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Effects of Environmental Conditions on Streptococcal Pyrogenic Exotoxins

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The effects of various environmental conditions on the production of streptococcal pyrogenic exotoxins (SPEs) was examined. The standard kinetics of SPE production in the strains T25$_3$ cured T12, 86-858, T18P, and NY-5 were established to determine the changes attributed to the experimental manipulations. SPE A was initially detected in late log phase and production ceased as the culture entered stationary phase. SPE B was initially detected in late log phase and production continued into stationary phase. No effect upon SPE A production was observed in conditions of selected temperatures, 7% carbon dioxide, and various pH ranges. SPE B production was increased in 1% bacto-tryptone and 7% carbon dioxide, while decreased in high glucose concentrations and aerated conditions. No effect on SPE B was observed at selected temperatures.

EFFECT OF ENVIRONMENTAL CONDITIONS ON GROUP A STREPTOCOCCAL PYROGENIC EXOTOXIN PRODUCTION

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This is to certify that I have examined this bound copy of a master thesis by

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and have found that it is complete and satisfactory in all respects and that any and all revisions required by the final examining committee have been made.

Patrick M. Schlievert

GRADUATE SCHOOL

ABSTRACT

The effect of various environmental conditions on the production of streptococcal pyrogenic exotoxins (SPEs) was examined. The standard kinetics of SPE production in the strains T253 cured T12, 86-858, T18P, and NY-5 were established in order to determine the changes attributed to the experimental manipulations. SPE A was initially detected in mid log phase and production ceased as the culture entered stationary phase. SPE B was initially detected in late log phase and production continued into stationary phase. In experimentally manipulated environments (1% bacto-tryptone, high concentrations of glucose, and aerated conditions), the rate of SPE A production was apparently reduced, yet the final concentration achieved was unchanged. No effect upon SPE A production was observed in conditions of selected temperatures (25° C, 37° C, and 40° C), 7% carbon dioxide, and various pH ranges (pH 5.5 to 6.5, pH 6.8 to 7.2, and pH 7.6 to 8.5) . Except for the decrease in rate, SPE A production was generally not effected by any environmental manipulations. SPE B production was increased in 1% bacto-tryptone, and 7% carbon dioxide, while decreased in high glucose concentrations and aerated conditions. No effect on SPE B was observed at selected temperatures. Proteinase activity was examined in all strains. Three distinguishable types of proteolytic activity were observed, two of which appeared to be distinct from SPE B. The appearance of a clear zone of surrounded by an opaque ring (generally accepted as a positive result) was evident in fractions from both 86-858 and T18P after preparative isoelectric focusing (IEF). 713 cloned SPE B was also found to possess some proteinase activity. Partial separation of SPE B and proteinase activity occurred after preparative isoelectric focusing. Strongly reactive IEF fractions were subjected to high pressure liquid chromotography (HPLC) resulting in a pattern of weak elution peaks which corresponded to SPE B reactivity and a pattern of strong elution peaks that correlated with SPE B and/or proteinase activity in both strains. The identity of SPE B and streptococcal proteinase precursor (SPP), which is reportedly the source of proteolytic activity, could not be firmly confirmed or negated without further investigation.

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INTRODUCTION

Streptococcus pyogenes, often referred to as group A streptococcus, are gram-positive, non-motile, chain forming bacteria. *S. pyogenes* can be quickly identified from other gram-positive bacteria because they are catalase negative, beta-hemolytic, and bacitracin sensitive. The primary source of energy for *S. pyogenes* is the fermentation of sugars resulting in the production of lactic acid. In media containing a high concentration of glucose, the accumulation of lactic acid and the consequential drop in pH will limit growth (76). Although most strains of *S. pyogenes* produce a capsule composed of hyaluronic acid, they are typically distinguished by their type-specific cell wall M-antigen (65). Over 55 immunologic types of M-antigen have been identified (65). *S. pyogenes* produce a number of extracellular products that include streptolysins (O and S), streptokinases (A and B), hyaluronidase, and streptococcal pyrogenic exotoxins (A, B, and C) (109). The streptococcal pyrogenic exotoxins (SPEs) belong to a group of bacterial proteins called pyrogenic toxins (PTs) which share common biological properties. Other PTs include staphylococcal enterotoxins (A, B, C1, C2, C3, D, E, and G), staphylococcal pyrogenic exotoxins (A and B), and toxic shock syndrome toxin-1 (10,63,109). Biological properties common to PTs include: non-specific T cell mitogenicity, superantigenicity, enhancement of endotoxin shock, induction of scarlet fever-like rash, and pyrogenicity (9,11,109). Moreover, the SPEs have been shown to be cardiotoxic (99) and are suggested to be involved with rheumatic fever, scarlet fever, and streptococcal toxic shock-like syndrome (TSLS) (19,30,66,103).

The research reported in this thesis was performed for several reasons. The information yielded from the characterization of conditions which affect toxin production may prove useful for the development of a model of SPE regulation. Since SPEs are believed to be involved in many streptococcal infections. such as TSLS, knowledge of conditions which confine or regulate toxin production may be beneficial in the treatment of these illnesses. In addition, each *S. pyogenes* carries a single copy of the *speB* gene (43,44,116), yet up to three major forms of SPE B can be recovered from the culture fluids. Thus, knowledge of environmental conditions which favor the production of SPE B would be of particular interest to future studies of SPE B synthesis and processing to yield the various forms seen. Finally, the variety of conditions studied may be utilized to further characterize the relationship between SPE B and streptococcal proteinase precursor (SPP), a protein postulated to be related to SPE B (32,107,114).

Characteristics of SPEs

The SPEs were first characterized by the Dicks in 1924 in group A streptococcal filtrates, which when applied to human skin, caused scarlet fever-like rashes (23). Subsequently, three serologically distinct types, designated SPE A, SPE B, and SPE C (109,110), have been reported. Hooker and Fullensby (49) described SPE A and SPE B while Watson (110) described SPE C. These toxins were demonstrated to be identical to the scarlet fever toxins (94).

Streptococcal pyrogenic exotoxin type A. In 1969, Nauciel et al. (79) reported that SPE A existed in two molecular forms based on charge differences. The estimated molecular weight (30,500) and immune reactivity of

both forms were identical. Furthermore, conversion of the two molecular species into a single form could be achieved by treatment with a reducing agent (79). Cunningham et al. confirmed the report of SPE A charge heterogeneity and identified isoelectrical points between pH 4.5 and 5.0 (20).

Frobisher and Brown (29) were the first to demonstrate that conversion of nonscarlatinal streptococci to toxigenic strains could be achieved by a filterable agent from scarlet fever isolates. Their results were confirmed Zabriskie (117) who showed that infection with a bacteriophage induced from streptococcal strain T12gl caused the nontoxigenic strain, T253, to elaborate type A toxin. Moreover, a return to the original phenotype was achieved through phage curing techniques. Further investigations confirmed these results (57,73,81,82).

A physical map of the lysogenic phage from T12gl was provided by Johnson and Schlievert. The phage was circularly permuted and 33 kb in size (55). Later studies by the same authors revealed that the phage carried the structural gene for SPE A (*speA*) which was subsequently cloned into *Escherichia coli* (56). The *E. coli* clone produced toxin comparable to streptococci-produced SPE A with respect to pyrogenicity, enhancement of susceptibility to endotoxin, lymphocyte mitogenicity, and alteration of immunoglobulin production. Weeks and Ferretti later confirmed these results (112). *speA* was also cloned in both *Bacillus subtilis* (62), and *Streptococcus sanguis* (112) and the resultant toxin was also demonstrated to be similar biochemically, biologically, and immunologically to streptococcal A toxin.

Johnson et al., as well as Weeks and Ferretti, sequenced *speA* (54,113). Results indicated that SPE A has a molecular weight of approximately 26,000 as a mature protein. Maturity is achieved after cleavage of a 30 amino acid signal

peptide. Charge heterogeneity is also displayed in cloned SPE A (56); two molecular forms with isoelectric points of 4.5 and 5.5 are observed.

Recently, SPE A has been associated with TSLS (46,78). The majority of the associated strains belong to M types 1 and 3. Interestingly, M1 isolates make very low levels or no detectable SPE A despite having the gene, whereas M3 isolates made large amounts of the toxin (15, 44, 66).

In 1981 Houston and Ferretti (51) reported that SPE A, as well as SPE B and SPE C, are produced throughout the growth cycle. These authors reported that SPE A production was not affected by temperature, glucose concentration, or metal ion concentration, although their published data indicated a 47% reduction in SPE A production in glucose enriched cultures (51). Recently, it has been shown that SPE A, when produced in *Staphylococcus aureus*, is under control of accessory gene regulator (*agr*), the global regulator of exotoxin production in that organism (P.M.Schlievert, personal communication). SPE A shares approximately 50% sequence similarity with staphlococcus exotoxin type B and C (SEB and SEC) both of which are *agr* regulated (43). It has thus been proposed that SPE A, SEB, and SEC have a fairly recent common ancestor and likely of staphylococcal origin. Since SEB and SEC are late log phase regulated proteins (61,85,86), rather than being made throughout the growth cycle, it may be hypothesized that SPE A should also be regulated similarly, in contrast to that seen by Houston and Ferretti.

Streptococcal pyrogenic exotoxin type B. The mechanism of regulation of SPE B production is unknown at this time. It has been reported that all but one group A streptococci carry a single copy of the gene *speB* which encodes for SPE B (28,44,116). For this reason, it is unlikely that *speB* itself is

carried by a bacteriophage, although Nida and Ferretti have suggested that SPE B production, in certain strains, is influenced by bacteriophage and transferred by lysogenic conversion as are A and C toxins (81). Like SPE A, SPE B also displays charge heterogenicity with isoelectric points of 8.0, 8.3, and 9.0 (2).

E. coli speB cloned from the streptococcal isolate 86-858 yielded toxin which retained its mitogenic properties (12). Subsequent studies inferred that *speB* encodes a 398 amino acid protein of approximately 43,000 molecular weight and containing a 27 residue signal peptide (45). The molecular weight of the 371 residue mature protein is 40,314. This mature protein can apparently later undergo proteolytic cleavage (44) to yield a final form with a molecular weight of 27,500. Comparisons between the inferred sequence of SPE B and that of streptococcal proteinase precursor (SPP) (46,107,114), an extracellular product of group A streptococci, demonstrate a close relationship. Also isoelectric point studies and serological assays between the two proteins suggest that they may be variants of the same protein. Gerlach et al. suggested that the charge heterogeneity of SPE B may be explained by its similarity to SPP which produces an isoelectric focusing pattern similar to that of SPE B (32). As a result of proteolytic cleavage all but one group A streptococci tested have DNA sequences which hybridize to a *speB*-specific probe (45,116); consequently, *speB* is not considered a variable trait. Although *speB*-like sequences are found in nearly all tested group A streptococci genomes, SPE B is detected in culture fluids from only half of the same streptococci (45).

Streptococcal pyrogenic exotoxin type C. Schlievert et al. initially characterized SPE C and determined its molecular weight to be 13,200 (91). Like

SPE A and SPE B, this toxin exhibits heterogenicity with isoelectric points of 6.7 and 7.0 (91). The process of lysogenic conversion in SPE C was first demonstrated by Colon-Whitt et al. (18) and Johnson (57) when both groups were able to initiate SPE C production in non SPE C-producin streptococcal strains through infection by phages induced from SPE C-producing streptococci strains. Furthermore, the high percentage of induced phage capable of causing this change indicated that lysogenic conversion, rather than specialized or generalized transduction, was taking place (57,36).

speC, the gene encoding SPE C, was cloned by Goshorn et al. (34) in *Escherichia coli* from group A streptococcal strain T18P which makes SPE C but not SPEs A or B. The cloned toxin was of the same size as streptococcal derived SPE C and possessed similar biological properties such as mitogenicity, pyrogenicity, and the ability to enhance susceptibility to endotoxin as those found in streptococcal-derived SPE C. *speC* was further subcloned onto a 1.7 kb fragment and a *speC*-specific DNA probe was constructed which was then hybridized to phage induced from CS112, a SPE C-producing streptococcal isolate (36). Infection with this phage resulted in SPE C production, however, it was specific for certain recipient strains (36). Moreover, all strains examined that made SPE C, even those strains that did not produce infectious *speC*-containing phage, were shown to possess *speC* adjacent to phage DNA. *speC* cloned from the CS112 phage produced toxin similar in size and serological properties as that cloned from T18P (34). These findings suggest that *speC* is associated with bacteriophage, but that the phages in many SPE C-producing strains are defective (36).

The nucleotide sequence of *speC* (34) indicates that mature SPE C, a 208 amino acid protein, has a molecular weight of 24,354. Like *speA*, it is a variable

trait. *speC* is found in approximately 50% of group A streptococci (116). The detection of SPE C is difficult due to its weak immunogenicity; thus it has not been determined whether the presence of *speC* is always associated with toxin production (91).

Structural Similarities of Pyrogenic Toxins.

Since pyrogenic toxins (PTs) share biological activities, it can be expected that they also share similar structures. The sequencing of PT genes allows for quantified analysis of such primary structural interrelatedness. Alignments of mature amino acid sequences, for example, indicate that SPE A, SEB, SEC1, SEC2, and SEC3 form a closely related cluster of toxins (34,43,44). SEA, SED, and SEE comprise another cluster (43,44). It has been suggested that all of the PTs may have evolved from a single precursor (43). This hypothesis is further supported by the fact that many PTs share a common mode of genetic regulation: *agr* controls the expression of *entB*, *entC*, *entD*, *tst*, and *speA* (when cloned into *S. aureus*) which encode for SEB, SEC, SED, TSST-1, and SPE A respectively (5,71,85,86). All PT genes except *speB* are variable traits and are carried by mobile elements such as phages in the case of *entA*, *speA*, and *speC* or large transposon-like elements as is the case with the other toxin genes(6,56,57).

Despite shared biological activities, some PTs exhibit relatively little primary sequence similarity with other members of the family. SPE B, TSST-1, and SPE C are examples of such toxins (43). It is surprising that no homology exists between even small regions (10 amino acids) of SPE B and TSST-1 and the other sequenced PTs (44).

Biological Properties of Streptococcal Pyrogenic Exotoxins.

Pyrogenicity. It has been demonstrated that the SPEs induce a fever response in rabbits which peaks at 4 hours and then returns to normal. This cycle remains the same regardless of the administration protocol used (93,98,110). Schlievert and Watson demonstrated the high diffusibility of SPE C by injecting rabbits intravenously, intramuscularly, intradermally, intracisternally (into the hypothalmus area), and subcutaneously and observing the same pattern of fever response (98). After a series of 5 to 6 injections every other day, the rabbits become immune to the pyrogenic activity. This is not so with endotoxin (98) where the agent causes fever responses that peak at 1 and 3 hours and in which it is possible to immunize and prevent only the 3 hour peak.

Schlievert and Watson reported that SPEs may be capable of crossing the blood-brain barrier (98). These reports suggest that the fever response is elicited by direct stimulation of the hypothalamus (98). Furthermore, SPE A has been shown to induce the release of tumor necrosis factor from monocyte cells (27). A small amount of this factor has been known to cross the blood-brain barrier. When produced centrally, tumor necrosis factor causes a pyrogenic response (7). It could be possible that SPEs cross the blood-brain barrier and elicit the release of tumor necrosis factor from a population of central nervous system cells, thus inducing fever in the host (7,98).

Enhanced susceptibility to endotoxin. Treatment with SPEs has been shown to enhance the host's susceptibility to other agents such as endotoxin and streptolysin O (98,99,109,110). The effects of endotoxins are magnified by SPEs

by as much as 100,000 fold (60,90). Rabbits injected with a sublethal dose of endotoxin 4 hours after an injection of SPE respond with fever, diarrhea, and death (98). It has been suggested that SPEs inhibit the reticuloendothelial clearance function due to transient inhibition of liver cell RNA synthesis and thus reduce the host's ability to clear the endotoxin from its circulatory system (40,95). Other investigators suggest that another factor besides reduced clearance must be involved (77).

Mitogenicity. The ability of SPEs to stimulate nonspecific lymphocyte production was demonstrated by Barsumian et al. in 1978 (3) in humans (cord and peripheral blood), rabbits, and guinea pigs. It is now known that this proliferation is limited to T cells (97). Other substances from streptococcal cells or supernates have been shown to resemble or be identical to the SPEs and to demonstrate the same ability to cause lymphocytes to proliferate nonspecifically. For example, blastogen A (37,87), a streptococcal product found in the extracellular culture fluid, possesses the same mitogenic attributes and has been found to be identical to SPE A (96). Recently, Leonard et al. reported that the SPEs are super-antigens because they cause proliferation of T cells bearing T cell receptors with certain variable region beta chain elements (67). Furthermore, antigen presenting cells and class II molecules are required for activity (67). Thus, each SPE will stimulate all T cells dependent on their having the 3 to 5 recognized beta chains. The mitogenic activity is nonspecific in that T-cells are stimulated to proliferate without regards to their antigenic specificity.

MATERIAL AND METHODS

Kinetics

Bacterial strains. Toxins for this study were obtained from *Streptococccus pyogenes* strains T25$_3$ cured T12 (M type 25), 86-858 (M type 12), T18P (M type 18), and NY-5 (M type 10 and 12). Through the use of Ouchterlony immunodiffusion and Western immunoblot assays, it has been shown that: T25$_3$ cured T12 produces detectable amounts of SPE A but not SPE B or SPE C; 86-858 produces only SPE B; T18P produces detectable amounts of SPE C but not SPE A or SPE B; and NY-5 produces detectable amounts of all three SPEs. Toxins for use as positive controls were purified from each of the primary producing strains or from cloned *E. coli* lysates. Streptococcal strains and clones were obtained from laboratory stocks.

Culture media, growth conditions and sampling. A standard or template streptococcal strain growth procedure for this study was established as follows: bacteria from stock cultures or stock blood agar plates (BAPs) were grown overnight at 37^{o} C in the presence of 7% carbon dioxide in Todd Hewitt broth (Difco Laboratories, Detroit, MI). The entire culture was used as an inoculum and grown to stationary phase in 100 ml of dialyzable beef heart medium supplemented with 5 ml/100 ml of glucose buffer, referred to as GB, (0.33 M glucose, 0.5 M sodium bicarbonate, 0.68 M sodium chloride, 0.12 M disodium

phosphate-heptahydrate, 0.027 M L-glutamine). This latter medium is referred to as complete beef heart medium. This culture becomes the source of inocula for each experiment and is referred to as the "stock" sample. Typically 1 ml of stock is added to 100 ml fresh complete beef heart medium and grown at 37° C in the presence of 7% CO_2. From this new culture, samples were periodically removed for testing. Part of each sample broth (2.5 ml) was used to monitor growth, colony forming units (CFUs), and pH. The remaining broth was then treated with 4 volumes of ethanol to precipitate toxins. Precipitates were sedimented by centrifugation (500 x g, 20 min), dried, resuspended in 1/10 volume of distilled water, insoluble material removed by centrifugation in a microfuge for 10 minutes, and stored at 4° C. The resulting concentrated (10x) supernate was used to determine toxin production and/or proteinase activity. Purity of the cultures was determined throughout growth and sampling by streaking a sample onto BAPs.

Toxin detection. The sample supernates were tested by dilution Ouchterlony immunodiffusion for reactivity with specific toxin antisera from hyperimmunized rabbits. The protocol for hyperimmunization is described by Schlievert et al. (91). Briefly, American Dutch belted rabbits were immunized subcutaneously with a mixture of equal volumes of purified toxin and Freund incomplete adjuvant. Rabbits were injected every 14 days and bled 7 days after each booster injection beginning on day 21. Blood was allowed to clot for 24 hours at 4° C, centrifuged for 30 minutes, and the serum tested for reactivity against known toxin. Toxin concentration in samples was estimated by determining the maximum dilution of supernate (20ul/well) which was detectable by Ouchterlony

immunodiffusion assay (the lower limit detection had been found empirically to be 6 ug/ml of the concentrated culture fluid, or 0.6 ug/ml in the original culture fluid).

Further concentration of some supernates was required. The concentrated (10x) samples were placed in a speed vac concentrator (SAVANT, Hicksville, NY), spun for 2 to 4 hours, and resolubilized in distilled water. The resulting concentrated sample which was 100 times concentrated with respect to the original culture was then tested by Ouchterlony immunodiffusion assay.

Monitoring growth and viability. Growth was monitored by measuring the absorbance of samples by use of a Beckman spectrophotometer model 34 (Irving, CA) at a wavelength of 655 nm (17). Sterile media with all appropriate buffers were used as reference samples. The method used to monitor cell growth and viability was by detection of colony forming units (CFUs). The sample broths were subjected to 10 fold serial dilutions, 100 ul plated on Todd Hewitt agar plates, the plates incubated at 37° C in the presence of 7% carbon dioxide for 24 hours, and the number of colonies determined.

Monitoring pH. The pH of sample broths was determined using Hydrion paper (Micro Essential Lab, Brooklyn, NY) of pH ranges 6.0 to 8.0, 3.0 to 5.5, or 3.0 to 10.0 as appropriate. Monitoring of large cultures were done with a Brinkman Instruments pH-101 meter (Westbury, NY).

Purification of control toxins. A standard purification protocol is described by Wannamaker and Schlievert (109). Briefly, bacteria were grown in complete beef heart medium as described previously. Culture supernates and

cells were precipitated with 4 volumes of ethanol and stored for 4 days at 4° C. The ethanol was then decanted, and the precipitate collected. The precipitate was resolubilized in acetate-buffered saline (ABS) (pH 4.5) and then centrifuged (10,000 x g, 20 min) to remove insoluble materials which include cell debris and irreversibly denatured protein. The supernatant was again treated with 4 volumes of ethanol, allowed to settle, and then the precipitate collected by centrifugation. The partially purified toxin was then resolubilized in distilled water, centrifuged, and dialyzed against distilled water for 2 days at 4° C. The resulting solution was concentrated approximately two-fold in dialysis tubing by evaporation and then lyophilized. The component proteins were separated by preparative thin-layer isoelectric focusing in pH gradients of 3.5 to 10 (Pharmacia LKB Biotechnology, Inc. Piscataway, NJ). Fractions were tested for the presence of toxin by Ouchterlony immunodiffusion as described above. Positive fractions were refocused in the appropriate narrow pH gradient corresponding to the reported isoelectric focusing point of the toxin 4 to 6 for SPE A, 6 to 8 for SPE C, and 7 to 9 for SPE B. Protein bands were harvested and dialyzed at 4° C for 4 days against distilled water to remove ampholytes. Toxin concentration was determined by both the Bradford protein assay (BioRad Laboratories, Richmond, CA) and dilution Ouchterlony immunodiffusion assay where the last visible dilution was defined as containing 6 ug/ml toxin.

SDS-polyacrylamide gel electrophoresis. The protocol used in this study for SDS-polyacrylamide gel electrophoresis was described by Laemmli (64). The discontinuous buffer system provided sufficient protein separation. Coomassie brilliant blue R250 staining was used to detect proteins. The molecular weight markers used were the BioRad (BioRad Laboratories, Richmond, CA) low range SDS-PAGE standards which include phosphorylase B, bovine serum

albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

Western immunoblot. For Western immunoblots, the proteins were first transferred from the polyacrylamide gels to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in a Trans-Blot cell (BioRad Laboratories) using standard transfer buffer (25 mM Tris buffer, pH 8.3, 192 mM glycine, and 20% [w/v] methanol) (43). Nitrocellulose membranes were incubated 1.5 hours with diluted hyperimmune rabbit antiserum (50 ul/100 ml) at room temperature, washed, and incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma) for an additional 1.5 hours. The protein - antibody complexes were detected by using the indolyl phosphate-nitroblue indicator system of Blake et al. (8). The molecular weight markers used in the Western immunoblots were the BioRad low range prestained SDS-PAGE standards which are identical to those listed above but have been conjugated to a dye.

