Development of improved anaerobic growth of *Bacillus mojavensis* strain JF-2 for the purpose of improved anaerobic biosurfactant production for enhanced oil recovery

Topical Report

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M. J. McInerney, M. Folmsbee, and D. Nagle

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Department of Botany and Microbiology
University of Oklahoma
770 Van Vleet Oval
Norman, OK 73019-0245

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Executive Summary

Our work focuses on the use of microorganisms to recover petroleum hydrocarbons that remain entrapped after current recovery technologies reach their economic limit. Capillary forces between the hydrocarbon and aqueous phases are largely responsible for trapping the hydrocarbons in the pores of the rock and large reductions in the interfacial tension between the hydrocarbon and aqueous phases are needed for hydrocarbon mobilization (1-3, 10, 11). Microorganisms produce a variety of biosurfactants (4), several of which generate the ultra low interfacial tensions needed for hydrocarbon mobilization (4, 5, 8). In particular, the lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the interfacial tension between hydrocarbon and aqueous phases to very low levels (<0.016 mN/m) (8) (9). *B. mojavensis* JF-2 grows under the environmental conditions found in many oil reservoirs, i. e., anaerobic, NaCl concentrations up to 80 g l^{-1}, and temperatures up to 45^{o}C (6, 7), making it ideally suited for in situ applications. However, anaerobic growth of *B. mojavensis* JF-2 was inconsistent and difficult to replicate, which limited its use for in situ applications.

Our initial studies revealed that enzymatic digests, such as Proteose Peptone, were required for anaerobic growth of *Bacillus mojavensis* JF-2. Subsequent purification of the growth-enhancing factor in Proteose Peptone resulted in the identification of the growth-enhancing factor as DNA or deoxyribonucleosides. The addition of salmon sperm DNA, herring sperm DNA, *E. coli* DNA or synthetic DNA (single or double stranded) to Medium E all supported anaerobic growth of JF-2. Further, we found that JF-2 required all four deoxyribonucleosides (deoxyadeonosine, deoxyguanosine, deoxycytidine and thymidine) for growth under strict anaerobic conditions. The requirement for the deoxyribonucleosides did not occur under aerobic growth conditions. DNA was not used as a sole energy source; sucrose was required for anaerobic growth and biosurfactant production in DNA-supplemented Medium E. In addition to DNA or deoxyribonucleosides, nitrate, amino acids and vitamins were all required for anaerobic growth of JF-2. *Bacillus mojavensis*^{T} (ABO21191), *Bacillus mojavensis*, strain ROB2 also required DNA or deoxyribonucleosides for anaerobic growth. The improved anaerobic growth of *Bacillus mojavensis* JF-2 was a prerequisite for studies that will lead to improved anaerobic biosurfactant production.

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Chapter 1. The anaerobic growth of *Bacillus mojavensis* requires DNA

Abstract

The goal of the following work was to improve the anaerobic growth of *Bacillus mojavensis* strain JF-2. JF-2 grows to an A_{600} of 0.8 to 1.0 in aerobic medium E but growth in this medium under strict anaerobic conditions was difficult to establish and replicate. Anaerobic growth of JF-2 was not improved by the addition of vitamins, amino acids, ribonucleic acids, polyglutamate, polyglutamine, polytryptophan, rumen fluid, fatty acids or Tween 80 to medium E. The addition of Tryptone, Proteose Peptone, Neopeptone, Tryptose or Casitone to medium E improved anaerobic growth of JF-2. Proteose Peptone was the most effective enzymatic digest at enhancing anaerobic growth. The growth-enhancing factor present in Proteose Peptone had an approximate molecular weight of 3900 g/mol. It was methanol insoluble and was retained by an anion exchange column. The growth-enhancing factor was acid and base stable, had a protein content of less than 4%, and exhibited a maximum UV absorbance of 260 nm. Salmon sperm DNA replaced the requirement for Proteose Peptone for anaerobic growth of *Bacillus mojavensis* JF-2. The type strain *Bacillus mojavensis*^{T} (ABO21191) and *Bacillus mojavensis* strain ROB2 also required DNA for anaerobic growth.

Introduction

Bacterial growth requires the application of appropriate environmental and nutritional conditions (46). Environmental conditions consist of temperature, pH, osmolarity, and redox potential. The effect of temperature on bacterial growth is due to the sensitivity of proteins to temperature. Most enzymatic reactions have a temperature optimum (for example, 37^{o}C for many biochemical reactions occurring in bacteria that grow in association with warm blooded animals). However, many proteins denature at temperatures 5^{o}C or more above the optimum (51). Therefore, the growth temperature must be high enough for bacterial biochemistry to proceed, but low enough to avoid denaturing the proteins involved. Bacteria and Archaea can grow at a diverse range of temperatures. For example, some bacilli, such as *Bacillus stearothermophilus* and *Bacillus palidus* grow at temperatures above 55^{o}C, whereas other bacilli such as *Bacillus subtilis* prefer to grow at temperatures ranging from 25-40^{o}C (42).

The effect of pH on bacterial growth is similar to that of temperature due to the effect that pH has on protein structure and activity (51). In general, microorganisms exhibit a broad pH optimum and the rate of growth declines gradually at pH's above or below the optimum. The pH of a bacterial growth medium is controlled with the addition of a buffer such as potassium phosphate or calcium carbonate. Many bacteria grow optimally at a pH close to neutrality but others, such as, Alicyclobacillus acidocaldarius,

grow in hot acid springs with a pH varying from 2 to 6 and at temperatures up to 70 oC (42).

The control of ionic strength is important to ensure that the target bacteria can maintain their internal osmotic pressure. If a bacterium growing under conditions of high osmolarity is abruptly placed in a solution of low osmolarity, excess water will flow into the cell, rupturing the cell. If the situation is reversed, water may flow out of the cell and dehydrate the cell.

Lastly, the redox state of the environment has a profound impact on what biochemical reactions (thus, bacterial growth and/or maintenance) can take place. All life depends on the flow of electrons from a donor to an acceptor to generate chemical forms of energy and many enzymes function only if provided with appropriate redox conditions. Obligate aerobes require oxygen for a terminal electron acceptor. Some enzymes, such as the Class I ribonucleotide reductase, require oxygen for activity (19). In contrast, obligate anaerobes such as methanogens cannot grow with oxygen and require a redox potential of –0.3 V since the enzymes involved in methane production only function at a low redox potential (13). The simple removal of oxygen from the medium is often not sufficient for obligate anaerobes to grow; the redox potential of the medium must be further reduced with a reductant (28).

In members of the genus *Bacillus*, the redox state of the environment controls some gene expression. The FNR protein is an anaerobic gene regulator (32). Target genes of FNR in *B. subtilis* are *nar*K and *nar*G (nitrite extrusion protiens), *ywi*D (probable transcription regulator) and *ywc*J (possible nitrite transporter) (32). The *fnr* gene can be induced in two ways, through self-induction or through the ResD-ResE signal pathway. In self-induction, the FNR protein induces the *nar*K operon promotor, which then stimulates *fnr*. (32). In FNR-independent induction, ResE, a histidine kinase and ResD, a response regulator, activate transcription of *fnr*. The ResD-ResE signal pathway controls gene expression in response to redox conditions by altering the relative kinase and phosphatase activities of ResE (33, 45). ResE acts as a kinase under anoxic conditions and as a phosphatase under aerobic conditions. The actual sensor molecule has not been identified, although it is thought not to be oxygen (32). Both ResD and ResE are required for aerobic and anaerobic respiration (32).

Aside from appropriate environmental conditions, the appropriate nutritional conditions must also be provided. Nutritional conditions provide energy and both macro and microelements needed to make bacterial cell components (13). The macro elements consist of carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, potassium, magnesium, calcium, iron, sodium and chloride. The first four comprise the main constituents of cell material. The next five are components of cofactors, enzymes or cell wall components. Sodium and potassium are important inorganic cations in the cell. Sodium is also involved in ATP synthesis and membrane transport processes. Chloride is an important inorganic anion. The microelements consist of metals, Zn, Mn, Mo, Se, Co, Cu, Ni, and W. All of these are important in key enzyme activities.

Some bacteria can make all of their cellular components from a single carbon source. Many bacteria, however, lack the ability to make some of their cellular components and require pre-made compounds (46). These medium components are called growth factors. Some bacteria have been shown to require complex medium containing more than one growth factor (5, 47, 49). Fatty acids for lipid synthesis, amino

acids for protein synthesis, vitamins for cofactor synthesis and purines or pyrimidines for DNA and RNA synthesis are examples of common growth factors required by some bacteria. A list of the more common vitamins and related compounds and their function in metabolism are listed in Table 1.1 (13). A few growth factors are described below.

Amino acids are the precursors to proteins and some bacteria require preformed amino acids (1) (25) (49). *Bacillus subtilis* requires pyruvate for anaerobic fermentation and this requirement has been reported to be replaced with amino acids (31). Some microorganisms have been known to require peptides as a source of amino acids. There are several reports of the utilization of peptides as growth factors by ruminal bacteria (1, 25, 52), lactic acid bacteria (20), *Listeria monocytogenes* (50) and *Bacillus subtilis* (23). Some have hypothesized that the uptake of oligopeptides supplied required amino acids in the absence of an uptake system for single amino acids (35, 50). However Amezaga *et al*. (27) suggested that peptides served as a mechanism to maintain turgor when growing at high osmolarity and not just as a source of amino acids.

Many bacteria require vitamins to make required cofactors, others have more unusual requirements such as the sugar inositol (24). Inositol is a six-carbon sugar that has been shown to improve the growth of some yeasts and fungi, but its exact role was unclear (24). Choline chloride is converted to betaine and functions as an osmoprotectant in medium of high osmolarity (34). Rumen fluid potentially contains fatty acids and other complex growth factors and Tween 80 provides some fatty acids in the form of oleic acid. Rumen fluid has been successfully used to stimulate anaerobic growth of some fastidious anaerobes (5). In 1974, Taylor *et al*., showed that a methanogen required coenzyme M (2-mercaptoethanesulfonic acid) which was found in rumen fluid (48).

In 1985, Javahari *et al*., reported the successful growth of *Bacillus mojavensis* JF-2 under anaerobic conditions (18). However, anaerobic growth was inconsistent and difficult to replicate. Occasionally JF-2 would grow to an optical density of 0.2 or so and at other times no growth was observed under anaerobic conditions. Therefore, studies were initiated to determine if the inconsistent growth of *B. mojavensis* JF-2 was the result of a nutritional deficiency.

Methods and Materials

Bacterial Strains

Bacillus mojavensis JF-2 was obtained from the stock culture collection of Bradley Jackson at the University of Oklahoma. *Bacillus mojavensis*^{T} ABO21191 was obtained from the American Type Culture Collection. *Bacillus mojavensis* strain ROB2 was obtained from the stock culture collection of Dr. Kathleen Duncan at the University of Oklahoma.

Growth Media

Medium E contained the following components per liter of water (final concentration in the medium, g/l): dibasic potassium phosphate (13.9), monobasic potassium phosphate (2.7), sodium chloride (50), sucrose (10), yeast extract (1), sodium nitrate (1), ammonium sulfate (1), magnesium sulfate (0.25) and 10 ml of a metal solution (6). The pH was adjusted to 6.8. The metal solution was a modification of Wolins' metal solution and was composed of the following components per liter of (final concentration of the metal solution, g/l): EDTA (1); $MnSO_4 \cdot H_2O$ (3); $FeSO_4 \cdot 7H_2O$ (0.1); $CaCl_2 \cdot 2H_2O$ (0.1); $CoCl_2 \cdot 2H_2O$ (0.1); $ZnSO_4 \cdot 7H_2O$ (0.1); $CuSO_4 \cdot 7H_2O$ (0.01); H_3BO_4 (0.01); $Na_2MO_4 \cdot 2H_2O$ (0.01); and $AlK(SO4)_2$ (0.01).

Where indicated, medium E was also supplemented with amino acids, nucleic acid bases, vitamins and fatty acids as described by Tanner *et al*. (47). The amino acid supplement contained (final concentration in the medium, g/l): Casamino acids (DF 0230-15-5) (4), glutamine (Sigma Corp, St. Louis, Missouri G7029) (0.1), tryptophan (Sigma T0254) (0.1), asparagine (Sigma 4284) (0.02), and methionine (Sigma M9625) (0.02). The nucleic acid base supplement contained adenine (Sigma A8626), cytosine (Sigma C3506), guanine (Sigma G0381), thymine (Sigma T0895) and uracil (Sigma U0750) each at a final concentration in the medium of 0.1 g/l. The vitamin supplement contained (final concentration in the medium, mg/l): biotin (Sigma B4501) (0.02), and folic acid (Sigma F7876) (0.02), pyridoxine-HCl (Sigma P9755) (0.1), thiamine-HCl (Sigma T4625) (0.05), riboflavin (Sigma R4500) (0.05), nicotinic acid (Sigma N3376) (0.05), calcium pantothenate (Sigma P2250) (0.05), paraminobenzoic acid (Sigma A9878) (0.05), lipoic acid (Sigma T5625) (0.05), and vitamin B_{12} (Sigma V2876) (0.001). The fatty acid supplement contained (final concentration in the medium, g/l): sodium formate (Fisher S-301) (2.5), sodium acetate (Fisher S-220-1) (2.5), propionic (Aldrich 10979-1) (1.0), butyric (Fisher A-81) (0.6), isobutyric (Fisher A-80), valeric (Sigma V9759) (0.2), isovaleric (Sigma I7128) (0.2), and 2-methylbutyric (Aldrich 19307-0) (0.2) acids.

Where indicated, individual amino acids, glutamate (Sigma G5513), glutamine (Sigma G3126), phenylalanine (Sigma P4905), tyrosine (Sigma T1145), tryptophan and methionine and poly-amino acids, such as polyglutamate (Sigma P4636), polytyrosine (Sigma P1800), polyarginine (Sigma P4663), polyasparagine (Sigma P8137) and polytryptophan (Sigma P0644), were added individually at the concentration of 0.1g/l.

Alternatively, 1 to 40g/l of yeast extract, Peptone (Difco Inc., Detroit, MI, DF 0118-15-2), Soytone (DF 0436-17-5), Neopeptone (DF 0119-17-9) Tryptone (DF 0123-15-5), Proteose Peptone (DF 0120-17-6), Proteose Peptone #2 (DF 0121-17-5) Proteose Peptone #3 (DF 0122-17-4), or Casamino acids were individually added to medium E prior to autoclaving the medium. Rumen fluid was obtained from a fistulated cow fed a diet with greater than 60% roughage and no antibiotics at the Animal Sciences Center of Oklahoma State University (Stillwater, OK); 5 to 50% rumen fluid was added (vol:vol) to medium E prior to autoclaving the medium. Proteose Peptone (30 g/l) and salmon sperm DNA (Sigma D-1626) (1 g/l) were added before autoclaving.

