MORAXELLA BOVIS PILI: PHASE ALTERNATION BETWEEN TWO TYPES FOR EACH STRAIN

by

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Two and only two distinct pilus types, α and β, were expressed by phase variants of single strains of M. bovis. Each strain expressed α and β pili which were unique for each strain. Strains were placed into groups according to similarity of α and β pili. The two pilus types of strain EPP63 had subunit molecular weights of 19,300 and 17,100, respectively. Static incubation in tryptose phosphate broth educed a number of different phases which were characterized by different colonial types. Both α and β pili were purified from a variety of these phase variants. Although pilus type could not be correlated with colonial type, piliated phases generally had different colonial morphology than non-piliated phases. Measurements of the serological cross-reactivity between α and β pili were confounded by the presence of both α and β pili in the preparations used to make pilus-specific antisera and in the test antigen preparations used to assay the antisera. The two pilus types of strain EPP63 had different peptide patterns when digested with the enzymes papain and V8 protease. Two types of pili were found on three additional strains of M. bovis: MED72 (4R), FLA64, and NTN63 (8033L). The two pilus types of strain MED72 appeared to have identical peptide digest patterns to the two pilus types of EPP63, so these two strains were placed in the same group. In ammonium sulfate, the two pilus types of EPP63 had different solubilities, and the two pilus types of FLA64 had nearly identical solubilities. There was a small but statistically significant difference in the growth rates on solid media of two colony types of EPP63 expressing different pilus types. Cultures of α and β piliated clones plated onto solid agar were reacted with α and β pilus antisera and the heterologous antisera reacted strongly with several individual colonies in both cultures, supporting the hypothesis that phase variation occurs within a culture. Preliminary experiments using a mouse model for infectious keratoconjunctivitis were attempted, but the eyes of mice could not be adequately infected to accomplish this type of experiment.

Forward

I wish to thank my research advisor, Dr. Charles Brinton, for the opportunity to work and study in his laboratory, for instruction in the scientific method, for continual encouragement and conveyance of his extraordinary enthusiasm for pili and piliation phase variation, for financial support, and for his assistance in the writing and editing of this thesis. I thank the other members of my thesis committee, Dr. R. Atchison and Dr. J. Armstrong. I am also grateful to Dr. Peter Fusco, Joan Silipigni Fusco, Dr. Sarah Wood, Jim Bryan, Marcia Hughes, Patricia Haneline and my other fellow graduate students and co-workers for assistance with experiments, friendship and comradery. For my preliminary education on Moraxella bovis and its pili, I am indebted to Dolores Vilella. I have special gratitude to Dr. Robert Yee for encouraging me throughout my graduate career.

I dedicate this thesis to my husband, Timothy Post.

TABLE OF CONTENTS

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[15] alternatate solubilization (in 0.01 M KPB)/crystalization (in 10% saturated ammonium sulfate) of pilus preparation as described in Section 2.6

LIST OF FIGURES

LIST OF TABLES

CHAPTER I.

INTRODUCTION

A. REVIEW OF THE LITERATURE

As the primary antigenic determinants, virulence factors and immunity factors on the cell surface of a number of species of Gram negative and some Gram positive bacteria, including the Enterobacteriaceae, Pseudomonadaceae and Neisseriaceae there has been much interest in the study of pili as organelles of adhesion and as vaccine components. Pili are the long, thin, rod-like protein structures that cover the bacterial surface or the polar ends of a bacterial cell. Pilus rods are composed of repeating monomers of the pilus major subunit, called pilin, arranged in a helical structure [7], and several minor proteins, including a tip adhesion protein (Hanson and Brinton, unpublished). Pili are of two general kinds: sex pili, which are required for the conjugal transfer of DNA among bacteria [13], and somatic pili, which are colonization, pathogenicity and immunity factors in bacterial diseases [11]. The molecular weights of somatic pilins ranges from about 17,000 to 22,000 daltons, depending upon the type of pili. Pili are different in structure, function, and are more numerous than flagella. The first detailed study of pili was done by Brinton and co-workers who demonstrated the existence of two distinct phases within one bacterial strain: piliated (p+), and non-piliated (p-) [12]. The word "pili" is derived from the Latin word meaning hair or hair-like structure. Pilus expression is controlled by phase variation; the switching on and off of certain genes. Fresh clinical isolates of bacteria are often in the p+ phase, but they may revert to the p- phase upon *in vitro* passage [5]. Since the observation that bacteria in the p+ phase bind to mammalian cells [26, 27], pili have been studied as organelles of adhesion. Bacteria in the p+ phase are virulent, having the ability to attach to the host's epithelium, and bacteria in the p- phase are generally avirulent [14, 44]. Recent comprehensive reviews of the literature on pili as adherence factors are included in Beachy [2], Hanson [37], and Old [57].

The bacterial species Escherichia coli expresses a number of different families[1] of pili. It is hypothesized that different types of pili of different families bind to different receptors of the epithelial cells of various organs [33, 41]. Pili of different families have been associated with diseases of different host species. These pilus families and hosts include: K88 in pigs [59, 75], 987 in pigs [55, 56], K99 in cattle [58, 74], F41 in cattle [54], NMS-H [34], and CFAII [31, 32] in human diarrhea isolates, P and p in human urinary tract infections [43, 48], and RDEC in young rabbits [22, 28]. Type 1 pili are virulence and immunity factors in human and animal diseases [33]. Type 1 pili characteristically bind to guinea pig red blood cells and cause hemagglutination that is inhibited by mannose [24, 26, 37, 69].

For the species E. coli, pili from more than one family can be present on an individual cell. This has been documented by immunoelectronmicroscopy and immunoelectrophoresis [34, 60]. Pilus genes can be either chromosomal or contained within a plasmid. Type 1 pili [6] and 987 pili are chromosomally controlled, although other families of E. coli pili such as non-mannose sensitive pili, [9], K88 [77] and K99 pili [58, 74] are controlled by plasmids. The genes of M. bovis pili are chromosomal [3]. Sex pili, of which F pili are one type, are contained on the F plasmid [13].

The pili from different strains of Neisseria gonorrhoeae are considered to be within the same family, because they all cross-react serologically [15]. However, different types of pili have been found in N. gonorrhoeae [11, 16, 46, 69]. Phase variants of N. gonorrhoeae were shown to produce two distinct types of pili. In the Lambden studies [46], the pili called α and β, were purified with exactly the same solubilization and crystalization procedure, developed in this laboratory [15].

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[1] Pili within a pilus family are serologically related. Pili are of different pilus types if they are less than 100% serologically related, or if they have different peptide digest patterns, or if they are chemically different in any way.

However, they differed in apparent subunit molecular weight MW_a[2] (19,500 and 20,500), and in ability to attach to buccal epithelial cells. The Salit and Lambden groups have reported that differences in pilus subunit size appear to correlate with differences in the gonoccal colony opacity Protein II phenotype. However, a more thorough and recent study has documented that there is no correlation between pilus subunit size and colony opacity phenotype (gain or loss of Protein II component of the cell membrane) [78].

1. Piliation Phase Syndrome

Bacteria in the piliated phase possess a group of properties that are not possessed by non-piliated bacteria [9, 14]. The piliation phase syndrome is the name given by Brinton to this group of properties. Many of the properties of the piliated phase syndrome can be considered as adaptive to the bacterium in its ability to colonize the host's epithelial cells. Some of these independently measurable properties are directly mediated by pilus rods, and others have been correlated with the piliated phase. These properties include: altered colony morphology, pilus specific antibody agglutinability, adhesion to vertebrate cells, auto-agglutination, pellicle formation in unshaken liquid medium, enhanced growth rate at low oxygen tension, pitting of agar beneath colonies, surface translocaton by twitching motility, and increased pathogenicity for natural hosts.

2. Piliation Phase Variation

Phase variation, the regulatory mechanism that directs the phenotypic expression of piliation in bacteria, acts as an on-and-off switch in individual cells of a bacterial population [5]. For one strain of E. coli under one set of growth conditions, the frequency of the p+ to p- switch was approximately 10^{-3}, and the

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[2] MW_a: apparent molecular weight: the molecular weights referred to in this study are considered apparent molecular weights, because mobility on an SDS gel may be affected by the chemical and physical arrangement of the amino acids in the pilin macromolecules, in addition to the absolute number of hydrophobic and hydrophilic amino acids. When molecular weight analysis by chemical (amino acid analysis) methods and gel molecular weights are compared, there is much less variability in the chemical molecular weights (Brinton, personal communication).

frequency of the p− to p+ switch was approximately 10^{-4} to 10^{-5} [5]. The molecular genetic mechanism of piliation phase variation has been partially characterized for E. coli Type 1 pili [29], and for gonococcal pili [51, 71]. The use of Southern blotting technique [76] has shown that pilus gene expression involves rearrangement of segments of chromosomal DNA. The movable genetic elements or transposons have the ability to become associated with specific genes, inducing translocations, inversions, deletions and insertions. Transposable elements were first described by McClintock [50] from her studies of the phenotypes of maize. In addition to pilus expression, the other procaryotic systems which are believed to regulated by transposable elements include the flagellar genes of Salmonella [73], the surface coat glycoproteins of Trypanosomes [85], and the virion proteins of Mu and P1 prophages [45]. A structural gene of M. bovis pili has been characterized [49], although there has been no documentation of genetic studies on piliation phase variation for M. bovis other than the thesis of D. Vilella [80].

Phase composition of a bacterial culture is affected by growth rate of different phases, as well as the phase switching probability and phase selection [9]. Phase selection is the outgrowth of certain phases over other phases. Phase switching is a random event that occurs with a predicted probability. Brinton [5] has shown that lower temperatures increase the rates of phase variation in one strain. The frequency of phase switching was measured by Brinton using the "zero−point method". Colony type variation is an *in vitro* manifestation of phase variation, and provides a means for detecting different phases. Studies on the phenotypes associated with phase variation include studies of colonial morphology associated with different phases. Brinton observed that p+ phase colonies produce smailer and smoother colonies on solid media than non−piliated colonies. Colonial morphology on solid media is affected by a variety of factors, some or all of which are associated with virulence including pili, growth factors, invasive enzymes, exotoxins, autoagglutination factors, cell motility factors, lipopolysaccharides, and other cell membrane components [11, 14].

3. Pilus Purification

Pili were first purified by Brinton [5], who demonstrated that pili can be crystalized under certain ionic conditions and solubilized under a different set of ionic conditions. Crystalized pili form needle-like aggregates called "paracrystals", which can be purified by centrifugation, filtration, sucrose gradients, or chromotagraphy. The standard criteria used in the Brinton laboratory for determining the purity of pilus preparations are electron microscopy, darkfield microscopy of paracrystals, ultraviolet absorption spectroscopy and SDS polyacrylamide gel electrophoresis [33]. Methods of pilus purification include the use of divalent cations (magnesium chloride), polyethylene glycol, (which affects the dielectric constant of water), and the adjustment of pH to the isoelectric point. These methods work because of the repeating subunit helical structure of pili and the specific surface properties of pilus rods. Pili have a natural tendency to aggregate with each other in a longitudinal way because of interaction between the identical pilin subunits which make up the pilus rods [7].

4. Pilus Vaccines

Brinton [8] proposed that pilus vaccines may be useful for the control of bacterial diseases, since pilus-specific antibodies can neutralize the pilus' function. The "direct and general approach" to bacterial disease control was developed in Brinton's laboratory [9, 11]. The direct approach to developing a pilus vaccine is as follows. First, a variety of bacterial isolates from disease sources are collected. Different colony types (phases) are isolated, and pili are purifed from the piliated phases. The purified pili of the different phases are serologically and biochemically typed, and the pili are grouped into pilus families and pilus types. It is determined whether pili are virulence factors by using phase cloned cultures in challenge experiments. It is determined whether pili are immunity factors by performing "challenge" experiments with heterologously and homologously piliated bacteria in immunized hosts. The "intact pilus vaccine", composed of whole pilus rods, includes pilus types from each of the major pilus families [11]. Brinton has discovered that disease protective antibodies are type specific for intact pilus vaccines so that such vaccines must be multivalent when more than one family or type of pilus is important for the same disease.

In a normal course of infection the bacterium comes into contact with the host's epithelial tissue. Upon contact, the bacterium readily attaches to receptors on the epithelium, using its pilus rods. Once attached, infection of the host occurs. However, if the bacterium is prevented from attaching to the epithelium, infection of the host cannot occur. Antibodies of the host can bind to the pilus rods; the pili are prevented from contacting the epithelium, and the host is not infected. There are other reasons why pilus antibodies are disease protective, which are reviewed in Brinton's recent work [11].

The pilus vaccines developed in the Brinton laboratory are of two classes: purified pilus vaccines, composed of intact pilus rods, and bacterins, composed of killed whole cells. Purified pilus vaccines are considered to be safe, because pili are non-toxic, although these vaccines may be more expensive to prepare. In vaccine research, purified pilus vaccines are necessary in order to assess immunogenicity of the vaccine components. Bacterins are considered safe in animals, although not suitable for use in humans.

E. coli diseases occur in human and animal hosts in numerous sites including the small intestine and urogenital tract. E. coli vaccines are under development in this laboratory for the control of human diarrhea, for urinary tract infections, and for diarrhea in newborn piglets, [19, 42, 53, 56]. Vaccines now in extended field use which were developed in this laboratory include the Piliguard Pinkeye Vaccine [80] for use in cattle. Other pilus vaccines under development include a vaccine for Pseudomanas aeruginosa infection in burn patients, [47, 72], the Scourmune vaccine for use in swine, and a vaccine for N. gonorrheae [14, 17, 18, 79].

5. Previous Work on M. bovis

Moraxella bovis is the etiologic agent of Infectious Bovine Keratoconjunctivitis (IBK), or "pinkeye" in cattle. IBK is the most important ocular disease of cattle. Although it is not a fatal disease, it has economic importance and occurs worldwide. Pinkeye is characterized by lachrymation, conjunctivitis, and keratitis. M. bovis was first associated with cattle pinkeye in 1915 in Bengal, India by Mitter [52], who identified the organism as the Morax-Axenfeld Bacillus. Moraxella species are

members of the Neisseriaceae family. M. bovis is a non-motile, non-flagellated, non-sugar fermenting Gram negative diplobacillus. It has been recognized that factors associated with M. bovis infection include infection by Mycoplasm bovoculi, and ultraviolet light [84]. Bovre and Froholm [3, 4], Pedersen [61] and Sandhu, White and Simpson [70] reported that colonial morphology of M. bovis is associated with piliation. Pedersen [61] has reported that only piliated strains are able to infect experimentally inoculated cattle. M. bovis produces a hemolysin that is required for virulence [62]. Reviews on M. bovis and IBK are in Babtista [1], Webber [81], and Wilcox [84].

There have been several attempts to develop an M. bovis vaccine against IBK. These vaccines include viable whole cells [39], non-viable (formalin, heat-killed) cells [38, 40], disrupted cells, a "purified pilus" vaccine [65, 67], and most recently a pilus vaccine enhanced with diptheria-tetanus toxoids and pertussis vaccine as adjuvants [66]. Pugh and Hughes reported a method of pilus purification [67]. However it was not demonstrated that any pili were actually purified. Proof of pilus purification should include electron micrographs of piliated cells and of pilus crystals, scans of the pilus preparation in the ultraviolet range showing maximum absorbance at 280 nm, and gel electrophoresis showing pilus proteins migrating in the molecular weight range of 20,000 daltons. None of these criteria of pilus purity were applied to the "pilus" preparations of Pugh and Hughes. None of their preparations were identified as pili by amino acid composition or sequence analysis. None of the experimental vaccines from this group has been successful.

In the Brinton laboratory, a method of purification of M. bovis pili was developed by Dolores Vilella [80], based on repeated cycles of solubilization/crystalization (as described in Section 2.3.) In addition to electron microscopy of piliated cells and pilus crystals and physico-chemical characterization of M. bovis pili, she classified five strains of M. bovis according to their serological cross-reactivity, and assigned each strain a rank-order, based on degree of cross-reactivity with the pili of other strains. EPP63 was included in the vaccine because it was the most highly cross-reactive among all of the strains, and FLA64 was

chosen because it appeared to be identical serologically to ATC(10900). Vilella also isolated different colony types within each strain. The work was continued by Haneline and Brinton [36] who found that the typing of pili from different strains of M. bovis could be duplicated using the Cleveland method of proteolytic digestion [23]. Pili were digested with enzymes, creating a peptide pattern for each of 16 kinds of pili. Some of the peptide patterns of strains were identical. The 16 strains were classified into eight groups, based upon similiarity of peptide patterns and serological cross-reactivity. The grouping of strains is shown in Table 1-1. This classification of strains is based on the properties of a single pilus type isolated from each strain.

Group	Strain
I	WSE64(2L)
	IBH68(712L)
II	EPP63
III	1965GLENN (5004)
	HIM63(5R)
IV	IVI64 (54L)
	MED72 (4R)
	MED72 (19L)
	WSE64(13R)
V	MAC74 (2554R)
	MAC74(2562R)
VI	FLA64
	ATC (10900)
	8613 (1)
VII	NTN63 (8033L)
VIII	GLN63

Table 1-1: Classification of strains of M. bovis by serological cross-reactivity and proteolytic digest patterns of a single pilus type per strain

Based on the results of the studies by Vilella, a bacterin vaccine against IBK was tested, produced and marketed by Bactex Inc. and Schering-Plough Corporation. "Piliguard Pinkeye" consists of piliated strains EPP63 and FLA64.

6. Identification of M. bovis

These criteria were suggested [63] as criteria for adequate identification of M. bovis from suspect cases of IBK. 1). Usually hemolytic, gram negative, non-motile diplobacillus. 2). Does not reduce nitrates to nitrites, or ferment carbohydrates. 3). Are proteolytic, oxidase positive, and produce a typical 3-zone reaction when grown in litmus milk. 4). Produces no surface growth in liquid medium but develops a course, flocculent sediment with little turbidity. 5). Does not grow on Herellia agar.

Different piliated colony types have been described, in addition to the p- colony type. Other workers have described at least two p+ colonial morphologies [4]. In the studies on M. bovis pili by Vilella, only one colony type (colony type 1) was used as a source of pili, although a total of 3 p+ colony types were described. The four colony types of EPP63 were: 1). p+: uniform dark color, rim not distinct, pits agar. 2). p-: spreading morphology, does not pit agar. 3). p+: lighter that colony type 1, rim more distinct, but not as distinct as colony type 4, pits agar. 4). p+: smaller than other colonies, lighter in color, rim very distinct, pits agar.