Proteinase assays. Hynes and Tagg (52) described an assay for detecting proteinase activity in liquid preparations. This well diffusion method used a 1:2 diluted Columbia agar base supplemented with 3% skim milk. The reducing agent (0.01 M 2-mercaptoethanol) was added to the samples prior to loading the wells. Their assay was modified to maximize detection of proteolytic activity at 6 hours. The base gel contained 0.75% agarose (Sigma Chemical Co., St. Louis, MO) and 1% skim milk (Difco). This mixture was slowly heated until molten. Immediately before pouring the 2.5 ml slides, 2-mercaptoethanol (Sigma) (20ul/10 ml agarose) was added to the molten agarose. After cooling, standard Ouchterlony wells were punched and unreduced culture samples (15 ul) were

directly loaded. The slides were routinely used within 24 hours, however, they still retained their sensitivity at 72 hours when stored at 4° C. The concentrated (10x) samples were incubated for 6 hours at 37° C in wells punched into the skim milk slides described above. Proteolytic activity was detected by the formation of clearing zones.

Manipulation of Environmental Conditions.

Protein level in media. The standard complete beef heart media was supplemented with 1% bacto-tryptone (w/v) (Difco). Standard procedures for sampling and testing were used.

Glucose level in media. To the standard glucose buffer described previously, additional amounts of glucose were added. The standard glucose buffer contains 0.33 M glucose, whereas the other buffers made contained 0.67 M glucose, 1.00 M glucose, 1.5 M glucose, and 2 M glucose. These concentrated glucose buffers (5 ml) were added to 100 ml of complete beef heart media. Standard growth protocol, sampling, and testing proceeded as previously described.

Incubation temperature of media. Temperatures selected were similar to those used by Houston and Ferretti (51) (i.e. 25° C, 37° C, and 40° C). Six 100 ml complete beef heart medium cultures were inoculated. A pair of cultures were designated for each temperature. One of each pair was incubated in the presence of 7% carbon dioxide. The other remained under stationary

conditions in a standard incubator. Sampling and testing proceeded as previously described.

Oxygen content of media. To change the oxygen content of the media, the standard protocol was modified and two methods were utilized. With the first method, pairs of cultures were designated. One culture was placed in an oscillating incubator (Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, NJ) (80 cycles/min) at 37° C. The other culture was placed stationary in the presence of 7% carbon dioxide at 37° C. In the second method, in order to distinguish the possible effects of carbon dioxide, triplicate cultures were designated. One culture was grown stationary in the presence of 7% carbon dioxide at 37° C. A second culture was grown stationary in a standard incubator at 37° C. The third culture was grown in an oscillating incubator (80 cycles/min) at 37° C. Sampling and testing procedures were as previously detailed.

pH of media. In order to manipulate the pH, the standard protocol was modified as follows: pH of media was adjusted to three ranges - acidic (5.5 to 6.5), neutral (pH 6.8 to 7.2), and basic (7.6 to 8.5). Adjustments were made after the addition of glucose-buffer, but prior to inoculation. The pH adjustment was made by dropwise addition of either 10 N sodium hydroxide or 12 M hydrochloric acid. To visualize the pH ranges, saturated phenol red solution (Sigma) was added to the cultures. Phenol red is yellow at pH < 6.4, orange near pH 7, and red at pH > 8.2. Cultures were grown in the presence of 7% carbon dioxide at 37° C. One milliliter samples of the cultures were taken after each pH adjustment to determine if the appropriate pH range for that culture had been achieved. Standard sampling and testing procedures were used.

Separation of SPE B from Proteinase Activity.

Strains T18P, (SPE B^-, proteinase$^+$) and 86-858 (SPE B^+, proteinase$^+$) were selected for the experiment based on preliminary proteinase and toxin assays. In order to compare the ammonium sulfate precipitation method of Elliott and coworkers (24,26,69) with the ethanol precipitation method of Wannamaker and Schlievert (109), the following protocol was designed. Strains were grown in duplicate in identical batches of 5 L complete beef heart media (7% CO_2) to stationary phase. One 5 L culture of each strain was subjected to 4 volumes of ethanol (e.g. 86-858 EtOH) and proteins separated by isoelectric focusing in a pH gradient of 3.5 to 10. The other 5 L culture was treated with ammonium sulfate (e.g. 86-858 A.S.) as follows: the 5 L culture was brought to 80% saturation (w/v) with ammonium sulfate and proteins allowed to settle for 24 hours at room temperature. Precipitates were collected by centrifugation (5000 x g,15 min), the liquid discarded, and precipitates resolubilized in 150 ml of pyrogen-free distilled water. Samples were dialyzed against water for 24 hours at room temperature with several changes of water. Insoluble materials were removed by centrifugation (10,000 rpm, 30 min) and the sample subjected for preparative isoelectric focusing gradient of 3.5 to 10.0 (as described above). The fractions from both EtOH and A.S. methods were harvested and tested for SPE B and proteinase activity as described above. Representative fractions were selected and subjected to high pressure liquid chromotography (HPLC). The protein peaks obtained were pooled and tested for SPE B and proteinase activity. Purified cloned SPE B (a lab stock) was subject to HPLC as a control.

RESULTS

Kinetics of SPE Production

T253 cured T12. The results of the baseline growth studies for strain T253 cured T12, which produces only SPE A, are shown in Figure 1a and 1b. A strong correlation was shown between determination of colony forming units (CFUs) and optical density (OD) as methods for monitoring growth. A linear drop in pH was observed which inversely corresponded to the bacterial growth. The Western immunoblot in Figure 1c illustrates the relationship of growth and toxin production of a typical trial. At the 24 hour sampling, a peak standard toxin concentration of 2.4 ug/ml was observed. The data indicate the majority of toxin was made just prior to stationary phase and thus the toxin was late log phase regulated.

86-858. The results from a representative baseline trial of 86-858 are shown in Figure 2a. Figure 2b depicts a Western immunoblot of SPE B production of a typical trial. SPE B is again only observed at detectable levels late in the growth cycle. Interestingly, addition of 1% tryptone significantly increased toxin production compared to standard conditions.

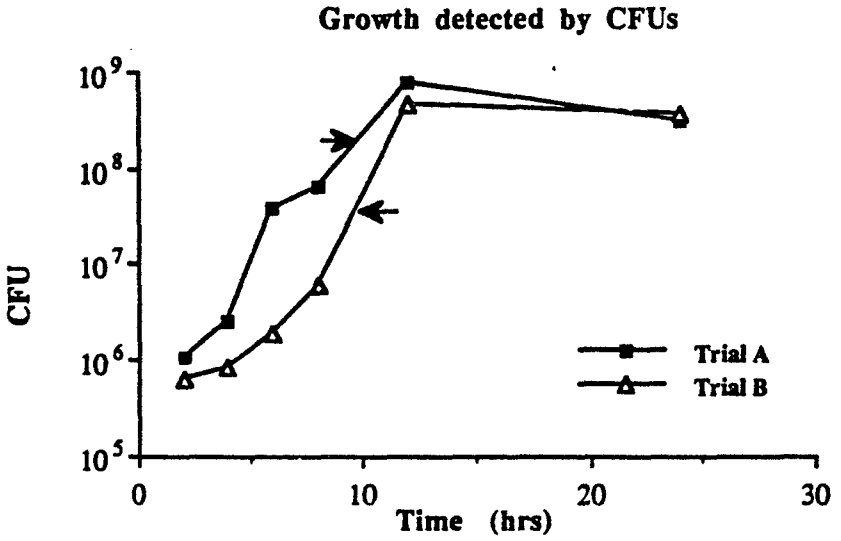
T18P. Figure 3 presents a typical example of trials for T18P which produces SPE C only. SPE C is typically not made in high concentrations. The Ouchterlony immunodiffusion results reported thus were obtained from 50 or

100 fold concentrated samples which were used if sufficient sample was available. T18P cultures did not grow to as high a density nor did they reach as acidic a culture pH level as did T253 cured T12 or 86-858. Again SPE C was only made in detectable amounts in late log phase of growth.

NY-5. Strain NY-5 is known to be a strong SPE A producer but also makes SPE B and SPE C (50,104,110). Figure 4a and 4b display representative trials of strain NY-5. Ouchterlony immunodiffusion results reported for SPE B and SPE C are from 50 or 100 fold concentrated culture fluids. As with strains that make only a single SPE all three were maximally made in late log phase in NY-5.

Increased inocula. Larger volumes of inocula were tried in order to increase toxin levels to within the sensitivity range of the antisera. This technique was applied to test further the possible relationship between cell density and toxin production. Although SPE B was detected earlier in the larger inocula trials, by 24 hours, the level of toxin was the same in all three cultures. Likewise, the time of production of toxin in the growth cycle was not altered with toxin being maximally produced in late log phase.

Figure 1a. Growth curves of $T25_3$ cured T12. Determination of colony forming units (CFUs)(above) and optical density (OD) (below) were used to monitor growth. All trials represent 1:100 inocula into 100ml of complete beef heart media grown at 37° C and 7% CO_2. Arrows indicate earliest detection of toxin.


Growth detected by CFUs
CFU
10^9
10^8
10^7
10^6
10^5
0
10
20
30
Time (hrs)
Trial A
Trial B

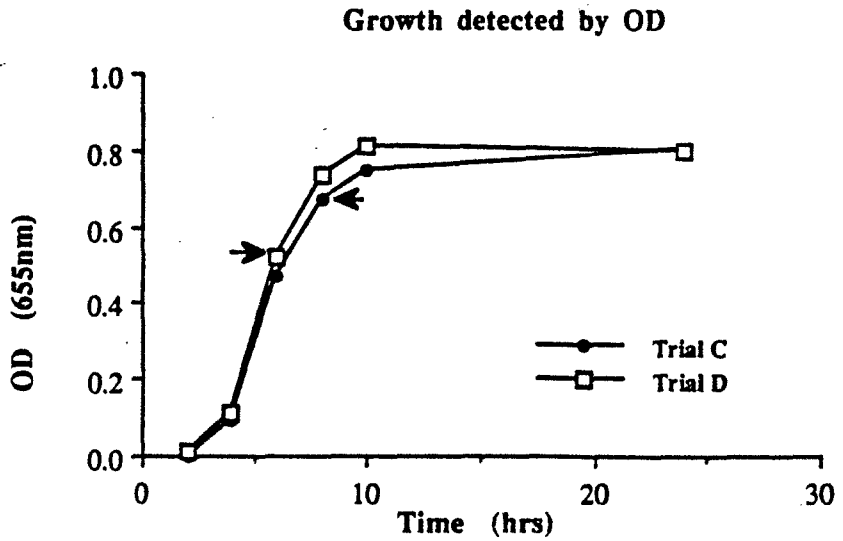
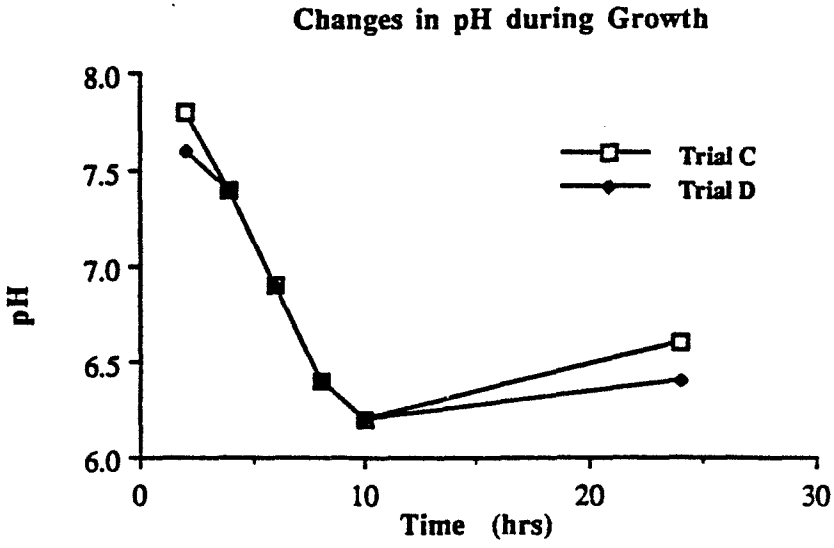

Growth detected by OD
OD (655nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (hrs)
Trial C
Trial D

Figure 1b. pH and SPE A production in T253 cured T12. pH changes (above) and SPE A production (below) are shown. Growth conditions are identical to Figure 1a. Toxin concentration of culture is represented as measured by Ouchterlony immunodiffusion.


Changes in pH during Growth
8.0
7.5
7.0
6.5
6.0
pH
0
10
20
30
Time (hrs)
Trial C
Trial D

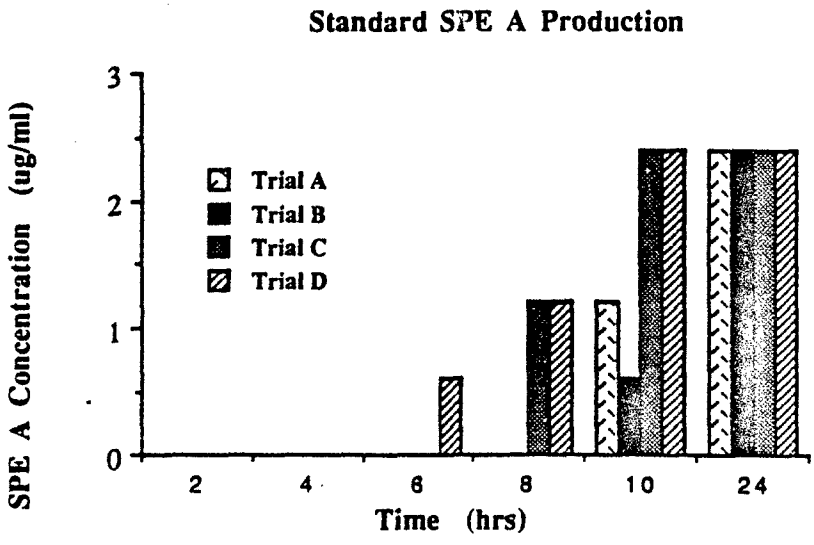

Standard SPE A Production
3
2
1
0
SPE A Concentration (ug/ml)
Trial A
Trial B
Trial C
Trial D
2
4
6
8
10
24
Time (hrs)

Figure 1c. Western immunoblot assay of SPE A production in $T25_3$ cured T12. The trial shown corresponds to trial A in Figures 1a and 1b. Peak of culture growth was at 12 hours, mid log corresponds to 8 hours. Ten microliters of 10 times concentrated culture fluid was loaded per lane.

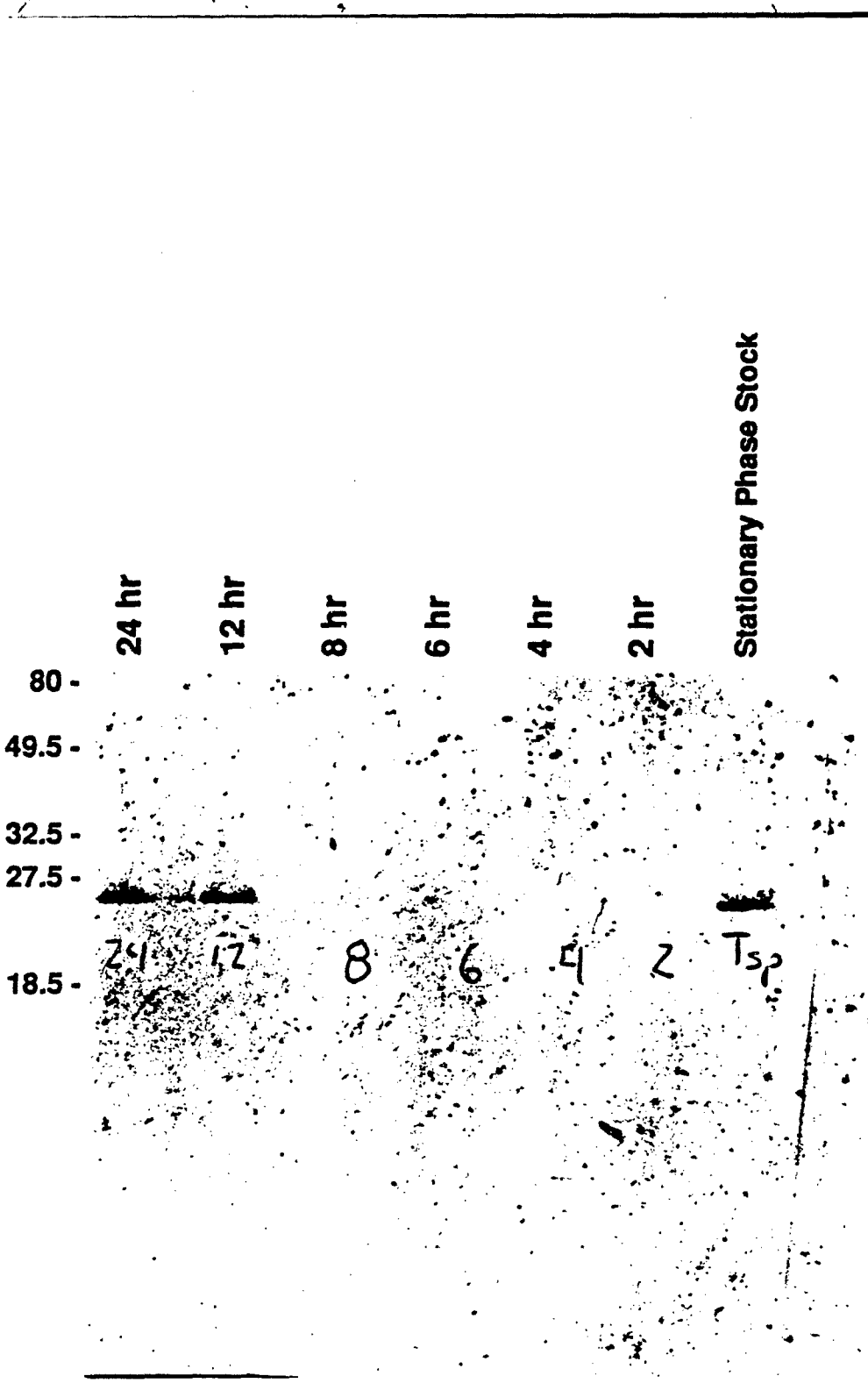
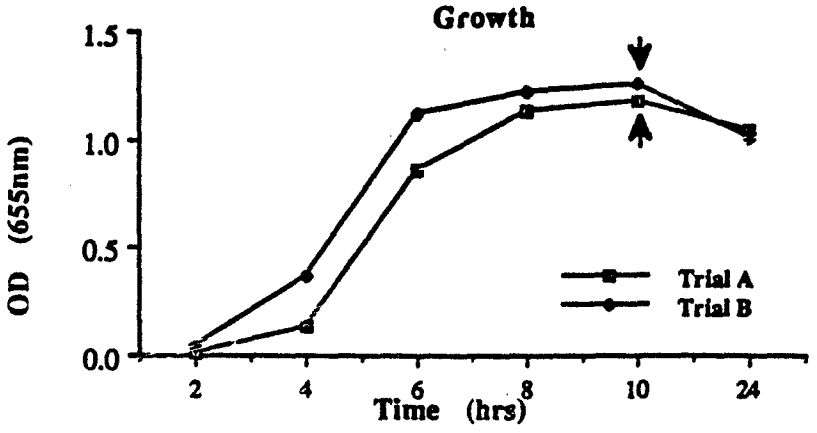
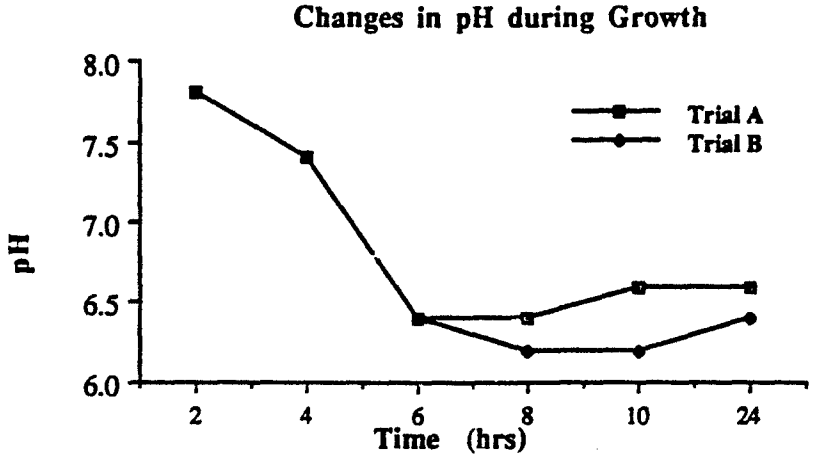

24 hr
12 hr
8 hr
6 hr
4 hr
2 hr
Stationary Phase Stock
80 -
49.5 -
32.5 -
27.5 -
18.5 -
24
12
8
6
4
2
Ts

Figure 2a. pH changes and toxin production during growth of strain 86-858. Determination of OD was used to monitor growth (top). All trials represent 1:100 inocula into 100ml of complete beef heart media grown at 37° C and 7% CO_2. Standard pH pattern (middle) and SPE B production (bottom) are shown. Arrows indicate the first detection of toxin.


Growth
OD (655nm)
1.5
1.0
0.5
0.0
2
4
6
8
10
24
Time (hrs)
Trial A
Trial B


Changes in pH during Growth
pH
8.0
7.5
7.0
6.5
6.0
2
4
6
8
10
24
Time (hrs)
Trial A
Trial B

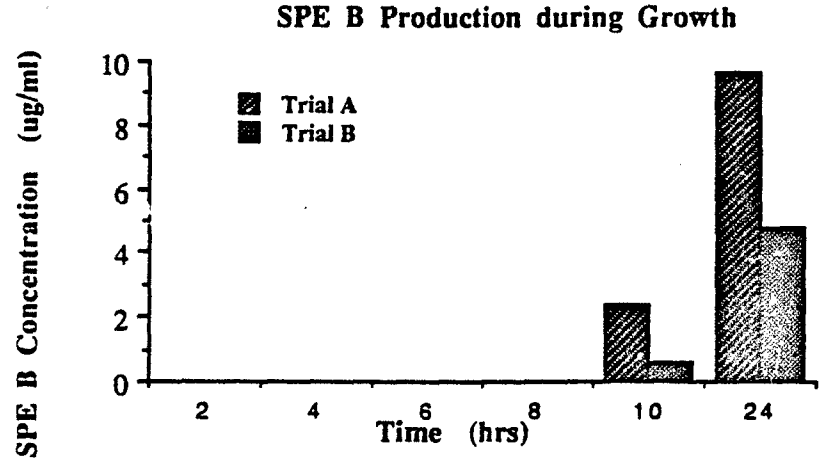

SPE B Production during Growth
SPE B Concentration (ug/ml)
10
8
6
4
2
0
2
4
6
8
10
24
Time (hrs)
Trial A
Trial B

Figure 2b. Western immunoblot assay of SPE B production in 86-858 in standard and 1% tryptone conditions. Twenty microliters of 10 times concentrated culture fluid was loaded per lane. Concentration of SPE B by Ouchterlony: stock 24 hour (1.2 ug/ml), standard 24 hour (4.8 ug/ml), and tryptone 24 hour (9.6 ug/ml).

