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Characterization of the 4.5S RNA molecule in Escherichia coli

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Abstract

The 4.5S RNA molecule of *Escherichia coli* is essential to cell viability. It has been shown that depletion of this molecule inhibits protein synthesis, induces the heat shock response, and generally slows cell growth. The molecule has also been implicated in protein secretion, as in cells depleted of 4.5S RNA, an unsecreted precursor to β-lactamase accumulates (pre-β-lactamase). A role in protein secretion is further supported by structural similarities with the 7S RNA molecule of eukaryotic SRP, specific binding to SRP54, and its homolog in *E. coli*, P48, and the ability of 7S RNA from certain archaebacteria to suppress 4.5S RNA depletion.

In this study I have utilized strains with mutant forms of the 4.5S RNA genes in order to study the effect of altered 4.5S RNA on cell physiology. These strains have their mutant 4.5S RNA under the control of the tryptophan synthetic operon. Decreased growth rates, inhibited cell division, and altered protein synthesis all result from these mutations.

Abbreviations used in text:

Amp	ampicilin
APS	ammonium persulfate
DDSA	dodecenyl succinic anhydride
DMP-30	Tris-dimethylaminomethyl phenol
EDTA	ethylenediaminetetraacetate
Kan	kanamycin
NBT	nitroblue tetrazolium salt
NMA	nadic methyl anhydride
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SRP	signal recognition particle
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tet	tetracyclin
Trp	tryptophan

Introduction

The molecule of concern in this study is 4.5S RNA. This is a small stable RNA molecule 114 nucleotides in length, found in the Gram negative bacterium *Escherichia coli* (see Figure 1). It is a rather abundant molecule, approximately 3000 copies present in each cell. The molecule has been sequenced (Hsu et al.), and has been shown to have significant secondary structure(see Figure 1). Approximately a decade ago, 4.5S RNA was shown to be an essential component of the cell; depletion of 4.5S RNA causes cell death(Brown, 1984). However, the reasons that 4.5S RNA is so vital have yet to be fully determined.

This molecule's function, or functions, has been very elusive because of the wide range of effects observed after a cell is depleted of 4.5S RNA. Early work suggested a role in initiation of translation (Bourgaize & Fournier, 1987). Using a strain with the 4.5S structural gene under the control of an induceable promoter, they showed that cell growth, DNA synthesis, and protein translation ceased when IPTG was depleted. They then isolated the site of inhibition as initiation of translation, by comparing the ability of cell extracts from 4.5S depleted cells to translate a poly(U) message and a natural messenger RNA. The extracts translated the poly(U) message which does not require the normal process of initiation, but were unable to translate the natural message, suggesting a role for 4.5S RNA in initiation of translation.

Shortly after initiation of translation was shown to be inhibited due to 4.5S RNA depletion, it was shown that 4.5S RNA transiently associates with the ribosome, and that this association is dependent on the presence of elongation factor G (Brown, 1987). It was shown

that strains with mutant forms of EF-G could grow normally at concentrations of 4.5S RNA which were lower than normal. Cellular extracts from these mutant strains contained 4.5S RNA which sedimented at a much higher rate than normal, a rate similar to whole ribosomes. By showing that 4.5S RNA's sedimentation reverted to normal in the presence of puromycin, an antibiotic which causes the nascent peptide chain to fall off of the ribosome, Brown was able to conclude that the 4.5S RNA was associating with the intact ribosome.

Although some of the details of the work by Bourgaize and Fournier conflict with those elucidated by Brown, certain aspects of 4.5S RNA action can be characterized. First, protein translation is inhibited by 4.5S RNA depletion. Second, 4.5S RNA associates with the ribosome directly, most likely in concert with EF-G. Bourgaize and Fournier later showed that depletion of 4.5S RNA induces the heat shock response in such a way that is nearly indistinguishable from a response due to elevated temperature, save for its elongated duration (Bourgaize et. al., 1990). Heat shock may be induced by a number of factors, including amino acid analogs, puromycin treatment, and ethanol treatment. All of these have also been shown to inhibit protein translation. From these facts Bourgaize et. al. (1990) concluded that the heat shock response in 4.5S RNA-depleted cells was a result of the translational inhibition caused by that depletion, and not a direct impact of the lack of 4.5S RNA.

Another important clue as to the function of 4.5S RNA was discerned by Brown (1991), when he showed that 7S RNA's of certain archaebacteria which contain a conserved 16 nucleotide hyphenated

sequence can complement deficiency of 4.5S RNA. This consensus sequence is also found in a number of eukaryotes, as well as in all archaebacteria screened (Brown, 1991). The human form of 7S RNA has been shown to partially suppress the lethality of 4.5S RNA depletion, presumably by replacing the function of 4.5S RNA (Ribes et. al., 1990). The 7S RNA's in eukaryotes have a known function in transport of proteins across the membrane of the endoplasmic reticulum. When performing this task, 7S RNA is bound to several other proteins to form what is referred to as the Signal Recognition Particle (SRP). The present working model describes the function of the SRP as recognizing a relatively long hydrophobic sequence of amino acids, the signal sequence, as it is translated. The SRP shuttles the polysome to the membrane, and interacts with a specific membrane protein, which shunts the hydrophobic signal sequence through the membrane, and the rest of the protein is transported co-translationally. A tantalizing piece of information is that homologs to certain SRP proteins are being discovered in *E. coli* (Ribes et. al.). This suggests that 4.5S RNA may have a role in protein secretion analogous to the role of 7S RNA in the eukaryotic SRP transport process. However, no evidence for an SRP-like particle in *E. coli* has been found.

This study was conducted to determine the essential function of the 4.5S RNA molecule, specifically examining its possible role in secretion. To that end, 15 mutant strains of *E. coli*, as well as a control strain have been utilized (see Table 3). Each strain has a particular mutation in the 4.5S RNA gene (See Figure 1), which is under the control of the tryptophan synthetic operon. Tryptophan is

a feedback repressor. When the concentration of tryptophan is high in a cell, the synthetic operon is turned off. The transcription of the 4.5S RNA can therefore be roughly regulated by growing the bacteria in the presence or absence of tryptophan.

The first step in characterizing the effect these mutations in 4.5S RNA have on cell physiology was to characterize their growth rates. This was an attempt to narrow down the field of mutants, singling out those with the most severely inhibited growth. In order to determine whether the inhibited function of the mutation was due to a gross change in structure of the RNA, we isolated and analyzed the RNA content of the cells. A significantly altered gel mobility of the 4.5S RNA would suggest a severe alteration of the 4.5S RNA molecule, while no change would suggest relatively normal structure of the mutant form of the molecule.

As stated above, depletion of 4.5S RNA causes an inhibition of protein translation, as well as induction of the heat shock response. To determine whether these mutations also exhibited this same condition, two-dimensional gel electrophoresis was conducted on all strains. This technique serves two purposes. First, it presents the general effect of the mutation on overall protein translation, not considering rate of translation. Secondly, and most important to this study, it gives an assay of heat shock response by showing the levels of production of the heat shock proteins.

Although a great deal of research has been done on the biochemical processes effected by depletion of 4.5S RNA, little has been done to show effects on general cell morphology. The mutant strains were viewed using both light microscopy and electron

microscopy, allowing some general characteristics of the morphology to be determined, as well as some more specific, ultra-structural aspects of the cell membrane and outer envelope.

While many of these techniques may result in data which is supportive of a role in protein secretion for 4.5S RNA, none of them will show any direct effect. In order to demonstrate a direct effect on the process of protein transport, immunoprecipitation experiments were done to detect the precursor to β-Lactamase. If build up of β-lactamase precursor could be shown to occur in these viable mutations, a direct role in protein secretion could be demonstrated.

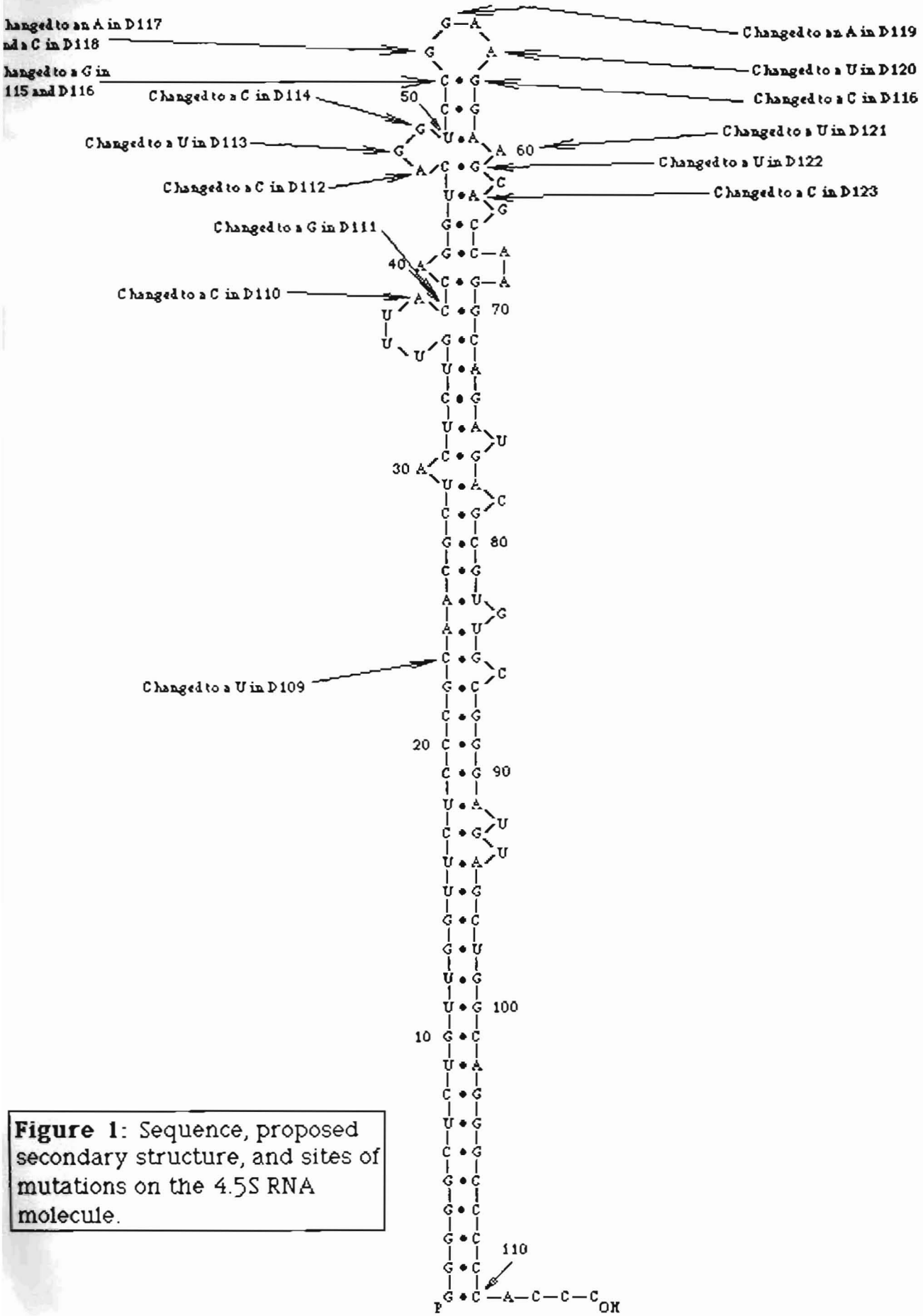


Figure 1: Sequence, proposed secondary structure, and sites of mutations on the 4.5S RNA molecule.

Materials and Methods

Table 1: Standard Buffers and Solutions

0.5X Wash Solution	0.5X SSC, 0.1% SDS
2X Wash Solution	2X SSC, 0.1% SDS
20X SSC	3 M NaCl, 300 mM sodium citrate, pH 7.0
L-Amino Acid Solution	4 µl/ml solution of all essential L-amino acids except cysteine, methionine, and tryptophan
APS	10% $(NH_4)_2S_2O_8$
Color Substrate Solution	45 µl NBT solution, 35 µl X-phosphate solution, in 10 ml Genius© Buffer #3
Epoxy Resin	6:4 Resin Mixture A:Resin Mixture B
Fluorography Mixture	25 ml Xylene, 0.36 g PPO, 50 ml Acetic Acid, 12 ml Ethanol
Gel Overlay Solution	8 M urea
Genius© Buffer #1	150 mM NaCl, 100 mM Tris-HCl, pH 7.5
Genius© Buffer #2	2% Genius© Blocking Reagent in Buffer #1
Genius© Buffer #3	100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5
Lower Gel Buffer	1.5 M Tris-HCl, 0.4% SDS, pH 8.9
Lysis Buffer	5.7 g urea, 2.0 ml NP40(10%), 0.5 ml β-mercaptoethanol, 0.4 ml pH 5-7 ampholines, 0.1 ml pH 3-10 ampholines, 2.4 ml H_2O
Maleate Buffer	100 mM maleic acid, pH 7.5, 150 mM NaCl
NBT Solution	75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide
Northern Prehybridization solution	5X SSC, 50% Formamide, 0.02% SDS, 0.1% N-laurylsarcosine, 2% Genius© Blocking Reagent, 20 mM sodium maleate, pH 7.5%
Northern Blocking Solution	2% Genius© Blocking reagent in Maleate Buffer
NP40	10% Nonidet P-40
Protein Gel Destain	5% Methanol, 7% Acetic acid
Resin Mixture A	11.0 g Epox 812, 16.6 g DDSA, 0.5 g DMP-30
Resin Mixture B	16.6 g Epox 812, 14.0 g NMA, 0.5g DMP-30

Resuspension Buffer, high salt	10 mM Tris-HCl, 1mM $MgCl_2$, 1 M NaCl
Resuspension Buffer, low salt	10 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM NaCl
RNA Loading Buffer	10 mM Tris-HCl, 1 mM $MgCl_2$, 10 mM NaCl, 20% Ficoll, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol
RNA Running Buffer	0.5X TBE
Sample Overlay	5.5 g urea, 5.0 ml H2O, 200 µl pH 5-7 ampholines, 50 µl pH 3-10 ampholines
SDS Destain Solution	7.5% Acetic Acid
SDS Lysing Solution	0.054 M Tris-HCl, pH 6.8, 2.6% SDS, 4.3% glycerol, 1.2 M β-mercaptoethanol
SDS Running Buffer(10X)	0.025 M Tris, 0.2 M Glycine, 0.1% SDS
SDS Sample Buffer	25 ml upper gel buffer, 20 g glycerol, 4.5 g SDS, 10 ml b-mercaptoethanol, 0.5% Bromophenol Blue
SDS Slab Acrylamide	30%: 146 g acrylamide, 4.0 g bis-acrylamide in 500 ml of H_2O
Sorensen Phosphate Buffer	36.0 ml 0.2 M Na_2HPO_4, 14.0 ml 0.2 M NaH_2PO_4 diluted to 100 ml with distilled H_2O, pH 7.2
Stain #1	1% TCA, 0.005% Coomassie Blue, 7.5% acetic acid, 50% ethanol
Stain #2	1% TCA, 0.005% Coomassie Blue, 7.5% acetic acid
10X TBE Buffer	990 mM Tris, 990 mM Borate, 20 mM EDTA
TE/SDS Buffer	10 mM Tris-HCl, pH 7.0-8.0, 1 mM EDTA, 0.1% SDS
Transformation Buffer	10 mM $CaCl_2$
Tris-SDS Buffer	1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA
Triton Buffer	2% Triton X-100, 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1 mM EDTA
Upper Gel Buffer	0.5 M Tris-HCl, pH 6.8, 0.4% SDS
X-phosphate Solution	50 mg/ml 5-bromo-4-chloro-3-indolylphosphate, toluidinium salt in 100% dimethylformamide

Media: Primarily two types of media were used in this study. Standard LB broth was used in preliminary growth measurements in agar plates. Minimal media (M9) was used for the majority of the study (See Table 2 below). Antibiotics were used at the following final concentrations: Amp 100 μg/ml; Kan 50 μg/ml; Tet 15 μg/ml.