7. Mouse Model for Keratoconjunctivitis

Pugh, Hughes and McDonald [64] reported that M. bovis infection could be established in the eyes of laboratory mice. Mice were challenged by flooding the open eye with a suspension of M. bovis, after the eyes were irradiated with ultraviolet light. In experiments on the effects of exposure and re-exposure to homologous and heterologous strains infection was established in 25-50 per cent of eyes challenged with M. bovis.

Chandler [20] reported that the C57Bl strain of mouse was more susceptible

than other strains of mice to M. bovis infection. Pretreatment with corticosteroid before infection aided in producing infection. Clinical signs included lachrymation, blepharitis, various degrees of opacity of the cornea, keratoconjunctivitis, corneal ulceration, and sometimes lenticular enucleation. In the Chandler studies, M. bovis was cultured from about 85 per cent of mice inoculated with strains previously shown to be pathogenic in cattle, and clinical signs were produced in 66 per cent of these mice. However, two strains previously shown to be pathogenic in cattle were not pathogenic in mice. Chandler [21] noted that there was a difference in the ability of corticosteroid preparations to predispose mice to IBK.[3] A variation of this mouse model was recently developed by Rosenbusch and Ostle [68]. Rather than pretreating the mice with corticosteroids, the mices' eyes are scarified, using a single stroke of small wire brush, and then the bacteria are introduced into the eye by touching a swab to colony growth and swabbing the eye. (Results from these studies are in publication by Rosenbusch). If this system can be optimized, then it would be useful in "fine-tuning" the vaccine for use in cattle. The serological relationships among different pilus types or combinations of pilus types, and their ability to produce a wide degree of protectiveness could be studied.

B. STATEMENT OF THE PROBLEM

In previous studies on M. bovis in this laboratory, there were a number of observations that were documented but not investigated. Two of these observations were the basis for the present study. The first observation was that all pilus preparations of M. bovis that were run on SDS polyacrylamide gels included a contaminant protein that was of close MW_a to the major pilus band. Because this

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[3] Betsovet is a veterinary corticosteroid preparation used in certain allergic and autoimmune conditions in animals. It is an injection formulation, obtainable as a ready-made suspension in five-ml vials containing the active ingrediants betamethasone adamantoate 20 mg/ml and betamethasone phosphate 1 mg/ml. Betsovet is a product of the Glaxovet company in Great Britain, and it is not available in the United States. In order for the product to be imported for research investigation, it is necessary to obtain an INADA license from the Food and Drug Administration. This information was kindly provided by Dr. Robert J. Fuentes, Manager, Science Information, Glaxo, Glaxo Inc. 5 Moore Drive, Research Triangle Park, North Carolina 27709.

protein seemed to have the same solubilization and crystalization properties as pili, and because it migrated with a slightly greater mobility than pili, it was hypothesized to be a pilin macromolecule from which a small peptide domain was missing. Alternatively, it was hypothesized that this protein band was an additional pilus type. The second observation was that multiple colony types of p+ morphology could be cloned from isolates from a bovine infected eye. Four colony types of strain EPP63 were isolated from a bovine infected eye. (The four clones were described in Section 1.1.7). Of the three p+ colony types, only colony Type 1 was used as a source of pili in the Vilella studies. In a preliminary experiment of the present study, pilus purification was done from each of the three p+ colony types of EPP63. Colony types 1 and 4 each had pili of different MW_a, and colony type 3 appeared to express both types of pili. When viewed under the darkfield microscope, the pilus crystals from the three pilus preparations had the characteristic appearance of pili. Although these pilus types appeared to have different subunit MW_a's from one another, there was no proof that the different colony types were variants of the strain EPP63. It was possible that the cow from which these bacteria were isolated were infected with two or more strains of M. bovis.

Based on the results of these preliminary experiments, as well as the knowledge of piliation in other genera of bacteria, the hypothesis was proposed: M. bovis possess at least two types of somatic pili. The purpose of the first phase of this study was to prove that colony types of one strain expressing different types of pili are phase variants of that strain. The purpose of the second phase of this study was to investigate several different systems for characterizing bacterial pili to determine what system or combination of systems can best be utilized to further understand the mechanisms of bacterial pathogenesis. Listed here are the experimental systems that were investigated.

1. *The identification of additional pilus types in additional strains of M. bovis*. Pili from strains of M. bovis were purifed and α and β pili from each strain were identified using MW_a as determined from SDS gel electrophoresis.

2. *Chemotyping by peptide mapping with proteolytic enzymes.* Chemical differences between pilus types could be demonstrated using this method which could not be demonstrated with MW_a comparisons.

3. *Immunological cross-reactivity.* Serological cross-reactivity between α and β pili of EPP63 was examined using rabbit antisera and the enzyme-linked immunosorbant assay.

4. *Growth rates of different clones of EPP63.* The number of cells within two colonies, Clone A (expressing α pili) and Clone B (expressing β pili) were compared to determine if there was a difference in the growth rate of two clones expressing different pilus types.

5. *Solubility studies in ammonium sulfate of the two different pilus types of two different strains of M. bovis.* The solubility is ammonium sulfate of α and β pilus types of strains EPP63 and FLA64 were characterized.

6. *Colony immunoblots.* Heterologous and homologous antisera was reacted with cultures of α and β piliated colonies to determine whether phase variation occured within a culture.

7. *Mouse model experiments.* An attempt was made to replicate a laboratory animal model for IBK. If this model could be replicated, then the model could be used to study the immunogenicity of a pilus vaccine, or to study the *in vivo* phase variation of M. bovis.

A number of important questions were raised in the process of doing these studies, but extensive experiments were not done in these areas. These are two questions which were not addressed in the present study:

- The effect of different growth media on the phase variation or phase switching rate of M. bovis.
- The relationship between different pilus types and different colony types.

CHAPTER II.

MATERIALS AND METHODS

A. IDENTIFICATION OF M. BOVIS

Strains of M. bovis were obtained from vials of frozen stocks that had been previously typed by workers in this laboratory. Many of them were obtained from Dr. David Hughes of the National Animal Disease Laboratory in Ames, Iowa. These criteria were used as positive identification of M. bovis: beta hemolysis of blood agar, oxidase positive, and Gram negative. However, more rigorous criteria were used to type unknown bacteria as M. bovis [63]. These criteria included liquification of gelatin, oxidation of nitrates to nitrites, penicillin sensitivity (sensitivity testing disks BBL), characteristic triple color layers in litmus milk and growth in Mueller Hinton Broth (Difco). Lack of reaction in an Enterotube (Roche Laboratories) was also noted.

B. GROWTH OF M. BOVIS

Bacteria were passed on Mueller-Hinton Agar [BBL lot #96242]. Plates were incubated for 20-24 hours in a Forma incubator at 37°C,[4] with 5% CO_2 and approximately 80% humidity. Cloning was done by picking a single, well isolated colony and streaking it onto a sterile MH plate. Cloning was done every 20-24 hours, to yield homogeneous and stable cultures. Longer incubation periods caused colonies to revert from piliated to non-piliated colony types. Cultures of M. bovis were stored at -70° in 8% glycerol in trypticase soy broth. A small sterile piece of filter paper was used to lift a single colony from an agar plate, and the filter paper was placed in the vial containing the TSB.

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[4] Temperature: all notations for temperature are in degrees Centigrade

The media used in the growth experiment in Section 3.2 were selected because they were readily available on the storeroom shelf. To inoculate liquid media, a suspension of bacteria was made, using several colonies from a plate of well-cloned bacteria. A turbid solution was produced, corresponding to a MacFarland Standard #1 (3 X 10^8 cells/ml). One drop (0.04 ml) was inoculated into each tube containing 10.0 ml of broth, and tubes were incubated for five days at 37°. After incubation tubes were vortexed to suspend cells uniformly, and broth was plated onto MH agar. When broth tubes were turbid with growth, the eye of the investigator was used to determine the degree of dilution necessary to produce a plate containing 100-1000 well-isolated colonies. Several different dilutions were done to achieve adequate growth on the plates. When no turbidity was present in the broth tubes, the broth was not diluted before spreading plates. Growth was recorded as (+) or (-). There were at least 2-3 colonies per plate for a broth type to be considered (+). All media were prepared according to manufacturers instructions. These media included: Bacto-peptone (Difco, cat. #0118-01), Bacto-protease peptone #3 (Difco, cat. #012201), nutrient broth (BBL, cat. #11479), Bacto-tryptone (Difco, cat. #0123-01), Bacto tryptose broth (Difco, cat. #704809), Bacto-tryptose phosphate broth (Difco, cat #704809), trypticase soy broth (BBL, cat #11768), trypticase soy broth (no Dextrose) (BBL, cat #H 8292-182502), and Mueller Hinton broth (Difco, cat. #0757-02). Z broth was prepared from tryptone (Difco) (10.0 g), yeast extract (BBL) (1.0 g), dextrose (1.0 g) and NaCl (8.0 g) per liter. For phase variation experiments, M. bovis was grown in tryptose phosphate broth (BBL) TPB at 37°.

To grow large batches of M. bovis the following procedure was used. Colonial growth from one or two MH plates was scraped into a flask containing 100 mls of 0.7% casamino acids. This bacterial suspension was inoculated onto sterile aluminum trays (36 x 26 cm) containing approximately 500 mls of Mueller Hinton solid media. Trays were incubated at 37° overnight in 5% CO_2 and approximately 80% humidity. Growth from trays was harvested with 15 ml of potassium phosphate buffer (KPB) μ = 0.01 pH 7.2 per tray and scraping the bacterial growth from agar surface with a glass projector slide (8.3 x 10.2 cm) (Kodak).

C. PURIFICATION OF M. BOVIS PILI

Purification of M. bovis pili was done using the method of Vilella [80]. Bacterial cells suspended in KPB were blended in an omni mixer at 10,000 rpm for two minutes to shear the pili from the cells. The suspension was centrifuged at 12,000 rpm for one hour. The supernatant containing soluble pili was removed from the cell pellet, and pili were crystalized by adding a 10% saturated ammonium sulfate solution in KPB. "Paracrystals" of aggregated pili were visible by darkfield microscopy at this step. After 20 minutes of mixing with a magnetic stir bar at room temperature, the pilus crystals were pelleted by centrifugation at 12,000 rpm for one hour. The supernatant was discarded, the pilus pellet was resuspended in KPB and the preparation was left stationary overnight at 4°. After overnight incubation, the pilus solution was centrifuged at 25,000 rpm for one hour in a Beckman ultracentrifuge. The ultracentrifugation was a clarifying step which removed particulate material that could not be removed at lower centrifugation speeds. In cycles after the Ist cycle, the centrifugation speed of this step was 12,000 rpm. The pellet of particulate contaminants was discarded. This procedure, of salting out the pilus crystals and resolubilizing pilus crystals, was repeated two or three additional times until the preparation was pure of major contaminants, by the criteria of SDS PAGE and UV light spectroscopy. Darkfield microscopy was used to assure that there was no gross bacterial contamination of the pilus preparations. 0.02% sodium azide was included in preparations of purified pili to inhibit bacterial growth.

The "quick-prep" method was used in order to screen multiple clones for pilus types, when only a small quantity of pili from each clone was required. Growth from 5-6 heavily inoculated plates was suspended in a volume of approximately 20 ml KPB. The pilus purification procedure is the same as the one outlined in the preceeding paragraph, except that the ultracentrifugation step was replaced with centrifugation at 12,000 rpm.

D. POLYACRYLAMIDE GEL ELECTROPHORESIS

Chemotyping and molecular weight experiments were done using SDS polyacrylamide gel electrophoresis. Materials for the gels included: running gel stock (pH 8.8) (TRIS (18.17 g/100 ml) and SDS (0.04 g/100 ml)), stacking gel stock (pH 6.8) (TRIS (6.06 g/100 ml) and SDS (0.04 g/100 ml)), acrylamide stock (acrylamide (30 g/100 ml) and bis acrylamide (0.8 g/100 ml) (stocks are filtered through a .45μ filter), 10% ammonium persulfate, and N,N,N,'N',–tetra–methylethylenediamine ("temed") (Eastman Chemicals). The lower "running" gel (15%) was prepared from 3.0 ml running gel stock, 6.0 ml acrylamide, 3.0 ml ddH_2O[5], 10 μl Temed, and 30 μl ammonium persulfate. The upper "stack" gel was prepared from 2.5 ml stack gel stock, 1.5 ml acrylamide, 6.0 ml H_2O, 10 μl Temed, and 30 μl ammonium persulfate. Protein samples at approximate concentrations of 1.0 mg/ml were diluted 1:4 in sample buffer. Sample buffer included 10% glycerol, 2% SDS, 0.02% bromphenol blue, 10% B mercaptoethanol, and 0.125 M TRIS HCl pH 6.8 in ddH_2O. Gels were run at 200 volts (constant) in running buffer pH 8.4 (0.192 M glycine, 0.025 M TRIS HCl, and 0.1% SDS). After running for the appropriate time (approximately 1 hour for mini–gels, about 3 hours for larger gels), the gels were stained with Coomassie blue (0.02% Coomassie brilliant blue in ddH_2O, acetic acid, methanol (5:1:5), or silver stained using the Bio–Rad silver staining kit. Coomassie blue stained gels were destained using a solution of 7.5% acetic acid and 5% methanol. For determination of MW_a, using the method of Weber [83] purified proteins of known molecular weights [bovine serum albumin (BSA) (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), myoglobin (17,200 daltons), and lysozyme (14,300 daltons)] were run on gels. Per cent mobility of the proteins was determined, by measuring from the dye front[6] running across the bottom of the gel, to the middle of the protein band. This distance was plotted against the $\log_{10}$ molecular weight of the protein, so that a straight line could be drawn through the points. A simple linear regression was used to determine the $\log_{10}$ molecular weight of the pilus proteins.

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[5] ddH_2O: distilled, dionized water

[6] On a gel, a dye front of sample buffer preceeded the proteins. When the dye front reached the bottom of the gel, electrophoresis was completed.

E. ANTISERA PRODUCTION

For antisera production, New Zealand white rabbits were injected subcutaneously with approximately 50–100 µg of pili per pound of body weight. The pili were mixed 1:1 with Freunds incomplete adjuvant (Sigma). The immunization schedule included a primary injection, followed by two boosters two and three weeks later. Prior to immunization the rabbits were bled from a lateral ear vein to obtain preimmune control serum. Rabbits were bled on the fourth week and sacrificed. The whole blood was allowed to clot overnight and the blood cells were removed by low speed centrifugation. Serum was stored at -20°.

F. ENZYME-LINKED IMMUNOSORBENT ASSAY

(This method was developed in the Brinton laboratory by Bryan and Brinton as a modification of the method of Engvall and Perlmann [30]) Soluble pili were diluted to a concentration of 0.01 mg/ml in 0.1 M bicarbonate pH 10.2 (50.9 ml 1M $NaHC0_3$, 16.3 ml 1M Na_2HC0_3 $10H_20$ to make 1 liter with ddH_20). Gluteraldehyde was added to a concentration of 0.25%. 1.0 µg of pili was delivered to each well in columns 2 through 12 of a Linbro non–sterile 96 well flat bottomed microtitration plate (Flow Laboratories Inc.). The plate was sealed with an adhesive cover and incubated 30', and then stored overnight at 4°. After overnight storage, the plate was allowed to equilibrate to room temperature, and then the antigen was shaken from the wells. The plate was washed two times with 0.1 M phosphate buffered saline (PBS) pH 7.0, and bicarbonate (0.15 ml per well) was added to each well and the plate was "prewashed" for 30' with rotation. Bicarbonate was shaken from the plate, and the plate was washed two additional times with PBS.

Sera dilutions were prepared on a "transfer" microtiter plate. 0.15 ml of PBS with 0.05% Tween 20 (Fisher) was placed in columns 4–12 as diluent, and 0.3 ml of each sera to be assayed were placed in the wells of column 3. To create a two–fold dilution in each well, 0.15 mls of sera were transferred to the wells of column 4, and the two–fold dilutions were continued across the plate. The starting dilutions of sera were selected so that a maximum number of points had absorbances within

the range of 0.3 to 2.0. 0.1 ml of each serum dilution was transferred from the "transfer" plate to the corresponding well of the antigen-coated "prewashed" plate, and the plate was incubated for 45 minutes with rotation. Sera was shaken from the wells, and the plate was washed three times with PBS + 0.5% Tween 20, pH 6.5. Conjugate (horseradish peroxidase conjugated anti-rabbit IgG heavy and light chains (Cappel Laboratories)) was added at 1:4000 dilution in PBS + Tween, and incubated for 15' with rotation. The conjugate was shaken from the wells, and the plate was washed three times with PBS + Tween. Equal volumes of dye (0.002% ortho-dianisidine dihyrochloride (Sigma)) and substrate (0.006% hydrogen peroxide (Fisher)) were mixed together. Standardization of results was achieved by including a standard serum on the last two rows of each plate. The standard serum was specific for the antigen bound to the plate. Plates were read on a Titertek Multiskan (Flow Laboratories) at 450 nm. A Titertek Multiskan Interface transferred absorbances to a Hewlett Packard 9815A calculator. A program (created by P. Fusco) according to the plotting method of Brinton [10] issued a printout that included absorbances, r^2, n, A max, KC, and titer. This program assumes that the single reciprocal plot drawn is approximated by a straight line. The program allowed standardization of the values to the standard serum on each plate. A Hewlett Packard 7225A Plotter was connected to the calculator. Another program (created by P. Fusco) allowed manual entry of the absorbances, along with dilution factors and blank absorbances for each serum dilution and the desired parameters for the x and y axis, so that a plot could be generated for each serum dilution. Examination of these plots illustrated that the distribution of points was not consistently linear. At very high dilutions, and very low dilutions, the points varied from linearity. Only the middle points that fell along a straight line were included in the final data analysis. A line was drawn by hand through as many points as possible. The x intercept was recorded as the titer. A single reciprocal plot is described by the equation: $A = (-1/k)(A/c) + Amax$, where A is proportional to the total number of antibody molecules bound to the antigen, Amax is the maximum absorbance at infinite serum concentration (proportional to the amount of antibody bound to antigen at saturation), k is the apparent average equilibrium binding constant of the antibody, and c is the concentration of antibody. The X intercept of the plot is the titer, being the product of Amax and kc. Titer is analogous to the traditional

endpoint titer of the traditional ELISA. Titer is a measure of the total ability of the antigenic determinants present in the well of the plastic plate to bind to the antibody added to the well. Figure 2-1 is an example of a single reciprocal plot, and illustrates the relationship among the parameters.