The cysteine-sulfide solution was prepared by adding 1.25 g of NaOH to 200 ml of Nanopure water. The alkaline water was boiled under nitrogen and allowed to cool before the anhydrous, cysteine·HCl (5 g) and sodium sulfide·$9H_2O$ (5 g) were added. Solutions of cysteine hydrochloride, dithiothreitol, dithionite solutions were prepared by addition of 5 g of the reductant to 100 ml of anoxic Nanopure water and the pH was

adjusted to 7.0 with 1 M NaOH. Titanium citrate solution was prepared as described by Moench and Ziekus (29). Reductant was added to the medium before autoclaving (final concentration of each reductant individually in the medium of 0.05 g/l).

All media and solutions were anaerobically prepared and used according to procedures described by Balch and Wolfe (3). Each medium was dispensed in 9 ml aliquots into culture tubes under a 100% O_2-free, N_2 gas phase. Cultures were brought to 10 ml with the addition of 1 ml of O_2-free water or the respective medium addition.

Inoculation Protocol

A serum bottle with 100 ml of anaerobic medium E was inoculated directly from a plate of *B. mojavensis* JF-2. The serum bottle was allowed to incubate for 24 hours at 37^{o}C. From this culture a 5%-10% inoculum was used for each tube in an experimental setup. Three replicates were inoculated and one replicate was left uninoculated for each condition. All tubes were incubated at 37^{o}C without shaking. Growth was measured by monitoring absorbance at 600 nm. The cultures were diluted when the absorbance was above 0.3.

Fractionation of Proteose Peptone with Sephadex G-25

Sephadex G-25 gel was soaked in water for 48 hours, the fines were decanted, and then the gel was degassed for 24 hours under 103 Kpa vacuum. The gel was poured into a 1-meter long column (final dimension of 90cm X 2 cm), partially filled with water. The column was equilibrated for 2 hours with water at a flow rate of 2 ml/min. The void volume was determined to be 31.5 ml by the elution of 1 ml of Blue Dextran in water at a flow rate of 1.5 m/min. After calibration, 2 ml of a 30% Proteose Peptone solution was injected at a flow rate of 1.5 ml/min. Thirty-two, five-ml fractions were collected. The fractions were pooled in the following manner: 7-11(F_A); 12-18 (F_B); 19-22 (F_C); 23-32 (F_D). A total of six milliliters of 30% Proteose Peptone was fractionated, lyophilized, pooled in four fractions and then returned individually to a volume of 6 ml with water. One ml of each of these solutions of pooled fractions was then added to 9 ml of medium. For a positive control, another 6 ml was fractionated, lyophilized, and then all fractions were recombined into a single pool and reconstituted with 6 ml of water. Cyanocobalamin (MW 1355); tryptophan (MW 204); aprotinin (MW 6500) were used as standards to determine the average molecular weight of the Proteose Peptone fractions.

Methanol Extraction of the Growth-enhancing Factor in Proteose Peptone

Proteose peptone (300 g) was stirred for 24 hours with 500 ml of methanol. The methanol insoluble fraction was filtered, dissolved in 100 ml of Nanopure water, boiled 5 min to remove traces of methanol, and then lyophilized. The 500-ml filtrate was combined with 500 ml of Nanopure water, boiled until less than 400 ml of liquid remained, and then lyophilized. The dry fractions were added to medium E to give a final concentration of 0.03 g (dry wt)/ml.

The methanol soluble and methanol insoluble fraction were size-separated on a Sephadex column and pooled as described above. The dry fractions were added to

medium E to give a final concentration of approximately 0.01 g (dry wt)/ml, except for the final pooled fraction, which was added at about 0.001 g (dry wt)/ml, due to the very small amount of material collected. The protein content of the size fraction that best supported growth (F_C) was measured by the Coomassie Blue and the BCA methods (17).

Ion Exchange Chromatography

Macro-Prep High S Cation Exchange Support and Macro-Prep High Q Anion Exchange Support (Bio-Rad) were hydrated, degassed and washed as recommended by the manufacturer. A 10-ml sample of 30% Proteose peptone was pulled through 100 ml of hydrated beads by vacuum filtration. The filtrate was lyophilized and added to medium E at the concentration of 0.03 g (dry wt)/ml.

Acid and Base Treatment of Proteose Peptone

Thirty grams each of Proteose Peptone was added to 90 ml of acidic Nanopure (pH 2 with HCl) (acid treatment), 90 ml of basic Nanopure (pH 12 with NaOH) (base treatment) and 90 ml of neutral Nanopure (pH 7) (neutral treatment). These were autoclaved for 20 min at 120^{o}C and 110 Kpa. After cooling, the pH was adjusted to 7 with HCl or NaOH and the volume was adjusted to 100 ml. As a control, thirty grams of Proteose Peptone was added to 100 ml of Nanopure and left untreated. These solutions were diluted 1/10 in medium E and added to medium E before autoclaving.

UV Spectrum

The UV absorbance scan was performed on a DU-64 Beckman Spectrophotometer at 750 nm/min from 400 to 200 nm. The sample was prepared first by the removal of the methanol soluble fraction from Proteose Peptone (as described above) and treatment by Sephadex G-25 column chromatography as described above. The F_C fraction was retained (F_C contained the highest specific activity, see Table 1.4) and the rest discarded. The F_C fraction was passed through a cation exchange column as described earlier and then scanned.

Results

Medium E was previously used for anaerobic growth of *B. mojavensis* JF-2 (18). However, although aerobic growth was robust in this medium (A > 0.8), anaerobic growth of JF-2 was poor (A_{max} <0.1) and difficult to replicate. In fact, it was observed that JF-2 only grew well in this medium if a small amount of oxygen was added. This could be accomplished by adding 3 ml of filter-sterilized air to reductant-free anaerobic medium E (A_{max} ~ 0.3), with stationary incubation of aerobic medium E (A_{max} > 0.8) or in aerobic medium with a nitrogen headspace (A_{max} > 0.8).

Medium E contains 1 g/l yeast extract, which could supply some growth factors but may not contain all possible vitamins and growth factors. Therefore, additional vitamins were added. However, the addition of a solution of Balch vitamins (described in the Methods and Materials section) (10 ml/ l), inositol (2 µg/l), choline chloride (2 µg/l), vitamin K (2 µg/l), or hemin (1 µg/l) to medium E did not improve anaerobic growth (Table 1.2). Further, anaerobic growth was not improved by the addition of increased amounts of yeast extract (up to 20 g/l), Casamino acids (up to 30 g/l), rumen fluid (up to 50%) or Tween 80 (3%) (Table 1.2).

The addition of 30 g/l Tryptone was successful at enhancing growth in anaerobic medium E (Figure 1.1). With the addition of Tryptone to medium E there was an almost 10-fold increase in growth compared to the growth of JF-2 in medium E without Tryptone. The previous growth factors described above were tested in anaerobic medium E with the oxygen removed and a nitrogen headspace, but without reductant since it was not clear that JF-2 would grow in the presence of a reductant. It was possible that JF-2 might grow well in the absence of oxygen (with the appropriate growth factors) so long as the redox potential was not poised at a very low value by the addition of a reductant. Once it was determined that JF-2 could grow in the absence of oxygen with an enzymatic digest of protein, then a reductant was added to the medium and it was determined that JF-2 could grow anaerobically with a reductant present. JF-2 was able to grow in Tryptone-supplemented medium E containing cysteine hydrochloride, dithiothreitol or dithionite as well as it grew in Tryptone supplemented medium E without a reductant (Table 1.3). The presence of titanium citrate or cysteine-sulfide resulted in slightly less growth. This data conclusively showed that JF-2 could grow under strict anaerobic conditions if the nutritional requirements were met.

Other enzymatic digests of protein were also tested for the ability to enhance anaerobic growth of JF-2. Proteose peptone (Proteose Peptone (DF 0120-17-6), Proteose Peptone #2 (DF 0121-17-5) and Proteose Peptone #3 (DF 0122-17-4), Neopeptone and Tryptose were effective in supporting anaerobic growth of JF-2 ($A_{max} > 0.5$) (Figure 1.2). Soytone, Casitone and Peptone were less effective ($A_{max} < 0.2$) at improving anaerobic growth of JF-2. The addition of Proteose Peptone #3 to medium E was the most effective at establishing reproducible anaerobic growth that did not decrease when the culture reached stationary phase (Figure 1.3)

In an attempt to replace Proteose Peptone with a complete combination of known growth factors, amino acids, nucleic acid bases, vitamins and fatty acids were added to medium E as described by Tanner *et al.,* (see the Methods and Material section) (47). In this supplemented medium E without Proteose peptone, anaerobic growth was very poor (A_{max} less than 0.1) (Figure 1.4). With Proteose peptone, anaerobic growth was consistently above an A_{max} of 0.6.

Several polypeptides were tested to determine if they could replace the requirement for Proteose Peptone. However, the addition of poly- amino acids, such as polyglutamate (glu_7), polyglutamine (gln_9), polytryptophan (try_{10}) polytyrosine (tyr_6), polyarginine (arg_7), or polyasparagine (asp_7) to medium E did not support anaerobic growth of JF-2 (data not shown).

In order to determine the nature of the component present in Proteose Peptone that was responsible for the growth stimulation, the components of Proteose Peptone were size-fractionated with a Sephadex G-25 column. This size-exclusion

chromatography resulted in the separation of the components in Proteose Peptone into three main size classes as determined by absorbance of the fractions at 280 nm (Figure 1.5). The approximate points of elution of aprotinin (MW 6500), cyanocobalamin (MW 1355) and tryptophan (MW 204) (the size standards) are given on the graph.

For ease of analysis, individual fractions were combined into four pools (F_A, F_B, F_C, F_D). Growth activity was determined by subtraction of the A_{max} resulting from growth in medium E from the A_{max} resulting from growth in medium E plus the respective size fraction.

A unit of growth-stimulating activity was defined as the amount of activity added to medium E that would lead to an increase in A600 maximum of strain JF-2 by 1.0 absorbance unit over medium E alone, in a 10 ml culture. Specific activity was defined as the number of units per assay per mg dry weight of sample added to the assay. The fraction that best stimulated the anaerobic growth of JF-2 was F_B (A_{max} ~ 0.6) (Figure 1.6). Most of the growth due to Proteose Peptone (0.38) was found in F_B (0.30) (Table 1.4). However the F_C fraction had a higher specific activity (as A_{600}/mg) than fraction F_B (0.0062 vs. 0.0036). F_C supported anaerobic growth of JF-2 for the first 40 hours (A_{max} ~ 0.22), but then growth rapidly declined thereafter (A_{max} ~ 0.1). The other fractions did not improve anaerobic growth of JF-2 over that produced in medium E. The total growth-enhancing activity due to Proteose Peptone was about 2.3 units, the total units of activity recovered from the sephadex column was about 2.9. This indicated that 130% of activity was found in F_A, F_B, and F_C. Recovery was probably somewhat overestimated because the amount applied was calculated for an assay within which the factor was essentially saturating. A standard curve was created using the molecular weight of aprotinin, cyanocobalimin and tryptophan and the ratio of elution volumn (V_e) to void volumn (V_o) of these compounds to determine the average size of the growth-supporting fraction. The fraction containing the highest concentration of growth-enhancing factor, F_B, had an average molecular weight of 3900 grams/mol, ranging from 2000 to 6000 g/mol.

The growth-enhancing factor was further characterized using methanol extraction and ion exchange chromatography. Crude purification revealed that the growth-enhancing factor was methanol insoluble (Figure 1.7), was retained by an anion exchange column, and eluted from a cation exchange column (Figure 1.8). The methanol insoluble fraction had 2.8X the growth-enhancing activity found in Proteose Peptone. This suggested that the growth-enhancing factor was very strongly hydrophobic and had an overall positive charge. Further characterization revealed that the growth-enhancing factor was acid and base stable (Figure 1.9) and low in protein content (Table 1.5).

Finally, a UV scan was performed on the growth-enhancing factor after methanol extraction of Proteose Peptone, size separation on a Sephadex gel G25 column, and passage through a cation exchange column. An absorbance peak at about 260 nm along with the low protein content, strongly suggested that the growth-enhancing factor consisted of, or contained, nucleic acids Figure (1.10). However, neither the addition of nucleic acid bases alone nor the combination of nucleic acid bases with peptides in the medium E replaced the requirement for Proteose peptone (data not shown). This was puzzling and the only apparent solution was to try another source of nucleic acids, DNA.

Salmon sperm DNA (1 g/l) replaced the requirement for Proteose Peptone for anaerobic growth of JF-2 (Figure 1.11). Salmon sperm DNA had 19X the growth

enhancing activity that Proteose Peptone had. With the addition of salmon sperm DNA to medium E, the growth of JF-2 reached a maximum absorbance of about 0.5. The addition of DNA plus 0.3 g/l Proteose Peptone slightly improved anaerobic growth (A_{max} ~ 0.6) over that of the addition of DNA alone. The addition of DNA plus 30 g/l Proteose Peptone to medium E further improved growth to an absorbance of about 0.7. These data show that Proteose Peptone could be replaced with DNA but also that Proteose Peptone supplied nutrients other than just DNA for growth. The additional nutrient requirements of JF-2 for anaerobic growth are described in Appendix I.

Since *Bacillus mojavensis* JF-2 required DNA for growth, other *Bacillus mojavensis* strains were tested for anaerobic growth requirements. Both the type strain *Bacillus mojavensis* ^{T} ABO21191 and *Bacillus mojavensis* strain ROB2 required DNA for anaerobic growth (Figures 1.12 and 1.13). The presence in of ribonucleosides (1 g/l each adenosine, guanosine, cytosine and thymidine) and 4 g/l Casamino acids in medium E did not replace the requirement for DNA.

Discussion

In 1995 Hoffman et al., (16) observed that the presence of a small amount of oxygen was enough to enhance "anaerobic growth" of *Bacillus subtilis*. Similarly, small amounts of oxygen enhanced growth of JF-2 compared to growth under strict anaerobic conditions in medium E. Therefore, medium E clearly supported aerobic growth of JF-2. JF-2 was able to obtain energy and synthesize all its growth requirements from a single carbon source, sucrose. Even a small amount of oxygen apparently created redox conditions appropriate for growth. However, the complete removal of oxygen from medium E strongly inhibited growth. These results suggested that either:

(1) JF-2 was an obligate aerobe (required oxygen as a terminal electron acceptor) or

(2) JF-2 was able to use an alternate electron acceptor (through respiration or fermentation) but was unable to make all the components needed for growth under anaerobic conditions in the same way that it was able to under aerobic conditions.

The addition of DNA or deoxyribonucleosides (Chapter 2) to medium E supported anaerobic growth of JF-2, confirming the second possibility. As far as I can tell, this is also the first report of a microorganism that has anaerobic growth factor requirements distinct from aerobic growth factor requirements. The possible reason for the DNA requirement is discussed in the following chapter.

