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PULSE FIELD GEL ELECTROPHORESIS FOR COMPARATIVE STUDIES OF BACTERIA DIVERSITY

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The technique of pulse-field gel electrophoresis (PFGE) makes use of restriction enzymes that cleave DNA at specific restriction sites, generating DNA fragments of different sizes. These fragments can be separated according to size by agarose-gel electrophoresis and visualized using DNA stains (e.g. ethidium bromide), giving a characteristic banding pattern. Given the high specificity of restriction enzymes, the banding pattern generated is specific to each DNA sequence. This technique is, therefore, widely used by microbial epidemiologists to differentiate between bacterial strains. In this study, an effective assay for the PFGE method was established for the potential analysis of the diversity within environmental nitrifying bacterial cultures.. To this end the *E. coli* strain MG1655 was used to test different parameters including: grade of agarose, cellular concentration, enzyme concentration, and wash volumes. The importance of each parameter is noted in this report. Restriction analysis computer software tools were used to predict banding patterns for the restriction digests. The banding patterns predicted *in silico* strongly correlate to those obtained empirically in this study. Moreover the banding pattern in our experiment was also remarkably similar to that one reported by Tenover *et al* for an identical *E. coli* strain (2). Although an effective assay was established in the last experiment of this study, reproducibility and importance of these parameters and additional, potential relevant parameters are yet to be determined.

Microbial species play an important role in maintaining the Earth's chemical balance and biogeochemical processes. One such family of microorganisms is the *Nitrobacteraceae*, more commonly known as nitrifying bacteria. Collectively, these chemoautotrophs cycle nitrogen by sequentially oxidizing ammonia to nitrite and nitrite to nitrate (4). Some known genera of ammonia oxidizers include: *Nitrosomonas, Nitrosocystis, Nitrosogloea, Nitrosospira*, and *Nitrosococcus*. Oxidation of nitrite to nitrate is carried out by nitrite oxidizing bacteria from the *Nitrobacter* and *Nitrocystis* genera (4). Although the metabolism of this group of bacteria is largely important, with many implications in both natural environments and wastewater treatment, the characteristics of these species have not been well delineated. The difficulty arises in part from the inability of cultivating such bacterial samples in a laboratorial environment. Long generation times and small cell size are limiting factors in the culture and analysis of these bacteria.

A tool commonly used by molecular epidemiologist to differentiate between strains of pathogenic and non-pathogenic bacteria is pulse-field gel electrophoresis (PFGE). In this type of bacterial analysis, unique restriction banding patterns are used to identify strains via PFGE (2). PFGE operates under the same principles as standard gel electrophoresis, where nucleic acid fragments are separated based on size in an agarose or polyacrylamide matrix by applying an electrical current. The advantage of PFGE is the ability to separate larger-sized fragments, beyond the 50 kb limit of unidirectional electrophoresis (3). The method of PFGE involves switching the direction of the electrical current in a set pattern (2), allowing for resolution of fragments up to 10 Mb (3). In this method, lysis and restriction is performed *in situ* by embedding cells into agarose plugs. This way, the integrity of bacterial genomes is preserved.

In order to apply PFGE to the analysis of *Nitrobacteraceae* diversity within samples, the system must be optimized. In this study, the *E. coli* strain MG1655 is the model species used for PFGE optimization.

MATERIALS AND METHODS

Media, Chemicals, and Solutions. Luria-Bertani (LB) broth was prepared by dissolving 5 g of Bacto-tryptone, 2.5 g of yeast extract, and 5 g of sodium chloride (NaCl) into 500 mL (total solution volume) of distilled water (dH_2O); the media was then autoclaved and stored at 4°C. A cell wash solution was prepared consisting of 75 mM NaCl, and 25 mM ethylenediaminetetraacetic acid (EDTA) in a total volume of 100 mL dH_2O; the pH of the solution was adjusted to 8.0 and it was stored at room temperature (RT). A solution of lysis buffer was prepared by dissolving 18.612 g of EDTA and 1 % w/v (1 g) N-lauroylsarcosine into 100 mL of dH_2O, and stored at RT; 1 mg/ml of Proteinase K (New England Biolabs, cat. #P8102S) was added to the lysis buffer immediately prior to use. A 100 mL solution of 10X Tris-EDTA (TE) buffer was prepared consisting of 100 mM Tris-base and 10 mM EDTA, pH was adjusted to 8.0. A solution of 10X Tris-boric acid-EDTA (TBE)

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buffer was prepared consisting 108 g of Tris-base, 55.0 g of boric acid, and 2 mM EDTA in 1 L of dH_2O. Solutions of TE and TBE were stored at RT. A 0.015 M solution of phenylmethyl sulfonyl fluoride (PMSF) was prepared by dissolving 0.02613 g in 10 mL of isopropanol, and stored at -20°C. Sterile dH_2O was prepared by autoclaving 100 mL of dH_2O.