Table 2: Amount of reagents per one liter of media:

LB media	10 g Triptone, 5 g yeast extract, 10 g NaCl*
M9 minimal media	6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 10 ml of 0.1M $CaCl_2$, 1 ml of 1 M $MgSO_4$, 10 ml of 20% Glucose, 1 ml of 1 mg/ml Thiamine, 10 ml L-amino acid solution*

*13 g agar added for every liter of media to pour plates

Strains[1] **and plasmids :** All strains and plasmids are described in Table 3.

Transformation of Strain D1: Approximately 50 ml of strain D1 was grown up to mid-log phase. The cells were pelleted at 8000 rpm for 5 minutes at 4°C in a Beckman JA-20 rotor. The cells were resuspended in 20 ml of Transformation Buffer, and allowed to equilibrate for 20 minutes. The cells were again pelleted, this time at 10,000 rpm for 10 minutes at 4°C. After centrifugation, the cells were resuspended in 1 ml of transformation buffer. At this point, two aliquots of 100 μl were transferred to two separate microcentrifuge tubes. To one of the tubes, a small amount of plasmid pPre4.5S was added, and to the other nothing was added. The tubes were incubated on ice for between 30 minutes and thirty hours, and then plated out on selective media.

[1]Strains D109-D123 kindly provided by Heather Wood

Table 3: Strains and Plasmids

Strain or Plasmid	Description[2]
D1	W3110 LacI^{Q} L8 (λnin#10) Kan^{R} Tet^{R} IPTG^{d} T^{S} recA l^{R}
D109	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG23U
D110	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pA39C
D111	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pC40G
D112	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pA47C
D113	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG48U
D114	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG49C
D115	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pC52G
D116	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pC52G & G57C
D117	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG53A
D118	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG53C
D119	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG54A
D120	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pA56U
D121	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pA60U
D122	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pG61U
D123	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pA63C
D124	W3110 LacI^{Q} L8 Kan^{R} Tet^{R} recA l^{R}/pPRE4.5S
pLH45-1[3]	pBR322 derivative containing 4 kb chromosomal region containing 4.5S RNA gene cloned into the tetracyclin-resistance gene
pPRE4.5S[4]	pLK9159 with 4.5S RNA structural gene under *trp* promoter control (from pDR720)

Curing of λ phage: Once transformed colonies had been isolated, they were cured of the λ phage. A small culture of approximately 2 ml was grown to mid-log phase, and then heat

[2]A letter followed by a number, and again by a letter indicates a single base change at that nucleotide number, e.g. C54G indicates a cytosine being replaced by a guanine residue at the 54th nucleotide

[3]Hsu et. al.

[4]Wood et. al.

shocked for 5 minutes in a 43°C water bath. The cultures were then immediately placed on ice for 5 minutes. The cultures were returned to the original permissive temperature(37°C) for 1 hour. Aliquots were then plated out on selective media at a selective temperature of 42°C.

Growth Curves: All fifteen strains (D109-D123) were revived from glycerol stabs and tested for antibiotic resistance markers. Each strain was then grown on M9 medium with and without tryptophan, and on LB medium at four separate temperatures: room temperature, 30°C, 37°C, and 42°C. After 48 hours the plates were observed and the relative growth of each strain recorded (see Table 5). From this data, those strains which exhibited unusual growth patterns were further studied. A growth curve for each of these strains, and later for the control strain D124, was determined in M9 media with and without tryptophan at 30°C and 42°C using a Klett meter (See Figures 3-8).

RNA Isolation and Analysis: Approximately 1 liter of M9 media was inoculated with 2-3 drops of an overnight culture. Each of the 1-liter cultures were allowed to grow to saturation and were then harvested by centrifugation in a Beckman JA-14 rotor, and transferred to a 50 ml Oakridge tube. They were spun down for 10 minutes at 5000 rpm at 4°C in a Beckman JA-20 rotor. The cell pellets were then resuspended in a minimum volume of high salt Resuspension Buffer (approx. 5 ml). An equal volume of phenol was added to the cell suspension, and vortexed. The suspension was then

centrifuged at 10,000 rpm for 5 minutes at 4°C, and the aqueous phase was recovered. The nucleic acids were precipitated out with $2\frac{1}{2}$ volumes of cold ethanol. Once the precipitate had formed, the solutions were centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was poured off, and the pellet dried in a Speedvac Concentrator (Savant©), and resuspended in 0.5 ml of low salt Resuspension Buffer.

Table 4: Gel Recipes

RNA 10% polyacrylamide Gel	Per Gel: 13.3 ml 30% Acrylamide (19:1 acrylamide: bis-acrylamide) 4.0 ml 10X TBE Buffer 22.7 ml H_2O & 300 µl 10% APS, 125 µl TEMED
First Dimension Tube Gels	Per Gel: 0.144 g urea 0.033 ml SDS Slab Acrylamide 0.05 ml NP-40 (10%) 0.05 ml H_2O 0.01 ml pH 5-7 ampholines 2.5 µl pH 3-10 ampholines & 0.25 µl 10% APS, 0.175 µl TEMED
Second Dimension Lower Gel	Per Gel: 7.5 ml Lower Buffer 11.5 ml SDS Slab Acrylamide 11 ml H_2O & 99 µl 10% APS, 15 µl TEMED
Second Dimension Upper Gel	Per Gel: 1.56 ml Upper Buffer 0.94 ml SDS Slab Acrylamide 3.75 ml H_2O & 18.75 µl 10% APS, 6.25 µl TEMED

The samples were then analyzed by gel electrophoresis. For each sample, 10-30 µl was mixed with an equal amount of RNA Loading Buffer. The samples were loaded onto a 10% polyacrylamide gel, and run at 40 volts overnight. The gel was stained with ethidium

bromide for approximately 15 minutes and then viewed and photographed on a UV-light box (see Figures 9 &10).

Electrophoretic Transfer and Hybridization : In order to specifically locate and quantify the gel mobility of each mutant form of 4.5S RNA found in the strains of interest, a Northern blot was obtained of the most interesting strains. Both native and denaturing acid-urea 10% polyacrylamide gels were run as described above for strains D124, D109, D112, and D114. Each gel was stained with ethidium bromide and photographed to display the mobility of 4.5S RNA in reference to the rest of the cellular RNA. The RNA from each gel was electrophoretically transferred onto a nylon membrane (Boehringer-Mannheim) using a Bio-Rad© electroblot apparatus. The electrophoretic transfer was conducted at 30 volts for 4 hours in 0.5X TBE. The RNA was crosslinked to the membrane using a UV crosslinker (Hoefer© Scientific). The membrane was stored at room temperature between two pieces of 3 MM paper.

In order to avoid the complications of radio-labeling, the probe (plasmid pLH45-1, which contains the wild-type gene for 4.5S RNA, see Figure 2) was labeled with the non-radioactive Genius© labeling kit (Boehringer-Mannheim). This was done by incubating approximately 3 mg of pLH45-1 with the following reagents: 10X Hexanucleotide mixture (2 µl), 10X dNTP labeling mixture (2 µl), distilled H_2O (to 19 µl), and Klenow Fragment (1 µl). This reaction mixture was incubated for 20 hours at 37°C. The reaction was terminated by adding 2 µl of 0.2 M EDTA to the solution, followed by 1 µl of 20 mg/ml glycogen in H_2O solution. To precipitate out the

labeled probe, 1/10 volume of 3 M sodium acetate, and $2\frac{1}{2}$ - 3 volumes of cold 100% ethanol were added to the reaction mixture. The precipitation was allowed to occur over 30 minutes at -80°C. The reaction mixture was warmed briefly to room temperature, and the centrifuged at 14,000 rpm for 15 minutes at 4°C. Supernatant was removed and the pellet washed with 100 µl of 70% ethanol, and again centrifuged at 14,000 rpm for 5 minutes at 4°C. The pellets were dried in a Speedvac Concentrator (Savant©) and resuspended in 50 µl of TE/SDS buffer. Samples were stored at -20°C for further use.

The labeling reaction was assayed using a quick colorimetric technique by comparing known concentrations of DNA with newly labeled probe. A known amount of dig-labeled DNA was diluted down to concentrations of 100 pg/µl, 10 pg/µl, 1 pg/µl, and 0.1 pg/µl. Four ten-fold dilutions of the newly labeled probe were made in siliconized microcentrifuge tubes. For each of the known DNA solutions, and for each of the four ten-fold dilutions of the newly labeled probe, 1 µl was spotted on a small piece of nylon membrane. The DNA was crosslinked to the membrane by UV light, and then wetted with a small amount of Genius© Buffer #1. The membrane was preincubated in Genius© Buffer #2 for 5 minutes, and then incubated with diluted (1:5,00) anti-DIG-alkaline phosphatase in Genius© Buffer #2 for 30 minutes. The membrane was washed twice for 5 minutes each with Genius© Buffer #1, and incubated for 2 minutes in Genius© Buffer #3 to activate the phosphatase. Genius© Buffer #3 was poured off, and the Color Substrate Solution was added. Color development was allowed to occur at room temperature

in the dark for 1 hour. The intensity of the spots was compared, and an approximate concentration of labeled probe was determined.

The hybridization was carried out according to the protocol for large DNA probes (>1 kb) as described in the Genius© kit instruction manual. The nylon membrane was placed in a hybridization bag containing 20 ml of prehybridization solution, and incubated with agitation for greater than 2 hours, up to an overnight incubation. Denatured probe was diluted to a final concentration of 5-20 ng/ml in prehybridization solution. The membrane was hybridized overnight at 42°C in this probe solution after the prehybridization step was complete. After the hybridization was complete, the probe solution was poured off into a sealable tube, and stored at 4°C for further use, while the membrane was washed twice with 2X wash solution for 5 minutes each time. The membrane was then washed twice more at 65°C in 0.5X wash solution for 15 minutes each time. After being transferred to a clean plastic tray, the membrane was washed for 1 minute in 50 ml of maleate buffer at room temperature. The membrane was incubated for 1 hour in 50 ml of Northern Blocking Solution, and then for 30 minutes in anti-DIG-alkaline phosphatase, diluted 1:10,000 in Northern Blocking Solution. The membrane was then washed twice with 200 ml of maleate buffer for 15 minutes each, and equilibrated in Buffer #3 for 5 minutes. Keeping the membrane wet, 2-3 sprays of Lumi-phos 530 were spread over the membrane by rocking the membrane. The membrane was placed between two sheets of acetate and then exposed to X-ray film for 1 hour.

Two-Dimensional Gel Electrophoresis: Saturated overnight cultures were diluted and grown up to mid-log phase in M9 media at 42°C. From each culture 1 ml of cells were transferred to microcentrifuge tubes, to which approximately 100 µCi of ^{35}S was added at 42°C. After 1 minute, 167 µl of 0.2 M methionine was added to chase the labeling reaction. The reaction tubes were incubated at 42°C for 3 minutes, and then centrifuged at 14,000 rpm for 20-40 seconds 4°C. The supernatant was poured off, and the pellet resuspended in 8 µl of SDS Lysis solution, and boiled for 4 minutes. After 1 minute on ice, 100 µl of SDS Lysis Buffer was added.

A sufficient number of tube gels were prepared according to Table 4, and placed in the first-dimension apparatus. The bottom of the apparatus was filled with 0.01 M H_3PO_4, and 20 µl of each protein extract was loaded onto the tube gels. The gels were overlaid with Sample Overlay, and the top of the apparatus was filled with 0.02 M NaOH. The tube gels were run at 400 volts overnight (12-15 hours). The next day, the gels were run for 1 hour at 1000 volts, removed from the apparatus, and extruded into approximately 5 ml of Sample Buffer, and allowed to sit for 15-30 minutes. The gels were frozen immediately, and stored at 20°C.

Sufficient polyacrylamide second-dimension gels were prepared according to Table 4. The tube gels were thawed and then laid on the stacking gel. The gels were overlaid with running buffer, and run at 40 volts overnight. The slab gels were removed from the apparatus and placed into Stain #1 for at least 3 hours(as long as overnight). After staining in Stain #1, the gels were placed in Stain

#2 for 15 minutes, then Destain solution for 15 minutes, and then they were immediately dried. The gels were exposed to X-ray film for 2-5 days.

Microscopy : Strains D124, D112, D114, and D122 were observed with a transmission electron microscope (TEM), while strains D124, D109, D112, and D114 were viewed by light microscopy. Preparation for the TEM was fairly standard. A 3 ml culture was grown up to log phase, and centrifuged to obtain a cell pellet. The cells were resuspended in approximately 1 ml of 3% Glutaraldehyde in Sorensen Phosphate Buffer, and allowed to fix for 1 hour. The cells were again pelleted, and washed three consecutive times for five minutes in Sorensen Phosphate Buffer, centrifuging between each wash. After the final wash and centrifugation, the cells were resuspended in 0.5 ml of a warm 1.5% agarose solution to suspend the cells in a solid media for sectioning. The agar was allowed to solidify, and was subsequently sliced up into small squares of approximately 1 mm^3. These pieces were post-fixed in 1% osmium tetraoxide in Sorensen Phosphate Buffer for 2 hours, and then rinsed with Sorensen Phosphate Buffer for 1 minute. The samples were then dehydrated by successive ethanol washes of increasing concentration, ending in four 100% ethanol washes of 10 minutes each. The ethanol was poured off, and the samples washed with 2 five minute changes of propylene oxide. In order to facilitate infiltration and embedding in epoxy resin, the samples were placed in a 6:4 resin:propylene oxide mixture, and incubated overnight to allow the propylene oxide to evaporate away, leaving epoxy

embedded samples. The next morning, samples were placed in a fresh resin mixture, and allowed to sit approximately 6 hours to complete the infiltration step. After the resin incubation, the samples were placed in labeled BEEM capsules and baked overnight at 60°C to cure them.

Sectioning was done two days after embedding, performed on a Ultracut E© microtome apparatus. Semi-thin sections were obtained from the blocks using a glass knife prepared just before sectioning. Once the blocks had been sufficiently faced-off, ultra-thin sectioning was performed using a Diatome diamond knife. Grids were stained first with 1% Uranyl Acetate, and then with 1.5% Lead Citrate for electron density. One day after staining, the grids were viewed on a Zeiss-EM109 transmission electron microscope at 50 kV.

For light microscopy, approximately 10 µl of a mid log-phase culture was dropped onto a ethanol cleaned slide. Once the cell suspension was dry, 2-3 drops of methanol were placed onto the cells to fix them. After a brief drying period, the slide was washed liberally several times with methanol. Once the methanol had evaporated away, 10 µl of Hoecht's dye was added to each slide, and a cover slip was placed on the slide. The slides were then viewed on a Zeiss-Axioscope© light microscope using an oil-immersion lens, as well as the necessary accessories to utilize the fluorescence of Hoecht's dye.

Immunoprecipitation : In order to detect β-lactamase, a protein known to be secreted, cellular proteins were labeled with ^{35}S. From a mid-log phase culture, 1.5 ml of cells were transferred to a 4

ml tube, incubated at 42°C. A sufficient amount of label (approximately 30 μCi/ml of cells) was added to the culture, and incubated for 30 seconds. After the 30 second incubation, the labeling reaction was chased with 1.5 ml of a 0.1% Methionine in M9 media solution. Immediately after the methionine solution was added, 1 ml of the solution was removed and added to 50 μl of 100% TCA, and then placed on ice. This was repeated at 1 minute and 5 minutes after the methionine solution had been added. These labeled samples were stored at 4°C.

The TCA-precipitated samples were first made 1% SDS by centrifuging at 14,000 rpm for 2 minutes at 4°C, washing the pellet with acetone, and then resuspending the pellet in 5 μl of Tris-SDS buffer. The samples were then boiled for 2 minutes followed by a 33-fold dilution, by adding 165 μl of cold Triton Buffer. The samples were centrifuged at 14,000 rpm for 10 minutes at 4°C, and the supernatant was transferred to a new microcentrifuge tube. At that point, 1 μl of β-Lactamase antibody (5'-3' Inc.) was added to the supernatant, and incubated overnight at 4°C.

The next morning, 5 μl of Protein A/Sepharose (Sigma) was added to the supernatant, and incubated for 2 hours at 4°C. The sepharose beads were pelleted by centrifugation and washed twice with approximately 100 μl of cold Triton Buffer, and once with a 10 mM Tris buffer, pH 8.0. The beads were resuspended in 100 μl of SDS sample buffer (same as sample buffer for two-dimensional gel electrophoresis), boiled for 2 minutes, and then stored frozen.