It should not be surprising that microorganisms have evolved strategies aimed at coping with fluxuating oxygen levels in their environment. Soil may be alternatively dry and aerated and subsequently flooded and anoxic or microaerophilic. It has also been shown that oxygen is undetectable 25 to 30 μm below the surface of some biofilms (53). Facultative anaerobes can sense the oxygen concentration and adjust their metabolism accordingly. Often changes in oxygen concentration are only considered with regard to the rate and route of carbon source utilization and the pathways of electron flow to

maintain the redox balance. However, a change in oxygen concentration may require more than just an ability to adjust bacterial metabolism for energy production; as this report shows, a change in oxygen may also require nutritional adjustments to supplement changing bacterial nutritional requirements. Therefore, nutritional requirements may determine if an organism is identified as an aerobe or an anaerobe as much as its energy metabolism.

No literature reports of a DNA-requiring facultative organism were found although in 1957 Hoff-Jorgensen did report of a *Lactobacillus* that required DNA for growth in aerobic medium (14). Most research concerned with DNA uptake relates to competence and bacterial transformation (4, 7-10, 12, 21, 22, 26, 39). Competence is a process whereby bacteria take up high molecular weight DNA. Double-stranded DNA is bound to the cell and one strand enters the cell while the other strand is degraded (9). Some bacteria, such as those in the genus *Haemophilus*, prefer to take up homologous DNA (40). Transformation is a process whereby bacteria exchange genetic information with the single strand of DNA resulting from competence. A minority of reports suggests that natural competence may have an auxiliary role in bacterial nutrition (36, 37, 41, 43). However, none of these reports consider the possibility of DNA or deoxyribonucleosides as a growth factor.

In 2001, Finkel and Kolter found that *Escherichia coli* could use DNA as a sole carbon and energy source although it does not require DNA for growth (11). They found *E. coli* competence gene homologs in many members of the γ subclass of the *Proteobacteria* and suggested that mechanisms for consumption of DNA may be widely conserved.

Bdellobivrios may use DNA or deoxynucleosides as a nutrient. The bdellobivrios are intracellular predators of Gram-negative bacteria (30, 44). They are a diverse group of microorganisms which are lumped together based on the ability to grow intracellularly rather than by natural phylogeny (2). They are generally cultivated in coculture with their prey, but host-independent bdellobivrios have been cultured (38). To grow them in the absence of prey requires rich medium supplemented with cell lysates of the host cell. The obligately intracellular life style suggests that these parasites might require growth factors found in the host (30). Apparently all known *Bdellovibrios, Coxiella, Rickettsia Chlamydia*, and *Plasmodium* can independently synthesize DNA, but no direct evidence was found in the literature for all of these organisms that conclusively shows that they make their own DNA precursors (30). Some may acquire them from the host cell. Although no DNA or deoxyribonucleoside-requiring intracellular parasites have been identified as yet, with DNA or ·HCl deoxyribonucleoside-supplemented medium it may be possible to isolate such parasites in axenic culture. Some intracellular parasites that have not been isolated independent of a host may require DNA or deoxyribonucleosides.

In summary, three strains of *Bacillus mojavensis* required DNA for anaerobic growth. This requirement does not exist under aerobic growth conditions. This is the first report of anaerobic growth factor requirements distinct from aerobic growth factor requirements. This is the first report of DNA as growth factor since Hoff-Jorgensen's report in 1952 (15).

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Table 1.1. Some vitamins and their function in metabolism (13)

Compound	Function in metabolism
p-aminobenzoic acid	Precursor of tetrahydrofolate, a coenzyme involved in transfer of one-carbon units
Biotin	Prosthetic group of enzymes catalyzing carboxylation reactions
Coenzyme M	Coenzyme involved in methane formation
Folic acid	Tetrahydrofolate is a coenzyme involved in transfer of one-carbon units
Hemin	Precursor of cytochromes
Lipoic acid	Prosthetic group of the pyruvate dehydrogenase complexes
Nicotinic acid	Precursor of NAD^+ and $NADP^+$
Pantothenic acid	Precursor of coenzyme A and of the prosthetic group of acyl carrier proteins
Pyridoxine (vitamin B_6)	Pyridoxylphosphate is a coenzyme for transaminases and amino acid decarboxylases
Riboflavin (vitamin B_2)	Precursor of flavin mononucleotide and flavin adenine dinucleotide
Cyanocobalamin (vitamin B_{12})	Involved in rearrangement reactions and precursor of deoxyadenosyl cobalamin
Vitamin K	Precursor of menaquinone

Table 1.2. The effect of various additions to medium E on anaerobic growth of *B. mojavensis* JF-2.

Addition to Medium E	Maximum Absorbance at 600 nm
No addition	0.10 ± 0.08
Oxygen (aerobic medium E)	0.80 ± 0.1
Balch vitamins	0.04 ± 0.003
Inositol + Choline chloride (2 μg/l each)	0.06 ± 0.03
Vitamin K (2 μg/l)	ND
Hemin (1 μg/l)	ND
Yeast Extract (up to 10 g/l)	0.091 ± 0.01
Casamino acids (up to 40 g/l)	0.12 ± 0.019
Rumen Fluid (up to 50%)	ND
Tween 80 (3%)	ND

ND = no growth detected,

Table 1.3. The effect of the addition of a reductant to medium E with 30 g/l Tryptone on anaerobic growth of *B. mojavensis* JF-2 (each at a final concentration in the medium of 0.05 g/l).

Reductant added to Medium E	Maximum Absorbance at 600 nm
No reductant	0.55± 0.1
Cysteine hydrochloride	0.56 ± 0.03
Dithiothreitol	0.49 ± 0.06
Dithionite	0.59 ± 0.13
Titanium citrate	0.35 ± 0.01
Cysteine/sulfide solution	0.29 ± 0.01

Table 1.4. The activity of Proteose Peptone in supporting anaerobic growth of *Bacillus mojavensis* JF-2, when fractionated on a Sephadex G-25 column and when extracted with methanol.

Medium Addition	A_{max}-A_{max} in medium E/10 ml culture	Specific Activity (units/mg)	Units Recovered
Medium E	0	0	0
Medium E + Proteose Peptone	0.38	0.0013	2.3
Medium E + F_A	0.10	0.0012	0.6
Medium E + F_B	0.30	0.0036	1.8
Medium E + F_C	0.08	0.0062	0.48
Medium E + F_D	0	0	0
Medium E + Methanol insoluble fraction	1.1	0.0037	2.8
Medium E + Methanol soluble fraction	0.105	0.0004	0

Table 1.5. The results of protein analysis of the methanol extracted, Sephadex G25 gel separated F_C component of Proteose Peptone.

Method of Protein Analysis	Dry weight (mg/ml)	Protein (mg/ml)	Percent Protein
Coomassie Blue	0.75 ± 0.35	0.012 ± 0.00078	1.83 ± 0.76
BCA	0.75 ± 0.35	0.019 ± 0.0014	2.81 ± 1.14

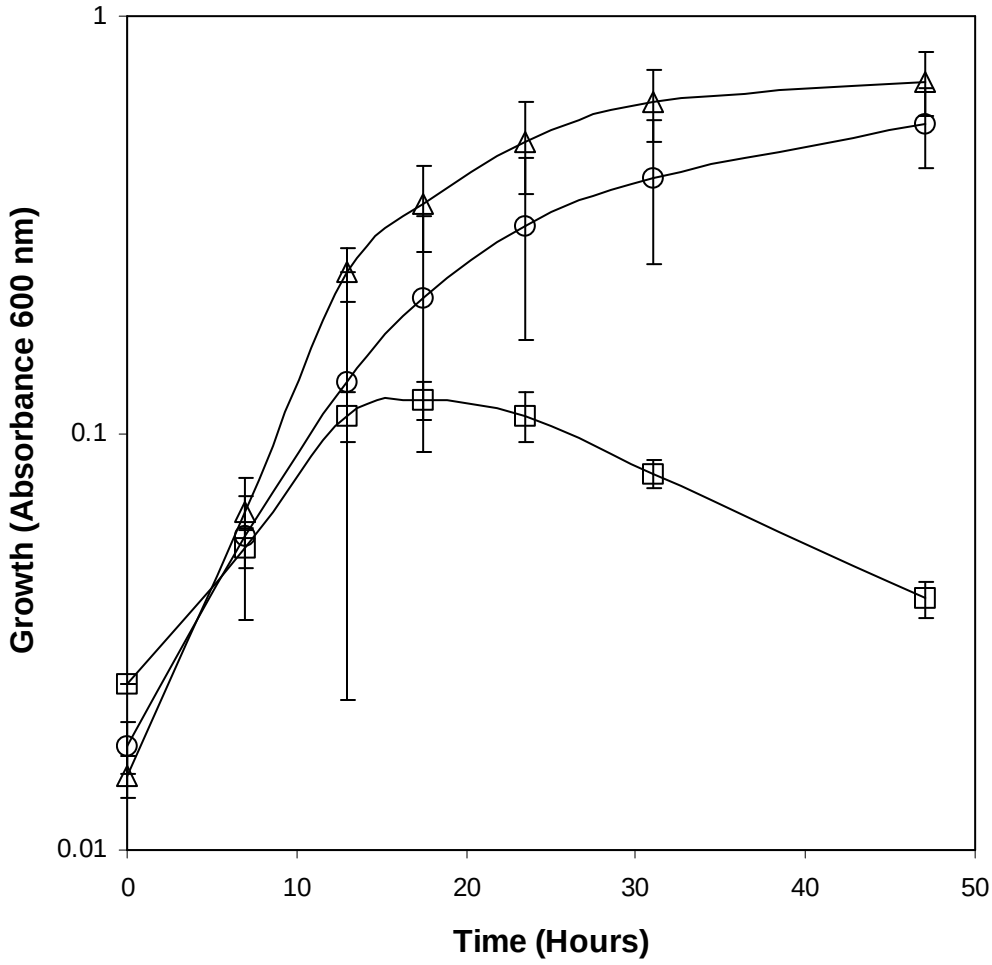

Figure 1.1. The effect of the addition of Tryptone to medium E on anaerobic growth of *B. mojavensis* JF-2.

Squares, medium E; circles, medium E plus 20 g/l Tryptone; triangles, medium E plus 45 g/l Tryptone. Bars indicate standard deviation for the average (n=3).

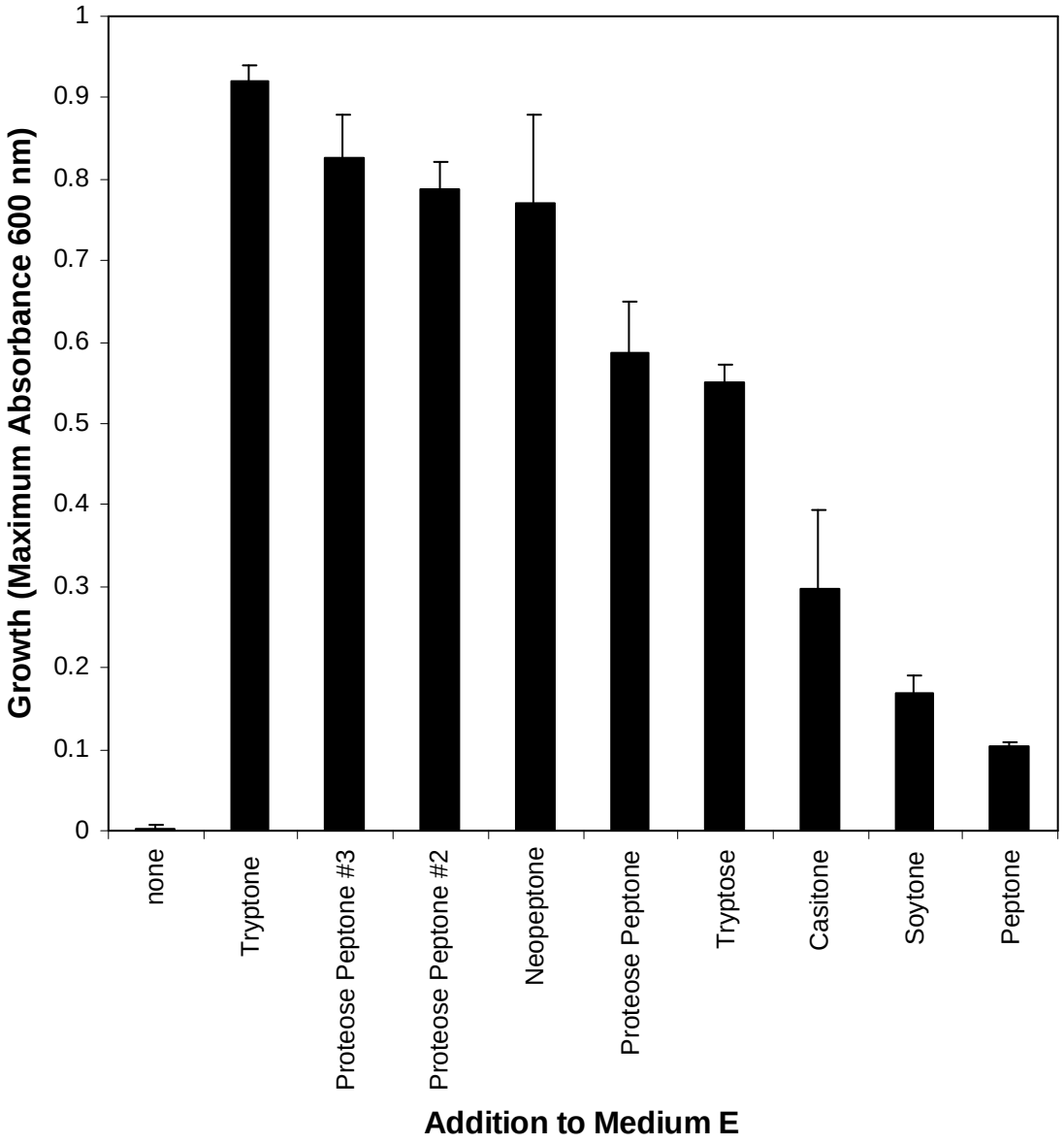

Figure 1.2. The effect of the addition of different enzymatic digests of protein to medium E on the anaerobic growth of *B. mojavensis* JF-2.