Culture of Bacteria. Liquid cultures of *Nitrosomonas* and plates of *E. coli* strain MG1655 were supplied (Department of Microbiology and Immunology teaching laboratory, the University of British Columbia). A loop of *E. coli* was inoculated into 10 mL of LB broth in a sterile 18 mm x 150 mm test tube and grown at 37°C under agitation in a water bath overnight (O/N). 2 mL of the O/N *E coli* culture was diluted by a factor of 1/5 via the addition of 8 mL of fresh LB broth in a sterile 18 mm x 150 mm test tube and grown to a measured OD_{610} of 1.2, at which time 1 mL samples were collected from the growing culture and placed in microfuge tubes. For gel #2, a loop of *E. coli* was inoculated into 10 mL of LB broth in 2 sterile 18 mm x 150 mm test tubes and grown at 37°C under agitation in a water bath overnight (O/N). 2 mL of each O/N *E coli* culture was diluted by a factor of 1/5 via the addition of 8 mL of fresh LB broth in 2 sterile 18 mm x 150 mm test tubes. The OD_{610} of both O/N and freshly inoculated samples was monitored over six hours, and 1 mL samples were removed at half-hour intervals.

Concentration of *Nitrosomonas* Cultures. A 50X cellular concentration was performed by filtering 50 mL of provided *Nitrosomonas* liquid culture with a 2 µm filter. Filtered cells were resuspended (with filter) in 1 mL of filtrate. A 250X concentration was also performed by filtering 250 mL of liquid culture and resuspending cells in 1 mL of filtrate.

Preparation of Agarose Plugs – Gel #1. *E. coli* samples collected from the growing culture were microcentrifuged at 16,100 x g for 10 min at RT. Upon removal of supernatant, pelleted cells were washed in 1 mL of the cell wash solution consisting of 75 mM NaCl and 25 mM EDTA. Cells were microcentrifuged at 16,100 x g for 10 min at RT and the wash solution was discarded. Washed pellets were then resuspended in an appropriate volume of cell wash solution to obtain twice the desired OD_{610} value of 1.2. This wash step was omitted for *Nitrosomonas* cultures. A 1% w/v solution of biotechnology grade agarose was melted in sterile dH_2O, this is incubated at 50°C prior to use. Cell resuspensions of both *E. coli* and *Nitrosomonas* were subsequently diluted by a factor of 1/2 by mixing equal volumes with liquid agarose. The agarose-bacteria mixtures were then dispensed into 1.5mm thick molds (Bio-Rad, cat. #170-3622XTU). The plugs were refrigerated at 4°C and allowed to solidify for 15 min, after which time, they were incubated in lysis buffer (0.5M EDTA, 1% N-laurylsarcosine, 1mg/ml ProteinaseK) overnight at 50°C.

Preparation of Plugs – Gel #2. Due to limited Nitrosomonas samples, experimentation was focused solely on *E. coli* MG1655 after the first gel run. In gel #2, the stage of growth and its relevance to *E. coli* PFGE was tested. The preparation of plugs follows that of gel #1 except that 0.5% chromosomal grade agarose (Bio-Rad, cat. #162-0135) was used in plug preparation and the cell culture parameters were changed as indicated above.

Preparation of Plugs – Gel #3. The preparation of plugs follows that of gel #2 except that the centrifugation time was reduced to 2 min during the cell washes. Cells were also resuspended in 1 mL of cell wash solution following washing, and instead an appropriate amount of agarose was calculated to achieve the desired OD_{610} values of: 0.6, 1.0, 1.25, and 1.6. Also, prior to mixing bacterial suspensions are incubated at 50°C in order to equilibrate temperatures.

