COVER SHEET

TITLE: Determining the minimal nutrient requirements for the swarming phenotype in *Escherichia coli* and other swarming bacterial species

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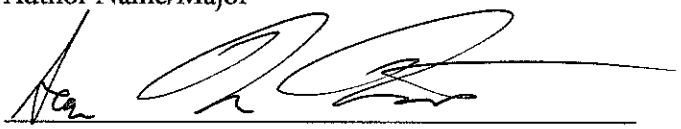

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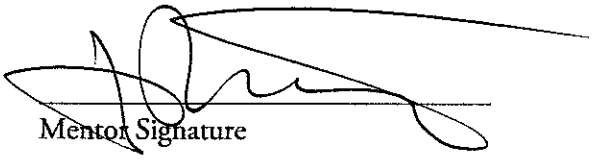

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This project lays the foundation for identifying chemical inducers and inhibitors in swarming cells of *Escherichia coli*. Swarming is a type of cell surface motility characterized by differentiated bacterial cells that have an elongated, hyper-flagellated, and multinucleated phenotype. This swarmer cell phenotype is thought to be the prevalent phenotype in the infection of urinary tracts and bladders by uropathogenic strains of *Escherichia coli* and *Proteus mirabilis*. The nutritional requirements necessary for differentiation and swarming motility are poorly understood. The goal of this project was to formulate and characterize nutrient media that has a well-defined set of components necessary to produce a robust swarming phenotype. This minimal swarm media was designed to satisfy the following characteristics: (1) chemical reproducibility, (2) defined composition, (3) production of the swarming, hyper-flagellated phenotype in *E. coli* and other swarming bacteria, (4) induction of surface motility, and (5) lack of auto-fluorescence. We suggest that the designed media could serve to standardize the swarming field of study and could provide a reproducibly defined platform on which to perform future examination of the swarming phenotype.

INTRODUCTION

Swarming cells are characterized by an elongated, hyper-flagellated, and highly motile phenotype (Figure 1; Harshey et al. 1994). Swarming has been observed in numerous bacterial strains and is correlated to pathogenicity. Examples of this phenomenon include the colonization of

the urinary tract and bladder during host infections by *E. coli* and *P. mirabilis* (Jacobsen et al. 2008; Justice et al. 2004). Though swarming is a medically relevant phenomenon, little is known about the nutritional requirements needed to produce this phenotype. Minimal medium have been quantitatively constructed for most model organisms making it possible to reproduce experiments while providing insight into the inner-workings of the organism. These medium are particularly informative because they eliminate the components that an organism can manufacture and provide those which must be available in the organism's environment (Neidhardt et al. 1974). More specifically, a defined medium does not exist for studying the swarming bacterial phenotype.

Current methods used to study both swarming *Salmonella* and *E. coli* use commercial nutrient broth consisting of peptone (1% w/v), beef extract (0.3% w/v), NaCl (0.5% w/v), glucose (0.5% w/v), and Eiken agar (0.45% w/v). The swarming substrates that are produced using these materials are inconsistent due to the heterogeneity of the ingredients, the techniques for preparing agar plates, and the fluctuation of the moisture content in the plates. The development of a controlled media would greatly further research efforts in this area and is key to understanding how chemical additives affect the swarming phenotype.

For example, Copeland et al. have used a microarray analysis to compare the regulation of genes in the swimming and swarming phenotype of *E. coli* strains. Their experiments revealed a two-to-nine-fold up-regulation in iron-uptake and iron-metabolism genes (Copeland et al. 2007, unpublished results). This data suggests that iron may play a pivotal role in triggering the swarming phenotype. Research with *Vibrio parahaemolyticus* demonstrates that an iron deficient medium acts

as a secondary signal for the production of lateral flagella and subsequent swarming (McCarter et al. 1989). In *E. coli*, the mechanism for differentiation is not fully understood.