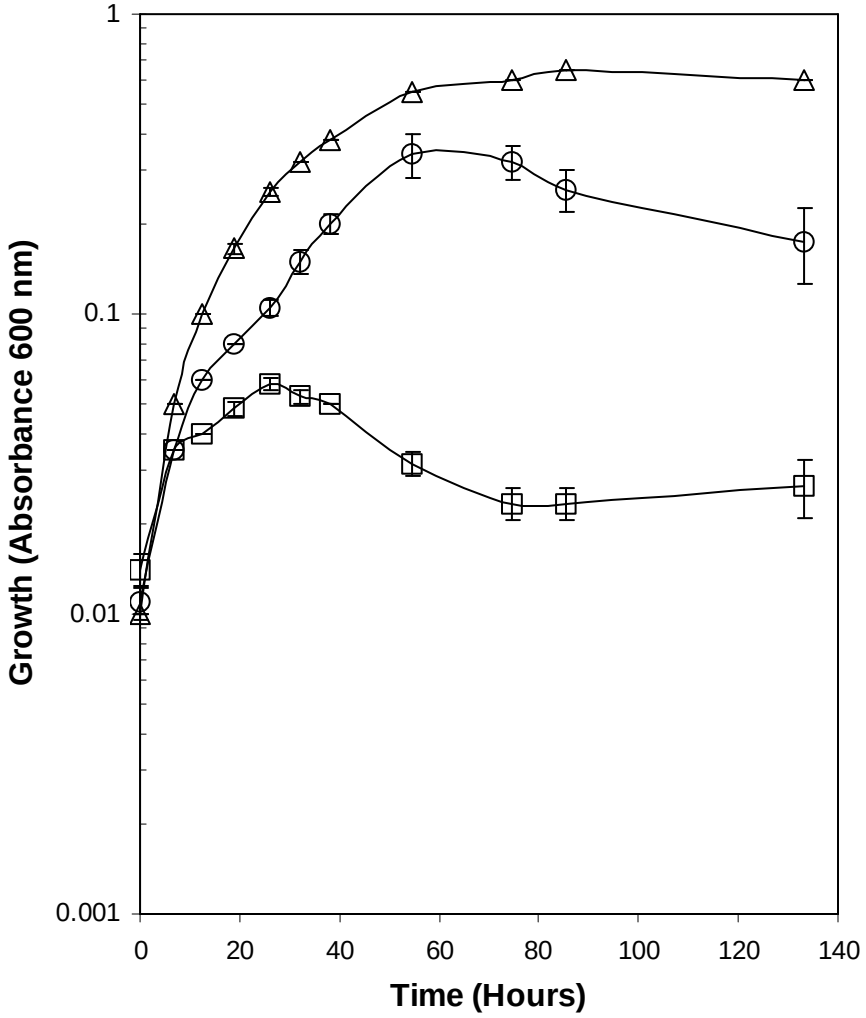

Figure 1.3. The effect of the addition of Tryptone and Proteose Peptone Medium E on the anaerobic growth of *B. mojavensis* JF-2.

Squares, medium E; circles, medium E plus 30 g/l Tryptone; triangles, medium E plus 30 g/l Proteose Peptone.

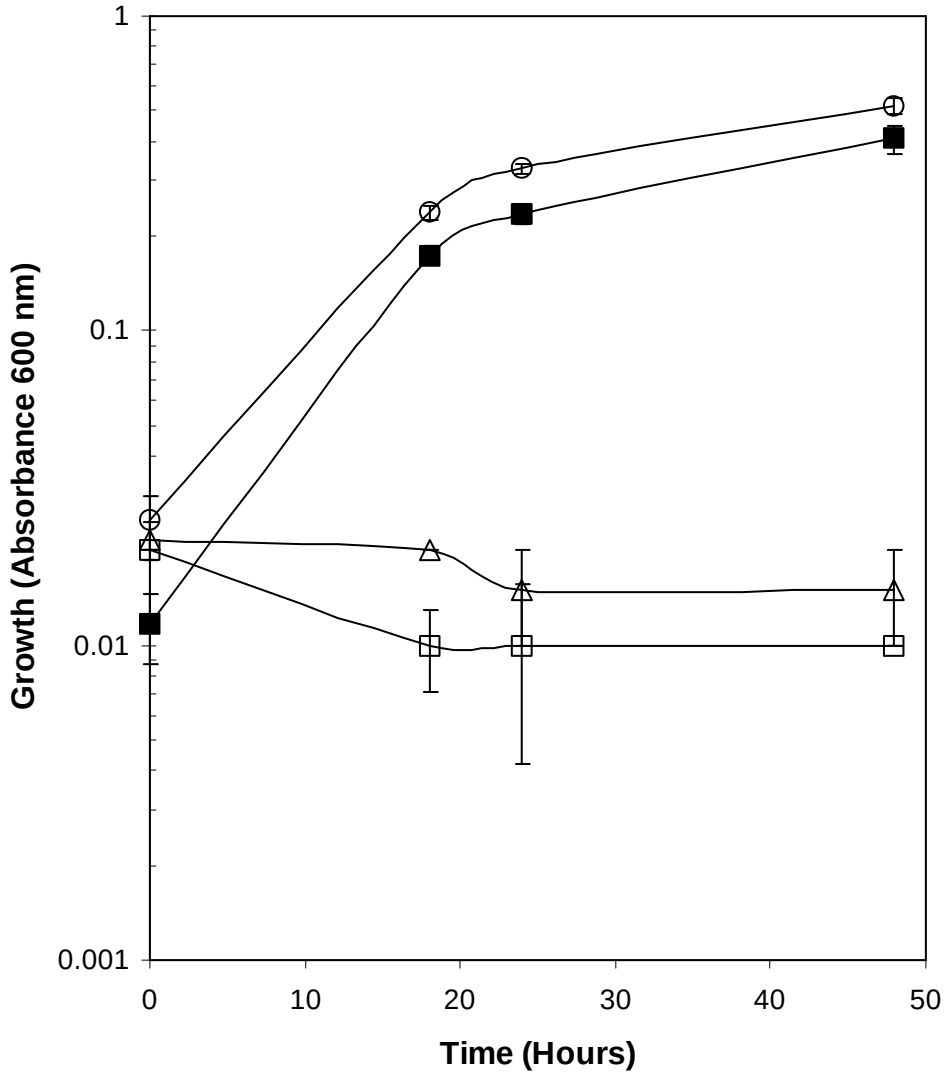

Figure 1.4. The effect of medium E supplemented with amino acids, vitamins, nucleic acids and fatty acids on anaerobic growth of *B. mojavensis* JF-2.

Squares, medium E; circles, medium E plus 30 g/l Proteose Peptone; triangles, medium E plus amino acids, vitamins, five nucleic acid bases and fatty acids; filled squares, medium E plus amino acids, vitamins, five nucleic acid bases, fatty acids and 30 g/l Proteose Peptone.

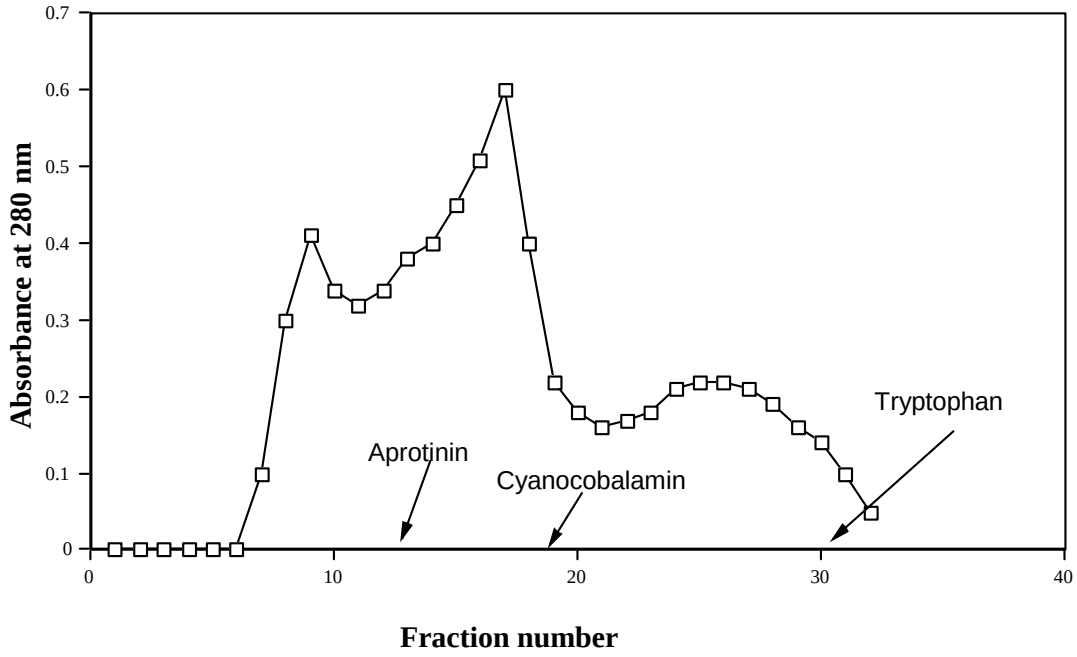

Figure 1.5. The UV absorbance of the fractions collected after fractionation of Proteose Peptone by size-exclusion chromatography.

The standards, aprotinen (MW 6500) cyanocobalamin (MW 1355), and tryptophan (MW 204) are shown at their point of elution. Fraction 7-11 (F_A), 12-18 (F_B), 19-22 (F_C) and 23-32 (F_D) were pooled, respectively.

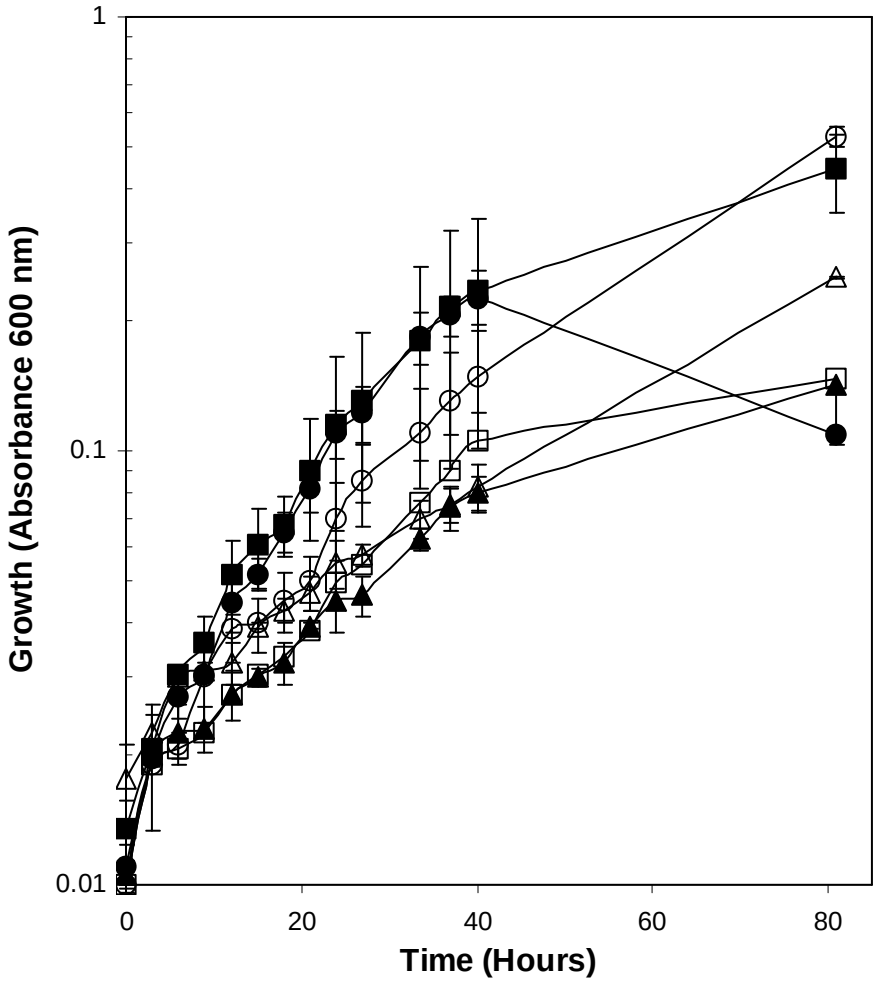

Figure 1.6. The effect of the addition of the pooled fractions (collected by size exclusion chromatography using Sephadex G25) to Medium E on anaerobic growth of *B. mojavensis* JF-2.

Squares, medium E; circles medium E plus 30g/l Proteose Peptone; triangles, medium E plus pooled fraction F_A; filled squares, medium E plus pooled fraction F_B; filled circles, medium E plus pooled fraction F_C; filled triangles, medium E plus pooled fraction F_D.

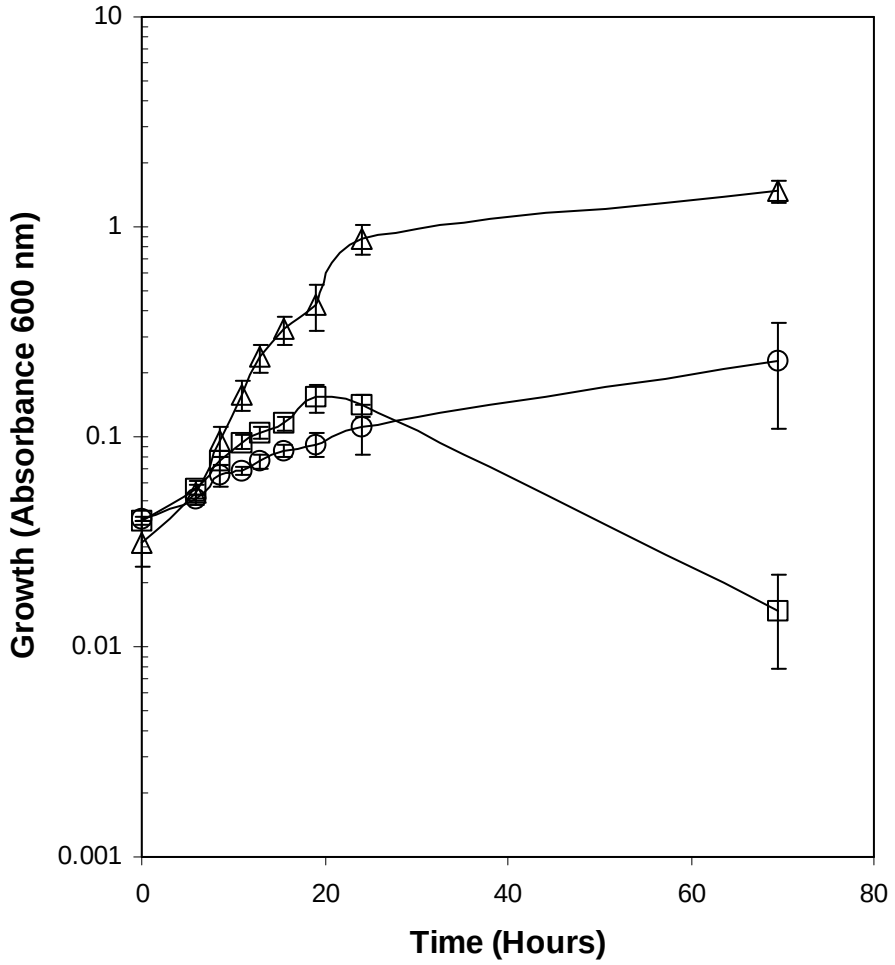

Figure 1.7. The effect of methanol soluble and methanol insoluble fractions on anaerobic growth of *B. mojavensis* JF-2.

Squares, medium E; circles, medium E plus 30 g/l methanol soluble extract; triangles, medium E plus 30 g/l methanol insoluble extract.