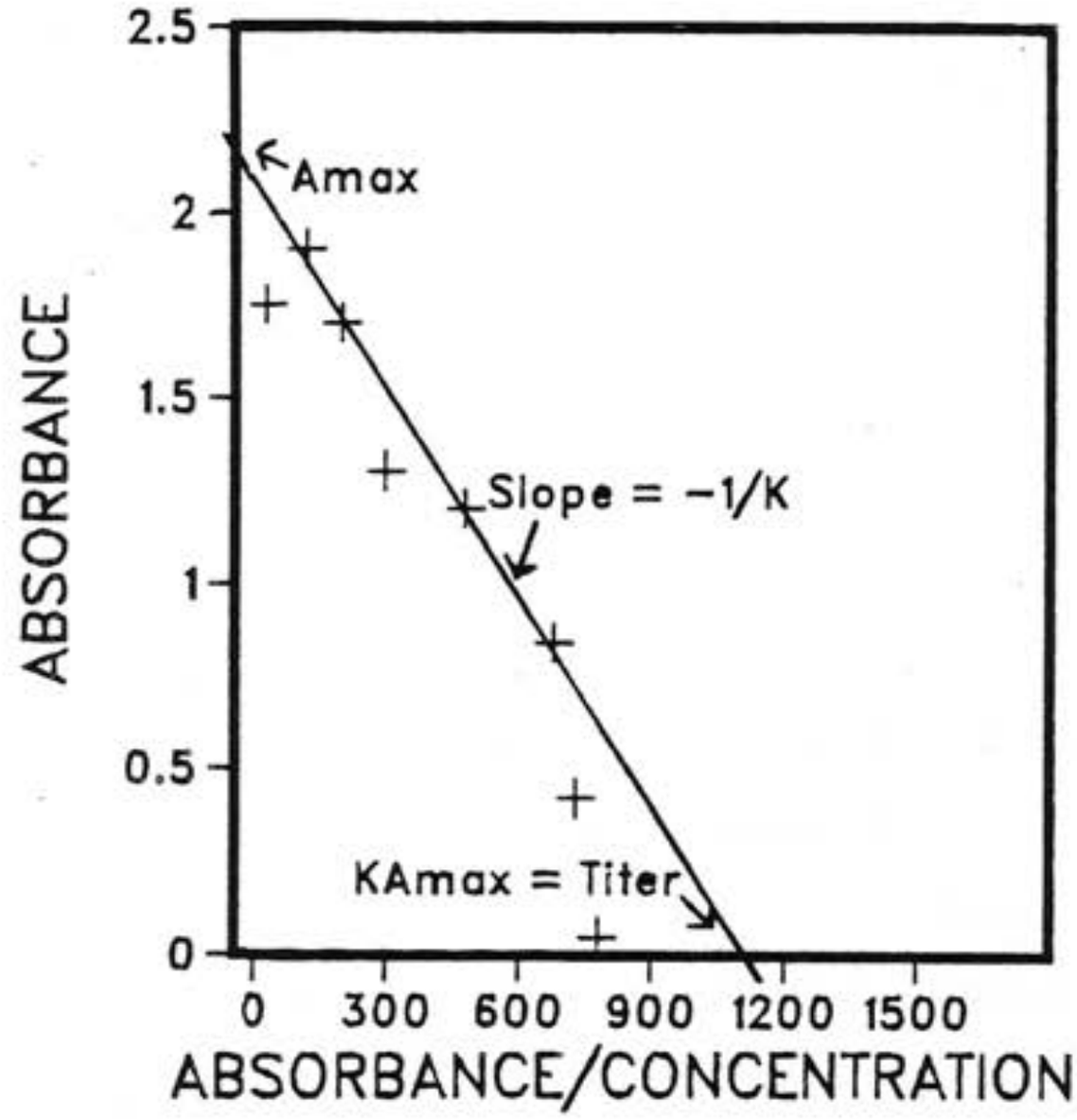

Figure II-1: Example of a single reciprocal plot showing parameters [Amax, slope, and titer]

G. CHEMOTYPING OF PILUS TYPES

Enzymatic digestions of purified pili were done for chemotyping experiments, using the method of Cleveland [23] adapted by Brinton [36] for the typing of pili. The three enzymes used were papain, S. aureus V8 protease, and chymotrypsin. Peptide mapping involves partial or complete enzymatic proteolysis in the presence of SDS, and analysis of the cleavage products on and SDS gel. S. aureus V8 protease specifically cleaves peptide bonds on the COOH terminal side of both aspartic and glutamic acids [86]. Papain is a major protein constituent of the latex and mellon-like green fruit of the solftwood tree Carica papaya. Papain has a

broad specificity [35]. Studies of the specificity of papain on a variety of synthetic substrates, as well as natural polypeptides show that papain hydrolizes the amides of alpha-amino-substituted arginine, lysine, glutamine, histidine, glycine, and tyrosine. With prolonged hydrolysis of peptides by papain, bonds involving other residues can be split as well. Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds involving L-isomers of tyrosine, phenylalanine, and tryptophan. In addition to bonds involving aromatic amino acids, chymotrypsin catalyzes at a high rate the hydrolysis of bonds of leucyl, methionyl, asparaginyl, and glutamyl residues and the amides and esters of susceptible amino acids.

The parameters used in these proteolytic digestions were devised for M. bovis pili by PG Haneline and CC Brinton [36]. Pilus proteins at 1 mg/ml concentration (0.05 mls) in digestion buffer (TRIS HCl (1.5 g), SDS (0.56 g), glycerol (11.1 ml), bromophenol blue (10.0 mg) in ddH_20 adjusted to pH 6.8 with 12N HCl and filtered) were placed into an Eppendorf tube and heated in a boiling water bath for 2'. 0.05 ml enzyme was added (1 mg/ml) (papain dissolved in ddH_20, V8 protease dissolved in ddH_20, chymoptrypsin dissolved in 0.001 N HCl) and vortexed. 0.05 ml digestion buffer was added to enzyme control. Samples were incubated (papain: 30' at 37°, V8 protease: 30' at 37°, chymotrypsin: 30' at room temperature with rotation), and then SDS concentration was increased from 0.5% to 2.0% by adding 0.01 ml of a 20% SDS stock to each sample. 0.12 ml of 10% B mercaptoethanol was added to each sample, and the enzymatic digestion was stopped by placing the samples in a boiling water bath for 2'.

H. SOLUBILITY STUDIES

For solubility studies, the dilutions of pili in saturated ammonium sulfate (pH 8.0) were prepared from an SAS solution in KPB, equilibrated to room temperature. Pilus concentration was adjusted to 0.02 mg/ml, and 0.5 ml aliquots were delivered to Eppendorf tubes. Pilus proteins were crystalized by adding SAS to make a 10% concentration (0.5 ml), and then Eppendorf tubes were placed in a microfuge and spun at 12,000 rpm for 15 min to pellet crystals. The supernates were removed from all tubes, and the appropriate diution of SAS was added to the tubes. Tubes

were vortexed to resuspend crystals, and then placed at 4° overnight. After overnight incubation, tubes were vortexed again, and then placed in the microfuge for 15 min at 12,000 rpm. The supernates were removed, and assayed by Lowry for protein concentration. A standard was included in the Lowry assay containing pili resuspended in KPB.

I. GROWTH RATE STUDIES

For growth rate experiments, plates were inoculated with a suspension of the strain EPP63 that had been phase cloned, and incubated at 37°. Plates were removed from incubation and examined for well-isolated, uniformly-sized colonies. Colonies were assayed for number of colony forming units after 13, 18, 23, 24, and 31 hours. The tip of a 26 gauge needle attached to a 1.0 ml syringe was used to pick a single, whole colony from the agar surface. The colony was suspended in a small volume of saline with the needle and syringe, and then transferred to a larger volume of saline. The 100 ml volume containing the suspended cells of one colony was thoroughly mixed, and then volumes of 0.01 ml were inoculated onto MH plates, and spread with a bent glass rod. Seven to ten plates were spread from each suspended colony. After overnight incubation the cfu's on the spread plates were counted and the total number of cfu's within each colony was calculated.

J. COLONY IMMUNOBLOT

For the colony immunoblot experiment, two strips of nitrocellulose (NC) were placed directly onto the agar surface with colonies, and then removed with forceps and placed in a solution of 1% BSA, 1% Tween, 0.01M TRIS HCl, 0.85% saline (pH 7.4) and incubated for 30 minutes. The BSA Tween was decanted, and antiserum (either anti α or anti β pili) was added at a dilution of 1:2000 in Tween TRIS buffer, (above buffer without BSA) and then incubated in serum for 45 minutes with rotation. The NC strips were washed for 30 minutes in Tween TRIS with three changes and then incubated with conjugate (horseradish peroxidase conjugated anti-

rabbit IgG heavy and light chains (Cappel Laboratories)) 1:2000 dilution in TRIS saline (above buffer minus BSA and Tween) for 45 minutes. The NC strips were washed in TRIS saline for 30 minutes with three changes, The NC strips were incubated in dye and substrate (80 mg 3-amino-1 ethyl carbozole, 20 ml N,N-dimethylformamide, 380 ml 50 mM Na Acetate pH 5.0, 0.2 ml 30% H_2O_2 filtered through a 0.45 µ filter) until color developed.

CHAPTER III.

RESULTS

A. PRELIMINARY EXPERIMENTS

1. Silver Staining of Cycled[7] Pilus Preparations

The purpose of this preliminary experiment was twofold; first, to compare the sensitivity of two gel staining procedures, Coomassie blue and silver stain, and second, to examine the effect of repeated purification cycles on a preparation of M. bovis pili.

M. bovis was grown and harvested by the method described in Section 2.5. Pili were purified, using the method described in Section 2.6. A sample of the crude pilus preparation was removed and cycled six additional times. The protein concentration of the seven samples was adjusted to 1.0 mg/ml, using an extinction coefficient of 0.59 mg/ml/absorbance unit [80]. Two mini-gels were prepared and loaded identically with 5 µl/well of the samples from each cycle. After electrophoresis was completed, one gel was stained with Coomassie Blue and one gel was silver stained. The stained gels are shown in Figure 3-1. The gel stained with Coomassie Blue revealed a single protein band (presumed to be pilin protein) of MW_a weight 19,300 daltons. There appeared to be no contaminant proteins on the Commassie blue stained gel, because the volume of protein placed in the wells of both gels was only 1 µl. It was necessary to use a very small volume, because a larger volume would have resulted in the silver stained gel being "overloaded". The silver stained gel revealed protein bands, in addition to the major band in cycles one

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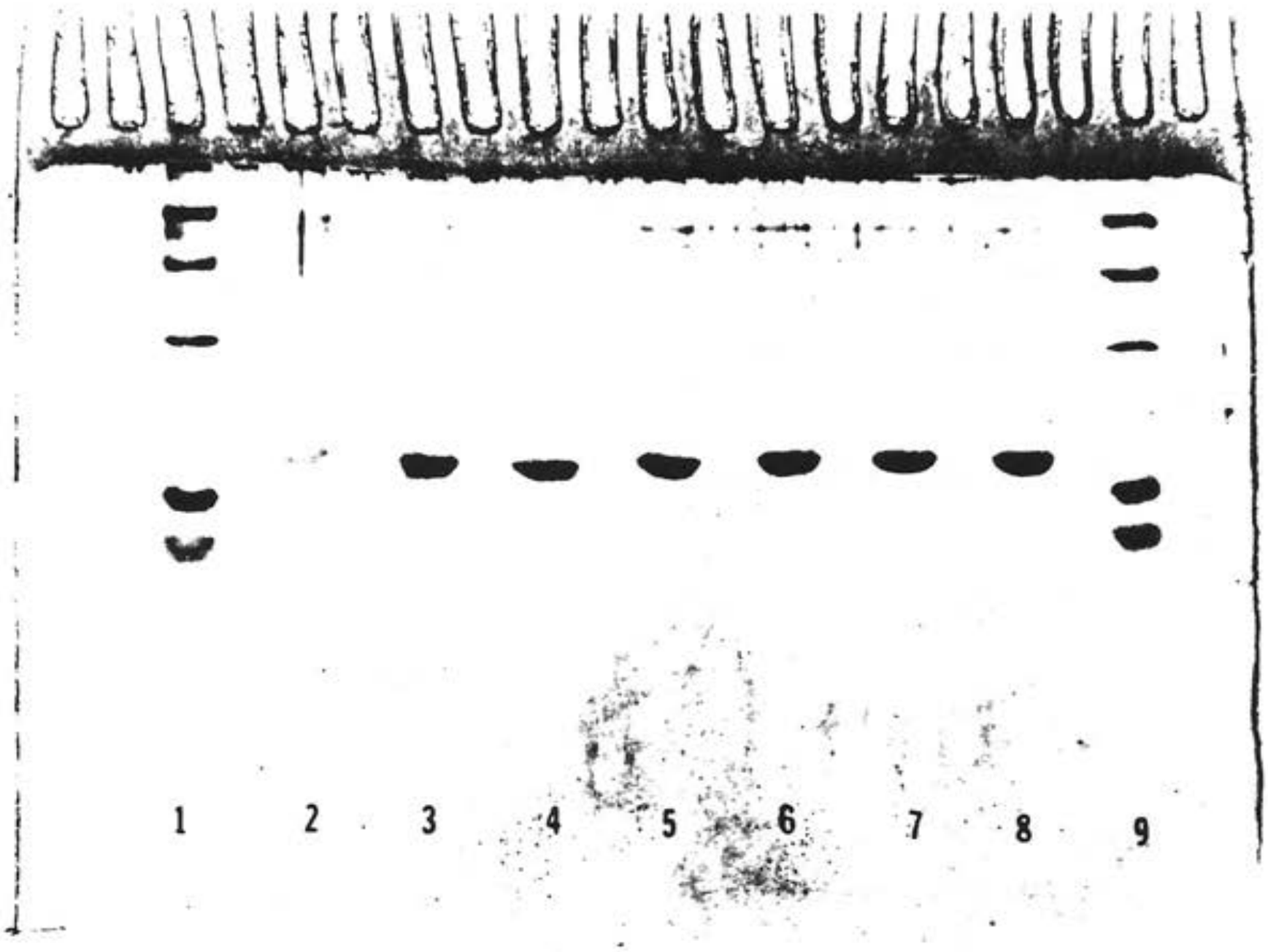
[7] alternatate solubilization (in 0.01 M KPB)/crystalization (in 10% saturated ammonium sulfate) of pilus preparation as described in Section 2.6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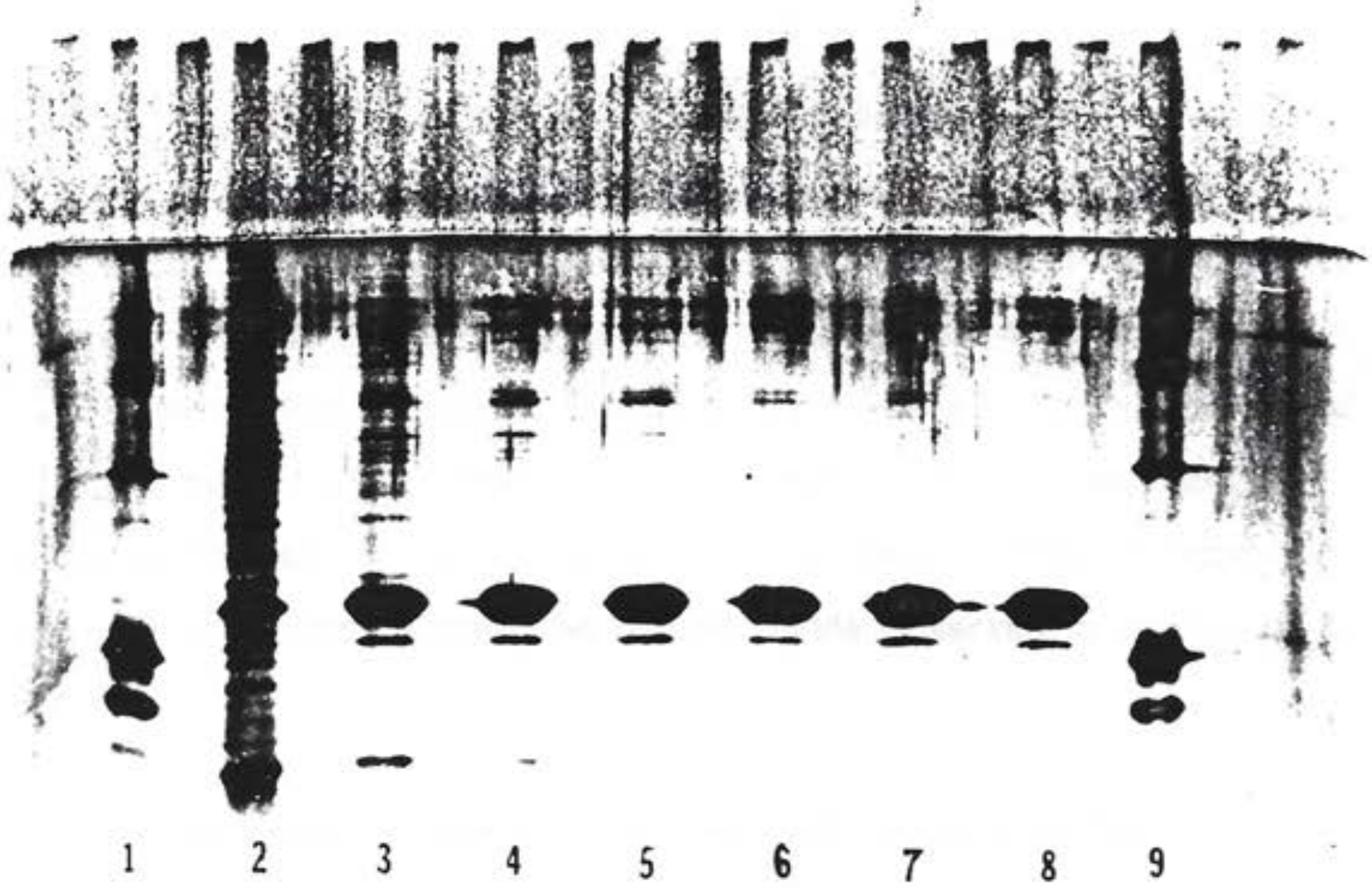
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Figure III-1: M. bovis pili from each of six cycles:
Gel A stained with Coomassie blue
Gel B stained with Silver Stain
M. bovis pili from each of six cycles:
lanes 1,9: molecular weight marker proteins
lane 2 crude pilus extract
lanes 3-8: cycles 1-6 of pilus preparation

