each gel lanes were loaded as follows: Lane 1, 3.7 ug protein, 1100 units of enzyme activity; Lane 2, 740 pg protein, 220 units of enzyme activity; Lane 3, 370 pg protein, 110 units of enzyme activity; Lane 4, 37 pg protein, 10 units of enzyme activity; Lane 5, 3.7 pg protein, 1 unit of enzyme activity. Gels were washed in either casein (9A and 9C) or isopropanol (9B and 9D) prior to activation as described.

Figure 9

A | **B**

1 2 3 4 5 | 1 2 3 4 5

B. gal.—

C | **D**

1 2 3 4 5 | 1 2 3 4 5

B.gal.—

33
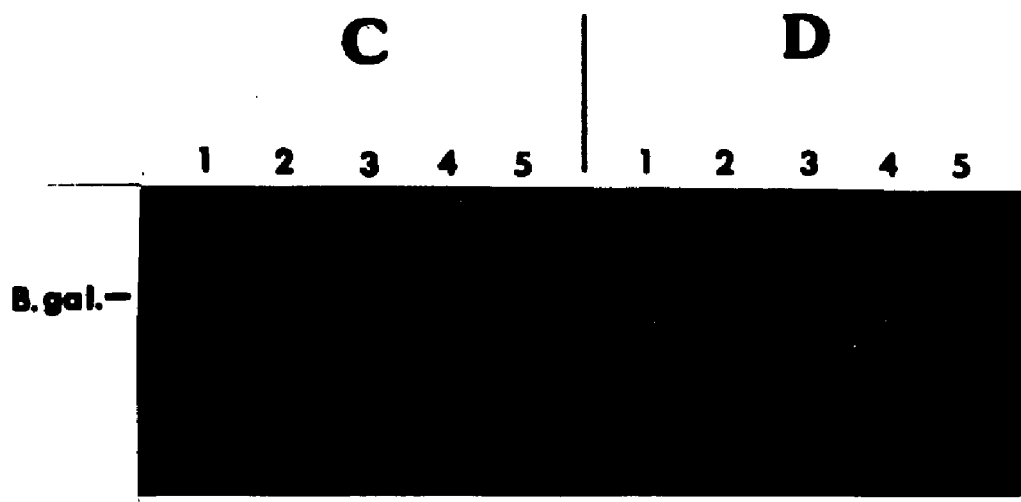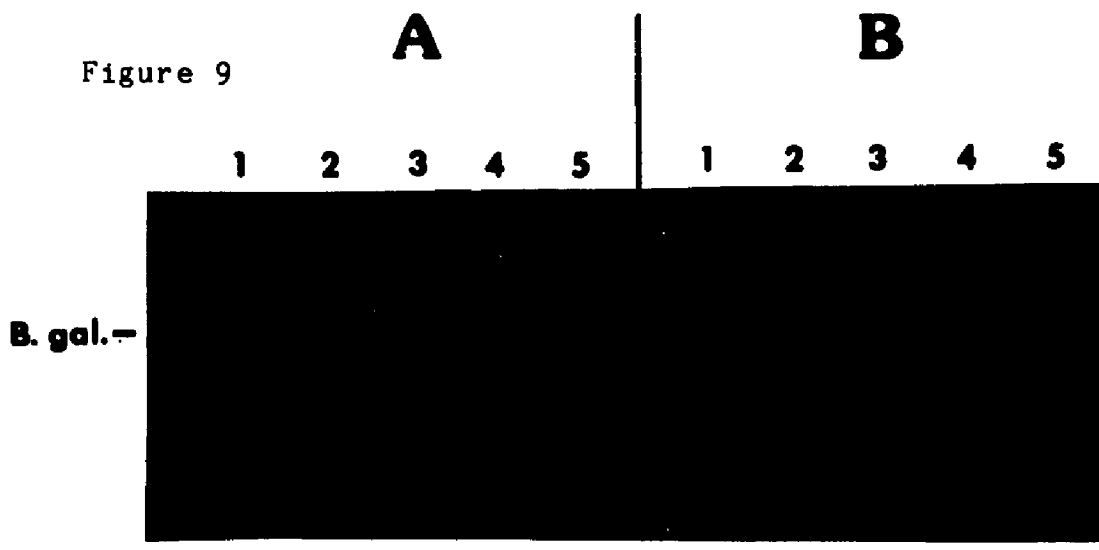
developed a precipitate upon the gel surface (Fig. 9B).

Gels prepared with Fisher SDS differed significantly in their sensitivities (Fig. 9C and 9D). Signal strengths of minor bands in each lane of isopropanol washed gels are weaker by comparison to identical lanes in the casein washed gel. In the isopropanol washed gel (Fig. 9D, lane 4) the activity of beta-galactosidase was not detected but it was detected in the corresponding casein washed gel (Fig. 9C, lane 4). Under conditions of low purity SDS, casein washing allowed 10 fold more sensitivity than isopropanol washing. Fig. 9C, lane 4 represents the detection of approximately 10 units of total beta-galactosidase activity or approximately 37 pg of purified enzyme.

Effect of BSA Concentration on ^{35}SDS Elution

^{35}S-labeled SDS nuclease detection gels containing BSA embedded in the matrix were washed and compared. In Figures 10 A and B it is seen that in a time-course wash experiment, gels containing 50 and 500 ug/ml BSA resulted in increased amounts of residual SDS remaining in the gel at equilibrium. It also appeared that the rate at which SDS eluted from the gel was slowed in comparison to 10 ug/ml BSA gels (Fig. 4).

Figure 10

Time course elution of ^{35} S-labeled SDS from polyacrylamide gels containing either 50 ug (Fig. 10A) or 500 ug/ml BSA (Fig. 10B). Gels were washed by in either buffer A (squares), Isopropanol buffer (triangles), or casein buffer (circles).

Figure
10A

Percent SDS Remaining

Figure
10B

Percent SDS Remaining

Time (min.)

36
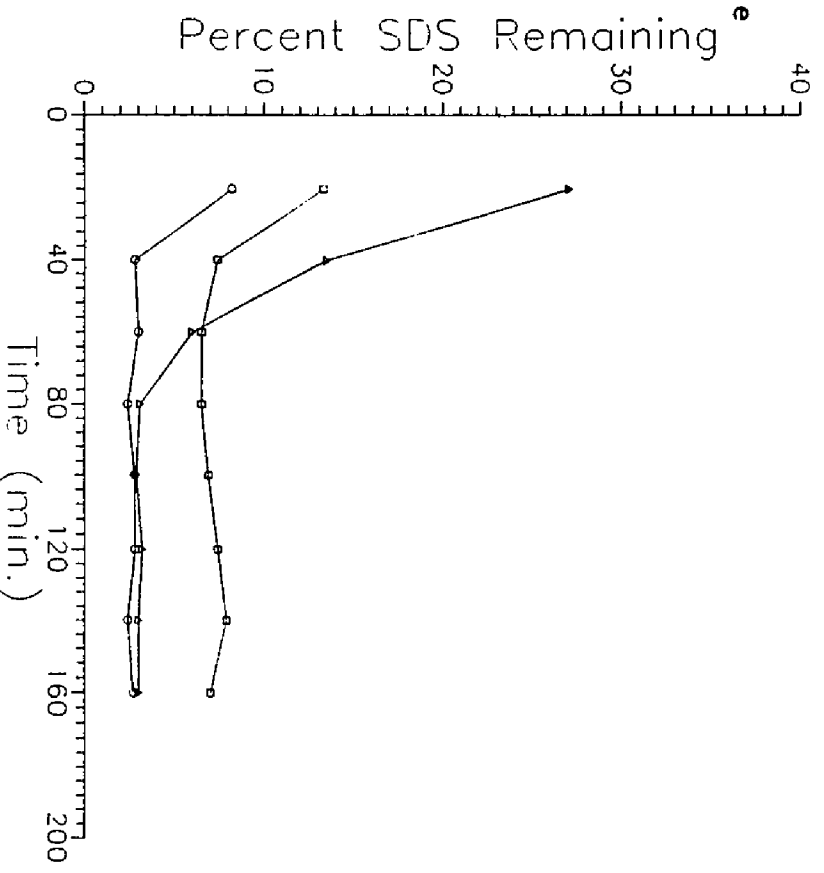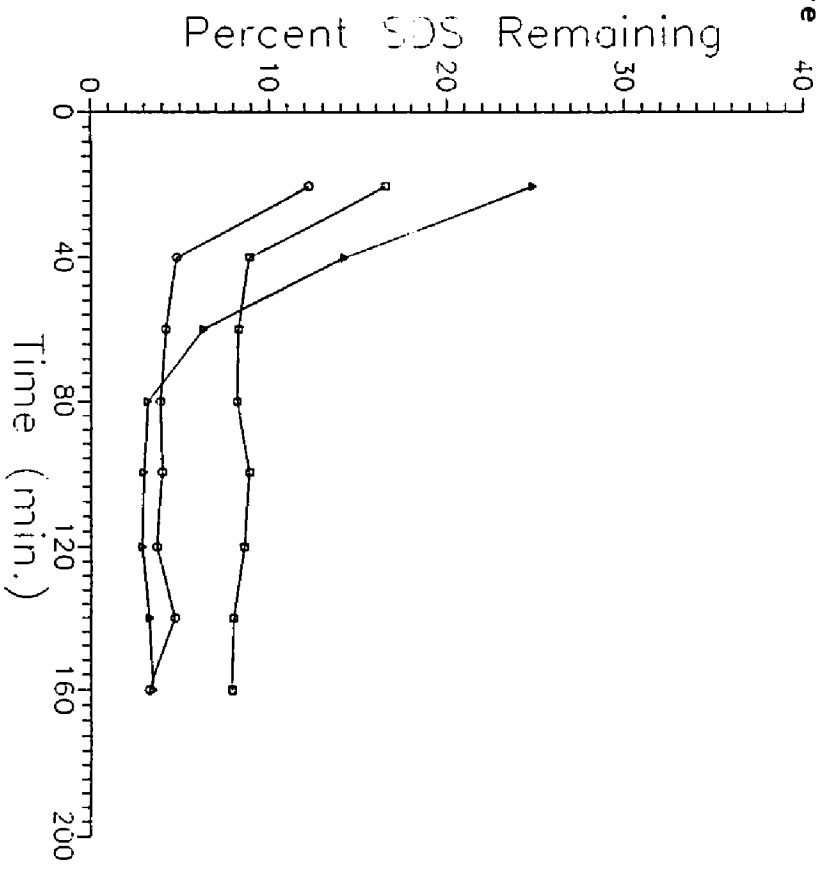
Effect of Increased Amounts of Gel Matrix BSA on Nuclease Gel Sensitivity

Gels containing 50 ug/ml BSA (Fig. 11A) showed no greater sensitivity for nuclease detection than those containing 10 ug/ml (Fig. 7A). However, in gels containing 500 ug/ml of BSA (Fig. 11B), DNAse I was not detectable in the 1.0 pg lane.

Figure 11

DNase I gel sensitivity gels as described in Figure 7 but containing 50 ug/ml (Fig. 11A) and 500 ug/ml BSA (Fig. 11B). Lanes A, B, C, and D contained 10.0 pg, 1.0 pg, 100 fg, and the 0g DNase I control, respectively. Both were washed in 1 liter casein wash buffer for 2 hours without buffer changes and activated for 24 hours in 2 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$.

Figure 11

A

10pg   1pg   100fg   0g

DNase — 1

B

10pg   1pg   100fg   0g

DNase — 1
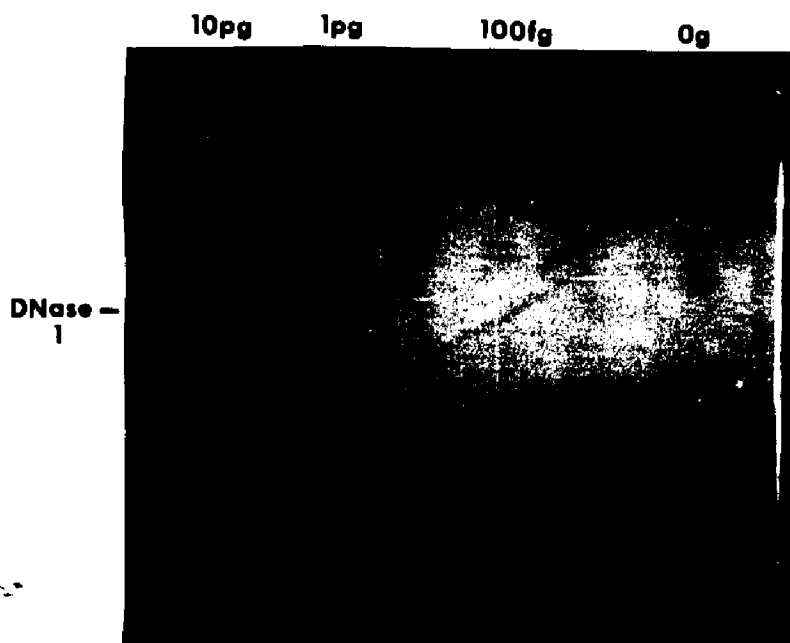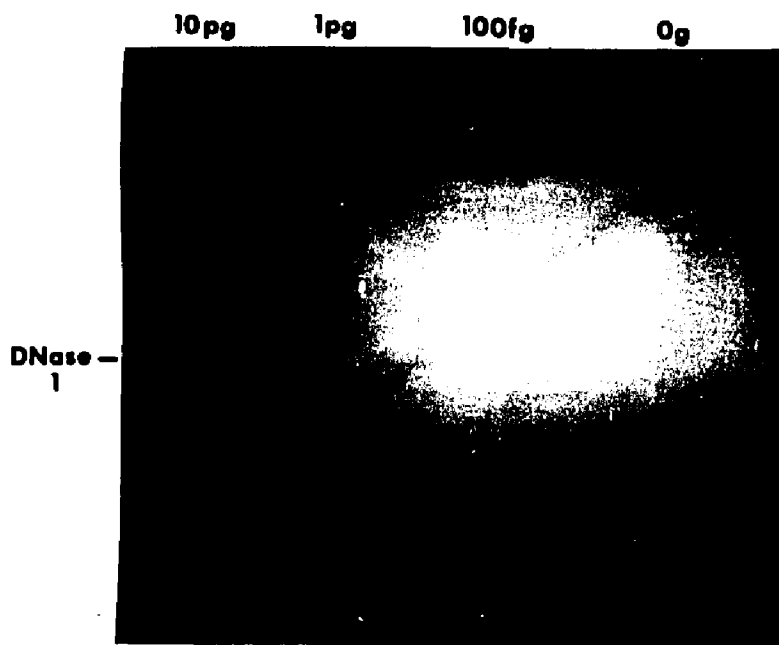
Discussion

Various washing procedures have been investigated
for their ability to improve post-electrophoresis
renaturation of enzymatic activities (2, 16). Two
aspects which are important in any wash method are
level of detection and breadth of application. The
casein wash procedure generally improves both by
rapidly reducing the amount of total residual SDS
within the SDS-PAGE gel, without the use of organic
solvents which may be detrimental to enzymatic
activities.

Casein wash buffer allows the detection of a
greater number of nucleases than the other methods
tested. All but three nuclease bands which appear in
casein washed gels appear on at least one other gel
washed by a different technique. This implies that
the detection of the majority of nucleases is probably
not the result of some unique interaction attributable
to the presence of casein. The three nucleases
detected only in casein-washed gels may be observable
because of: i) casein/substrate interaction that
permits nuclease activity , ii) a casein/enzyme
interaction that augments nuclease activity, or iii)
increased sensitivity for nuclease detection not
obtainable by other wash methods.

Incubation of casein wash buffer with plasmid

pBR322 indicated that no nuclease activity exists which could alter the mobility of either the linear, nicked relaxed, or supercoiled forms of the plasmid in agarose electrophoretic gels. Therefore it seems unlikely that the casein wash buffer is modifying the DNA substrate.