The identification of a minimal swarming nutrient medium would allow for the study of chemical interactions that induce the signal cascade responsible for differentiating cells into the swarming phenotype. Moreover, this becomes feasible because a defined medium will not contain contaminating amounts of the chemical of interest; for example, iron is found in both peptone and beef extract, making the study of iron's role in swarming difficult to ascertain when using these heterogeneous compounds. This highly quantified broth will satisfy any requirements for nutrition, surfactant, and inorganic metals. Analytical characterization of this medium may further our understanding of the essential metabolic requirements needed to stimulate bacterial swarming and allow for uninhibited study of iron's role in inducing and maintaining the swarming phenotype.

Finally, the creation of a medium to study the swarming phenotype across bacterial species is in the best interest of the field. Cross species analysis could elucidate true inducers and inhibitors, streamlining the swarming bacteria field. Meta-analyses of swarming experiments performed on the same platform may provide insight to a common gene ontology and ancestral lineages, revealing permutations in the cellular regulation of swarming. In this paper, I delineate a quantified media which supports a robust swarming phenotype across bacterial species. This presents a unique opportunity for a field to employ a common platform to study a phenotype using a cross-phylogenetic approach.

RESULTS

Media

Current media, hereby called nutrient broth (NB), is composed of peptone (1% w/v), beef extract (0.3% w/v), NaCl (0.5% w/v), and glucose (0.5% w/v), and Eiken agar (0.45% w/v). Through an iterative process, the Modified MOPS Media (MMM) was created (Table 1) to satisfy all nutrient requirements normally found in NB. Subsequent qualification and characterization was necessarily conducted to verify the utility of the MMM as a replacement for NB.

Macroscopic Observations

Spot inoculated plates were observed macroscopically 18 hours post-inoculation. The rate of *E. coli* strain RP437 swarm progression on MMM (Figure 2A, 2B, 3A) was significantly slower than that observed on the positive control NB plates (Figure 2C and 2D). Wanting to see if this observation held true for other swarming species, we ran the same experiment using *Proteus mirabilis* (ATCC 7002), *Salmonella enterica typhimurium* (ATCC 19585), and *Serratia marcescens*. In fact, this decrease in ring expansion appeared to hold true for *S. marcescens* (Figure 3D) but was not observed on plates inoculated with *P. mirabilis* (Figure 3C) or *S. enterica typhimurium* (Figure 3B), where the rate of swarm ring expansion on MMM was nearly equal to that observed on NB. To identify whether the macroscopic observation of limited swarm expansion was a result of the cells not adopting the swarming phenotype, we observed the inoculated plates microscopically.

Microscopic Observations

Microscopically, it appears that both point inoculated MMM (Figure 4A) and NB (Figure 4B) swarm plates exhibit the swarming phenotype, although it appears that the cell density on the

MMM plates is greater than the NB plates. This greater cell density is in agreement with the macroscopic data which suggests that the colony is stacking vertically as the cell population grows and divides, instead of the typical growth and cell division which leads to spreading of the swarm colony across the agar surface. Video which captured the observed cell migration from which Figure 4 screen shots are derived can be found on the Weibel Lab Server.

To best study the progression from swimming cells in a liquid culture to the swarming phenotype, we conducted experiments where plates were microarray inoculated (See Methods & Materials) such that a confluent swarm arises after about three hours of incubation. This allowed us to truly observe the developmental stages of swarm development on the two mediums and denote any differences. No apparent differences in swarm development or the timeline of development were observed between the two mediums and are representative of five independent experiments. These videos are not shown but can be found on the Weibel Lab Server.

For both forms of inoculation, it appears that the rate and cooperativity of swarming cells are nearly indistinguishable between MMM and NB plates. Likewise the length of the cells appears to be very comparable between the experimental condition and positive control. Thus, from a microscopic perspective, RP437 adopts a full swarmer phenotype, as previously defined, just as well in MMM as it does in NB.

Comparison of Growth Rates

Having verified that the decrease in swarm ring expansion is not due to the lack of adoption of the swarming phenotype, we next examined the relative growth rates of RP437 in both MMM and NB. Growth curves completed for eight hours revealed an attenuation in the maximum growth

denisty possible for RP437 in MMM equal to only two-thirds the maximum density reached in NB (Figure 5). This factor is not of much concern because we are not looking at the point of confluency on the swarm plates; instead, the progression of the swariming colony is of interest to us. Thus, the maximum rate of growth was found through extrapolation of the linear region observed during the exponential growth phase; the maximum rate of growth of RP437 in a liquid MMM culture was 86% of the growth of RP437 in liquid NB culture (Figure 6). This small decline in growth rate does not fully account for the decreased ring expansion that was observed macroscopically for RP437 on MMM swarm plates because the decline in ring expansion between swarm colonies on MMM agar plates versus NB agar plates is greater than 14%.