Restriction of Plugs with XbaI. *E. coli* plugs in gel #1 and gel #2 were digested with an old batch of XbaI restriction enzyme (Invitrogen, cat. #15226-012) of unknown quality and age. The final PFGE (gel #3) was performed with a fresh batch of enzyme (Invitrogen, cat. #15226-012), which was purchased new. To prepare plugs for restriction enzyme digestion, lysis solution was aspirated and the plugs were rinsed with gentle agitation in TE (10mM Tris, 1mM EDTA (pH 8.0) containing 1.5 mM PMSF twice for 15 min each time at room temperature. The plugs were then washed with TE alone four times for 15 min each time at room temperature. There was approximately 250 µL of wash for each 80 µL plug (four plugs were placed in a weigh boat with 1 mL of solution) prepared for gel #1 and gel #2. In gel #3, this ratio was increased to approximately 1 mL of wash solution per plug. Washed plugs were incubated in 1X REAct buffer (Invitrogen, cat. #15226-012) for 30 min at RT under gentle agitation. The buffer was then replaced with fresh buffer containing either 50 units of XbaI restriction enzyme, as indicated. Plugs were incubated in the restriction enzyme solution for 4 h at 37°C. After restriction digestion, plugs were washed in 0.5X TBE (45mM Tris-boric acid, 1 mM EDTA) solution for 30 min and then resolved by PFGE.

Pulse-field Gel Electrophoresis of Plugs. Both undigested and digested samples were resolved in 1% Seakem Gold agarose (Fisher Scientific, cat. #BMA 50152) in gel #1 and gel #2. The restriction enzyme step was simply omitted for undigested samples. All plugs were digested for gel #3. Plugs were melted at 80°C and 25 µL of each plug was loaded into gel #1 and gel #2. In gel #3 solid plugs were loaded into 1% pulsed field agarose (Bio-Rad, cat. # 62-0137XTU) and were sealed with 0.5% chromosomal grade agarose (Bio-Rad, cat. #162-0135) melted at 50°C. The gels were run in 0.5X TBE buffer at a voltage 6 V/cm for 22 hours at 14°C using a CHEF DR-II electrophoresis apparatus (Bio-Rad, cat. #70-3615XTU) with an initial switch time of 5 sec and final switch time of 50 sec. Yeast chromosomal (New England BioLabs, cat. #N0345S) lambda concatemers (New England BioLabs, cat. #N0340S) molecular weight markers were used as standards. Following electrophoresis, the gels were stained in a 0.2 µg/ml solution of ethidium bromide for 30 min and subsequently destained in distilled water overnight. Visualization of gels was performed with Alphaimager software (Alpha Innotech).

Genomic Analysis of Restriction Enzyme Fragments. Genomic sequences were obtained from NCBI's GenBank database (Accession Version Numbers: *E. coli* MG1655: U00096.2; *N. europaea*: AL954747.1; *N. eutropha* C91: CP000450.1). Fully sequenced genomes were entered into pDRAW 32 DNA analysis software (AcaClone software) and analyzed for XbaI restriction sites.

RESULTS

Optimization of PFGE assay for DNA restriction analysis of *E. coli* strain MG1655. Gel #1 and gel #2 produced DNA smears or banding patterns with poor resolution (FIG. 1 and FIG. 2). Due to the limited supply and slow generation time of *Nitrosomonas* cultures, experimentation after gel #1 was focused only on *E. coli* until a reproducible assay could be established. In gel #1, only the molecular weight standards were resolved (FIG. 1). After analysis of this gel, the problem was thought to be due to the quality of the agarose used for plug preparation. To address this issue, chromosomal grade agarose grade was used in gel #2.

No useful information about the varying growth stages of *E. coli* could be inferred from gel #2 due to poor resolution (FIG. 2). Although better resolution of melted plugs compared to solid plugs was observed by Volar (3), a problem in this study was inferred to be potential shearing of genomic DNA due to high temperatures (80°C used to liquefy plugs). The agarose matrix could be another variable restricting resolution. Additionally, the enzyme and digestion of plugs could be ineffective; this was inferred from the large

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molecular weight band approximately 680 kb in size that is present in all lanes, which is likely undigested DNA (FIG. 2).