The samples were run on a standard protein gel (same as second-dimension gel of 2-D gel electrophoresis). Along with 10 μl of a pre-

labeled (methylene blue) size-standard protein solution (), 20 µl of each sample was loaded onto the gel. The gels were run overnight at 40 volts, using SDS running buffer (as per 2-D gels). Gels were fixed in standard protein destain solution for 4 hours. In order to detect the small amount of labeled β-Lactamase, fluorography was performed on the gels. After fixation, the gels were soaked in Fluorography mixture under the hood for 1 hour. The gels were then washed twice with distilled water for 10 minutes and 15 minutes respectively, and then dried at 60°C for approximately 2 hours. Gels were exposed to X-ray film at -80°C for 1-3 days depending on strength of signal.

Results

Growth Measurements : The growth rates of each of the strains described in this study were characterized. The first, rough estimate of growth characteristics was done on solid media. A number of interesting growth characteristics can be seen in Table 5. Some of the mutants showed no significantly altered or inhibited growth. However, the inhibited growth that was observed was a result of two major factors: temperature and the presence of tryptophan. In general, none of the mutants grew well, if at all, on the rich medium, presumably from the high concentration of tryptophan present. Several of the strains showed a similar effect on M9 medium with tryptophan present. While most strains grew more slowly on the minimal media with tryptophan, strains D112 and D114 were particularly affected, while D111 and D122 showed a smaller effect. Along with the tryptophan effect, for some of the strains there is a temperature dependent effect, characterized by little to no growth at 30°C, but high growth at 42°C. Strains D109 and D114 on M9 without tryptophan are an obvious examples of this, while a similar, but lesser effect can be seen in strains D111 on minimal media without tryptophan, D115 on rich medium, and D118, D119, D120, and D123 on M9 with tryptophan, However, the temperature effect was not as clear as that caused by the presence of tryptophan, so the tryptophan effect was investigated first.

Based on these results, we concluded that strains D109, D111, D112, D114, and D122 would be the first strains to be further studied. Growth curves for each of these strains as well as the

control strain D124 were determined at 30°C and 42°C, as well as at 42°C in the presence of trp (see Figures 2-7).

Table 5: Qualitative growth measurements of strains D109-D123

	M9			M9 +TRP			LB		
	30 C	37 C	42 C	30 C	37 C	42 C	30 C	37 C	42 C
D109	1	4	5	1	5	3	0	2	2
D110	3+	3	5	1	4	3	0	1	3
D111	2	5	5	1	3	3	1	3	4
D112	0	1	2	0	0	0	0	0	0
D113	1	4	2	0	1	2	0	0	0
D114	1	5	5	0	0	0	0	0	0
D115	4	3	4	1	5	3	0	1	4
D116	2	5	3	1	5	2	0	2	3
D117	3	3	4	1	4	2	1	1	3
D118	2	2	4	1	5	5	1	1	3
D119	2	3	3	1	2	5	1	2	2
D120	2-	4	<#>	1	<#>	5	1	3	3
D121	1	5	<#>	1	<#>	2+	1	2	2
D122	2	5	3	1-	1	2	0	0	0
D123	2	5	3	1	2	5	0	1	4

The cultures were rated for growth on a scale of 0 to 5
(0 = no growth; 5 = confluent growth in streaked area)
<#> indicates crossover of growth between strains on the plate

Table 6: Summary of Growth Curve Data

Strain	Doubling Time at 30°C(min)	Doubling Time at 42°C(min)	Doubling Time at 42°C with trp(min)
D124	-	81.9	91.4
D109	111.5	77.2	-
D111	106.0	88.8	81.8
D112	124.4	90.9	174.0
D114	117.6	76.8	129.8
D122	117.6	80.7	90.0

All of these strains exhibited their smallest doubling time, and therefore their highest rate of growth at 42°C (see Table 6).

Figure 2: Growth Curves for Strain D124 at 42°C and 42°C with tryptophan

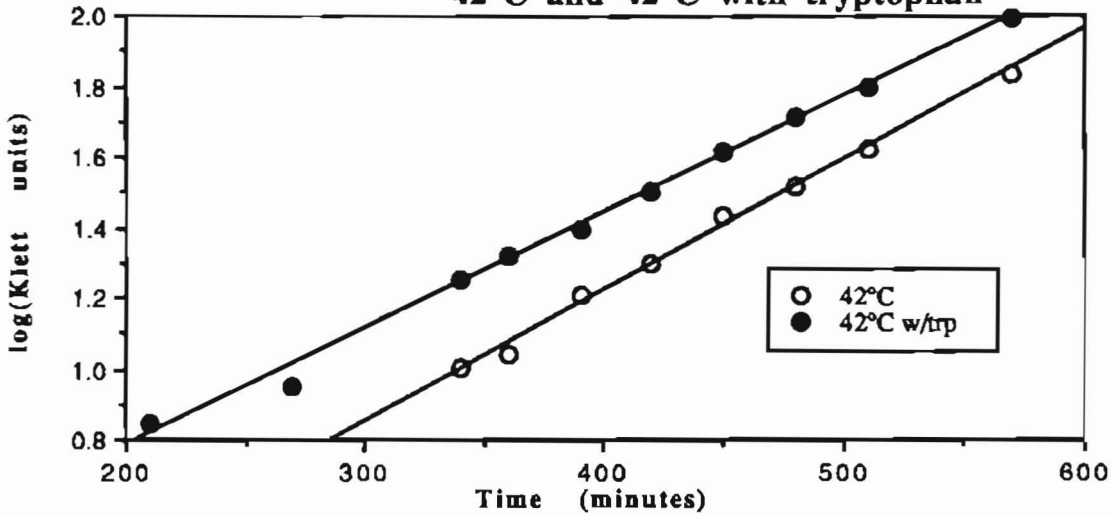


Figure 3: Growth Curves of Strain D109 at 30°C and 42°C

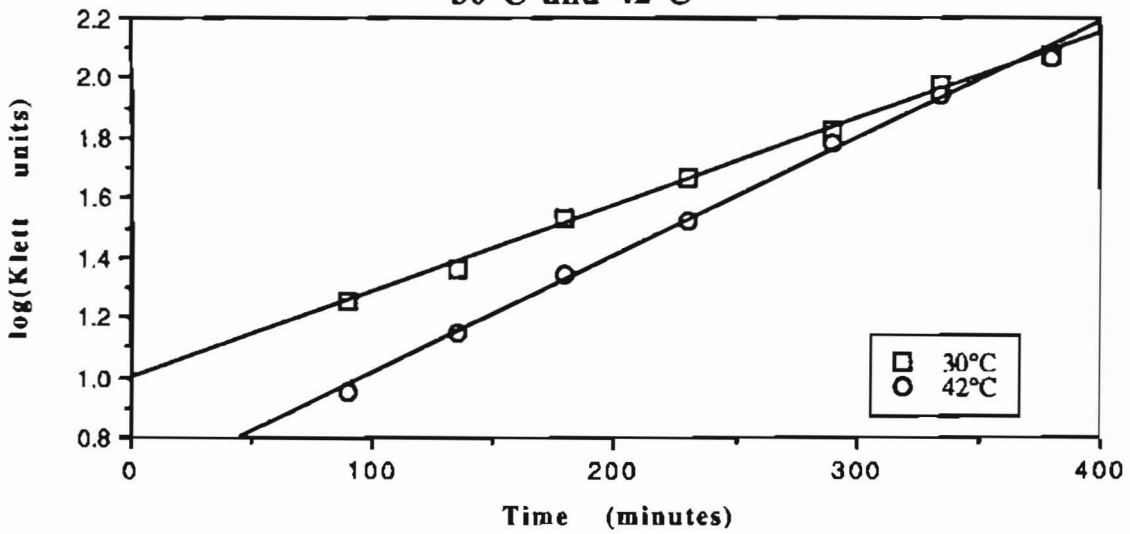


Figure 4: Growth Curves for Strain D111 at 30°C, 42°C, and 42°C with tryptophan

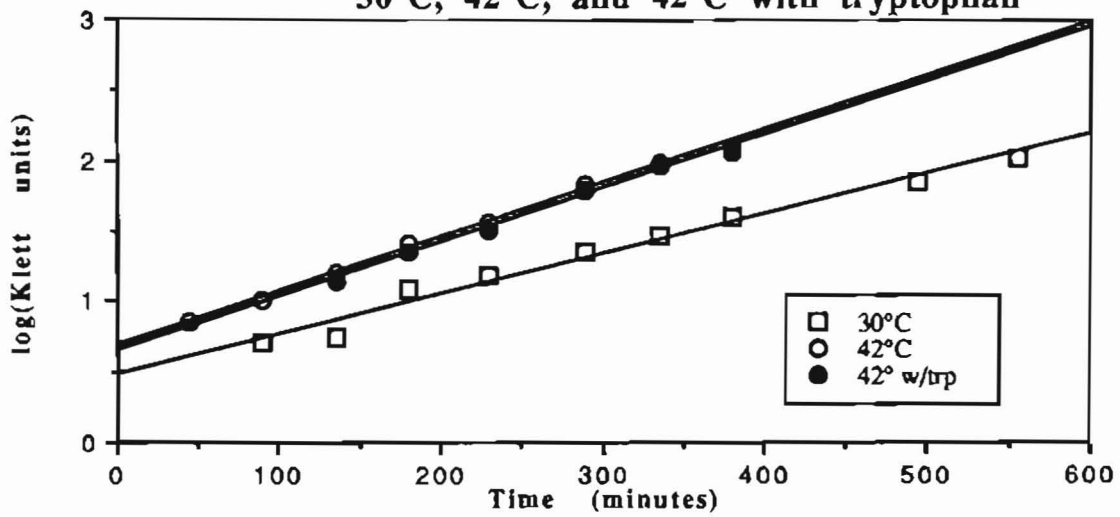


Figure 5: Growth Curves for Strain D112 at 30°C, 42°C, 42°C with tryptophan

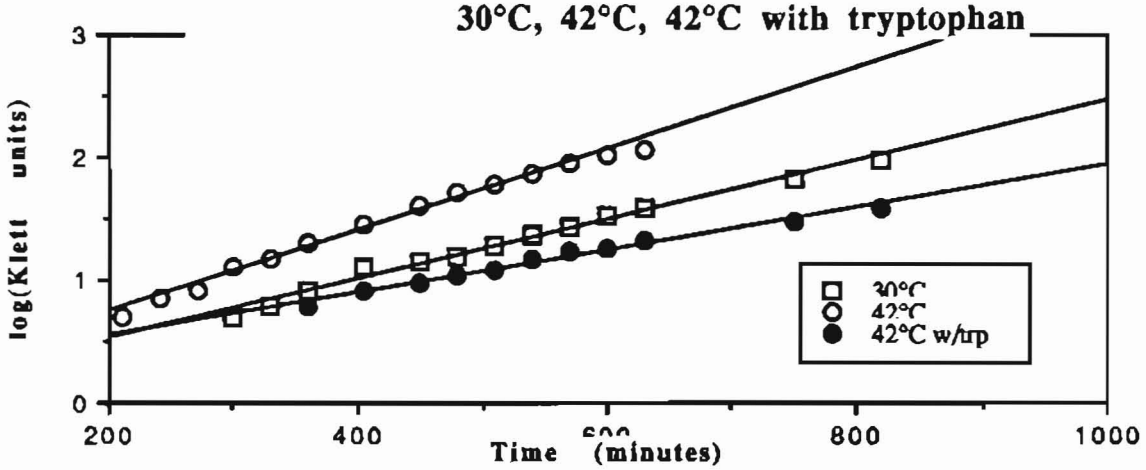


Figure 6: Growth Curves for Strain D114 at 30°C, 42°C, and 42°C with tryptophan

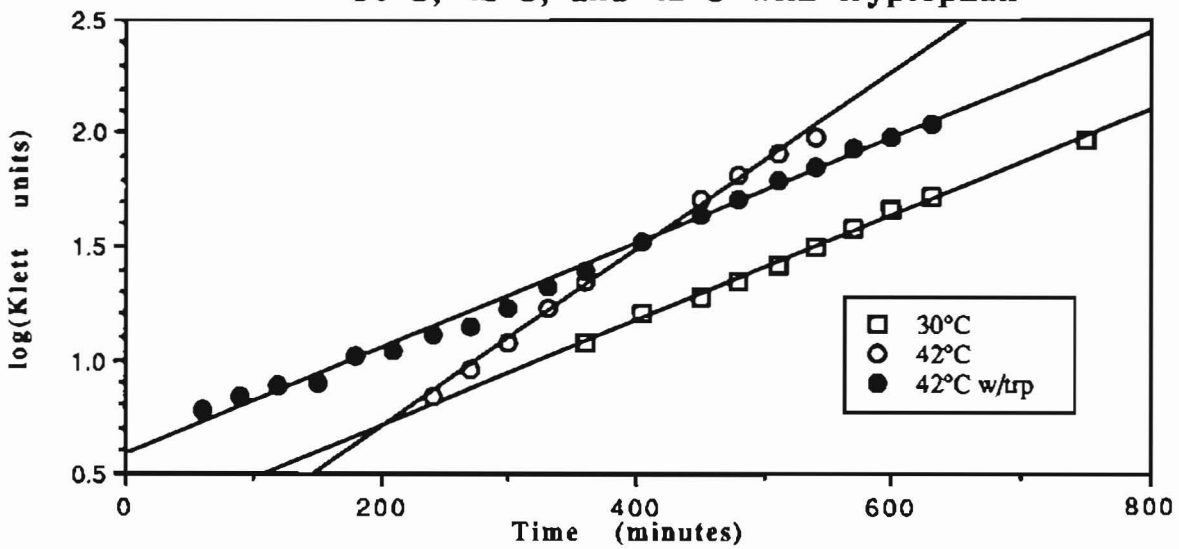
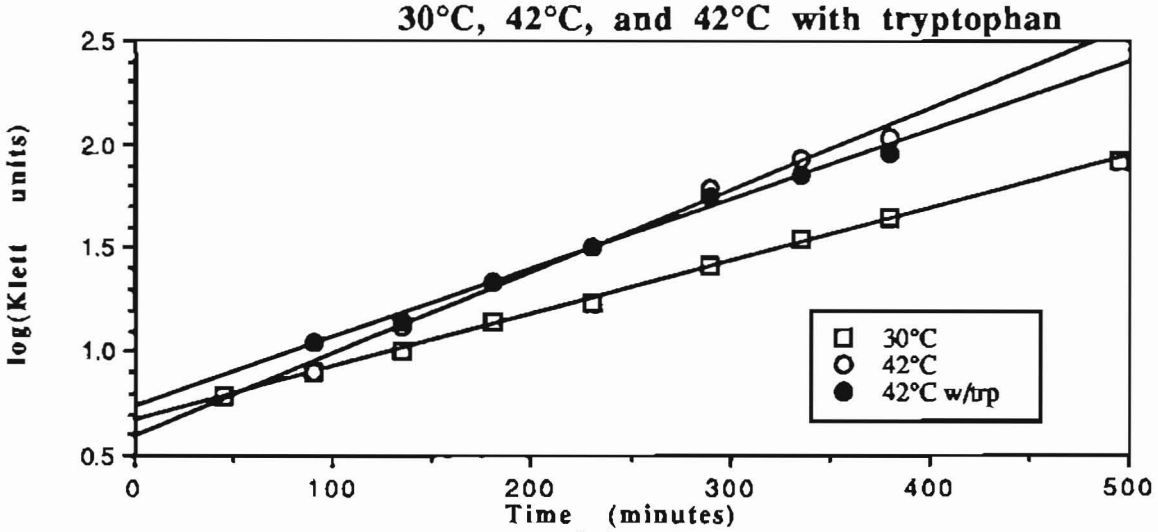


Figure 7: Growth Curves for Strain D122 at 30°C, 42°C, and 42°C with tryptophan



However, when strains D112 and D114 were grown in the presence of tryptophan, their doubling times increased by 91% and 69% respectively. The other strains exhibited no significant changes in their doubling times in the presence of tryptophan.

4.5S RNA analysis : By examining the gels run on the nucleic acid extracts of strains, we can see that there is little difference in the gel mobility of any of the 4.5S RNA molecules extracted from any of the strains. This can be seen in both the native and denaturing gels (only native gels are shown, Figures 8-9). Further analysis of the RNA of strains D124, D109, D112, and D114 by the northern blot confirmed this yet again (Figure 10).