Sources of Limited RP437 Swarm Ring Expansion

There appears to be a surfactant in NB which has not been fully characterized in the quantified MMM. An approach to test this possibility was to add a surfactant or wetting agent to the media composition and observe changes in swarm ring expansion. It appears that the addition of Tween 80, a surfactant and surface wetting agent, promotes macroscopic swarm ring expansion and subsequent microscopic cell translocation. This observation was made when adding Tween 80 to both MMM (Figure 7C and 7D) and NB (Figure 7A and 7B) swarm plates, allowing the plates to become confluent in ten hours, nearly half the time it takes for swarm plates to normally reach confluency on NB agar plates. However, this increased rate of ring expansion was accompanied by the induction of cell filamentation to a greater extent than plates not containing Tween 80 (Videos not shown but can be found on the Weibel Lab Server). Such results implicate the reduced wetness of MMM swarm plates as the primary cause for decreased swarm ring expansion.

DISCUSSION

Future Directions

Further work must be completed to ensure the full functionality of the MMM. Initial experiments with Tween 80 (Figure 7) demonstrate that improved surface wetness is necessary to link both the microscopic swarming phenotype currently supported by MMM and the macroscopic spreading of the swarm ring. Additionally, if wetting agents are used, then it must be ensured that any introduced artifacts are taken into account and ultimately limited. The preliminary experiments with Tween 80 support the conclusions found in Niu et al. (2005) that minute additions of Tween 80 promote macroscopic expansion of the swarm ring. The filamentation observed in RP437 is a novel artifact not previously detected and could be the result of incorporation of the hydrophobic end of Tween 80 molecules into the cell membrane which may disrupt cellular regulation and initiation of cell division. Testing of other surfactants or wetting agents would best vet this possibility. Moreover, it has been observed that many other species either produce surfactants or secrete osmotic agents such as *Proteus mirabilis* to reduce surface tension and promote cell spreading, thereby increasing the amount of liquid on the surface of the agar plate and promoting swarming (Rauprich et al. 1996).

Additionally, to further eliminate any heterogeneity in the swarm plate composition and address the surface chemistry of the plate, a synthetic polymer, such as polyacrylamide, could be used as a defined platform that replaces agar and provides the desired surface chemistry to promote cell translocation. Thus, combining both the defined media described up to this point with this defined

surface would truly grant the swarming field stringent control over the introduction of unknown variability in the study system.

Finally, the MMM can be used to identify chemical inhibitors and inducers of the swarming phenotype. More specifically, preliminary gene expression analysis of RP437 implicates iron in the development of the swarming phenotype. It has been shown that *E. coli* cells depend on external sources of iron in order to maintain homeostasis (Semsey et al. 2006). The MMM can be used to control or ultimately limit specific concentrations of iron in the media to study its impact on the swarming phenotype.

Conclusions & Implications

While the outlined and characterized medium, the Modified MOPS Media, in itself does not provide further distinct insight to the field of swarming bacteria, it does provide a characterized platform capable of inducing the microscopic swarming phenotype while not conferring any negative artifacts on the study system. This platform, on which future experiments can be conducted, becomes increasingly important when completing gene regulation studies, especially those which employ microarray analyses. Furthermore, the broad applicability of this media across bacteria species provides an opportunity for conducting mechanistic and phenotypic studies across the field, using cross-species research to aid in fully understanding the swarming phenotype and ultimately aiding in the unification of swarming studies.

METHODS & MATERIALS

Bacterial Strains

Bacteria strains used were *Escherichia coli* (RP437), *Proteus mirabilis* (ATCC 7002), *Salmonella enterica typhimurium* (ATCC 19585), and *Serratia marcescens*. Fresh overnight cultures were started for each experiment. Cultures used for plate inoculation were started the day of the experiment from an overnight culture and grown to an OD_{600} between 0.4 and 0.8 to ensure that cells were in mid-log phase prior to plate inoculation. All bacterial cultures and inoculated plates were grown at 30°C. Liquid cultures were incubated in a shaking incubator, while swarm plates were grown upright in stationary incubators.