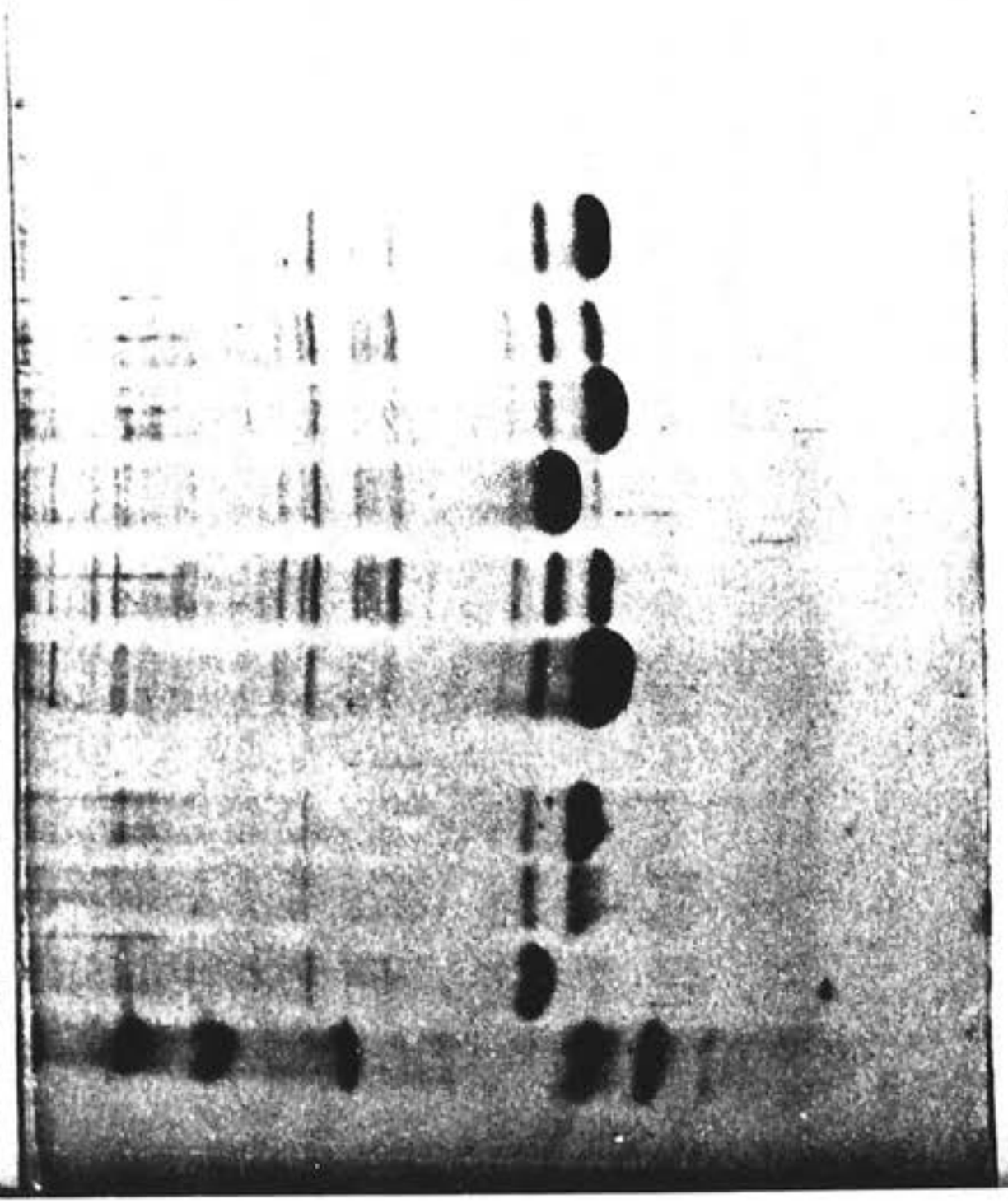
and two. In cycles three through six, it was apparent that most of the additional bands were eliminated with the repeated cycling. However, there was also a persistent band, having a molecular weight of approximately 17,000 daltons, which was not reduced in cycles three through six. It was also noted that both the Coomassie blue stained gel and the silver stained gel revealed artifactual bands at the very top of the running gel, which are not related to pilus proteins or molecular weight standard proteins. There are two conclusions from this experiment. First, silver staining was a more sensitive stain than Coomassie Blue, and second there was an additional protein band at 17,000 daltons that co-purified with the major pilus band.

2. Pilus Purification from Three P+ Clones of EPP63

In a second preliminary experiment, pili were purified (cycled three times) from each of the three p+ clones of M. bovis EPP63 that had been previously identified and cloned by Vilella [80]. The three clones used in this experiment had been named EPP63 1, EPP63 3, and EPP63 4, by Vilella, and are described in Section 1.6. EPP63 1 was further designated "Type A" colony type, and EPP63 4 was designated "Type B", since the two clones had distinctly different colony types. EPP63 3 was designated EPP63 INT for this experiment, since it appeared to be intermediate in colonial morphology between EPP63 1 and 4. (It was labled as EPP63 INT on the gel in Figure 3-2.) EPP63 1 and EPP63 4 appeared to yield pili of distinctly different MW_a, and both preparations contained both a heavier and a lighter molecular weight band. The more concentrated band of one sample corresponded to the less concentrated band of the other. EPP63 3 appeared to contain equivalent amounts of both the protein bands observed in clones EPP63 1 and EPP63 4.

For this experiment, it was hypothesized that unique pilus types may be associated with different colony types, and that the different colony types may be phase variants of a single strain of M. bovis. The presence of two distinct bands indicated that a rapid phase equilibrium might exist between the two phases of the same strain of M. bovis, one expressing pili of 19,700 daltons, and the other pili of 17,100 daltons.

PURIFIED PILI FROM DIFFERENT CLONES OF EPP63



Derived from EPP63 CLONE INT

CLONE 6
CLONE 5
CLONE 4

CLONE 3

CLONE 2
CLONE 1

CLONE B
CLONE INT
CLONE A

Mol. Wt. Markers

Figure III-2: Pilus preparations from three piliated phase variants with different colonial morphologies and pilus preparations from six piliated phase variants isolated from a TPB culture of EPP63
lane 1: molecular weight markers
lane 2: pilus preparation from EPP63 1
lane 3: pilus preparation from EPP63 3
lane 4: pilus preparation from EPP63 4
lanes 5-10:
pilus preparations from 6 p+ clones

B. PHASE VARIATION IN LIQUID MEDIA

From extensive examination of the p+ and p− colony types of M. bovis strain EPP63 on MH agar, it was concluded that p+ and p− clones retained their piliated and non−piliated colonial morphology when passed on solid MH media. Colonial morphology was maintained by picking a single clone and restreaking onto fresh media. The only observable type of phase variation that regularly occurred was a switch in the p+ to p− direction. Switching in the p− to p+ direction was never observed on solid agar.

Brinton and his colleagues have shown that even small variation in the growth environment of bacteria can have profound effects on the phase composition of bacterial cultures, by modifying both phase−switching probabilites and phase growth rates in a phase−specific way (Brinton, unpublished observations). They have shown that a large number of different phases of a single species can be found if one gives the strain a variety of different conditions and isolates all the different colonial types found in such cultures. Brinton has coined the term phase "eduction" (uncovering something hidden) for this procedure.

Incubation in liquid media was tested as a growth environment for educing the expression of multiple p+ phases from p+ and p− clones of M. bovis EPP63. Eleven different broth media were tested. The broth media were inoculated and incubated as described in Section 2.4. After five days static incubation in 10.0 ml of broth, the turbidity of the broth was noted, and 0.05 ml of the cultures were plated out onto each of ten MH plates. Variation in colonial morphology was observed after growth in several of the broth media. Growth and variation in colonial morphology are presented in Table 3−1. Photographs of the variation in colony morphology on MH agar plates inoculated with growth from TPB cultures inoculated with EPP63 3 are shown in Figure 3.3 and Figure 3.4.

The significance of this experiment was that a large number of new phases of M. bovis could be educed by enrichment in unshaken broth culture that were never seen during transfer on MH agar. The colony types varied in size, color, edge morphology and surface texture. When colony types were passed on MH

Broth	Presence or Absence of Growth
1. Peptone	–
2. Protease Peptone #3	–
3. Nutrient Broth	–
4. Tryptose	–
5. Tryptose Broth	+
6. Tryptose Phosphate Broth	+
7. Tryptone	+
8. Trypticase Soy Broth	+
9. Trypt. Soy Br. (no dextrose)	+
10. Mueller Hinton Broth	+
11. Z Broth	–

Table III-1: Presence or absence of growth in eleven different broth media after five days static incubation at 37° determined by plating cultures onto MH plates.
(-)<2 cfu's/plate, (+)>2 cfu's/plate.

Figure III-3: Multiple colony type phase variants derived from a single cell of EPP63 after five days static incubation in TPB.

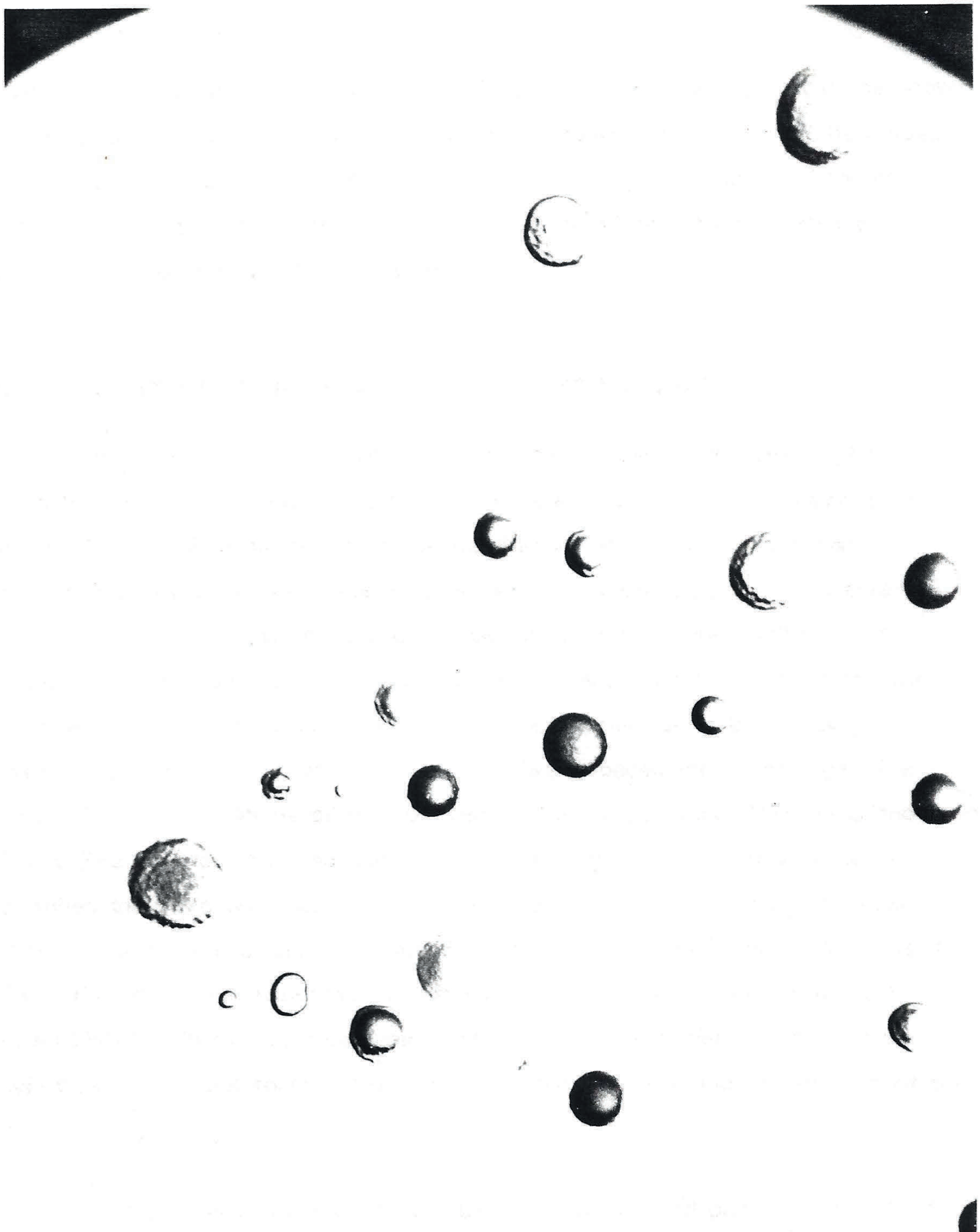


Figure III-4: Details of colony type phase variants derived from a single cell of EPP63 after five days static incubation in TPB.

agar, they did not all retain their primary morphology.[8] It was not within the scope of this study to "enumerate" all of the different phases, since some of the phases were stable, others were unstable, and there was a large number of different phases. No further experiments were done on the effect of environmental conditions as an educer of phase variants.

C. PILUS PURIFICATION FROM M. BOVIS PHASE VARIANTS

This experiment was performed to determine whether the unique phase variants isolated from static incubation in TPB were associated with unique pilus types. From a MH plate of phase variants (having variation like that shown in Figures 3-3 and 3-4), six phase variants having p+ morphology were selected. The six p+ clones were randomly chosen to be different from one another in colony morphology. The six clones were passed on MH agar, and the stablility of their colonial morphology was ascertained. Pili were purified, using the "quick-prep" method described in section 2.11.3. The pili were loaded onto a mini-gel after one cycle. The concentrations of the six preps were not adjusted. This gel is shown in Figure 3-2. It was observed that the pili purified from the six new phases all exhibited the same two bands as the three phase variants isolated by D. Vilella, although the relative amounts of each band varied among the phases. As a result of these findings, it was hypothesized that two pilus types could be expressed by strain EPP63. These two pilus types were assigned the names α and β. Two hypotheses were put forth at this time to explain the differential expression of the two pilus types.

1. Rapid phase variation occurs between two piliated phases, α and β. A bacterial culture contains bacteria in each of the two phases, each of which expresses one pilus type.

2. M. bovis may express two or more pilus types simultaneously on its cell surface, and the relative amounts of each type vary among the preparations.

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[8] primary morphology: morphology of a colony when first inoculated onto MH agar after liquid culture.

D. CHARACTERIZATION OF ALPHA AND BETA PILI OF EPP63

The purpose of the following experiments was to demonstrate the uniqueness of the two pilus types. The criteria used were: MW_a as determined by SDS PAGE, immunological cross-reactivity as determined by ELISA, and proteolytic peptide typing ("chemotyping").

1. MW_a Determination of Alpha and Beta Pili

The MW_a's of α and β pili were obtained using SDS PAGE with standard molecular weight marker proteins, as described in Section 2.4. The $\log_{10}$ of the molecular weights of the five proteins were plotted against their relative mobilities on an SDS gel. A linear regression[9] done for each experiment, and the mean molecular weight was determined from seven experiments (r^2 =.9873).[10] The calculated molecular weights for α pili was 19,700 daltons, and for β pili was 17,100 daltons. A linear regression line of $\log_{10}$ molecular weight vs. relative mobility is shown in Figure 3-5. Gels showing the five marker proteins and the two pilus types for each clone are shown in Figure 3-2. Although the relative amounts of protein in each of the two bands of a pilus preparation is variable, the molecular weights of each band are constant for the pilus preparations from each of the six clones.

2. Immunological Cross-Reactivity of Alpha and Beta Pili

ELISA serological cross-reactivity studies between EPP63 α and β pili was additional evidence that the pilus types were distinct from one another. The data is presented in non-normalized (absolute titer) form in Table 3-2 and normalized[11] form in Table 3-3. As a negative control, an ELISA was also done, using whole

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[9] a simple linear regression was done: $y=mx+b$, where x was the mobility of the protein, and y was the calculated molecular weight

[10] r^2 is the percentage variance accounted for from the linear regression

[11] To normalize data: homologous titers are given a value of 100%, and other titers are calculated as ratios of 100%

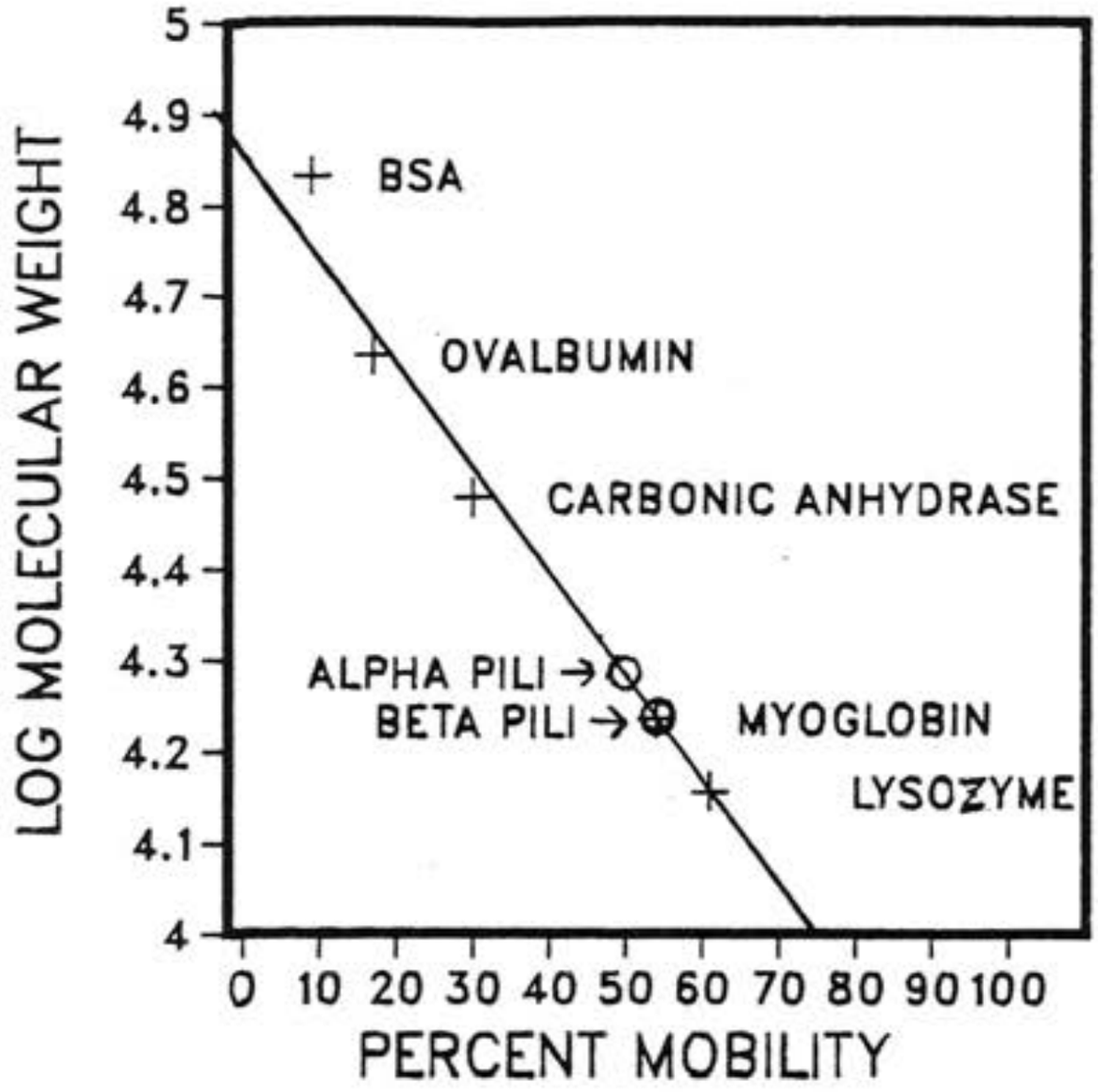

Figure III-5: Regression plot of log 10 molecular weight vs. per cent mobility on polyacrylamide gel with molecular weights of two pilus types extrapolated from mobilities.

pilus rods of N. gonorrheae and E. coli (Type 1) pili (obtained from J. Bryan), the corresponding antisera, and EPP63 α and β pilus antisera. The cross-reacting titers between the gonococcal and E. coli pili and the M. bovis antisera were equivalent to pre-immune titers. The pre-immune titers for the four rabbits were: (1) 9, (2) 4, (3) 7, (4) 2. The method for determination of titer is described in Section 2.6. Although the titers are not statistically different from one another, (t=.6965 for α sera and t=.7299 for β sera, p>.5 for both sera)[12] with a larger sample size it is

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[12] Statistical analysis of the data was done using a Student's t-test with comparison of the means as two independent samples, using a pooled estimate of variance.

