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# *BACILLUS MOJAVENSIS* AND *BACILLUS SUBTILIS* REQUIRE DNA OR DEOXYRIBONUCLEOSIDES FOR ANAEROBIC GROWTH

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By

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# *BACILLUS MOJAVENSIS* AND *BACILLUS SUBTILIS* REQUIRE DNA OR DEOXYRIBONUCLEOSIDES FOR ANAEROBIC GROWTH

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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# Abstract

*Bacillus mojavensis* strain 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 is 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 enzymatic digests of protein to medium E improved anaerobic growth of JF-2. DNA from various sources replaced the requirement for enzymatic digests of protein for anaerobic growth of *Bacillus mojavensis* JF-2 and two other *B. mojavensis* strains. The addition of a mixture of four deoxyribonucleosides to medium E replaced the requirement for DNA. The addition of a mixture of five nucleic acid bases, four ribonucleotides or four ribonucleosides to medium E did not replace the requirement for four deoxyribonucleosides. 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 carbon and energy source.

Five *Bacillus subtilis* strains also required DNA or deoxyribonucleosides for anaerobic growth. *Bacillus subtilis* 168 required deoxyribonucleosides at a concentration approximately 10 times the theoretical requirement. *Bacillus subtilis* JH642 required about one quarter of the deoxyribonucleosides required by *Bacillus subtilis* 168. Amino acids were also required by *Bacillus subtilis* 168 for anaerobic growth. The addition of pyruvate to the medium did not replace the requirement for deoxyribonucleosides or amino acids. The addition of glutamine or nitrate did not replace the requirement for amino acids. Microaerophilic growth of *Bacillus subtilis* 168 and *Bacillus subtilis* JH642

was inhibited by 0.1 g/l hydroxyurea and growth inhibition by hydroxyurea was overcome with the addition of deoxyribonucleosides. Microaerophilic growth of *B. sonorensis* and *B. licheniformis* was not inhibited by 0.1 g/l hydroxyurea. A BLAST (NCBI) search failed to identify a Class II or a Class III ribonucleotide reductse in the *Bacillus subtilis* subsp. *subtilis* strain 168 genome and no *nrd*D or *nrd*J has been annotated for *B. subtilis*. All other *Bacillus* genomes available on the NCBI website had at least a putative *nrd*D gene (at least 32% identical and 49% similar) and most were annotated as anaerobic ribonucleotide-triphosphate reductases.

# Preface

The intent of the first chapter is to describe how anaerobic growth of JF-2 was improved with nutritional supplements. This chapter illustrates the attempt to improve anaerobic growth of JF-2 with many different nutritional supplements and then finally describes the isolation of the growth-enhancing factor, DNA. During the time of this research, undergrad students, Warren Frey and Michelle Staudt worked on determining the aerobic requirements for growth and biosurfactant production. Warren Frey conducted the experiments performed to determine the relationship between optical density and dry weight of *Bacillus mojavensis* JF-2, this data was later used to determine the dry weight of *Bacillus subtilis* cells as described in the third chapter. The second chapter is focused on describing how the requirement for deoxyribonucleosides was discovered and why this specific anaerobic requirement might exist. Chapters one and two were combined into a paper submitted to Applied and Environmental Microbiology.

Chapter three describes how the results of Chapters one and two were applied to explore the anaerobic growth of another bacilli, *Bacillus subtilis*. Cindy Russell provided a *B. subtilis* strain; Dr. Kathleen Duncan provided three *B. subtilis* strains and the *B. licheniformis* and *B. sonorensis* strains for this work. An undergraduate student, Ashley Geddie contributed to the initial BLAST searches for the Class III ribonucleotide reductase gene.

Appendix I is the paper submitted to Biotechnology. This paper describes the work done by Petroleum Engineering graduate student, Sai Maudgalya, and myself on oil recovery in sand packs. I cultured JF-2 to generate the biosurfactant solution; I measured

biosurfactant activity (surface tension) and I measured biosurfactant concentration on the HPLC. I modified an HPLC assay reported in the literature for biosurfactants for this assay. Sai Maudgalaya made the sand packs and did all of the other sand pack work.

Appendix II describes my further work in determining the anaerobic growth requirements of JF-2. This work was conducted to determine all the anaerobic growth factor requirements of JF-2 so as to eventually make it possible to introduce a limiting nutrient. I was specifically interested in limiting nitrogen (for potentially influencing biosurfactant production) so I wanted to eliminate any unnecessary sources of nitrogen, if possible.

Appendix III is a record of the data obtained from anaerobic growth experiments performed with *Bacillus licheniformis* and *Bacillus sonorensis*. I wanted to show that not all the bacilli I worked with required DNA or deoxyribonucleosides, given that so many did.

Appendices IV through VII show the results of the BLAST searches performed on various genomes. This was done to test the hypothesis that *B. subtilis* did not have any other ribonucleotide reductase gene other that for the Class I enzyme.

# General Introduction

Microbially enhanced oil recovery (MEOR) focuses on the use of microorganisms to re-establish oil flow from an oil well after current recovery technologies reach their economic limit (8). In one application of MEOR, microbial biomass is used to selectively plug high-flow sections of an oil well, forcing oil out of low-flow sections (5, 11). Alternatively, microbially-produced biosurfactants can be used to improve oil recovery (see Appendix I). Petroleum is trapped in porous rock by capillary forces and biosurfactants can generate the low interfacial tensions needed for the release of petroleum from an oil-bearing rock formation (1-3, 10, 12). The lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the interfacial tension between the hydrocarbon and aqueous phases to very low levels (<0.016 mN/m) (7, 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, and temperatures up to 45$^{o}$C (4, 6), making it ideally suited for in situ applications. However, anaerobic growth of *B. mojavensis* JF-2 is inconsistent and difficult to replicate, which limits its use for in situ applications. Here, I show that DNA or deoxyribonucleosides are required for the anaerobic growth of three *B. mojavensis* strains including strain JF-2, and five *Bacillus subtilis* strains. Understanding the anaerobic growth requirements of JF-2 will lead to a better understanding of 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 $^{o}$C (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 premade 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 |

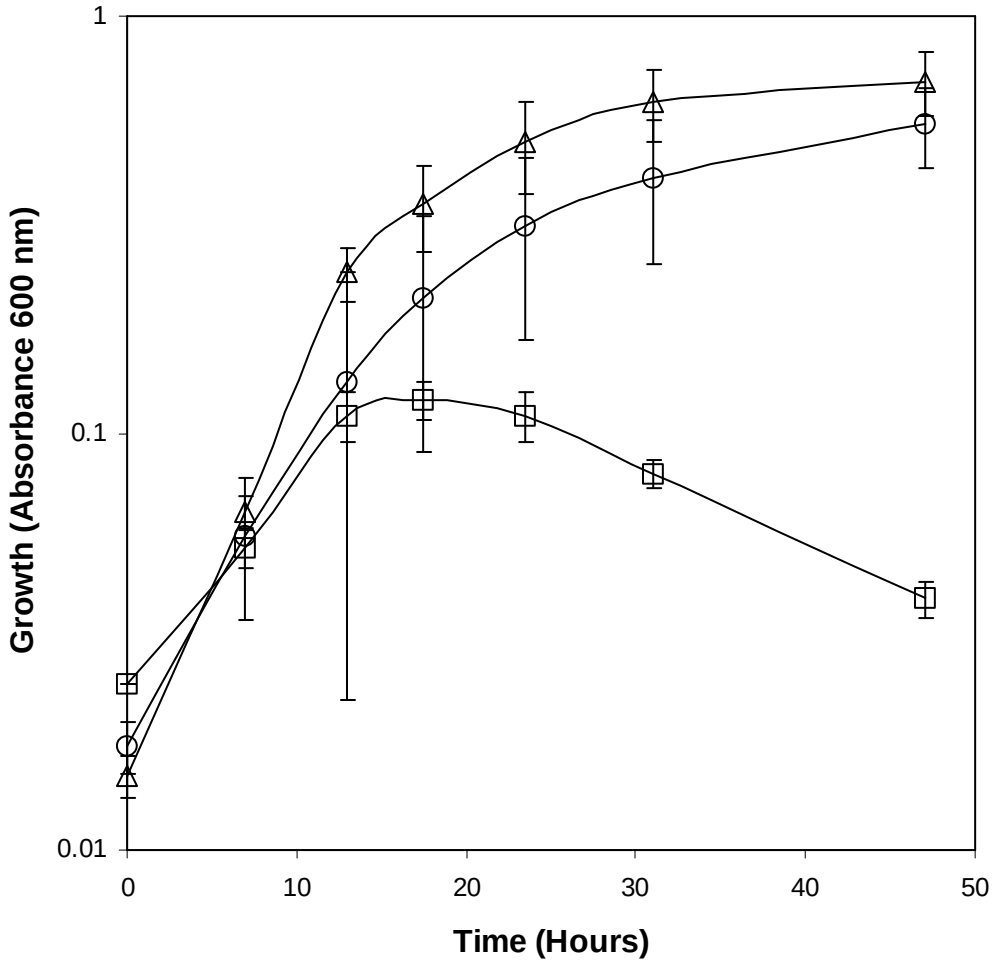


**Figure 1.1.** The effect of the addition of Tryptone to medium E on anaerobic growth of *B. mojavensis* JF-2.

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).