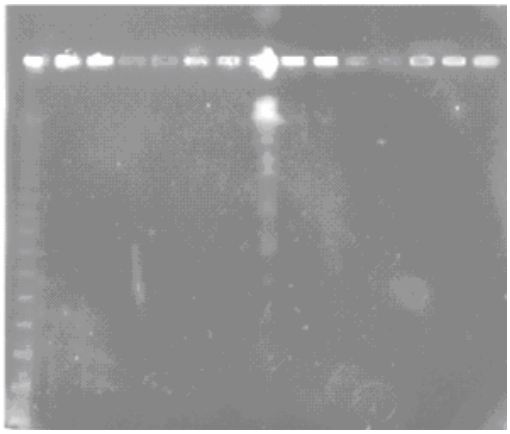

FIG. 1 PFGE of plugs prepared using 1% biotechnology grade agarose and run on a 1% (w/v) SeaKem Gold agarose gel. Plugs were digested with an old batch of XbaI. All plugs were melted at 80ºC in a heating block. Undigested samples are loaded in lanes 2-7, digested samples in lanes 9-14. Molecular weight markers are observed in lanes 1 (concatamers) and 8 (yeast standard).

It is possible that the volume of wash solutions could be insufficient if the plug volume acts to dilute solutions. To resolve these issues, a new batch of XbaI (Invitrogen, cat. #15226-012) was purchased, the ratio of solution to plug volume was increased, solid plugs were loaded instead of melted plugs, and the type of agarose used in the PFG matrix was changed. To test if enzyme and/or cell concentrations are also relevant variables, OD_{610} values were varied and restriction enzyme reactions with 10 units or 50 units were performed and compared.

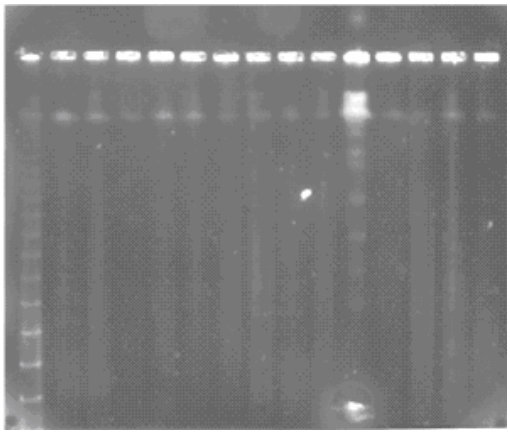

FIG. 2. PFGE of plugs prepared using 0.5% (w/v) chromosomal grade agarose and run on a 1% (w/v) SeaKem Gold agarose gel. Plugs were digested with an old batch of XbaI. All plugs were melted at 80ºC. In lanes 2-4 O/N samples were loaded. In lanes 5-10, and 12-15, freshly inoculated plug samples were loaded. All plugs were digested with the exception of lane 15. Molecular weight markers are observed in lanes 1 (concatamers) and 11 (yeast standard).

Only in gel #3, the last PFGE experiment, was a distinguishable banding pattern observed (FIG. 3). In the samples where only 10 units of enzyme were used (lanes 4, 7, 11, 14, and 15) a large band about 680 kb in size and a fainter 1100 kb band appear on the gel. The desired banding pattern is mainly observed in samples where 50 units of XbaI were used (lanes 3, 5, 9, and 10). Anomalies in this trend are seen however, in lanes 2, 6, and 12, where 50 units of enzyme still produced no banding or faint banding.

Restriction Enzyme Banding Pattern Analysis. The final cell concentrations (calculated OD_{610} values), in agarose plugs were also varied. A banding pattern was observed in lanes of OD_{610} values of 0.6, 1.0, and 1.25. The optimal (strongest) observed pattern being at an OD_{610} of 1.0 in the agarose plugs. There was either no banding or very weak banding at highest concentration of cells with an OD_{610} of 1.6 (lanes 12, 13, 14, and 15). The banding patterned obtained (FIG. 3) visibly correlates with the *E. coli* MG1655 XbaI restriction enzyme digest observed by Tenover *et al.* (FIG. 4).