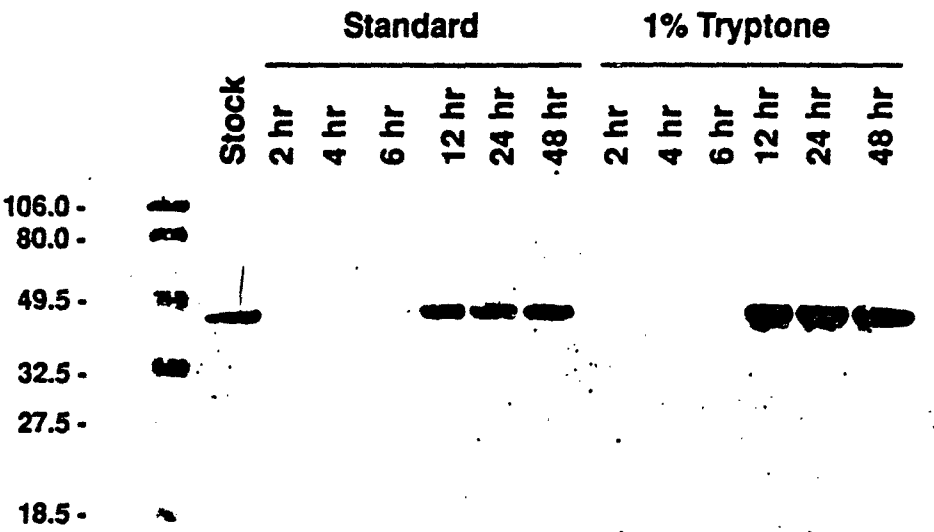
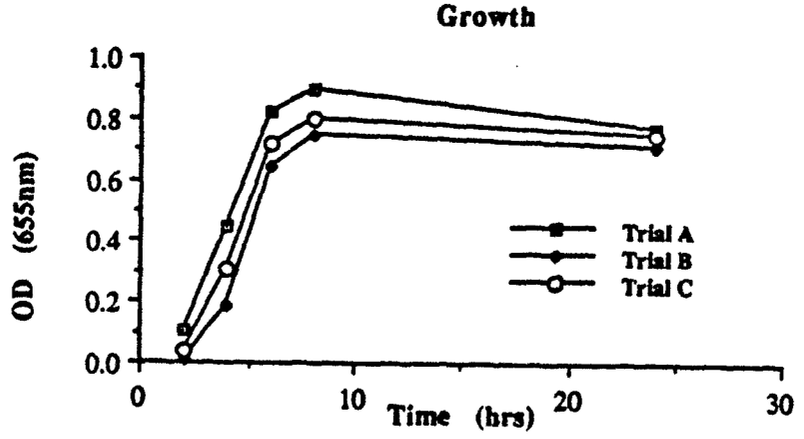
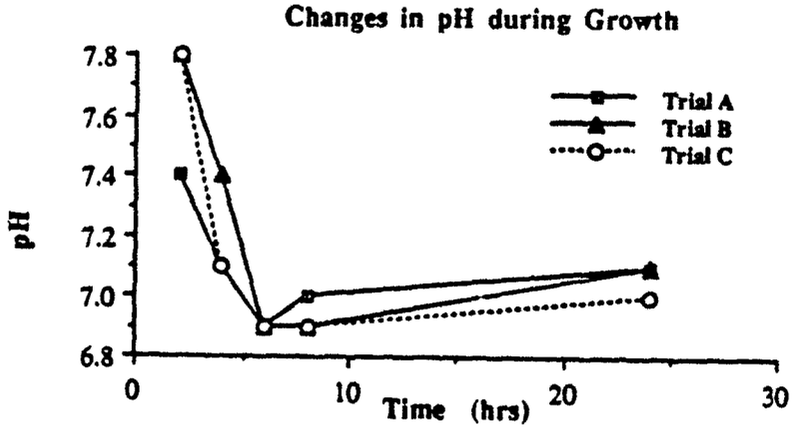

Standard
1% Tryptone
Stock
2 hr
4 hr
6 hr
12 hr
24 hr
48 hr
2 hr
4 hr
6 hr
12 hr
24 hr
48 hr
106.0 -
80.0 -
49.5 -
32.5 -
27.5 -
18.5 -

Figure 3. pH changes and toxin production during growth of strain T18P. Determination of OD was used to monitor growth (top). All trials represent 1:100 inocula into 100ml of complete beef heart media (5% GB) grown at 37° C and 7% CO_2. Standard pH patterns (middle) and SPE C production (bottom) are shown.


Growth
OD (655nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (hrs)
Trial A
Trial B
Trial C


Changes in pH during Growth
pH
7.8
7.6
7.4
7.2
7.0
6.8
0
10
20
30
Time (hrs)
Trial A
Trial B
Trial C

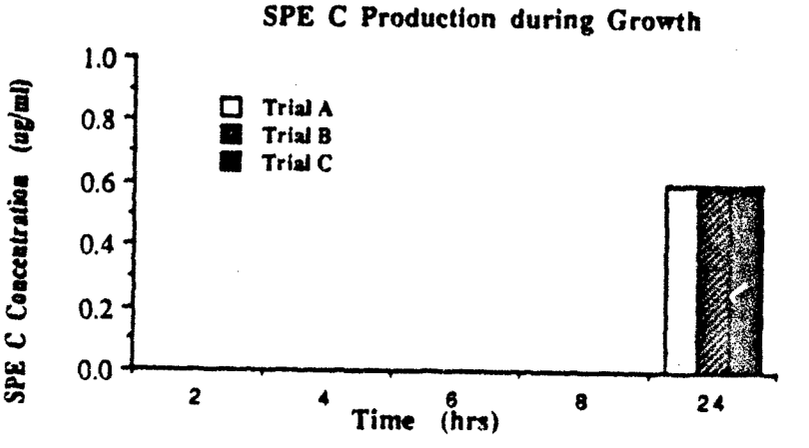
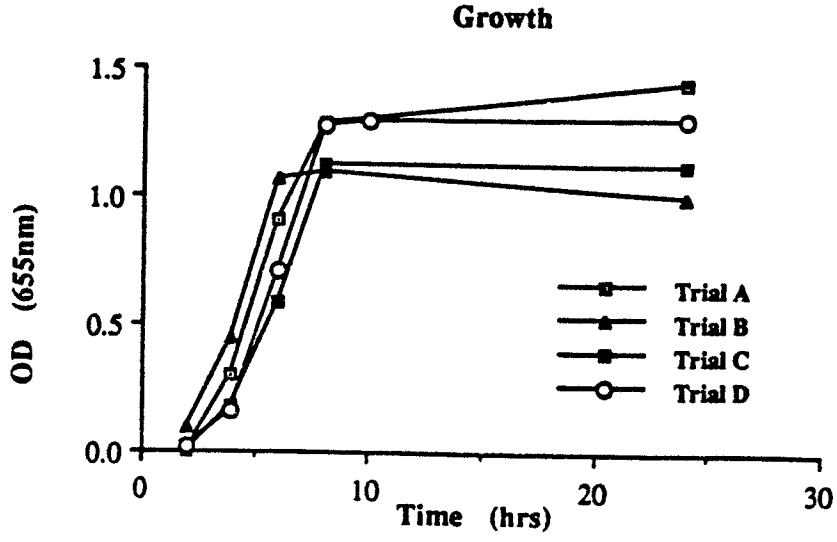

SPE C Production during Growth
SPE C Concentration (ug/ml)
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
24
Time (hrs)
Trial A
Trial B
Trial C

Figure 4a. pH changes during growth of NY-5. Determination of OD was used to determine growth (above). All trials represent 1:100 inocula into 100 ml of complete beef heart media grown at 37° C and 7% CO_2. Standard pH patterns are shown (below).


Growth
1.5
1.0
0.5
0.0
OD (655nm)
0
10
20
30
Time (hrs)
Trial A
Trial B
Trial C
Trial D

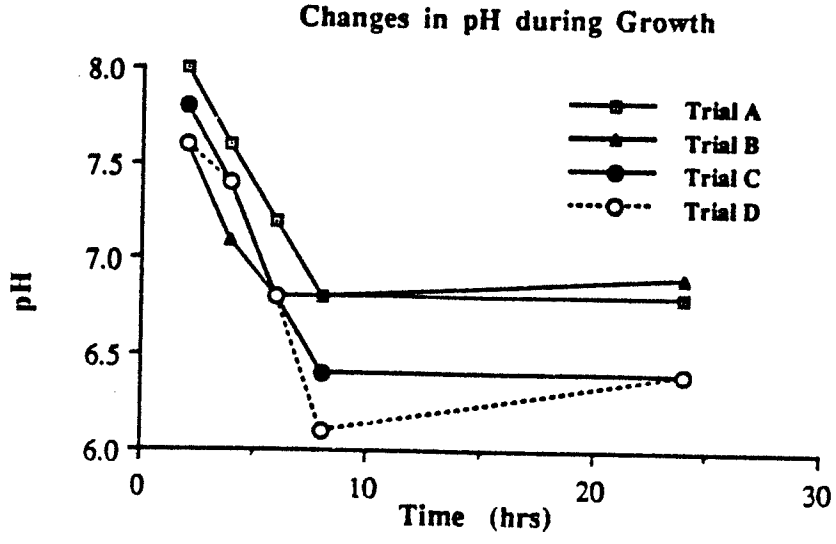
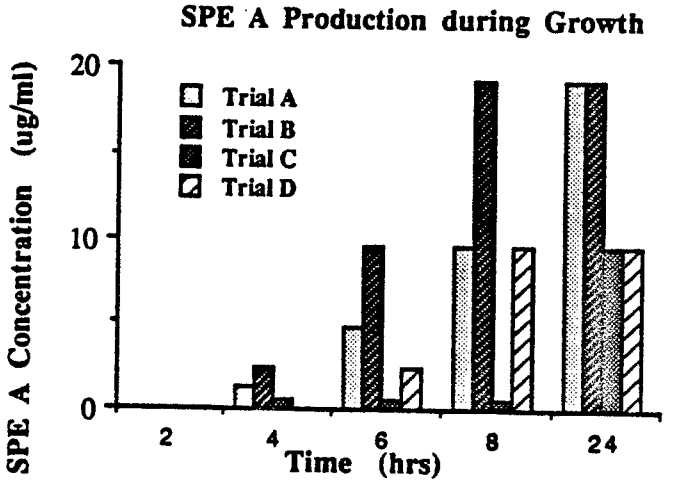
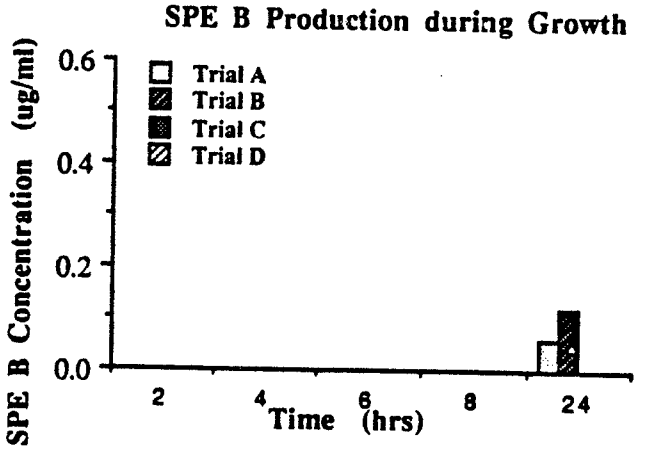
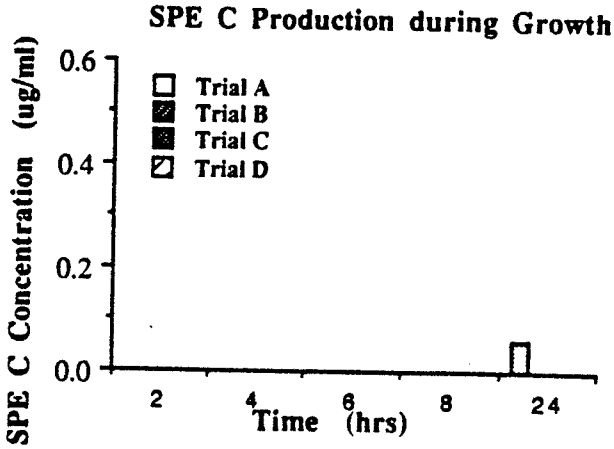

Changes in pH during Growth
8.0
7.5
7.0
6.5
6.0
pH
0
10
20
30
Time (hrs)
Trial A
Trial B
Trial C
Trial D

Figure 4b. Toxin production in NY-5 during growth. SPE A (top), SPE B (middle), and SPE C (bottom) production are shown. Results reported for SPE B and SPE C are from 100 fold concentrated samples. SPE A production is much stronger than either SPE B or SPE C.


SPE A Production during Growth
SPE A Concentration (ug/ml)
20
10
0
Trial A
Trial B
Trial C
Trial D
2
4
6
8
24
Time (hrs)


SPE B Production during Growth
SPE B Concentration (ug/ml)
0.6
0.4
0.2
0.0
Trial A
Trial B
Trial C
Trial D
2
4
6
8
24
Time (hrs)


SPE C Production during Growth
SPE C Concentration (ug/ml)
0.6
0.4
0.2
0.0
Trial A
Trial B
Trial C
Trial D
2
4
6
8
24
Time (hrs)

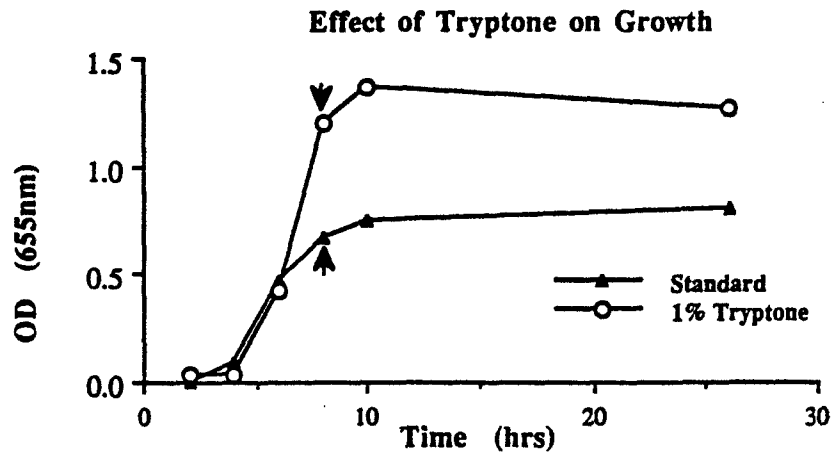
Manipulation of Environmental Conditions.

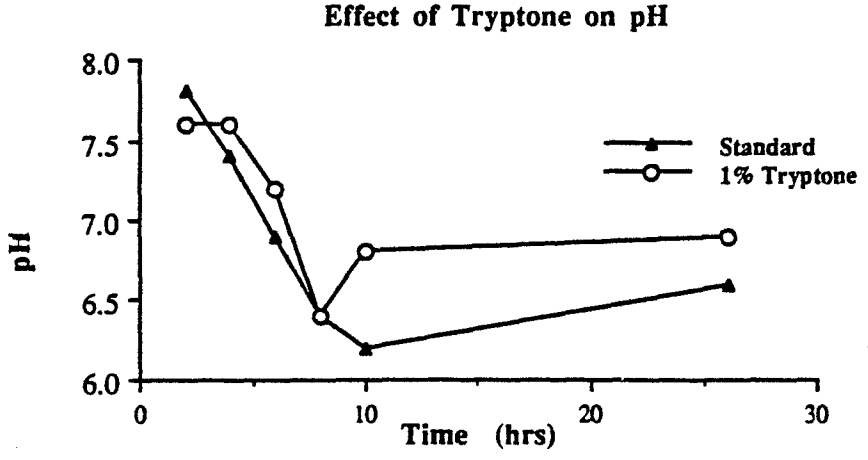
Protein enriched media. The results of paired cultures with and without bacto-tryptone are depicted for strains T253 cured T12, 86-858, and T18P strains in Figures 5a, 5b, and 5c. For all three strains, the protein enriched culture exhibited a longer lag phase followed by more rapid and extensive growth than the standard culture of the same strain. This was also seen for strain NY-5 which makes all three toxin (data not shown). The pH of the media became increasingly more acidic in proportion with the growth of the culture. Enriched cultures characteristically produced twice the amount of SPE B and demonstrated a decrease in the rate of SPE A production as compared to the standard cultures. The decrease in SPE A production was not seen after 24 hours of culture of T253 cured T12 but was in NY-5 (data not shown).

Glucose enriched media. To increase the glucose content of the culture media, five separate stocks of glucose were made ranging in concentration from 0.33 M to 2.0 M glucose. Strains T253 cured T12 and 86-858 were grown at 37° C in the presence of 7% carbon dioxide and representative trials are logged in Figure 6 and 7 respectively. Glucose delayed toxin production by T253 cured T12 at concentrations above 16.7 mM did not result in maximal SPE A until 24 hours whereas 16.7 mM peaked at 10 hours. SPE B gave similar results but production of toxin was below detectable levels by Ouchterlony immunodiffusion assays in 4 of 5 glucose strengths. Therefore, tryptone (1% [w/v]) was then used to enhance SPE B production of 86-858 to detectable levels. The results are recorded in

Figure 8a. The inverse correlation between glucose and SPE B is most clearly shown in Figure 8b. In all trials, a significant drop in pH was observed.

Figure 5a. Effect of protein enriched media on growth, pH, and toxin production in T253 cured T12. Determination of OD was used to monitor growth (top). The trials represent 1:100 inocula into 100 ml of complete beef heart media with or without 1% tryptone (w/v) and grown at 37° C and 7% CO_2. Standard and tryptone supplemented culture pH (middle) and toxin production (bottom) patterns are shown. Arrows indicate initial toxin detection.


Effect of Tryptone on Growth
OD (655nm)
1.5
1.0
0.5
0.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone


Effect of Tryptone on pH
pH
8.0
7.5
7.0
6.5
6.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone

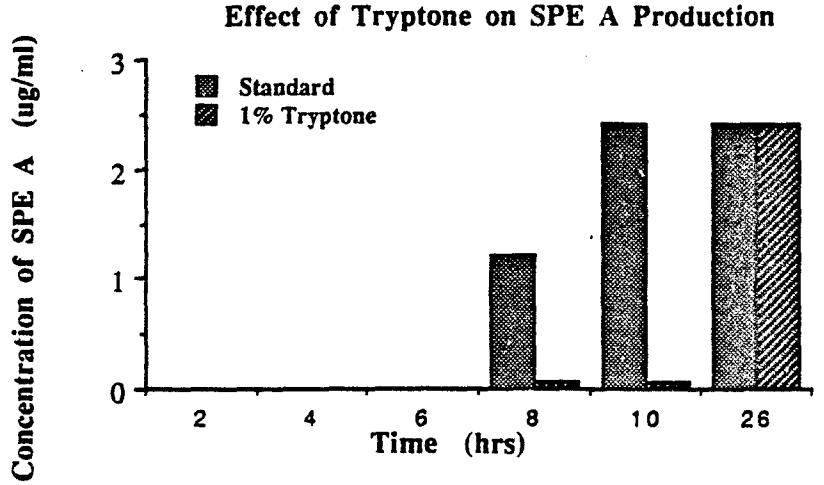
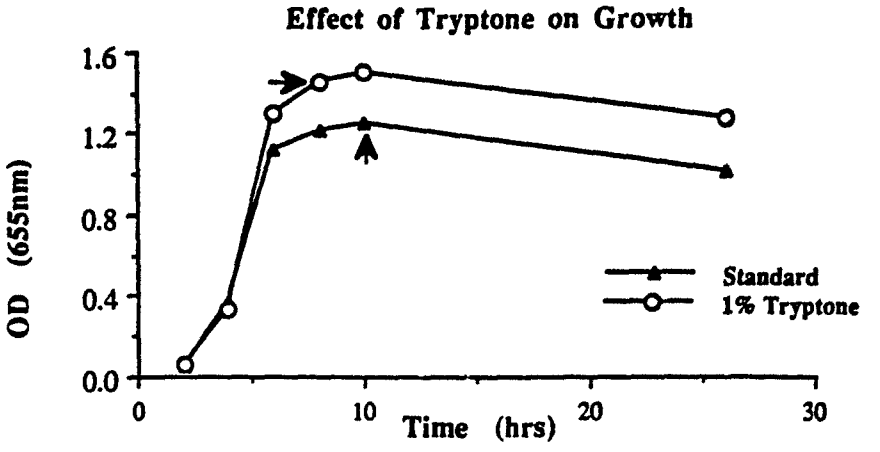
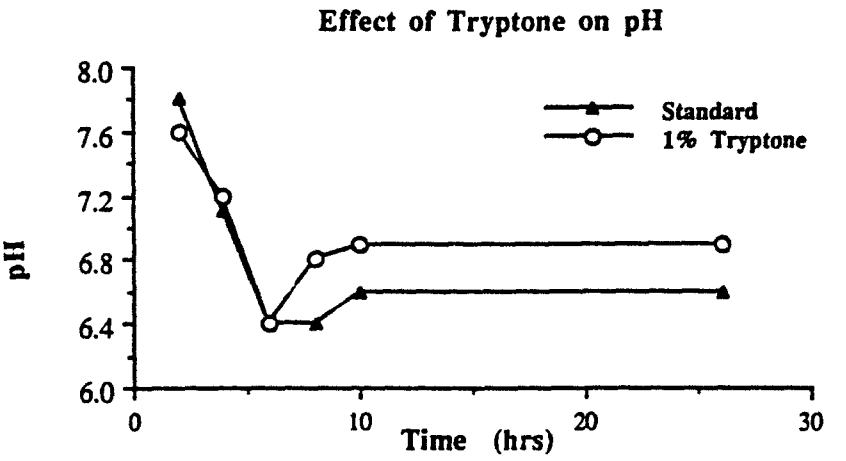

Effect of Tryptone on SPE A Production
Concentration of SPE A (ug/ml)
3
2
1
0
2
4
6
8
10
26
Time (hrs)
Standard
1% Tryptone

Figure 5b. Effect of protein enriched media on growth, pH, and toxin production in 86-858. Determination of OD was used to monitor growth (top). All trials represent 1:100 inocula into 100 ml complete beef heart media with or without 1% tryptone (w/v) and grown at 37° C and 7% CO_2. Standard and tryptone supplemented patterns of pH (middle) and SPE B production (bottom) are shown. Arrows indicate initial toxin detection.


Effect of Tryptone on Growth
OD (655nm)
1.6
1.2
0.8
0.4
0.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone


Effect of Tryptone on pH
pH
8.0
7.6
7.2
6.8
6.4
6.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone

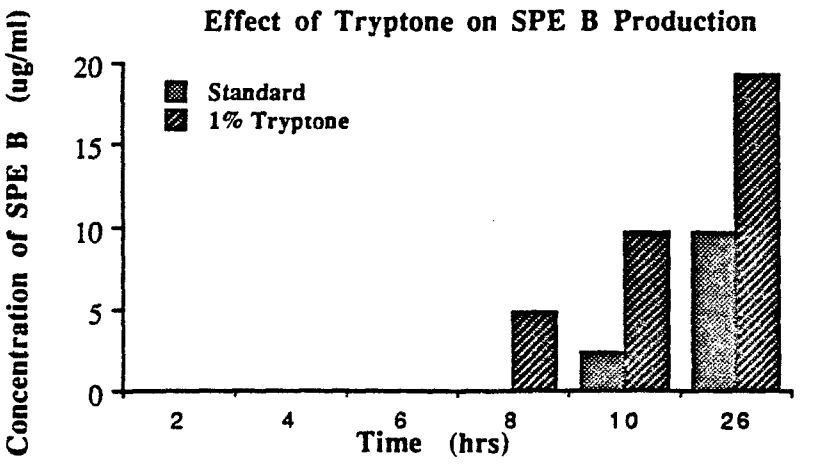
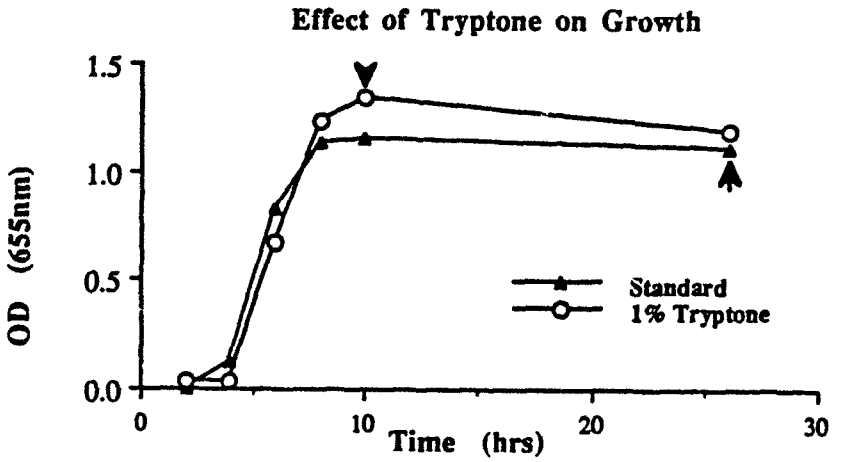
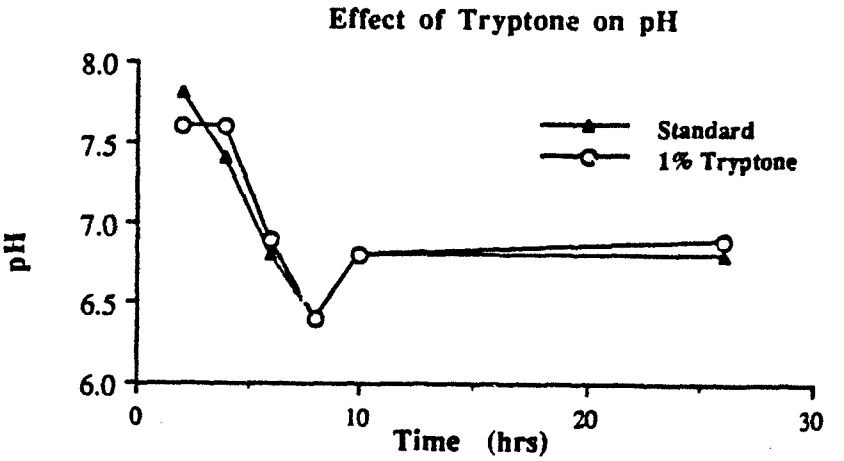

Effect of Tryptone on SPE B Production
Concentration of SPE B (ug/ml)
20
15
10
5
0
2
4
6
8
10
26
Time (hrs)
Standard
1% Tryptone

Figure 5c. Effect of protein enriched media on growth, pH, and toxin production in T18P. Determination of OD was used to monitor growth (top). All trials used 1:100 inocula into 100 ml complete beef heart media with or without 1% tryptone (w/v) and grown at 37° C and 7% CO_2. Standard and tryptone supplemented patterns of pH (middle) and SPE C production (bottom) are shown. Arrows indicate initial toxin detection.