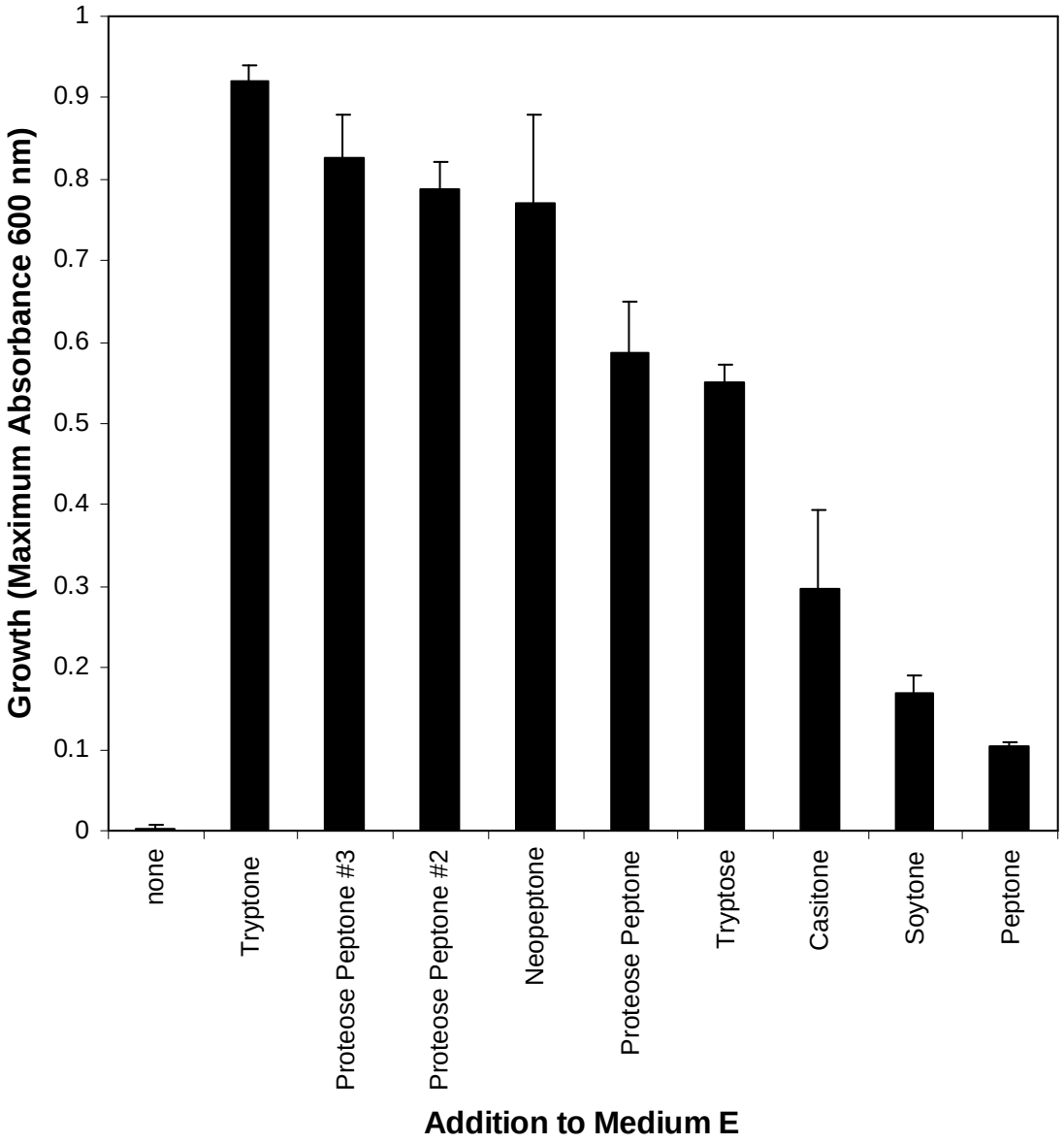


**Figure 1.2.** The effect of the addition of different enzymatic digests of protein to medium E on the anaerobic growth of *B. mojavensis* JF-2.

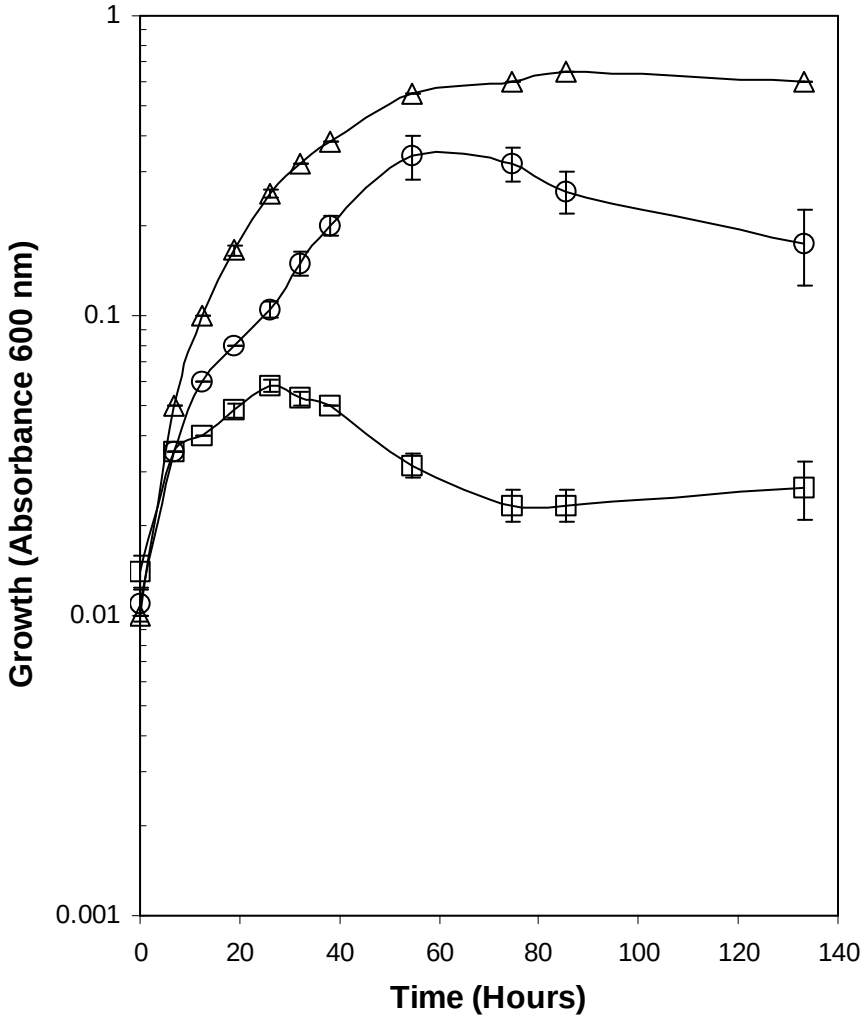


**Figure 1.3**. The effect of the addition of Tryptone and Proteose Peptone Medium E on the anaerobic growth of *B. mojavensis* JF-2.

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

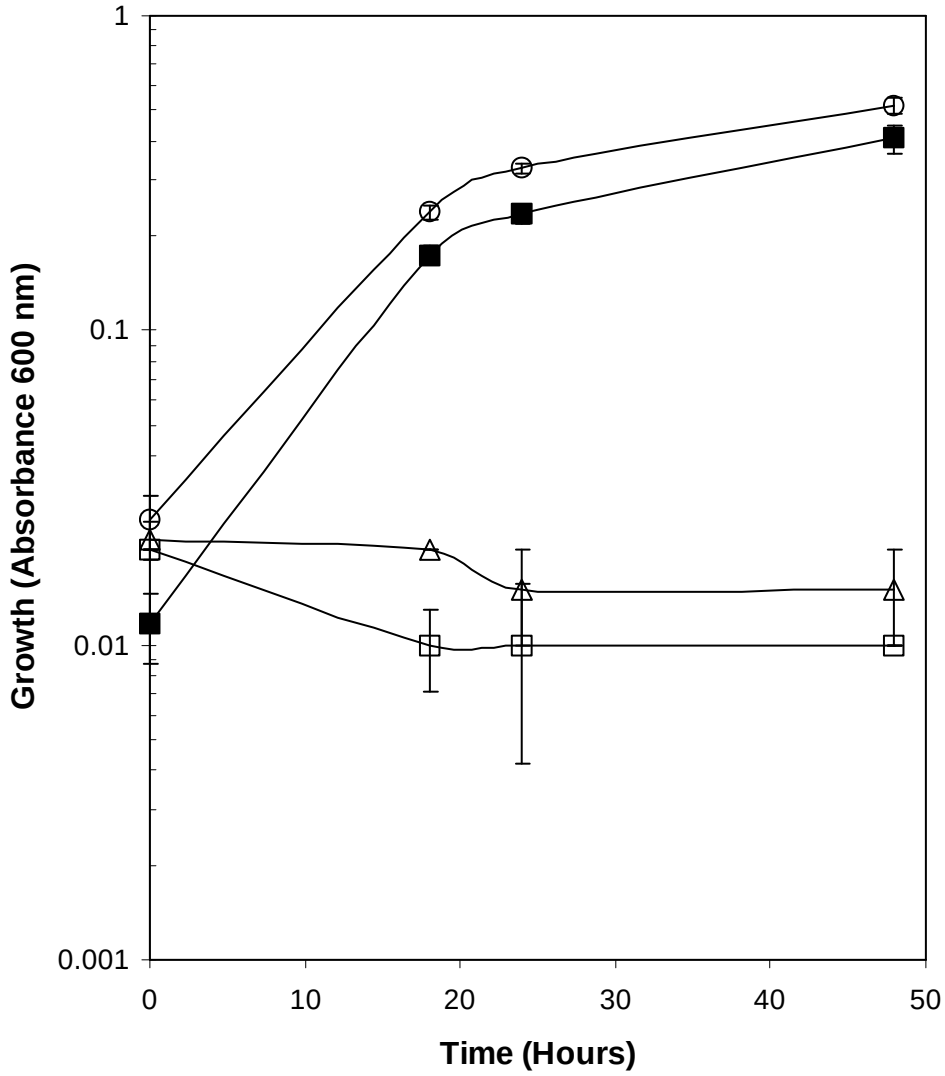


**Figure 1.4**. The effect of medium E supplemented with amino acids, vitamins, nucleic acids and fatty acids on anaerobic growth of *B. mojavensis* JF-2.

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.

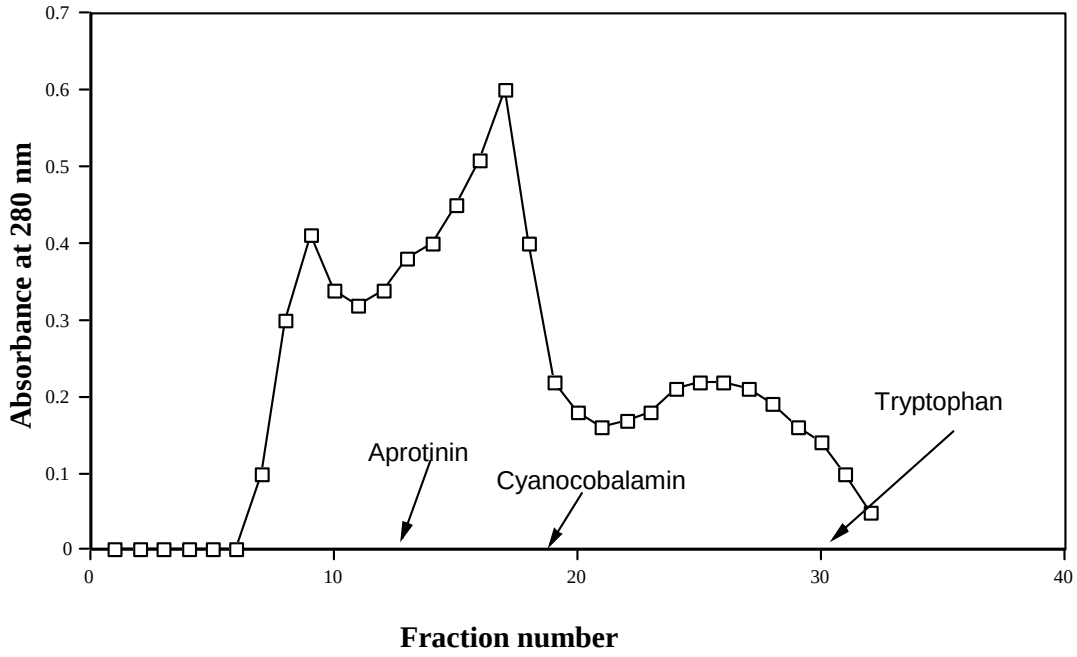


**Figure 1.5.** The UV absorbance of the fractions collected after fractionation of Proteose Peptone by size-exclusion chromatography.