Antisera	Antigen on Plate	
	α pili	β pili
(α sera)		
Rabbit #1	10,000	4,185
Rabbit #2	19,641	4,151
Mean Absolute Titer	14,821	4,168
(β sera)		
Rabbit #3	3,502	3,925
Rabbit #4	5,706	10,000
Mean Absolute Titer	4,604	6,963

Table III-2: Absolute titers of α and β antisera against α and β pili as determined by ELISA

Antisera	Antigen on Plate	
	α pili	β pili
α pili	100%	28%
β pili	68%	100%

Table III-3: Mean Normalized titer of α and β antisera against α and β pili as determined by ELISA.

expected that the titers would be statistically different. The degree of serological homology cannot be accurately determined from this experiment, because the pilus preparations used to inoculate rabbits and to coat wells of the ELISA plates were "contaminated" with the heterologous pilus type. The SDS gels of the α and β pili used to make the antisera are shown in Figure 3-2, (lanes two and four).

3. Proteolytic Digests of EPP63 Alpha and Beta Pili for Chemotyping

Pilus preparations of EPP63 α and β pili were enzymatically cleaved into peptide fragments using papain and V8 protease in the presence of SDS. After digestion with one of these enzymes, digested pili were loaded on a gel, and run as described in Section 2.4. Each of the enzymes revealed different peptide cleavage patterns for both of the pilus typess, indicating that α and β pilus type are unique. These results are shown in Figure 3.6.

E. CLASSIFICATION OF EPP63 BETA PILI WITHIN THE PILUS TYPING SYSTEM

In this experiment, enzymatically treated EPP63 β pili were compared to enzymatically treated pili from eight previously typed pilus groups. The eight pilus groups, each containing between one and four strains of M. bovis are listed in Table 1–1. The strains were placed in these groups according to the peptide digest patterns of their pili. EPP63 α pili were classified in Group II. For each of the eight pilus groups, the pili from a single strain were used to represent the entire group, because all of the strains within each group had been shown [36] to have identical peptide digest patterns. The eight digested pili from the eight groups[13] were placed on 15% gels in order of their decreasing MW_a. These gels are shown in Figure 3–7 (V8 protease) and Figure 3–8 (chymotrypsin). For both enzymes, the β pili of EPP63 had a peptide digest pattern identical to Group IV pili. It was concluded that strain EPP63 could be classified in both Group II (α pili) and Group IV (β pili). It was further concluded that reclassification of all strains should be done after all the strains had been educed to express both α and β pili.

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[13] The proteolytic digestion of all pilus types except EPP63 b pili was done by P.G. Haneline.

PAPAIN AND V8 PROTEASE DIGESTION OF ALPHA AND BETA PILI OF EPP63

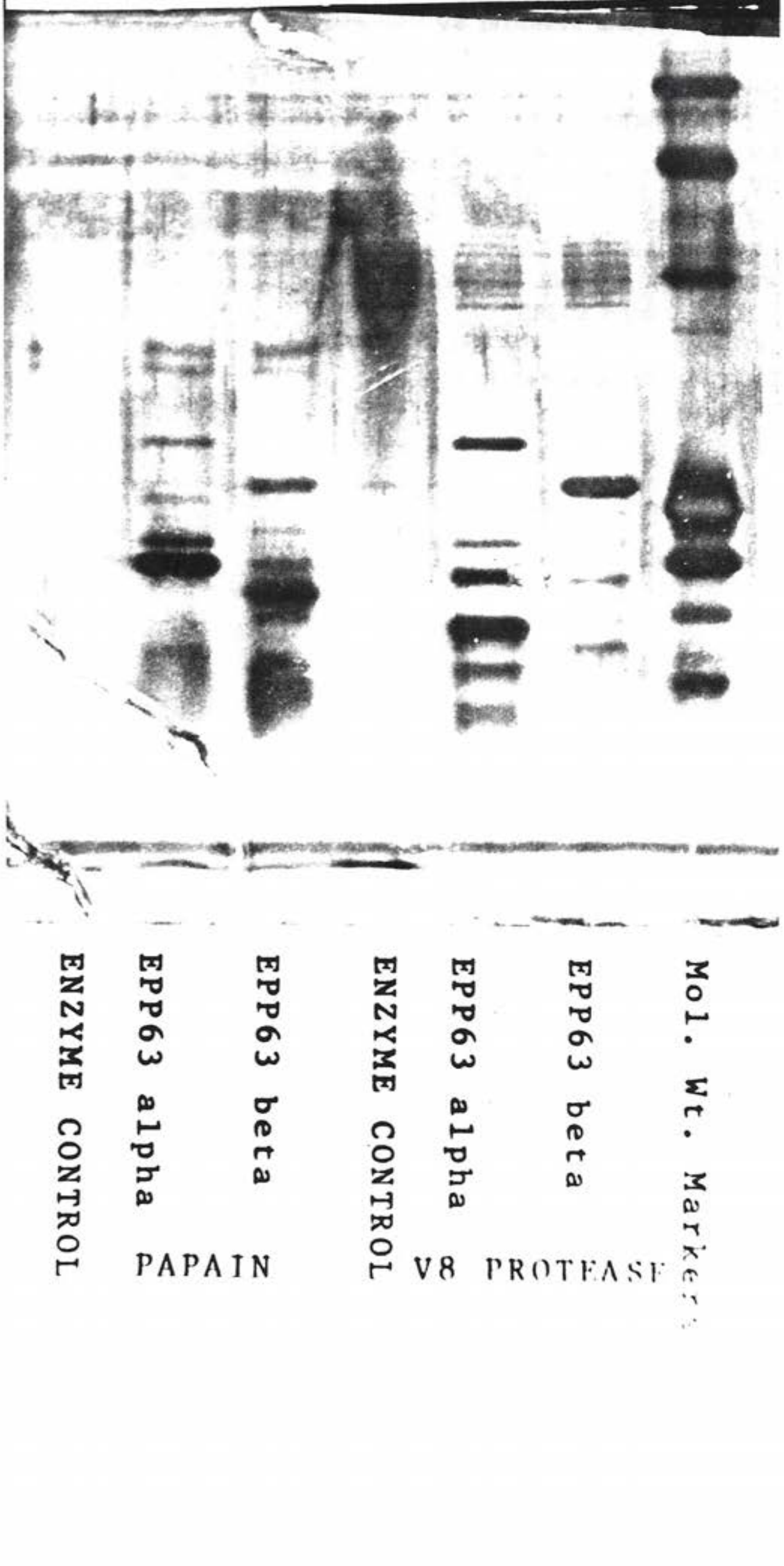
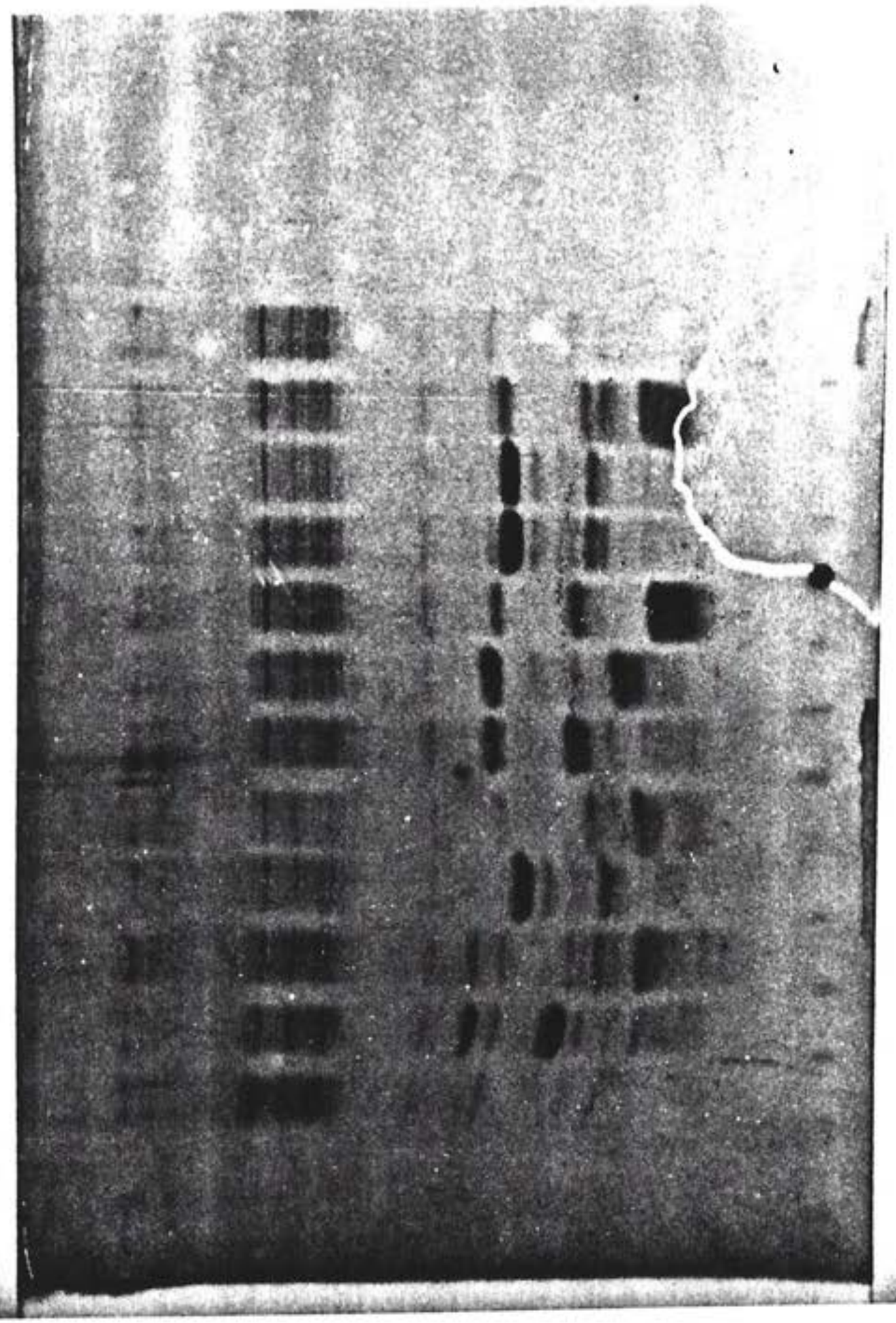


Figure III-6: Papain and V8 protease digestion of α and β pili of EPP63 [from left to right: papain enzyme control, EPP63 alpha, papain, EPP63 beta, papain, V8 protease enzyme control, EPP63 alpha, V8 protease, EPP63 beta, V8 protease, molecular weight markers]

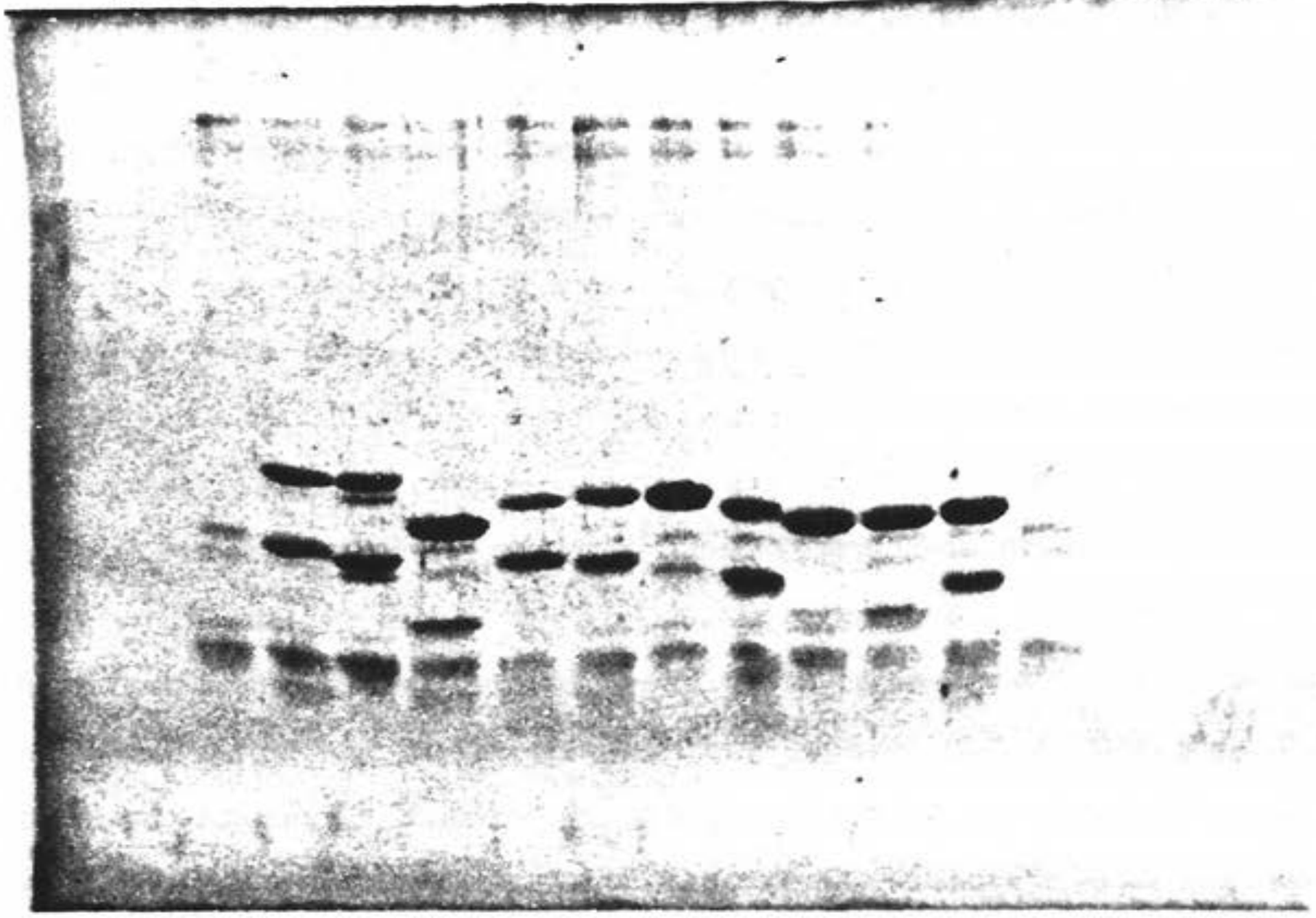
V8 PROTEASE DIGESTION OF PILI FROM EIGHT GROUPS AND EPP63 ALPHA AND BETA PILI



ENZYME CONTROL
FLA64 (V)
EPP63 beta
WSE64 (13R) (IV)
1965GLENN (5004) (II)
IBH68 (712) (I)
GLN63 (VI)
NTN63 (8033L) (VIII)
EPP63 beta
EPP63 (II)
MAC74(2554) (IV)
ENZYME CONTROL

Figure III-7: V8 protease digestion of pili from eight groups with EPP63 β pili [from left to right:
V8 protease enzyme control,
MAC74 2554 group IV,
EPP63 group II,
EPP63 beta pili,
NTN63 8033I group VIII
GLN63 group VI,
IBH68 712 group I,
1965GLENN 5004 group II,
WSE64 13R group IV,
EPP63 beta pili,
FLA64 group V,
V8 protease enzyme control]

CHYMOTRYPSIN DIGESTION OF PILI FROM EIGHT GROUPS AND EPP63 ALPHA AND BETA PILI



ENZYME CONTROL

FLA64 (V)
EPP63 BETA
WSE64 (13R) (IV)
1965GLENN (5004)
IBH68 (712) (I)
NTN63 (8033L) (VI)

EPP63 beta
EPP63 (II)
MAC74(2554) (VI)

ENZYME CONTROL

Figure III-8: Chymotrypsin digestion of pili from eight groups with EPP63 β pili [from left to right: chymotrypsin enzyme control, MAC742554 group IV, EPP63 group II, EPP63 beta pili, NTN63 8033L group VIII, GLN63 group VI, IBH 712 group I, 1965GLENN 5004 group II, WSE64 13R group IV, EPP63 beta pili, FLA 64 group V, chymotrypsin enzyme control]

F. IDENTIFICATION OF ADDITIONAL PILUS TYPES IN STRAINS OF M. BOVIS

The preliminary hypothesis, stated in Section 3.1.2, though unsupported with experimental data, was that the α pilus type could be correlated with a particular colony type "Type A", and β pilus type could be correlated with "Type B" colony type. In the N. gonorrheae system, Type 1 colonies and Type 2 colonies always yield pili of different types (Brinton, unpublished results). In addition, the experiment described in Section 3.1.2 suggested this hypothesis, since the two pilus types were isolated from colonies of different morphologies. The approach taken in this experiment was as follows: from the classification scheme of the strains of M. bovis, a representative strain was picked from each group as a source of pili. The colonial morphology of the clones of these strains were of the "Type A" colony type, which was like that of EPP63 I, in the experiment in Section 3.1.2. It was hypothesized that pili resulting from a "Type B" colony type would be of lower molecular weight than the pili purified from "Type A" colony types, since this was the case with EPP63. Each of the strains was thawed, and then inoculated into TPB and incubated at 37° for five days. It was hypothesized that after five days static incubation in TPB, the plated-out growth would contain colonies of "Type B" morphology. If no "Type B" colonies were present in the growth, the procedure could be repeated. A colony or colonies which appeared to be the most "Type B"-like, or unique from the rest of the culture was selected and reinoculated into TPB culture, and incubated for an additional five days. Sometimes this procedure was repeated a third and forth time, until a clone having a "Type B" colony morphology was isolated. There were several factors, however, which were discovered during the course of this procedure which disproved the initial hypothesis. They were: 1. colony types were not always stable when passed on MH agar. 2. there were different variations of "Type B" and "Type A" colony types. 3. even when a "Type B" colony type was isolated, cloned, and remained stable, the purified pili could be of the same MW_a as the pili of a "Type A" colony type.