Because in situ beta-galacosidase activity was also enhanced in casein-washed gels, it is unlikely that any nuclease-specific interaction has occurred to augment activity in nuclease gels. Therefore, it is probable that casein wash buffer enhanced the sensitivity of the nuclease detection gels beyond that of gels washed by other methods.

Detection enhancement by casein working in an SDS-removal capacity is infered from our [35]SDS elution studies. In all gel wash studies (using 50 ug/ml BSA or less in the gel matrix), casein wash buffer attained an equilibrium SDS concentration below the others investigated. This concentration was also achieved in a shorter amount of time. Only in gels where matrix protein was high (500 ug/ml) was the isopropanol wash able to reach a lower equilibrium SDS concentration. However, the equilibrium concentration acheived by the isopropanol wash occurred 40 minutes after the casein wash buffer had reached a comparable equilibrium concentration.

We propose that casein binds SDS soon after it elutes from the gel. This maintains a low effective SDS concentration in the gel wash buffer allowing diffusion to proceed at a maximal rate. This aspect is also important for removing the reported SDS-contaminants which bind more tenaciously to proteins than does SDS (16,20,10,11). Removal of contaminants which have a low dissociation rate is slower unless competitive binding for the free molecules occurs.

Addition of various proteins to the gel matrix has been shown to aid enzyme reactivation. Addition of BSA (21,16,2) or fibrinogen (15) to SDS gels yields better enzymatic activity than gels devoid of them. The mechanism responsible for this effect is not clear although roles in the scavanging of lipophilic inhibitors or in providing a microenvironment for protein refolding have been suggested (15). With our experiments we were unable to address these questions. But we have determined that there is a point of diminishing returns with respect to the concentration of BSA which can be tolerated. Concentrations in excess of approximately 50 ug/ml BSA within a gel reduce the amount of renatured DNase I activity. Our radioisotope studies suggest that a larger concentration of residual SDS remains in gels containing 500 ug/ml BSA after extensive washing.

Although there was a 10-fold higher BSA concetration within the gel seemingly to bind SDS to spare DNase I from denaturation, this effect seems to be out-weighed.

It is possible that at high protein concentration in the gel, and low concentrations of SDS, residual bound SDS may be shared between individual protein molecules (29). Such sharing between a BSA and DNase I molecule may result in enzyme inactivation.

In addition to the improvement in sensitivity, the casein wash procedure offers significant economic advantages. The isopropanol wash procedure while offering similar sensitivity is not only more cumbersome but three-fold more costly per gel than the casein procedure.

The enhancement of nuclease activity is most pronounced in those gels prepared with poor quality SDS. However, the greatest sensitivity is still obtained in gels prepared with high purity SDS for which there is no substitute.

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II. <u>Nuclease</u> <u>Expression</u> <u>in</u> <u>Competence-Induced</u> <u>Mutants</u>
<u>of</u> <u>Bacillus</u> <u>subtilis</u>

<u>Bacillus</u> <u>subtilis</u> is capable of undergoing two
types of developmental changes which greatly increase
its ability to survive. The formation of a dormant
endospore allows the cell to survive adverse
environmental conditions. The attainment of
competence, the ability to take up exogenous DNA to
become genetically transformed, acts to increase the
genetic diversity of the species and increases the
gene pool from which important functions may be
selected. Both of these processes can be induced by
changes in a culture's growth rate or the nutritional
state.

Elucidation at the molecular level of the
regulatory mechanisms responsible for both sporulation
and competence are complicated by many factors: i.)
Sporulation and competence induction occur at the same
time in a growing culture, at late logarithmic/early
stationary phase when a large number of physiological
changes due to culture growth rate, nutritional state,
and/or cell density are expected to occur (1, 2, 25,
29). ii.) In the case of competence, only 10-20% of
the culture express competence (26, 29). Thus

regulatory molecules which are normally produced in minute quantities are extremely difficult to detect, isolate and characterize. iii) Many of the regulatory changes occur very early in competence and sporulation and their effects are not observed until later in development (25, 26, 29, 52). iv.) Mechanisms required for either sporulation or competence may also include some normal vegetative systems. For example many competence functions probably interconnect with processes such as cell division, DNA repair, DNA synthesis, etc. (1, 27, 28). v.) The regulatory mechanisms at work to ensure the temporal coordination of the greater than 80 loci responsible for sporulation are complex (1, 6). Certain of these affect the development of competence.

The Importance of Competence and Sporulation Mutants

A large number of sporulation (spo) mutants have been isolated and well characterized. These fall within seven recognizable morphological stages which can be observed over the 8-10 hour period comprising sporulation (1, 3). They reveal a complex array of regulatory interactions which allow the cell to control the temporal expression of important sporulation genes. Regulatory mechanisms involving DNA-directed RNA polymerase sigma factor cascades (1,

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7, 8, 19, 21, 24, 29, 49, 57, 56), "helix-bending regulatory proteins" (60), promoter switching and overlap (20, 22, 29, 56, 59), activation of sigma factors by proteolytic cleavage (12, 18) coupled to septation events (1, 58, 59) and methods of signal transduction and environmental sensing (7, 10, 46) have all been found to act in sporulating cells. In addition, Stragier et al. have found that during later stages of sporulation a mother-cell chromosomal rearrangement occurs which joins together two separate spo genes, spoVCB and spoIIIC (65). The two loci respectively code for the amino- and carboxy-terminal portions of the complete, mother-cell specific, Sigma K (Sigma 27).

Culture conditions which stimulate sporulation relative to competence, and vice versa (25, 44) are useful to investigate either process. By inducing sporulation under such conditions, separation of sporulation from competence functions is partially achieved. Sporulation inducing methods have resulted in the development of spo mutants which fail to proceed past various stages of sporulation, the earliest of which is stage 0.

Other mutants have been identified in which sporulation is not inhibited by normal repressive conditions (15) such as high glucose medium. Some of

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these mutants have been recognized as partial suppressors of the more pleiotropic sporulation mutations and have helped elucidate the location, mode of action and interaction of various spo genes (1, 9, 13, 14, 15, 16, 25, 43, 52, 53, 61).

Competence (com) mutants have also been generated with characteristic phenotypes. They have been categorized by their differing abilities to bind exogenous transforming DNA, internalize it, and recombine it with their genome. Differences in host cell ability to become transformed by chromosomal DNA compared to plasmid DNA are also commonly observed.

Other characteristics have been used to categorize com mutants in the laboratory of D. M. Green. These characteristics include: ability to be transfected, marker rescue, ability to solublize single stranded DNA, ability to solubilize double stranded DNA, presence of a surface endonuclease capable of inactivating phage DNA, ultraviolet sensitivity or resistivity, ability to form heat resistant spores, and the presence in cellular extracts of calcium, magnesium, and manganese-activated deoxyribonuclease biochemical markers. Observable variations in com mutant phenotype led to the identification of enzymatic changes associated with a given mutation.

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Investigative Approaches to Study Competence

Because all of the cells in a competence-induced culture are not competent, three approaches can be used to study alterations in gene expression specific to competence development.

First, separation of competence-induced culture into heavy and light density fractions by density gradient fractionation appears to enrich the percentage of competent cells. Cells from the light fraction are more readily transformed and a greater percentage of a previously recognized set of <u>com</u> genes become expressed (25). This method is variable in its results (26). The possibility remains that some of the cells in the non-competent heavy fraction are actually pre- or post-competent and share many biochemical features with the light fraction.

A second method is the comparison of heterogeneous competence-induced whole cultures with non-induced whole cultures. The observed differences are assumed to be largely associated with the competent fraction of cells. Because media or growth regime must vary to some degree between induced and non-induced cultures, true control experiments cannot be performed. Many differences observed may be brought on by nutritional changes possibly unrelated

to the competent state.

I have used a third method in this investigation. By comparing competence-induced wild-type cells with competence-induced com and spo mutants in a two dimensional zymogram (2DZ) assay for deoxyribonucleases (62, 73), the biochemical phenotypes characterizing mutants can be more closely associated with competence development than by other means of investigation. By 2DZ analysis, I am able to directly observe alterations in nuclease production found in competence-induced com and spo mutants. Because the donor DNA undergoes endonucleolytic and exonucleolytic cleavage during the transformation process, it is believed that changes in cellular nuclease activity are implicated in competence development.

Previous Work using the 2DZ Procedure for Studying Nucleases of Bacillus subtilis

The 2DZ nuclease detection system is extremely sensitive. More than 80 previously unreported nuclease activities have been detected in B. subtilis lysates (39, 62). Coughlin observed a competence specific low molecular weight nuclease (17 kilodaltons and approximate pI 4.8) as well as other competence associated nucleases and newly synthesized peptides when comparing wild-type competence-induced with wild-

type vegetatively growing cultures. The same proteins were affected in the limited survey of com mutants investigated. The competence-specific nuclease was designated nuclease 2 by a numbering system devised by Coughlin to describe the deoxyribonuclease forms identifiable by 2DZ. A nearly identical enzyme released into the media of sporulating cells has also been isolated and carefuly described by Akrigg (63, 64), and is probably the extracellular version of the competence-specific enzyme which has been the focus of numerous studies by Venema (32, 33, 34, 35, 36, 37, 38, 40). Because they have similar molecular weights, pI, and cation requirement, I suggest that both the Venema and Akrigg enzymes are identical to nuclease 2.

Focus on Mutants

In this study, I have examined 2DZ gels of 56 different competence-induced com or spo mutants and compared them to wild-type cells grown in the competence regime. Of the 83 reported nucleases reported by Coughlin, my attention has focused upon the 22 which appear to be associated with competence. These 22 nucleases were selected by three criteria:

1. Those nucleases previously described as competence specific or competence associated.

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2. Those nucleases determined to be associated with vegetative growth.

3. After careful inspection of the 2DZ gels, as many additional readily identifiable nuclease entries as the database would allow.

The selected nucleases are commonly but not invariably detected in all mutants, increasing the possibility of assigning a specific mutant genotype with nuclease phenotype. The nuclease expression data were used to compare each with an individual nuclease's expression. Use of this data permitted the detection of patterns of nuclease expression that were previously unrecognized.

Materials and Methods

Sodium dodecyl sulfate (lot #M2276), urea and acrylamide were electrophoresis grade from Biorad corporation. Ampholytes were from LKB; N,N,N',N'-tetramethylenediamine, N,N'-methylenebisacrlyamide, and ammonium persulfate were from Eastman Kodak; calf thymus DNA (D-1501), Trizma base, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, N-ethylmaleimide, and purified casein (# C-5890) were from Sigma; Nonidet NP-40 was from Bethesda Research Laboratories; bovine serum albumin (BSA) was Pentax fraction V; All other chemicals were reagent grade.

Database computer comparisons of nuclease gel data were prepared utilizing Ashton-Tate's dBase II on an IBM compatible microcomputer.

List of Strains

The following strains were a kind gift from Terrance Leighton (Department of Microbiology and Immunology, University of California, Berkeley):

Simplified Designation	Original Designation(13,14)	Genotype
TL-1	RS5052	spo0K crsA47
TL-2	1S28	spo0K trpC2
TL-3	RS5008	spo0F221 crsA47 pheA1

TL-4	1S19	spo0F221 trpC2 pheA1
TL-5	RS2112	spo0F221 rvtA11 lys1
TL-6	not available	spo0B^{ts} crsA47
TL-7	not available	spo0B^{ts} rvtA11
TL-8	not available	spo0B^{ts} lys1
TL-9	not available	spo0E11 rvtA11 lys1 pheA1
TL-10	RS5022	spo0E11 crsA47 pheA1
TL-11	1S17	spo0E trpC2 pheA1

Strains of the "KB" series were the kind gift of Kenneth Bott of Stanford University, Palo Alto, California and represent an isogenic series originating with Zuber:

KB-449	spo+
KB-451	spo0B136
KB-452	spo0C92
KB-453	spo0E11
KB-454	spo0F221
KB-455	spo0H81
KB-456	spo0J93

Isolation of Sporulation-Associated Nuclease

Two liters of 37°C Akrigg sporulation media (63)
were seeded with 30 ml of a saturated, overnight
culture of wild-type Bacillus subtilis SB-25. After
the culture reached a Klett 660 value of 75, the cells
were pelleted at 4°C and the media collected into
spectrapor 8 kDalton molecular weight cut-off dialysis
tubing. The media was dialyzed against Buffer A, (0.5
M Tris/HCl pH 8.1, 1 mM EDTA), overnight before bulk-
adsorbtion to 25 grams of DE-52 ion exchange resin.
The resin was washed extensively with Buffer A, poured
into a 50 ml syringe as a column, and eluted with 250
ml of Buffer A containing 0.4 M NaCl. The collected
eluate was dialyzed against three 2-liter changes of
Buffer A over 48 hours at 4° C. The dialyzate was
loaded onto a 80 ml bed volume of DE-52 and eluted
with a 300 ml total volume linear gradient of 0 to 0.4
M NaCl in Buffer A. Fractions were assayed for
manganese and cadmium nuclease activity by reduction
in DNA-intercollated ethidium bromide fluorescence as
observed with a Turner fluorometer, (330 nm
excitation, 548 nm emission). Pooled fractions were
assayed for endonuclease activity by hydrolysis of
supercoiled pBR322 in the presence of manganese and

cadmium. Pooled fractions were equilibrated in 0.025 M histidine/HCl pH 6.2 prior to separation on 30 ml bed volume of chromatofocusing resin (Polybuffer exchange PBE-94). Samples were eluted with 400 ml of polybuffer 74/HCl pH 4.0 and assayed for nuclease activity as described. Active fractions were pooled and separated on a 500 ml bed volume of Pharmacia G-100SF in Buffer A containing 0.1 M NaCl. Fractions (5 ml) were collected at 4°C into tubes preloaded with 1 ml of 75% glycerol, 36uL of 0.5 M reduced glutathione, and 6uL of PMSF. Fractions were gently mixed soon after their collection. After nuclease assay, pooled fractions were dialysed into 0.05 M Tris pH 7.9, 25% glycerol 0.1 mM EDTA, 0.5 mM glutathione and 0.1 mM PMSF. The fractions percolated through a 6.5 ml bed volume column of heparin-agarose and collected as effluent. The bed was washed with 60 ml of eqilibration buffer and the effluents were pooled. The pooled effluents were dialyzed against three 500 ml changes of buffer A and concentrated on 2.5 gms of DE-52. The final active fraction was eluted by placing the resin into a 10 ml centrifuge tube, eluting with 5 ml of Buffer A containing 15% glycerol and 0.25 M NaCl, and collecting the supernatant liquid after centrifugation. Samples were stored at 4°C in this elution buffer after the addition of reduced

glutathione to 3 mM.

Preparation of Competent Cell Lysates

Mutant and wild-type strains of Bacillus subtilis deficient in sporulation or competence where grown in the growth competence inducing regime prescribed by Spizizen (69) and modified by Kohn and Green (70). This protocol is routinely used for inducing competence in wild type cells. Briefly, cells are grown for 4.5-5 hours to late log phase in HSC media (39) and a fraction seeded into a fresh secondary medium, LSC, (39) for 90 minutes. At the end of this period a portion of a wild type culture becomes physiologically competent to take up and express exogenous DNA.

Induction of Competence

Cells were made competent by the method of Spizizen (69) as modified by Kohn and Green (70).

DNA preparation

Calf thymus DNA was prepared for embedding in SDS-PAGE gels as previously described (62).

Two Dimensional Zymography

Two dimensional zymographs were prepared by subjecting cellular lysates to both non-equilibrated

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pH gradient electrophoresis, SDS-polyacrylamide gel electrophoresis followed by detection of nuclease activities within the gel. In the remainder of this text the entire process will be abbreviated "2DZ".