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.

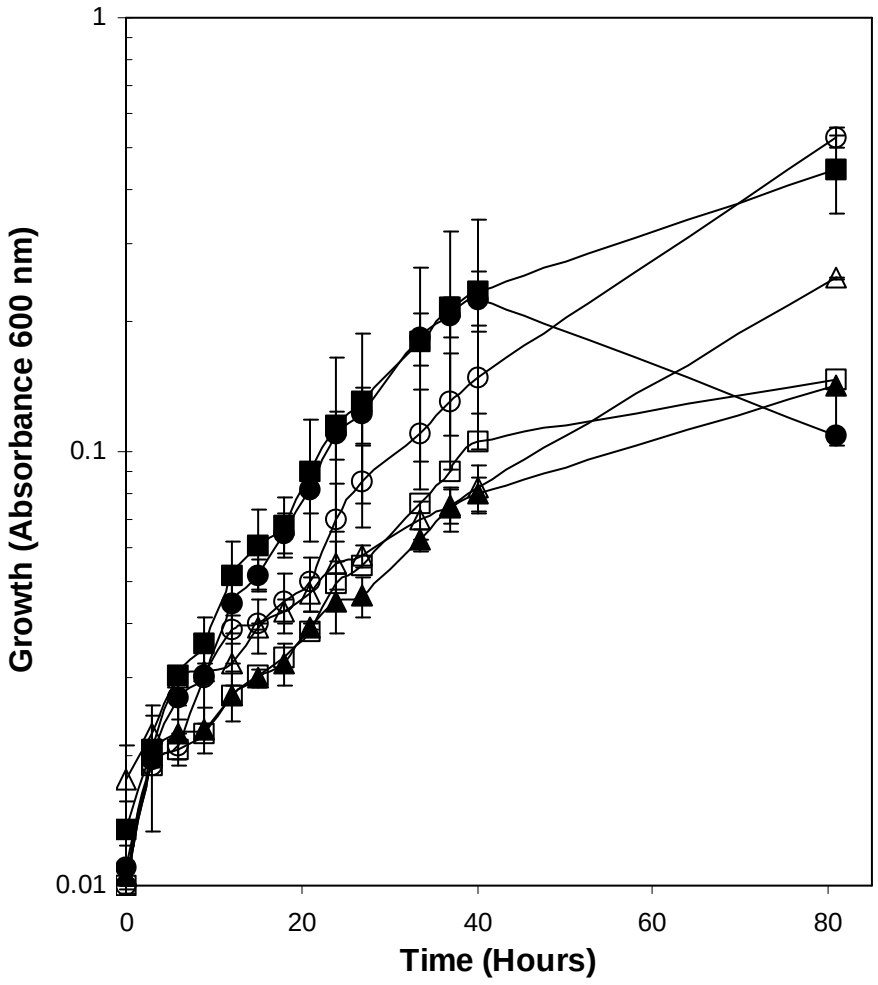


**Figure 1.6.** The effect of the addition of the pooled fractions (collected by size exclusion chromatography using Sephadex G25) to Medium E on anaerobic growth of *B. mojavensis* JF-2.

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 $F_D$.

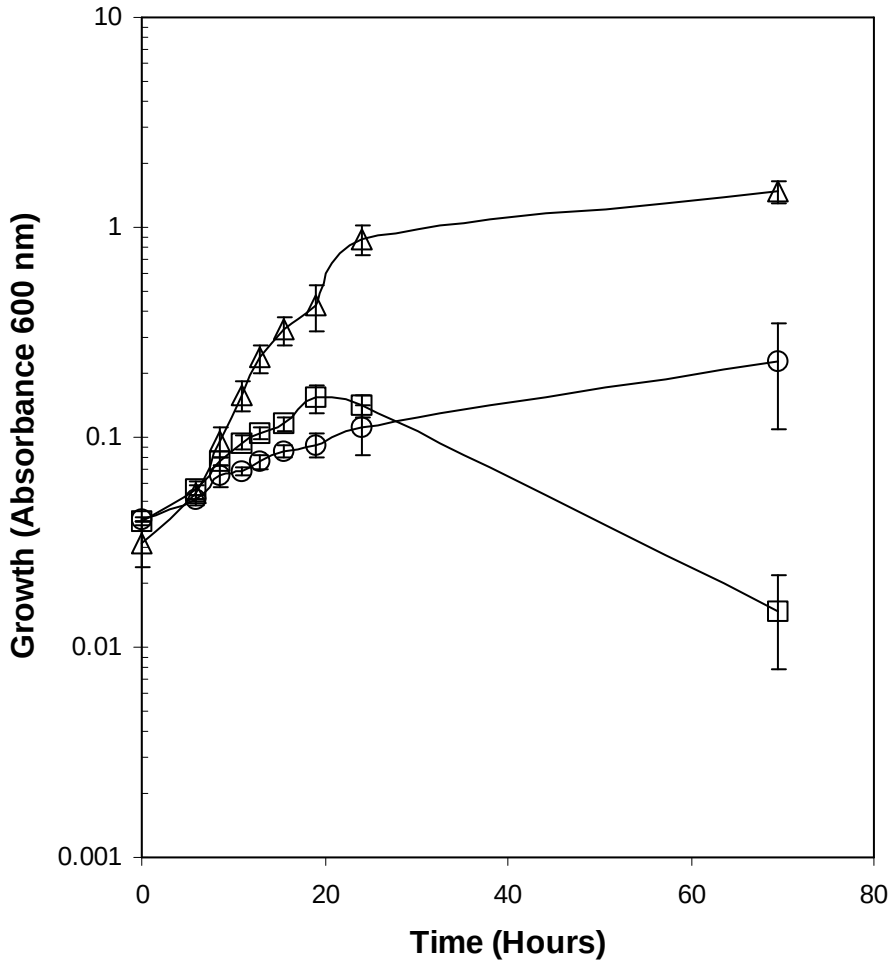


**Figure 1.7.** The effect of methanol soluble and methanol insoluble fractions on anaerobic growth of *B. mojavensis* JF-2.

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

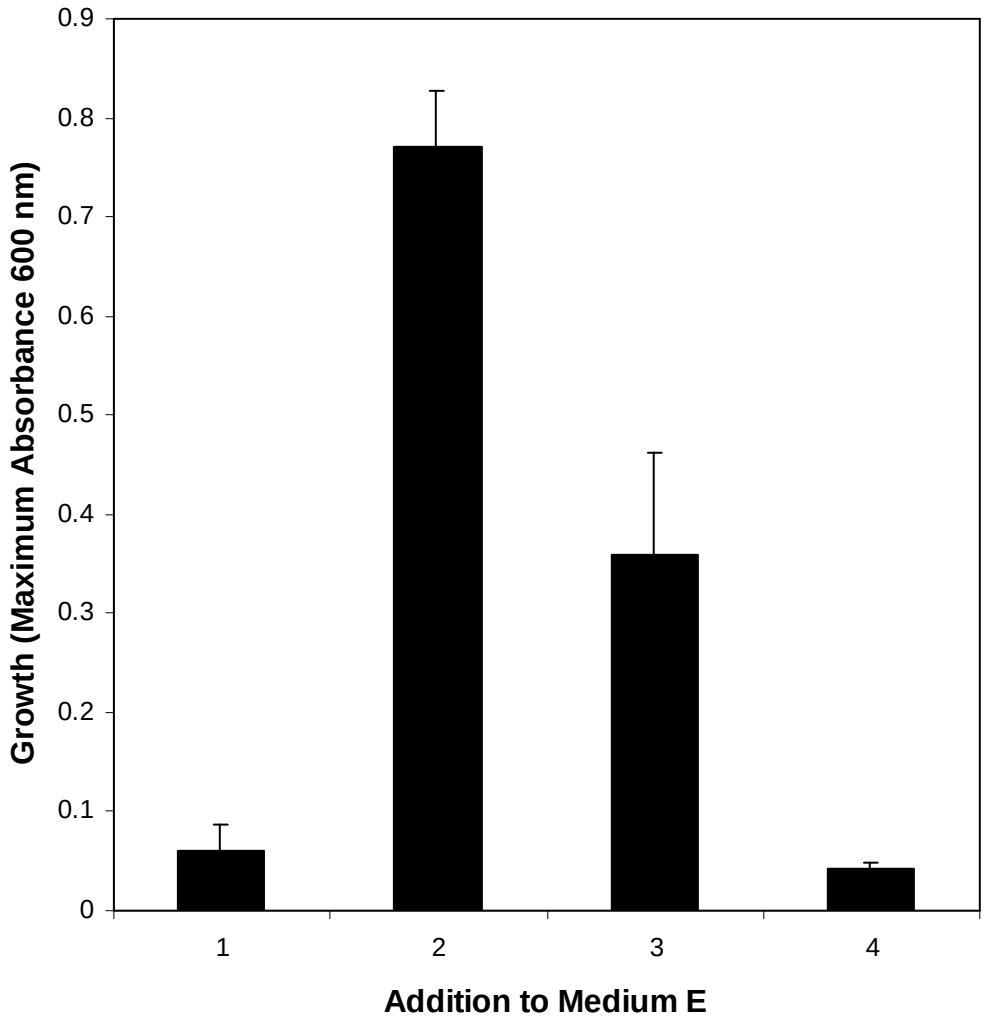


**Figure 1.8**. The effect of the addition of ion exchange fractions to Medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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