Effect of Tryptone on Growth
OD (655nm)
1.5
1.0
0.5
0.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone


Effect of Tryptone on pH
pH
8.0
7.5
7.0
6.5
6.0
0
10
20
30
Time (hrs)
Standard
1% Tryptone

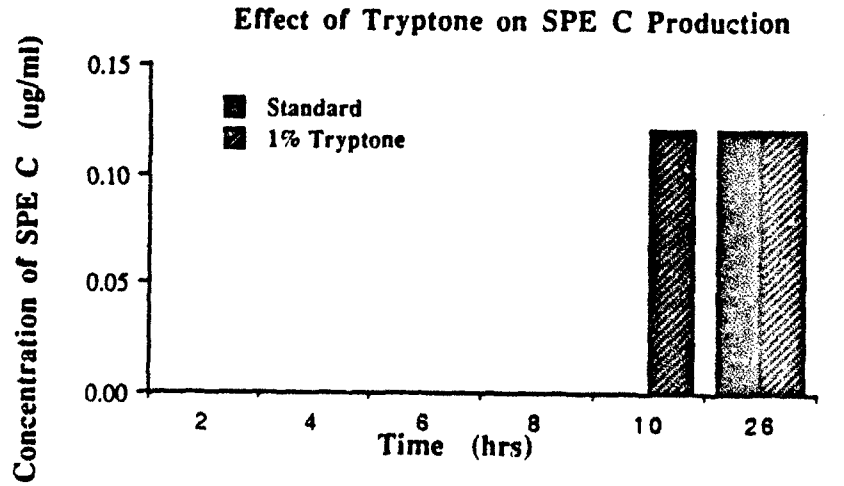
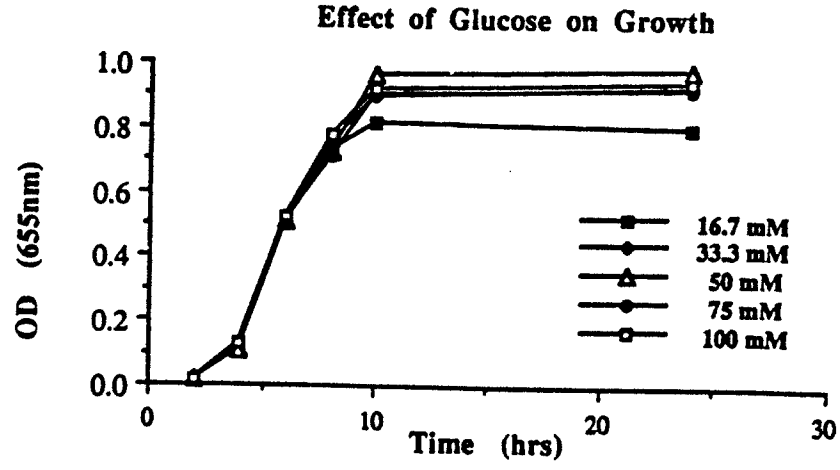
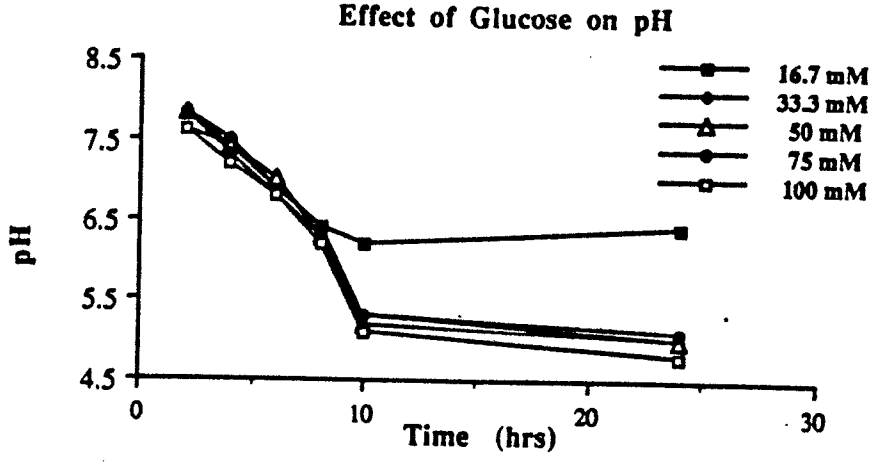

Effect of Tryptone on SPE C Production
Concentration of SPE C (ug/ml)
0.15
0.10
0.05
0.00
2
4
6
8
10
26
Time (hrs)
Standard
1% Tryptone

Figure 6. Effect of glucose enriched media on growth, pH, and toxin production in T253 cured T12. Determination of OD was used to monitor growth (top). All trials represent 1:100 inocula into 100ml complete beef heart media (with indicated glucose concentrations) grown at 37° C and 7% CO_2. Standard and enriched patterns of pH (middle) and SPE A production (bottom) are shown. Concentrations of glucose shown indicate final culture concentrations.


Effect of Glucose on Growth
OD (655nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (hrs)
16.7 mM
33.3 mM
50 mM
75 mM
100 mM


Effect of Glucose on pH
pH
8.5
7.5
6.5
5.5
4.5
0
10
20
30
Time (hrs)
16.7 mM
33.3 mM
50 mM
75 mM
100 mM

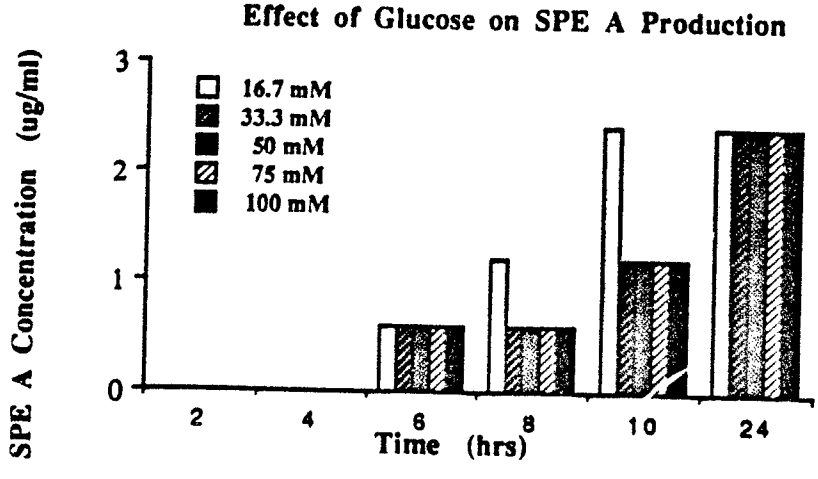
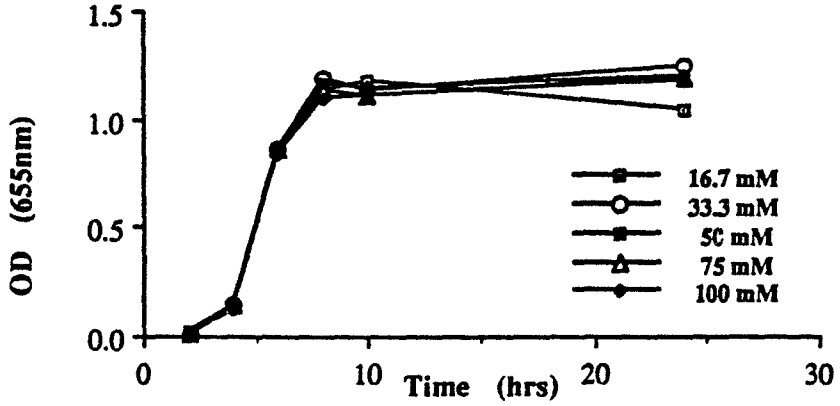
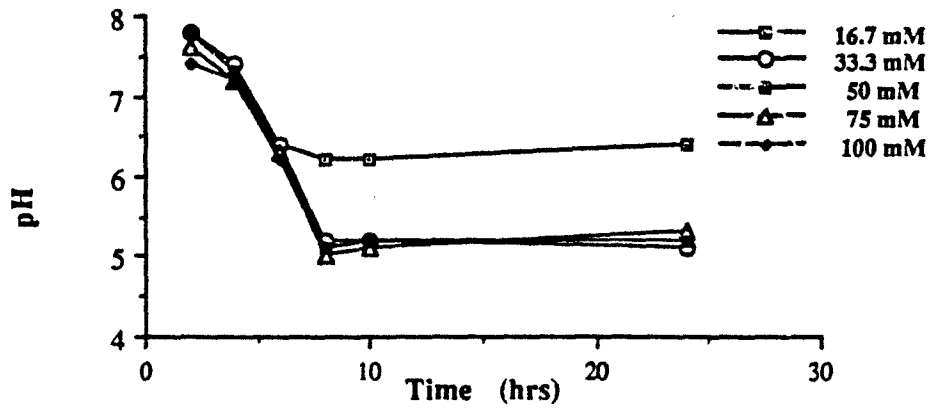

Effect of Glucose on SPE A Production
SPE A Concentration (ug/ml)
3
2
1
0
2
4
6
8
10
24
Time (hrs)
16.7 mM
33.3 mM
50 mM
75 mM
100 mM

Figure 7. Effect of glucose enriched media on growth, pH, and toxin production in 86-858. Determination of OD was used to monitor growth (top). All trials represent 1:100 inocula into 100 ml complete beef heart media (with indicated glucose buffers) grown at 37° C and 7% CO_2. pH patterns (middle) and SPE B production (bottom) in both standard and enriched media are shown. Concentration of glucose shown indicate final culture concentrations.

Effect of Glucose Concentration on Growth



Effect of Glucose Concentration on pH



Effect of Glucose on SPE B Production

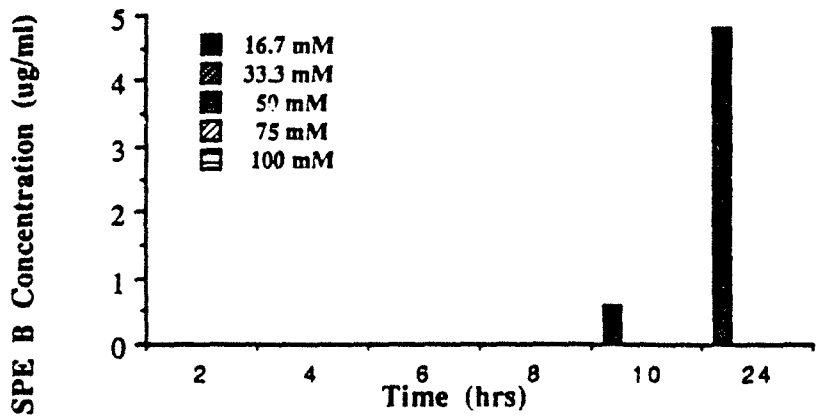
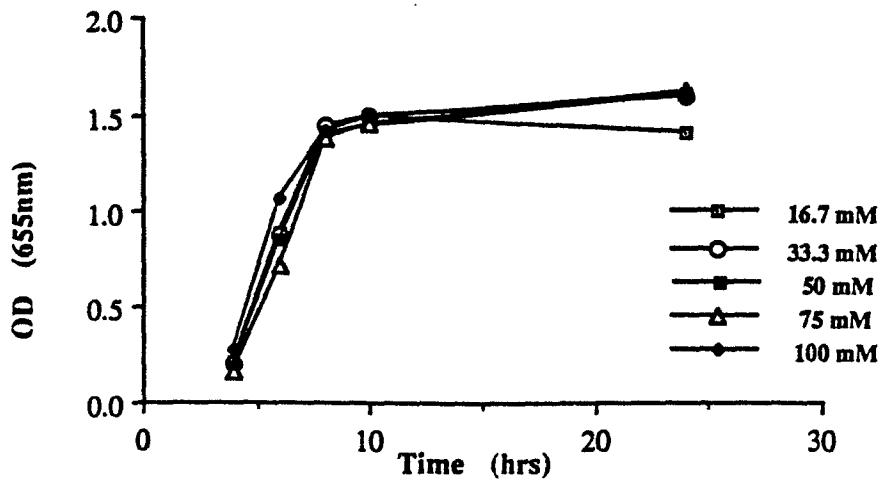


Figure 8a. Effect of glucose and tryptone enrichment on growth and toxin production in 86-858. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart media (with indicated glucose concentrations) supplemented with 1% tryptone (w/v) and grown at 37° C and 7% CO_2. SPE B patterns of production are shown (bottom). Concentration of glucose shown indicate final culture concentrations.

Effect of Glucose and Tryptone on Growth



Effect of Glucose and Tryptone on SPE B

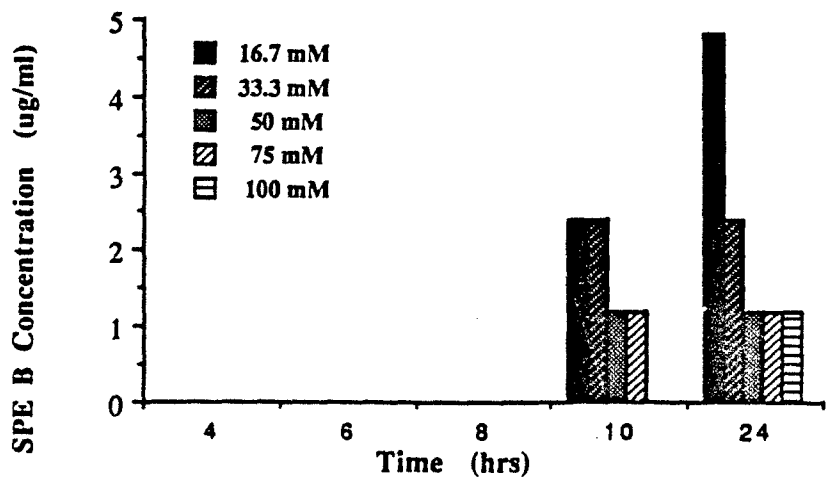
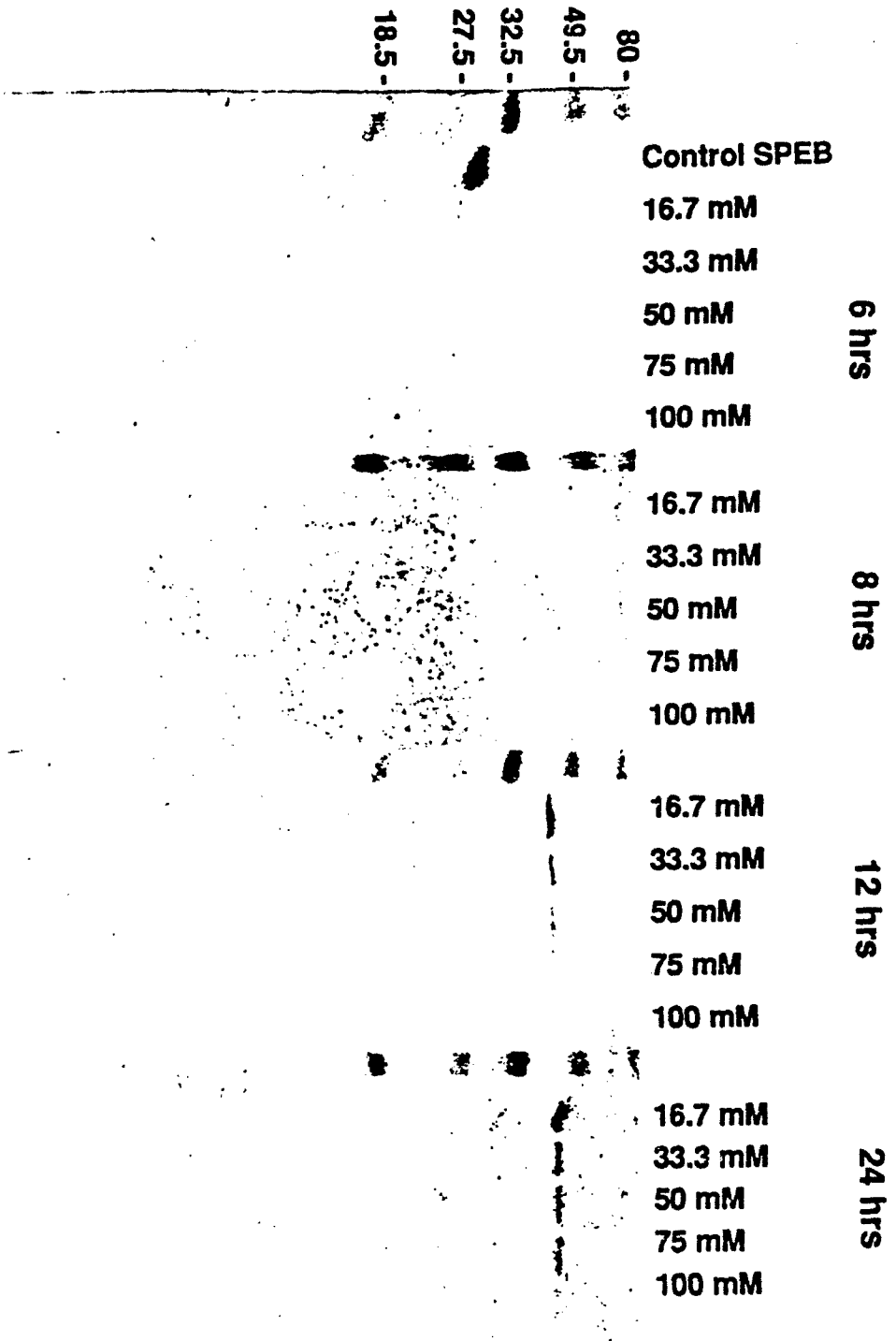


Figure 8b. Western immunoblot assay on the effect of glucose and tryptone enrichment on SPE B production in 86-858. Glucose concentrations indicated reflect final culture concentrations. Trials are identical to Figure 8a. Twenty microliters of 10 times concentrated culture fluid was loaded per lane. Concentration of SPE B was determined by Ouchterlony immunodiffusion assay: control contained 25 ug/ml at 24 hours, experimental samples contained 4.8 ug/ml, 2.4 ug/ml, 1.2 ug/ml, 1.2 ug/ml, and 1.2 ug/ml of toxin at 24 hours corresponding to glucose levels of 16.7 mM, 33.3 mM, 50 mM, 75 mM, and 100 mM respectively.


80 -
49.5 -
32.5 -
27.5 -
18.5 -
Control SPEB
16.7 mM
33.3 mM
50 mM
75 mM
100 mM
6 hrs
16.7 mM
33.3 mM
50 mM
75 mM
100 mM
8 hrs
16.7 mM
33.3 mM
50 mM
75 mM
100 mM
12 hrs
16.7 mM
33.3 mM
50 mM
75 mM
100 mM
24 hrs

Temperature of media. Six cultures of T253 cured T12, 86-858, and NY-5 were incubated at different temperatures (25° C, 37° C, and 40° C) with and without the presence of 7% carbon dioxide. T253 cured T12 demonstrated reduced growth and a proportionally reduced level of toxin production at 25° C with and without carbon dioxide through 24 hours; all other T253 cured T12 cultures reached the standard toxin levels within 10 hours. 86-858 demonstrated reduced growth and SPE B production at 24 hours in 25° C and 40° C. A unique band pattern for 86-858 was observed (Figure 9) in that an extra band was seen at 43,000 molecular weight. Strain NY-5 had a similar growth and toxin production pattern.

Oxygen content of media. To discern if the concentration of oxygen is involved in toxin production, all four strains were incubated at 37° C in either the presence of 7% carbon dioxide or aerated as described in Materials and Methods. Each strain was compared to its standard (7% CO_2) for growth and toxin production (Figures 10a, 10b, and 10c). Aerated 86-858 strain did not produce detectable SPE B; the rate of SPE A production of T253 cured T12 was reduced, but was compensated for by the 24 hour sample, whereas NY-5 SPE A production remained reduced (data not shown).

A second method was designed in order to test the effects of aeration. Bacto-tryptone was added to enhance toxin production. T253 cured T12 (Figure 11) and 86-858 (Figure 12) were run in triplicate: one culture in the presence of 7% carbon dioxide, one in stationary conditions without shaking, and one exposed to the air while shaken (80 cycles/min) (referred to as aerated). T253 cured T12 aerated produced twice the amount of SPE A compared to stationary and 7% CO_2

conditions. Similar results were observed in other experiments with NY-5 (data not shown). Conversely, 86-858 stationary and 86-858 aerated resulted in reduced amounts of SPE B 86-858 incubated in 7% CO_2.

pH of culture media. The pH of the culture media was adjusted by addition of sodium hydroxide or hydrochloric acid into three ranges: acidic (pH 5.5 to 6.5), neutral (pH 6.8 to 7.2), and basic (pH 7.6 to 8.5). T253 cured T12 (Figure 13) did not grow well in an acidic environment, yet still produced a low level of SPE A in the 6 hour sampling; normal levels of SPE A were achieved under basic and neutral conditions. Likewise, 86-858 (Figure 14) showed reduced growth in acidic conditions. SPE B was not detected at any time point in cultures under manipulated pH conditions.

Table 1 summaries the effects of environmental conditions on the growth and toxin production by the strains tested.

Figure 9. Effects of temperature on SPE B production in 86-858. Trials represent 1:100 inocula into 100 ml complete beef heart media grown as indicated. Apparent molecular weight of reactive bands is indicated at right. Twenty microliters of 10 times concentrated culture fluid was loaded per lane.