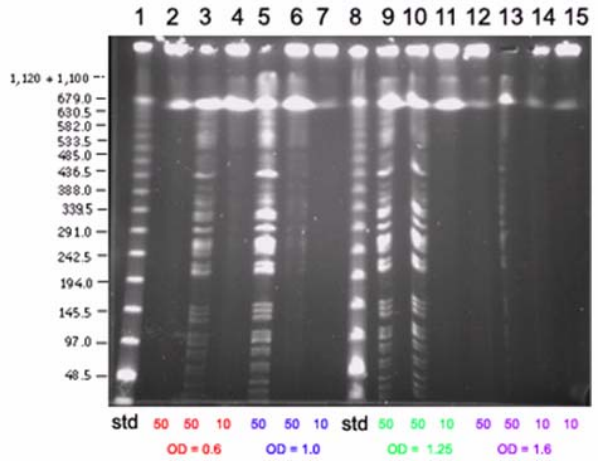

FIG. 3. PFGE of plugs prepared using 0.5% chromosomal grade agarose and run on a 1% BioRad PFGE agarose gel. Plugs were digested with a new batch of XbaI at a concentration of 50 units or 10 units. Varied cell concentrations are represented in the gel based on initial OD_{610} readings and diluted with 0.5% chromosomal grade agarose to the final calculated OD_{610} values (0.6, 1.0, 1.25 and 1.6). shown in the figure above. Molecular weight markers are observed in lanes 1 (concatamers) and 8 (concatamers).

In addition to literature, the expected number and size of fragments was obtained via genomic analysis of sequenced strains from NCBI's Genbank database. Using pDraw32 DNA analysis software, it can be seen that there are 39 predicted XbaI restriction sites, which would produce 38 nucleic acid fragments in the circular chromosome of *E. coli* MG1655 For the two

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Nitrosomonas sequences, *Nitrosomonas europaea* and *Nitrosomonas eutropha* C91, 26 and 91 XbaI restriction sites are expected for each species respectively (Table 1).

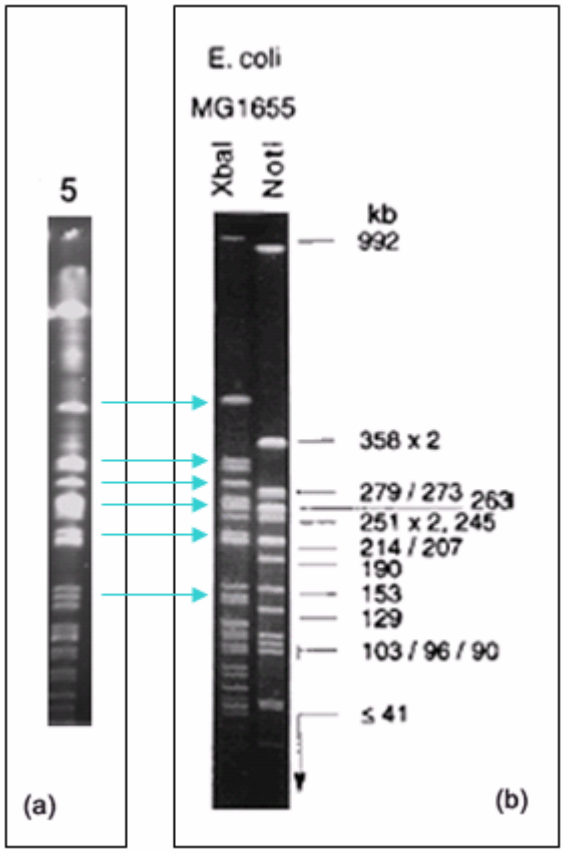

FIG. 4. Comparison of (a) restriction enzyme banding pattern from PFGE of *E. coli* MG1655 (lane 5 of gel above) with that of (b) a similar digest in previously published work (adapted from FIG. 3 of Tenover *et al.*, 1995 (2)).

Table 1. Number of Xba1 restriction enzyme sites determined for sequenced bacterial species.

Species	Accession Version Number	Size of genome (kb)	Number of XbaI restriction sites
E. coli MG1655	U00096.2	4639.675	39
N. europaea	AL954747.1	2852.034	26
N. eutropha C91	CP000450.1	2561.057	90

The predicted sizes of each restriction enzyme fragment were calculated from differences in the specified XbaI cleavage sites indicated in Table 1. Predicted restriction enzyme fragment sizes range from 2kb to 440kb. Fragments smaller than 40kb were not considered as they are too small to be retained on the gel .The estimated resolution between two bands of similar size is approximately 2kb. Taking the above limitations into consideration, a total of 24 distinct bands were expected (Table 2).

Table 2. Predicted XbaI Restriction Enzyme Fragment Sizes calculated from pDraw analysis.

Restriction Fragments smaller than 40 kb were not retained in the gel.

Fragments sized within 2 kb of each other resolved together as one band on the gel as indicated by arrows.