Media & Plate Assembly

The base of the media was created using a version of the MOPS Media outlined in Neidhardt (1974) which did not contain the suggested supplements. This base was created according to Neidhardt (1974) and stored appropriately prior to use. It included Dipotassium Hydrogen Phospahte, Ammonium Chloride, Potassium Sulfate, Calcium Chloride, Magnesium Chloride, Sodium Chloride, Ferrous Sulfate, Tricine, and MOPS.

Examination of Difco Manuals provided the necessary characterization of the heterogeneous components of the NB media, primarily the beef extract and peptone. Reagent grade chemicals were added to fulfill the nutrient requirements of and replace both beef extract and peptone; these compounds included amino acids (Alanine, Arginine, Aspartic Acid, Cysteine, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine), vitamins (Pyridoxine, Calcium Pantothenate,

Thymidine, Riboflavin, Para-Amino Benzoic Acid, Biotin, Thiamine, Choline Chloride, Folic acid, Inositol, and Nicotinic Acid), and inorganics (Ammonium Molybdate, Boric Acid, Cobalt Chloride, Cupric Sulfate, Manganese Chloride, and Zinc Sulfate). Concentrations and addition of both glucose and agar were maintained from that used in NB plates. Agar type and final agar concentrations used for experiments varied for *Escherichia coli* (Eiken Agar 0.45% w/v), *Proteus mirabilis* (Difco Agar 1.5% w/v), *Salmonella enterica typhimurium* (Difco Agar 0.6% w/v), and *Serratia marcescens* (Difco Agar 0.8% w/v). In later experiments, Tween 80 was added as a wetting agent at a final concentration of 0.01% v/v. Final chemical concentrations necessary for the creation of the Modified MOPS Media are detailed in Table 1.

Media and plates were created fresh for each experiment from refrigerated stock solutions. All components except for the glucose, vitamins, and Neidhardt 10x MOPS stock were added prior to autoclaving. Plates used for spot inoculation were inoculated one hour after pouring with a 1:10,000 dilution of a bacteria culture with an OD_{600} ranging from 0.4 to 0.8. Examination by macroscopic and microscopic observation was conducted beginning 14 hours post-inoculation. Plates used for microarray inoculation or swarm confluence studies were inoculated 24 hours after pouring with a bacteria culture ranging in OD_{600} from 0.4 to 0.8. Five milliliters of said culture was poured onto the plate, let sit for five minutes, and then poured off the plate, making sure to wick excess liquid from the surface of the plate. Microscopic examination began after two hours and typically lasted no more than five hours.

Treated in all extents like an experimental condition, positive control NB plates were constructed with peptone (1% w/v), beef extract (0.3% w/v), NaCl (0.5% w/v), glucose (0.5% w/v), and Eiken agar (0.45% w/v). All plates were made in triplicate at a minimum.

Macroscopic Observations

Macroscopic pictures were taken with a Nikon digital camera. The radius of the swarm ring on each MMM plate was measured and compared with positive control NB plates to measure the ability of MMM to promote swarming surface colonization. Macroscopic pictures were taken of each plate prior to microscopic observation. This data was used to help determine the swarm-rate of the experimental replicates and positive control.

Microscopic Observations

Plates were examined by phase contrast brightfield microscopy to directly observe and measure individual cell motility via rate of edge expansion and scoring of the wild-type swarming phenotype. All microscopy work was carried out on an upright Nikon Eclipse 80i microscope using a total magnification of both 400x (40x objective) and 600x (60x objective). Microscopy data was collected on a charge-coupled device for further review.

Bacteria on each MMM plate was qualitatively compared to those on the positive control NB plates to detect the hallmarks of cooperative motility in highly differentiated cells at the swarm edge. The rate of expansion of the swarming colony under the microscope was recorded in a qualitative manner. Iterative adjustments to the chemical and physical composition of the MMM agar plates ensured true optimization such that successive permutations continued to adopt

characteristics of NB agar plates until an absolute defined media was established. Successful assays produced swarming cells that were identical to the cells on the positive control NB agar plates.