Nonequilibrium pH gradient electrophoresis

O'Farrell's procedure for non-equilibrium pH gradient electrophoresis (NEPHGE) (17) was used as described (62).

SDS-PAGE gel electrophoresis

Two-dimensional gel electrophoresis was performed using the discontinuous system of Laemmli as described by O'Farrell (17) and modified by Coughlin (62).

SDS Removal and Nuclease detection.

The basic procedure described by Coughlin (62) was used for SDS removal, nuclease activation and detection within the gels. However, in this study I have added casein to the SDS removal buffer (buffer A), as described in chapter I to enhance nuclease detection within our SDS-PAGE gels. All gel wash incubations were performed at 37°C with agitation on a New Brunswick Scientific gyrotory shaker rotating at 50-60 rpm.

To prepare SDS removal buffer , buffer A (0.04 M Tris-HCl pH 9.0, 0.02% sodium azide, 2 mM EDTA

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prepared in autoclaved distilled deionized water) was
made 1% in casein and autoclaved (20 psi, 121 $^{\circ}$ C) for
30 minutes to denature contaminating enzymes.

Following SDS removal, gels were placed into 20
gel volumes pre-warmed activation buffer B (0.04 M
Tris-HCl pH 7.6, 0.02% sodium azide) containing 2 mM
$CaCl_2$, $MgCl_2$ and $MnCl_2$. Gels where incubated at 37°C
until additional nuclease activities could no longer
be detected (5-7days). In situ DNA hydrolysis was
terminated and the results photographed as previously
described (62).

Evaluation of 2DZ Gel Nuclease Activities for use in
Databases

Activated 2DZ gels were scored for nuclease
activity at a given position relating to the map and
numbering system of Coughlin (39). Conclusions drawn
in this study were only derived from those cases when
a nuclease was altered in its ability to be detected,
either present when normally absent, or vice versa.
Such "on/off" comparisons were made to eliminate
subjective observer error.

A qualitative value was assigned to each database
nuclease representing the quantity of detected
activity as described below. These "quantity of
activity" values may be useful in future

investigations by selecting a single nuclease as a internal standard to which all other quantities of nuclease activity are compared.

Nuclease Scoring Legend

"++++"=overexpressed, greater than expected
when compared to wild-type.

"+++"=wild type expression.

"++"=underexpressed, less expression than wild-type.

"+"=detectable, far less expression than wild-type.
"-"=not detectable, missing from nuclease gel.

Scoring Other Fields

The following fields were scored and data provided by D.M. Green concerning phenotypic characteristics of <u>Bacillus</u> <u>subtilis</u> competence mutants.

In fields MRESCUE, TFORMATION, AND TFECTION: "0"=wild-type;

"2"=400% more than wild type; 20% to 400% of wild type="1

In fields XOS and XOD, "+"=wild type; "H"= high and "L"= low falling above or below two standard errors of the mean.   "ND"=no data.

In fields CALENZYME, MAGENZYME, AND MANENZYME; the "H" and "L" are values falling outside two standard errors of the mean judged from nine wild type

extracts.

"Sen" is the ability to inactivate phage DNA. Those less than 25% able to inactivate compared to wild-type are scored "-".

"UVS"= 50% of wild-type survival to UV; UVR cells show 50% more survival than wild-type.

"SPO (-)" are mutants which fail to form heat resistant spores.

Database comparisons of nuclease gels

Databases were created as outlined below to allow the rapid comparison of mutant phenotype with nuclease expression. Note that in order to simplify the use of each database, titles of phenotypes, nucleases, or groups of nucleases were given abbreviated names. These abbreviated names are reported here and will be used in the text so that interested users may easily access the databases in the future.

MUTBASE.DBF

Mutbase.dbf = a root data base consisting of the following fields:

FIELD	DESCRIPTION
MUTANT	mutant designation
BINDING	ability of cell to bind DNA
UPTAKE	ability of cell to take in DNA

MRESCUE   ability of cell to restore internalized DNA to a form that can be rescued by a super infecting phage

TFECTION   ability of cell to be transfected

TFORMATION   ability of cell to be transformed

XOS   extracellular single stranded nuclease

XOD   extracellular double stranded nuclease

CALENZYME   extracellular calcium activated nuclease

MAGENZYME   extracellular magnesium activated nuclease

MANENZYME   extracellular manganese activated nuclease

SEN   surface endonuclease inactivation of phage DNA

UV   resistance or sensitivity to UV light

FACE   presence of nucleases 6-9 in nuclease gels

LOGNUKES   nucleases 3,4,10,12,21,26,29 in nuclease gels

LITTLENUKES   M.W. 12 Kdal nuclease in nuclease gels

TWOTAIL   presence of basic "tail" on nuclease 2

NUKE2   presence of nuclease 2 in nuclease gels

NUKEBASE.DBF

NUKEBASE.DBF is a major "root database" which is used to directly relate the appearance of specific nucleases with transformation and sporulation

associated phenotypes of <u>Bacillus</u> <u>subtilis</u> mutants.
Nucleases were identified and numbered as recommended
by Coughlin (39). NUKEBASE.DBF contains the following
fields:

FIELD NAME	DESCRIPTION
MUTANT	All descriptions have been made
NUKE2	in "MUTBASE.DBF" except that
NUKE3	"NUKE#" as appears in this database
NUKE4	refers to the presence or absence
NUKE6	of said nuclease in association with
NUKE7	any designated mutant.
NUKE8	
NUKE9	
NUKE10	
NUKE12	
NUKE14	
NUKE16	
NUKE17	
NUKE21	
NUKE24	
NUKE25	
NUKE26	
NUKE28	
NUKE29	
NUKE35	
NUKE37	(continued next page)

NUKE40

NUKE66

BINDING

UPTAKE

MRESCUE

TFECTION

TFORMATION

SPO

UV

XOS

XOD

All scoring rules are described under "MUTBASE.DBF".

SPORT.DBF

SPORT.DBF is a smaller version of NUKEBASE.DBF that contains only spo mutants of the "KB" series or "TL" (Terrance Leighton) series. Subsorts of the database are named SPO2, SPO3 SPO5...etc., meaning for example; SPO2.DBF = "SPORT.DBF sorted with respect to NUKE2. The fields in SPORT.DBF are as follows:

SPO

NUKE2

NUKE3

NUKE6

NUKE7

NUKE8

NUKE9

NUKE10

NUKE12

NUKE16

NUKE21

NUKE24

NUKE25

NUKE29

NUKE35

NUKE66

MUTANT

TWOTAIL

LITTLENUKE

KBASE.DBF and TLBASE.DBF

KBASE.DBF and TLBASE.DBF are two smaller sub-
databases which are built around the individual spo0
strains. They are also used to directly relate cell
phenotype to nuclease expression.

Identifying Nucleases Associated with a Given Phenotype

To reveal nuclease associations, each data base
was sorted with respect to each field. For example:
If an association was suspected between low ability of
a cell to bind DNA and the appearance of XOD activity;

first NUKEBASE.DBF is sorted with respect to XOD activity and named XODSORT.DBF. Using XODSORT.DBF, comparisons of the expression of each individual nuclease was easily accomplished.

Nucleases Strongly Associated by Co-expression

With the aid of NUKEBASE.DBF, percentages of co-expression were calculated as described below. The data base was sorted to determine how many times a nuclease, (the primary nuclease), was expressed under competence-inducing conditions in 56 mutant lysates. The number of times any other nuclease, (the secondary nuclease), was coexpressed was recorded. Dividing the number of times the secondary nuclease was expressed at the same time as the primary nuclease, by the number of times the primary nuclease was expressed, gives a fraction of the times that the secondary nuclease was expressed with respect to the primary. Fractions were converted to percentages by multiplying by 100. These percentages are designated "percentages of co-expression".

Properties of the Isolated Sporulation-Associated Nuclease

The nuclease I isolated from sporulating Bacillus subtilis cultures had an approximate molecular weight of 15 kiloDaltons. It was shown to hydrolyze native calf thymus DNA in the presence of 2mM manganese, and to a lesser degree in 2 mM magnesium. Minimal activity was observed in the presence of cadmium and no detectable activity in calcium. (Figure 12). Its activity is both endo- and exonucleitic (Figures 13 and 14), and it's capable of hydrolyzing supercoiled, linear-duplex and single-stranded DNA. It was not active on RNA. Its mobility within our two-dimensional gel system was comparable to that of competence-associated nuclease 2 (Figure 15). It was shown to be identical to the enzyme described by Akrigg (63) by enzymatic inhibition useing antisera directed against the Akrigg nuclease (Figure 16).

Two-dimensional Electrophoretic Analysis of Non-competent mutants of Bacillus subtilis

Nucleases Strongly Associated by Co-expression

By cross-sorting the information reported in database NUKEBASE.DBF for the screened com and spo0

Figure 12:

One dimensional SDS-PAGE nuclease-detection gel
analysis of sporulation medium (lanes A-D) and
purified sporulation nuclease (lanes E-H). Individual
gel slices were incubated for 4 days at $37^{\circ}C$ in
activation buffer containing either 2 mM $CaCl_2$ (lanes
A and E), 2 mM $MgCl_2$ (lanes B and F), 2 mM $MnCl_2$
(lanes C and G), or all three combined (lanes D and
H).

Figure 13:

Agarose gel (1%) separating the products of a plasmid
pBR322 digestion with purified sporulation nuclease.
Time course hydrolysis products were produced by
incubation of plasmid DNA with sporulation nuclease in
the presence of either 2 mM $CdCl_2$, $CaCl_2$, or $MnCl_2$.
Bracketed lanes, from left to right, represent
digestion products after 0, 0.5, 1, 2, and 4 minutes
of hydrolysis, respectively.

Figure 12

A B C D   E F G H

— 66

— 45

— 36

— 29

— 24

— 20.1

— 14.2

Figure 13

Cd   Ca   Mn

70
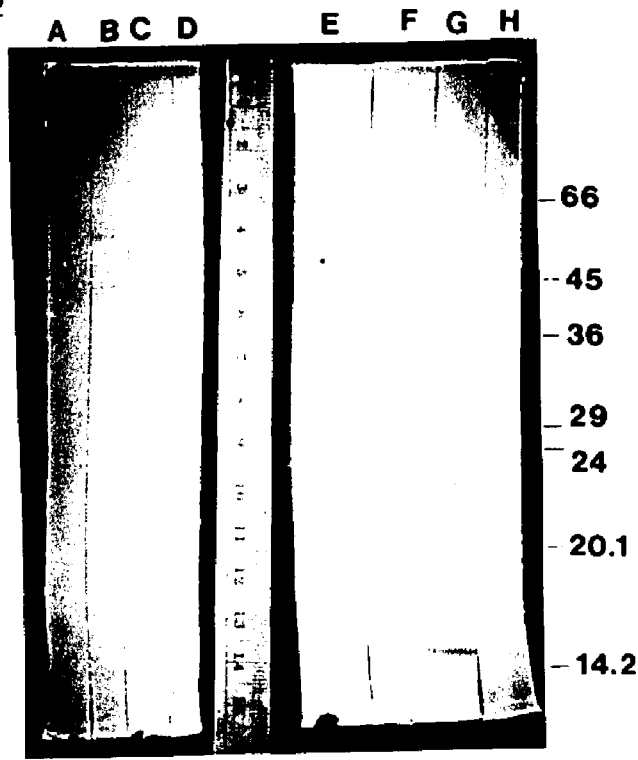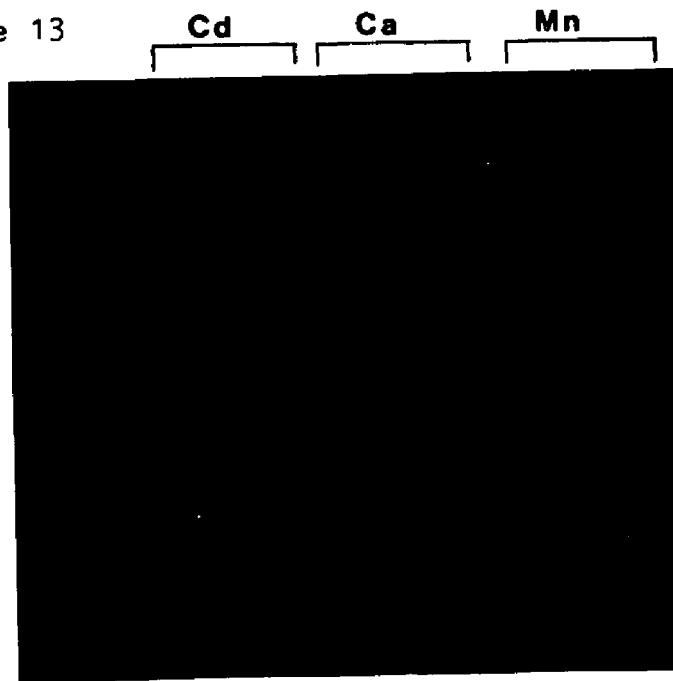
Figure 14:

Agaorse gel (1%) separating the products of sporulation nuclease time-course digestion of either plasmid pBR322, M-13 single -stranded circular DNA, or poly-(G-U)$_{60}$ RNA. Bracketed lanes represent, from left to right, digestion products after 0, 0.5, 1, and 2 minutes of digestion at 37°C in activation buffer containing 2 mM MnCl$_2$.

Figure 15:

Two-dimensional nuclease detection gel analysis of purified sporulation nuclease. The gel was incubated in activation buffer containing 2 mM MnCl$_2$, 2 mM MgCl$_2$, and 2 mM CaCl$_2$.