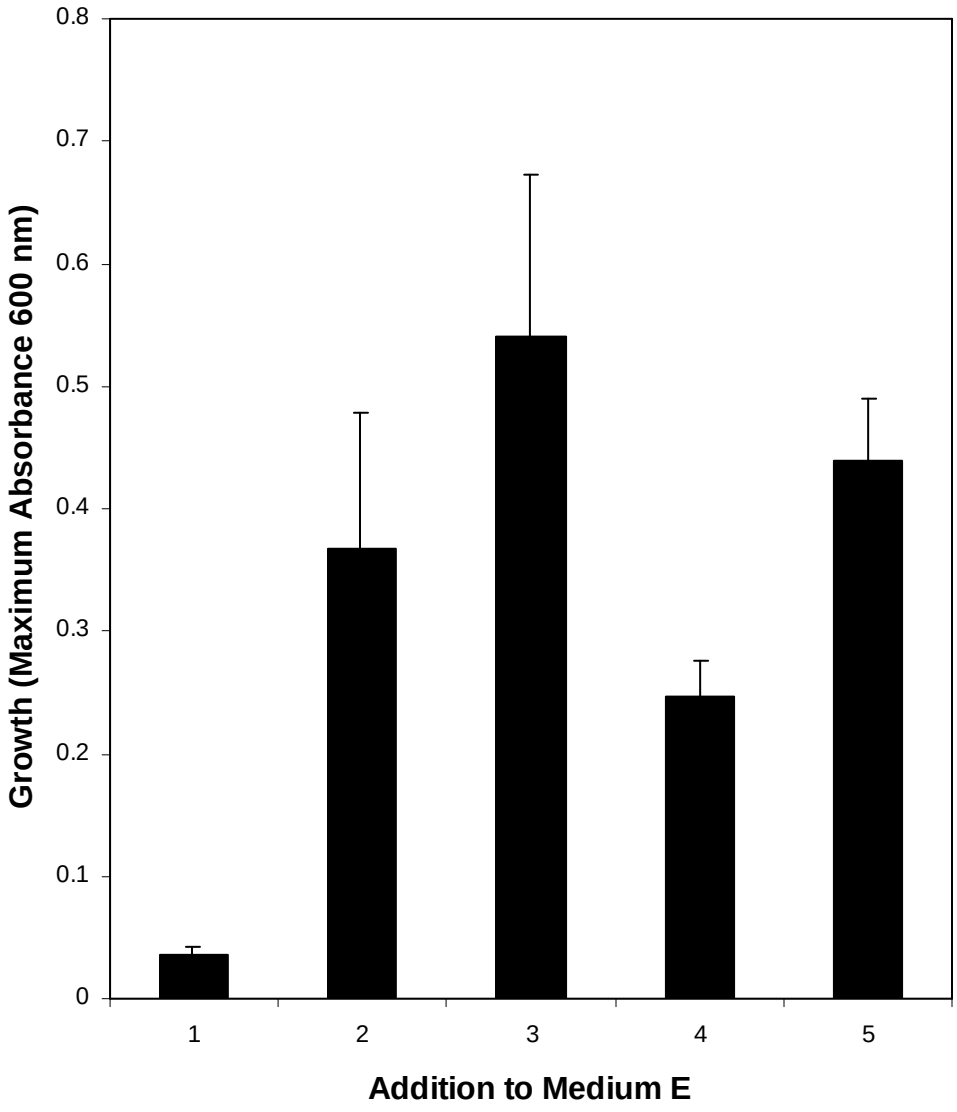


**Figure 1.9.** The effect of the addition of neutral, acid and base treated Proteose Peptone to Medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

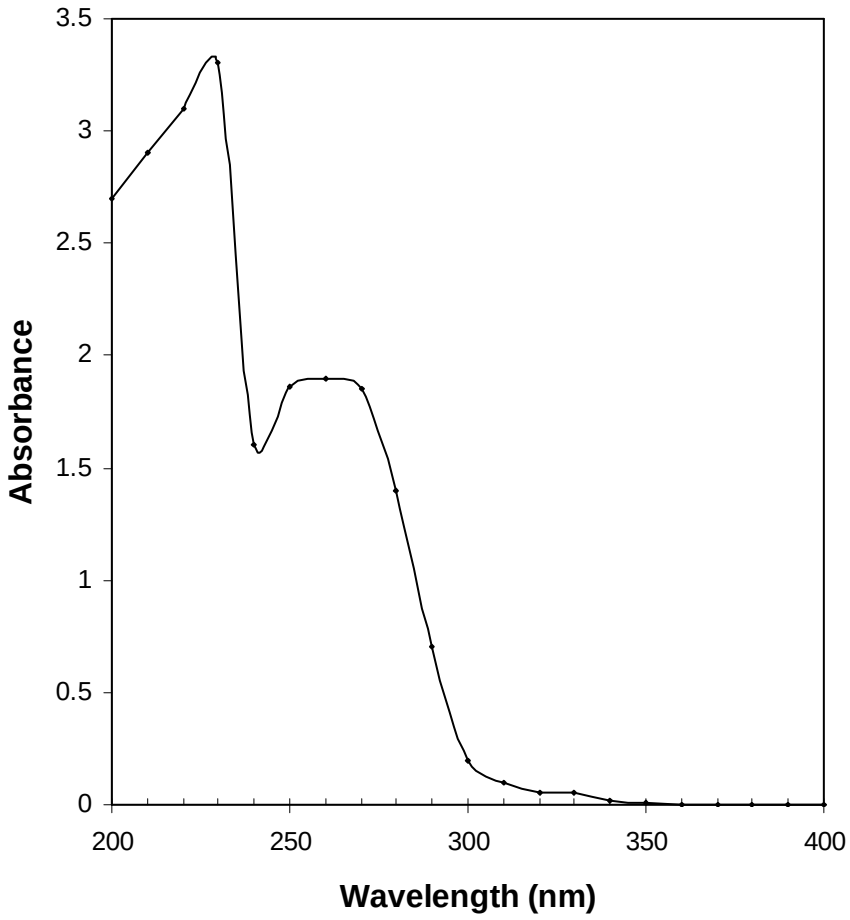


**Figure 1.10.** UV scan of the growth enhancing factor in Proteose Peptone after methanol extraction, size fractionation, and passage through a cation exchange.

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

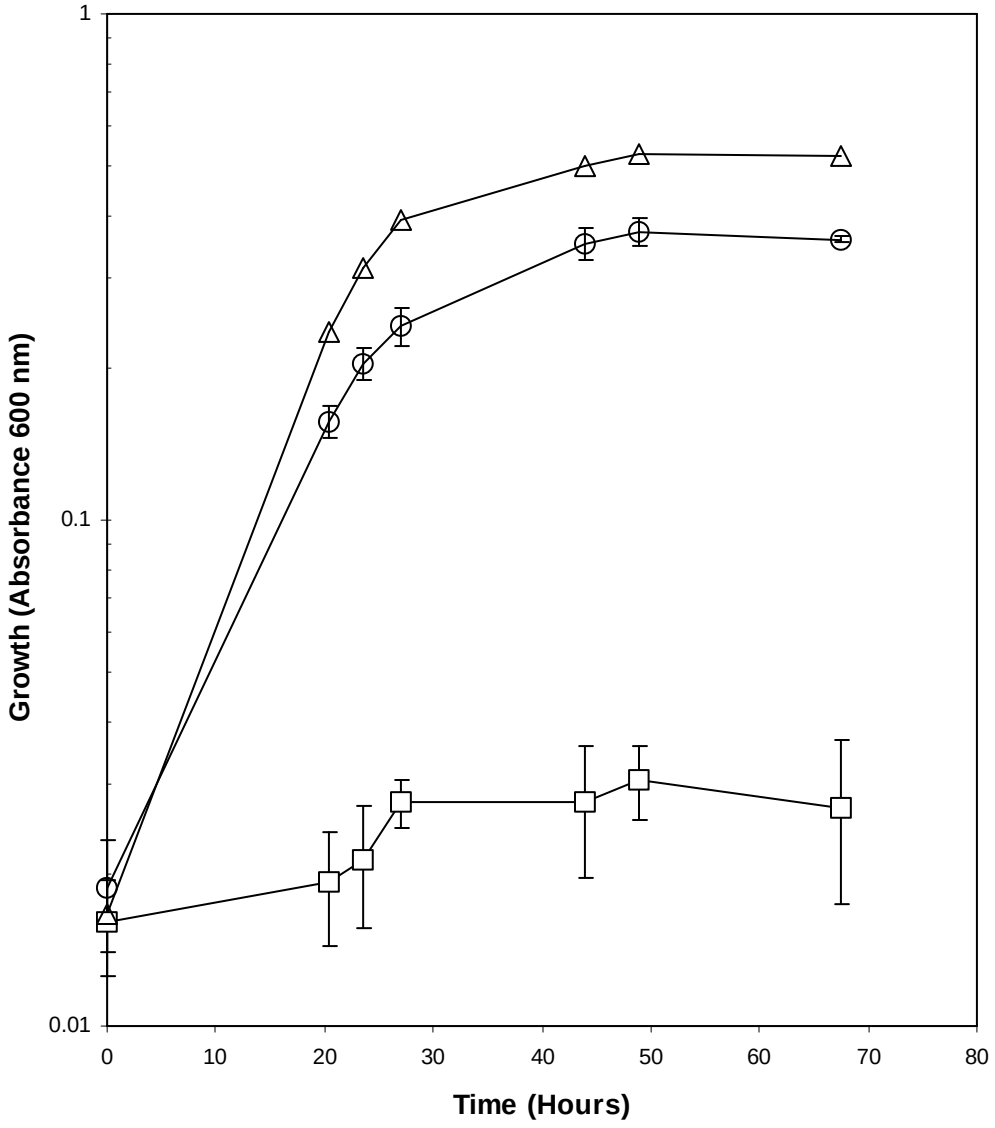


**Figure 1.11.** The effect of the addition of salmon sperm DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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