Growth Rates

Overnight cultures of RP437 were used to inoculate respective triplicate cultures of MMM and NB such that the starting optical density at 600nm (OD_{600}) for each culture was equal. Using a nanodrop spectrophotometer, each culture density was measured periodically for 7.5 hours. Experimental data was plotted with a sigmoid curve fit derived from the program Igor. To best identify the maximum rate of growth, the linear section of the exponential growth phase was examined. The slope of the linear fit provided a relative rate of growth comparison between the mediums, as reported.

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FIGURES & TABLES

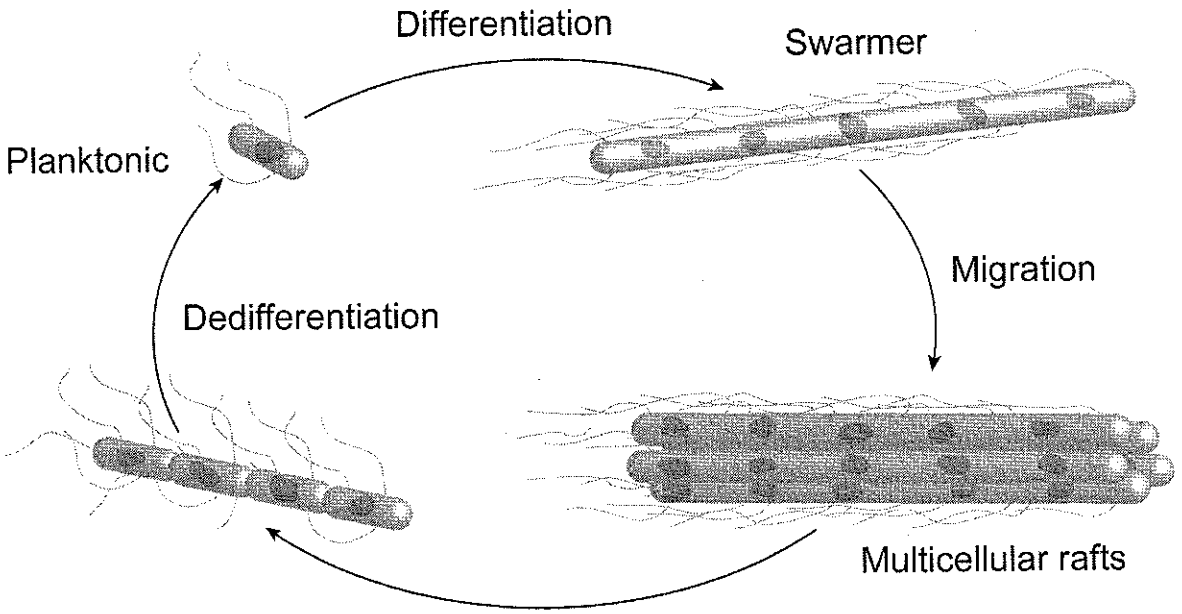

Figure 1: The swarming cycle by which cells acquire the swarming phenotype and subsequently dedifferentiate into normal cells.