Microscopy: The light microscopy revealed the presence of elongated, or filamentous cells in strains D109, D112, and D114 (Figures 14-22). The average cell was significantly longer then those observed of the D124 strain (Figures 11-13). These elongated cells exhibited a light and dark banding pattern, which corresponded to the location of nucleoids within the cells; the nucleoids located in the light bands, other cytoplasmic material in the dark. The cells appeared to replicate and segregate the chromosomes, but were then unable to form septa (the invagination of the cell membrane which marks the site of new cell wall formation) and divide.

The electron micrographs of strains D124, D112, D114, and D122 unveiled some interesting effects of the mutations on the cellular ultra-structure. Both the cell membrane, and the outer membrane were intact in all strains viewed. The periplasmic space seemed to

Figure 8: 10% polyacrylamide gel of the RNA content of strains D110-11, D113, D115-23, and a standard. Lanes (l-r): std, D110, D111, D113, D115, D116, D117, D118, D119, D120, D121, D122, and D123. The arrow denotes the 4.5S RNA band.

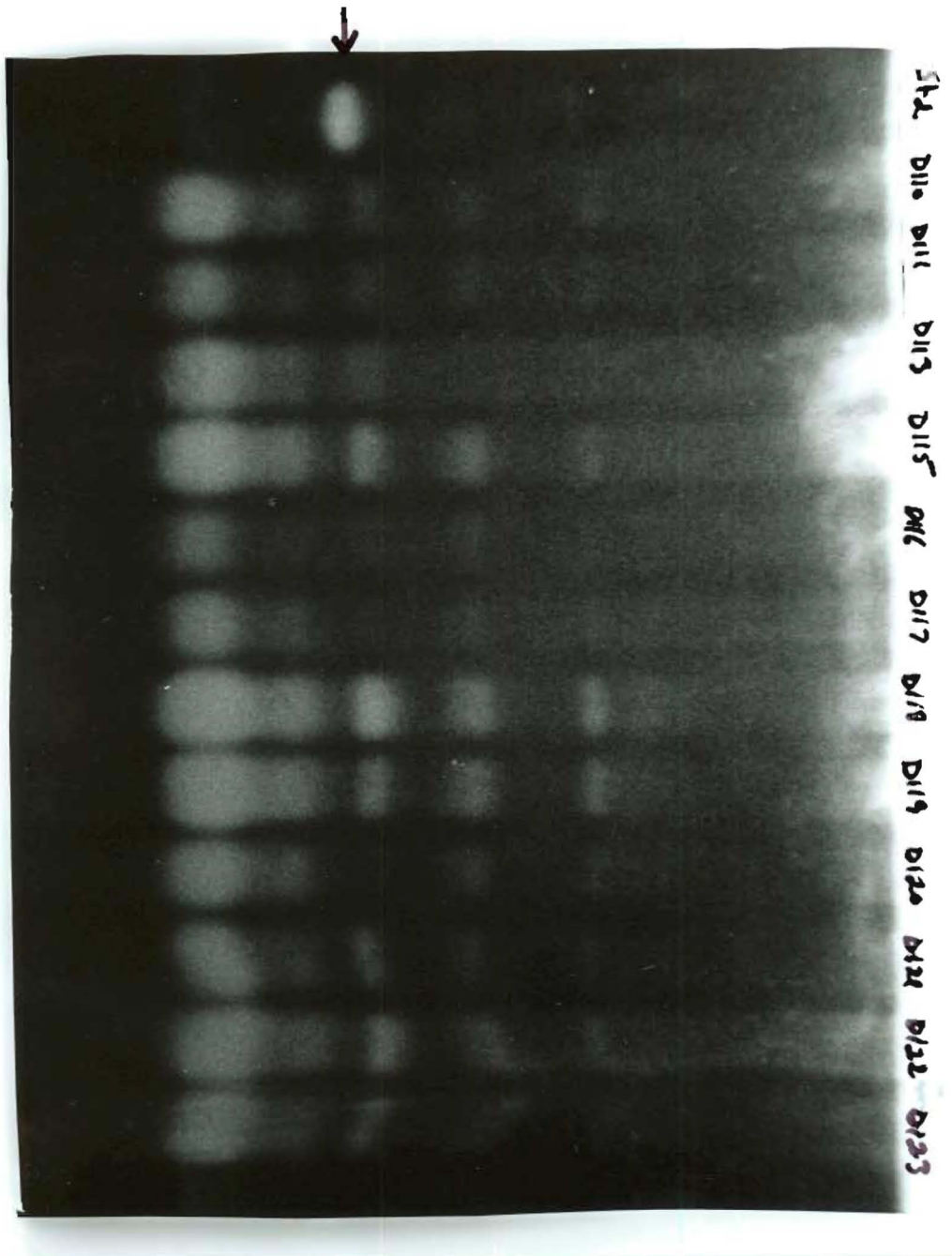


Figure 9: 10% polyacrylamide gel of the RNA content of strains D109, D112, D114, and a standard. Lanes (l-r): D124, Std, D109, D112, and D114. The 4.5S RNA band is at approximately 73 mm.

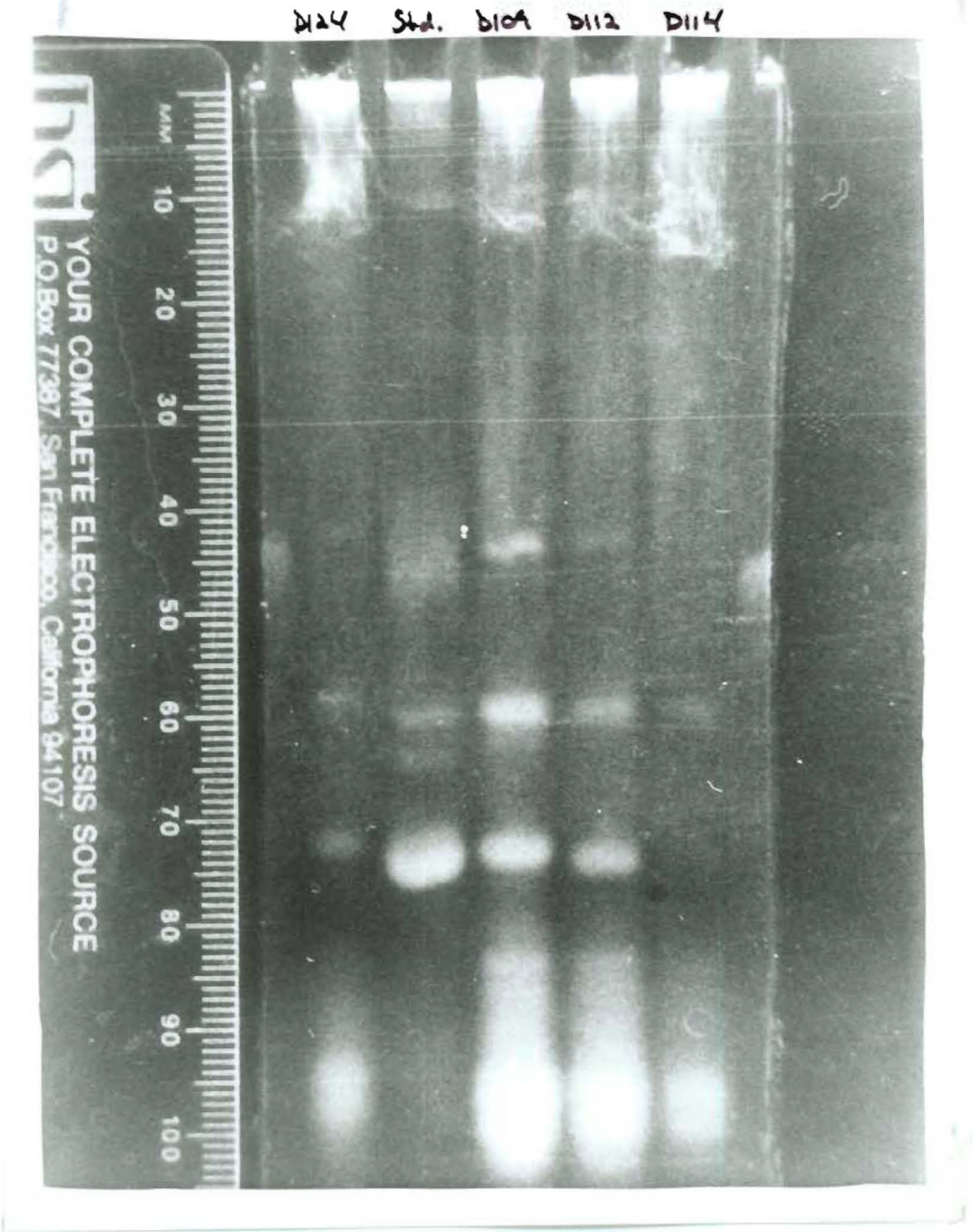
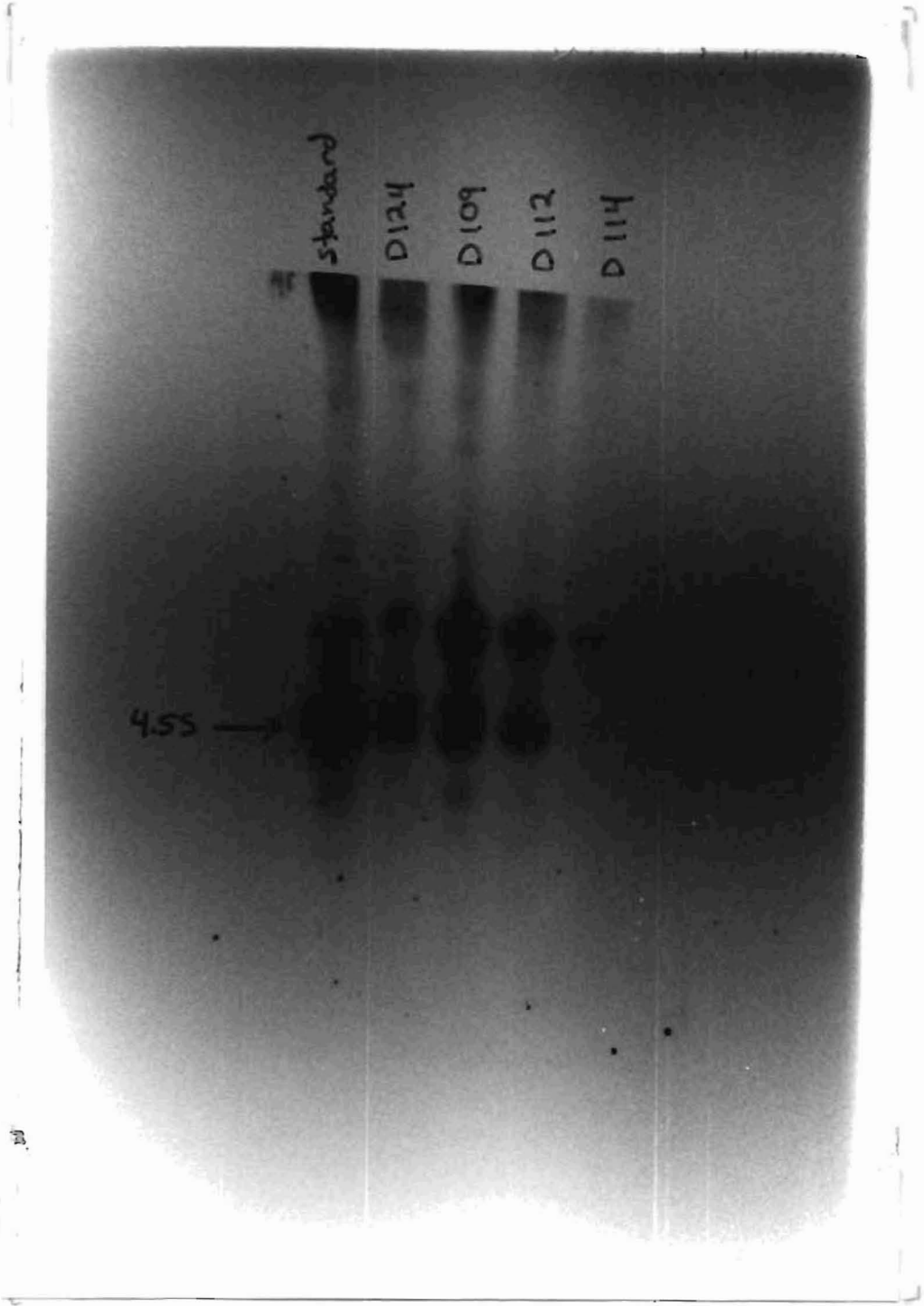


Figure 10: X-ray film of the hybridization experiment. The lanes and the location of the 4.5S RNA band are denoted on the gel. The other bands are due to the presence of 5S RNA, which the probe also detects.



be normal. However, in many cases, a large area of electron transparent substance could be seen along the inside of the cell membrane. In standard TEM staining procedures, the only substances which remain electron transparent are nucleic acids and lipids. We have assumed that the substance in these mutants is a lipid-based substance since nucleic acids tend to be much more diffuse, and would have electron-dense ribosomes associated with them. In some cases, this material appears to be exerting abnormal pressure on the cell membrane and outer membrane causing a disruption in the uniform shape of the bacteria (Figures 24-30). Since we see no such structural alteration or electron transparent region in the control strain D124 (Figure 23), we can assume that it is not an artifact of the embedding and staining process.

Two-Dimensional Gel Electrophoresis: This technique displays the general pattern of protein synthesis in the cell. The X-ray films of many of these mutant strains show some variations of the overall pattern of protein synthesis from the control. This is especially evident in strains D112, and D114 (Figure 31). The altered pattern of protein synthesis in these strains may be attributed to the mutated form of the 4.5S RNA molecule.

Immunoprecipitation: Due to technical problems, I have been unable to submit data regarding this aspect of the study.

Figure 11: Light Micrograph of Strain D124, grown in M9-glucose media at 30°C.

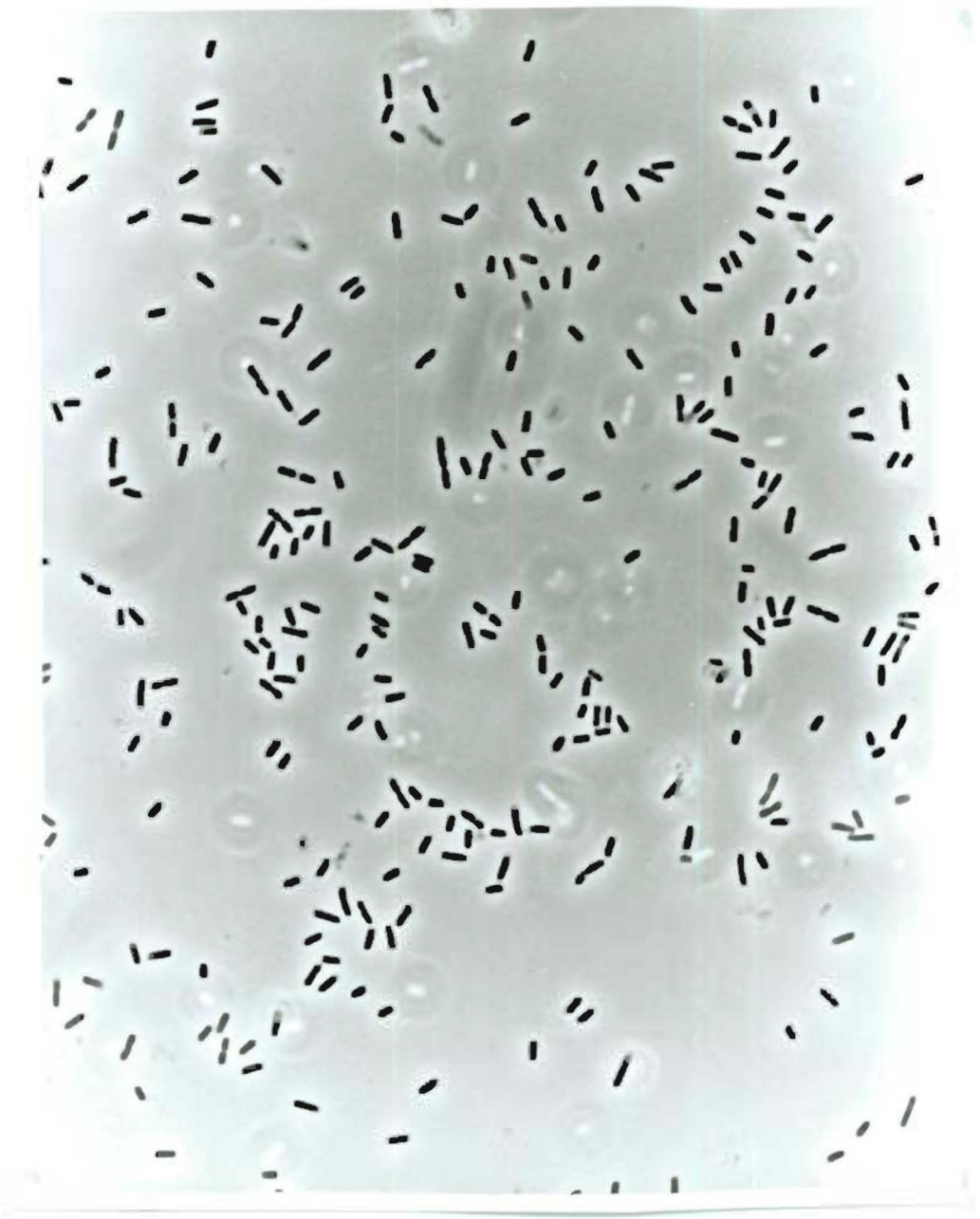


Figure 12: Light Micrograph of Strain D124, grown in M9-glucose media at 42°C.