The only strain from which an additional pilus type was isolated from a "Type B" colony type was strain MED72 (4R). However, this new pilus type had a greater MW_a (19,300 daltons) than the original pilus type, so this new pilus type, purified

from a "Type B" colony type, was actually an α pilus type. (The name α was assigned to the pilus type of a strain having the higher MW_a, and the name β was assigned to the pilus type having the lower MW_a.)

In an attempt to isolate additional pilus types from additional strains of M. bovis, the protocol was modified. Instead of isolating only "Type B" colony types, a variety of colony types was randomly selected, cloned, and pili was purified from them, whether or not their colony morphologies remained stable. Using this random selection method, two unique pilus types were isolated from strains FLA64 and NTN63 (8033L). The calculated MW_a's for each pilus type of the four strains are shown in Table 3-4.

Strain	α pili	β pili
EPP63	19,300	17,100
MED72(4R)	19,300	17,100
FLA64	18,300	17,500
NTN63	18,300	17,500

Table III-4: MW_a of two pilus types of four strains of M. bovis as determined by SDS gel electrophoresis.

It was concluded that colony type is not a good marker for pilus type in M. bovis.

1. Correlation Between EPP63 Pilus Types and MED72 (4R) Pilus Types

After it was determined that EPP63 β pili had identical peptide digest patterns to Group IV pilus types, an second pilus type was isolated from strain MED72 (4R). This second pilus type of MED72 (4R) had a MW_a of 19,300, corresponding to the MW_a of EPP63 α pili. The α and β pilus types of MED72 (4R) and EPP63 were digested with enzymes, and it was shown that the chemotypes of MED72 (4R) α and β pili were identical to the chemotypes of EPP63 α and β pili. The gels of the

digested pilus types are shown in Figure 3-9 and Figure 3-10. (The "MED72 original" pilus preparation in both gels shows that pilus type purified from the clone used in the Vilella study was of the β pilus type.) It was concluded that EPP63 should be reclassified as a member of Group IV, because of its identical pilus types with strain MED72 (4R).

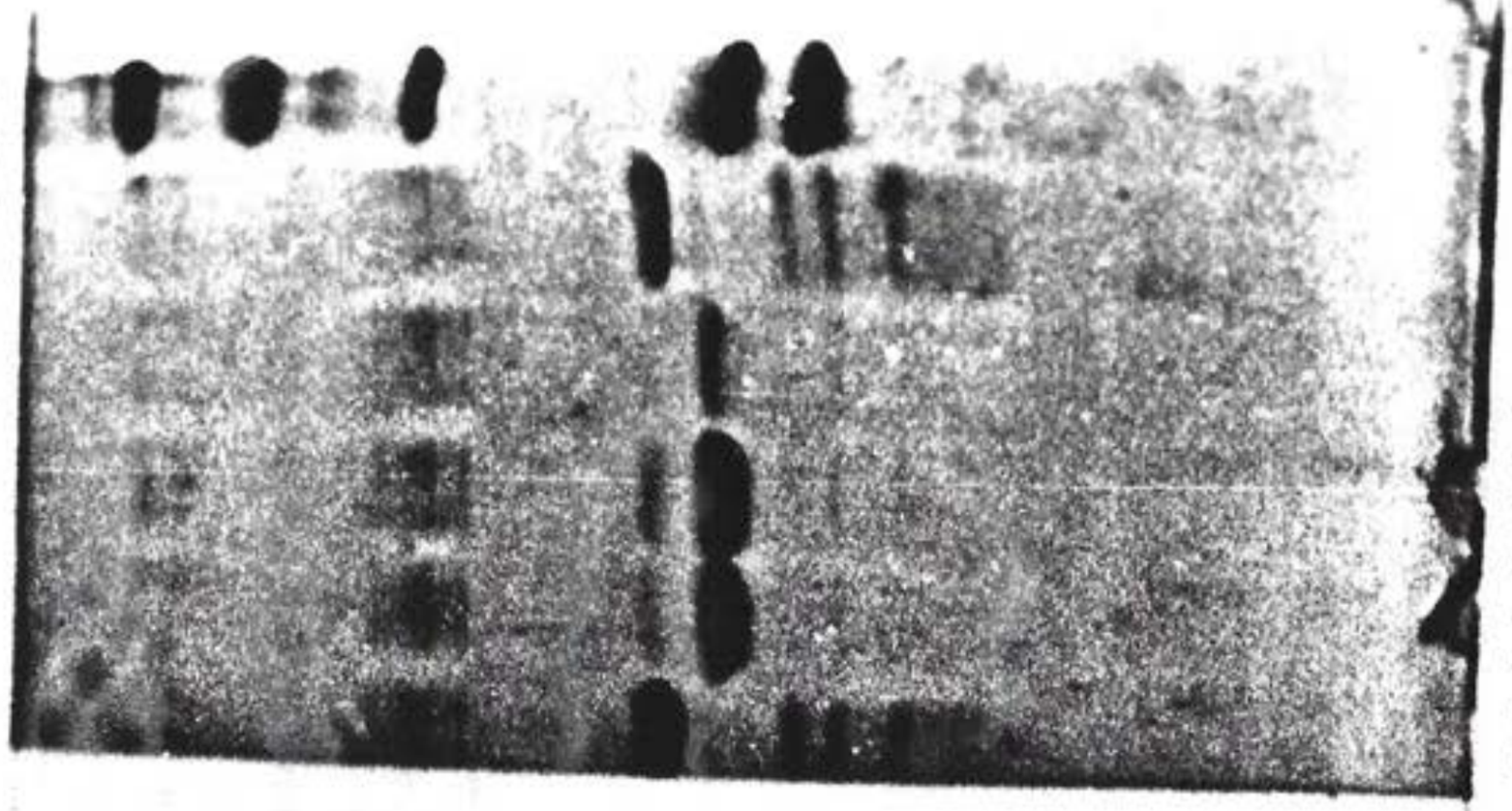
2. Additional Pilus Types Isolated from Strains FLA64 and NTN63 8033L

An additional type of pilus was found on each of the strains NTN63 (8033L) and FLA64. For FLA64, the new pilus type was an α type, with MW_a of 18,300, and for NTN63 (8033L) the new pilus type was a β type with MW_a of 17,500. The MW_a of the two pilus types of the two strains correspond, as shown in Table 3-4. Since these two sets of MW_a's for these two strains appeared to be identical, it was hypothesized that the two strains could be placed into a single group, as was done with MED7 (4R) and EPP63. However, the results of the chemotyping digest experiments revealed unique peptide digest patterns for all four pilus types. Thus, the identity between the two pairs of MW_a values was fortuitous. The gels of the digested pilus types are shown in Figure 3-11. From the results of these experiments it was concluded that it is important to isolate both pilus types from each strain of M. bovis, so that accurate classification of strains into groups can be done.

G. SOLUBILITY CURVES OF PILUS TYPES FROM EPP63 AND FLA64

In this experiment, the solubility in ammonium sulfate of pairs of pili, α and β, from the two strains of M. bovis EPP63 and FLA64 was investigated. The purpose of this study was to establish protocols for purifying pili, so that two pilus types of a strain could be purified from one another, or so that two pilus types could be co-purified. Co-purification of pili could be done in the preparation of a vaccine, when it is necessary to purify all of the pilus types from a single clone. Solubility is mainly a function of the number, type, and arrangement of ionizable groups on the surface of a pilus rod, although the number and arrangement of any hydrophobic groups on the surface may also be important. The solubility curves are

V8 PROTEASE DIGESTION OF
TWO TYPES OF EPP63 PILI
AND TWO TYPES OF MED72 PILI



Mol. Wt. Markers

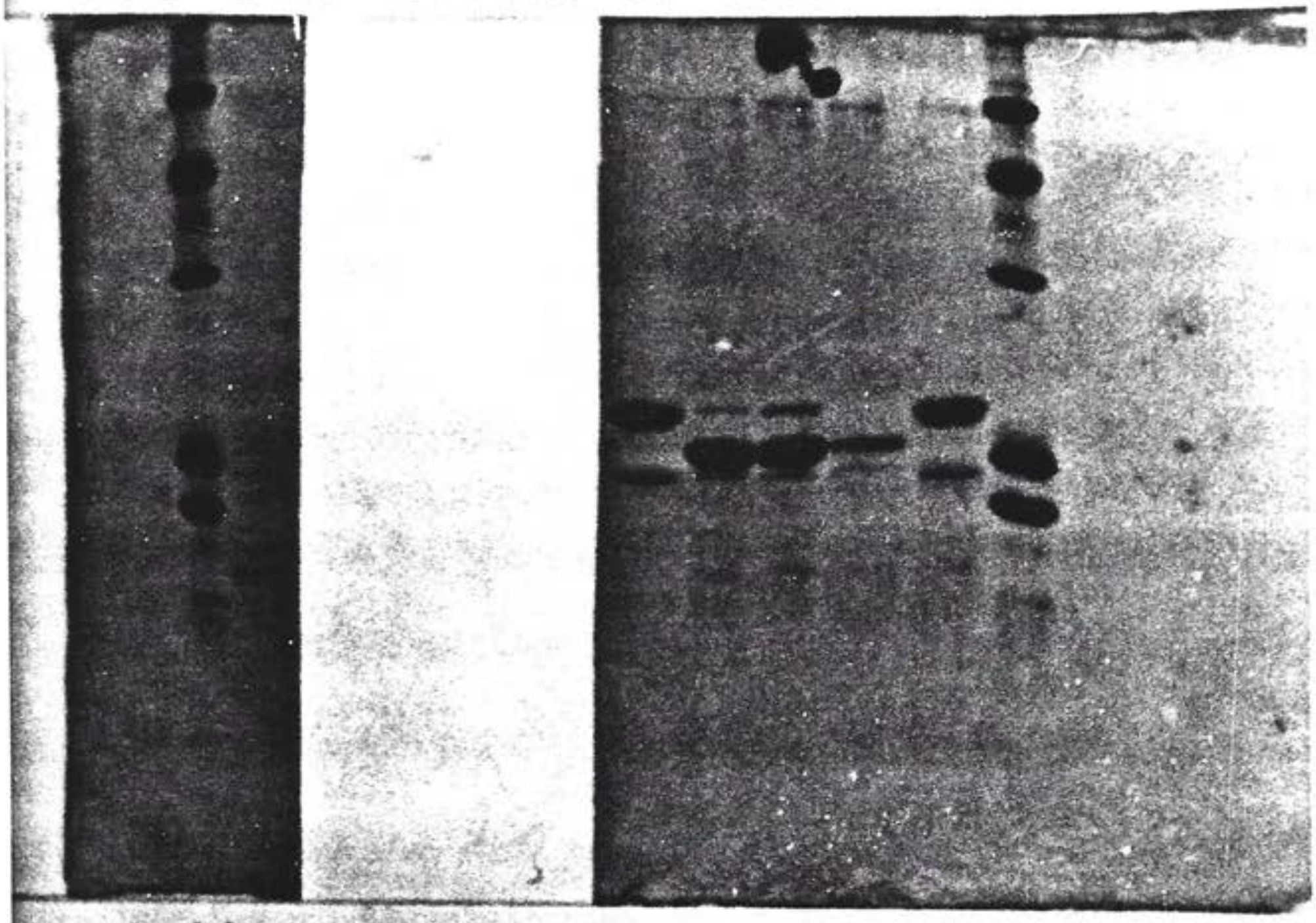
MED72 alpha

MED72 beta
MED72 (original)
EPP63 beta
EPP63 alpha

ENZYME CONTROL
Mol. Wt. Markers

Figure III-9: V8 protease digestion of two types of EPP63 pili and two types of MED72 4R pili
[from left to right: molecular weight markers
V8 protease enzyme control
EPP63 alpha pili
EPP63 beta pili
MED72 4R beta pili original preparation
MED72 4R beta pili
MED72 4R alpha pili
molecular weight markers]

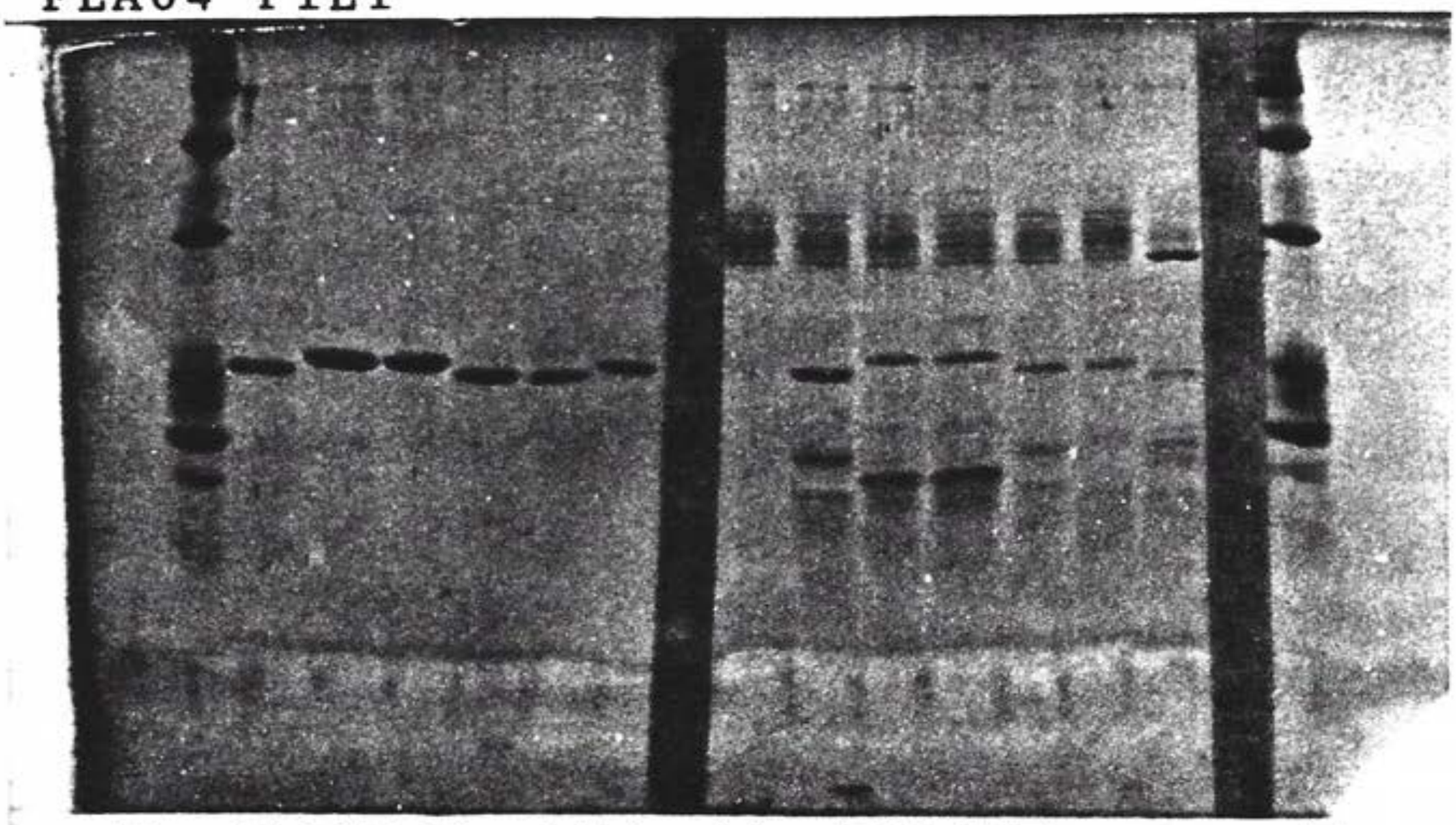
CHYMOTRYPSIN DIGESTION OF TWO TYPES OF EPP63 PILI AND TWO TYPES OF MED72 PILI



ENZYME CONTROL
Mol. Wt. Markers
MED 72 alpha
MED72 beta
MED72 (original)
EPP63 beta
EPP63 alpha
Mol. Wt. Markers

Figure III-10: Chymotrypsin digestion of two types of EPP63 pili and two types of MED72 4R pili [from left to right: molecular weight markers
chymotrypsin enzyme control
EPP63 alpha pili
EPP63 beta pili
MED72 4R beta pili original preparation
MED72 4R beta pili
MED72 4R alpha pili
molecular weight markers]

V8PROTEASE DIGESTION OF FOUR QUICK-PREPS of NTN63 (8033L) PILI AND TWO TYPES OF FLA64 PILI



Mol. Wt. Markers
FLA64 alpha
FLA64 beta
NTN63(8033L)
NTN63(8033L)
NTN63(8033L)1
ENZYME CONTROL
FLA64 alpha
FLA64 beta
NTN63(8033L)4
NTN63(8033L)3
NTN63(8033L)2
NTN63(8033L)1
Mol. Wt. Markers

Figure III-11: FLA64 pili and NTN63 pili:
undigested and digested with V8 protease
[from left to right: molecular weight markers
NTN63 8033L clone 1
clone 2
clone 3
clone 4
FLA64 beta pili
FLA64 alpha pili
V8 protease enzyme control
NTN63 8033L clone 1 V8 pr
clone 2 V8 pr
clone 3 V8 pr
clone 4 V8 pr
FLA64 beta pili
FLA64 alpha pili
molecular weight markers]

shown in Figure 3-12. There was a marked difference in the solubility of the two EPP63 pilus types: the inflection point of solubility for α pili was 1% SAS, and for β pili was 3% SAS. In contrast, both types of FLA64 pili had a solubility inflection point of about 1% SAS. The relative solubility was determined as a percentage of the maximum solubity, as determined from a control pilus preparation that had not been recrystalized and resuspended in buffer.