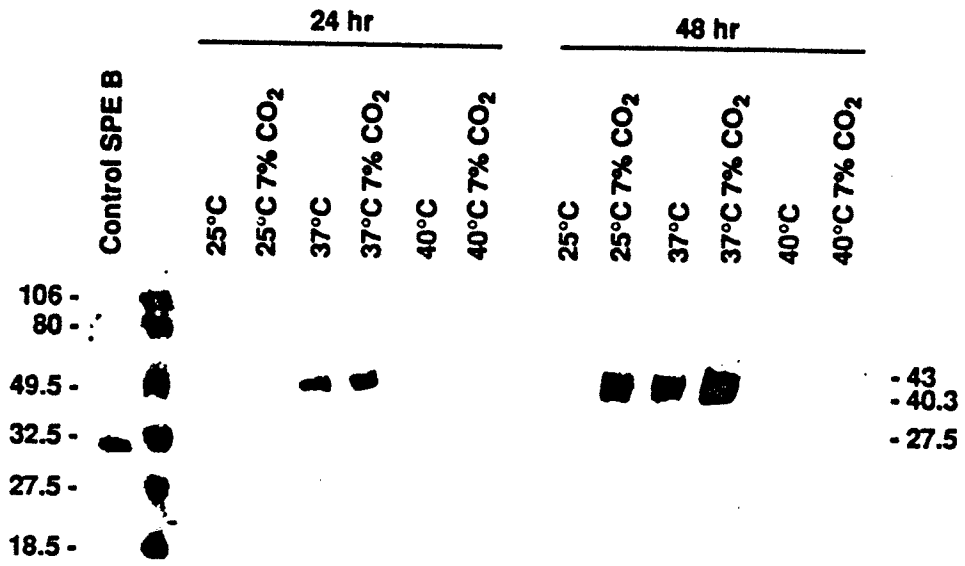
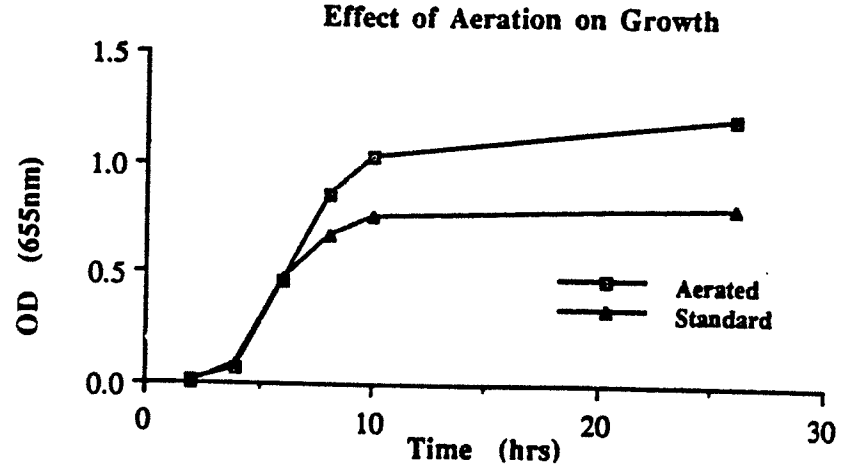
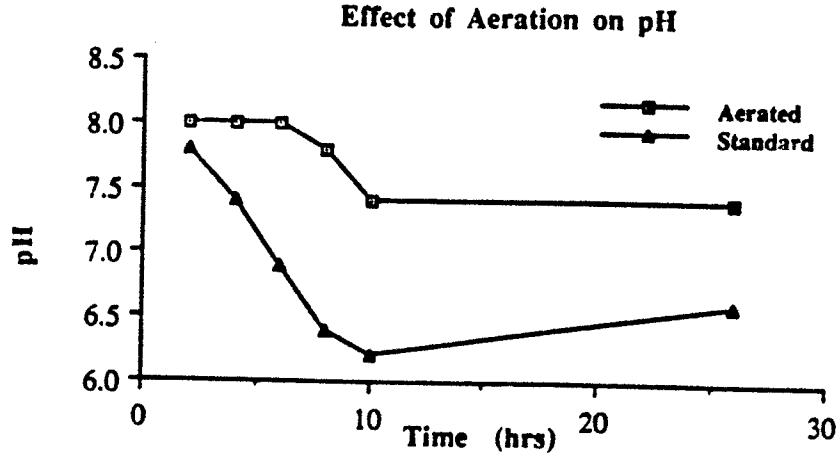

24 hr
48 hr
Control SPE B
25°C
25°C 7% CO2
37°C
37°C 7% CO2
40°C
40°C 7% CO2
25°C
25°C 7% CO2
37°C
37°C 7% CO2
40°C
40°C 7% CO2
106 -
80 -
49.5 -
32.5 -
27.5 -
18.5 -
- 43
- 40.3
- 27.5

Figure 10a Effects of aeration on growth, pH, and toxin production in T253 cured T12. Determination of OD was used to monitor growth (top). Effects on pH (middle) and SPE A production (bottom) are shown. Trials represent 1:100 inocula in 100 ml complete beef heart media grown at 37° C. Standard trials were grown in 7% CO_2. Aerated samples were placed on a mechanical shaker (80 cycles/min).


Effect of Aeration on Growth
OD (655nm)
1.5
1.0
0.5
0.0
0
10
20
30
Time (hrs)
Aerated
Standard


Effect of Aeration on pH
pH
8.5
8.0
7.5
7.0
6.5
6.0
0
10
20
30
Time (hrs)
Aerated
Standard

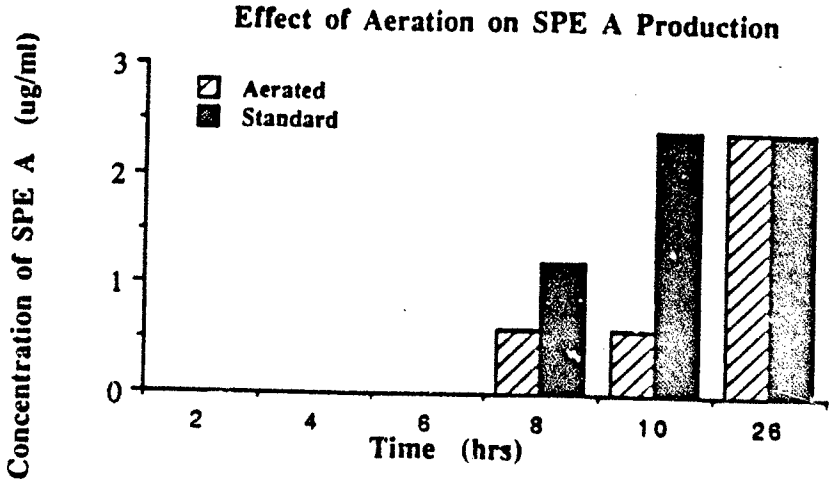
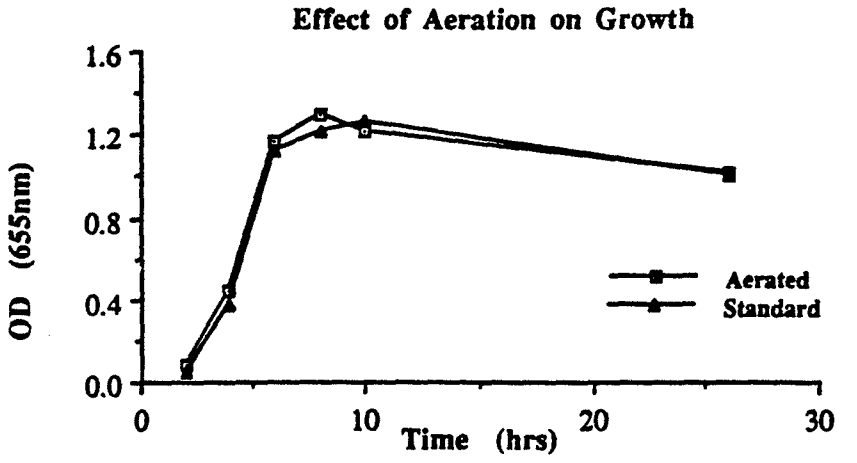
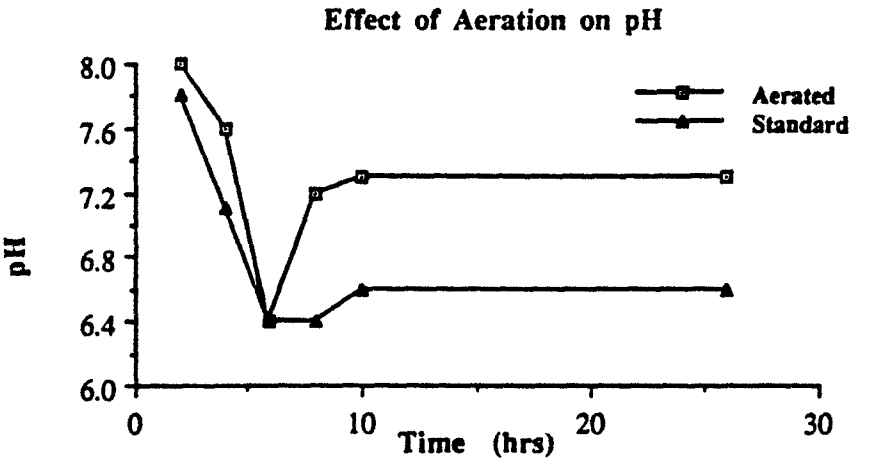

Effect of Aeration on SPE A Production
Concentration of SPE A (ug/ml)
3
2
1
0
2
4
6
8
10
26
Time (hrs)
Aerated
Standard

Figure 10b. Effects of aeration on growth, pH, and toxin production in 86-858. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula in 100 ml complete beef heart media grown at 37° C. Standard trials were grown in 7% CO_2. Aerated samples were placed on a mechanical shaker (80 cycles/min). Effects on pH (middle) and SPE B production (bottom) are shown. SPE B production did not reach detectable level.


Effect of Aeration on Growth
OD (655nm)
1.6
1.2
0.8
0.4
0.0
0
10
20
30
Time (hrs)
Aerated
Standard


Effect of Aeration on pH
pH
8.0
7.6
7.2
6.8
6.4
6.0
0
10
20
30
Time (hrs)
Aerated
Standard

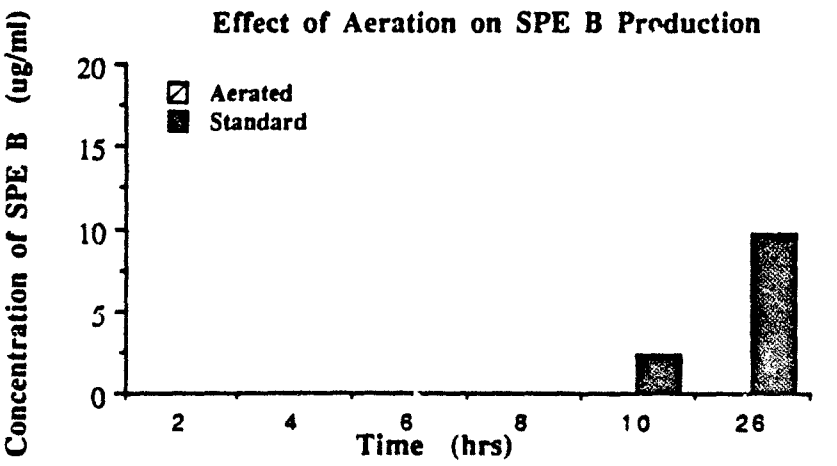
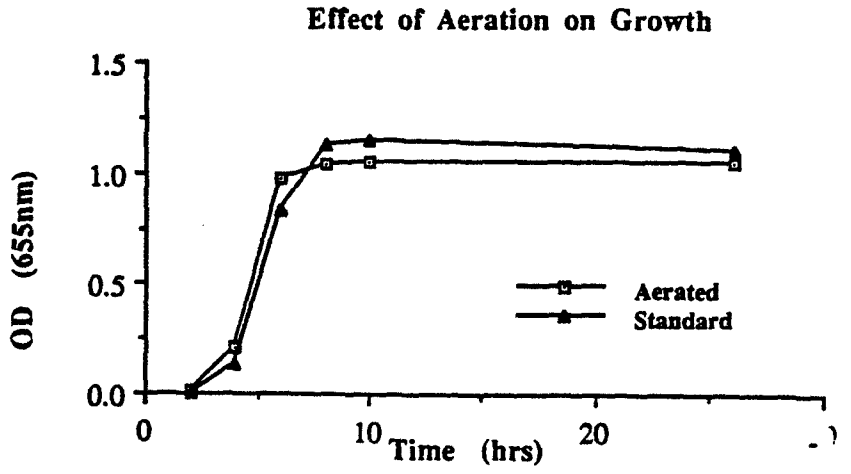
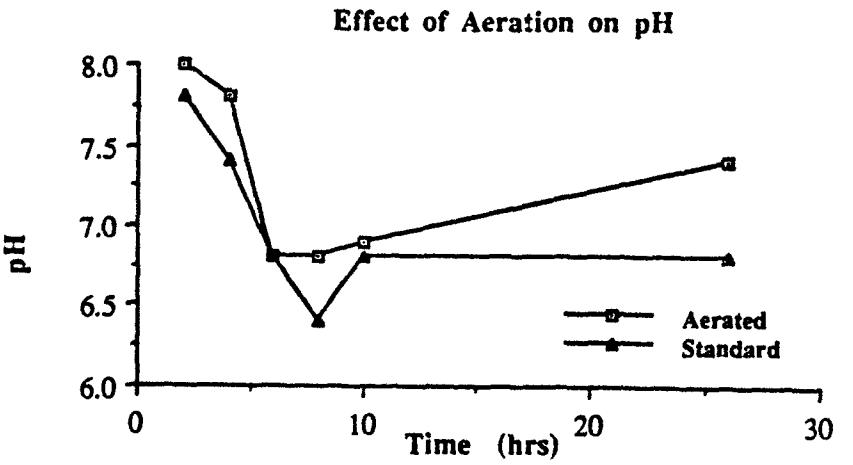

Effect of Aeration on SPE B Production
Concentration of SPE B (ug/ml)
20
15
10
5
0
2
4
6
8
10
26
Time (hrs)
Aerated
Standard

Figure 10c. Effects of aeration on growth, pH, and toxin production in T18P. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart media grown at 37° C. Standard trials were grown in 7% CO_2. Aerated samples were placed on a mechanical shaker (80 cycles/min). Effects on pH (middle) and SPE C production (bottom) are shown.


Effect of Aeration on Growth
OD (655nm)
1.5
1.0
0.5
0.0
0
10
20
Time (hrs)
Aerated
Standard


Effect of Aeration on pH
pH
8.0
7.5
7.0
6.5
6.0
0
10
20
30
Time (hrs)
Aerated
Standard

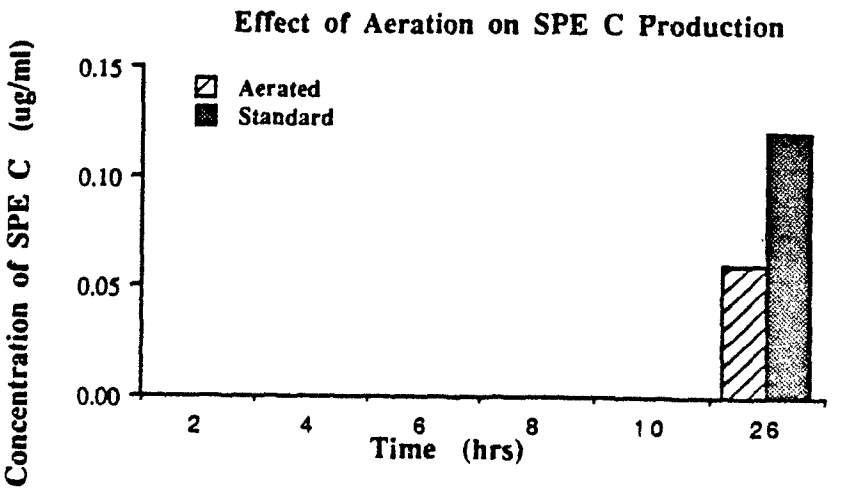
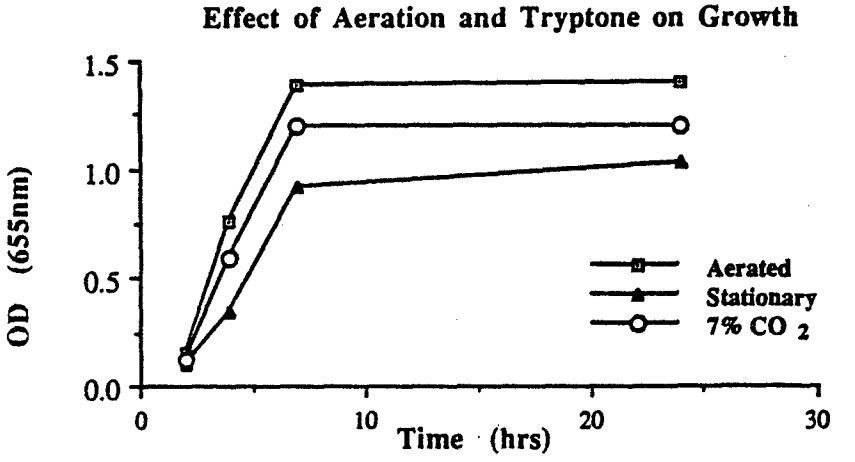
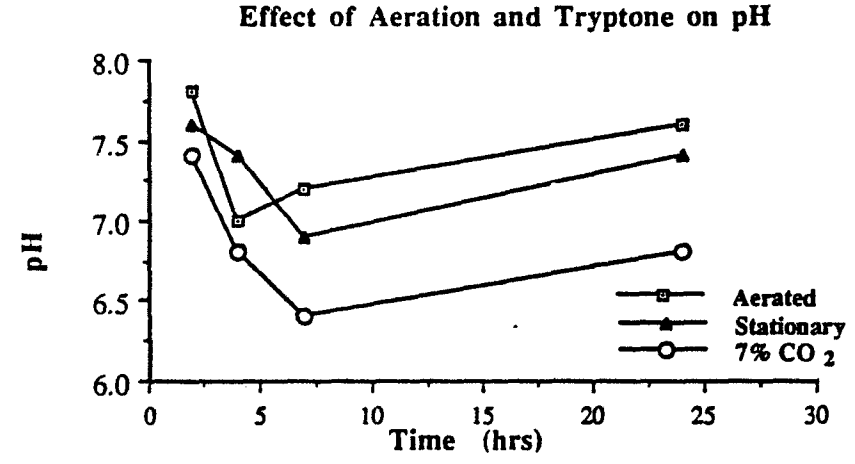

Effect of Aeration on SPE C Production
Concentration of SPE C (ug/ml)
0.15
0.10
0.05
0.00
2
4
6
8
10
26
Time (hrs)
Aerated
Standard

Figure 11. Effect of aeration and tryptone on growth, pH, and toxin production in T253 cured T12. Determination of OD was used to determine growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart media supplemented with 1% tryptone (w/v) and grown at 37° C. Standard sample was grown in 7% CO_2. The aerated sample was exposed to air and placed in a mechanical shaker (80 cycles/min). The stationary sample was exposed to the air but not shaken. pH patterns (middle) and SPE A production (bottom) are shown.


Effect of Aeration and Tryptone on Growth
OD (655nm)
1.5
1.0
0.5
0.0
0
10
20
30
Time (hrs)
Aerated
Stationary
7% CO 2


Effect of Aeration and Tryptone on pH
pH
8.0
7.5
7.0
6.5
6.0
0
5
10
15
20
25
30
Time (hrs)
Aerated
Stationary
7% CO 2

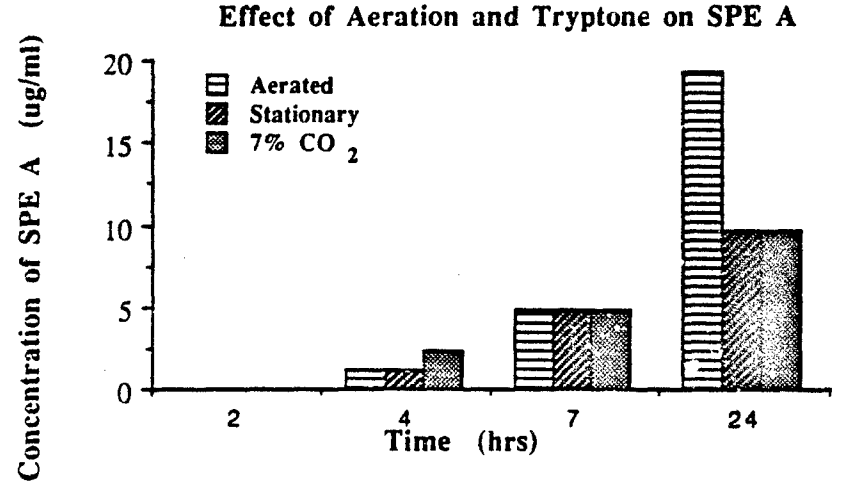
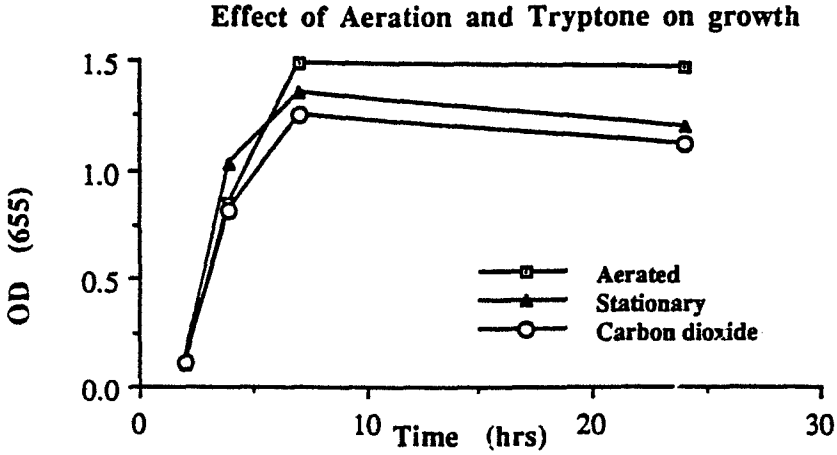
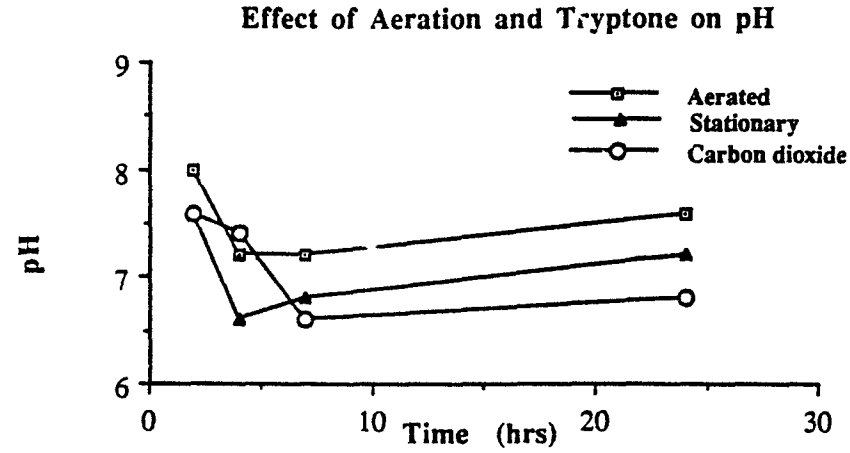

Effect of Aeration and Tryptone on SPE A
Concentration of SPE A (ug/ml)
20
15
10
5
0
2
4
7
24
Time (hrs)
Aerated
Stationary
7% CO 2

Figure 12. Effect of aeration and tryptone on growth, pH, and SPE B production in 86-858. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart media supplemented with 1% tryptone and grown at 37° C. Standard sample was grown in 7% CO_2. Aerated sample was placed in a mechanical shaker (80 cycles/min). Stationary sample was exposed to air but not shaken. pH patterns (middle) and SPE B production (bottom) are shown.


Effect of Aeration and Tryptone on growth
OD (655)
1.5
1.0
0.5
0.0
0
10
20
30
Time (hrs)
Aerated
Stationary
Carbon dioxide


Effect of Aeration and Tryptone on pH
pH
9
8
7
6
0
10
20
30
Time (hrs)
Aerated
Stationary
Carbon dioxide

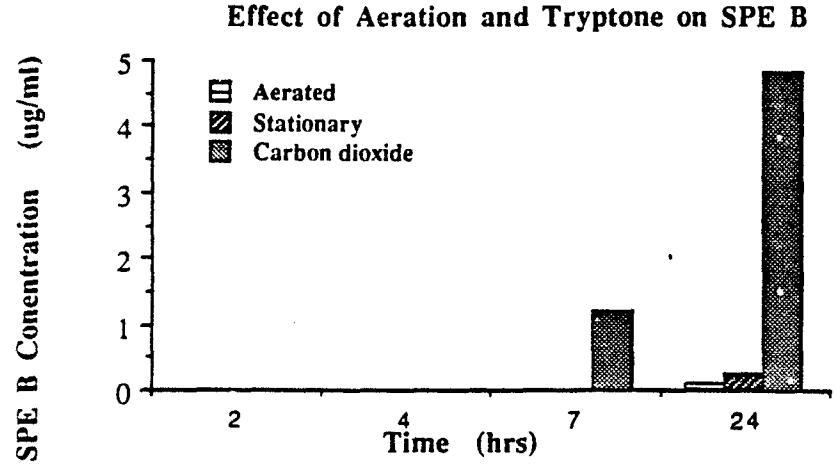
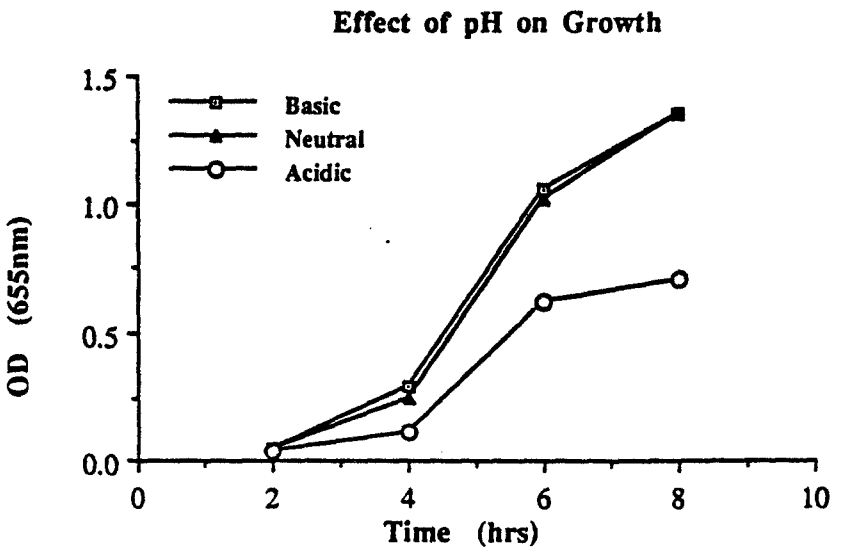

Effect of Aeration and Tryptone on SPE B
SPE B Conentration (ug/ml)
5
4
3
2
1
0
2
4
7
24
Time (hrs)
Aerated
Stationary
Carbon dioxide

Figure 13. Effect of pH on growth and toxin production in T25$_3$ cured T12. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart grown at 37^o C and 7% CO_2. pH was adjusted with either 10 N NaOH or 12 M HCl to maintain samples in specific ranges: acidic - pH 5.5 to 6.5, neutral - pH 6.8 to 7.2, and basic - pH 7.6 to 8.5. Production patterns of SPE A are shown.