Figure 14

pBR322　　M - 13　　poly-(G-U) RNA
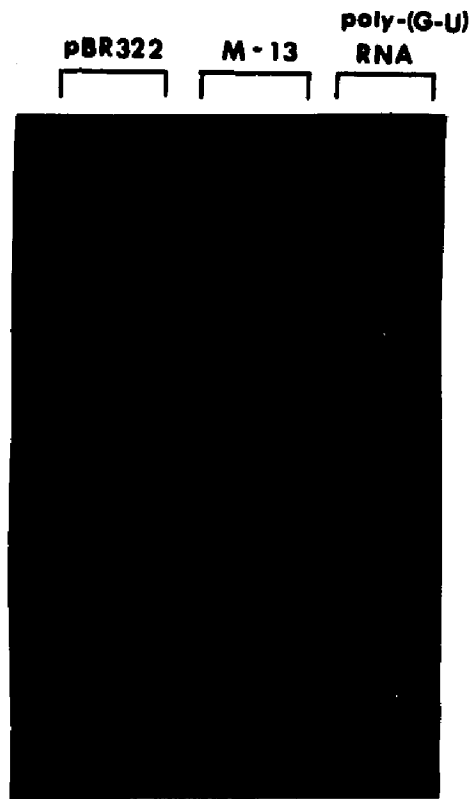
Figure 15

Figure 16:

Inactivation of sporulation nuclease enzymatic
activity by antiserum directed against the Akrigg
sporulation nuclease. Plasmid pBR322 was incubated
int the presence of one or more of the following:
Buffer (control) lane 1. Sporulation nuclease, lane 2.
Sporulation nuclease and Akrigg antiserum, lane 3.
DNase I, lane 4. DNase I and Akrigg antiserum, lane 5.
Akrigg antiserum (control), lane 6.Each reaction was
performed for one minute at 37°C in the presence of 2
mM MnCl$_2$.
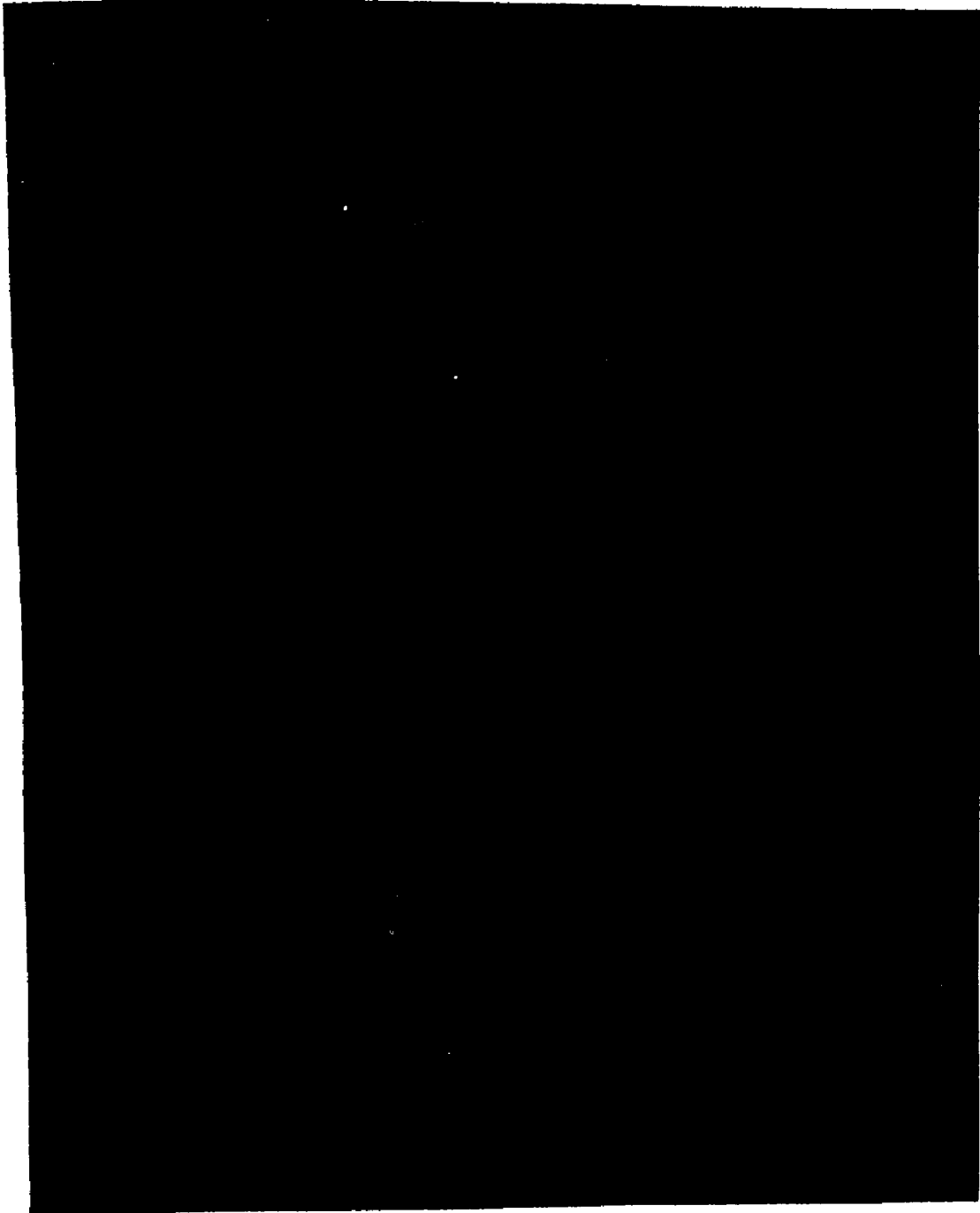
Figure 16

mutants, a "Percentage of Co-expression table" was calculated as described (Table I). (Also see Figure 17 for Coughlin's <u>Bacillus</u> <u>subtilis</u> nuclease map). From this table the following relationships can be observed:

1.   Although nuclease 2 is present in all gels, the other 19 nucleases studied are individually co-expressed in 89% or less of the mutants. This suggests nuclease 2 is probably produced in a different mode compared to the others and will be discussed later.

2.   Nuclease 3 is associated with nucleases 4, 10, 12, and 16 more than 95% of the time.

3.   Nuclease 8 is associated with nucleases 3, 6, 10, 12, and 16 more than 95%. of the time.

4.   Nuclease 10 is associated with nucleases 12 and 16 more than 95% of the time.

5.   Nuclease 16 is associated with nucleases 10 and 12 more than 95% of the time.

6.   Nuclease 21 is associated with nucleases 10, 12, 16, and 26 more than 95% of the time.

7.   Nuclease 26 is associated with nucleases 12, 16, 21, and 29 more than 95% of the time.

From these observations four clusters of co-expressed nucleases are apparent:

Cluster A = nucleases 3 and 4.

Table 1

Per cent Co-expression Table

Co-Expression (per cent of mutants in which
top row nuclease was associated with
those of each database).

Fraction of mutants that nuclease appeared	Nuclease Database used for comparison	Co-expressed Nuclease																		
		2	3	4	6	7	8	9	10	12	16	17	21	24	25	26	28	29	35	66
64/64	2Base	X	84	83	64	63	67	66	89	89	·89	83	83	61	84	83	27	80	48	80
54/64	3Base	100	X	98	72	70	76	72	98	98	98	65	93	67	83	91	26	87	56	83
42/64	8Base	100	95	93	95	93	X	93	100	100	95	76	93	69	83	88	29	86	62	86
57/64	.10base	100	93	91	72	70	75	72	X	98	95	61	89	65	84	88	25	84	51	·81
56/64	16Base	100	93	93	68	68	71	70	95	95	X	61	93	64	80	91	25	88	52	82
52/64	21Base	100	94	94	73	73	77	73	ℂ6	96	100	67	X	67	79	96	25	92	56	79
50/64	25Base	100	84	82	62	60	66	64	88	90	86	48	78	54	X	80	26	76	44	88
52/64	26Base	100	92	92	69	69	73	71	94	96	98	65	96	69	81	X	25	96	56	79
51/64	66Base	100	88	86	69	67	·73	69	90	92	90	61	82	59	92	82	24	78	55	X

Figure 17:

Reproduction of Coughlin's two-dimensional zymogram map of <u>Bacillus</u> <u>subtilis</u> deoxyribonucleases which are active on native calf thymus DNA (39). Nuclease 2 and co-expressed nuclease Clusters A, B, C, and D are circled.
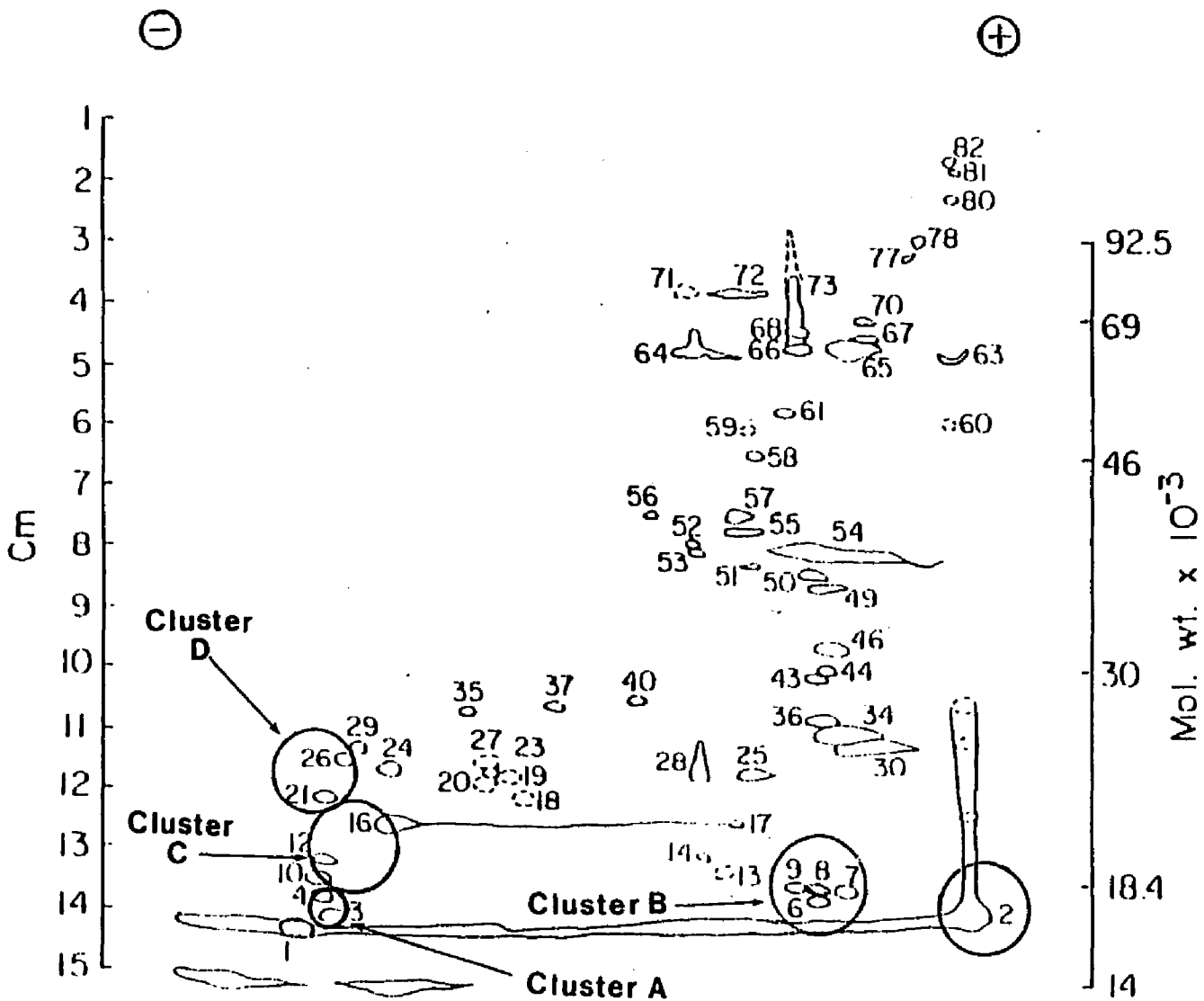
Figure 17

Cluster B = nucleases 6, 7, 8, and probably 9.

Cluster C = nucleases 10, 12, and 16.

Cluster D = nucleases 21 and 26.

Within each cluster, nucleases appear related in molecular weight and pI. Relationships within a cluster can also be seen with respect to cation activation and nucleic acid substrate preference. It is possible that some members of a given cluster are truncated or modified forms of a pro-enzyme.

Coughlin's nuclease data shows that within clusters A and B, the member nucleases are related in their substrate and cation requirements (39). Cluster A nucleases are manganese activated and prefer native DNA as substrate. They are similar to one another in molecular weight (approximately 17,000 Daltons) and are basic in pI.

Cluster B enzymes are manganese activated though they can use magnesium for cation activation and can hydrolyze both native and denatured DNA. Cluster B nucleases are close in pI and molecular weight (approximately 18,000 Daltons). com mutants 903, 1501, and 2203 each possess normal nuclease 9 activity while nucleases 6, 7, and 8 are reduced. Cluster B nucleases are never seen in the absence of nuclease 9. Therefore, it is possible that nuclease 9 is the major precursor nuclease from which the other members of

Cluster B are derived.

Within Cluster C the nucleases differ slightly in their activation profile (39). Nucleases 10 and 12 are strictly activated by manganese and hydrolytic only on native DNA. In contrast, the third member of cluster C, nuclease 16, is activated primarily by manganese but can also use magnesium (39). Its substrate can be both native and denatured DNA. On a 2DZ gel, its pI is significantly more acid than the other members of the cluster and is larger in molecular weight, (21,000 Daltons for 16 compared to approximately 19,000 Daltons for 10 and 12).

The two member cluster D nucleases degrade native and denatured DNA but differ in their cation requirements (39). Nuclease 21 can be activated by calcium, magnesium or manganese whereas nuclease 26 has a strict requirement for manganese. Their molecular weights are approximately 23,000 Daltons and their pI's are basic.

A 35,000 Dalton Nuclease is Secreted by Competence Mutant NH2203

A Petri dish nuclease-secretion survey was performed upon 23 Sen+ Bacillus subtilis com mutants. A halo of nuclease activity, (Figure 18), was observed surrounding NH2203. By 2DZ analysis,

Figure 18:

BHI-agar plates containing calf-thymus DNA stabbed
with wild-type and <u>com</u> mutants of <u>Bacillus</u> <u>subtilis</u>.
After overnight incubation at room temperature, plates
were stained with ethidium bromide prior to
photography.

Figure 18

(Figures 19 and 20), this nuclease was found to have a molecular weight of 35 kiloDaltons, a pI of approximately 5.0, and be able to hydrolyze native calf thymus DNA when activated by manganese. It is not known whether other cations are capable of activating this enzyme or whether it is active on single stranded DNA. However, using Coughlin's nuclease map as reference, (Figure 17), it was determined that the intracellular nuclease is likely to be nuclease 49. The rationale for this determination is as follows.

On the Coughlin 2DZ nuclease map there are five possible nucleases which could be the 2203-secreted nuclease. Based upon similar molecular weight and pI, these candidates are nucleases 46, 49, 50, 51, and 54. However, nucleases 46, 50, and 51 strictly require calcium for activation. Both nucleases 49 and 54 are most active in the presence of calcium but can also use manganese. In addition, nuclease 54 is activated at a low level by magnesium. Nuclease 54 is reported to have a molecular weight of 37,000 Daltons. Nuclease 49 has a molecular weight of 35,000 daltons; closer to that of the secreted nuclease. In addition, nuclease 54's mobility in the NEPHGE dimension is greater than that of nuclease 49, and usually appears as a smear rather than a well focused area of DNA hydrolysis. The 2203-secreted nuclease appears well

Figure 19:

Two-dimensional zymograph of analysis of BHI-agar media supernates from <u>Bacillus</u> <u>subtilis</u> wild-type. Gels were incubated for 5 days at $37^{\circ}C$ in the presence of activation buffer containg 2 mM $MnCl_2$.

Figure 20:

Two-dimensional zymograph of analysis of BHI-agar media supernates from <u>Bacillus</u> <u>subtilis</u> NH-2203. Gels were incubated for 5 days at $37^{\circ}C$ in the presence of activation buffer containg 2 mM $MnCl_2$.

Figure 19   **SB—25**

Figure 20

NH—2203
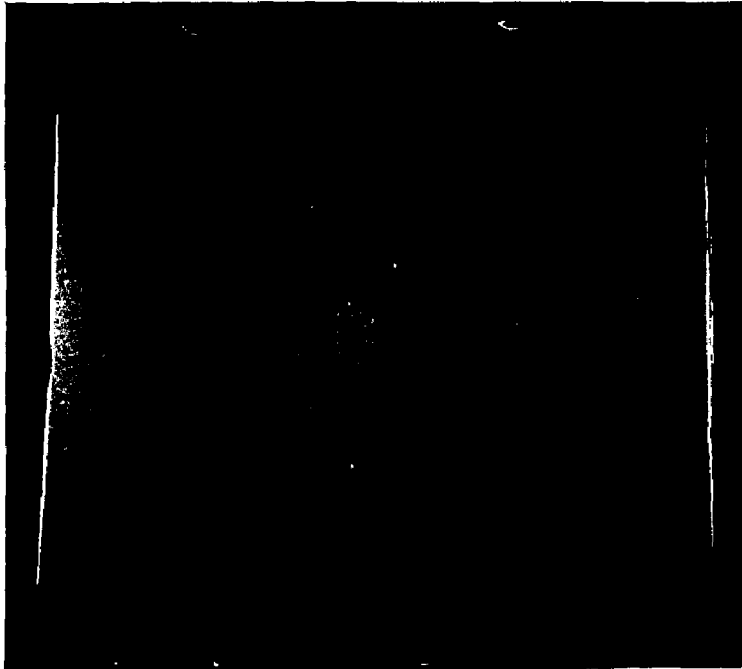
—35kDaltons

Table 2

Mutation	Competence rel. to wt	Nuclease 2[*]	Cluster[*]			
			A	B	C	D
spo0A12 (spo0C9v)	0.020-0.002	N	R	R	N	N
spo0B136	0.028-0.005	R	R	R	R	R
spo0B136 crsA47	0.0015	I	I	I	I	I
spo0B136 rvtA11	0.066	R	A	A	R	R
spo0E11	1.04	N	R	R	R	R
spo0E11 crsA47	0.0063	N	I	N	I	I
spo0E11 rvtA11	0.0007	N	I	N	I	I
spo0F221	0.73	N	N	N	N	N
spo0F221 crsA47	0.001	R	I	I	I	I
spo0F221 rvtA11	0.81	I	I	I	I	I
spo0J87	0.22	I	I	I	I	I
spo0H81	0.03-0.005	N	R	R	N	N
spo0K141	0.01	R	N	N	N	N
spo0K141 crsA47	0.0006	R	I	I	I	I

Abbreviations[*]:
A = activity is absent compared to wild-type.
I = activity is increased compared to wild-type.
N = activity is normal compared to wild-type.
R = activity is reduced compared to wild-type.

focused as does nuclease 49. For these reasons I tentatively identify the 2203-secreted nuclease as nuclease 49.