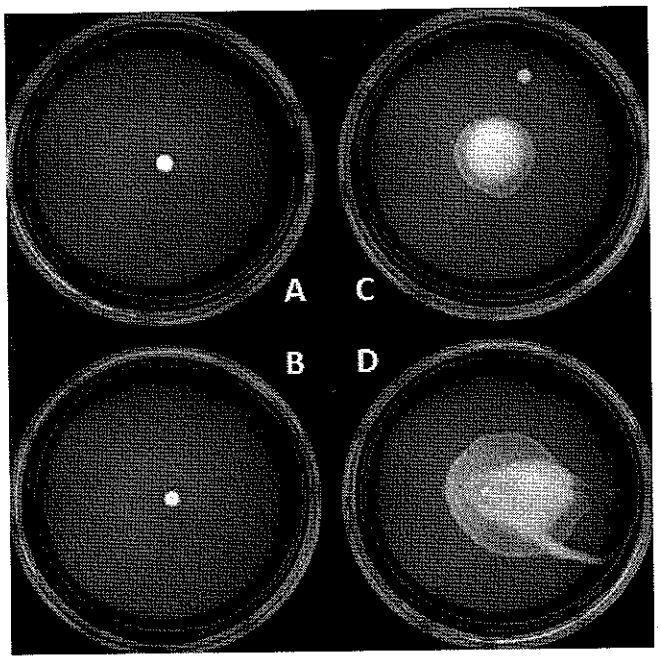

Figure 2: RP437 inoculated MMM plates (A and B) and NB plates (C and D) 18 hours post-inoculation. The swarm rate is significantly slower on MMM plates as compared to NB plates. These results are representative of five experiments.

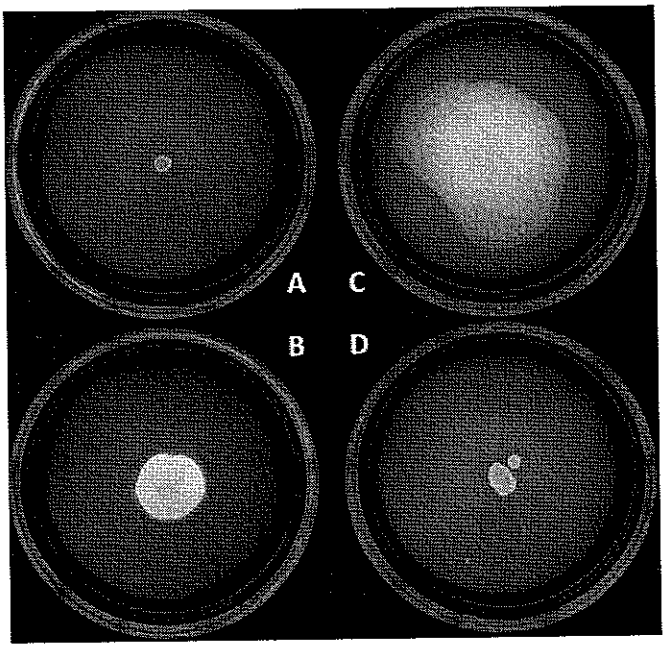

Figure 3: *MMM swarm plates 18 hours post-inoculation. (A)* Escherichia coli *(RP437), (B)* Salmonella enterica typhimurium, *(C)* Proteus mirabilis *(ATCC 7002), and (D)* Serratia marcescens. *These results are representative of two experiments.*