Effect of pH on Growth
Basic
Neutral
Acidic
OD (655nm)
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Time (hrs)

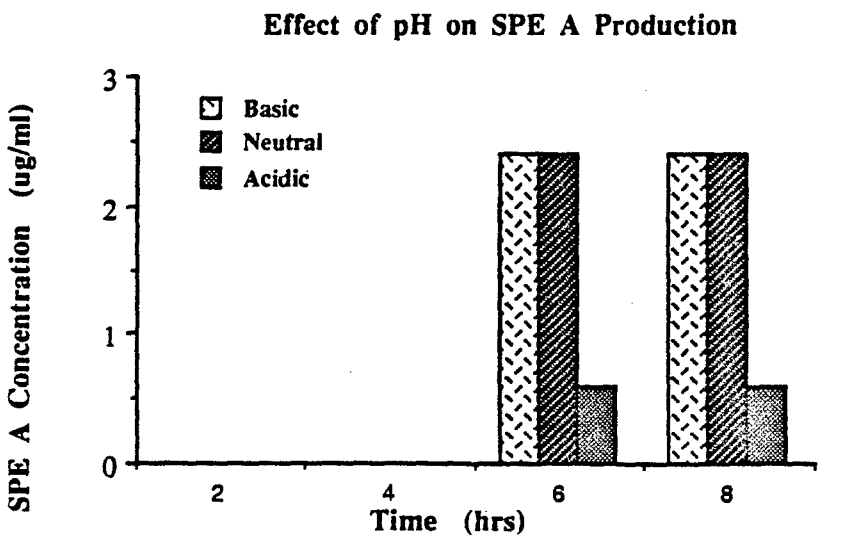
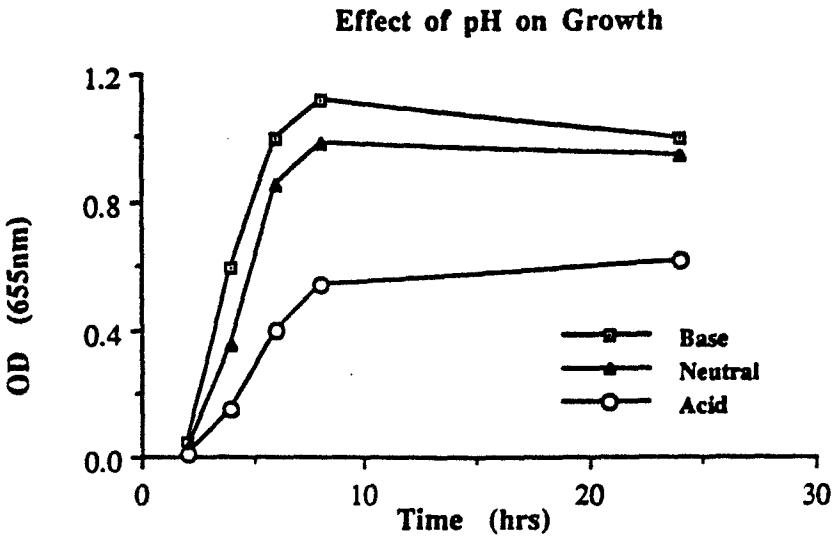

Effect of pH on SPE A Production
Basic
Neutral
Acidic
SPE A Concentration (ug/ml)
3
2
1
0
2
4
6
8
Time (hrs)

Figure 14. Effect of pH on growth and toxin production in 86-858. Determination of OD was used to monitor growth (top). Trials represent 1:100 inocula into 100 ml complete beef heart media grown at 37° C and 7% CO_2. pH was adjusted either 10 N NaOH or 12 M HCl to maintain samples in specific ranges: acidic - pH 5.5 to 6.5, neutral - pH 6.8 to 7.2, and basic - pH 7.6 to 8.5. SPE B (bottom) was not detected at any time point under any any pH condition.


Effect of pH on Growth
OD (655nm)
1.2
0.8
0.4
0.0
0
10
20
30
Time (hrs)
Base
Neutral
Acid

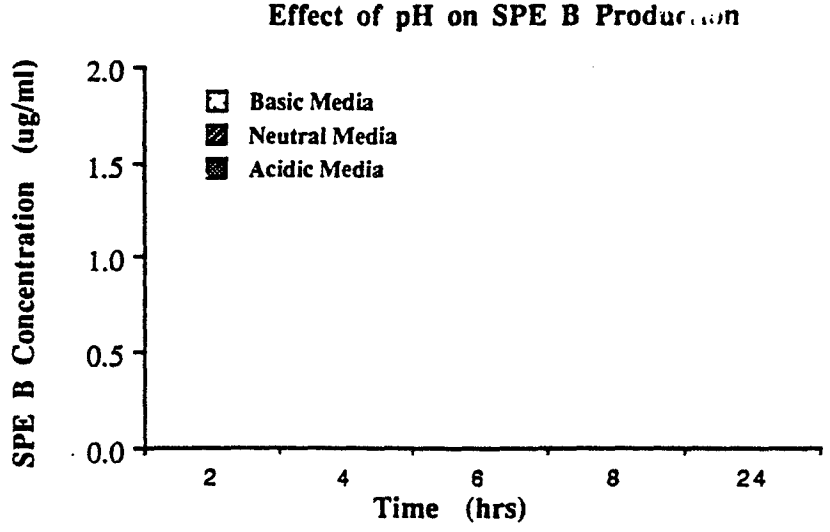

Effect of pH on SPE B Production
SPE B Concentration (ug/ml)
2.0
1.5
1.0
0.5
0.0
2
4
6
8
24
Time (hrs)
Basic Media
Neutral Media
Acidic Media

Table 1.
Summary of Environmental Influences on SPE Production.

	Growth	SPE A	SPE B	SPE C
Tryptone	++	delayed[1,2]	++	none[4,5]
Glucose	+	delayed [1]	- -	N.D.
Glucose + Tryptone	+	N.D.	-	N.D.
Temperature	varied	none	none	none
Aeration	none[3]	delayed[1,2]	- -	delayed[4]
Aeration + Tryptone	++	++	-	N.D.
7% Carbon Dioxide	+	none	++	none
pH	varied	none	N.D.[6]	N.D.

symbols: ++ = strongly positive
+ = positive
- = negative
-- = strongly negative
N.D. = not determined
none = no effect

1. Delayed production, but not final level in T253 cured T12.
2. Delayed production and final level in NY-5.
3. Aeration had a strongly positive effect on T253 cured T12.
4. SPE C was undetectable in NY-5.
5. Results standardized for growth.
6. Further investigation required.

Heterogenicity of SPE B

In this study, SPE B has been observed by Western immunoblot assay in three forms which appear to have the approximate molecular weights of 43,000, 40,000, and 30,000 mw by comparison of migration to standard markers. It has been previously reported (45) that the proteolytically cleaved form of SPE B (estimated weight of 27,500 mw) migrates at approximately 30,000 mw. In fact, this observed shift in migration is a common characteristic of all PTs. Thus, this third band will be referred to as the 27,500 mw band. Figure 15 contains a composite of Western immunoblots which helps to illustrate the different forms. The distribution of the forms varied between experiments: only 40,000 mw forms are observed in Figure 2b; 40,000 mw and 27,500 mw forms in Figures 8b and 15; and apparently all three forms are visible in Figure 9. Strong bands of the 27,500 mw form were typically observed in cultures where an acidic environment of pH 6.6 or less was achieved.

Proteinase Activity

Representative proteinase assays for strains T18P, NY-5, and 86-858 are shown in Figure 16. Different amounts and types of proteinase activity were observed, chiefly: partial clearance or haze, complete clearance, and complete clearance within a precipitant ring. Both the amount and type of proteinase activity changed during growth. The third type of activity appears to correspond with SPE B production. The effect of pH on proteinase activity was observed and recorded in Figure 17. Basic conditions produced the greatest proteinase activity and the most complete clearance, even more activity than the stock samples. In

acidic conditions, proteinase activity was not detected in the 6, 8, and 24 hour samples. No activity of the third type and no detectable SPE B was observed.

Figure 15. Western immunoblot assay of selected stationary phase 86-858 cultures. Twenty microliters of each culture fluid was loaded per lane. The SPE B control sample was loaded in lane 1 and 6. Lanes 2 and 3 depict 24 hour samples of the same stationary phase stock grown under identical conditions. Lane 4 is a sample of a 24 hour stationary phase stock culture; lane 5 is a 24 hour sample of a new culture inoculated with the stock indicated in lane 4. Lanes 7 through 9 represent duplicate 24 hour samples from cultures used in the tryptone enrichment experiment. Standard trials are represented in lanes 7 and 9 while tryptone enriched samples are shown in lanes 8 and 10.

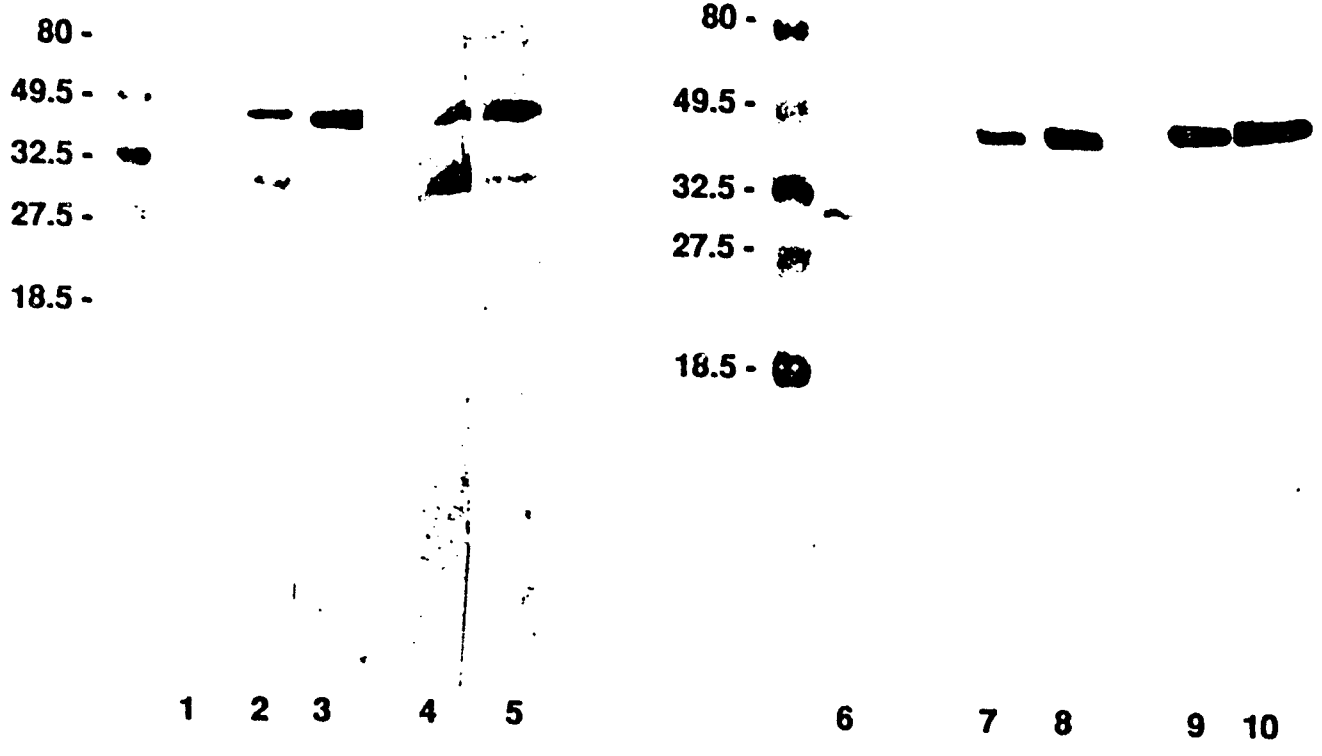

80 -
49.5 -
32.5 -
27.5 -
18.5 -
80 -
49.5 -
32.5 -
27.5 -
18.5 -
1 2 3 4 5 6 7 8 9 10

Figure 16. Selected proteinase assays for strains 86-858, NY-5, and T18P. Samples (15 ul) were loaded in each well on skim milk agarose slides (2-mercaptoethanol [20 ul/10 ml agarose], 0.75% agarose [w/v], and 1% skim milk [w/v]). Plates 1 and 2 represent samples from the 86-858 tryptone enrichment experiment. Plates 3, 4, and 5 represent samples from the standard kinetics experiment of the indicated strains. Time of sampling is denoted below each well.

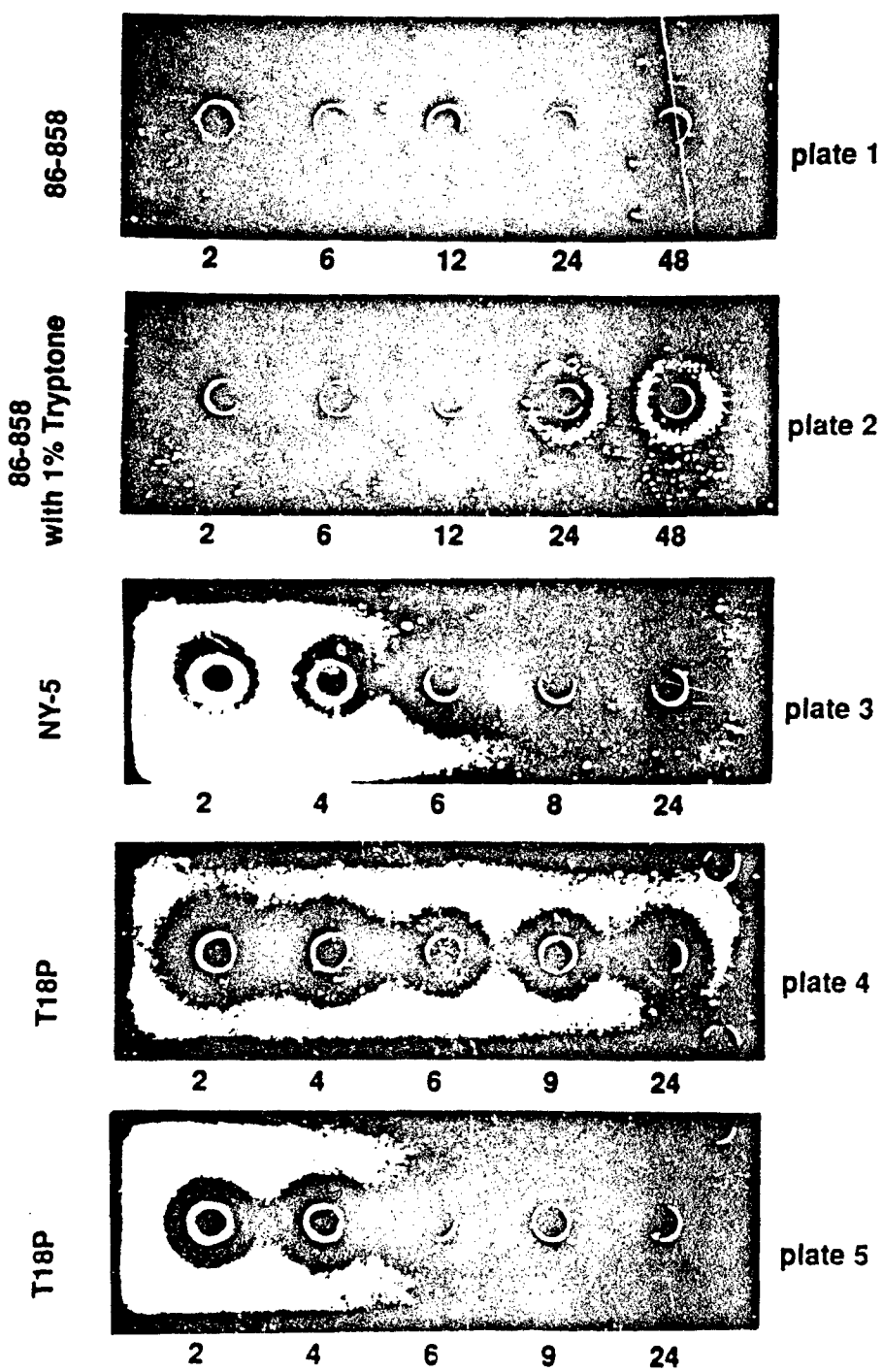
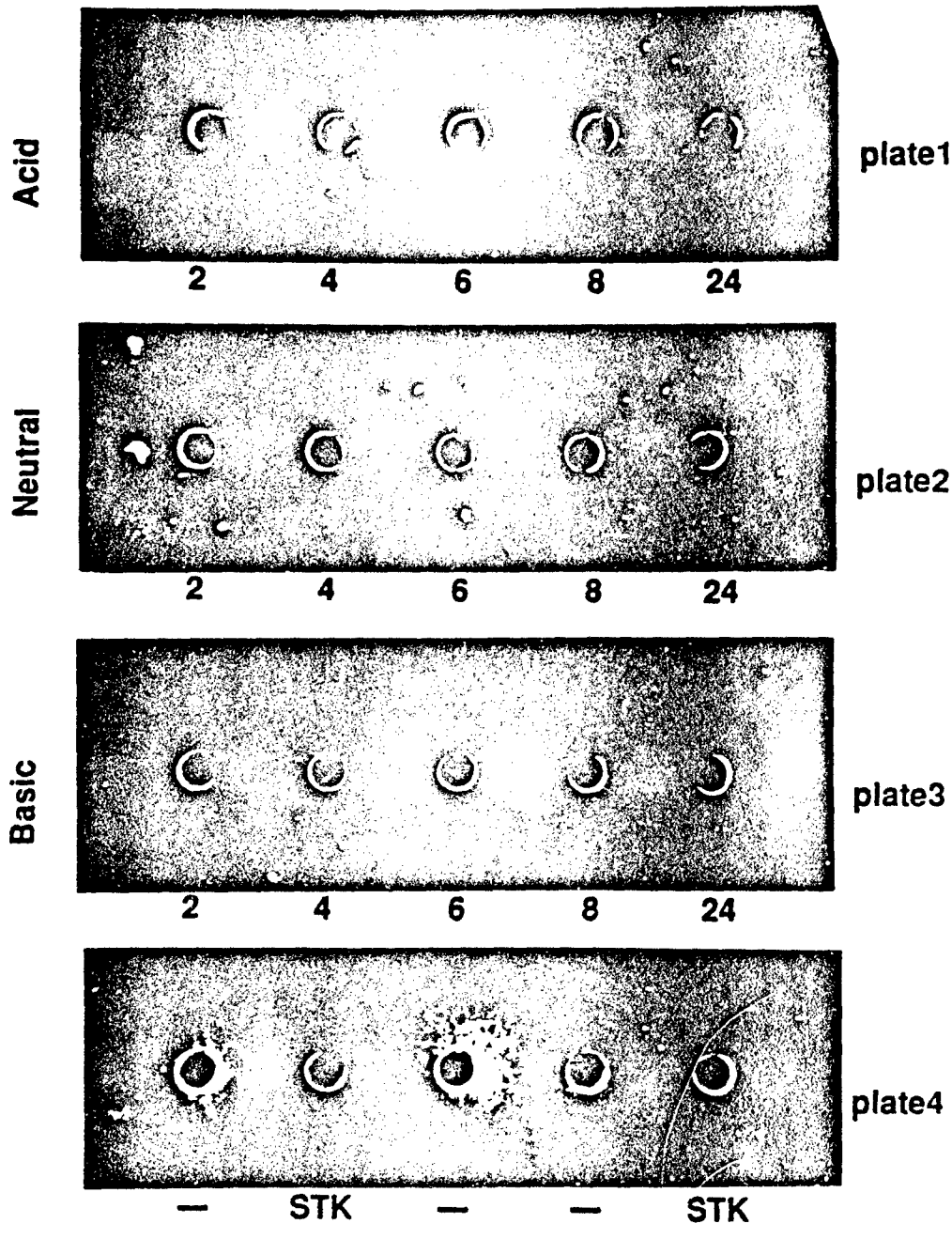

86-858
2 6 12 24 48
plate 1
86-858
with 1% Tryptone
2 6 12 24 48
plate 2
NY-5
2 4 6 8 24
plate 3
T18P
2 4 6 9 24
plate 4
T18P
2 4 6 9 24
plate 5

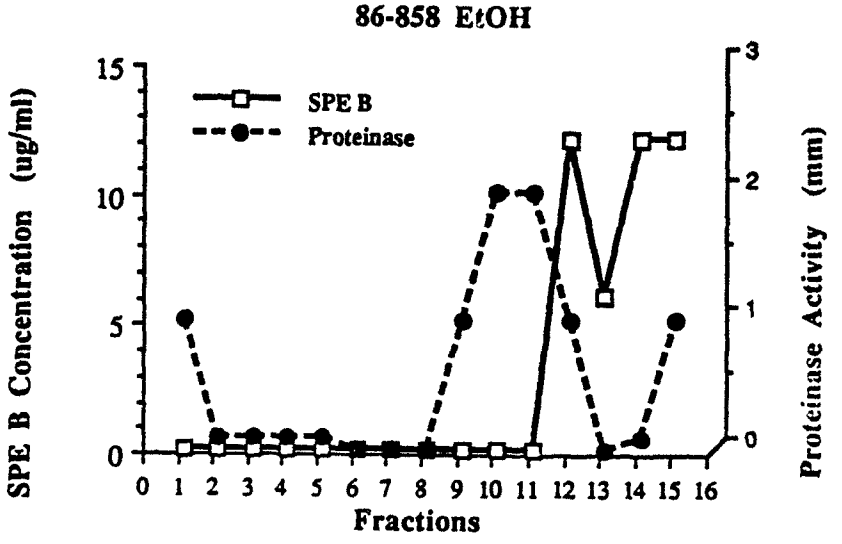
Figure 17. Effect of pH on Proteinase Assays. Samples (15 ul) were loaded in each well on skim milk agarose slides (2-mercaptoethanol [20 ul/10 ml agarose], 0.75% agarose [w/v], and 1% skim milk [w/v]). Samples represented correspond to those in Figure 14. Plate 4 contains duplicate stationary phase stock samples (STK).


Acid
plate1
2
4
6
8
24
Neutral
plate2
2
4
6
8
24
Basic
plate3
2
4
6
8
24
plate4
—
STK
—
—
STK

Separation of SPE B from Proteinase Activity.

In both strains 86-858 and T18P, the ammonium sulfate samples accumulated a larger amount of precipitate compared to the ethanol treated samples. The results of toxin and proteinase assays from the isoelectric focusing fractions are shown in Figure 18a and 18b. Proteinase activities are displayed for the IEF fractions of 86-858 EtOH and T18P (Figure 19). T18P clearly demonstrates complete clearance with a surrounding opaque ring yet does not make detectable SPE B. Cloned 713 SPE B (500 ug/ml), 86-858 A. S. fraction 10 (96 ug/ml SPE B), and 86-858 EtOH fraction 10 (less than 6 ug/ml SPE B) were concurrently tested for proteinase activity (Figure 20). 86-858 EtOH sample exhibited slightly more proteinase activity than did 713 cloned SPE B. From 86-858 fractions 10 and 14 and from T18P fractions 4 and 10 were selected for HPLC separation. Peak similarities are shown in Table 2. Values reported in the columns labelled "SPE B" and "proteinase activity" were determined prior to HPLC separation. Proteinase and Ouchterlony assays were performed on the collected HPLC peaks: all peaks were negative for proteinase and SPE B. Peak samples were then lyophilized and brought up to 100x concentration. These samples were tested again: no positive activity was found.