Two-dimensional Electrophoretic Analysis of spo0 Mutants of Bacillus subtilis

The Rationale Behind the Investigation

Because many of the early events in the establishment of competence are shared in the development of sporulation, relating nuclease expression to known spo0 mutants may be useful in understanding the complex regulation mechanisms of both processes. For this purpose spo0 mutants were grown in the competence regime to compare their two dimensional gel patterns with wild-type and competence-deficient strains of Bacillus subtilis. Each gel was scored for presence of each nuclease or nuclease cluster. Data were tabulated, (Table 2), and analyzed as described to determine whether any particular nuclease or cluster of nucleases were specifically associated with a unique spo0 mutation.

Nuclease Production in spo0 Mutants

KB-452, spo0C, (Figure 21 and Table 2), showed nearly wild-type (KB-449) levels, (Figure 22), of nuclease production. In KB-449, (wild-type), cluster

Figure 21:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
KB-452 (spo0C92). Hydrolysis of Native calf thymus
DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM
$MgCl_2$, and $CaCl_2$.

Figure 22:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
KB-449 (wt$^+$). Hydrolysis of Native calf thymus DNA
was detected in the presence of 2 mM $MnCl_2$, 2 mM
$MgCl_2$, and $CaCl_2$.

Figure 21   **KB-452**

Figure 22   **KB-449**

89
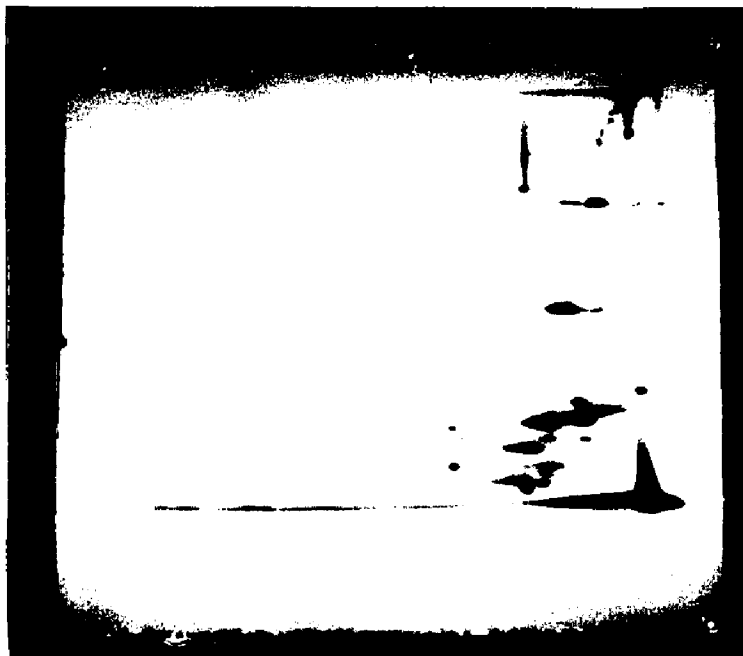
D nucleases are not produced. This is also true for the KB452 (spo0C) strain. However, clusters A and B which are expressed in wild-type is partially reduced in spo0C. A 14,000 Dalton nuclease was found below and to the basic side of nuclease 2 as in wild-type. (Table 3).

In both the KB-451, (Figure 23), and TL-8, (Figure 24), spo0B mutants, repression of all nucleases occurs leading to minimum detectable amounts; except for nuclease 2 which is only slightly below wild-type activity. The low molecular weight, basic nucleases, clusters A, C, and D, are reduced in TL-8 strain and absent in KB-451. Cluster B is present in TL-8 but not in KB-451. (See Table 2). A 14,000 Dalton nuclease found in wild-type below and to the basic side of nuclease 2 was not found in either spo0B strain (Table 3).

For the spo0F mutations the nuclease patterns differ in the KB-454, (Figure 25), and TL-4, (Figure 26), backgrounds. In KB-454 clusters A, C, and D are present but cluster B is absent. Nuclease 2 activity is normal and cluster D enzymes are enhanced. All other nucleases seem to be produced at near wild-type levels, including the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 3). In contrast, TL-4, (spo0F trp- phe-), nucleases are near normal but

Figure 23:

Two-dimensional zymogram of <u>Bacillus subtilis</u> mutant KB-451 (spo0B136). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 24:

Two-dimensional zymogram of <u>Bacillus subtilis</u> mutant TL-8 (spo0spo0B^{ts}). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 23

KB—451

Figure 24

TL—8

Table 3

Strain	Mutation	14,000 Dalton Nuclease
KB-449	spo$^+$	present
KB-452	spo0C92	present
KB-451	spo0B136	absent
TL-8	spo0B^{ts}	absent
KB-454	spo0F221	present
TL-4	spo0F221	absent
KB-453	spo0E11	absent
TL-11	spo0E11	absent
KB-455	spo0H81	present
KB-456	spo0J93	present
KB-457	spo0K141	absent
TL-2	spo0K141	absent
TL-3	spo0F221,crsA47	absent
TL-6	spo0B^{ts},crsA47	absent
TL-10	spo0E11,crsA47	absent
TL-1	spo0K141,crsA47	absent
TL-5	spo0F221,rvtA11	absent
TL-7	spo0B^{ts},rvtA11	present
TL-9	spo0E11,rvtA11	present

Figure 25:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant KB-454 (spo0F221). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 26:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant TL-4 (spo0F221). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 25

KB—454
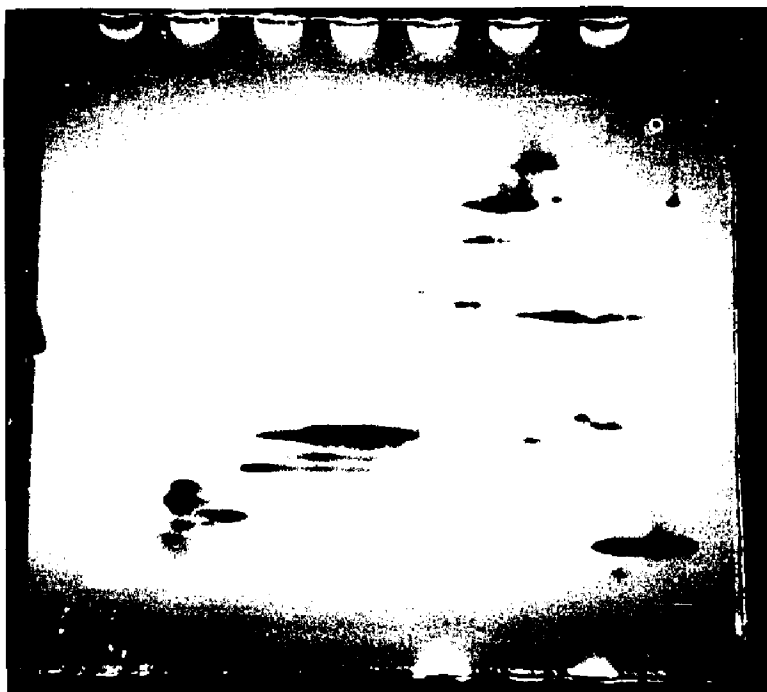
Figure 26

TL—4
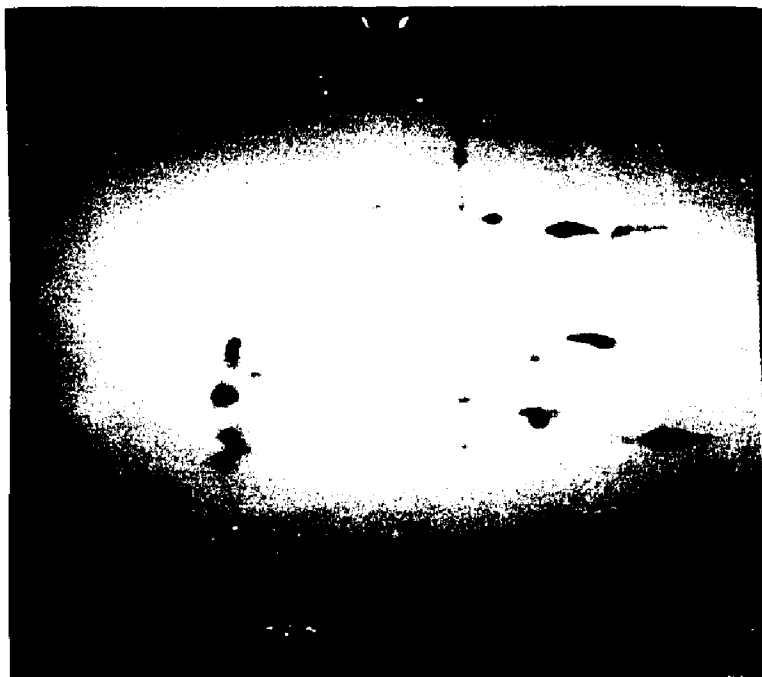
nuclease 2 is slightly repressed. (See Table 2). The
14,000 Dalton nuclease normally found below and to the
basic side of nuclease 2 in wild-type was absent form
TL-4 (Table 3).

The two strains of spo0E mutants used in this
study were KB-453, (Figure 27), and TL-11, (spo0E trp-
, phe-), (Figure 28). In KB-453, the 2DZ showed
repressed levels of only the cluster B enzymes. In
contrast, TL-11 was heavily repressed in virtually all
nuclease production except for nuclease 2. (See Table
2). The 14,000 Dalton nuclease, found in wild-type,
was not found below and to the basic side of nuclease
2 in either spo0E strain (Table 3).

Nuclease production in KB-455, (Figure 29),
appears only slightly altered in this spo0H mutant
when compared to the spo[+] KB-449. Clusters A and B
nucleases are slightly less prominent. Nuclease 2
production seems unaffected. (See Table 2). The
14,000 nuclease was detected below and to the basic
side of nuclease 2 (Table 3).

In KB-456,(spo0J93), (Figure 30), production of
all nucleases is increased comnpared to wild-type
(Table 2). The 14,000 Dalton nuclease was also present
below and to the basic side of nuclease 2.

spo0K strains reveal almost total repression of
nuclease 2 production in the KB-457 background,

Figure 27:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant KB-453 (spo0E11). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 28:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant TL-11 (spo0E11). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.
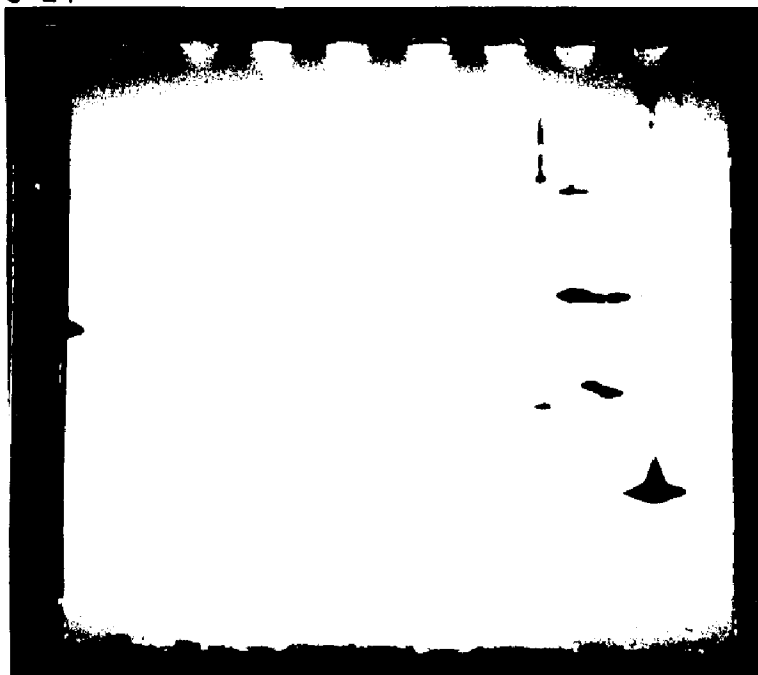
Figure 27

KB—453

Figure 28

TL—11

Figure 29:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant KB-455 (spo0H81). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 30:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant KB-456 (spo0J93). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 29

KB—455

Figure 30

KB—456

(Figure 31), and decreased expression in the TL-2 strain, (Figure 32). However, in both strains, nucleases of all four clusters (A, B, C, and D) are expressed at or above wild-type levels.

These are the only spo0 strains in which nuclease 2 expression is nearly absent (KB-457) or repressed (TL-2) while all four clusters remain unchanged. (See Table 2). The 14,000 Dalton nuclease, normally found in wild-type, was not found below and to the basic side of nuclease 2 in either spo0K strain (Table 3).

Suppressors of spo0 Mutations and their affect on Nuclease Production in spo0 Strains

TL-series spo0 mutants containing either the crsA47 or rvtA11 suppressor mutations were grown in the competence regime. These mutations allow the cell to by-pass or compensate for the loss of certain spo0 functions (53). Their 2DZs were compared to those generated by their respective spo0 suppressor-less parent strains, and to wild-type.

Nuclease Production in spo0 Strains in a crsA47 Background

Analysis of nuclease zymograms of spo0B, spo0E, spo0F, and spo0K showed the crsA47 mutation generally resulted in increased production of the enzymes of

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Figure 31:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant KB-457 (spo0K141). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 32:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant TL-2 (spo0K141). Hydrolysis of Native calf thymus DNA was detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 31   **KB—457**

Figure 32

TL—2

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clusters A, B, C, and D.

Nuclease 2 production remained unchanged in TL-4, (spo0F), with respect to wild-type. However, in TL-3, (spo0F crsA47), it was depressed, (Figure 33 and Table 2). The crsA47 mutation did not allow the expression of the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 3).

Nuclease production in TL-8, (spo0B), was poor as described previously. However, the spo0B crsA47 suppressor strain, TL-6, showed heavy production of all nucleases including nuclease 2, (Figure 34 and Table 2). The crsA47 mutation did not allow the expression of the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 4).

crsA47 partially suppresses the sporulation defect in spo0B (9,14) but not the competence deficiency (Green, personal communication).

Nuclease production in the parent spo0E strain, TL-11, showed normal nuclease 2 production and repressed levels of clusters A, B, C, and D. In contrast, TL-10, (spo0E crsA47), (Figure 35), showed normal levels of cluster B enzymes and heavy production of clusters A, C, and D. (See Table 2). The crsA47 mutation did not allow the expression of the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 3).