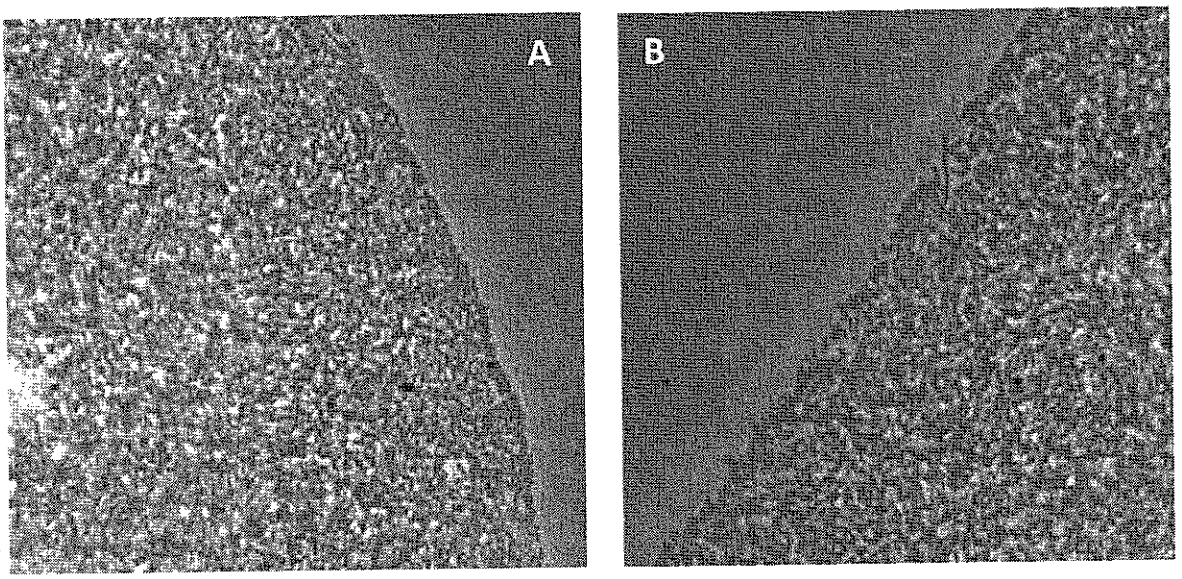

Figure 4: *Swarm edge of RP437 inoculated MMM (A) and NB (B) swarm plates 18 hours post-inoculation. Individual cell phenotype appears to be comparable at swarm edge between the two conditions. These results are representative of five experiments.*

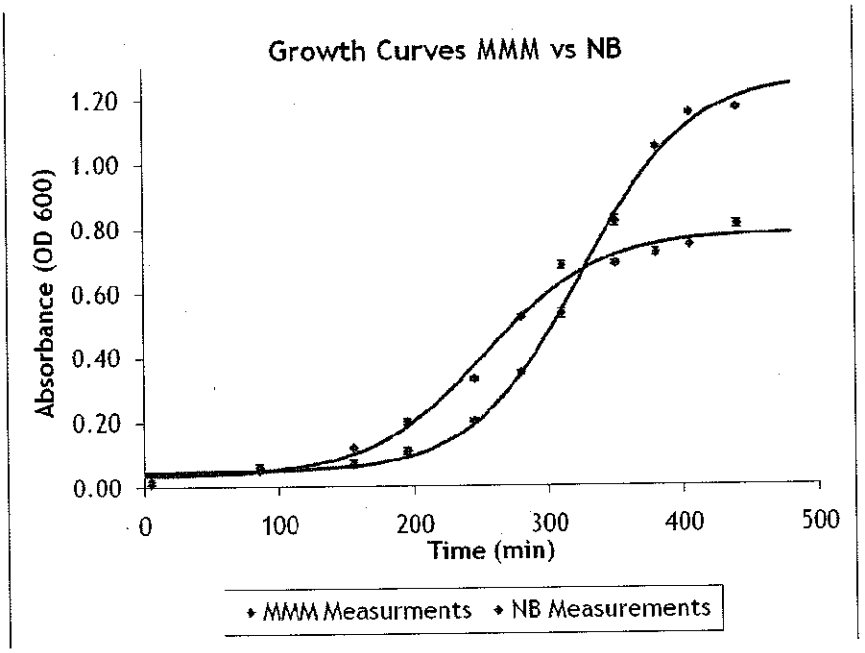

Figure 5: Growth curves comparing rate of growth of RP437 in MMM and NB. Maximum culture density appears to be significantly different when comparing the two mediums. These results are representative of two experiments.