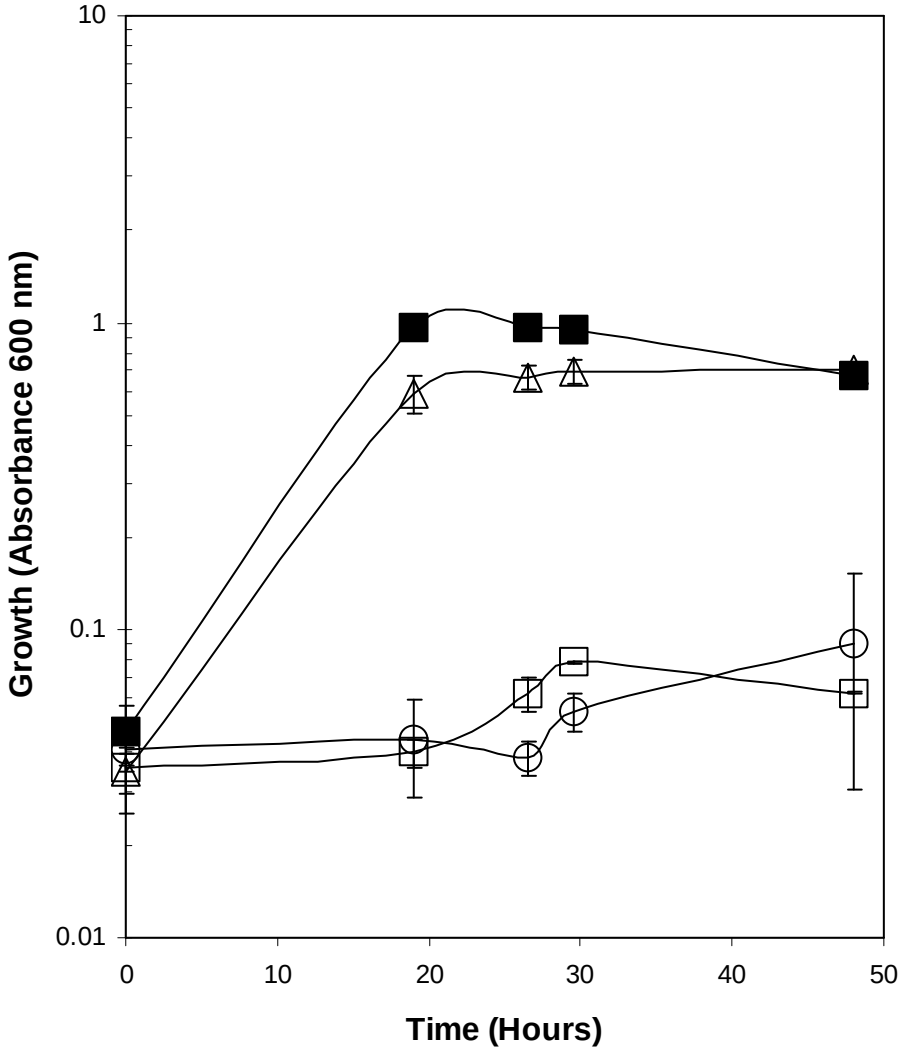


**Figure 1.12.** The effect of the addition of DNA to medium E on anaerobic growth of *Bacillus mojavensis* (ATCC AB021191).

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.

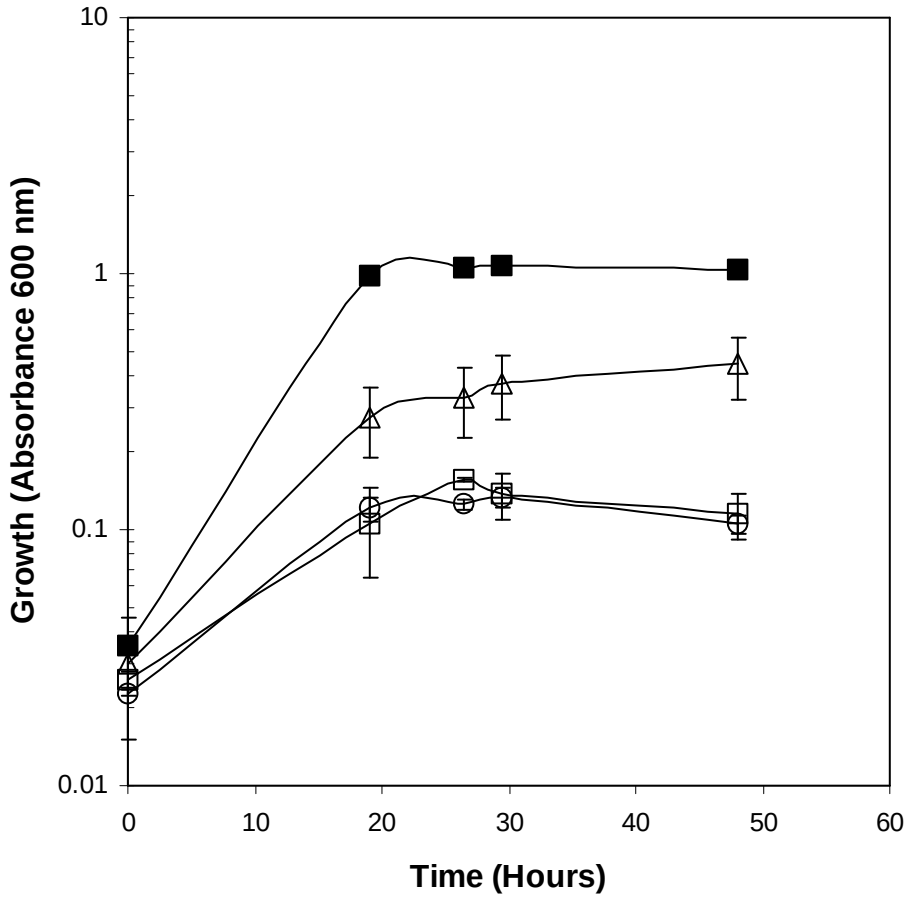


**Figure 1.13.** The effect of the addition of DNA to medium E on the anaerobic growth of *Bacillus mojavensis* ROB2.

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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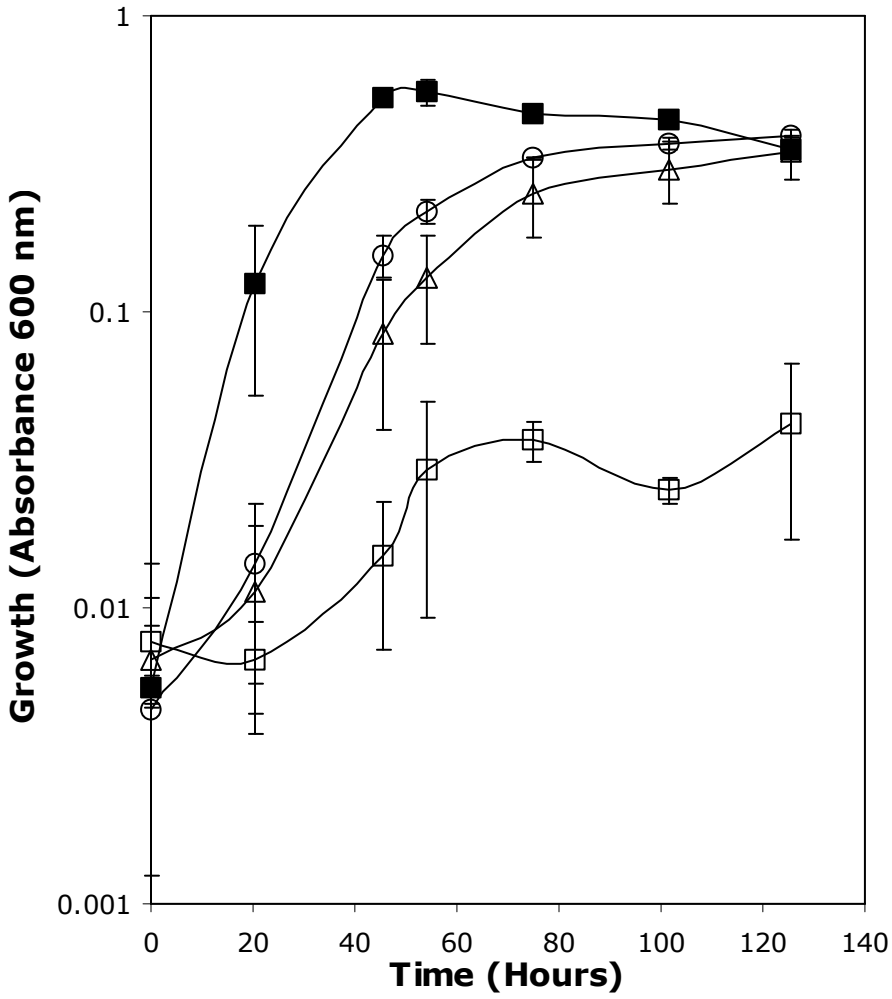