Figure 33:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-3 (spo0F221, crsA47). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 34:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-6 (spo0B^{ts},crsA47). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 33 TL-3

Figure 34

TL-6

Figure 35:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-10 (spo0E11, crsA47). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 36:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-1 (spo0K141, crsA47). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
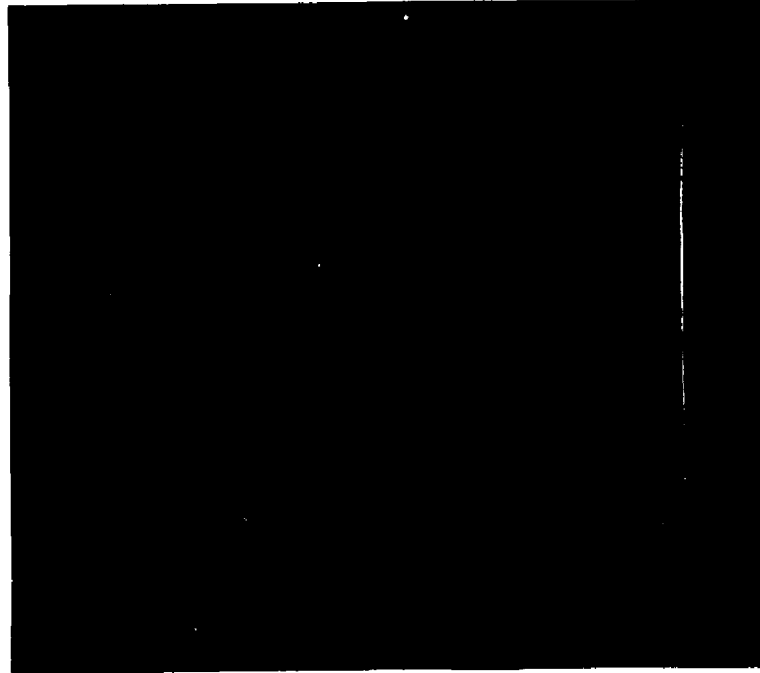2 mM $MgCl_2$, and $CaCl_2$.

Figure 35   **TL-10**
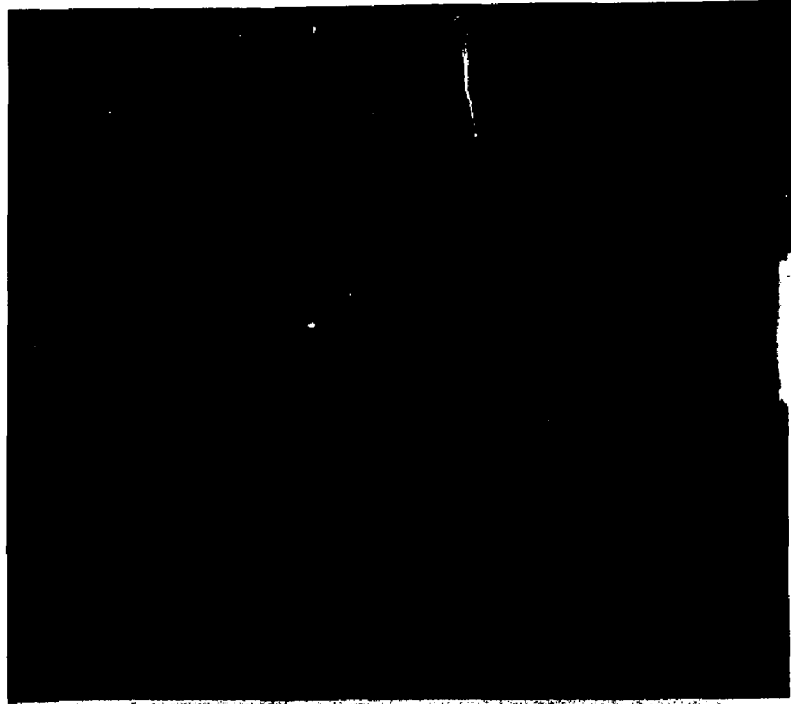
Figure 36
                    TL-1

Nuclease production in TL-2, (spo0K), was normal or slightly repressed. Only nuclease 2 was repressed.

The suppressor mutant, TL-1, (spo0K crsA47), showed heavy production of all nucleases except nuclease 2. (Figure 36 and Table 2). The crsA47 mutation did not allow the expression of the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 3).

Nuclease Production in spo0 Strains in a rvtA11 Background

Nuclease production in the spo0F parental strain, TL-4, was near normal for all nucleases except nuclease 2 which was slightly repressed. The rvtA11 mutation introduced into the spo0F background, TL-5, greatly increased production of all nucleases to above wild-type levels. (Figure 37 and Table 2). However, the rvtA11 mutation did not allow the expression of the 14,000 Dalton nuclease found in the vicinity of nuclease 2 (Table 3).

Production of nuclease 2 is repressed and the four co-expressed clusters are poorly detectable in the parent strain, TL-8, spo0B (Table 2). Nuclease 2 production in TL-7, (spo0B rvtA11), (Figure 38), is enhanced and a 14,000 dalton nuclease is seen in its vicinity. All other nucleases remain at low level. This is in contrast to the spo0B crsA47 strain where

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Figure 37:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-5 (spo0F221, rvtA11). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 38:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-7 (spo0B^{ts}, rvtA11). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 37   **TL−5**

Figure 38   **T L− 7**

the 14,000 dalton nuclease is not found and all other nucleases are expressed above the parent strain. (See Tables 2 and 3).

TL-11, spo0E showed repressed activity in all nucleases except nuclease 2, (Table 2). When the rvtAll mutation was added, strain TL-9, all four clusters became heavily expressed and the 14,000 dalton nuclease near nuclease 2 was present, (Figure 39 and Table 3).

Two-dimensional Electrophoretic Analysis of
other Mutants of Bacillus subtilis

Nucleases Associated with Prophage

Figure 40 is a representation of nuclease activities associated with Bacillus subtilis YB-886,(trp C2, met B5, amy E, sig B, xin-1, rec$^+$, comp$^+$, phage SP beta$^-$, non-inducible for endogenous prophage PBSX)(28). By comparison 19 nucleases usually present in competent cells are notably absent. Nucleases 30, 34, 36, 37, 40, 43, 44, 46, 49, and 50-60 are missing.

Proteolytic Modification of Nucleases

The presence of all the clusters in strain BG-2097, (Figure 41), is evidence against post-translational proteolytic cleavage by either alkaline

Figure 39:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
TL-9 (spo0E11, rvtA11). Hydrolysis of Native calf
thymus DNA was detected in the presence of 2 mM $MnCl_2$,
2 mM $MgCl_2$, and $CaCl_2$.

Figure 40:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
YB-886 (rec$^+$, com$^+$, phage SP beta$^-$, non-inducible for
PBSX). Hydrolysis of Native calf thymus DNA was
detected in the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$,
and $CaCl_2$.

Figure 39

TL-9
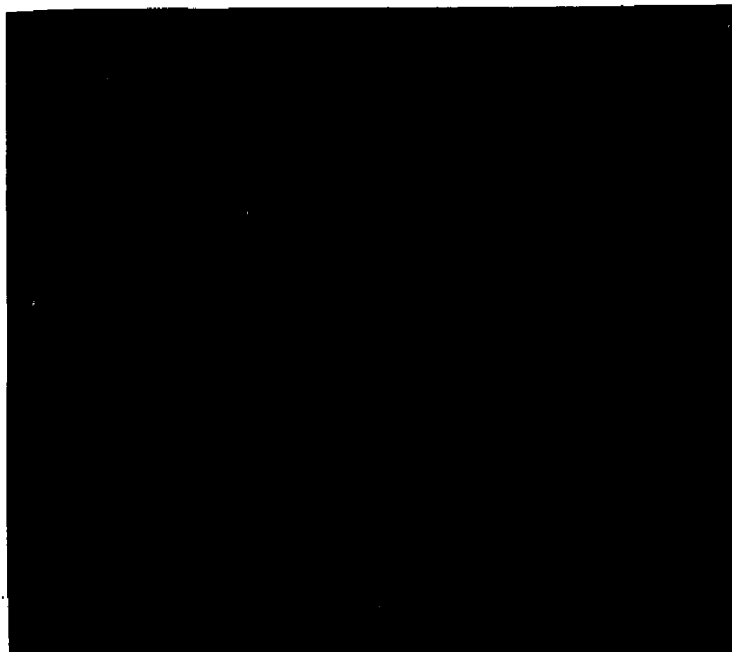
Figure 40

YB-886

Figure 41:

Two-dimensional zymogram of <u>Bacillus</u> <u>subtilis</u> mutant
BG-2097 (contains deletion mutations of <u>apr</u> and <u>npr</u>
(alkaline and neutral proteases). Hydrolysis of
Native calf thymus DNA was detected in the presence of
2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

BG-2097

Figure 41

or neutral protease. BG-2097 is deficient in both proteases and therefore those nucleases with unaltered mobilities are not normally proteolytically modified by this enzyme.

Nuclease 2 does not possess its "tail" pattern and the 14,000 dalton form is absent in BG-2097. This may indicate that proteolytic modification is required for the "tailing" pattern or 14,000 dalton form of nuclease 2 to be observed.

Discussion

Two-dimensional Electrophoretic Analysis of
Non-competent mutants of Bacillus subtilis

The Role of Nuclease 2 in Bacillus subtilis Competence

There is evidence that nuclease 2, reported by Coughlin (39), and a 17,000 Dalton, competence associated, membrane-bound nuclease reported by Venema et al., (32-38), are identical. Both nucleases: i) are approximately 17,000 in molecular weight, ii) are activated by manganese or magnesium, iii) have acidic pI's of approximately 4.8, and iv) are active on native DNA. In addition, production of both nucleases increases at the time of competence and is missing or repressed in wild-type, logarithmically-growing, non-competent cells (32, 33, 34, 35, 39). This led Coughlin (39) and Venema (32), independently, to suggest that the 17,000 Dalton nuclease has direct involvement in the transformation process. For ease of nomenclature, the 17,000 Dalton competence associated nucleases of both Coughlin and Venema shall be considered one, and will continue to be called nuclease 2 in this discussion.

In cultures grown in competence inducing conditions, 2 dimensional zymogram (2DZ) gels showed that nuclease 2 activity was present to varying

degrees in all 38 of the competence mutants I investigated, (data not shown). Although in some mutants nuclease 2 was heavily expressed, and in others hardly detectable, it always appeared.

Its presence in all mutants suggests two possibilities: i.) it is required for cellular functions other than competence and is required in conjunction with other competence specific proteins for transformation or, ii) that it is not required for competence but is expressed temporally with competence specific polypeptides.

Temporally controlled expression of many protein products is required during vegetative growth as well as during sporulation and development of competence (6, 26, 41). Proteins responsible for inducible DNA repair have been shown to participate in transformation (27). Proteins associated with cell septation during cell division can be tied to morphological changes during sporulation (12). Therefore, it can be expected that nuclease 2 may have a normal cellular function which is required in conjunction with other highly regulated developmental changes to allow transformation.

Nuclease 2 is increased during development of competence. This suggests it has a direct involvement in the transformation process. However,

the necessity of its role is brought into question by reports that insertion mutants devoid of nuclease 2 and an accompanying "regulatory subunit" retain transformation frequency which is 5% that of wild-type (38). When the associated regulatory subunit is absent, cells bind more DNA but its entry is 30% that of wild-type. Thus, if nuclease 2 is involved in a DNA binding and uptake process, then another mechanism must also be available to fill its role when it is defective. The existence of an alternative pathway for binding and uptake has been suggested (38).

Early events in sporulation occur at the same time (1) in which competence is developing and it is possible that nuclease 2 is produced in response to those regulatory signals. In my study, two spo0K mutants, KB-457 (spo0K141) and TL-2 (spo0K trp-) were strongly inhibited in their capacity to produce nuclease 2. spo0K mutants are transformation deficient (44) which suggests that nuclease 2 production is required for sporulation as well as competence.

Expression of a Smaller form of Nuclease 2

When the cloned 17,000 Dalton nuclease 2 is expressed in E. coli and B. subtilis, an additional 14,000 Dalton nuclease activity is detected (37, 38).

In E. coli the smaller nuclease was produced in nearly equal quantities as the 17,000 dalton nuclease, but in Bacillus subtilis the smaller form predominated. Both nucleases were absent or reduced in activity in a series of Bacillus subtilis competence mutants studied by Vosman et al. (38).

The origin of the smaller nuclease has been proposed to be the result of a second translational start codon downstream in the same reading frame of the RNA transcript coding for nuclease 2 (37, 38). Whether the 14,000 Dalton nuclease is present in vivo or is a proteolytic degradation artifact generated during lysate preparation is unknown.

In 2DZ gels of B. subtilis SB-25 grown in the competence regime, I detected a 14,000 Dalton nuclease. It was not observed prior to the adoption of the casein wash procedure for sodium dodecyl sulfate removal (see chapter I). Because the 14,000 Dalton nuclease was absent from TL-4 (spo0F) and TL-11 (spo0E) which are both near normal in transformation, it is probably not required for transformation. The nuclease is manganese activated and possesses a pI close to that of nuclease 2. This nuclease appears to be identical to the reported 14,000 Dalton form of nuclease 2 (38).

The following observations, while supporting

this relationship, suggest a different origin: The 17,000 Dalton enzyme contains 25 acidic side chain amino acids and 18 basic ones making the enzyme highly acidic at neutral pH (38, 39). The secondary translation start site proposed by Vosman (38) would reduce these amounts to 20 acidic residues and 13 basic ones. Thus the net charge of the two forms will be the same. However they are not when observed in 2DZ gels. If the secondary translation start site were at nucleic acid 156 (38), (N-terminal valine rather than methionine), the smaller nuclease would have one additional positive charge located at its amino terminus. The result would be a nuclease with slightly greater mobility in both the first and second dimension separations in the 2DZ gel. This is the pattern observed for both nuclease 2 and the smaller 14,000 Dalton nuclease in our 2DZ gel system.

Analysis of a 12,000 Dalton Nuclease Secreted during Sporulation

It is possible that the 12,000 Dalton nuclease that Akrigg and Mandelstam isolated from the media of sporulating cells (63, 64) is identical to either nuclease 2 or the 14,000 Dalton nuclease discussed above. All three are nearly identical in pI, substrate preference, and cation activation profile. Differences in reported molecular weight may be

attributable to different methods of molecular weight determination and will be addressed later.

Secretion of this nuclease is one of the several biochemical marker events in early sporulation (63). I isolated its nuclease activity from sporulating cultures and found it was activated by manganese, magnesium (not shown) and cadmium, but not calcium (Figures 12 and 13). It was capable of hydrolyzing native super-coiled and single-stranded DNA but had no activity on RNA (Figures 13 and 14). Its mobility within our 2DZ gel system approximated that of both nuclease 2 forms (Figure 15). Gel filtration of the spore media nuclease by both G-100SF and HPLC resulted in a calculated molecular weight of approximately 12,500 (not shown). Molecular weight determination by SDS gel consistently resulted in a higher estimate of approximately 15,000 Daltons (Figure 15). Nuclease 2 usually migrates in our SDS gel system at a molecular weight of approximately 16-17,000 Daltons. The smaller molecular weight form of nuclease 2 migrates at approximately 14,000 Daltons. The sporulation associated nuclease had a molecular weight closer to the 14,000 dalton form of nuclease 2. Anti-sera raised against the sporulation nuclease, which was graciously provided by Akrigg and Mandelstam, specifically inhibited nuclease isolated

from sporulating cultures (Figure 16).

Comments on the forms of Nuclease 2 and its relationship to Competence and Sporulation

The production of the two forms of nuclease 2 raises interesting regulatory possibilities. One possibility is that they may be produced by a variation in the translation process. If nuclease 2 is normally present during both the early stages of competence and sporulation, a translational control mechanism could be used to alter the product of the nuclease gene appropriately for either developmental process. By altering the nuclease translationally, the cell could continue to use the same transcriptional apparatus without affecting the expression of many early sporulation or competence genes. Within clusters A and B, the nucleases possess like cation activation and substrate specificity profiles. They are also similar in molecular weight and pI. It is possible that nucleases within these clusters may be subject to this type of translational regulation.