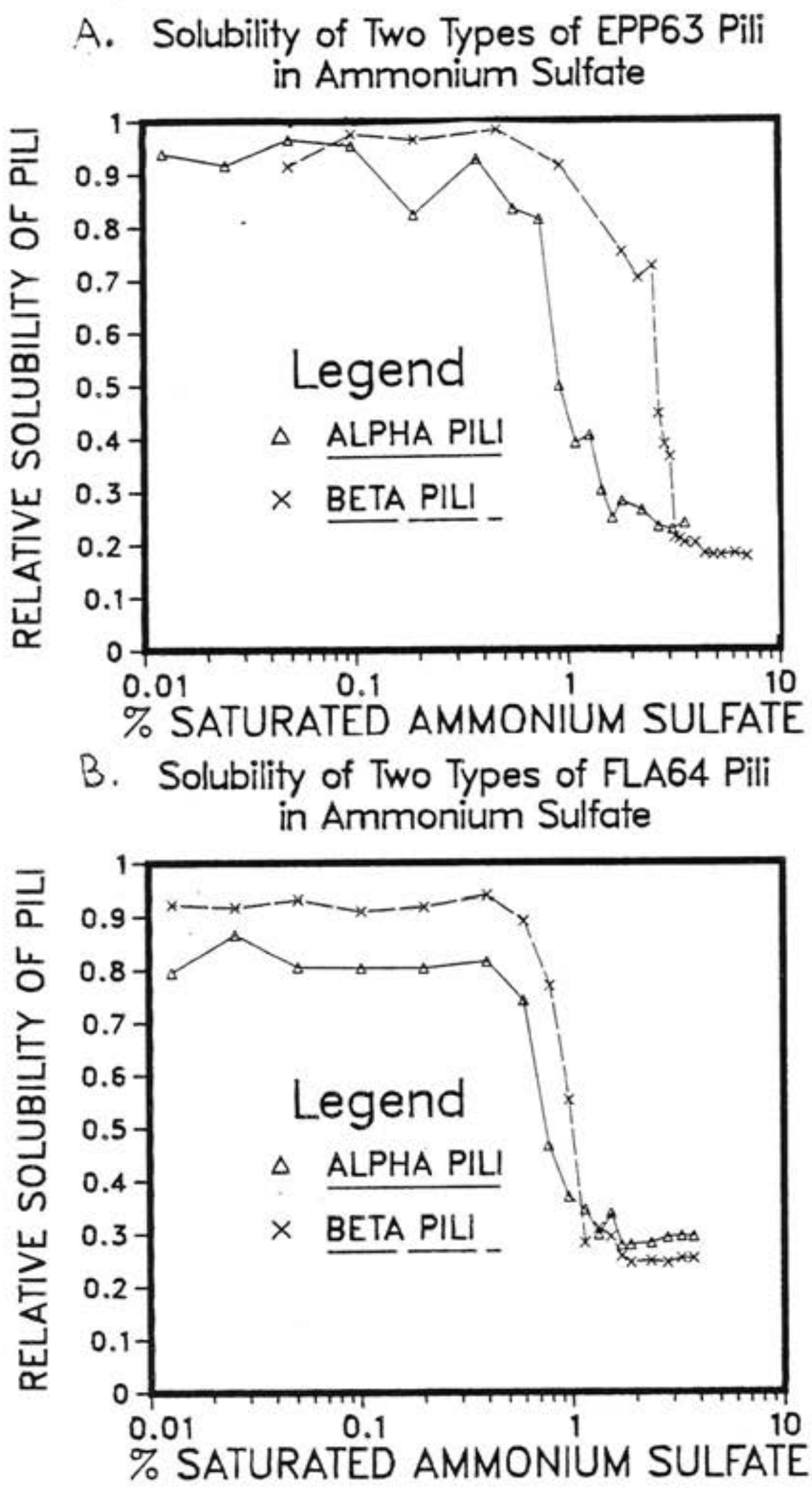


Figure III-12: Relative solubility of α and β pilus types as a function of percentage saturated ammonium sulfate used for recrystalization.
A. EPP63 α and β pili.
B. FLA64 α and β pili

H. GROWTH CURVES OF DIFFERENT PHASES OF EPP63

The purpose of this experiment was to determine whether two phase variants expressing different p[ilus types grow at different rates. As described in Section 3.1.2, it was initially hypothesized that "Type A" and "Type B" colony types expressed specific pilus types represented specific phases, expressing α and β pilus types, respectively. A sketch showing differences in size and texture of the two colony types is shown in Figure 3-13.

Clone A

Clone B

Figure III-13: Two colony types of EPP63 used in growth rate experiment

If different phases have different growth rates, then the phase composition of a bacterial culture will be a function of the relative growth rates of the different phases. This experiment was done on solid agar, rather than in broth medium, for the following reasons. First, colony type could be closely monitered as an indicator of phase type. Second, a single colony is derived from a single cell. The two colony types "Type A" and "Type B" colonies were grown for a specified length of time on MH agar, and the entire colony was lifted from the agar surface and suspended in 100 mls of saline with a syringe and 26 gauge needle. The time points in this experiment were 13, 18, 23, 24, and 31 hours. Colonies were not visible before 13 hours. Aliquots were plated out, and the number of colony

done on the data sets at the time points at 18 hours (t=7.958, p<.001), 24 hours (t=8.807, p<.001), and 31 hours (t=3.884, p<.025). The differences in the numbers of cfu's at these three time points was statistically significant.

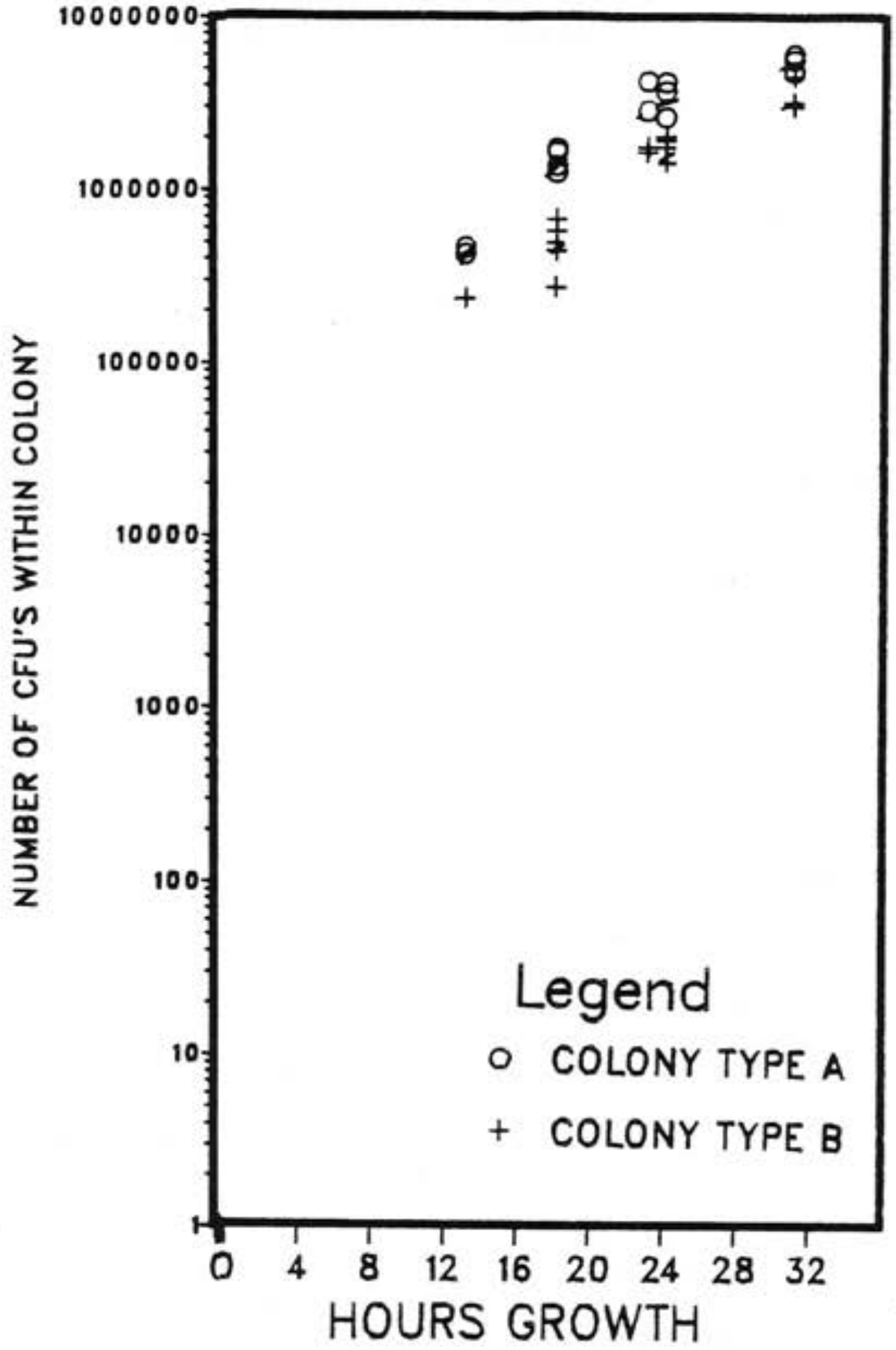


Figure III-14: Growth curves of two phase variants of strain EPP63 assayed on solid media. The number of cfu's within each colony was determined by resuspending the colony and counting the number of cfu's using dilutions and replating. Colonies were not visible on solid media at less than twelve hours.

forming units (cfu's) were counted on the following day. A Student's t-test was

Hours Growth	Clone A	Clone B	
13	42,46	23	
18	163,124,171 136,124	27,44,57 67,49	p<.001
23	282,419	163,173	
24	458,366,414	174,142 190,200	p<.001
31	556,600,471 445	314,299	p<.025

Table III-5: Colony forming units (X 10,000) within colonies after specific incubation periods

I. COLONY IMMUNOBLOT OF ALPHA AND BETA PILIATED CLONES WITH HETEROLOGOUS SERA

The purpose of this experiment was to determine whether or not all colonies, spread from a single clone onto solid MH media, reacted uniformly with both α and β antisera. A uniform reaction would be one in which all of the colonies on the plate yielded the same color reaction on NC after immunoblotting. This would indicate that all the colonies on a plate were identical, and that all of the individual cells within a colony expressed identical amounts of both pilus types on their cell surfaces. Differential reactivity of colonies between α and β serum would mean that phase variation within a culture occurred, and that an individual bacterium, or the resulting homogeneous colony, expressed either one or the other type of pili, but not both. For the experiment, cultures plated onto solid media, known to yield mostly α pili or mostly β pili were blotted with NC, and a part of each culture blot was reacted with one of the antisera. Each culture blot reacted strongly with the homologous serum, so that the size and shape of the individual colonies could be detected on the NC blot. However, when each culture was reacted with the heterologous serum, there was a small, but significant number of individual colonies that reacted positively. The majority of the colonies reacted only slightly with the

heterologous serum, probably because of the antibodies to contaminant pilus protein of the opposite type. The results of this experiment indicates a rapid phase change from α to β phase and the reverse, and explains the difficulty in obtaining pure α or β pili. Photographs of these blots are shown in Figure 3-15.

J. LABORATORY MODEL FOR INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

1. Experiment 1

The purpose of this experiment was to duplicate the procedure of Ostle and Rosenstriech for inducing IBK in laboratory mice. Five mice were lightly etherized, and the right eye of each was abraded by a single light stroke with a small wire brush[14]. A swab was touched to an agar plate containing M. bovis α piliated colonies, so that 4-5 colonies adhered to the swab. The swab was brushed against the abraded eye, so that a clump of bacteria was visible on the surface of the eye. The left eye was left undisturbed. The mice recovered from the ether after a few moments, and were returned to their cages. After 24 hours, the mices' eyes were examined for signs of inflammation. One mouse of the five had a noticeably watery discharge, although there was no corneal opacity present. In this experiment, the eyes were not cultured for M. bovis.

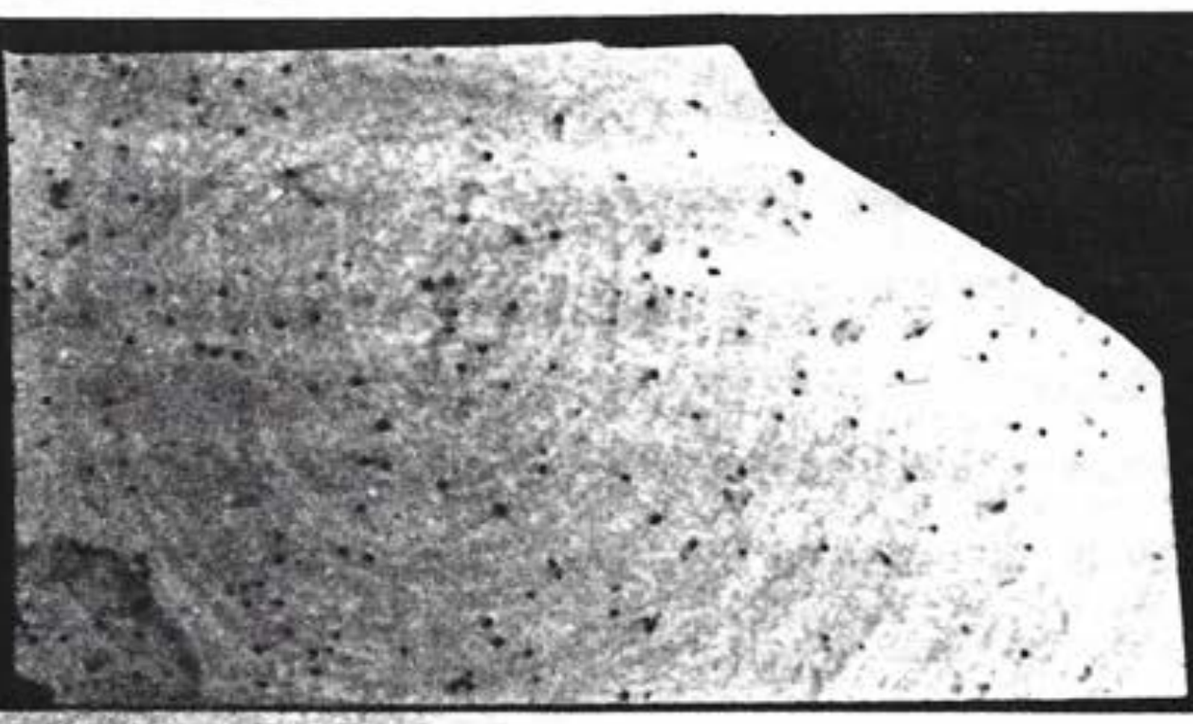
2. Experiment 2

Two aspects of the procedure were altered in this second experiment. The mice were injected intraparentaneally with 0.3 ml of 10% Nembutal solution, rather than anethetizing them with ether. Instead of using a swab to deliver colonies to the mouses' eyes, a cell suspension of 0.01 ml (containing approximately 3×10^6 bacteria) was delivered with a micropipetman to the mouses' eyes. Since the mice were under anethesia with their eyes open, the droplet of suspended bacteria remained undisturbed as a miniscus for approximately 75 minutes. After 24 hours,

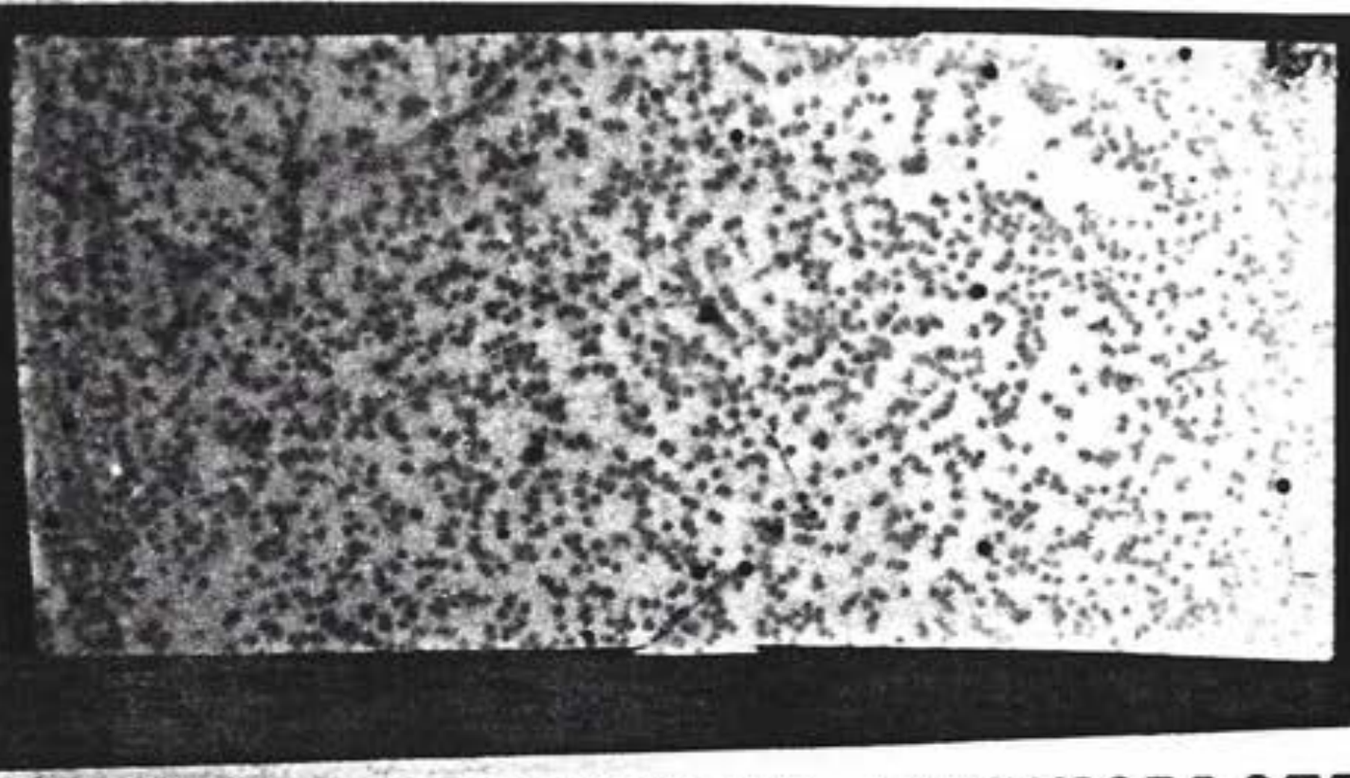
---

[14] The small wire brush was made from a two inch section of rubber-coated copper wireing that was purchased in a hardware store. Approximately twelve thin wires were enclosed within the wire tubing. The rubber was peeled away from one end to expose the copper wires, to create the brush.