**Figure 2.1.** The effect of the addition of herring sperm DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

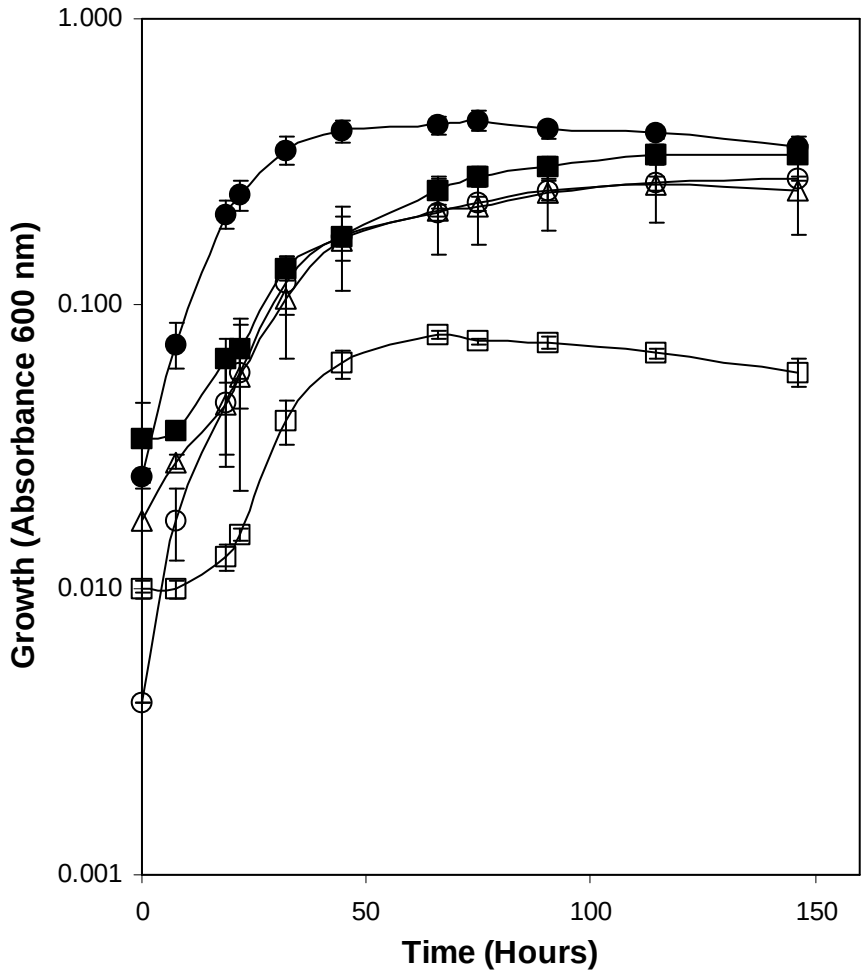


**Figure 2.2.** The effect of the addition of *Echerichia coli* DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

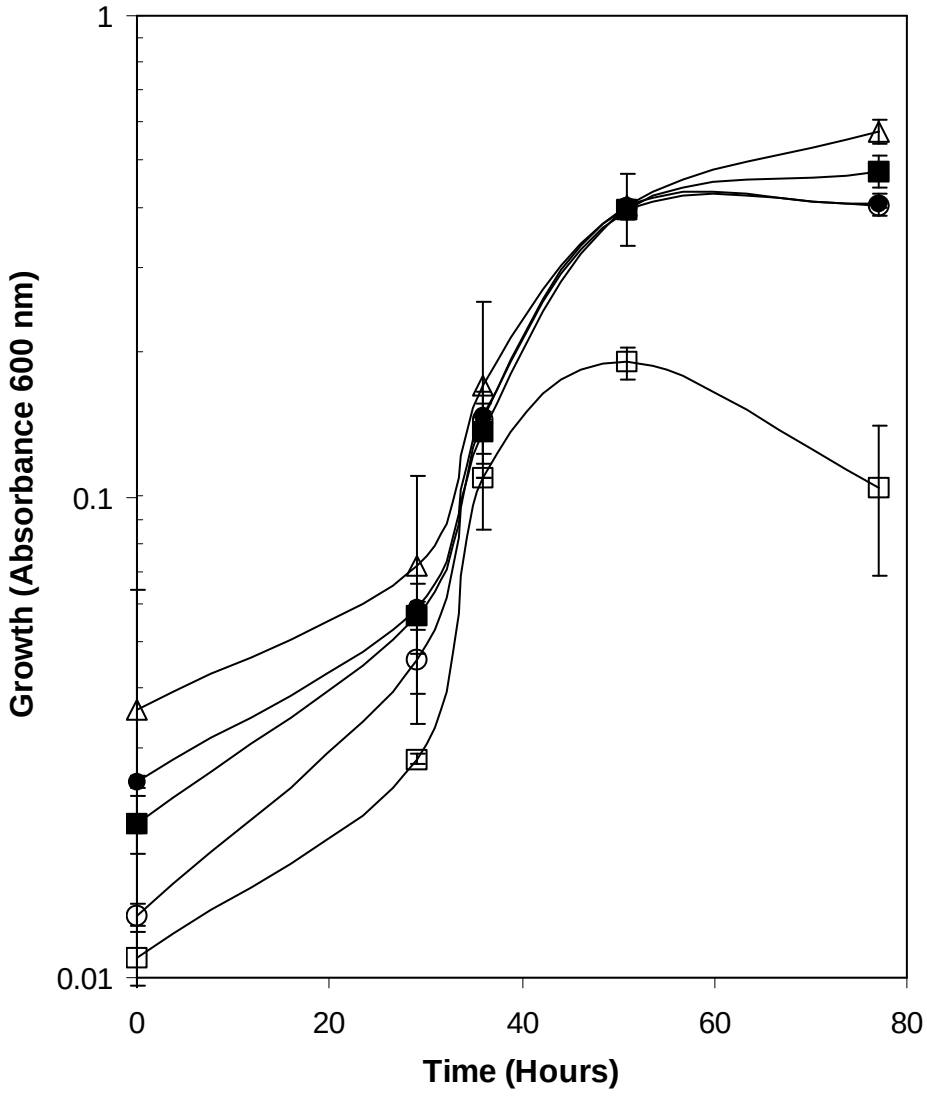


**Figure 2.3.** The effect of the addition of synthetic single-stranded and double-stranded DNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

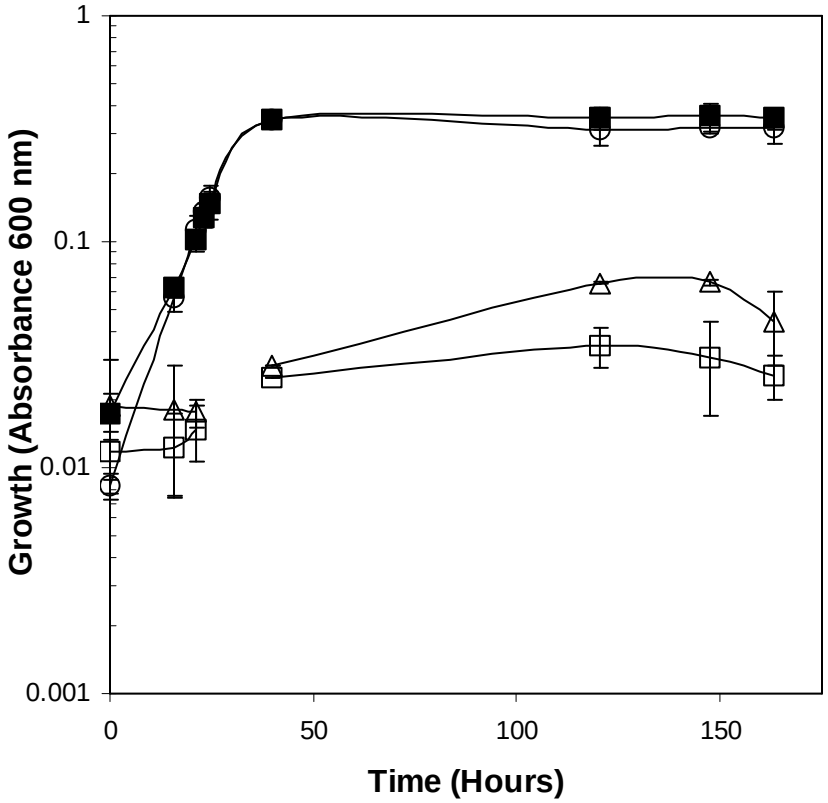


**Figure 2.4.** The effect of the addition of RNA to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

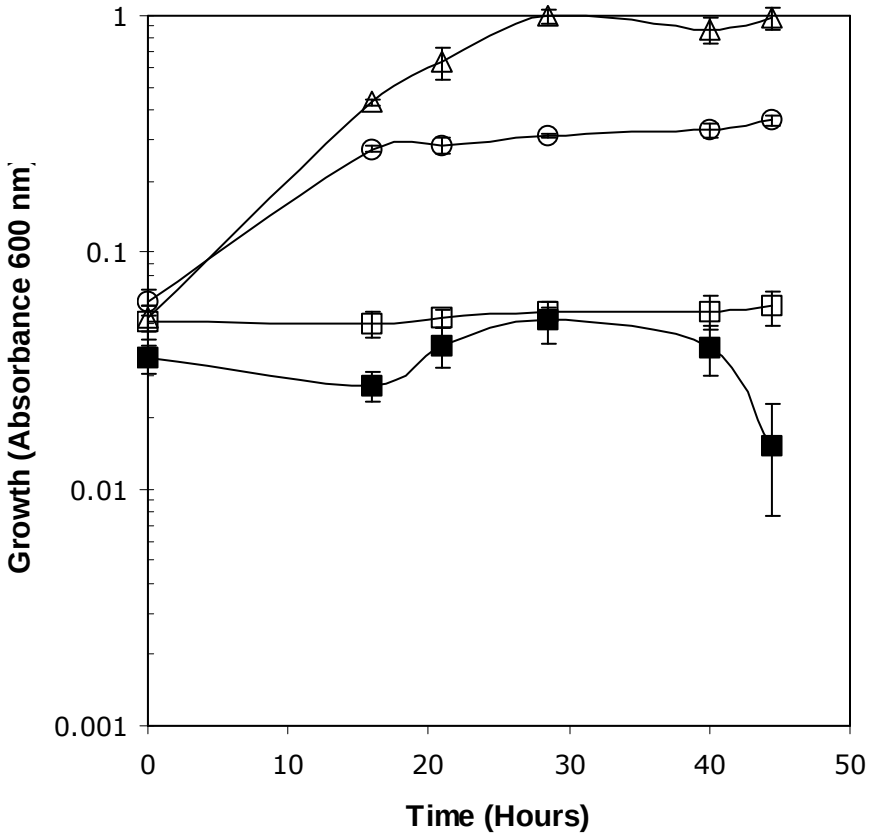


**Figure 2.5.** The effect of the addition of deoxyribonucleosides or ribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

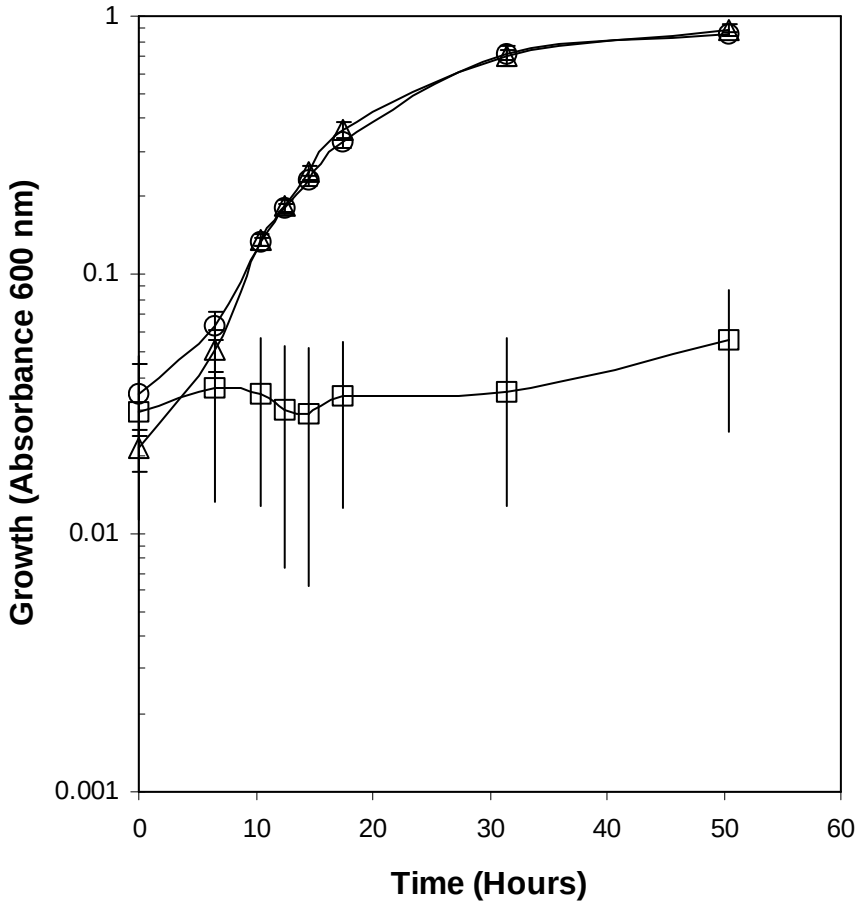


**Figure 2.6.** The effect of the addition of unequal amounts of deoxyribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