Anomalous Behavior of Nuclease 2 in certain com Mutants

Cell wall chemistry has been the subject of numerous studies by investigators of both competence

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and sporulation. Cell wall charge was found to become more negative during competence development (42). The effects of cell wall precursor molecules on the attainment of competence (41) led to the hypothesis that the forespore septum may be a site associated with DNA entry (45). Sadaie and Kada have shown that formation of competent cells requires cell wall synthesis and that materials necessary for competence are associated with cell division (44). Therefore, an interaction between cell envelope structures and the competence associated nuclease are not unexpected.

Membrane-bound nuclease 2, from competent SB-25, normally possesses a tail-shaped nuclease activity pattern in 2DZ gels. This "tail" streak from its acidic gel position to the basic side of the gel is the result of charge variation among the nuclease 2 species as it is focused in the first dimension (NEPHGE). However, its molecular weight in the second dimension is constant.

This behavior may involve the interaction between nuclease 2 and a membrane structure, or its putative regulatory protein subunit (37) that may be poorly denatured by urea and NP-40 in the first dimension. The absence of "tailing" in the second dimension indicates that this association is disrupted by the presence of SDS and/or 2-mercaptoethanol in the second

dimension.

Venema reported that nuclease 2 is part of a larger 75,000 Dalton tetrameric membrane-bound holoenzyme which has two nucleolytic and two regulatory subunits (molecular weight 18,000 Daltons) (34, 35, 36). This heterotetramer complex could be responsible for the "tailing" phenomenon in the following way. As the first dimension NEPHGE gel separation begins, the enzyme complex may enter the low percentage (4.06%) acrylamide gel due to its net negative charge supplied by the regulatory subunit. However, subunit dissociations result in movement of the basic monomeric regulatory subunit toward the cathode and movement toward the anode by the released nuclease subunit. Because the gel pH gradient is not allowed to come to equilibrium in a NEPHGE separation, and movement of nuclease 2 to its focused position is incomplete, the resultant pattern of nuclease activity is a "tail". Those nuclease subunits released early in the separation are well focused at the acidic end of the gel.

Of interest is that "tailing" occurs towards the basic side of the gels. The regulatory subunit of the 75,000 Dalton complex, if highly basic in nature, may allow the highly acidic nuclease component to more easily associate with the negatively charged cell

wall. Cellular synthesis of the positively charged regulatory subunit could not only control nuclease specificity but appoint its cellular location.

. An analysis of the association of com mutant phenotypes with the presence or absence of "tailing" was undertaken. Fourteen of the com mutants investigated showed no "tailing".

The com mutant phenotypes in this study are based on either ability to become transformed or nucleolytic activity of the cell surface or cell lysate. Neither of these aspects were found to be associated with alterations in the "tailing" phenomenon. It may be more informative to compare "tailing" with other cellular processes such as cell wall make-up and synthesis than the traits investigated.

Vosman et al. cloned a region conjectured to code for the nuclease 2 regulatory protein (37, 38). The cloned region contains two overlapping open reading frames, one of which undoubtedly codes for nuclease 2 and its smaller form. However, the second proposed to be that of a "regulatory protein" region (38) is suspect for the following reasons.

Nuclease 2 and its smaller form were cloned and expressed in E. coli. On stained SDS-PAGE gels of the expressed proteins, an accompanying 18,000 Dalton protein was observed and was assumed to be the

regulatory subunit. However, the so-called regulatory region codes for a smaller protein, (15,000 Dalton molecular weight), than the 18,000 Dalton protein actually synthesized (38). Vosman et al. proceeded to create insertion mutations in the 18,000 Dalton region and found that DNA binding is not affected and DNA uptake is 25 percent of wild-type. It is possible that in the mutant they studied the nuclease 2 enzymes were partially inactivated and the 18,000 Dalton regulatory subunit, which is coded for elsewhere in the genome, was unaffected.

Nucleases Strongly Associated by Co-expression

The independent appearance of nuclease clusters A, B, C, and D in different mutants suggests individual modes of regulation for the production of each cluster set. It is possible that each nuclease cluster is transcribed from a single operon coding for more than one nuclease. Alternatively, each cluster may be part of separate regulons responsive to the same cellular stimuli. Multiple forms of each nuclease could also be expressed from a single transcript using independent translational start sites as proposed for the nuclease 2-related 14,000 Dalton nuclease (37).

Proteolytic modification in vivo or during lysate

preparation may be responsible for producing multiple forms of nucleases in the 2DZ gels. However, because each mutant has been equally treated with known protease inhibitors and protein denaturants to reduce the possibility of degradation this is unlikely. In addition, BG-2097, a strain lacking alkaline and neutral protease activities, produced all four of these clusters when grown in the competence regime (discussed later).

Covalent modification is also a plausible explanation for the observed charge differences between cluster members. Protein kinase activity may be responsible for alterations of cluster pI without affecting observed molecular weight in 2DZ gels. Experiments in which I treated lysates with bacterial alkaline phosphatase failed to affect any nucleases 2DZ mobility (data not shown). However, additional experiments should be performed before excluding phosphorylation as a form of nuclease modification. Cluster B nucleases may be found to be modified forms of a precursor nuclease, possibly nuclease 9. This is supported by observations that nuclease 9 is the major form in cluster B of com mutants NH-903, NH-1501, and 2202 (Figure 42 A, B, C). In all other strains, cluster B nucleases are not expressed in the absence of nuclease 9.

Figure 42:

Two-dimensional zymograms of <u>Bacillus</u> <u>subtilis</u> <u>com</u>
mutants NH-903 (A), NH-1501 (B), and NH-2202 (C).
Hydrolysis of Native calf thymus DNA was detected in
the presence of 2 mM $MnCl_2$, 2 mM $MgCl_2$, and $CaCl_2$.

Figure 42

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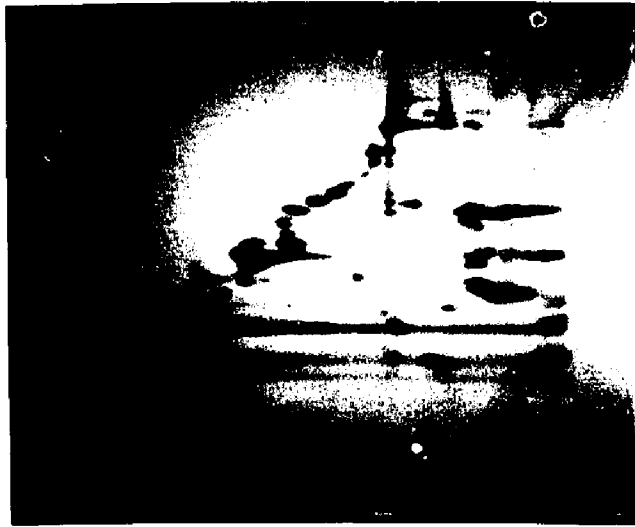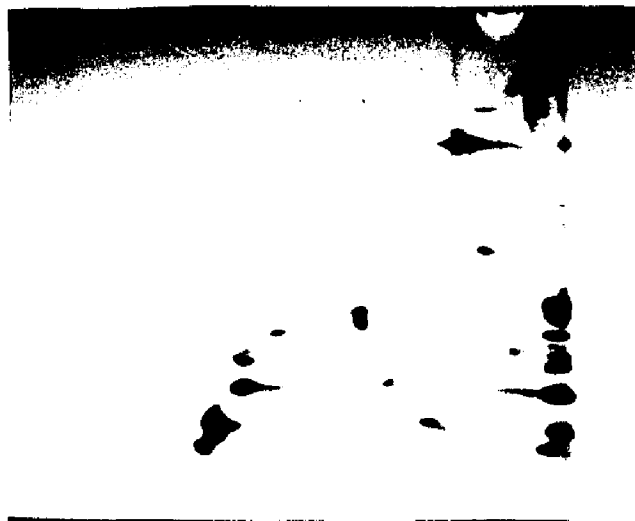
A 35,000 Dalton Nuclease is Secreted by Competence Mutant NH2203

NH2203 is a competence mutant which is defective in DNA uptake. Its cellular lysates are reduced in a previously detected calcium-activated nuclease (Green, personal communication). It is possible the reduction of intracellular calcium enzyme compared to wild-type lysates is due to its inappropriate export from the cell. Extracellular nuclease may result in poor transformation of NH2203 by disrupting a required transformation mechanism or more simply by hydrolyzing transforming DNA before its uptake. The second possibility may be examined by transforming with increased amounts of donor DNA. If secreted nuclease 49 is interfering with transformation by hydrolyzing donor DNA, there may be an observable DNA concentration effect on the NH2203 transformation.

A second possible explanation for NH2203's poor transformation is that nuclease 49 is a phage-associated nuclease (Figure 40). If phage-induction is associated with competence, nuclease 49's overexpression may result in loss of competence.

Two-dimensional Electrophoretic Analysis
of spo0 mutants of Bacillus subtilis

Differences in spo0 Genetic Backgrounds

The "KB" and "TL" strains of spo0 mutants differed in their nuclease production phenotypes (Figures 21-40, Tables 2 and 3). Specific differences between individual spo0 mutants in each background will be discussed in this section. However, the existence of such differences must be explained.

A complex regulatory system which coordinates major cell themes such as vegetative growth, DNA repair, cell division and sporulation control is especially sensitive to variations in genetic background. An additional unidentified single mutation in any of the loci responsible for these closely tied processes has the potential to effect the timing of competence, sporulation, or other cellular functions. Competence is associated with the disruption of cell wall synthesis and cell division. Similarly, sporulation mutations can alter membrane components (47) while sporulation events are temporally regulated by spore wall structures (12). Therefore, direct comparison of spo and com mutant phenotypes in two different parental backgrounds are not likely to yield identical results. Only mutants compared in isogenic backgrounds are likely to permit

uncomplicated interpretation of the effects of mutations at <u>com</u> and <u>spo</u> loci.

Nuclease Production in spo0 Mutants

Development of the mature <u>Bacillus</u> <u>subtilis</u> spore proceeds through seven stages designated 0 - VII, delineated by morphological or biochemical marker events. Mutants can be isolated representative of nearly each of the seven stages.

Vegetative cells are at "stage 0" of the developmental pathway which leads to sporulation. Mutants which fail to respond to sporulation inducing conditions are known as stage 0 mutants or spo0. These mutants fail to enter the earliest stages of sporulation which result in metabolic slow-down and condensation of the bacterial chromosome into the "axial filament" characteristic of stage I.

spo0 mutations have been mapped in seven unlinked loci. They are spo0A (spo0C), spo0B, spo0E, spo0F, spo0H, spo0J, and spo0K. Of these spo0A and spo0B are the most pleiotropic in their effects on vegetative and sporulating cells, followed sequentially by spo0F, spo0E, spo0H , spo0J and spo0K (23). My data on the effect of spo0 mutations upon competence-induced nuclease expression is discussed in the that same order.

Because spo0C and spo0A are alleles (67), my observations of spo0C competence-induced nuclease expression can probably be related to reported findings for spo0A.

Cluster A and/or B nuclease activities may be required for competence. This is suggested by two findings. spo0C (Green, personal communication) and spo0A (25, 44) are low in transformation, and spo0C is low in nuclease clusters A and B. Hence, the spo0C mutation affects the production of cluster A and B nucleases during the induction of competence.

Cluster B nucleases have previously been implicated as occuring during establishment of the transformation process. Coughlin found that Cluster B nucleases are absent from non-competent cells and temporally expressed at the time of competence (39).

spo0A (spo0C) and another very early sporulation mutant, spo0B, have the most pleiotropic effect upon sporulating cells (23). Both are expressed before the induction of sporulation (1, 66). Early gene functions required for the regulation of sporulation may coincide with those of competence development. Therefore, spo0A (spo0C) and spo0B mutants should be expected to be repressed in competence function. Repression of competence has been reported by Sadaie (44) and this lab (Green, personal communication).

Note that Albano et al. report normal transformation in a spo0B deletion mutant (25). Their strain of spo0B must be evaluated by our transformation method and by 2DZ to determine if nuclease production in their strains is affected by the spo0B deletion.

In the spo0B mutants that I investigated, KB-451 and TL-8, strong repression of all nuclease production occurred (Figures 23 and 24). Although clusters A, B, C, and D are repressed, nuclease 2 production remains normal. This suggests that spo0B action is not required for nuclease 2 production and that nuclease 2 is regulated differently than nucleases clusters A, B, C, and D.

Disagreement also exists concerning spo0F's ability to be transformed. Albano (25) and Green (personal communication) report that spo0F is hardly affected in competence while Sadaie et al. report (44) it competence-deficient. It is possible that differences in reported spo0F competence are the result of the genetic backgrounds in which the spo0F mutation is placed or the method of assay (to be discussed later). Strain differences are apparent between KB-454 and TL-4 as shown in their 2DZ nuclease patterns (Figures 25 and 26).

Nuclease 2 and clusters A and B are expressed normally in TL-4 (spo0F) (Figure 26), consistent with

our finding it is nearly normal in transformation (Table 2).

The TL-11 (spo0E) 2DZ results, (Figure 28, Table 2), corroborate the theory that nuclease 2 expression is regulated independently of the nuclease clusters. Only nuclease 2 is expressed at wild-type levels, the others are repressed.

In the presence of the spo0E mutation, up to 20% of the culture can sporulate (23). This "leaky" aspect of the spo0E mutation makes it difficult to attribute a particular observation to the non-sporulating fraction of the culture. If spo0E initiates sporulation while grown in the competence regime, only a portion of the cells may contribute to the nucleases expressed. Differences in growth regime may be responsable for different reports concerning competence.

If cluster A and B nucleases are required for transformation as suggested, spo0E should be competence-deficient. Sadaie (44) reports this, but Green and Albano find spo0E mutants to be normal in competence. These differences may be attributed to the means by in which transformation is induced or by the DNA concentration used in the assay.

Albano induced competence by growing cells in a single media without passage. Another widely used

practice involves a two-stage growth regime in which the culture in late log phase growth is passed from one medium into another. Because both competence and sporulation can be induced by varying media nutrient content and culture growth rate (Birrer and Chesbro, manuscript in preparation), it is possible that discrepancies between laboratories concerning spo0E's ability to transform or sporulate may reflect induction methods.

DNA concentrations used in transformation assays performed by each laboratory differ and prevent direct comparison of transformation data between investigators. Green commonly uses 0.25ug/ml a just-saturating concentration of transforming DNA, while Albano and Sadaie use, respectively, 1.0 and 5.8ug/ml, (a 4 and 23.2-fold increase). DNA excess in the transformation assay could reduce the detection of variations between com mutants. For example, certain mutations may result in lowered efficiency of DNA binding or fewer entry sites on the cell surface. Such phenomenon may be masked by saturating amounts of transforming DNA in the assay. In addition, a com mutant such as NH2203, which secretes a calcium/manganese activated nuclease, might be overlooked by another laboratory. If NH2203 is competence-deficient due to hydrolysis of substrate

prior to transformation, higher concentrations of DNA in the assay might result in increased transformation frequencies.

spo0H ranks second only to spo0A in loss of sporulation-associated properties (23). It has no known suppressors (1) and is thought to be a very early spo gene which may help the cell decide when to sporulate. (19). Albano et al. reported that the spo0H gene is required for full expression of com genes (25). spo0H mutants are 16 to 1000-fold less capable of being transformed and fail to divide into high an low buoyant densities in a renograffin gradient separation of competent from non-competent cells. The spo0H gene product has been identified as a minor vegetative and early sporulation sigma factor, sigma factor H (24). Sigma H, (formerly sigma 30), is required for the transcription of certain genes by giving Bacillus subtilis DNA-directed RNA polymerase specificity for the proper promoters involved in sporulation (24). Three different studies observe that the competence of spo0H mutants ranged from 6.0 to 0.2% that of wild-type (25, 44, Green personal communication). Their conclusion was spo0H, (sigma H), is required for transformation.