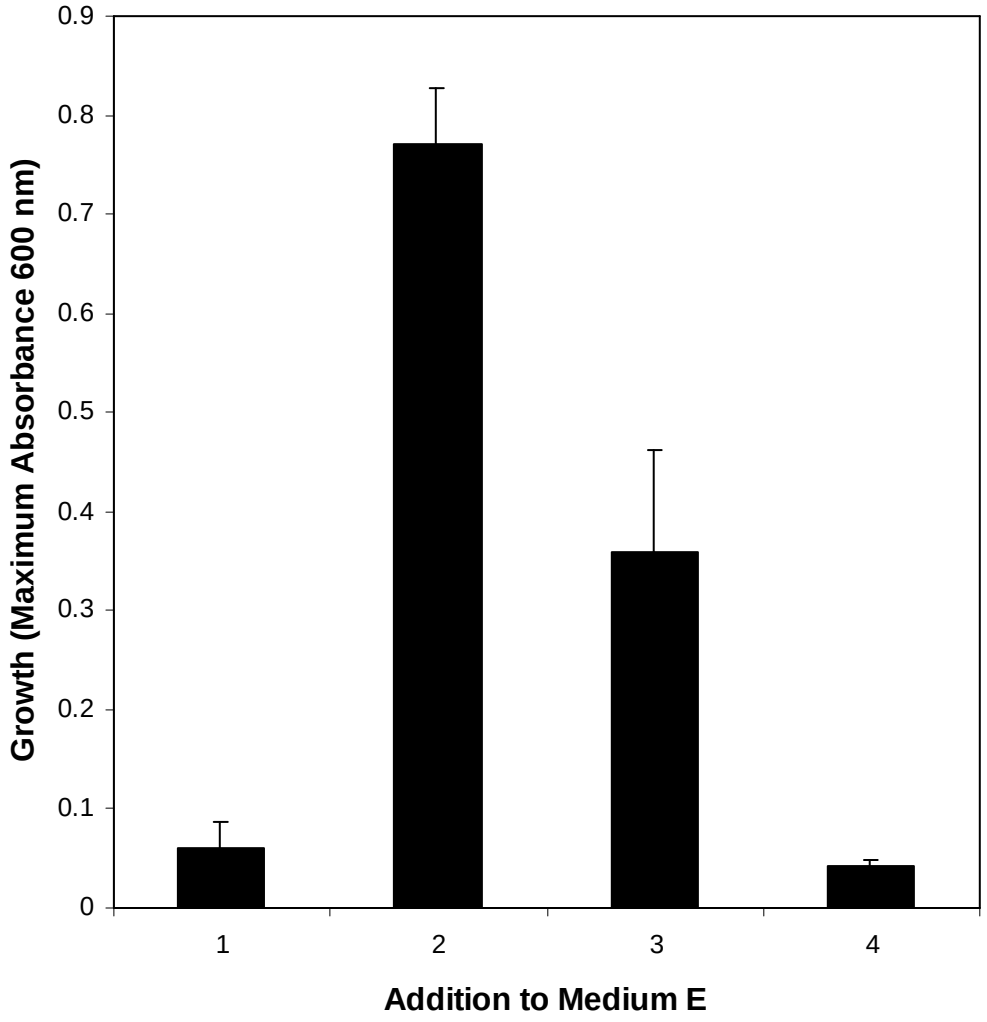

Figure 1.8. The effect of the addition of ion exchange fractions to Medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Column one, medium E; column 2, medium E plus 30 g/l Proteose Peptone; column 3, medium E plus 30 g/l lyophilized cation exchange eluate; column 4, medium E plus 30 g/l lyophilised anion exchange eluate

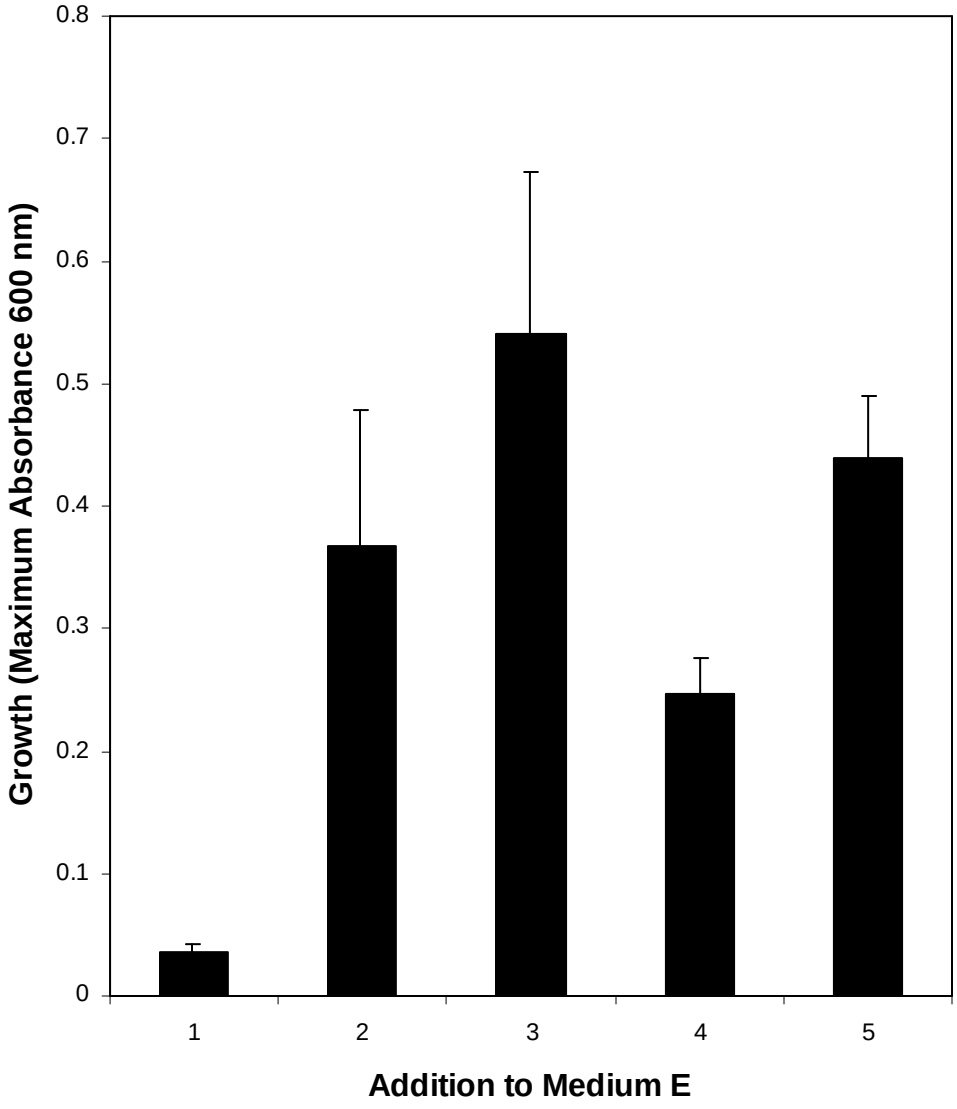

Figure 1.9. The effect of the addition of neutral, acid and base treated Proteose Peptone to Medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Column 1, medium E; column 2, medium E plus 30 g/l Proteose Peptone; column 3, medium E plus 30 g/l lyophilized product of the neutral-treated Proteose Peptone; column 4, medium E plus 30 g/l lyophilized product of the acid-treated Proteose Peptone; column 5, medium E plus 30 g/l lyophilized product of the basic-treated Proteose Peptone.

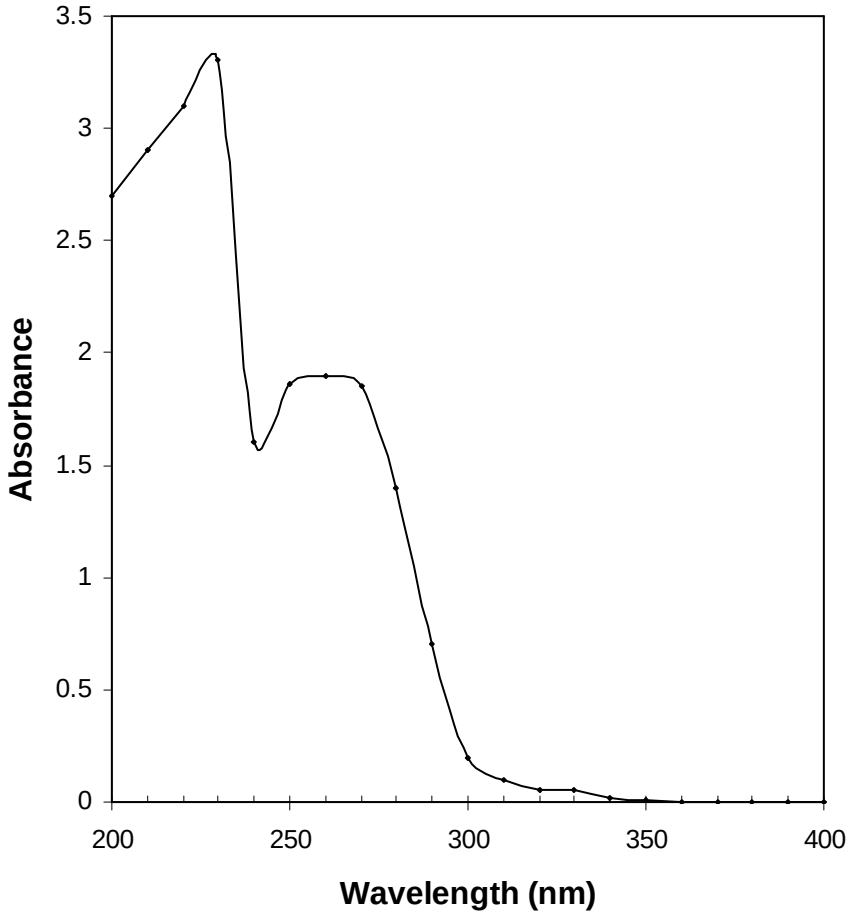

Figure 1.10. UV scan of the growth enhancing factor in Proteose Peptone after methanol extraction, size fractionation, and passage through a cation exchange.

First the methanol soluble faction was removed from Proteose Peptone, then size fractionated and the size fraction (Fc) was passed through a cation exchange.

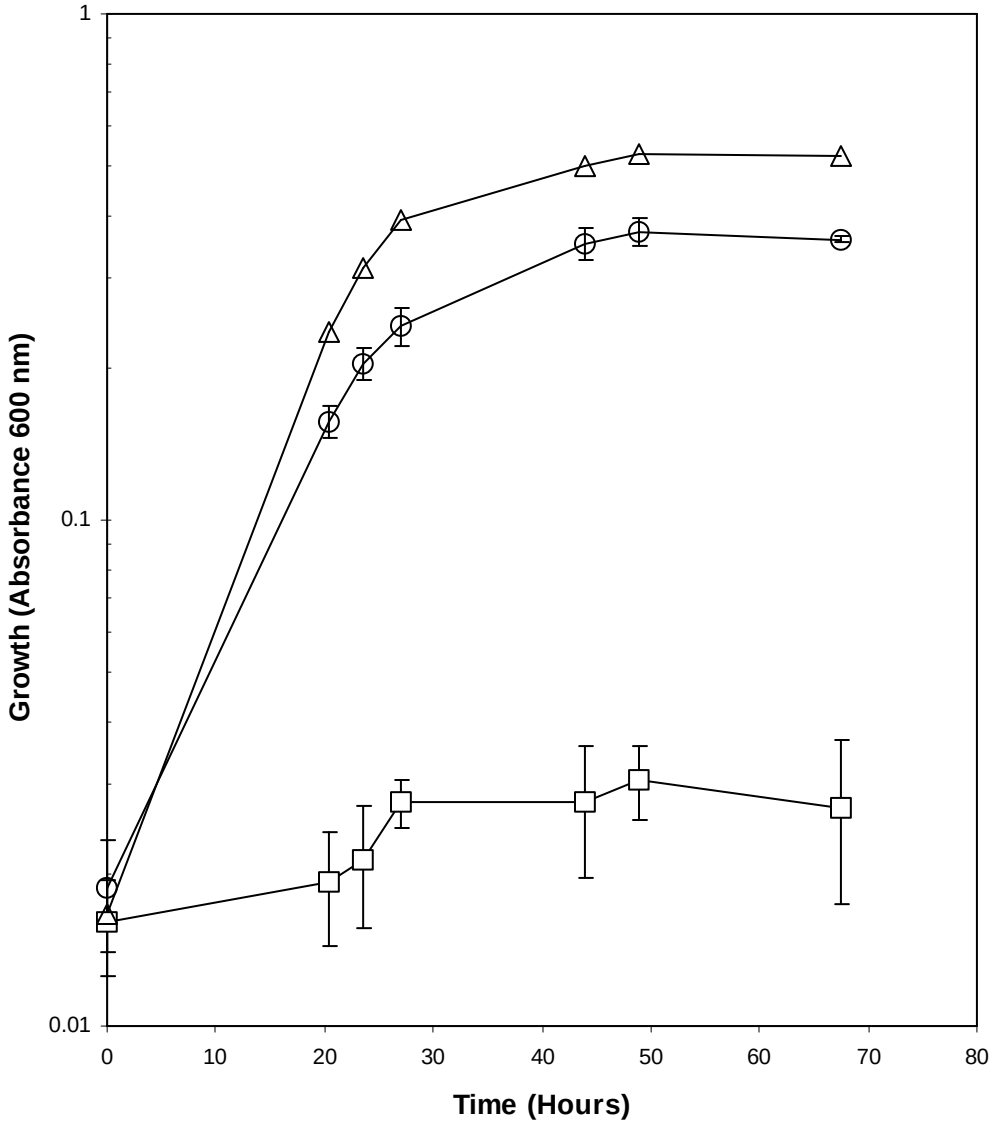

Figure 1.11. The effect of the addition of salmon sperm DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 1 g/l salmon sperm DNA; triangles, medium E plus 30 g/l Proteose Peptone.