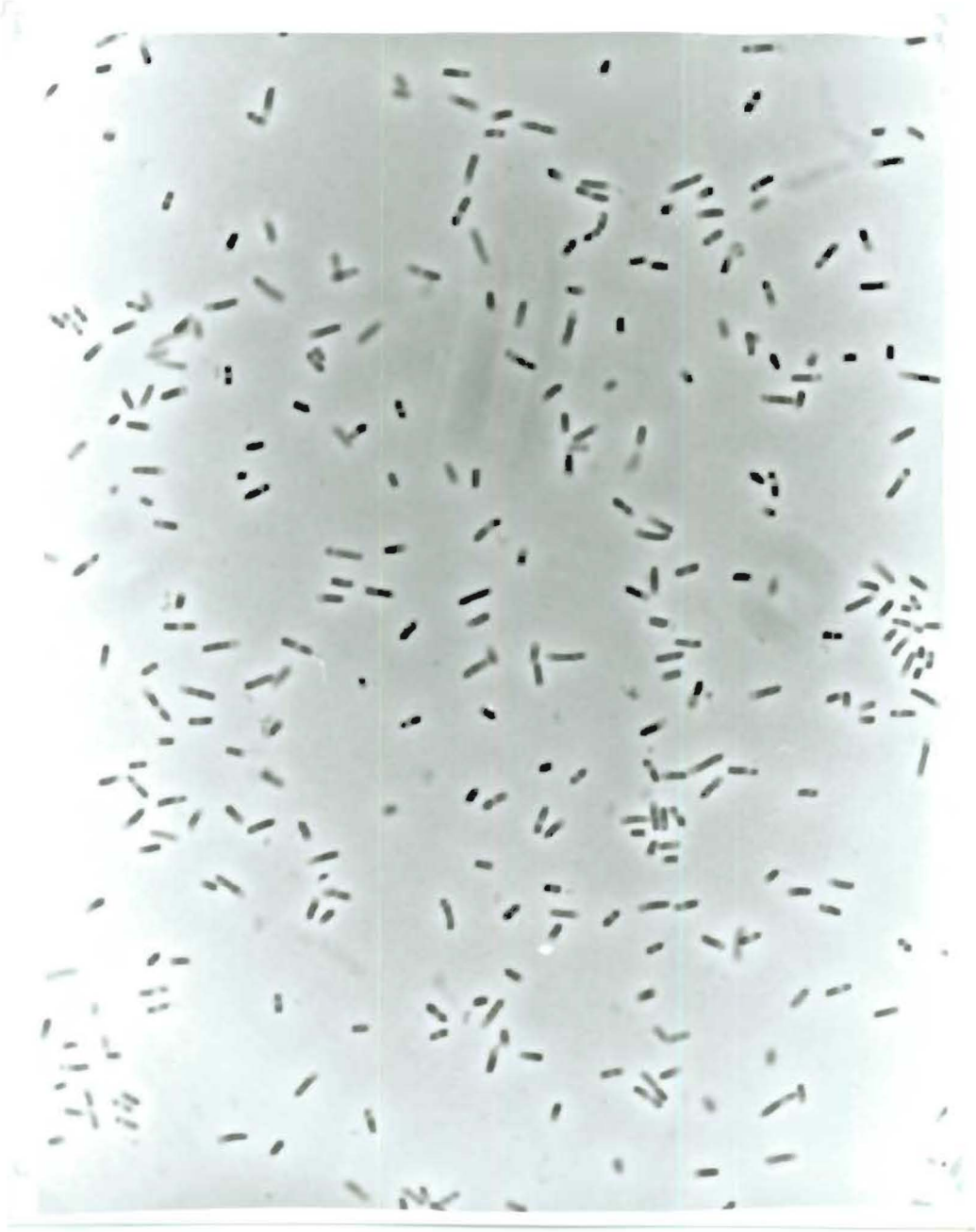


Figure 13: Light Micrograph of Strain D124, grown in M9-glucose media with tryptophan at 42°C.

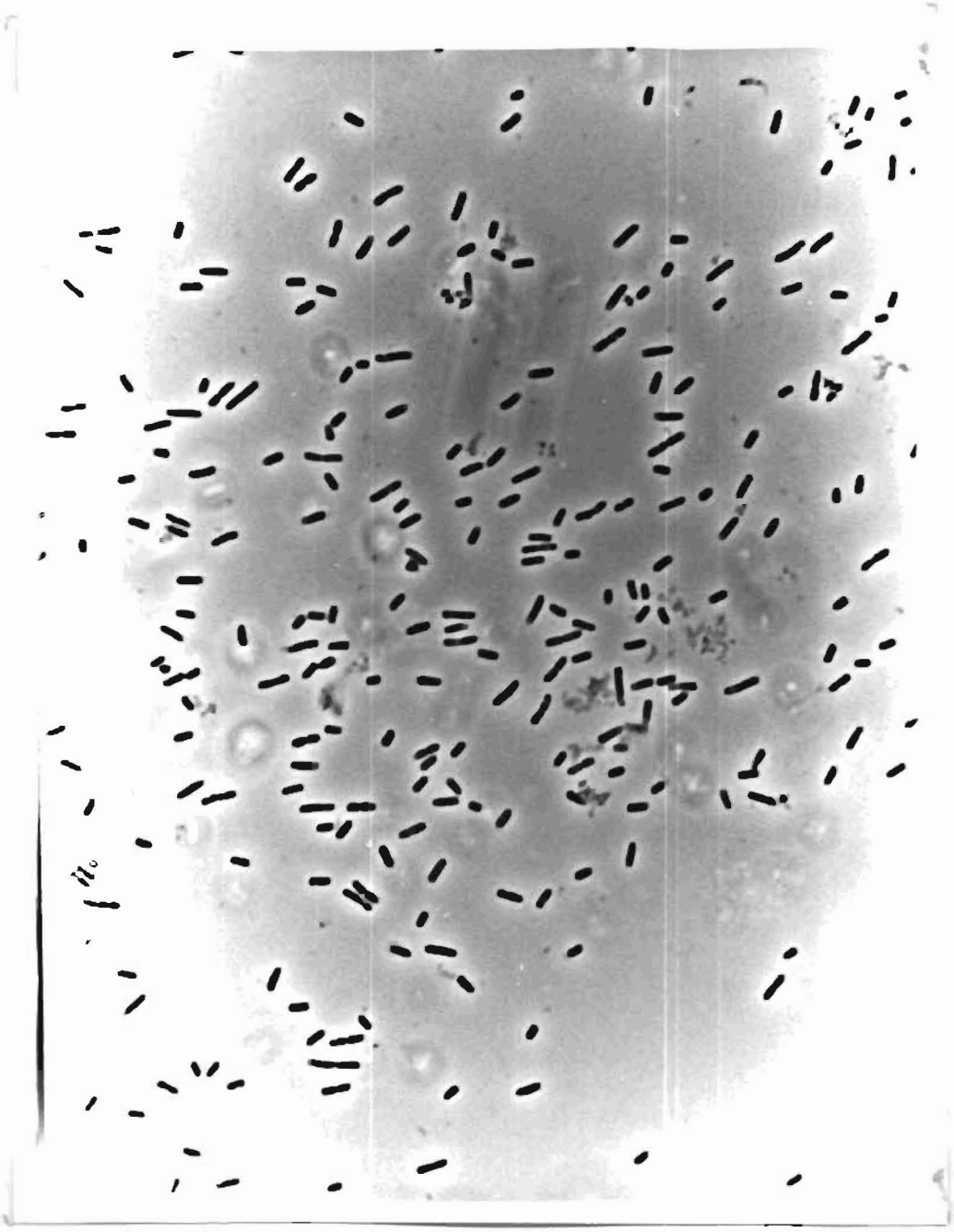


Figure 14: Light Micrograph of Strain D109, grown in M9-glucose media at 30°C.

Figure 15: Light Micrograph of Strain D109, grown in M9-glucose media at 42°C.

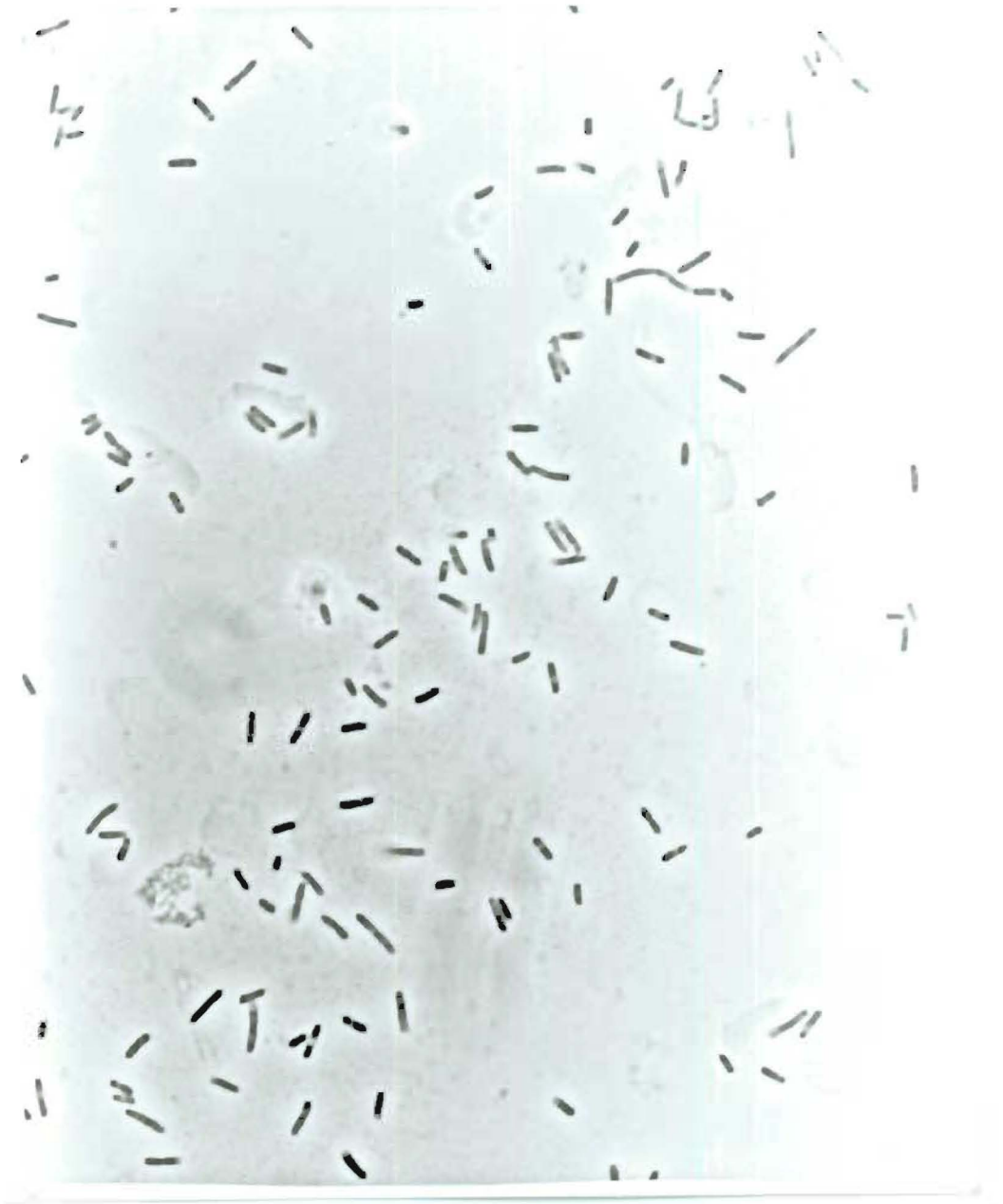


Figure 16: Light Micrograph of Strain D109, grown in M9-glucose media with tryptophan at 42°C.

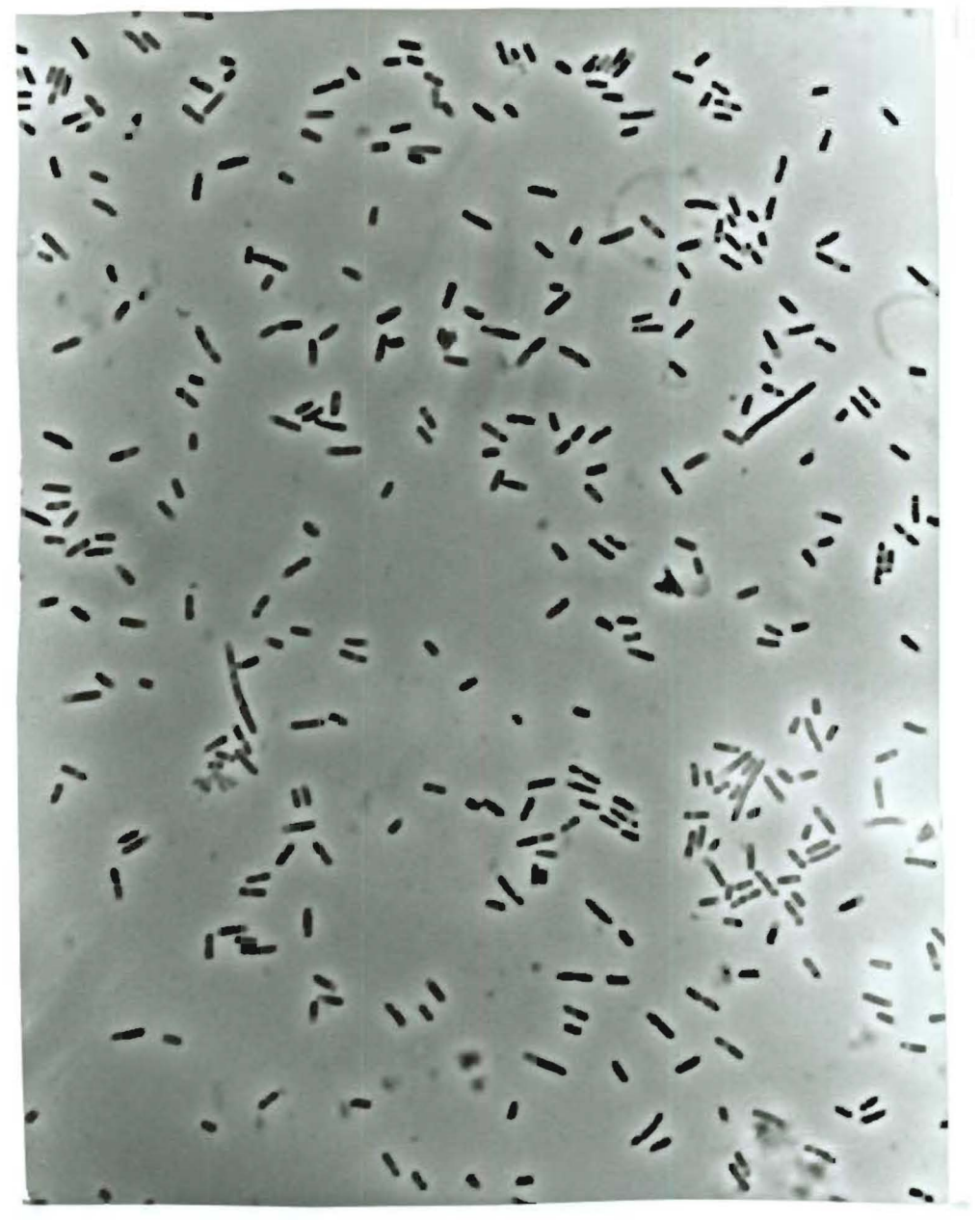


Figure 17: Light Micrograph of Strain D112, grown in M9-glucose media at 30°C.