Sigma factor H is not needed for the production of nuclease 2 and its smaller form as well as cluster

C and D nucleases in KB-455 (Figure 29). Poor competence may be the result of its reduction of clusters A and B (Table 2). Conversely, sigma H is required for nuclease cluster A and B expression.

Although nuclease production in KB-456, spo0J93, is excessive, the spo0J93 mutant is normal in transformation (Green, personal communication) (25,44). Normal spo0J function is not required for any aspect of competence. This suggests that nuclease overexpression may not necessarily lead to loss of transformation due to nucleic acid degradation. Mechanisms which protect absorbed DNA within the cell prior to recombination are known to exist (28, 29) and their functions need not be compromised by mutations resulting in increased amounts of cellular nuclease.

Additional evidence that nuclease 2 expression differs from that of other nucleases comes from two spo0K strains. Almost total repression of nuclease 2 production is seen in spo0K strain KB-457, (Figure 31), and partial reduction in TL-2, (Figure 32). However, nucleases of all four clusters (A, B, C, and D) are expressed at or above wild-type levels. These are the only spo0 strains where nuclease 2 expression is repressed while all four clusters are elevated or normal. This confirms that not only is nuclease 2 regulated by a means different than that of clusters

A, B, C, and D, but mutation at spo0K alters that mechanism.

Repressed nuclease 2 production in spo0K is associated with loss of competence (44, and Green, personal communication). As suggested by Coughlin (39), nuclease 2 function may be required for transformation. Because spo0H and spo0C mutants are normal in nuclease 2 but deficient in cluster A and B nucleases and competence, it is again inferred that cluster A and/or B nucleases are also required for competence.

Suppressors of spo0 Mutations and their affect on Nuclease Production in spo0 Mutants

The Effect of crsA47 on Various spo0 Mutants

The mutation crsA47 was originally discovered by Takahashi by its ability to allow cells to sporulate in media containing high concentrations of glucose. High glucose conditions usually inhibits sporulation of wild-type Bacillus subtilis (15). The phenotype, "catabolite resistant sporulation", (crs) was found to suppress the inhibition of sporulation in early spo mutants spo0B, spo0E, spo0F, spo0K and maybe spo0J (9, 14). The spo0A and spo0H mutations are not suppressed by crsA47 (9, 14).

The crsA47 mutation maps at rpoD, the site for

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the sigma factor A structural gene (9, 16). How this alteration acts at the molecular level is unknown but it may affect sigma A interaction with the RNA polymerase holoenzyme resulting in a new response to transcriptional regulators. This notion is partly defended by the occurrence of another crs mutation, crsE, which maps to rpoBC. rpoBC codes for the beta and beta-prime subunits of the RNA polymerase holoenzyme.

Analysis of nuclease zymograms of spo0B, spo0E, spo0F, and spo0K, (Figures 33-36), showed the crsA47 mutation generally resulted in increased production of enzymes of clusters A, B, C, and D. When these spo0 mutations are suppressed by the crsA47 mutantion, overproduction of nucleases occurs in conjunction with a dramatic reduction in competence (Green, personal communication) (Table 2). However, increase in nuclease production need not lead to a significant reduction in competence. Evidence of this comes from spo0J93, and spo0F rvtA11 zymograms in which production of all nucleases are increased; yet their competence, respectively, is 58 and 81% that of wild-type (Green, personal communication). Hence, the crsA47 mutation probably results in two separate effects, loss of competence and nuclease over-production.

In both spo0B and spo0F backgrounds, crsA47 diminished transformation frequency below that of each parent strain, (Table 2) (Green, personal communication). In spo0B transformation decreased from 2.6-11.5% to 0.2% in spo0B crsA47. In spo0F an analogous decrease from 5-16.4% to 0.01% occurred. This reduction in competence may signify that crsA47 is deleterious to competence. Investigations into its affect upon competence should be pursued.

Nuclease 2 production in spo0B crsA47 was heavy compared to its reduced expression in spo0B. In spo0F, nuclease 2 expression was normal but repressed in spo0F crsA47. This substantiates that nuclease 2 is regulated in a manner different than the four nuclease clusters mentioned.

In spo0E crsA47 and spo0K crsA47, nuclease 2 expression remained the same in comparison to their respective parent strains. It can therefore be concluded that nuclease 2 production in these two strains was not responsive to catabolite control mechanisms. Clusters A, B, C, and D nucleases were all heavily expressed in all spo0 crsA47 strains examined and are therefore subject to repression in high glucose media conditions in wild-type cells.

The Effect of rvtA11 on Various spo0 Mutants

The mutation rvtA11, was originally isolated as an extragenic suppressor of spo0F. It is similar to sof-1 an allele of spo0A (1).

The rvtA11-containing strains suppress spo0F, spo0B and spo0E sporulation phenotype to different degrees. Ability to sporulate in the presence of rvtA11 is best in spo0F (near 100%), followed by spo0E (near 50%), and then spo0B (2.5%) (13). By 2DZ, I found nuclease production followed the same order, greatest in spo0F, followed in decreasing order by spo0E and then spo0B. Competence is normal in the spo0F rvtA11 mutant (81%) but deficient in spo0B rvtA11 (6.6%) and spo0E rvtA11 (0.007%) (Green, personal communication).

In both spo0F and spo0E, (Figures 26 and 28), nuclease activity in clusters A, B, C, and D were increased. However, in the spo0B mutant, (Figure 24), only clusters C and D were slightly enhanced. Nuclease 2 was normal in all of the rvtA11 strains investigated (Figures 37-39) which verifies that nuclease 2 is regulated independently of the other cluster nucleases.

Of eleven TL strains investigated, the 14,000 Dalton form of nuclease 2 was induced only in spo0B rvtA11 and spo0E rvtA11 (Table 3, Figures 38 and 39).

In the KB mutants, it was found in KB-449 (wild-type), KB-452 (spo0C), KB-454 (spo0F), KB-455 (spo0H), and KB-456 (spo0J93), but not in spo0K, spo0B, and spo0E. This suggests that spo0B, spo0E, and spo0K are required for its production. rvtAll can suppress the spo0B and spo0E phenotypes and lead to 14Kdal nuclease production.

If, as previously suggested, the mode of action for the regulated expression of the 14,000 Dalton nuclease involves recognition of a alternate translational start site on the nuclease 2 transcript, the normal spo0B, spo0E or spo0K proteins are probably required for the utilization of such a site. The suppressive effects of rvtAll also suggest a role for spo0A.

Two-dimensional Electrophoretic Analysis of other Mutants of Bacillus subtilis

Nucleases Associated with Prophage

The absence of 19 nucleases from YB-886 (Figure 40) suggests that during the induction of competence either i.) the absent nucleases are expressed by genes of lysogenized phage or, ii.) they are induced in the host due the phage presence within the cell.

The nucleases found to be associated with prophage were absent from TL-8 and TL-11, (Figures 24

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and 28)(spo0B and spo0E). They were also missing from their corresponding crsA47 and rvtA11 strains (Figures 34, 35, 38, and 39)(Table 2). Hence, it can be concluded that not only are the phage-associated nucleases dependent upon spo0B and spo0E for activation during the induction of competence, but both the crs and rvt suppressors fail to allow their production.

Two sigma factors, sigmas B and D, are known to be affected by spo0E and spo0B mutations.

Transcription from sigma B promotors strictly requires the action of normal spo0A, spo0B, spo0E, spo0F and spo0H genes before it becomes active (48). If prophage induction required sigma B, all of these mutants should lack the prophage associated nucleases. However, only spo0B and spo0E mutants are devoid of them.

Sigma D transcriptional activity is shut down early in sporulation (71). Transcription from this minor vegetative factor's promotors is influenced by normal spo0A, spo0B, spo0E, spo0F and spo0H function (47) but does not strictly require them. Each of these spo0 gene products could potentially effect prophage-associated-nuclease induction. Because spo0B and spo0E mutants are the only ones which lack the prophage-associated nucleases, it is possible that

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these nucleases are sigma D transcribed and regulated by normal spo0B and spo0E.

The DNA sequences characteristic of sigma D promotors resemble those of E. coli heat-shock promotors (71, 72, 77). In Bacillus subtilis such promotors might not only express "emergency" proteins to deal with heat-shock, but might also activate lysogenized phage.

Sigma D's amino acid sequence has carboxy-terminal similarity to E. coli FlbB, a regulatory protein required with FlaI for flagellar synthesis (51). Bacillus subtilis cells which contain an interrupted sigma D gene become filimentous and fail to produce flagellin. Helmann et al. (51) suggest that FlaI may function as a sigma factor with FlbB acting as a positive regulator, or that the two proteins act together as a sigma. They compare this type of association with two regulatory proteins required for late gene expression of Bacillus subtilis phage SPO1, gene products 33 (12 kDa) and gene product 34 (24 kDa) (74). Gene product 33 and 34 simultaneously bind the RNA polymerase holoenzyme to recognize late phage promotors (76).

If sigma D does in fact transcribe flagellar synthesis genes, as well as heat-shock and phage genes, an interesting scenario can be proposed.

Cellular stress, (heat-shock), may activate phage and flagellar synthesis. Because certain phage are flagellotropic, [for example PBS1 (75)], not only are phage being synthesized inside the host, but infective phage from outside the host may be allowed in and thereby increase the chances of phage genetic variation by recombination.

The crsA47 suppression, which is due to a mutation in sigma A, would not be expected to affect prophage-associated nuclease expression. The rvtA11 suppressor acts essentially as a functional analog to spo0F but in this case may not be able to reinstate nuclease production to spo0E and spo0B mutants.

Differences exist between the "TL" and "KB" backgrounds regarding the expression of the prophage-associated nucleases during development of competence. The presence of prophage-associated nucleases in all other spo0 mutants investigated besides spo0B and spo0E, suggests that lack of these nucleases in spo0B and spo0E is probably not due to the strain being "cured" of phage. In the "KB" background, prophage-associated nuclease activation can proceed in spo0B and spo0E strains during transformation but in the "TL" background does not.

Proteolytic Modification of Nucleases

The presence of nuclease clusters A, B, C, and D in strain BG-2097, (Figure 41), is evidence against post-translational proteolytic cleavage by the major alkaline or neutral proteases. BG-2097 is alkaline and neutral protease-free and therefore those nucleases with 2DZ mobilities identical to those from wild-type are not normally proteolytically modified by these enzymes.

Nuclease 2 does not possess its "tail" pattern in BG-2097 and the 14,000 dalton form is absent. This suggests that these phenomenon require proteolytic action at some step for their appearance.

Possible role of spo0F in Competence-Induced com and spo0 Mutants

spo0B has been reported to precede spo0F in the sporulation pathway (23), and is required along with spo0F and spo0A for the transcription of spo0H (23, 52). This order may be the result of a system other than cascades of regulatory proteins (8) used by Bacillus subtilis for temporal expression of sporulation genes.

Investigation into the mechanism of cellular response to nutrient and environmental stimuli (7, 10, 47) has resulted in a proposed model for a 2-component signal transduction system which could

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allow the cell to elicit a rapid response to environmental changes (10).

In the 2-component model, there are two distinct classes of proteins, a proposed sensor class and a regulator class. The sensor class proteins found in the NtrB, OmpP, and CheY systems in E. coli for nitrogen source evaluation, osmotic regulation and chemotaxic response respectively (46), have two regions of hydrophobicity in the C-terminal region. These are proposed to enable the protein to insert through the cell membrane into the periplasmic space. The C-terminus has a 200 amino acid which is highly conserved. This portion of the protein is found on the cytoplasmic side of the cell membrane. The N-terminus is believed to sense an environmental change, possibly by binding a ligand, which generates a conformational change on its C-terminal end. The C-terminus, now "activated", is able to interact with the second member of the two component model, the regulator.

The regulator domains also have a region of highly conserved amino acid sequence, (approximately 120 amino acids long), as well as a region less highly conserved and one of variability. The region of variability is at the C-terminal end and the highly conserved region near the N-terminus.

Because the homologous regions of sensors and regulators are found in the same positions on the 2-component regulatory proteins of a number of environmental sensors from different species, it is believed that the common regions have analogous functions whereas the variable areas provide for specific ligand binding, catalysis of covalent modification and regulatory interaction.

The model proposes that ligand binding at the N-terminus of the sensor protein causes the "activation" of its C-terminal end. The activated end then catalyzes the covalent modification of the regulator protein allowing it to affect the appropriate cellular response. It is possible by such a system to sense external environmental stimuli and directly or indirectly transduce the information to RNA polymerase sigma A to introduce an appropriate response. (47). It is interesting to note that both competence and sporulation are induced by nutritional signals (1).

Both spo0A and spo0F code for proteins which have N-terminal homology to the regulator class of proteins in the 2 component model (10). Specifically enteric bacterial chemotaxis signal processing genes, cheY and cheB. It is also predicted that another set of proteins will be identified that resemble the sensor class (46). Mutations in either the sensor or

regulator class may have the capability of rendering a set of genes either non-inducible or constitutive (10). Hoch et al. suggest that the sof-1 gene, an allele of spo0A, codes for a product which no longer requires interaction with spo0B, spo0E, and spo0F to be active (68).

Grossman and Losick (17) showed that Bacillus subtilis is capable of secreting an extracellular differentiation factor (EDF) which can induce a late log phase culture to begin sporulating. Both spo0A and spo0B fail to produce EDF (17). spo0F and spo0H fail to sporulate after the stage when spo0A or spo0B mutations act. Thus, these may be genes which respond to EDF.

Both spo0F and spo0A share N-terminal sequence homology with regulator proteins and at is possible that this feature is involved in EDF response. Although spo0A mutants do not produce EDF, EDF production is restored when the abr partial sporulation-suppressor is introduced. The failure of the abr spo0A strain to sporulate could result from failure to properly respond to EDF, or other pleiotropic affects of abr.

The proposal that the spo0F gene product is involved with EDF response fits previous observations that spo0F is required for the expression of sigma

factors H, B, and D.

Sigma factor H, (sigma 30), is a minor vegetative and early sporulation sigma factor indicated as a portion of the mechanism which induces sporulation (19). Its production requires a wild-type spoOF and spoOA gene to be expressed in vegetatively growing cells (1, 52).

Sigma factor D (sigma 28) is also a minor vegetative sigma responsible for control of protease genes that is influenced by spoOF (47)

Sigma factor B (sigma 37) is a minor vegetative and early sporulation sigma which strictly requires the spoOF gene product as well as those from spoOA, spoOB, spoOE, and spoOH (48). This sigma factor along with sigma C can transcribe a later spo gene, spoVG from one of 2 promoter sites (22). It is interesting to note that competence-associated clusters A and B require normal spoOH, spoOB, spoOE, and spoOC(spoOA) as does sigma 37 for their production (Table 2).

Therefore, the cell's inability to respond to EDF, due to spoOF mutation, may translate to inability to express crucial sigma factors responsible for middle sporulation functions.

This model of spoOF action also allows for the existence of suppressor mutations. The suppressor rvt was initially isolated as a suppressor of spoOF.

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rvtA11 is related to sof-1, an allele of spo0A. Both spo0F and spo0A are related to the 2-component environmental sensing system described earlier in this paper. Because rvt may be functionally analogous to spo0F, a mutation in wild-type rvt may suppress spo0F. Its action could be one of restoring spo0F function by correcting a faulty response to EDF. The rvtA11 mutation only partially suppresses spo0B sporulation functions (13). spo0B effects occur early in the sporulation developmental pathway. This is consistant with the model since failure to suppress spo0B effects may be due to spo0B failure to produce EDF. The rvtA11 partial suppression can be interpretted if rvtA11 is capable of partially initiating some of the responses normally carried out by the normal spo0F in sporulating cells, without EDF stimulation. The result could be partial suppression in a spo0B strain.

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