Fragment Size (kb)			Fragment Size (kb)	
2.07			94.06	
6.06			100.78	
12.22			**105.75**	
12.52			**105.76**	←
14.08			**106.07**	
21.78			117.29	
26.50			149.21	
28.54			155.30	
28.99			197.15	
33.54			206.62	
39.70			214.86	←
41.40			224.94	
48.42			248.42	←
48.82			254.64	
57.69			262.13	
61.00			269.55	
66.88			323.97	
72.56			335.01	
79.55			440.06	

DISCUSSION

In the first PFGE experiment, the type of agarose used in plug preparation was deemed unsuitable (1%(w/v) biotechnology grade was used instead of 0.5%(w/v) chromosomal grade). No bands were visualized on the gel besides that of the molecular weight standards. It is inferred that the grade of agarose used in plugs may potentially restrict large DNA fragments from moving out of wells. Also, incomplete lysis of cells and/or incomplete restriction enzyme digestion may also restrict DNA resolution. Chromosomal grade agarose potentially allows improved diffusion of solutions for both *in situ* lysis and restriction. It is possible that chromosomal grade agarose facilitates the movement of large molecular weight fragments out of the wells, or is otherwise implicated in the diffusion of solutions for proper lysis and/or restriction prior to PFGE.

The second PFGE experiment used chromosomal grade agarose in plug preparation at a concentration of 0.5%(w/v) rather than 1%(w/v). In this gel, DNA is seen as smears and indistinguishable banding patterns. There is a larger band approximately 680kb in size that is consistent across all lanes. This band is hypothesized

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to be the undigested bacterial chromosome. Since *E. coli* MG1655 has a genome length of about 4600kb, the circular chromosome likely exists in the form of super-coiled DNA, which would effectively migrate at a faster rate than a linear uncoiled DNA size equivalent. It can be inferred from this result that incomplete digestion of plugs could be the cause of the problem. It is also possible that DNA shearing occurred during the handling of plugs (physical damage or heating to melt plugs at high temperatures 80^{o}C).

The last PFGE experiment produced a visible banding pattern, distinct to *E. coli* MG1655, and comparable to banding observed by Tenover *et al.* (2). In this gel, a fresh batch of newly purchased XbaI was used for the restriction enzyme digestion. This is important if the incomplete restriction of genomic DNA did result in poor band resolution, and if the enzyme was in fact ineffective. Another variable that was changed was the volume in which plugs were incubated in during wash and lysis steps. In gel #1 and gel #2, the plug to solution ratio was approximately 1:3. In this case, the water present in the agarose plug volumes may have acted to effectively dilute solutions. This may have contributed to the reduced effectiveness of lysis and/or inactivation of proteinase K with PMSF during the washes. As a serine protease, the presence of proteinase K in subsequent reactions could have potentially reduced the effectiveness of XbaI restriction enzyme activity. In the last gel, solution volumes were at least twice the total volume of plugs in incubation to prevent this. Also, the brand of pulse-field gel (PFG) agarose used in the matrix was changed from Seakem Gold to Biorad. The significance of PFG matrix grade does not seem to be as significant, as molecular weight standards were resolved in either case.

Contradictory results were obtained for resolution of melted vs. solid plugs from those observed by Volar (3). In our study, plugs were melted at extreme temperatures of 80^{o}C, which were inferred to be detrimental to chromosomal integrity, and produced smearing on the gel. It is not specified what temperature plugs were melted at prior to loading in Volar's study (3), which could explain this point of differentiation. A lower temperature may in fact result in better resolution of DNA compared to solid plugs. It is still yet to be proven whether or not melted plugs do in fact result in superior PFGE resolution, or the contrary, DNA smearing in gels.

In the interest of time, multiple parameters were modified in our last study which were thought to contribute to the optimization of the assay. It is therefore difficult to assign the improvement of the assay to a single factor. It is also possible that all parameters modified are important for optimal assay results. A direct comparison of digests generated using fresh or old XbaI restriction enzymes was not run; it is therefore unclear whether the replacement of the enzyme contributed to the improvement of the assay. In future experiments it would be important to test viability of restriction enzymes before proceeding with the digestion of the chromosomal DNA. Agarose and solution volumes are other factors to consider, particularly the grade and percent agarose used to make the plugs, which seems essential to the movement of material out of wells.

Chromosomal integrity is conserved via embedding cells in the agarose matrix. Lysis of cells prior to this essential step could potentially lead to genomic DNA degradation and poor PFGE resolution. In gel #3 cell resuspensions were equilibrated to 50^{o}C prior to agarose addition, also the centrifugation time was reduced to 2 min, which was sufficient to pellet cells. These two precautions may have decreased premature lysis of cells and may have potentially improved resolution as observed in gel #3.