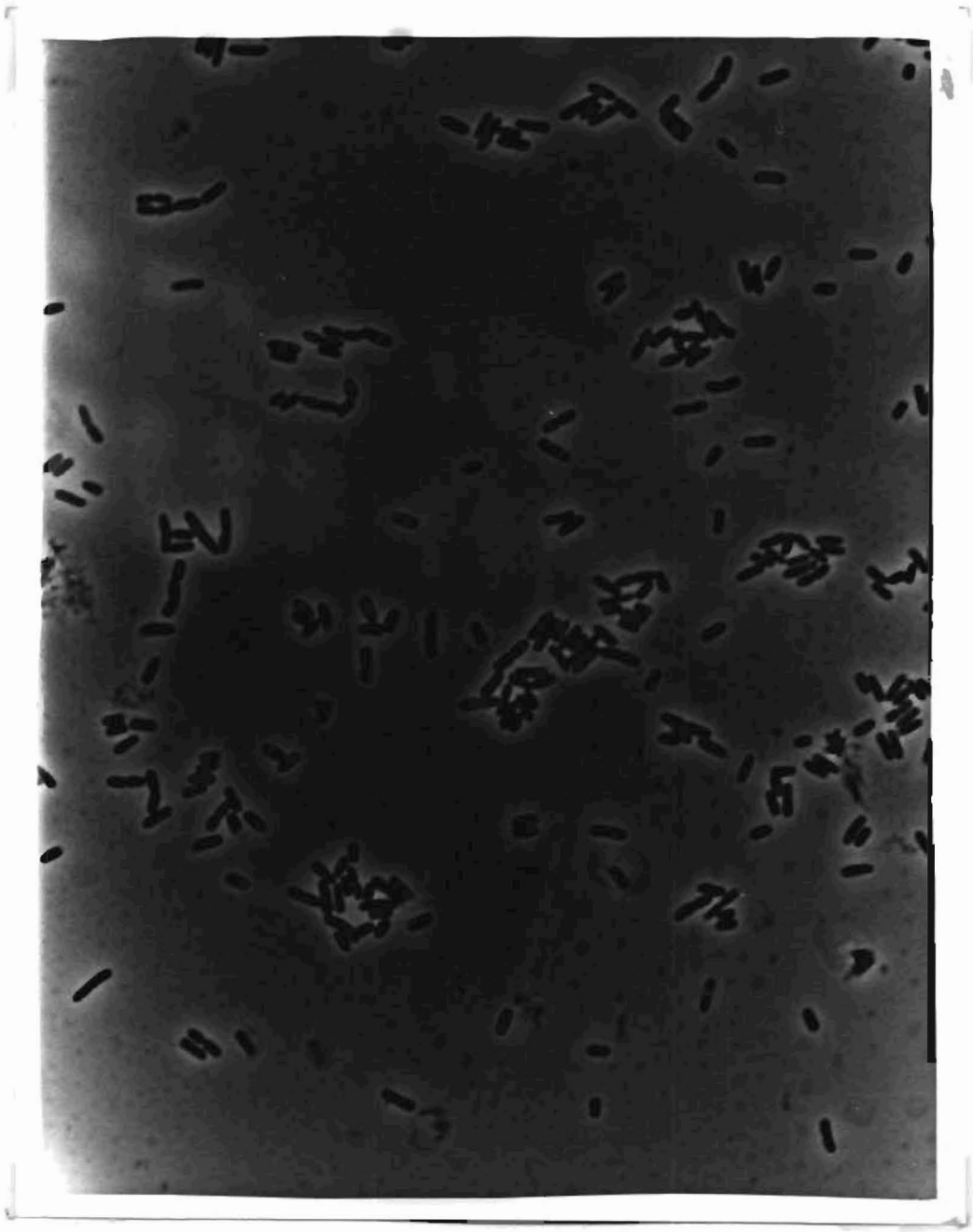


Figure 18: Light Micrograph of Strain D112, grown in M9-glucose media at 42°C.

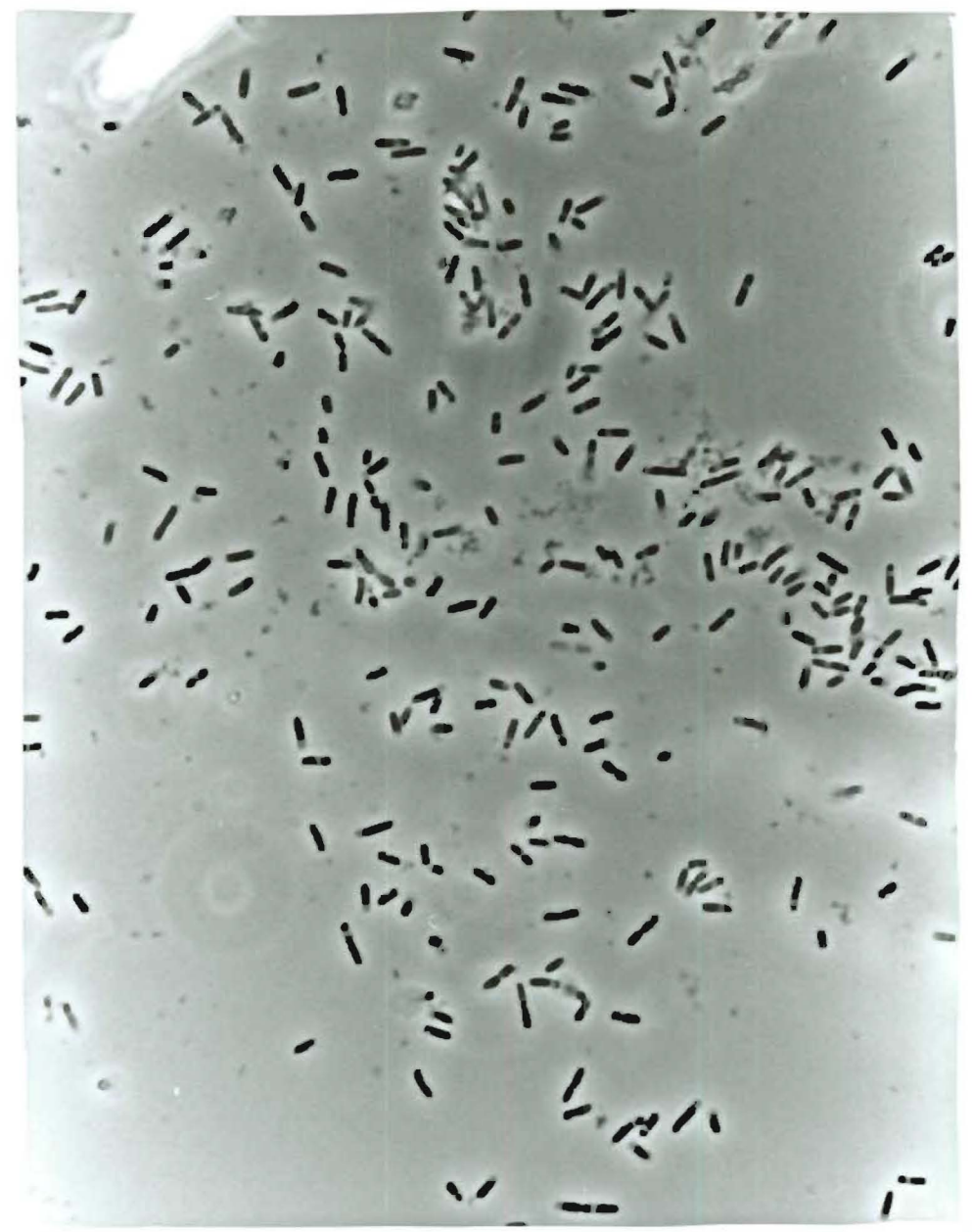


Figure 19: Light Micrograph of Strain D112, grown in M9-glucose media with tryptophan at 42°C.

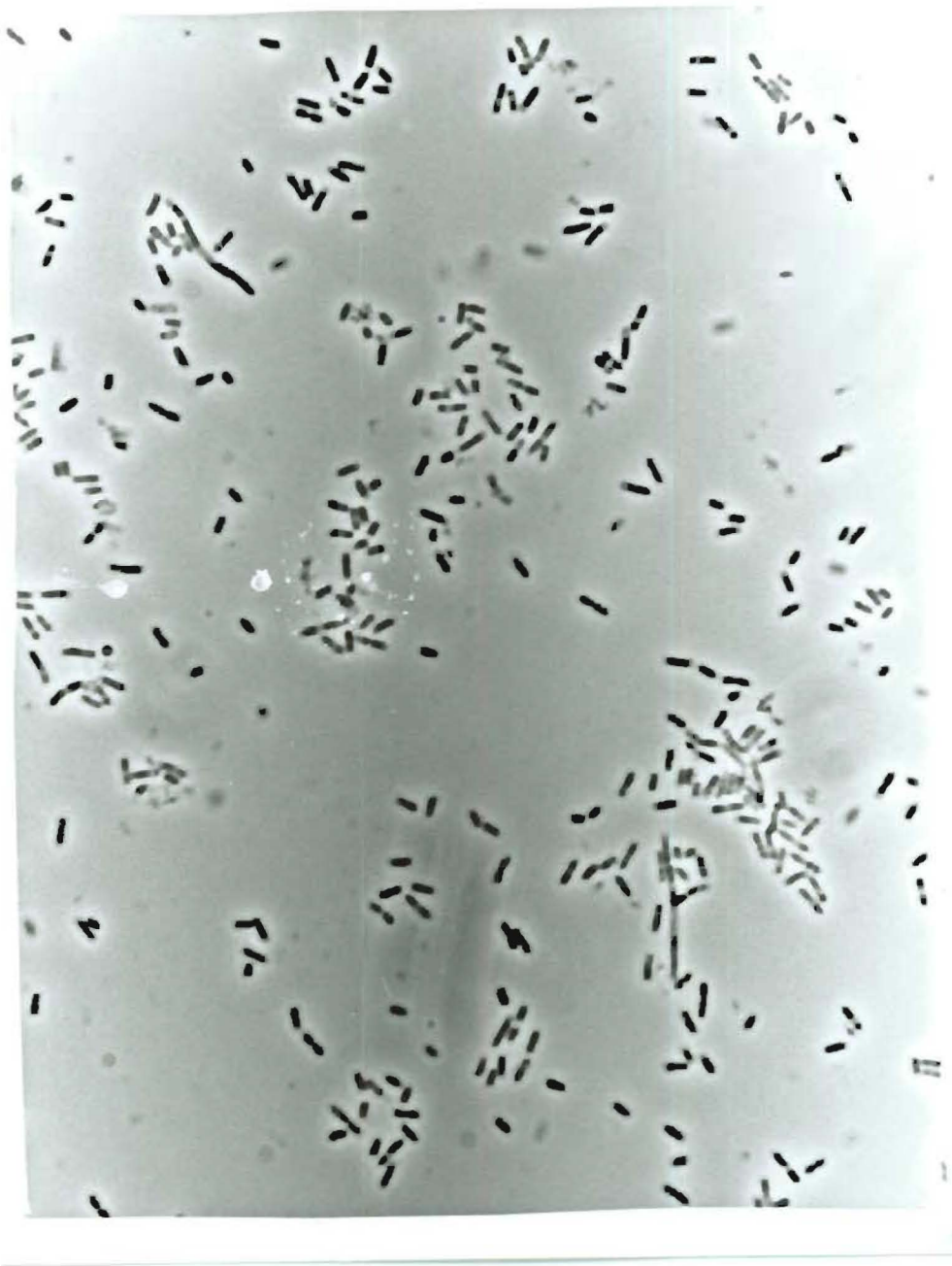


Figure 20: Light Micrograph of Strain D114, grown in M9-glucose media at 30°C.

Figure 21: Light Micrograph of Strain D114, grown in M9-glucose media at 42°C.

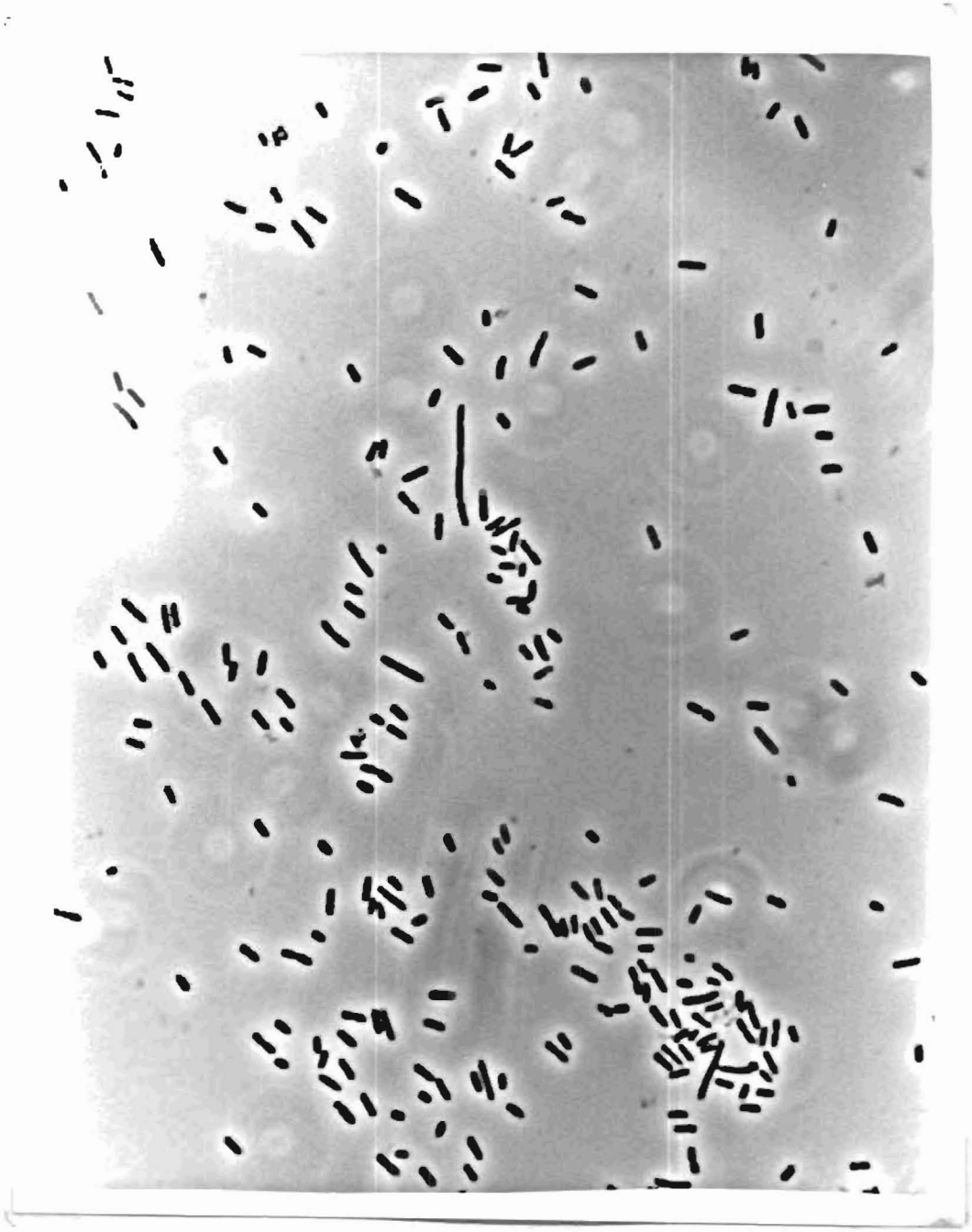


Figure 22: Light Micrograph of Strain D114, grown in M9-glucose media with tryptophan at 42°C.