BETA PILIATED COLONIES IMMUNOBLOTTED
WITH ALPHA PILI ANTISERA



ALPHA PILIATED COLONIES IMMUNOBLOTTED
WITH BETA PILI ANTISERA



ALPHA PILIATED COLONIES IMMUNOBLOTTED
WITH ALPHA PILI ANTISERA

BETA PILIATED COLONIES IMMUNOBLOTTED
WITH BETA PILI ANTISERA

Figure III-15: Colony immunoblots of two "pure" α and β piliated cultures of EPP63 reacted with heterologous antisera and with homologous antisera as a control.

the mices' eyes were examined for signs of corneal opacity, lachrymation, and conjunctivitis. One mouse of the five showed signs of lachrymation, and a slight conjunctivitis. Cultures were taken after 24 hours from the five mice, by swabbing the eye and inoculating a sterile MH plate with the swab. Both eyes of all five mice were cultured. A variety of different colonial types were present after overnight incubation. Those colonial types which had the appearance of M. bovis were isolated, and subjected to identification tests. Isolates from the eyes of two mice were positively identified as M. bovis. From the two experiments, clinical signs were observed in 20% of the infected mice, and M. bovis was recovered from 40% of the infected mice.

Total Number of Mice	Lachrymation Conjunctivitis	Corneal Opacity	Cultural Recovery
5	1/5	0/5	ND
5	1/5	1/5	2/5

Table III-6: Results of two experiments using the method of Rosenbusch and Ostle to acheive M. bovis infection in laboratory mice

CHAPTER IV.

DISCUSSION

A. SUMMARY OF RESULTS

The nature and expression of two unique pilus types, α and β, of M. bovis was studied. It was demonstrated that each strain of M. bovis expressed two pilus types, which are unique for each strain. Strains were placed into groups according to which two pilus types they expressed. The purified pili of three colony types of M. bovis strain EPP63 had different subunit MW_a values. It was demonstrated that static growth conditions in tryptose phosphate broth allow a single clone of M. bovis to switch to phase variants with different colony types, when plated onto solid media. These colony types were different phase variants of a single strain. However, it was determined that pilus type could not be correlated with colonial morphology. Pili were purified from six of the p+ phase variants, and the pili were run on SDS polyacrylamide gels. Two types of pili, each with a characteristic MW_a, were found on all six phases. Some colony types expressed more of one pilus type than the other, and other colony types expressed nearly equal amounts of both types of pili. α and β pili of EPP63 were characterized by SDS gel electrophoresis with the enzymes V8 protease, papain, and chymotrypsin. The two types of EPP63 pili were compared to pilus types of other strains of M. bovis. It was determined that α and β pili of EPP63 had identical proteolytic digest patterns to α and β pilus types of MED72 (4R). Further attempts were made to purify and characterize additional pilus types from phases of additional strains of M. bovis. α and β pili were purified and chemotyped from each of the strains NTN63 and FLA64. The α and β pili of strains NTN63 and FLA64 were different from each other and from those of other strains included in this study. In a colony immunoblot experiment using α and β antisera on plated cultures of "pure" α and β phases of strain EPP63, it was demonstrated that a single bacterium and the resulting nearly pure

phase colony expresses either α or β pili, but not both types simultaneously. Solubility studies in ammonium sulfate were done on α and β pilus types of the strains EPP63 and FLA64. The two pilus types of strain EPP63 had different solubility characteristics, and the two pilus types of FLA64 had nearly identical solubility characteristics. It was established that two different p+ phases of one strain had different growth rates on solid media. In a laboratory model for infectious bovine keratoconjunctivitis, different techniques of infecting mices' eye were attempted. Clinical signs of infection were observed in 20% of the infected mice, and M. bovis was recovered from 40% of infected mice.

B. DISCUSSION OF RESULTS

1. Phase Variation of M. bovis

This study documents the identification of two types of pili, α and β, expressed on different phases of the same strain of M. bovis. The immunoblot experiment is evidence that a strain of M. bovis has two piliated phases, which alternate like the two flagellar phases of Salmonella [73]. Phase switching occurs between the two phases, so that a single cell expresses either one or the other pilus type, but not both simultaneously. The results of this study suggest that there are no more than two pilus types expressed by a single strain of M. bovis. Several pieces of evidence support this hypothesis. First, all of the colonies having piliated (p+) morphology which were subjected to the pilus purification procedure yielded pili; pilus crystals could be seen by darkfield microscopy, and the protein migrated on an SDS gel with a MW_a that was characteristic of M. bovis pili. Second, in all of the pilus preparations (close to 100 phase variants were assayed for pili), no phase variants showed any more than two pilus bands on a gel. Finally, there is precedence in other procaryotic systems for phase switching between two and only two antigenic determinants. For instance, there are only two flagellar genes for Salmonella, H1, and H2.

There may be other pilus types or families expressed by M. bovis, that are not expressed under the environmental conditions used in this study, or that are not

soluble under the conditions used in this study. In these experiments, M. bovis pili were purified using only one set of ionic conditions for crystallization of pilus rods. Also, it must be remembered that other bacterial species express multiple pilus types and families. E. coli is known to express a number of pilus families that are expressed under different environmental conditions and purified using different ionic conditions [33]. N. gonorroheae expresses pili of varying antigenic types, having a range of solubility properties and in some cases, a unique purification method must be worked out for each pilus type [11]. Although the results presented in this study indicate that a strain of M. bovis expresses two pilus types and perhaps only two types, the possibility must be reserved that a strain can express more than two types.

There are two theories to explain the advantage of phase variation between pilus types of pathogenic bacteria. The first is that bacteria have multiple phases so that they can escape the immune system of the host. The second theory is that different pilus types have different functions and different receptor specificities. At different stages of infection, as bacteria infect different microenvironments within a cow's eye, different pilus types may be expressed. *In vivo* experiments may be able to correlate specific pilus types with specific stages of infection.

In this study no two strains were found which shared one pilus type, but not the other. The two strains, EPP63 and MED72 (4R), expressed identical pilus types. The two other strains which were found to express α and β pilus types were FLA64 and NTN63 (8033L). From these results it may be hypothesized that the α and β pilus genes are transmitted *in toto*. If an α and β pilus type were found from any other strain within Group IV, it is expected that the two pilus types would be like the two pilus types of FLA64.

In this study, pilus type was never correlated with a particular colony type. There are three alternative hypotheses to explain why pilus type was not correlated with colony morphology. The first hypothesis is that additional types of phase variation are occuring concurrently with pilus type phase variation, so that the additional types of phase variation are "superimposed" onto pilus phase variation,

obscuring any morphological characteristics that may be associated with pilus type variation. The second hypothesis is that the characteristics associated with pilus type phase variation are so subtle that they may not be recognized. The third hypothesis is that pilus type may not affect colonial morphology at all. However, it should be noted that the presence or absence of pili was associated with colony morphology differences.

2. Grouping of M. bovis Strains According to Pilus Chemotype

With the identification of α and β pilus types for the strains EPP63, MED72 4R, NTN63 (8033L), and FLA64 of M. bovis, the grouping of strains presented in Table 1-1 was redistributed. The new grouping of strains is shown in Table 4-1. Since the strain EPP63 and the strains of groups IV expressed the same pilus types, the number of groups was reduced from eight to seven. The vaccine, Piliguard-Pinkeye, developed in the Brinton laboratory (with animal testing carried out collaboratively with Schering Animal Health Laboratories, Elkhorn, Nebraska) contains pili of strains EPP63 and FLA64. As shown in Table 1-1, with the eight groups, classified according to the pilus types isolated from a single phase of each strain, the vaccine would appear to contain the pilus types of 4/16 (25%) of the grouped strains. When strain EPP63 is included in the group containing MED72 (new Group III), (because of its identical α and β pilus types), 8/16 (50%) of strains are included in Groups III and V. Groups III and V include the two strains contained in the pinkeye vaccine. Anecdotal field data from users of the vaccine (to CC Brinton, from M. Schwartz, personal communication) indicate that the vaccine's efficacy is about 50% in the field. This 50% efficacy of the vaccine corresponds to the percentage of strains which are included in Groups III and V. When a more extensive analysis of pilus grouping for M. bovis is completed, a few main groups may emerge which contain the majority of naturally occurring strains.

From the new grouping in Table 4-1 and Brinton's finding that disease-protective antibodies are pilus specific for intact pilus vaccines [11], it is hypothesized that the efficacy of an M. bovis vaccine for pinkeye in cattle could be increased from about 50% to about 90% if three more strains (from groups I, II,

Group		Strain	
I		WSE64 (2L)	
		IBH68(712L)	
II		1965GLENN (5004)	
		HIM63(5R)	
III		IVI64 (54L)	
		MED72 (4R)	(α and β)
		MED72 (19L)	
		WSE64 (13R)	
	*	EPP63	(α and β)
IV		MAC74 (2554R)	
		MAC74 (2562R)	
V	*	FLA64	(α and β)
		ATC (10900)	
		8613 (1)	
VI		NTN63 (8033L)	(α and β)
VII		GLN63	

Table IV-1: New classification of strains of M. bovis by serological cross-reactivity and proteolytic digest patterns of α and β pili [* denotes strains included in vaccine]

and IV) were included in the bacterin and growth of the cultures were managed so that both α and β pili were expressed in sufficient quantities by each of the five strains.

3. Serological Cross-Reactivity of Pilus Preparations

The ELISA experiment demonstrates that for serological studies to be valid, the pilus preparation used for antisera production and as antigen on ELISA plates must not be contaminated with any other pilus types. The serological data presented in this study indicates that the two pilus types of EPP63 have different serological determinants, although the actual degree of serological cross-reactivity between the two pilus types cannot be abstracted from the data presented in this

study, because the pilus preparations were not pure. However, the antisera was specific enough to be used in the colony immunoblot experiment.

The production of uncontaminated pilus preparations could be facilitated by biochemical methods for separating one pilus type from another pilus type. For example, the results of the SAS solubility experiments presented in this study indicate that the two pilus types of EPP63 could be separated from one another by recrystalization with 0.9% SAS. For other pilus types which have negligible differences in solubility, the electrophoresed pilus bands could be extracted from an SDS gel and pilus-specific antisera could be produced. The antisera could be used to purify a pilus preparation by adsorption of a contaminant pilus type. Alternatively, more highly purified pilus preparations could also be obtained by controlling the phase composition of single phase clones, doing more extensive phase cloning of inocula, and using new methods (as yet undeveloped) of suppressing phase switching and growth of phases expressing the alternative pilus type. These methods would aid in the elimination of contaminating pili of the opposite type.

4. Tryptose Phosphate Broth Culture as a Medium for Eduction of Phase Variants

A set of environmental conditions (static incubation in tryptose phosphate broth) was established which allows the eduction of multiple p+ phases, as well as phase variation in the p− to p+ direction. In contrast, transfer of colonies on solid MH agar did not educe piliated from nonpiliated phases, and only resulted in slight variations in piliated colony morphology. The reasons for the differential effect of environmental conditions were not defined in this study. These environmental effects could be cell density, type of medium, the contrast of liquid vs. solid medium, increased cell assortment, or aging of the culture with limiting growth conditions.

"Phase eduction potential" is a property of a particular environment which causes a proliferation of different phases to occur [11]. It is likely that a major reason for the appearance of new phases after incubation in TPB is an increase in

phase switching probability or phase selection under these growth conditions. As discused in Section 1.1.2, the phase composition of a bacterial culture is dependent upon the phase switching rate and phase selection. It was not determined in these studies whether the increase in phase eduction potential to piliated phases when transfering from isolated colonial growth on MH agar to several days of liquid growth in tryptose phosphate broth was because of cell density, type of medium, liquid vs. solid medium, increased cell assortment, or aging of the culture with limiting growth conditions.

5. Solubility Characteristics of Different Pilus Types

Solubility curves in ammonium sulfate suggest that the two pilus types of EPP63 have different surface properties, and the two types of pili isolated from FLA64 have similar surface properties. The solubility of a pilus is a function of the number, kind and arrangement of ionizable groups (hydrophilic) as well as hydophobic groups on the surface of the pilus proteins. Pilus rods, like many other protein macromolecules, are quaternary structures, so that the hydrophobic groups are contained on the inside, and hydrophilic groups, which are involved in pilus–pilus interactions, pilus–water and pilus–ion interactions occur on the outside. The strength of the pilus–pilus side by side adhesion in aqueous solutions is the cause of solubility or loss of solubility under a given set of ionic conditions.

The solubility experiments show that the two pilus types of EPP63 can be purified from one another using varying amounts of SAS. However, the two pilus types of FLA64 could not be easily purified from one another using this technique. If pilus types could be classified according to solubility properties, this evidence would support the theory that different pilus types have different functions in pathogenicity. However, the only correlation that can be made from the four pilus types studied in this experiment concerns the differences in solubility and the differences in MW_a. The two types of FLA64 had a difference in solubility of 0.2% SAS and a difference in MW_a of 800 daltons. The two pilus types of EPP63 had a difference in solubility of 2.1%, and differed in MW_a by 2,200 daltons. However, a given MW_a was not correlated with solubility at a given percentage of SAS.

6. Growth Rate of Two p+ Phases on Solid Media

The difference in growth rates of two clones of EPP63 was statistically significant at three time points. It was hypothesized that Clone A would have a higher growth rate than Clone B, because the size of the colonies of Clone A appeared to be larger on examination under the stereo-microscope. It was originally hypothesized that α and β pilus types could be isolated from Type A and Type B colony types, respectively, but this hypothesis was not substantiated by subsequent experiments. The experiment was done on solid media, because the starting inoculum (exactly 1 cell/colony) could be more easily controlled than in liquid media. In addition, phases could be maintained, and the actual increase in colony size over time could be visibly observed. This experiment was limited because colonies below a certain size could not be assayed, since they were too small to be visualized in the stereo-microscope. It is likely that as soon as colonies became visible on solid agar, the growth rate of the bacteria was no longer exponential. Also, the lag time before cell division occurs cannot be established using this procedure. Hence, there was no meaningful way to measure the slope of the exponential portion of the growth curves. The most practical way to analyze the data points was to apply a Student's t-test to the sets of data points at each time point. This test determined that there was a statistically significant difference in the number of cfu's within the two clones of the different phases. If some phases have faster growth rates than other phases, then phase growth rates is one of the determining factors of phase composition of a bacterial culture.

For growth rate experiments to be considered in conjunction with phase variation experiments in liquid media, the growth rate experiments should be done in liquid medium. The growth rate experiments that were attempted in liquid medium were unsuccessful, because of difficulties with inoculation of varying numbers of cells. Growth rate has been shown to be dependent upon inoculation number, with smaller numbers of cells inoculated resulting in a lower overall growth rate [25].

7. Laboratory Model for IBK

The purpose of these experiments on a laboratory model for IBK was to replicate a method that has been reported by other workers. There were no formal "no-treatment" control groups included in these experiments, because a limited number of mice were available. In both experiments in this study the percentage of mice that showed clinical signs of infection was 20%, and M. bovis was isolated from 40% of the infected mice. However, the clinical signs that were observed in this study were not as definitive as those reported by other workers in previous studies [21]. The other studies reported corneal opacity as a sign of M. bovis infection, but corneal opacity was not noted in the present study. Although each of the three previous studies [21, 65, 68]reported that the method of pretreatment was adequate to establish IBK in a certain percentage of the mices' eyes, it may be that a combination of the three pretreatments (steroids, scarification, and UV irradiation) are necessary to cause IBK in a high percentage of animals. Another factor that can be manipulated is dosage of bacterial cells to the mices' eye. In the first experiment, although the pretreatment was less severe (one stroke with a wire brush instead of 5-6 strokes), the dosage of bacteria delivered to the mices' eye was not as closely controlled as in the second experiment.

In these experiments, identification of M. bovis from eye cultures was difficult. Without a selective media, representative colonies from each eye culture had to be individually typed. Much time was spent subculturing M. bovis and the M. bovis may have been overgrown by other organisms present in the mices' eye. In future experiments, it may be useful to use a semiselective media for M. bovis, containing cloxacillin, as described by Webber [82].

There are several issues which could be addressed using a mouse model.

1. Phase variation *in vivo* could be studied. Bacteria in a known phase could be inoculated into a mouse's eye, and cultures over the course of infection could be assayed for phase type.

2. The mouse model could be used to study protection by a vaccine, and to

correlate antibody levels to a certain pilus type with protection against homologous or heterologous challenge.

3. It could be determined whether a bacterin, prepared from killed bacteria cloned for a certain pilus type confers better immunity than a purified pilus vaccine. Immunity could be assessed by ability to protect against homologous and heterologous challenge.

4. The immune response, both humoral and cellular, could be studied in response to vaccination as well as infection. All of these issues could be addressed using the UV-irradiated cattle-eye model for IBK [80] but they could be done more economically and rapidly if a valid mouse model could be developed.

C. DIRECTIONS FOR FUTURE STUDY

There are three general directions for future study:

1. *To improve the pinkeye vaccine:* The isolation of α and β pili from additional strains of M. bovis could be done, to produce a more comprehensive grouping of strains of M. bovis according to pilus types. A representative strain from each major group could be incorporated into a multivalent pilus vaccine. As outlined in Section 1.1.4, this is the "direct and general approach" of Brinton's to vaccine development.

2. *To understand phase variation better:* A method has been presented in this study which allows phase variation to be readily observed. Colony blots could be used in association with incubation in liquid tryptose phosphate to study the relationship of phase switching pathways, and with their probabilities among α, β, and the p- phase(es).

3. *To understand pathogenesis better:* The mouse model could be improved or modified to study phase variation *in vivo,* and to study the effectiveness of certain pilus types as vaccines, and the immune response to vaccination and infection.

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