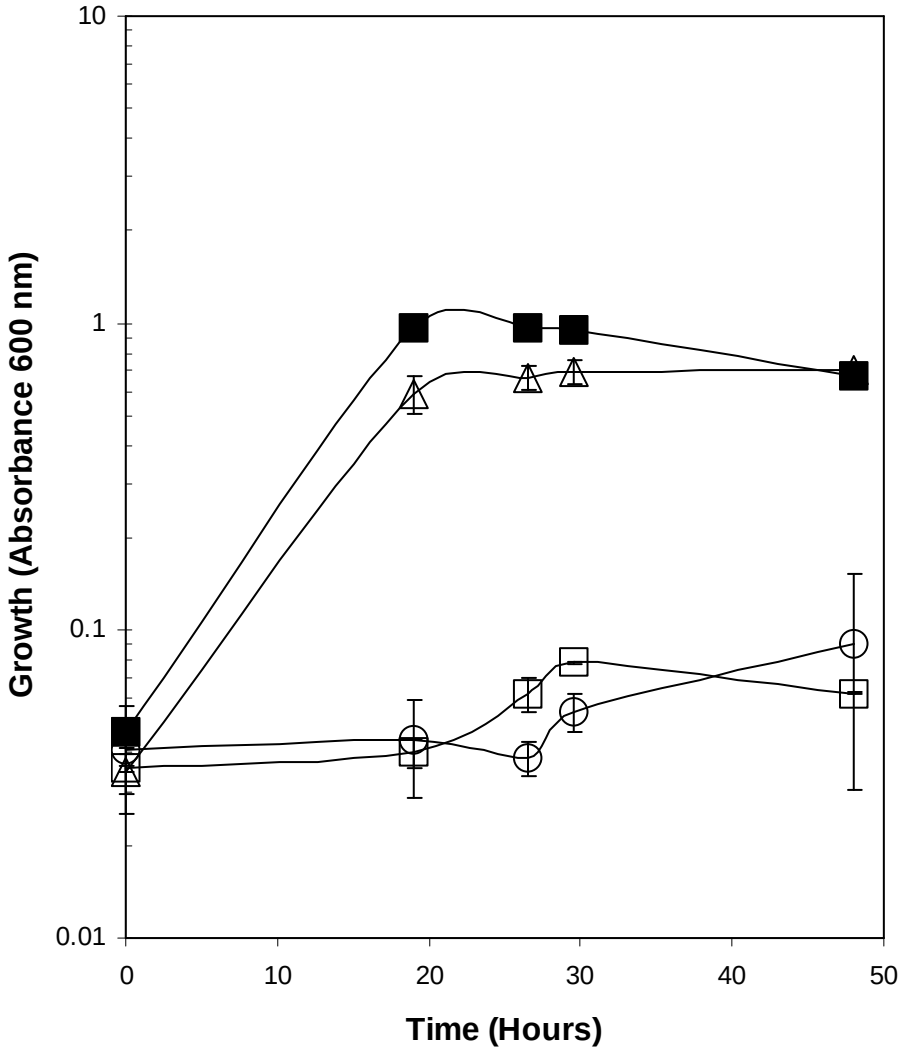

Figure 1.12. The effect of the addition of DNA to medium E on anaerobic growth of *Bacillus mojavensis* (ATCC AB021191).

Squares, medium E; circles, medium E plus ribonucleosides (0.1 g/l each adenosine, guanosine, cytidine and thymidine) and 1 g/l Casamino acids; triangles, medium E plus 0.5 g/l herring sperm DNA; filled squares, medium E plus 0.5 g/l herring sperm DNA and 10 g/l Proteose Peptone.

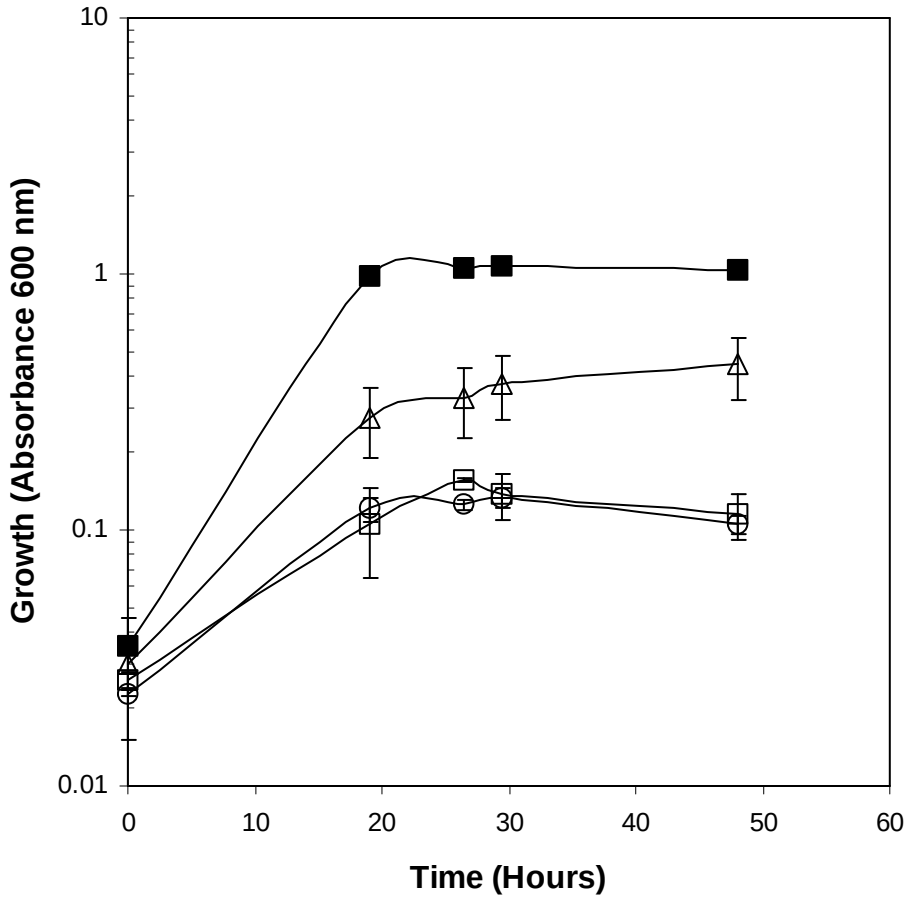

Figure 1.13. The effect of the addition of DNA to medium E on the anaerobic growth of *Bacillus mojavensis* ROB2.

Squares, medium E; circles, medium E plus ribonucleosides (0.1 g/l each of adenosine, guanosine, cytidine and thymidine) and 1 g/l Casamino acids; triangles, medium E plus 0.5 g/l herring sperm DNA; filled squares, medium E plus 0.5 g/l herring sperm DNA and 10 g/l Proteose Peptone.

Chapter 2. Anaerobic Growth of *Bacillus mojavensis* JF-2 requires DNA or deoxyribonucleosides

Abstract

Bacillus mojavensis JF-2 required four deoxyribonucleosides or DNA for growth under strict anaerobic conditions. The requirement for the deoxyribonucleosides or DNA did not occur under aerobic growth conditions. The addition of a mixture of five nucleic acid bases, four ribonucleotides or four ribonucleosides to the medium E did not replace the requirement for four deoxyribonucleosides. However, the individual addition of salmon sperm DNA, herring sperm DNA, *Echerichia coli* DNA and synthetic DNA (single or double stranded) to the medium E supported anaerobic growth. The addition of four deoxyribonucleosides to aerobic medium rescued *B. mojavensis* JF-2 from hydroxyurea-induced aerobic growth inhibition. DNA was not used as a sole energy source.

Introduction

Ribonucleotide reductase genes are considered essential to all organisms to regulate and maintain the deoxyribonucleotide pool necessary for DNA synthesis (4, 9, 15, 19, 21, 22). Recently, the minimal essential genes of *Bacillus subtilis* were narrowed to only 192 "essential" genes out of 4100 genes (10) and included the Class I ribonucleotide reductase genes, *nrd*E and *nrd*F, part of the deoxyribonucleotide biosynthetic pathway. The possibility that the deoxyribonucleotide pool can be regulated without the activity of a ribonucleotide reductase been largely discounted, in spite of the fact that, in 1952, *Lactobacillus johnsonii* was identified that required at least one externally supplied deoxyribonucleoside and was reported to lack ribonucleotide reductase activity (7). The idea that other organisms may depend on externally supplied deoxyribonucleosides was never pursued. In some cases, this lack of interest was probably because the requirement for a deoxyribonucleotide turned out in reality to be a requirement for thymine (3, 11, 18).

The ribonucleotide reductase enzymes are use to make deoxyribonucleotides regardless of whether the *de novo* pathways or the salvage pathways of DNA biosynthesis are used (15, 20). The *de novo* pathways make DNA from metabolic precursors produced in the anabolic pathways of metabolism. The salvage pathways use exogenously supplied bases and nucleosides or recycle endogenously produced bases and nucleosides (14).

In order to use deoxyribonucleosides as DNA precursors, an organism must be able to phosphorylate the deoxyribonucleoside to a deoxyribonucleotide. *Echerichia coli* and *Salmonella typhimurium* do not have the deoxyribonucleoside kinases necessary for

converting deoxyribonucleosides into deoxyribonucleotides and therefore cannot utilize exogenously supplied deoxyribonucleosides as sole DNA precursors (15). However, *Bacillus subtilis* and lactobacilli do have all the kinases necessary to phosphorylate the deoxyribonucleosides to deoxyribonucleotides and could potentially use them as sole DNA precursors when growing without the benefit of ribonucleotide reductase activity (20).

Three main classes of ribonucleotide reductases have been identified. One important difference among the reductases is their relationship to oxygen (9, 23). The class I ribonucleotide reductase requires oxygen to generate the tyrosyl radical needed to reduce the ribonucleotide to the deoxyribonucleotide (9). This class of ribonucleotide reductases is inhibited by hydroxyurea since hydroxyurea inactivates the tyrosyl radical. The class II ribonucleotide reductase neither uses oxygen nor is sensitive to oxygen and functions under both aerobic and anaerobic conditions (9). The class III ribonucleotide reductase has a glycyl protein radical that is destroyed by oxygen and thus functions only under anaerobic conditions (9). Each class of ribonucleotide reductase generates all four deoxyribonucleotides from the respective ribonucleotides di(or tri) phosphate substrates.

The apparent ubiquitous nature and mechanism of action of ribonucleotide reductases has led to the speculation that ribonucleotide reductases play a central role in cell metabolism and in cell cycle control both in eukaryotic and prokaryotic cells (5, 19). In eukaryotic cells, ribonucleotide reductase apparently stimulates DNA synthesis before and during hypoxic stress and interacts with other cell cycle control proteins, influencing the progression of the cell cycle (5). In prokaryotes, ribonucleotide reductases control the amount of ribonucleotides that are pulled from RNA production into DNA production (1, 2). The inference then is that cells cannot grow without ribonucleotide reductases and that the deoxyribonucleotide pool cannot be balanced exclusively by exogenously supplied deoxyribonucleosides.

However, during attempts to grow JF-2 under strict anaerobic conditions in defined medium, we found strong evidence that deoxyribonucleotide pools must be maintained through exogenously supplied deoxyribonucleosides. This suggests that other proteins, not solely ribonucleotide reductases, can regulate the intercellular balance of ribonucleotides and deoxyribonucleotides, and that ribonucleotide reductases may not be essential to the cell cycle.

Methods and Materials

Medium Additions

Adenosine (Sigma A4036), cytidine (Sigma C4654), guanosine (Sigma G6264), and thymidine (Sigma T1895) were combined to provide a ribonucleoside stock solution and adenosine monophosphate (ICN 100080), cytidine monophosphate (Sigma C1006), guanosine monophosphate (Sigma G8577), and thymidine monophosphate (Sigma T7004) were combined to provide a nucleotide stock solution. The deoxyribonucleoside solution consisted of deoxyadenosine (Sigma D8668), deoxyguanosine (BioChemika 31070), deoxycytidine (Sigma D0776) and thymidine (Sigma T1895). The final

concentration of each ribonucleoside, deoxyribonucleoside and ribonucleotide in the medium was 0.10 g/l. An unbalanced pool was achieved by adding 0.1 g/l of three deoxyribonucleosides and 0.2 g/l of the fourth deoxyribonucleoside. For the latter experiment, each deoxyribonucleoside was tested in excess separately, since similar absorbances were obtained in growth experiments for each condition, the results were averaged together to give the results shown on the graph.

Salmon sperm DNA (Sigma D-1626), herring sperm DNA (Sigma D-3159), and *Echerichia coli* DNA (Sigma D-2001) (individually added at 1.0 g/l) were added directly to the medium before autoclaving. RNA (Sigma R-6625) (1 g/l) was added after autoclaving.

Synthetic DNA: A random sequence of 50 nucleotide bases was generated and then tested for hairpin turns and self-annealing sequences with the oligonucleotide properties calculator found at www.basic.nwu.edu/biotools/oligocalc.html. Selected bases were changed until a sequence was generated that did not contain hairpin turns, or self-annealing areas, and was about 50% GC. The final sequence, named JF-2 SS, was TGG CGA AGG ATG CTG GCT ACA CTG CAG TTA TCT CTC ACC GTT CTG GCG AA. A DNA sequence that was complementary to JF-2 SS, named JF-2 COM was also generated and tested. DNA was obtained from Integrated DNA Technologies (IDT). To determine if single-stranded or double-stranded DNA supported anaerobic growth, three tubes of medium E with 0.5 g/l each of JF-2 SS, JF-2 COM and JF-2 SS plus JF-2 COM were used.

Agarose Gel Electrophoresis

E. coli and herring sperm DNA were run on a 30-ml, 1.0% Agarose gel with 2 µl of ethidium bromide (1 µg/ml) and PCR markers (1000 to 50 BP) to determine the size of the oligonucleotides. The gel was run for 30 minutes at 96 volts. The gel was viewed under UV light and recorded using a Nucleocam by Nucleotech Imaging (San Mateo, Ca).

Results

Salmon sperm DNA replaced the requirement for Proteose peptone during anaerobic growth of JF-2 in reduced medium E (Chapter 1). Other sources of DNA were also tested for the ability to support anaerobic growth of JF-2. The addition of herring sperm DNA (oligonucleotide length of ~50 bp as determined by gel electrophoresis) (Figure 2.1) or *E. coli* DNA (1000-750 bp) (Figure 2.2) to medium E also supported anaerobic growth of JF-2. As little as 0.08 g/l of *E. coli* DNA was saturating for growth in medium E. These sources of DNA were extracted and purified (to various degrees of purity) from cells. It was possible that the growth-enhancing factor did not consist of DNA but rather an impurity retained in the process of DNA isolation. To exclude that possibility, synthetic DNA was obtained and tested for the ability to support anaerobic

growth of JF-2. The addition of single stranded or double stranded synthetic DNA (50 bp) to medium E also supported anaerobic growth of JF-2 (Figure 2.3).

The addition of RNA did not support growth (Figure 2.4). Further, the addition of 2-deoxyribose combined with nucleic acid bases did not support anaerobic growth (data not shown).

A mixture of deoxyribonucleosides consisting of deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine (each at 0.1 g/l) replaced the requirement for DNA, whereas the corresponding ribonucleosides did not (Figure 2.5). Subsequent studies showed that even when one deoxyribonucleoside was added at twice the concentration of the other three deoxyribonucleosides (0.1g/l of three deoxyribonucleosides and 0.2 g/l of 1) anaerobic growth proceeded in a manner indistinguishable from that of anaerobic growth in medium containing an equal concentration of each deoxyribonucleoside (Figure 2.6). The individual addition of a single deoxyribonucleoside to medium E supported growth to an absorbance less than 0.2 (Figure 2.7).

Aerobic growth of JF-2 in medium E was greatly reduced in the presence of 15 mM hydroxyurea. In the absence of hydroxyurea, the maximum absorbance was 1.3 compared to less than 0.2 in the presence of hydroxyurea. The addition of all four deoxyribonucleosides to medium E containing hydroxyurea resulted in growth similar to that of the control without hydroxyurea (Figure 2.8).

With the addition of up to 1g/l of DNA to medium E, it was possible that the DNA was used as a carbon source and not simply as a growth factor. However, Figure 2.9 shows that DNA did not serve as a sole carbon or energy source for JF-2. No growth occurred when medium E lacked sucrose, but had 1g/l DNA.