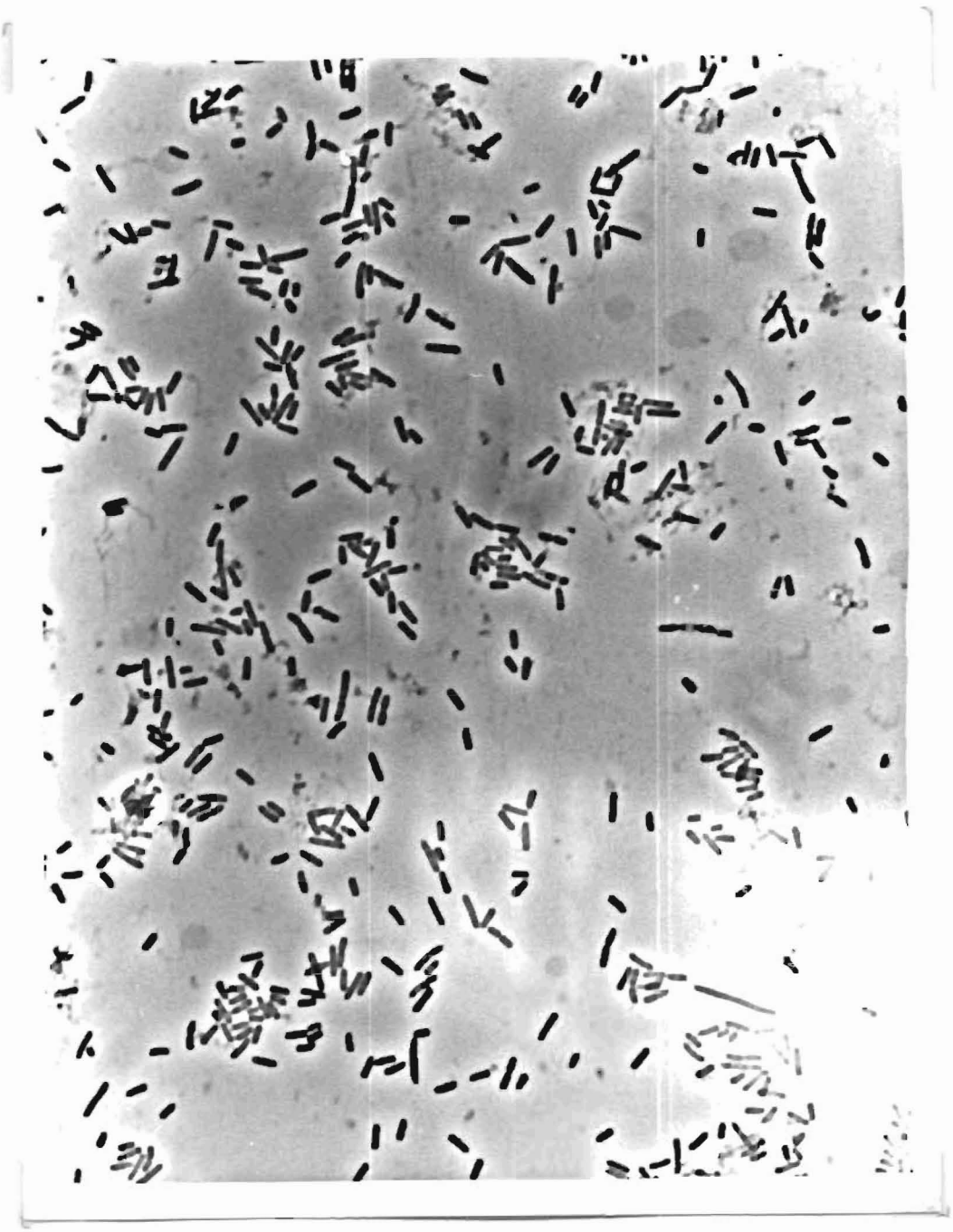


Figure 23: Electron Micrograph of Strain D124, grown in M9-glucose media at 42°C.

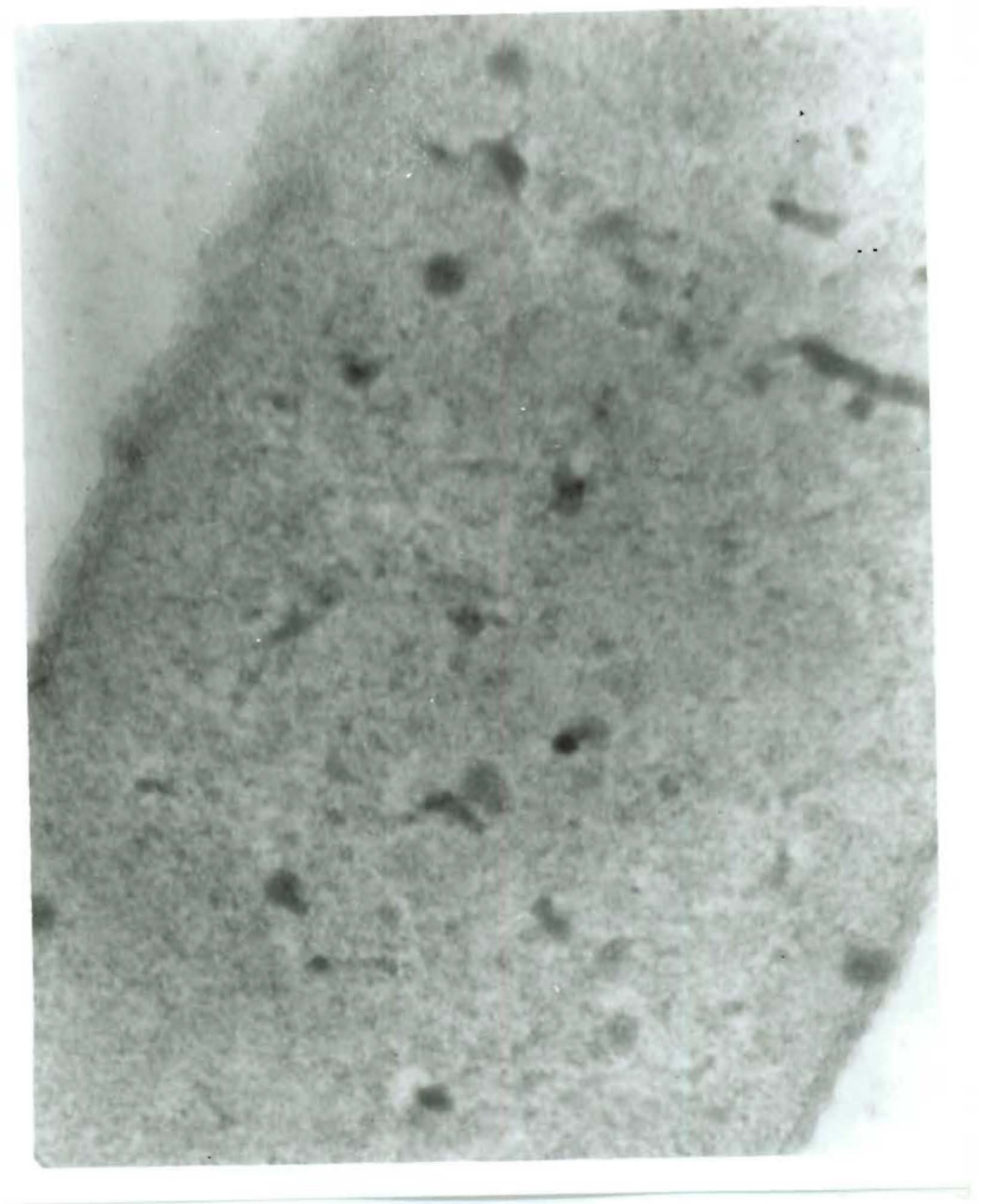


Figure 24: Electron Micrograph of Strain D112, grown in M9-glucose media at 42°C.

Figure 25: Electron Micrograph of Strain D112, grown in M9-glucose media with tryptophan at 42°C.

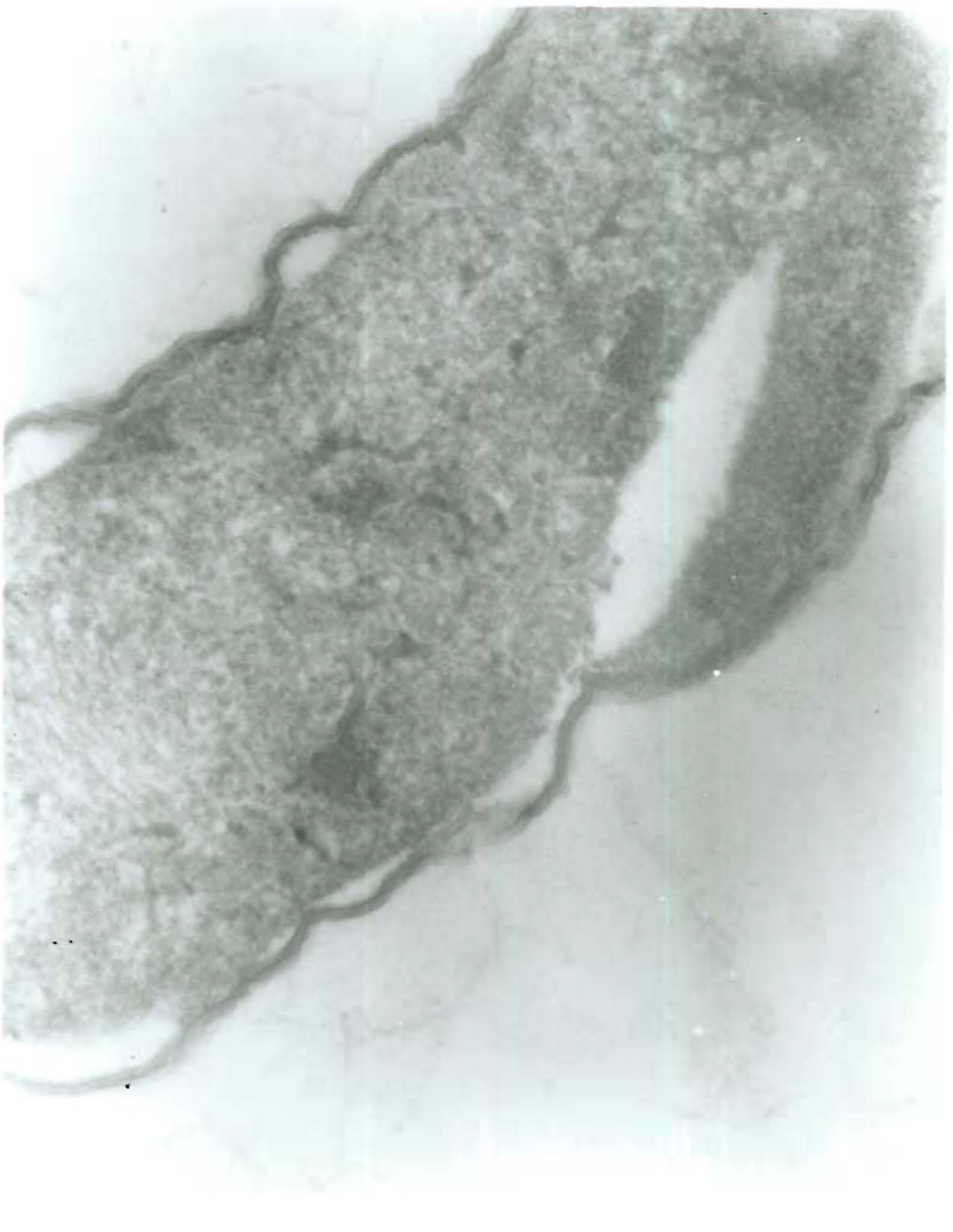


Figure 26: Electron Micrograph of Strain D112, grown in M9-glucose media with tryptophan at 42°C.

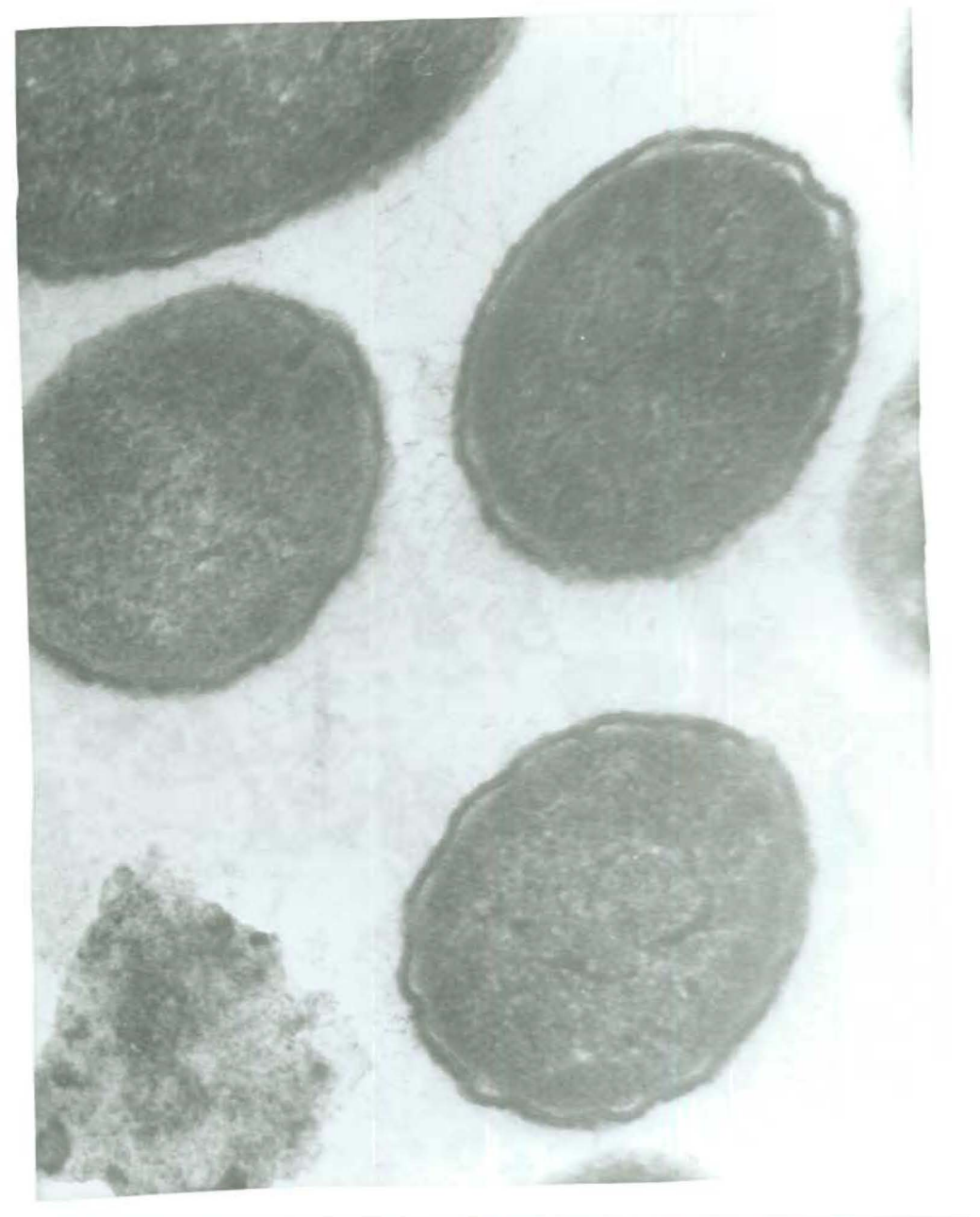


Figure 27: Electron Micrograph of Strain D114, grown in M9-glucose media at 42°C.