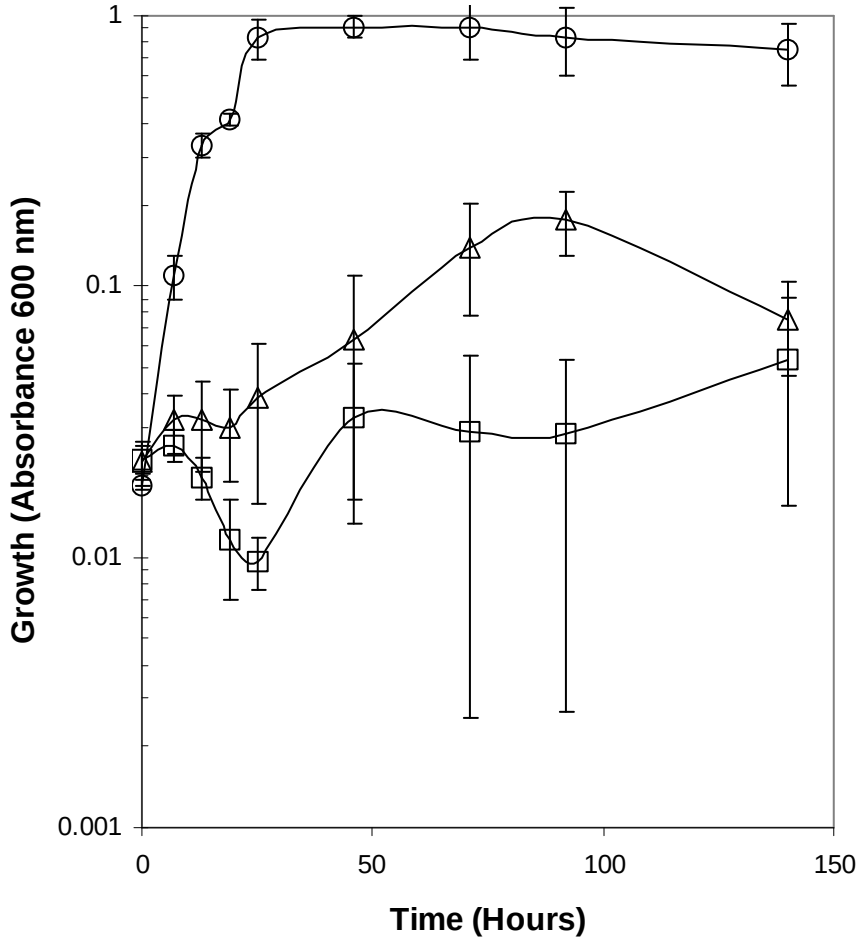


**Figure 2.7.** The effect of the addition of individual deoxyribonucleosides to medium E on the anaerobic growth of *Bacillus mojavensis* JF-2.

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.

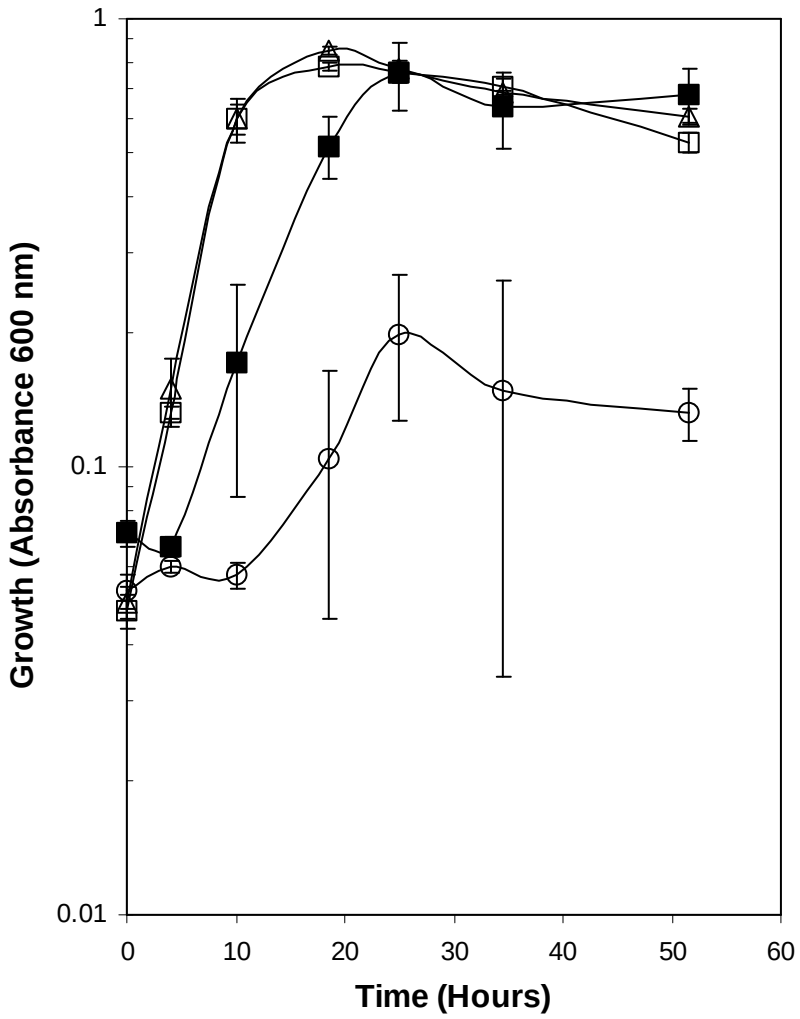


**Figure 2.8.** The effect of the addition of hydroxyurea and deoxyribonucleosides to Medium E on the aerobic growth of *Bacillus mojavensis* JF-2.

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.

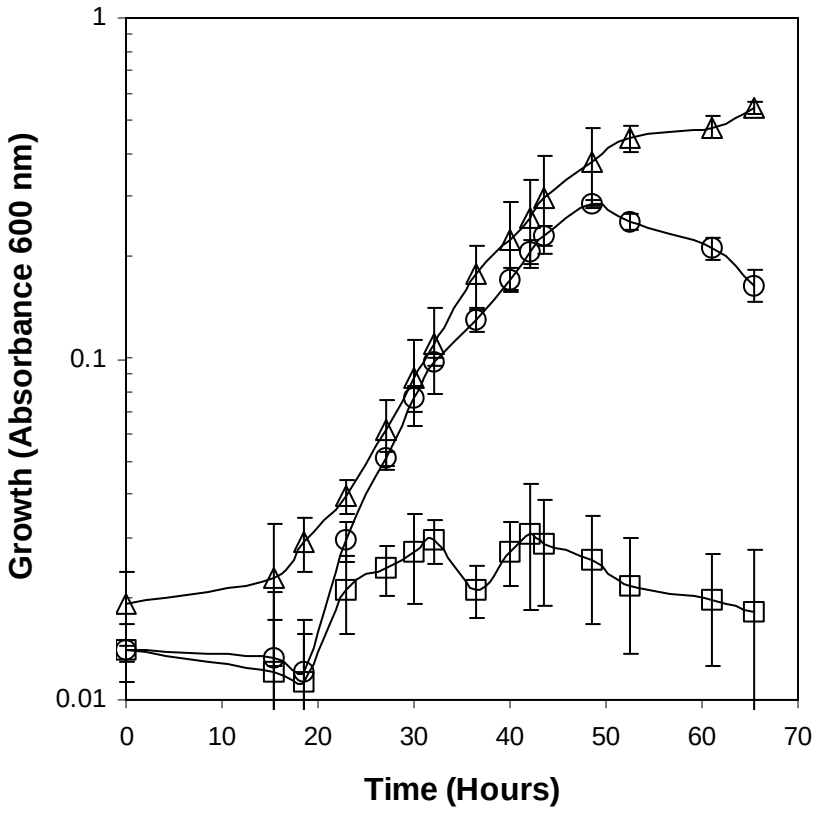


**Figure 2.9.** The effect of sucrose on anaerobic growth of *B. mojavensis* JF-2 in medium E supplemented with 1g/l DNA.

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.

# Chapter 3. *Bacillus subtilis* requires DNA or deoxyribonucleosides for Anaerobic Growth


## Abstract

*Bacillus subtilis* 168 (ATCC 23857), whose complete genomic sequence has been obtained, *Bacillus subtilis* (ATCC 6051), *Bacillus subtilis* 1A700 (Bacillus Stock Culture Collection in Iowa), *Bacillus subtilis* (ATCC 12332), and *Bacillus subtilis* JH642 all required DNA or deoxyribonucleosides for anaerobic growth. Two strains of *Bacillus licheniformis* and one strain each of *Bacillus sonorensis* and *Bacillus cereus* (ATCC 14579) did not require DNA for anaerobic growth. *Bacillus subtilis* 168 required deoxyribonucleosides at a concentration approximately 10 times the theoretical requirement. *Bacillus subtilis* JH642 required less than that required by *Bacillus subtilis* 168. The addition of pyruvate to the medium did not replace the requirement for deoxyribonucleosides or amino acids. The addition of glutamine did not replace the requirement for amino acids. Microaerophilic growth of *Bacillus subtilis* 168 and JH642 was inhibited by 0.1 g/l hydroxyurea and growth inhibition by hydroxyurea was overcome with the addition of deoxyribonucleosides. Microaerophilic growth of *B. sonorensis* and *B. licheniformis* was not inhibited by 0.1 g/l hydroxyurea. A BLAST (NCBI) search using the amino acid sequence of the Class III ribonucleotide reductase (*nrd*D) from *Bacillus cereus* (AAP10536) failed to find a Class III ribonucleotide reductase in the genome of *Bacillus subtilis* subsp. *subtilis* strain 168; although a *nrd*D gene (at least 97% identical and 99% similar) was found in *Bacillus cereus* and *Bacillus anthracis*. Similar results were obtained when the search was performed with the *nrd*D

gene of *Escherichia coli* (AE000495). A BLAST search using the protein sequence of the Class II ribonucleotide reductase gene (*nrdJ*) of *Lactobacillus leichmannii* also failed to find a Class II ribonucleotide reductase in the genome of *Bacillus subtilis*. A similar BLAST search using the amino acid sequence of the Class I ribonucleotide reductase of *Bacillus cereus* did identify a Class I ribonucleotide reductase in the genome of *Bacillus subtilis*.