Although the concentration of cells used in plug preparation is important, there seems to be a range of values between 0.6 and 1.2 OD_{610} that are expected to produce successful gels. In the PFGE of *E. coli* MG1655, a final cell concentration correlating to the calculated OD_{610} of 1.0 seems to be optimal. Higher cell concentrations beyond OD_{610} values of 1.2 did not produce visible banding. A weak band was observed in lane 13, which shows that some band resolution is possible at the higher cell concentration. This indicates the reproducibility of such results may be questionable. Although an overall trend in cell concentration is observed with strong banding for OD_{610} values of 1.0-1.2, it is still yet to be proven that this is an essential component to PFGE that can be reproduced. Discrepancies in individual cellular kinetics (cellular growth stage) not represented simply by optical density values should be investigated. The poor results of highly concentrated plugs are likely due to the inability of complete cell lysis. This arises from potentially limited cell-solution contact *in situ*.

Two concentrations of XbaI were investigated in the last PFGE experiment. From the results it is observed that 50 units of enzyme are required for complete restriction and proper banding resolution. An enzyme concentration of 10 units is potentially not sufficient because the reaction must take place within an agarose matrix, which is more restrictive of diffusion than in the standard case of liquid restriction digests. Some samples digested with 50 units still failed to produce a strong banding pattern as expected. These anomalous results could indicate that an even higher enzyme concentration is required. It was also observed that results were hard to reproduce, even from the established parameters of Volar (3), thus all samples should be performed in multiples as the reproducibility of this technique may be problematic.

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Based on the pDraw analysis of *E. coli* MG1655 sequence data and calculated XbaI fragment sizes, there should be 24 distinct DNA fragments on a gel ranging in size from 40-440kb. The banding pattern obtained from PFGE of *E coli* MG1655 is mostly consistent with this result as there are 24 bands visible in this range. Results differ from theoretical expectations in the fact that there are poorly resolved bands in the gel which are larger than 440kb. In addition to these larger bands, there is a strong band at 680kb, and a less intense band at 1100kb, which appears consistently across lanes. These bands, at 680kb and 1100kb are hypothesized to be undigested super-coiled and semi-super-coiled forms of the bacterial chromosome. Slight discrepancies of banding within the 40-440kb range can be attributed to differences in sequence information, limited gel resolution and subjective interpretation, or the possibility of horizontal gene transfer and genetic drift within strains of *E. coli* MG1655. Also incomplete digestion and band smearing are addition sources of discrepancies from published banding of *E. coli* MG1655 (2).

FUTURE EXPERIMENTS

In this study, many parameters of PFGE were explored to produce an effective procedure for *E. coli* MG1655. Further optimization of the PFGE can be determined by varying incubation times of lysis and restriction enzyme digests. Another investigation could delve into the importance of initial cell concentration (stage of cellular growth – lag, log, stationary, or death phases) prior to mixing with plug agarose. This was attempted in gel #2 of this study, but results were indeterminable due to poor resolution and lack of distinguishable banding. However, the reproducibility of the assay is still yet to be firmly established, based on the parameters discussed in this study. Time constraints did not allow for the repetition of parameters established in the last effective experiment (gel #3). This experiment should be repeated in order to confirm its reproducibility.

Once reproducibility is firmly established, this system can be used as an initial point for experimental specifications, and PFGE can be eventually applied to the analysis of *Nitrobacteracea,* as was the original intent of this research. In terms of restriction enzyme choice, XbaI is a good candidate to use in the analysis of samples containing *Nitrosomonas europaea* as it only restricts this genome 26 times based on pDraw analysis. XbaI is a less ideal to use for *Nitrosomonas eutropha* C91, since cleavage of this species will result in up to 90 fragments, which are likely too numerous to be resolved in a gel.

Although the principle of PFGE genomic analysis will be directly applicable to these bacterial species, there are many practicalities that must be considered when working with Nitrifying bacteria. One such consideration is the extremely small cell size of these bacteria. Since plug cell concentration is a factor in the success of gels, a method of quantifying *Nitrobacteracea* must be also be established. *Nitrosomonas* species are not effectively centrifuged out of solution. Filtration is an alternative method of separating cells from media that can be explored. The initial filtration performed in this study produced very low yields (data not shown). Thus, various methods of filtration may be investigated in order to maximize cellular yields.

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