Figure 18a. Isoelectric focusing of 86-858. Identical cultures of 86-858 were partially purified by two methods: ethanol precipitation (identified as 86-858 EtOH) and ammonium sulfate (80%) precipitation (identified as 86-858 A.S.). Samples were subjected to IEF across a pH gradient of 3.5 to 10. Fractions were collected. SPE B concentration (ug/ml) and proteinase activity (mm) were assayed for 86-858 EtOH (top) and ammonium sulfate (bottom). Fractions are numbered (1 = acidic to 15 = basic).


86-858 EtOH
SPE B
Proteinase
SPE B Concentration (ug/ml)
Proteinase Activity (mm)
Fractions
0
5
10
15
0
1
2
3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

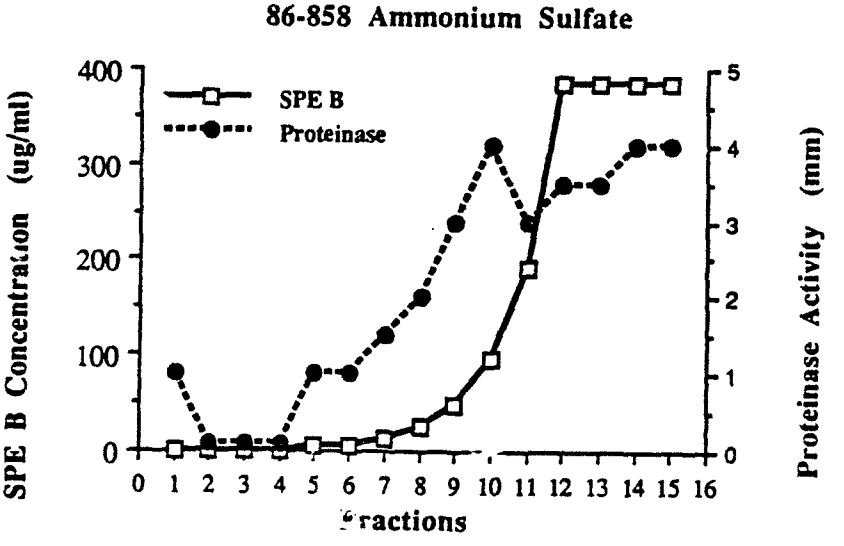
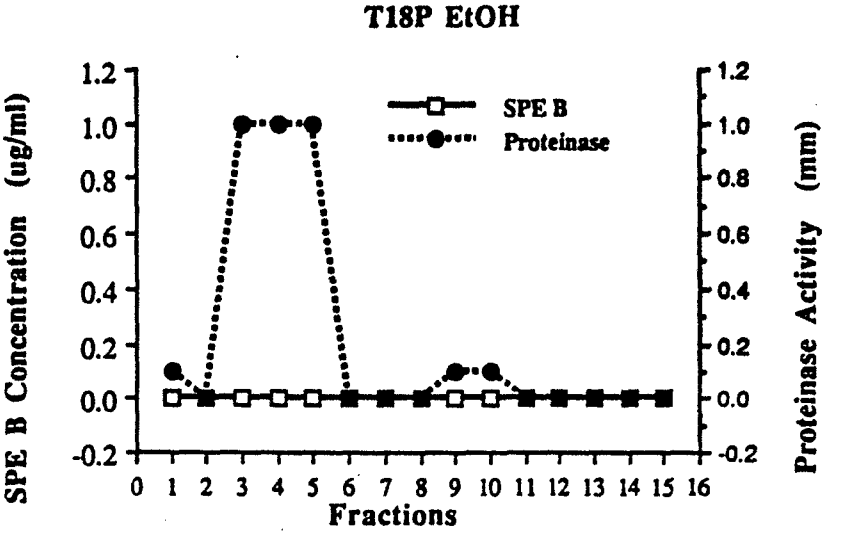

86-858 Ammonium Sulfate
SPE B
Proteinase
SPE B Concentration (ug/ml)
Proteinase Activity (mm)
Fractions
0
100
200
300
400
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 18b. Isoelectric focusing of T18P. Identical cultures of T18P were partially purified by two methods: ethanol precipitation (identified as T18P EtOH) and ammonium sulfate (80%) precipitation (identified as T18P A.S.). Samples were subjected to IEF across a pH gradient of 3.5 to 10. Fractions were collected. SPE B concentration (ug/ml) and proteinase activity (mm) were assayed for T18P EtOH (top) and ammonium sulfate (bottom). Fractions are numbered (1 = acidic to 15 = basic).


T18P EtOH
SPE B
Proteinase
SPE B Concentration (ug/ml)
Proteinase Activity (mm)
Fractions
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

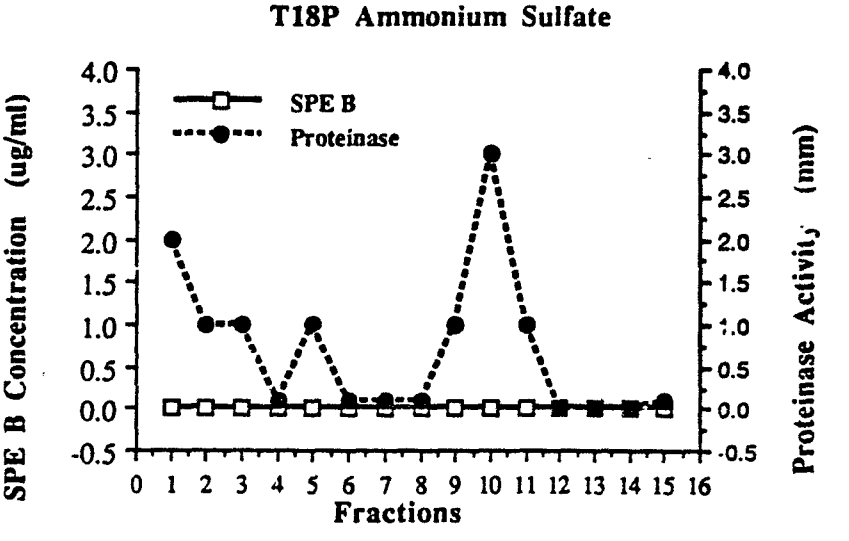

T18P Ammonium Sulfate
SPE B
Proteinase
SPE B Concentration (ug/ml)
Proteinase Activity (mm)
Fractions
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 19. Distribution of type and degree of proteinase activity after isoelectric focusing. Samples (15 ul) were loaded in each well of skim milk agarose slides (2-mercaptoethanol [20 ul/10 ml agarose], 0.75% agarose [w/v], and 1% skim milk [w/v]). Proteinase activities are displayed for the IEF fractions of 86-858 EtOH (top) and T18P (bottom). Fractions are numbered (1 = acidic to 15 = basic) below each well..

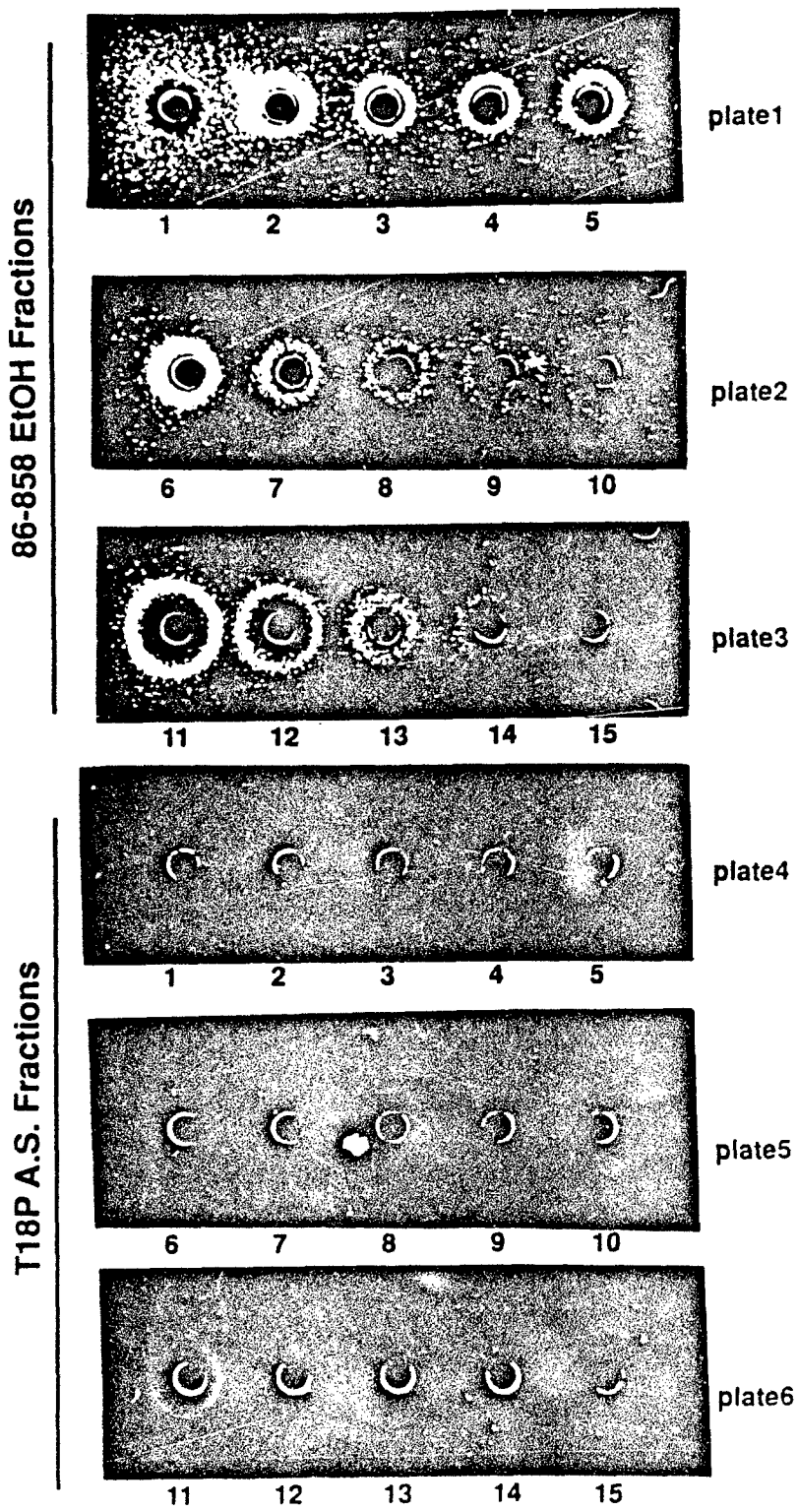
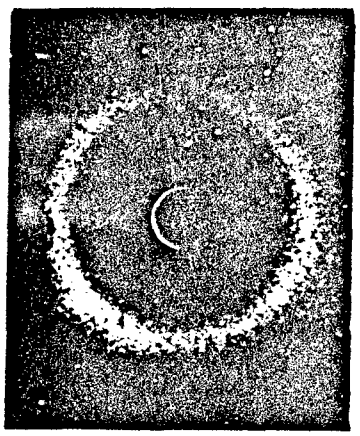

86-858 EtOH Fractions
T18P A.S. Fractions
plate1
1 2 3 4 5
plate2
6 7 8 9 10
plate3
11 12 13 14 15
plate4
1 2 3 4 5
plate5
6 7 8 9 10
plate6
11 12 13 14 15

Figure 20. Comparison of SPE B concentration to proteinase activity. Proteinase activity of 713 cloned SPE B from *E. coli* (500 ug/ml), 86-858 EtOH fraction 10 (less than 6 ug/ml SPE B), and 86-858 A. S. fraction 10 (96 ug/ml SPE B) are shown.

713
Cloned
SPE B

A

86-858
EtOH
fraction 10



B

86-858
A.S.
fraction 10

C

Table 2.

Summary of HPLC Purification of Selected 86-858 and T18P

	Proteinase[a] Activity	SPE B[b]	HPLC Elution Peaks[c] #1	#2	#3	#4	#5	#6
713 SPE B	5.0	500	12	32	33	**36**	**39**	
86-858								
EtOH #10	2.0	-[d]				**36**	**39**	
A. S. #10	4.0	96		30	31	**36**	38	43
86-858								
EtOH #14	0.1	12			31	**36**	39	
A. S. #14	4.0	384		29	31	**35**	38	43
T18P								
EtOH # 4	1.0	-				34	**36**	
A. S. # 4	0.1	-	**9**			36	38	**41**
T18P								
EtOH #10	0.1	-				37	**38**	
A. S. #10	3.0	-				**36**	39	43

a. Proteinase activity detected on skim milk agarose slides. Values are reported as mm of clearance.

b. Concentration of SPE B determined by Ouchterlony immuno-diffusion assay and reported as ug/ml.

c. Peaks reported as ml of elution. Bold print indicates strongest peak(s) in sample.

d. Negative result (i.e. < 6 ug/ml).

DISCUSSION

Since the first description in the mid 1920s by Dick and Dick (23), three serotypically distinct streptococcal pyrogenic exotoxins (SPEs) have been isolated, characterized, and sequenced: SPE A (5,49,80,110), SPE B (49,104,110), and SPE C (6,4,35,110). Their proposed involvement with streptococcal illnesses such as rheumatic fever, scarlet fever, and toxic shock-like syndrome (TSLS) (19,30,66,103) underscores the clinical significance of the production of these extracellular proteins. Moreover, recent molecular epidemiological studies (116) have confirmed previous work from this laboratory (43,44) which found that nearly 100% of the group A streptococcal isolates probed contained the gene encoding SPE B (*speB*), yet only 50% expressed SPE B (43,44,116). Also, studies have failed to show a 1:1 correlation between *speA* and SPE A (44). This research sought to establish basic knowledge of environmental stimuli which act upon exotoxin production in order to form a foundation for further molecular studies of SPE regulation.

Considering that streptococci are non-motile and thus unable to move toward favorable or away from harmful surroundings, the bacteria must be able to quickly respond to environmental changes if they are to survive. Also, group A streptococci are pathogens of humans only and thus typically find themselves in what is perceived to be a hostile environment. It is, therefore, reasonable to postulate that exotoxin production may be influenced by or respond to environmental conditions. Earlier work (17,24,33,50,76,104) reported a variety of isolated conditions and observations which provided a source of parameters for

this study. In order to identify trends in toxin production which were a result of experimental environmental manipulations, it was necessary to first establish the "normal" parameters of toxin production within laboratory strains (T253 cured T12, 86-858, T18P, and NY-5).

T253 cured T12 (M type 25) produces detectable SPE A, but not SPE B or SPE C. Figure 1a shows both methods of monitoring growth: colony forming units (CFUs) and optical density (OD) at 655 nm. Since the two methods correlate well, the OD method established by Cohen (17) was used throughout this study because it was less prone to error. Houston and Ferretti (51) have reported SPE A regulation to be constitutive. If SPE A production is constitutive (i.e. always on), then toxin level is determined chiefly by the number of viable cells and the length of time they are allowed to grow. That is to say, the more viable cells present, the more SPE A should be produced. However, SPE A, in this study, was not detected until late log phase regardless of the length of lag phase during trial. For example, trial B in Figure 1a demonstrated a long lag phase in growth and SPE A was not detected until the 12 hour sample which represented the end of log phase and the peak of growth. Moreover, trial A grew 10 fold better than trial B, achieving 8 X 10^7 CFUs at 8 hours, yet SPE A was still not detected until late log phase (12 hour sample). This argues against the accumulation of toxin simply due to growth or number of viable cells. Other experiments showed SPE A being detected as early as 6 hours after inoculation which argues against the accumulation of toxin simply as a process occurring over time. Consistent with previous reports (51), once stationary phase of growth has been reached, the level of SPE A plateaus; this level was found to be 2.4 ug/ml SPE A in T253 cured T12. Taken together, these observations suggest that SPE A is positively regulated during late log phase, when the bacterium begins to be stressed or nutrient starved, and negatively regulated

during the shift to stationary phase, when the bacterium is clearly stressed and nutrient starved.

86-858 (M type 12) produces SPE B, but not SPE A or SPE C. SPE B production corresponds to late log phase growth as detected by Ouchterlony immunodiffusion assay (Figure 3) and seen on a representative Western immunoblot assay (Figure 4). Unlike SPE A, the concentration of SPE B continued to increase while bacteria were in stationary growth, reaching a stable, final concentration by 24 hours. These observations indicate that SPE B is positively regulated as the bacterium is well into stationary phase in response to environmental conditions which limit growth.

For the experiments with SPE C, strain T18P (M type 18), which produces only SPE C, was selected. Initial results were inconsistent and the source of the problem apparently was the antisera. The reliability of the rabbit antisera against SPE C used in this study is uncertain; duplicate assays often resulted in different results. Consistent positive detection of SPE C could only be achieved down to a concentration of 20 ug/ml of control SPE C. To overcome the sensitivity problem, potentially SPE C positive samples, typically the 24 hour samples, were selected and concentrated an additional 5 or 10 fold resulting in 50x or 100x solutions. In the kinetics experiments, SPE C was only detected at 24 hours using 100x samples for Ouchterlony immunodiffusion assays. Since the gene encoding for SPE C is carried on a bacteriophage (18,55) like SPE A, it is easy to postulate that they are regulated in the same manner. Initial experiments with T18P supported the parallel model of SPE C and SPE A and thus only limited experiments were done with the T18P strain.

NY-5 was selected in order to compare SPE production in a strain which produces multiple SPEs. NY-5 has been shown to produce more SPE A than SPE B

or SPE C (50,104,110). To overcome the inherent weak production of SPE B and SPE C, the samples were concentrated an additional 5 or 10 fold which quickly exhausted the samples, thus again limiting the number of duplicate assays which could be done on each of the SPEs. Priority of usage favored SPE A and SPE B due to the unreliability of SPE C antisera. During the trials, the NY-5 strain exhibited two different types of culture pH patterns (Figures 4a and 4b). In one pattern, the pH of the culture did not drop below 6.8, whereas, in the other pattern, a drop in pH of 6.4 and below occurred. The trials with the more neutral pattern produced roughly twice the amount of SPE A as did the acidic pattern. The earlier observed plateau effect was evident: SPE A concentration leveled off once stationary phase was achieved. Interestingly, the relative amount of SPE B produced by the bacteria did not correlate proportionately to the amount of SPE A between trials, suggesting that the two toxins are under different regulatory control.

Larger inocula volumes were tested in order to achieve higher toxin yields and to distinguish if the final toxin level or plateau effect was simply the accumulation of toxin over time or if the phenomenon was linked to the growth phase of the bacteria. The assumption of the experiment was this: if the amount of toxin produced was dependent upon the number of viable streptococci per unit time, as would be expected from a constitutively regulated system, then increasing the inocula volume from the stationary phase stock culture should result in an increase in toxin production at 24 hours. However, if toxin production is triggered as a response to a limiting factor in the media, (i.e. when bacterial growth enters stationary phase), equal volumes of identical media will result in the same level of toxin regardless of inocula. Furthermore, since the given volume of media can only support a finite number of bacteria, larger inoculum sizes should reach stationary phase before smaller inocula. This was illustrated in

strain 86-858 where 2ml, 3ml, and 5ml of inocula were added to 100ml of fresh complete beef heart media. These cultures were designated: 2:100, 3:100, and 5:100 respectively. Each case resulted in a final concentration of 1.2 ug/ml SPE B at 24 hours. The sequential increase of SPE B initially detected by Ouchterlony immunodiffusion assay is apparent. The 5:100 sample was detected at 4 hours, 3:100 at 6 hours and the 2:100 at 8 hours. The plateau effect of the media is also evident. Similar results were found with T253 cured T12 and NY-5. The data suggest that inocula volume does not have an effect on the quantity of toxin produced, but rather that growth phase of the culture is involved with the initiation of toxin production.

The plateau effect observed with larger inocula suggests that a component in the media may be limiting and thus inducing stress on the bacteria in the late log phase. The addition of nutrients may delay the introduction of stress that may be affecting toxin production. The effect of neopeptone (Difco) on streptococcal growth and proteinase synthesis had been described by Cohen (17). The effect of bacto-tryptone was reported by Elliott and Dole in 1947 (25). In this study, bacto-tryptone (Difco) was selected to supplement the culture media because its composition as reported in the Difco manual is similar to neopeptone, yet is more basic (pH 7.2) in a 1% solution than is neopeptone (pH 6.8). Furthermore, bacto-tryptone (referred to as tryptone) had been previously used at this laboratory to facilitate staphylococcal and streptococcal strain growth. The .1% tryptone enrichment of the media caused a longer lag in growth in all strains (Figures 5a, 5b, and 5c) followed by a rapid and more extensive growth than the standard culture. An example of the effect of tryptone enriched media on SPE B production in 86-858 is shown by Western immunoblot in Figure 2b.

SPE A levels, whether from T253 cured T12 or NY-5, when tryptone enriched, were lower than standard in cultures throughout growth, suggesting a decrease in the rate of toxin production. In T253 cured T12, this reduction was compensated for by the 24 hour time point. Since the tryptone enriched culture grew decisively better than the standard culture, the observed recovery in production rate may be a byproduct of more growth. In fact, if the amount of SPE A is standardized for growth, an overall decrease in SPE A production results which is similar to that seen in NY-5. The tremendous growth of the enriched culture may have masked the effect of tryptone on SPE A production, however. Due to the plateau effect previously observed, SPE A production may reach a threshold concentration and induce negative regulatory effects. In this case, extent of growth is irrelevant, and the tryptone did not affect toxin production. A clear picture cannot be drawn from this experiment alone.

SPE B production, on the other hand, increased in both 86-858 and NY-5 resulting in 2 and 4 fold more SPE B, respectively, at 24 hours. In 86-858, SPE B was also detected earlier in growth than in the standard culture, but this may reflect the difference in speed of growth of the two cultures. The overall increase of SPE B in the culture, however, was not proportional to the improved growth. This implies that tryptone positively stimulated an unknown regulation system resulting in the observed increase. An unknown negative regulation mechanism apparently functions to normalize SPE B production, because the enriched culture also demonstrated a plateau effect in SPE B concentration, albeit at a higher level.

In T18P, SPE C production reached detectable levels at 10 hours with 1% tryptone, whereas, in the standard culture, 24 hours were required. Like the pattern observed with SPE A, the final level of SPE C was equal (0.12 ug/ml) in both standard and tryptone enriched cultures. This similarity supports the

postulate that SPE A and SPE C may be regulated in the same fashion. The apparent enhancement of toxin production by tryptone proved to be a useful tool in later experiments.

Early studies in the 1930s with scarlet fever toxins (49,50,76) (which were shown to be identical to the SPEs [94]) reported that, by adding glucose as a source of fermentable energy directly to the media, bacteria growth was enhanced and, therefore, toxin production increased. In strain NY-5, Hooker and Follensby also noted that the addition of 2% glucose did not effect SPE A production, but that SPE B was not detected after 8 hours (49). Experiments were therefore designed in order to quantify this reported effect using the strong toxin producing strains of T253 cured T12 and 86-858.

The first attempt to enrich for glucose simply involved increasing the volume (5ml, 10 ml, 20 ml, 30 ml, and 50 ml) of the glucose-buffer added prior to inoculation. Alterations in the toxin production pattern of both strains tested (T253 cured T12 and 86-858) were observed in conjunction with an increase in glucose buffer. The samples which contained 30 ml and 50 ml of glucose buffer in both strains demonstrated an extensive lag in growth. The T253 cured T12 cultures did however make toxin during late log phase. The 86-858 cultures did not. This procedure produced two inconsistencies: first, the concentrations of secondary salts from the buffer were not constant between cultures; secondly, the increase of additive volume affected the inocula ratio. As the amount of glucose buffer added changed the inocula ratio, the expected delay in toxin detection was observed. Moreover, the apparent dilution effect only reinforced the previous experiment in which inocula volumes were increased. However, the recovery of SPE A production in T253 cured T12 suggests that ion concentration in the media does not have regulatory influence upon SPE A. In fact, the observation

corresponds to the lack of effect of metal ion concentration on SPE A production reported earlier (51). The lack of recovery of SPE B in 86-858 suggests that ion concentration may also influence SPE B production, however, this aspect was not further pursued.