Discussion

The requirement for DNA or deoxyribonucleosides as a growth factor for anaerobic growth is unusual especially since these compounds are not required for aerobic growth. We have found that several strains of *Bacillus mojavensis* and several strains of *Bacillus subtilis* require deoxyribonucleosides of DNA for anaerobic growth (Chapter 3). Thus, this requirement is not the result of a mutation in a single laboratory strain since it has been detected in several strains of bacteria. The only other organism that has been reported to require deoxyribonucleosides is *Lactobacillus acidophilus* R-26 (7). *Lactobacillus acidophilus* R-26 requires deoxyribonucleosides even for aerobic growth. This organism was used in a DNA bioassay in 1952 and was rather extensively studied some 50 years ago (6, 8, 12, 13, 17).

Although it is clear that *B. mojavensis* JF2 requires deoxyribonucleosides for anaerobic growth, it is not yet clear how the deoxyribonucleosides are taken up. The bacilli are well known for their ability to uptake DNA by way of the competence system, although this usually involves long strands of double stranded DNA (20). The herring sperm, *E. coli*, and synthetic DNA, all of which satisfied the requirement for DNA, consisted of short oligonucleotides (from 50 to 1500 bases). The synthetic DNA was

effective in enhancing growth either as single-stranded or as double-stranded DNA. These data argue that the competence system may not be involved in the uptake of DNA for anaerobic growth. The possibility that secreted nucleases lyse DNA to individual deoxyribonucleotides is more likely. This hypothesis is supported by the fact that deoxyribonucleosides replaced the requirement for DNA.

Why deoxyribonucleosides are required by JF-2 for anaerobic growth is unclear, but may involve whether JF-2 has the ribonucleotide reductase necessary to make deoxyribonucleotides anaerobically. If JF-2 has only the Class I ribonucleotide reductase and not one of the other classes, it would be unable to reduce the ribonucleotide to the deoxyribonucleotide under anaerobic conditions. Thus, it would have to synthesize deoxyribonucleotides from exogenously supplied deoxyribonucleosides. This hypothesis is supported by the fact that the addition of deoxyribonucleosides, but not ribonucleosides to medium E supported anaerobic growth. The fact that the addition of deoxyribonucleosides rescues JF-2 from hydroxyurea growth inhibition also supports the notion that only the Class I ribonucleotide reductase is found in this organism.

The addition of only one deoxyribonucleoside to medium E does not support anaerobic growth of JF-2; all four deoxyribonucleosides are required. This supports the hypothesis that externally supplied deoxyribonucleosides are required to maintain internal deoxyribonucleotide pools. JF-2 is apparently unable to make all four DNA precursors from a single deoxyribonucleoside as theoretically would be possible with a deoxyribosyltransferase. Deoxyribosyltransferases are enzymes that can exchange one purine or pyrimidine base attached to a deoxyribonucleoside for another. In fact deoxyribosyltransferases have been found in *Lactobacillus* and *Streptococcus* species but have not been reported in *Bacillus* species (16). It is also interesting that an unbalanced pool where one deoxyribonucleoside is present at a 2-fold higher concentration supports anaerobic growth. This suggests that the organism has a mechanism for selective uptake of nucleosides or is able to balance the internal pool of deoxyribonucleotides in spite of an unbalanced external supply of deoxyribonucleosides.

It is possible that the requirement for DNA is a phenotypic characteristic of anaerobic growth of some bacilli and aerobic growth of some lactobacilli. However, other organisms may also have this requirement. Most bacteriological growth media, with the exception of Luria-Betani medium, do not contain deoxyribonucleosides (as indicated by typical analysis reports). As a result, bacteria that require deoxyribonucleosides would be unlikely to initiate or maintain growth on common laboratory media and may represent an important class of uncultured microorganisms. These data also suggest that currently unidentified proteins in addition to ribonucleotide reductases regulate the intercellular balance of ribonucleotides and deoxyribonucleotides. Further, ribonucleotide reductases may not (always) be essential to the cell cycle or an essential part of the *Bacillus* minimal genome.

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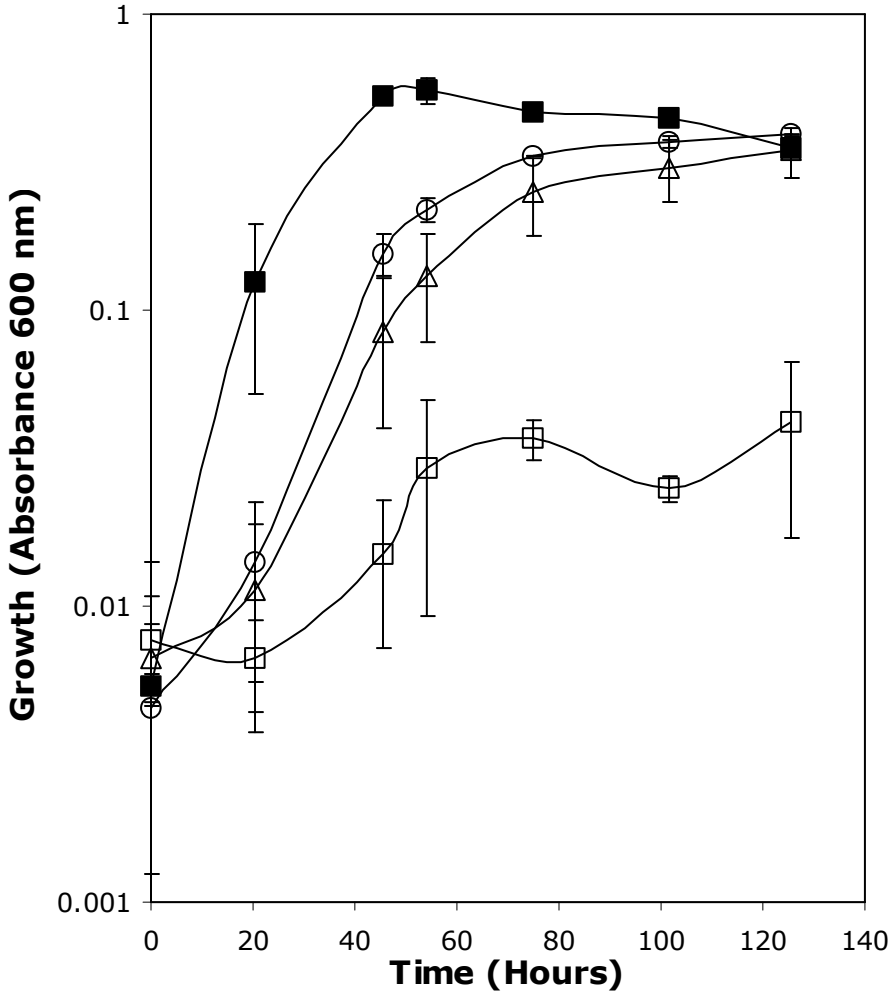

Figure 2.1. The effect of the addition of herring sperm DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 0.5 g/l of herring sperm DNA; triangles, medium E plus 1 g/l of herring sperm DNA; filled squared, medium E Plus 10 g/l of herring sperm DNA.

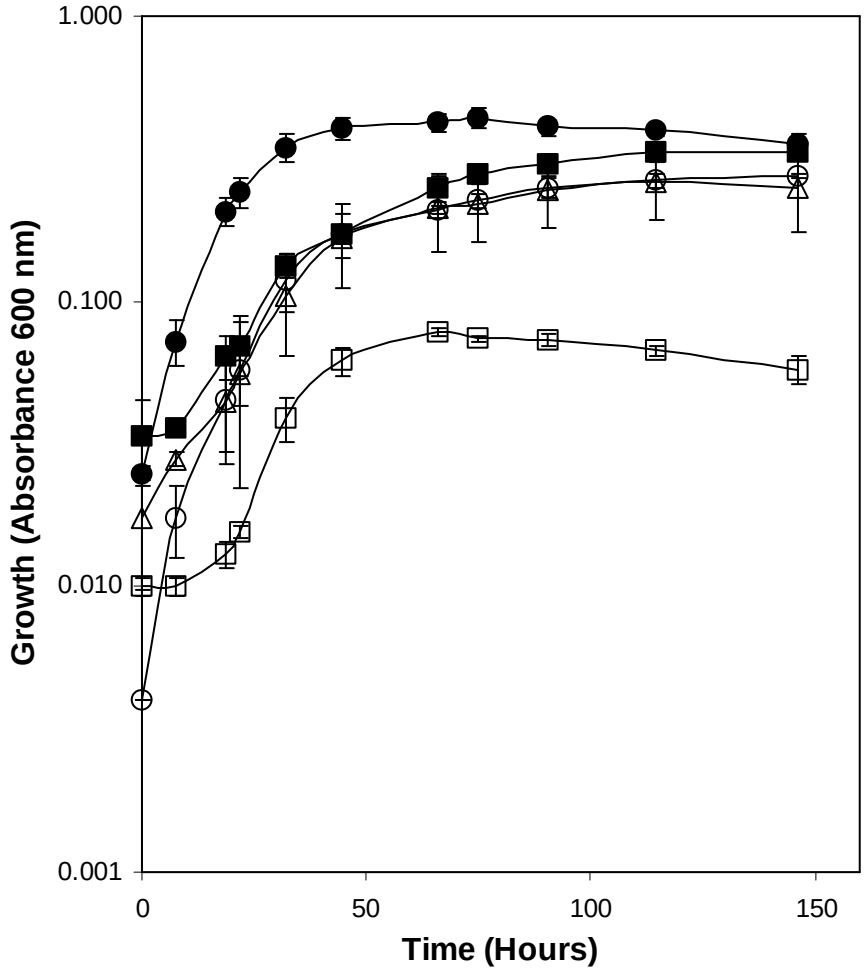

Figure 2.2. The effect of the addition of *Echerichia coli* DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 0.08 g/l of *E. coli* DNA; triangles, medium E plus 0.2 g/l of *E. coli* DNA; filled squares, medium E Plus 1 g/l of *E. coli* DNA; filled circles, medium E plus 1 g/l of herring sperm DNA.

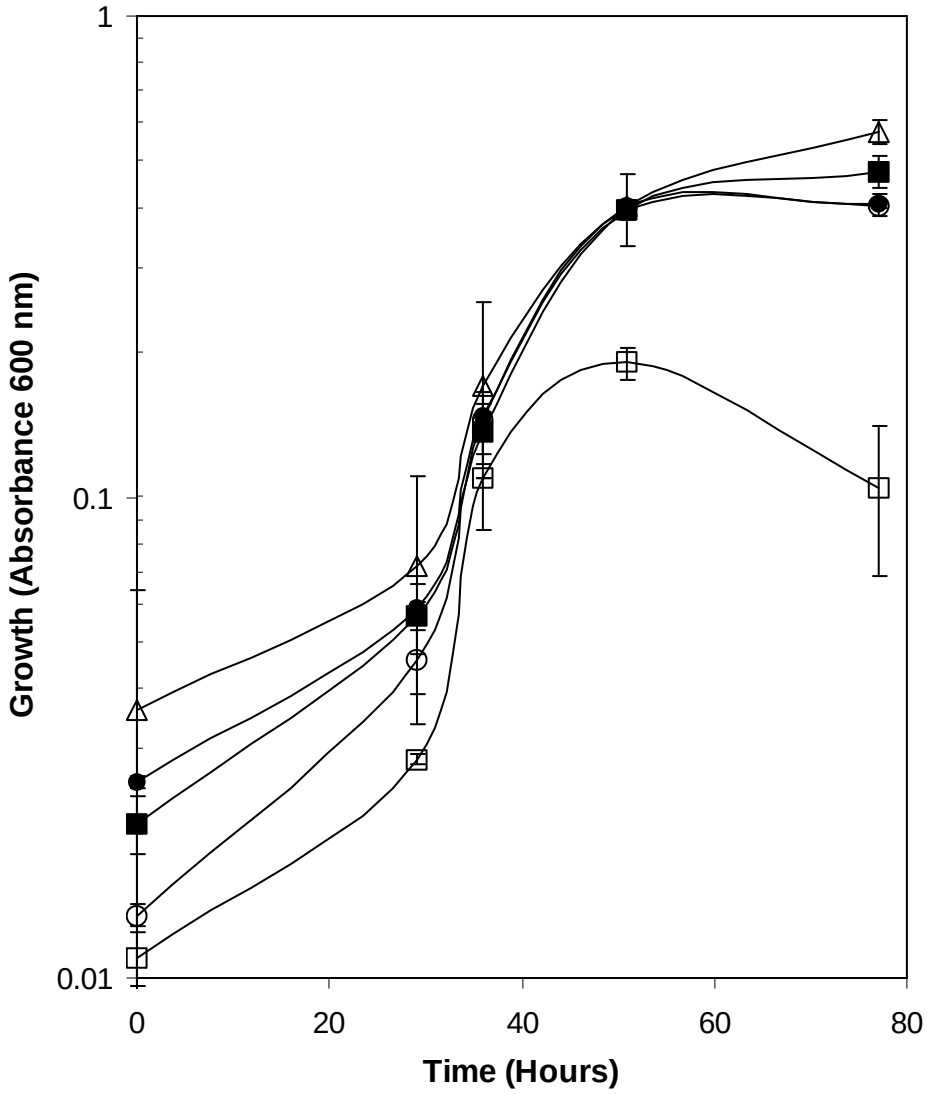

Figure 2.3. The effect of the addition of synthetic single-stranded and double-stranded DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 0.5 g/l of herring sperm DNA; triangles, medium E plus 0.5 g/l of single stranded DNA; filled squares, medium E Plus 0.5 g/l of single stranded (complementary strand) DNA; filled circles, medium E plus 0.5 g/l of double stranded DNA.

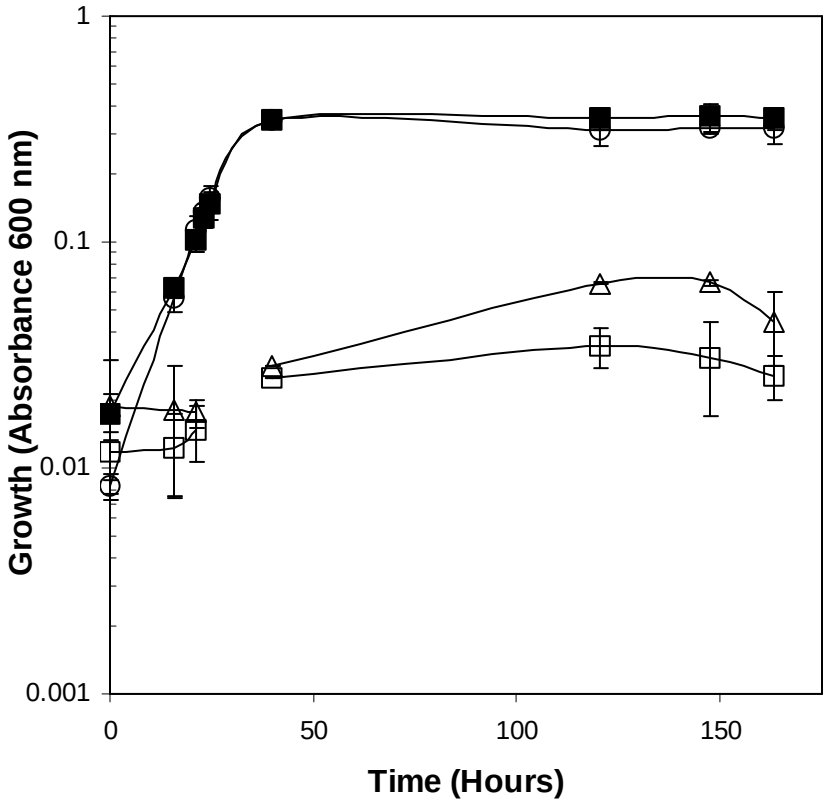

Figure 2.4. The effect of the addition of RNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 1 g/l of herring sperm DNA; triangles, medium E plus 1 g/l of RNA; filled squares, medium E Plus 1 g/l each of DNA and RNA.