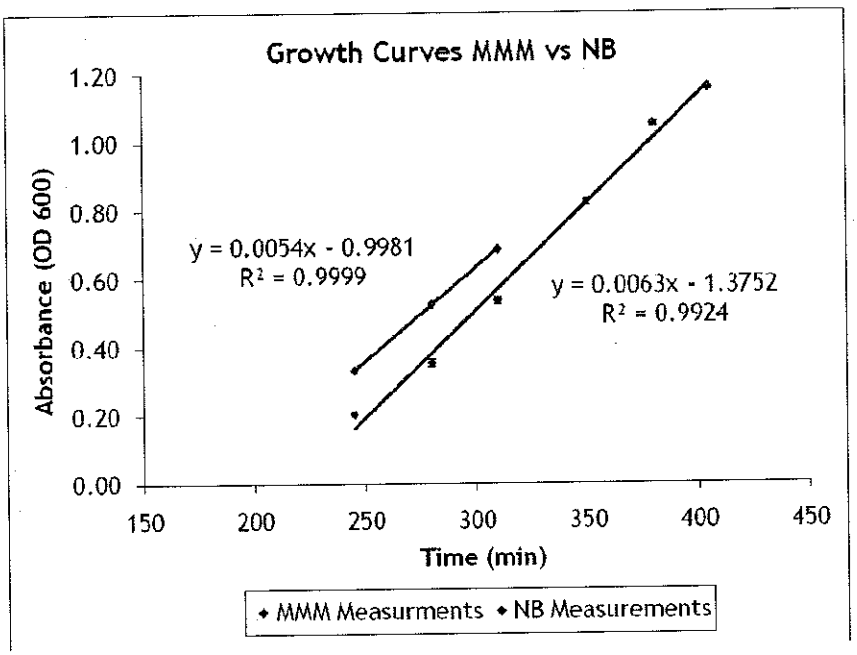

Figure 6: Extrapolated rate of RP437 growth in MMM or NB. Rate of growth does not appear to be significantly different between the two mediums. These results are representative of two experiments.

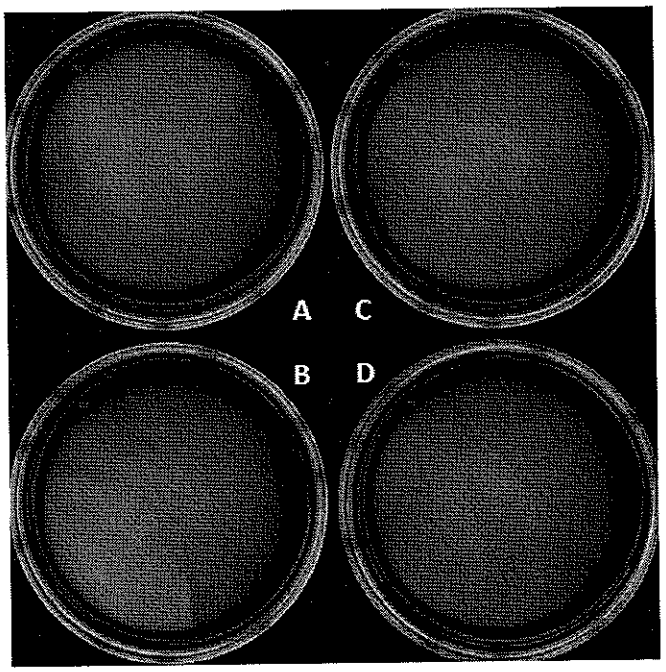

Figure 7: RP437 inoculated NB (A and B) and MMM (C and D) plates with 0.01% v/v Tween 80 added as a wetting agent. After ten hours, plates are confluent. These results are representative of two experiments.

Chemical	[Final]
Eiken Agar	0.45% (w/v)
Glucose	0.5% (w/v)

Amino Acid	[Final] (mM)
Alanine	15.98
Arginine	5.36
Aspartic Acid	5.99
Cysteine	9.69
Glutamic Acid	10.82
Glycine	35.87
Histidine	0.97
Isoleucine	1.67
Leucine	3.62
Lysine	3.43
Methionine	1.15
Phenylalanine	1.64
Proline	12.02
Serine	4.02
Threonine	2.21
Tryptophan	0.54
Tyrosine	0.58
Valine	3.16

Salts	[Final]
Dipotassium Hydrogen Phospahte	1.32 mM
MOPS	40 mM
Tricine	4 mM
Ferrous Sulfate	10 μM
Ammonium Chloride	9.5 mM
Potassium Sulfate	0.276 mM
Calcium Chloride	0.5 μM
Magnesium Chloride	0.525 mM
Sodium Chloride	50 mM
Ammonium Molybdate	2.91 nM
Boric Acid	0.40 μM
Cobalt Chloride	0.30 μM
Cupric Sulfate	96.1 nM
Manganese Chloride	0.81 μM
Zinc Sulfate	97.4 nM

Vitamins	[Final]
Pyridoxine	236 nM
Calcium Pantothenate	210 nM
Thymidine	20.6 μM
Riboflavin	133 nM
PABA	1.09 μM
Biotin	16.4 nM
Thiamine	296 μM
Choline Chloride	179 μM
Folic acid	11.3 nM
Inositol	172 μM
Nicotinic Acid	5.28 μM

Table 1: *Modified MOPS Media (MMM) chemical composition for use in* E. coli *RP437. The only modification for use with other bacteria species is the agar type and concentration, as outlined in the Methods and Materials section.*