New buffers were made which varied only in the concentration of glucose. The glucose concentration in the buffers was 0.33 M, 0.67 M, 1.0 M, 1.5 M, and 2.0 M which resulted in a final sample concentration of 16.7 mM, 33.3 mM, 50 mM, 75 mM, and 100 mM glucose. This equates to 0.3%, 0.6%, 0.9%, 1.4%, and 1.8% respectively. 2% glucose had been demonstrated as being in excess (49,76,104). As shown in Figure 6, T253 cured T12 grew equally well in all buffers without the lag previously observed, thus supporting the premise that dilution was the main cause of the lag. The pH of the cultures became drastically more acidic, presumably from the production of lactic acid, with increasing amounts of glucose, but standard SPE A levels were achieved by 24 hours. This suggests that SPE A production and culture growth through the 24 hour sampling were not affected by the acidic environment or by glucose concentration. The production of SPE B in 86-858 (Figure 7) was reduced under the same conditions to undetectable levels by the standard Ouchterlony immunodiffusion assay. To enhance SPE B production, the culture media used for the second 86-858 trial was supplemented with 1% tryptone. The 86-858 strain in the tryptone enriched media demonstrated a pattern of decreasing SPE B production (Figure 8a) which correlated to the increase of glucose in the media. This pattern is clearly seen on a Western immunoblot assay (Figure 8b) even at 24 hours which is typically the production peak in strain 86-858. The results from these experiments indicate that SPE A production is not effected by glucose concentration, but that SPE B

production is greatly reduced, even with the addition of tryptone which has been shown to enhance SPE B elaboration.

Temperature of the culture media during incubation was tested for its effect on toxin production. Temperatures were selected to represent potential environments encountered by the bacteria: 25° C corresponds to environments outside the host; 37° C to environments within the host; and 40° C to the upper limit of streptococcal viability during fever within the host. These temperatures are similar to those used by Houston and Ferretti (51).

Even though the range of temperatures produced varying degrees of growth in each strain, the corresponding toxin levels proportionately responded to the changes in growth. This suggests that temperature has no effect on toxin production which is distinguishable from growth. During this experiment, two interesting observations were made. First, three bands reacted with antisera against SPE B in the 86-858 temperature trial (Figure 9). These bands apparently correspond to the three possible forms for SPE B (43,000, 40,000, and 27,500mw). The observation is more fully discussed later in this section. Secondly, some variation in toxin production was exhibited between the stationary non-CO_2 cultures and those in 7% carbon dioxide. This indicated a potential involvement of oxygen or carbon dioxide and warranted further investigation.

The apparent effect of 7% carbon dioxide on growth and toxin production compared to aerated conditions was studied. First, identical cultures of all strains were incubated at 37° C in 7% carbon dioxide or aerated by mechanical shaking. The effect of aeration on growth and toxin production (Figure 10a, 10b, and 10c) were compared to a standard control trial of each strain. All strains except T253 cured T12 grew as well in aerated conditions as in carbon dioxide. T253 cured T12 grew markedly better aerated than under carbon dioxide. The apparent

equality of SPE A production in T253 cured T12 (Figure 10a) at 26 hour sampling is due to the extensive growth of the culture under aerated conditions. Both SPE A and SPE B production in all strains was reduced in aerated conditions as compared to standard 7% carbon dioxide. Even SPE C production in T18P (Figure 10c) showed a decrease of toxin production. This suggests that oxygen may negatively influence the production of all three SPEs.

To further distinguish the effects of oxygen and carbon dioxide on streptococci, three identical cultures were incubated at 37° C: one in 7% carbon dioxide, one in stationary non-CO_2 conditions, and one aerated on a mechanical shaker. To enhance toxin production,1% tryptone was added to the cultures. In T253 cured T12 (Figure 11), the aerated sample grew better, maintained a more neutral pH, and produced more SPE A than under stationary non-CO_2 conditions or in 7% carbon dioxide. The 7% carbon dioxide sample grew slightly better than samples in stationary non-CO_2 conditions, yet produced equivalent amounts of SPE A; this implies that carbon dioxide has no effect on SPE A production. Similar results were obtained for SPE A in NY-5. In 86-858 (Figure 12), the aerobic culture grew better and maintained a more neutral pH, but still produced less SPE B than under 7% carbon dioxide. The 86-858 culture under stationary non-CO_2 conditions also produced minimal amounts of SPE B. Uniquely, the combination of aeration and tryptone in 86-858 did not result in enhanced toxin production as was seen in T253 cured T12; this supports the postulate that SPE A and SPE B could be under different regulatory mechanisms. Furthermore, when compared to stationary non-CO_2 conditions, CO_2 substantially enhances SPE B production. From this experiment, it is suggested that oxygen and tryptone apparently work synergistically to positively regulate SPE A production, whereas carbon dioxide and tryptone apparently work in unison to positively regulate SPE B production.

Since several patterns of culture pH had been observed in the previous experiments, the effect of the pH of the culture media was studied by adjusting the pH into three ranges during growth: acidic (pH 5.5 to 6.5), neutral (pH 6.8 to 7.2), and basic (pH 7.6 to 8.5). Previous studies (76) reported that a neutral pH was optimum for growth. Others, however, have suggested that an acidic environment is best for toxin production (32). To determine the effect of each range, three identical cultures were adjusted to the appropriate pH range and incubated at 37° C under 7% carbon dioxide. In a separate experiment, it was determined that the pH of media alone under 7% CO_2 would only drop from pH 7.8 to 7.2 in 24 hours; therefore, the drastic drops in pH were due primarily to bacterial growth. In $T25_3$ cured T12 (Figure 13), the basic and neutral cultures grew and expressed SPE A equivalently. The decrease in growth and subsequent toxin expression in the acidic culture was expected since it is known that, as streptococci grow, the pH of the culture drops and, if the media is acidic, the growth of the culture will be limited (49,76,104). In 86-858 (Figure 14), the highest growth was seen in basic conditions, while acidic conditions demonstrated markedly reduced growth. From previous experiments, it was expected that SPE B production would be lower in cultures under acidic conditions as compared to more neutral conditions (as seen in Figures 2a, 4a, 5b, and 7). Surprisingly, in pH manipulated conditions (Figure 14) SPE B was not detected in any of the cultures, even after concentrating the sample to 100x. To compensate, 1% tryptone was added to the cultures to enhance SPE B production. Unfortunately, the tryptone enriched cultures grew so quickly that the pH ranges were not maintained or that the extreme growth required a large volume of concentrated reagents which lysed bacteria. When less concentrated reagents were used, the additional volume required to pH adjust the media altered the overall inocula ratio in the culture.

This change shifted the growth cycle of the individual cultures and decreased the validity of direct comparisons between the cultures. Repeated attempts to grow three cultures of 86-858 side by side failed and the partial data varied due to the differing culture volumes. Therefore, the effect of pH on SPE B production was not assessed. SPE A production, however, appears not to be effected by pH.

Although regulation mechanisms of SPE production have not yet been fully characterized, a considerable amount of research has been directed toward the regulation of most of the other PTs (5,6,43,71,75,81,85,86). A common observation concerns the regulation of differing staphylococcal exoproteins by the accessory gene regulator (*agr*) locus (61,85). The *agr* locus has been reported to positively regulate the production of hemolysins (alpha, beta, and delta), SEB, SEC, SED, and TSST-1 and to negatively regulate protein A and coagulase elaboration, yet has no effect on SEA (61,71,84,85). It has been postulated that *agr* exercises an action similar to a two component system, and a model of regulation has been suggested (71). Briefly, as the bacteria experience a decline in exponential growth, a signal is generated and then transmitted through multiple transducing components and finally results in the production of RNA III which has been shown to directly regulate exoprotein expression (71). This product of *agr*, RNA III, was reported to be a transacting, DNA binding molecule which regulates exoprotein at the level of transcription (84,85). This model was supported by further studies (5,71) which identified DNA binding sites near the location of transcription initiation. Interestingly, *agr* has been shown to influence cloned SPE A production in *Staphylococcus aureus* (54).

Another common mode of regulation is catabolite repression. In general, when bacteria are grown in standard media in which the amount of glucose is limiting, a signal is produced which, through multiple transduction and activator

molecules, typically cyclic AMP and CAP, promotes the transcription of necessary genes for catabolism or other functions. The presence of high concentrations of glucose decreases the amount or availability of the signal molecules which in turn decreases the transcription of the regulated genes. This form of negative regulation has been shown to effect the production of SEA, SEB, and SEC (71, 84,85) and the amount of steady-state mRNA in SEC (86). In *E coli*, the catabolite repression system depends on cyclic AMP and the catabolite gene activator protein (CAP) (71,84,86), but a cyclic AMP independent repression system has been suggested for *Staphylococcus aureus* (86) since staphylococci do not appear to have cyclic AMP. The method of catabolite repression in streptococci is not well defined.

A mode of SPE production regulation can be hypothesized from the kinetics experiments and a summary of environmental influences on elaboration (Table 1). It has been observed that SPE A is detected in late log phase and that production ceases as the culture enters stationary phase. On the other hand, SPE B is detected in late log phase and production continues until stationary phase is well established. The switch near late log phase to turn on toxin production corresponds to the expected pattern if regulated by *agr*, but the continued expression of SPE B well into stationary phase does not fit the pattern. This regulatory effect of *agr* could be easily assayed by the introduction of cloned SPEs into agr^- and agr^+ bacteria. The results under similar conditions used in this study would directly test the hypothesis of *agr* regulation. Studies have shown that *speA* in *S. aureus* is under *agr* control (P.M. Schlievert, personal communication). Studies have been done to clone SPE B into such strains, and none of the resultant clones expressed SPE B. It is possible that *S. aureus* proteinases destroy any SPE B made.

SPE B production is apparently also regulated by catabolite repression. The separation of regulatory effects on other PTs from *agr* and glucose has been previously established (86). SPE B production was drastically decreased with the addition of glucose to the media; this response exemplifies regulation by catabolite repression. Even after the addition of 1% tryptone, a demonstrated SPE B enhancer, the levels of production were markedly reduced and the glucose concentration dependency evident (Figures 8a and 8b). Although this alone strongly supports the postulate, additional experiments using glucose analogs (such as glycerol, maltose, or pyruvate) would solidify the correlation. Another regulation effect upon SPE B production similar to that of glucose was observed in aerated samples. This effect is attributed to the presence of oxygen which greatly reduced the levels of SPE B as compared to standard cultures. The addition of tryptone did not fully compensate for the loss of production.

Throughout this study, a variety of conditions were imposed upon the bacterial strains and numerous SDS gels and Western immunoblot assays have been run. From this pool of data, SPE B has been observed in three different forms which migrate uniquely. When compared to standard molecular weight markers, the forms migrated at approximately 43,000, 40,000, and 30,000 mw. The 713 SPE B control, and HPLC purified SPE B clone from *E. coli*, also migrated at approximately 30,000 mw. The molecular weight of 713 SPE B has been estimated to be approximately 27,500 mw(45) and has been previously shown to migrate near 30,000 mw(46). This smallest size band was thus referred to as the 27,500 form. The 43,000 form corresponds to the translated protein with its 27 residue signal peptide; the 40,000 form to the mature expressed protein; and the 27,500 form to the proteolytically cleaved protein (43,44). Some cultures only expressed detectable levels of the 40,000 form (Figure 2b and 15), whereas others expressed

the 40,000 and 27,500 forms (Figure 8b and 15), or apparently all three forms (Figure 15). The cloned 713 SPE B control demonstrated only the 27,500 form.

The higher molecular weight bands observed in Figure 9 (lanes 4, 5, 9, 10, and 11) could be the 43,000 and 40,000 forms of SPE B. However, it is unlikely that the bacterium would accumulate the fully translated yet untransported 43,000 form. Furthermore, this pattern was only observed in this particular experiment when identical conditions (as in lane 5 and 11) were used in over twenty trials with the same strain. Since both bands are equally strong within each lane, it is most likely an artifact caused during the running of the gel. However, the possibility of the appearance of the 43,000 form cannot be completely ruled out since crude cell lysates were tested.

It has been suggested that proteolytic cleaving, which causes the 27,500 form, occurs during toxin purification (43), however, in this study, partial cleavage has been occasionally observed in the single ethanol precipitated crude samples. The comparison of stationary phase crude samples of 86-858 (Figure 15) revealed that the proportional distribution of 40,000 and 27,500 forms varied between experiments. Two cultures of 86-858 simultaneously grown in identical media under the same conditions produced two patterns: one with both 40,000 and 27,500 forms (lane 2) and another with only the 40,000 form (lane 3). This difference suggests that environmental conditions alone are not responsible for the cleavage. Likewise, proportional distribution of the inoculum does not determine the distribution of the toxin. For example, a stock culture which contained proportionally more 27,500 form (Figure 23, lane 4) produced a culture with more 40,000 than 27,500 form (lane 5). This change may be due to the concentration or the difference in efficiency of proteinases in the culture. The addition of neopeptone (17) and some forms of bacto-tryptone (25) have been

shown to effect proteolytic activity in streptococci. In this study, the addition of Difco-brand bacto-tryptone apparently did not negatively effect the proteinase activity needed to produce the 27,500 form (lanes 9 and 10) nor does the addition of tryptone solely initiate the activity required to produce the smaller form (lane 7 and 8). The appearance of the lower molecular weight band corresponds with those cultures in which the pH dropped to below 6.5 during growth. This observation is supported by previous studies (17) which reported streptococcal proteinase only to be produced or activated at pH < 6.5. This active streptococcal proteinase may therefore be involved in the appearance of the 27,500 proteolytic form of SPE B. Furthermore, previous work in this laboratory has shown that multiple molecular weight forms of SPE B appear when iodoacetic acid, a proteinase inhibitor, is added (43). It was therefore necessary to investigate the proteolytic activity.

It has been previously shown (109) that group A streptococci produce many extracellular proteins, some of which possess proteolytic activity. In 1945, Elliott and co-workers (24,25,68) characterized an extracellular protein which was reported to be the source of proteinase activity. This protein was found to be produced as an inactive zymogen (approximately 36,700mw) which, in sufficient reducing conditions, undergoes proteolytic cleavage resulting in an active proteinase (approximately 32,000mw) (45, 114). Gerlach et al. (33) have suggested that the zymogen, designated streptococcal proteinase precursor (SPP) (107) and streptococcal pyrogenic exotoxin B (SPE B) are the same protein. Comparison of the published inferred amino acid sequences of SPE B and SPP (26,107,114) demonstrate a high degree of homology between the two proteins differing at 13 amino acid positions. Furthermore, the similar isoelectrical and serological characteristics of the proteins suggest that they may exist as variants of

the same protein (2,32,33,45). In 1969, Cohen demonstrated the relationship of "streptococcal proteinase" production and environmental effects (17). Proteinase production was shown to be inhibited under three conditions: 0.5% neopeptone, low carbohydrate level at neutral pH, and by preventing the pH of the culture from dropping below pH 6.7. Elliott and Liu (26,68,69) have reported streptococcal proteinase elaboration to begin at late log phase and to be inactivated by iodoacetic acid, iodoacetamide, metal ions (Cu^{++} and Ag^{+} for instance) and atmospheric oxygen. It has also been suggested that streptococcal proteinase is a sulfhydryl enzyme, containing half of a cystine residue per molecule (68).

During screening the samples for proteinase activity using well diffusion on skim milk agarose slides, as described in Materials and Methods, three distinguishable proteolytic activities were noted. The first activity was a partial clearance of the skim milk protein resulting in a light haze. The second was a complete clearance of all milk protein forming a clear circle on the slide. Thirdly, a complete clearance surrounded by a brighter, denser ring of precipitated milk proteins occurred. For ease of discussion, the activities will be referred to as type 1, type 2, and type 3 respectively. In previous studies, type 1 and type 2 activity have been considered the result of acidic samples in similar assays (17). However, the pH of each sample tested in this study was determined and was in a range of pH 6.9 to 8.0. When sterile culture media containing 5% glucose-buffer was pH adjusted (range 8.5 to 6.0) then tested for proteinase activity, a pH below 6.6 was required for clearance. Therefore, the proteinase activity observed in the tested samples (Figure 16) was apparently from proteinase within the sample and not just caused by acidic effects. Moreover, both type 1 and type 2 activity have been observed to decrease or increase with strain growth (Figure 16) implying that the causative substance is being negatively or positively regulated. Duplicate

cultures of the same strain have been observed with and without type 1 or type 2 activity (Figure 16, Slide 1 and 2). Still, the possibility that type 1 and type 2 activity may be varying degrees of the same proteolytic activity cannot be ruled out. Type 3 activity (complete clearance with an opaque ring) has been previously reported (52) as the indication of positive proteinase activity. As shown in Figure 16, observed type 3 activity generally correlated to the presence of higher concentrations of SPE B: 4.8 ug/ml SPE B in the 24 hour sample of 86-858 (Slide 1) shows type 3 activity as compared to 0.12 ug/ml SPE B in the 24 hour sample of NY-5 (Slide 3) which shows type 2. From these results, type 3 proteinase activity was apparently a marker for accumulated SPE B.

The report (17,33) that streptococcal proteinase is produced only at pH < 6.5 was then tested (Figure 17). The apparent type 1 or type 2 activity was well pronounced in the basic and neutral samples, but it was lost as the culture grew in the acidic environment. An extremely small, faint opaque ring around the 24 hour acidic sample may indicate weak type 3 activity. Because type 1 and type 2 proteinase activity were clearly evident in the basic and neutral cultures, Cohen must have been considering type 3 as being a positive. Since SPE B was not detected (down to 0.06 ug/ml) in any of the three conditions, the correlation of type 3 proteinase activity and SPE B appeared to be correct.

This correlation of type 3 activity and SPE B, however, was weakened by other observations. For example, the stationary stock samples (Figure 17) produced detectable SPE B (at least 0.6 ug/ml) but did not exhibit a type 3 profile. Furthermore, when comparing the type of proteinase activity in the isoelectric focusing samples, it was found that both 86-858 (SPE B^+) and T18P (SPE B^-) possess fractions with type 3 activity (see Figure 19). Thus, SPE B may not be the source of type 3 proteinase clearance.

Separation of SPE B from proteinase activity was then attempted through isolation and purification. Strains T18P (SPE B^-, $proteinase^+$) and 86-858 (SPE B^+, $proteinase^+$) were selected. In order to directly compare previous published findings, the ammonium sulfate precipitation method of Elliott and coworkers (24,26,29) and the ethanol precipitation method of Wannamaker and Schlievert (109) were both incorporated into the protocol. The strains were grown using the same preparations of media and buffers to eliminate possible differences due to reagents. The first level of separation after isolation was thin layer preparative isoelectric focusing with a pH gradient of 3.5 to 10.0. The plate was divided into 15 fractions (acidic = 1, basic = 15) and tested for SPE B and proteinase activity (Figure 18a and 18b - 86-858 and T18P respectively). Three peaks of proteinase activity (centered on fractions 1, 10, and 14) could be seen in the 86-858 samples. Although the T18P samples exhibited other proteinase peaks, they possessed only two of the three peaks (fractions 1 and 10) observed in 86-858.

The first two proteolytic peaks (fractions 1 and 10) were found to be different from SPE B. If SPE B is solely responsible for all proteinase activity, then all proteinase positive fractions would contain SPE B in some form. However, this is unlikely because the polyclonal antisera used to detect SPE B should have reacted with all forms of SPE B and the peaks (fractions 1 and 10) did not react with the antisera. Because both 86-858 EtOH fraction 10 and T18P A. S. fraction 10 show a high degree of proteinase activity, yet exhibit less than 6 ug/ml SPE B, this implies that SPE B cannot be solely the cause of this proteolytic clearing. In order to define the relationship between SPE B concentration and proteinase activity, both fraction 10 samples (EtOH and ammonium sulfate) from 86-858 were tested concurrently with 713 cloned SPE B for activity (Figure 20). 713 cloned SPE B (500 ug/ml) exhibited slightly less type 3 activity than 86-858 EtOH

fraction 10 (less than 6 ug/ml SPE B), thus it appears that the concentration of SPE B is independent of the degree of proteinase activity in a sample. Taken as a whole, the observations in this study demonstrate a partial divergence of proteolytic activity and the presence of SPE B as tested by Ouchterlony immunodiffusion. The presence of similar types of proteinase activity in isoelectric focusing fractions of both T18P and 86-858 (Figure 19), the non-correlation between concentration of SPE B and degree of proteinase activity (Figure 20), and the lack of polyclonal antisera activity with the proteinase-positive IEF fraction 10 support the postulate that SPE B is not solely responsible for all of the streptococcal proteinase activity.

Conversely, the third proteinase peak (fractions 14 and 15) observed in 86-858 may be linked to SPE B. This third peak was evident in the 86-858 (SPE B^+) samples (both EtOH and ammonium sulfate), but it was not seen in the T18P (SPE B^-) samples (Figures 18a and 18b). Since 713 cloned SPE B (Figure 20) has been shown to possess some proteolytic activity, it seems reasonable that SPE B in fractions 14 and 15 may contribute to the proteinase activity observed in the samples. Furthermore, in the 86-858 ammonium sulfate sample, both high levels of SPE B and proteinase activity were found in fractions 12 through 15. It was therefore necessary to attempt further separation of proteinase from SPE B by an alternative method.

Fractions with strong proteinase activity and those with highest concentrations of SPE B were subjected to HPLC in order to identify potential differences in composition as compared to 713 cloned SPE B. The results of the experiment are summarized in Table 2. The ammonium sulfate fraction of both strains characteristically contained an additional peak (eluting between 41 to 43) which did not correlate to SPE B or proteolytic activity. A direct correlation does

apparently exists between prior detection of SPE B and peaks that elute between 29 and 33. However, these peaks are small, suggesting they are not solely responsible for the prior reactivity of the protein with SPE B antisera. Unfortunately, the proteinase and SPE B tests on the collected HPLC peaks were all negative. Even after lyophilization and concentration of the samples 100 fold, the tests for SPE B and proteinase were still negative. The loss of activity may be a result of the HPLC process itself. Since the 713 cloned SPE B sample had been previously run through HPLC, the possibility of complete inactivation does not appear likely.

Overall, my studies indicate that there are likely three forms of proteinase (types 1-3), two of which (IEF fractions 1 and 10) are not related to SPE B and one of which (fraction 14-15) appears to be SPE B. In addition, several other important points link some degree of proteinase activity to SPE B. First, HPLC purified 713 cloned SPE B from *E. coli* would be expected to only contain the protein encoded by the introduced gene (*speB*) and exhibits proteinase activity (Figure 20). Although unlikely, *E coli* derived proteinases cannot be discounted. Second, since SPP and SPE B share an extremely high degree of sequence homology (45) and only a single copy of the *speB* gene was found (43), it seems reasonable to postulate that both proteins may be from this gene. Finally, SPE B from strain 86-858 appears to have a rearranged proteinase active site (45), which may explain its reduced activity. Taken together, these facts strongly point to a single protein which possesses, singly or together, SPE B reactivity and proteinase activity.

Subsequent experiments to resolve the relationship of SPE B and SPP should be directed towards inactivating the SPE B gene in streptococcal strains and evaluating production of SPP and SPE B by those strains. If the proteins are the same, inactivation of *speB* should then result in concurrent loss of the proteinase

activity which was seen in IEF fractions 12 through 15. Conversely, if the proteins are different (i.e. from separate genes), inactivation of *speB* should not alter proteinase activity.

It is also possible that some fractions of SPE B exhibit strong Ab reactivity yet weak proteolytic and that others are weak Ab and strong proteolytic due to cleavage of the proteinase precursor to active proteinase. This may explain the data obtained in Figures 18a and 18b where SPE B and proteinase activities are displayed in IEF profiles. It is possible to test this hypothesis by physically manipulating the size of the protein produced from 713 cloned SPE B through specific deletion mutations of the *speB* gene. The resulting mutants could be cloned in *E. coli*, expressed, and subjected to preparative isoelectric focusing. It is practical to predict that various distributions of the forms (pI 8.0, pI 8.3, and pI 9.0) will be observed. Depending on the clone, each form can then be screened for proteinase activity and SPE B reactivity as a function of protein concentration.

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