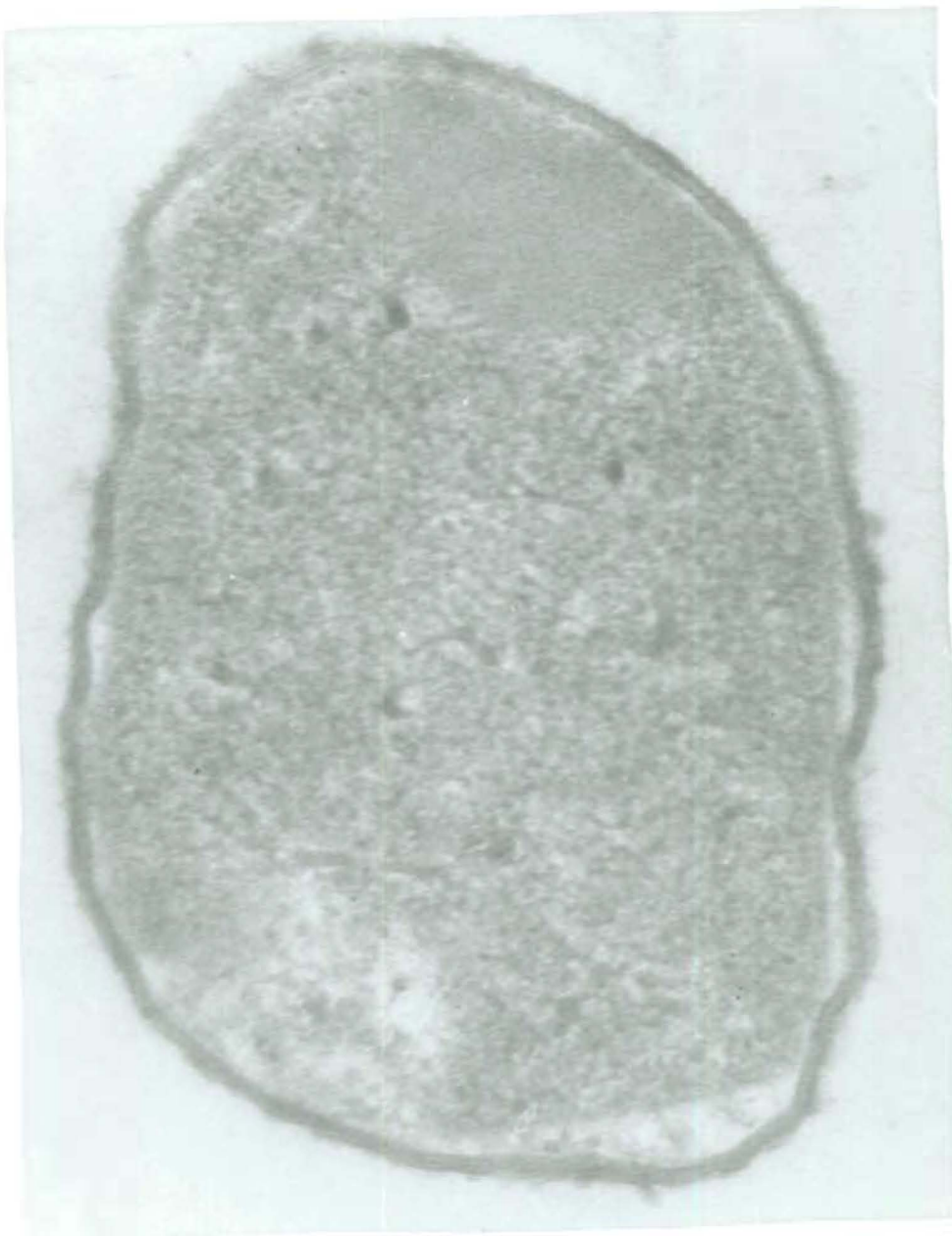


Figure 28: Electron Micrograph of Strain D114, grown in M9-glucose media at 42°C.

Figure 29: Electron Micrograph of Strain D122, grown in M9-glucose media at 42°C.

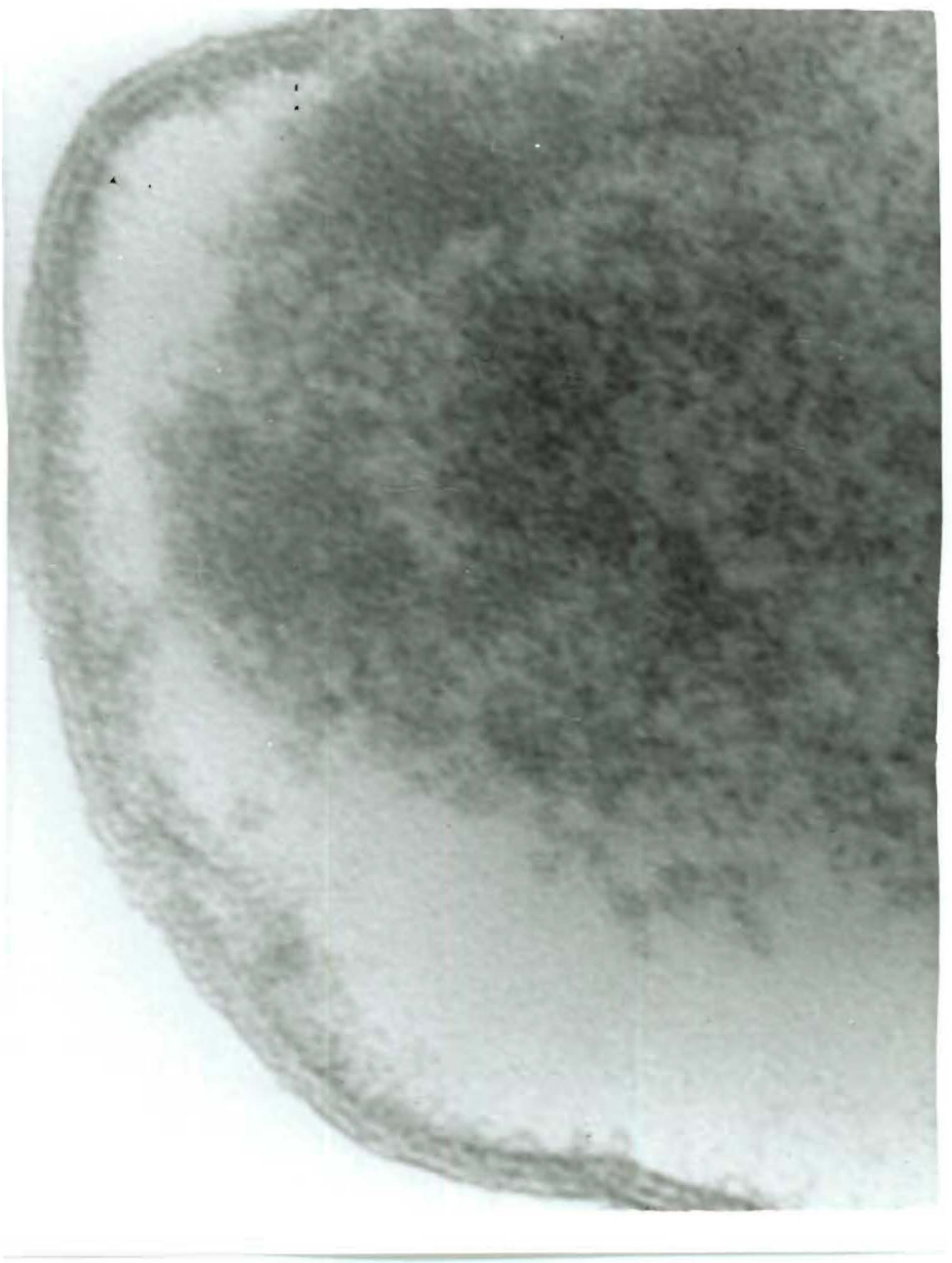
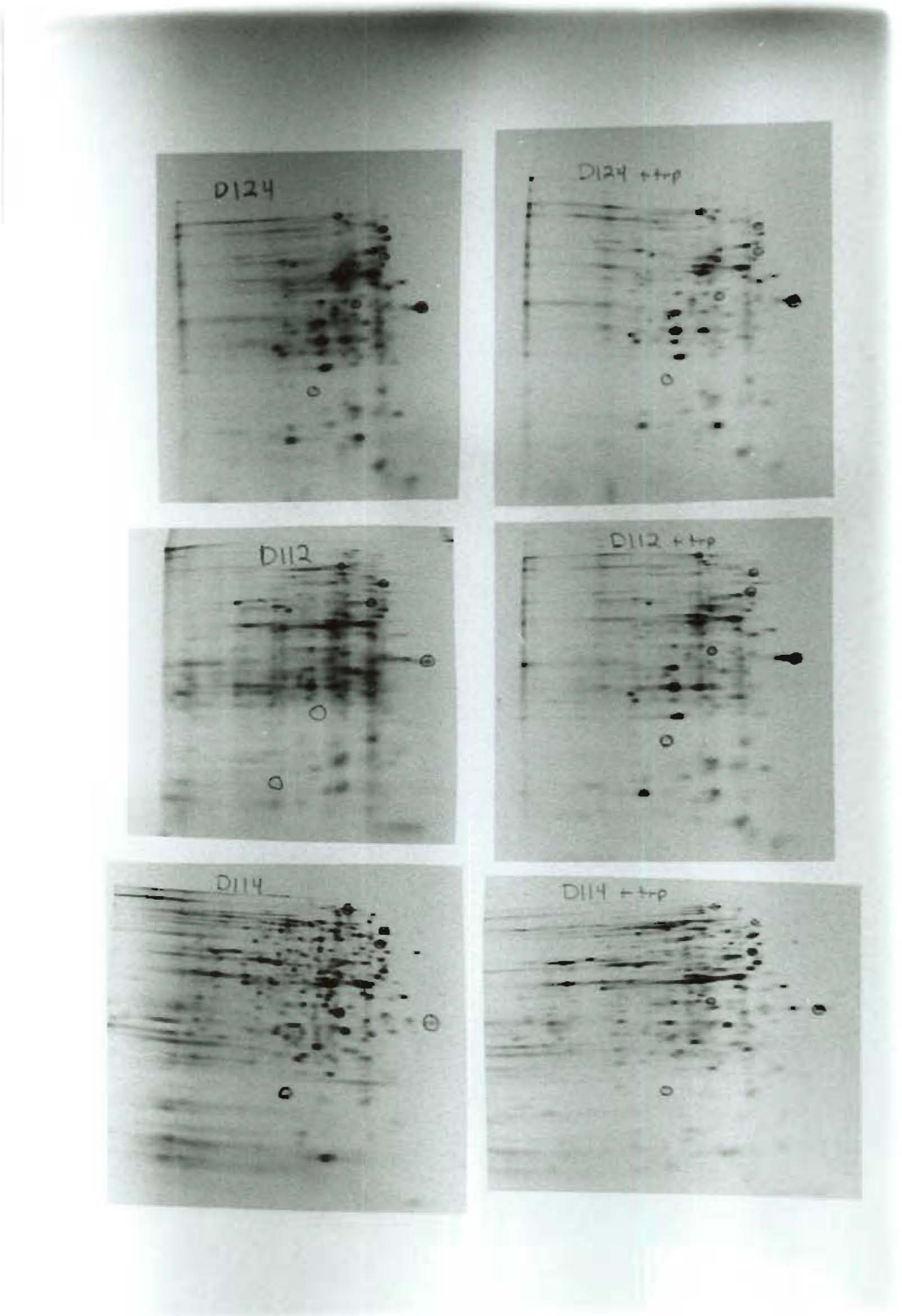


Figure 30: Electron Micrograph of Strain D122, grown in M9-glucose media with tryptophan at 42°C.

Figure 31: X-ray films of the two dimensional gels of Strains D124, D112, and D114 grown in M9-glucose media with and without tryptophan at 42°C.



Discussion

The function of the 4.5S RNA in *E. coli*, remains an unanswered question. Much of the data gathered and described in this study supports a generally accepted role in protein secretion. However, in all cases, this information only supports the hypothesis, failing to give concrete proof of a direct role in protein secretion.

The growth curve data confirms the fact that these are 'sick' cells, with some serious ailment in a fairly central cellular progress. These cells grow very slowly, especially in the presence of tryptophan, which causes a decreased production of 4.5S RNA in these mutants. However, we cannot assume that this inhibited growth is due to depletion of 4.5S RNA alone, since the control strain D124 does not exhibit a similarly decreased growth rate in the presence of tryptophan. The effects of tryptophan on D124 is a mere 11% increase in the doubling time, while in strains D112 and D114 for instance, the doubling times increase 91% and 69% respectively. Obviously, a large portion of the effect is due to the specific mutation of each of those strains.

A more specific physiological effect is the elongation of cells in the mutant strains, as seen in the light micrographs. As stated above, the cells exhibited a banding pattern which indicated the presence of multiple copies of the chromosome. The cells were sometimes 2-5 times their normal length, containing a corresponding number of chromosomes. They were all properly segregated, but the cell did not form a septum and divide. The inhibition of septum formation could be caused by a number of things, including a secretory problem. The production of cell wall and septum depend largely on

secreted proteins. If those proteins needed for septum formation could not be secreted due to a mutation in 4.5S RNA, then the cell would be unable to divide, resulting in the filamentous cells observed.

The analysis of the 4.5S RNA molecules in the strains examined in this study allows to determine the nature of the structural effects of these mutations. The mutations used in this study are small, differing by only one or two bases from the wild-type copy of the 4.5S RNA gene. If the reduced function of the 4.5S RNA were due to a severe disruption in the three-dimensional structure of the molecule, we would expect to see altered gel mobility on a native polyacrylamide gel, but we do not. We can also assume that the processing of these particular mutants is not significantly affected by the mutation, since there seems to be no significant change in the gel mobility of these molecules on a denaturing gel which separates molecules based on size, or length of the nucleic acid. From the data collected, it seems that the inhibitory effects of the mutations comes from a sequence-specific interaction being disrupted, not an overall structural change of the molecule.

Continuing in that vein, we may now look at those strains which exhibit the most interesting characteristics: especially inhibited growth, severe morphological changes, and altered patterns of protein synthesis. We then can identify the mutation (see Figure 1), and make some general assumptions about functionally significant sites on the molecule. Strain D112 for example exhibits the slowest growth of all the strains. In both the light and electron micrographs the D112 possessed a severely altered morphology. From those

observations we can conclude that the mutation in D112 affected an important site on the molecule. The 4.5S RNA in D112 differs from that in the control strain only by nucleotide number 47, which has a cytosine substituted for an adenine. Judging from the fact that this strain exhibited the lowest growth rate, significant accumulation of a lipid-based substance in micrographs, and altered protein synthesis, we can conclude that the identity of this nucleotide is very important to the function of the molecule. Similar conclusions can be made regarding nucleotide number 49(D114), 23(D109), and possibly 61(D122). The fact that these nucleotides have been found to be important is not a surprise since they all lie in the area of the molecule which contains the conserved sequences with other small RNA's, is the site of presumed functional importance, and has been shown to be essential by Wood et. al..

When examining the information in the two-dimensional gel electrophoresis experiments, we see a definite change in the overall pattern of protein synthesis. The mutations in the 4.5S RNA molecule are in some way altering that pattern. This is not an unexpected result, since depletion of 4.5S RNA has been shown to inhibit translation (Bourgaize and Fournier, 1987; Brown, 1987).

The transmission electron micrographs introduce a new aspect to the problem. What is the substance which accumulates just inside the cell membrane? It is electron transparent, leading us to conclude that it is either lipid or nucleic acid. We would expect a nucleic acid to be much more diffuse, and to have electron-dense ribosomes associated with it, thus we believe it is a lipid-based substance. Assuming that the mutations in 4.5S RNA have caused a significant

inhibition of the secretory process, including the secretion of secreted proteins necessary for outer membrane synthesis, it would be reasonable to hypothesize that the electron transparent material is a lipid-based substance destined for the outer membrane. Further investigation is necessary to identify the substance and determine whether it is a direct result of 4.5S RNA mutation.

A number of important areas need to be addressed before the function of this molecule is determined. Experiments conducted in this study need to be repeated on those mutants exhibiting a temperature-dependent effect to better characterize the specific mutations. More immunoprecipitation experiments need to be conducted in order to better understand the function of 4.5S RNA in secretion, if any direct function exists. Studies of a more structural nature may be useful in determining possible active sites, as well as confirming hypothesized sites of importance. If the molecule is a part of some type of primitive SRP-like system, then it may be helpful to do an *in situ* hybridization using a biotinylated probe in an attempt to see if the 4.5S RNA is associated with the membrane as it would be if it were a member of an SRP-like system. Until these and other experiments are conducted, 4.5S RNA will remain to be a molecule without an identified function.

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