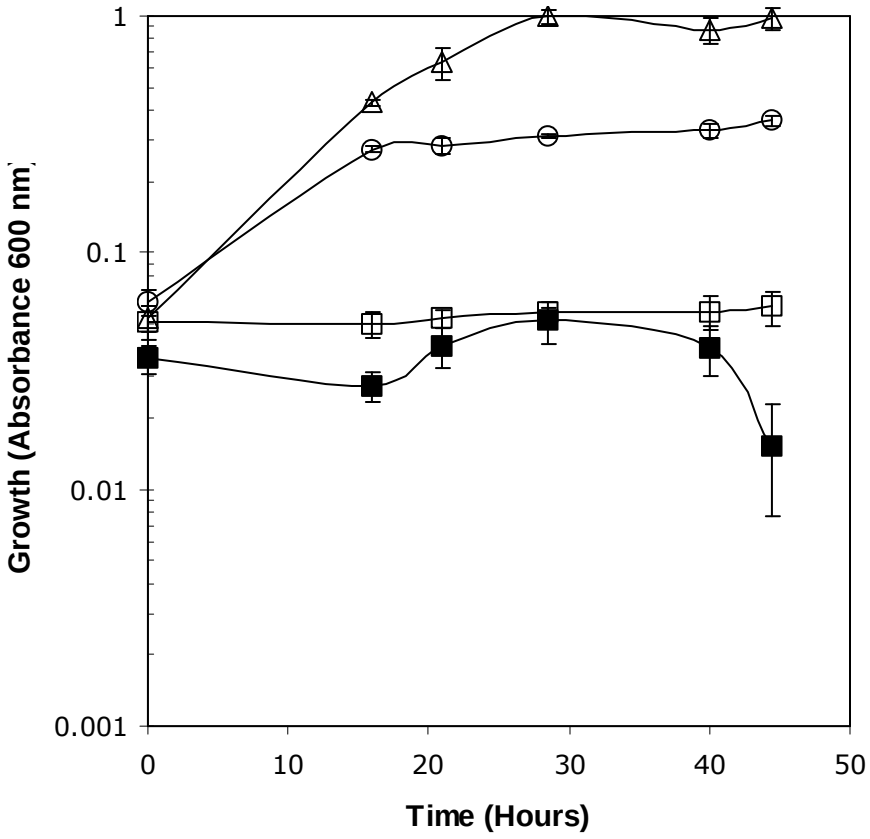

Figure 2.5. The effect of the addition of deoxyribonucleosides or ribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 1 of g/l herring sperm DNA; triangles, medium E plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; filled squares, medium E Plus 0.1 g/l each of adenosine, guanosine, cytidine and thymidine.

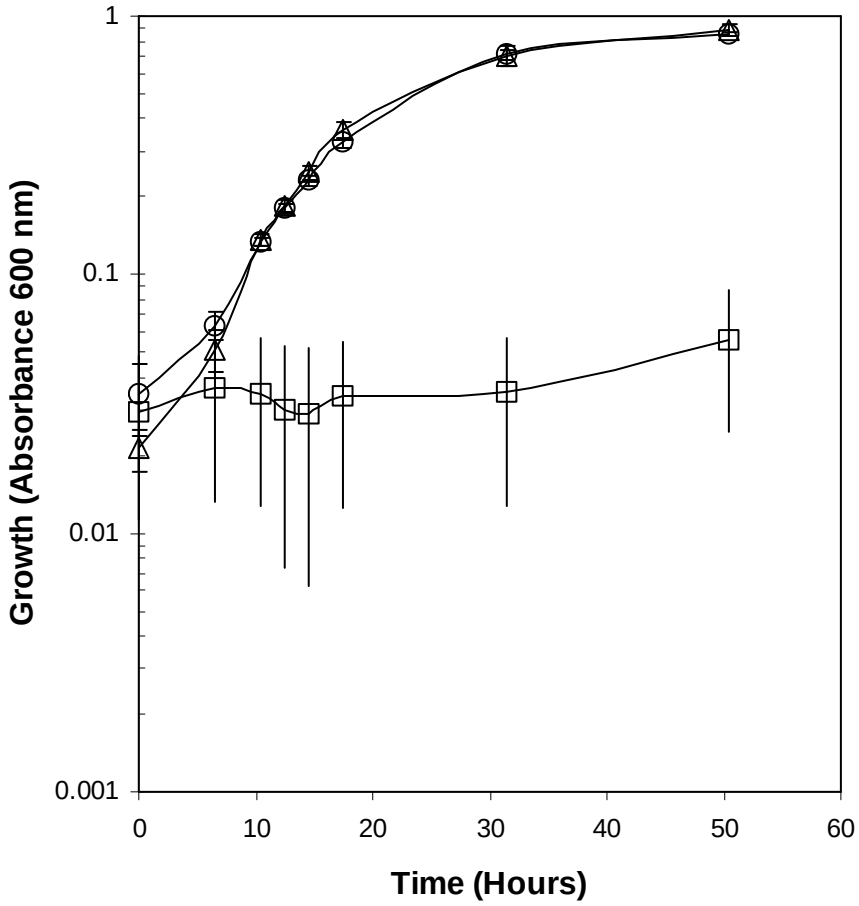

Figure 2.6. The effect of the addition of unequal amounts of deoxyribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, medium E plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; triangles, medium E plus 0.1 g/l each of three deoxynucleosides and 0.2 g/l of the fourth (average of all four possible combinations). Each deoxyribonucleoside was tested in excess separately but the results were similar and the four data sets were averaged to give the results shown above.

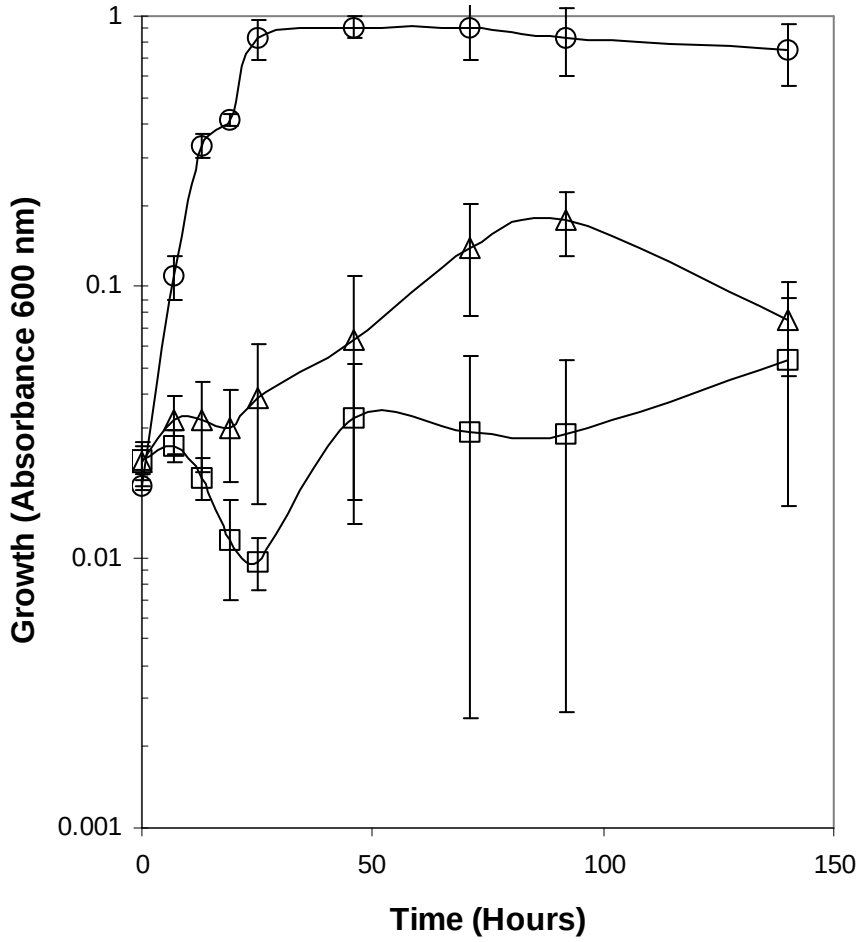

Figure 2.7. The effect of the addition of individual deoxyribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

Squares, medium E; circles, Medium E plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; triangles, medium E plus 0.1 g/l of a single deoxyribonucleoside. Individual deoxyribonucleosides were tested separately, but the results were similar and the four data sets were averaged to give the results shown for the curve represented by open triangles.

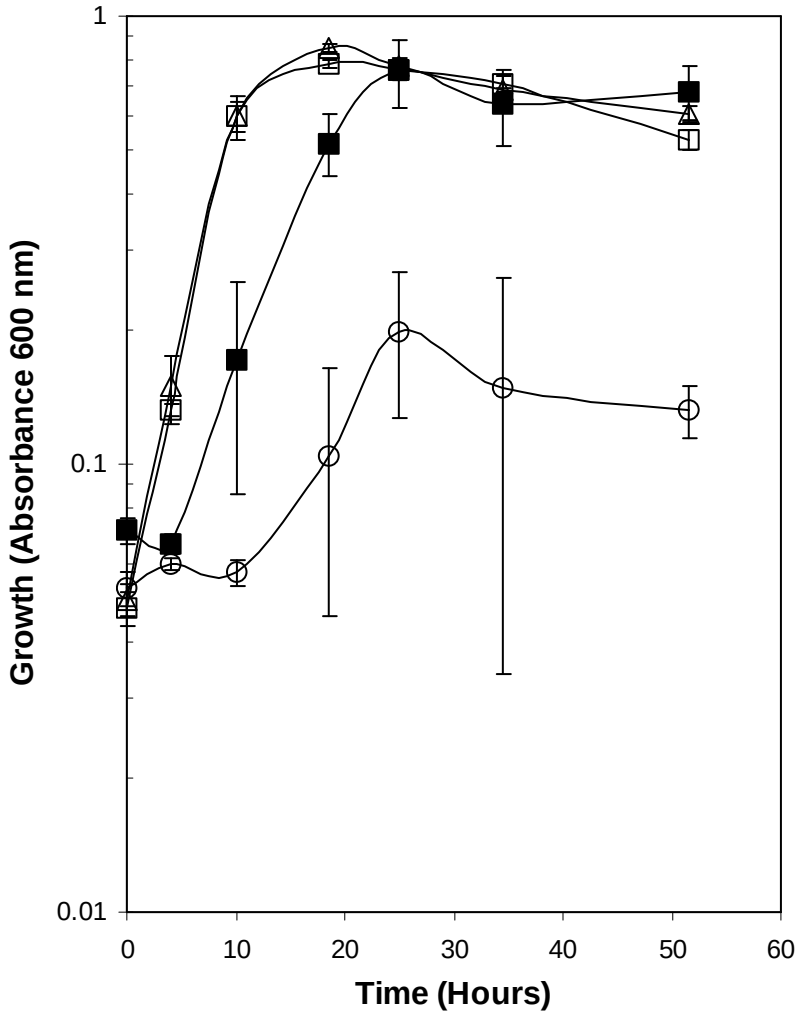

Figure 2.8. The effect of the addition of hydroxyurea and deoxyribonucleosides to Medium E on the aerobic growth of *Bacillus mojavensis* JF-2.

Squares, aerobic Medium E; circles, aerobic Medium E plus 0.1 g/l hydroxyurea,; triangles, Medium E Plus plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; filled squares, aerobic Medium E plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine, thymidine and hydroxyurea.

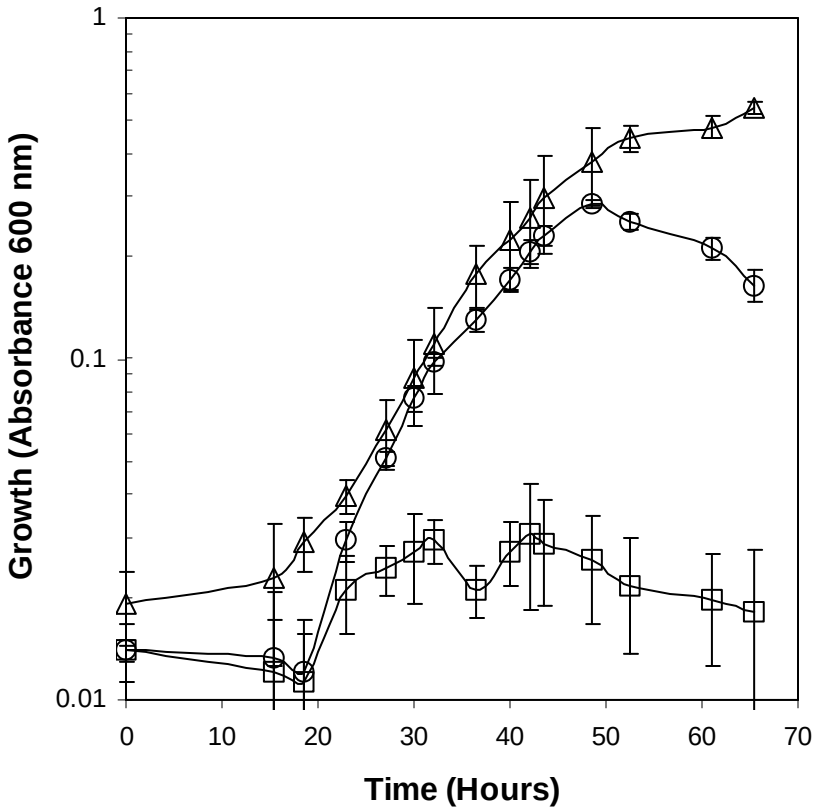

Figure 2.9. The effect of sucrose on anaerobic growth of *B. mojavensis* JF-2 in medium E supplemented with 1g/l DNA.

Squares, medium E with 1 g/l DNA without sucrose; circles, medium E with 1 g/l DNA plus 5 mM sucrose; triangles, medium E with 1 g/l DNA plus 20 mM sucrose.