## Introduction

Some bacilli such as *Bacillus licheniformis* and *Bacillus sonorensis* are known to grow well anaerobically with nitrate as an electron acceptor (44). Until just a few years ago *Bacillus subtilis* was considered an obligate aerobe. Since then it has been determined that *B. subtilis* grows anaerobically by nitrate reduction or by fermentation. *B. subtilis* uses only nitrate or nitrite as an electron acceptor and not any other electron acceptors and unlike *B. licheniformis*, *B. subtilis* is not a denitrifier (15, 40). Fermentation by *B. subtilis* is a little more difficult to establish but Nakano *et al*. (22), reported that pyruvate stimulated fermentation and this requirement could be replaced with the addition of amino acids. It was not clear why pyruvate or amino acids stimulated fermentation, but it was suggested that this was due to the fact that *B. subtilis* lacks pyruvate-formate lyase and instead uses pyruvate dehydrogenase when growing anaerobically (22). Growing anaerobically, *B. subtilis* carries out mixed acid-butanediol fermentation (22, 33).

The earliest report of nitrate reduction by *B. subtilis* under microaerophilic conditions was in 1970 by Michel (21). One of the first reports of anaerobic growth of *B. subtilis* was by Priest in 1993, however the conditions under which anaerobic growth

were established was not given (31). Since then, numerous reports of anaerobic growth of *B. subtilis* can be found (Table 3.1) (8, 10, 17-19, 22, 24-27, 31-33, 38, 40, 45). However, a review of this literature reveals that microaerophilic growth conditions may have been used or that the medium had the inadvertent addition of DNA or deoxyribonucleosides (with the addition of Tryptone). Many studies use static incubation of aerobically-prepared medium, aerobically-prepared medium with an oxygen-free headspace or aerobic medium enclosed in anaerobic jars (see Table 3.1). Anaerobic jars can contain as much as 1% oxygen (12). These conditions are not truly anaerobic and the small amounts of oxygen that are present provide growth conditions distinct from growth in reduced media. In 1995, Hoffman observed that the presence of a small amount of oxygen is enough to change anaerobic behavior of *B. subtilis* (ie. that a small amount of oxygen led to subsequent anaerobic growth) (15). Some studies used Luria-Bertani broth as a growth medium and removed oxygen by boiling and the headspace was oxygen-free (Table 3.1) (15, 18, 23). Luria-Bertani broth contains 10 g/l Tryptone. As described in Chapter 1, Tryptone supported anaerobic growth of JF-2 and was later replaced with DNA.

Although in 1952 Hoff-Jorgensen identified a *Lactobacillus* strain that required DNA for aerobic growth, which was subsequently used for a DNA assay (13), up to now, DNA has not been considered as a growth factor (46). However, the fact that *Bacillus mojavensis* JF-2 required DNA for anaerobic growth (Chapter 2), suggested that other bacilli might also require DNA for anaerobic growth. The fact that microaerophilic conditions led to improved anaerobic growth and that only Luria-Bertani broth supported strict anaerobic growth of *B. subtilis* suggested the possibility that *B. subtilis* also

required deoxyribonucleosides for anaerobic growth. Studies were performed to determine if *B. subtilis* also required deoxyribonucleosides for anaerobic growth.

## Methods and Materials

### Bacterial Strains

*B. subtilis* 168 ATCC 23857, *B. mojavensis*$^{T}$ ABO21191, and *B. cereus* ATCC 1457 were obtained from the American Type Culture Collection (ATCC). *B. subtilis* ATCC 6051 was obtained from the bacterial stock culture collection of the Department of Botany and Microbiology at the University of Oklahoma. *B. subtilis* 1A700 was obtained from the Bacillus Genetic Stock Center (BGSC Iowa). *B. subtilis* JH642, *B. subtilis* ATCC 12332, *B. mojavensis* strain ROB2, *B. licheniformis* (strains T68-25 and T68-35) and *B. sonorensis* T68-8 were obtained from the stock culture collection of Dr. Kathleen Duncan at the University of Oklahoma.

### Growth Media

Medium F 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 (5), sucrose (10), sodium nitrate (1), magnesium sulfate (0.25), Casamino acids (4), glutamine (0.1), tryptophan (0.1), asparagine (0.02), methionine (0.02), biotin (0.02), folic acid (0.02), pyridoxine-HCl (0.1), thiamine-HCl (0.05), riboflavin (0.05), nicotinic acid (0.05), calcium pantothenate (0.05), paraminobenzoic acid (0.05), lipoic acid (0.05), vitamin $B_{12}$ (0.001), deoxyadenosine (0.0125), deoxyguanosine (0.0125), deoxycytidine (0.0125) and thymidine (0.0125) and 10 ml of a metal solution (5). 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).

Sucrose (10 g/l)- and nitrate-supplemented Tryptic Soy Broth was prepared by adding to water (g/l): Tryptic Soy Broth (BD 211825) (30), sucrose (10) and $NaNO_3$ (2). Deoxyribonucleosides (1 g/l each) were added before autoclaving when indicated. Luria-Bertani broth consisted of (g/l): Tryptone (10), yeast extract (5), sodium chloride (5), sodium nitrate (2) and glucose (10) in one liter of water. Glucose was added after autoclaving. Hydroxyurea (0.1 g/l) was added before autoclaving. Low strength LB broth consisted of (g/l): $KH_2PO_4$ (1.3) $K_2HPO_4$ (0.27), Tryptone (1), yeast extract (0.5), sodium chloride (5), sodium nitrate (2) and glucose (10) in one liter of water.

Aerobic medium was prepared by dispensing aerobically-prepared medium and then sealing with an air headspace. Microaerophilic conditions were established by dispensing aerobically-prepared media under a constant stream of nitrogen. Each tube was flushed with nitrogen for about 30 seconds before sealing.

Anaerobic medium was prepared by boiling the medium under nitrogen, allowing it to cool and then dispensing under nitrogen. Once cool, 0.025 g/l cysteine HCL was added from an anoxic stock solution (2).

**Electronic gene search**

The electronic search for ribonucleotide reductatase genes in *B. subtilis* was performed at the web site of the National Center for Information (NCBI) using the

microbial BLAST program (1). The Class III ribonucleotide reductase BLAST search was performed using the amino acid sequence of the *Bacillus cereus nrdD* and the amino acid sequence of the *Echerichia coli nrdD* (948755) online at www.ncbi.nlm.nih.gov/sutils/genom_table.cgi against microbial genomes provided by the site. The expected number of random matches was set at 10. The genomes selected were Completed *Bacillus anthracis* str. A2012, Completed *Bacillus anthracis* str. Ames, Completed *Bacillus cereus* ATCC 10987, Completed *Bacillus cereus* ATCC 14579, Completed *Bacillus halodurans*, Completed *Bacillus subtilis* subsp. subtilis str. 168, Completed *Lactobacillus johnsonii*NCC 533, and Completed *Escherichia coli* K12.

The Class II ribonucleotide reductase BLAST search was performed using the amino acid sequence of the *Lactobacillus leichmannii nrdJ* (Q59490). The Class II ribonucleotide reductase of *Lactobacillus leichmannii* was used because no *Bacillus* sp *nrd*J was found. The genomes selected were *Prochlorococcus marinus* str. MIT 9313, *Synechococcus elongatus* PCC 7942, *Bacillus anthracis* str. A2012, *Bacillus anthracis* str. Ames, *Bacillus anthracis* str. Ames 0581, *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 14579, *Bacillus cereus* G9241, *Bacillus halodurans* C-125, *Bacillus subtilis* subsp. subtilis str. 168, *Lactobacillus gasseri*, *Lactobacillus johnsonii* NCC 533, *Lactobacillus plantarum* WCFS1, and *Escherichia coli* K12. *Prochlorococcus marinus* str. MIT 9313 and *Synechococcus elongatus* PCC 7942 were selected as positive controls since they each have an annotated Class II ribonucleotide reductase gene.

The Class I ribonucleotide reductase BLAST search was performed using the protein sequence of the *Bacillus cereus nrdE* (NP_977791). The genomes selected were

*Bacillus cereus* ATCC 10987, *Bacillus subtilis* subsp. subtilis str. 168 and *Escherichia coli* K12.

**The *B. cereus* nrdD protein sequence used was (1205948):**

MLQ NNT RGE ELM KVF ETI VHG NEQ DLM QEN ANV DGR SPM GVM GTF ASE SAK YYA VEN LLS DQV KKA INE NIL YPH DLD FYA TGT TTC SQI PLA QML ANG FHT GHG HMR QPQ DIK SAL ALS SII FQA NQN MQH GGQ SFA LFD IDL APY VRK TIE RHK KRL QSY PLK KEQ IEE FAW KET END TYQ ACE AFV HNS NSM HSR GGG QVP FIS INY GTD TSK EGR MLI KQL LKA TQA GLG KGE TPI FPI QIF KMK KGV NFE ESD PNY DLF ELA LET TAE RLF PNF SFL DAP FNA VHY DGR PES EVC YMG CRT RVM SNI HGE ETA IGR GNL SFT SIN LVK LAL ISG SKE AFF EAL NYY LDL GIK QLL ERY AYQ CTK RAR DFQ FLY SQG VWR GGE TLQ PED SVA SIL KQG TLS IGF IGL AEC LVA LTG KHH GED EES WKL GYE IIS FMR DRM DKA TEE HQL NFS VIA TPA EGL SGK FVK KDR KEF GVI SGI TNH NYY TNS FHI PVY YNI QAI NKI RLE GPF HAL CNG GHI TYI ELD GAA MHN KKA LKQ IVQ AMA EHG VGY GSI NHP VDR CKC CSY HGV IGN ECP SCG NED EAN IER IRR ITG YLV GDM SKW NSA KRS EEI DRV KHK

**The *Escherichia coli* nrdD protein sequence used was (948755):**

MTP HVM KRD GCK VPF KSE RIK EAI LRA AEV DDA DYC ATV AAV SEQ MQG RNQ VDI NEI QTA VEN QLM SGP YKQ LAR AYI EYR HDR DIE REK RGR LNQ EIV VSE QMQ GRN QVD INE IQT AVE NQL MSG PYK QLA RAY IEY RHD RDI ERE KRG RLN QEI RGL VEQ TNA SLL NEN ANK DSK VIP TQR DLL AGI VAK HYA RQH LLP RDV VQA HER GDI HYH DLD YSP FFP MFN CML IDL KGM LTQ GFK MGN AEI EPP KSI STA TAV TAQ IIA QVA SHI YGG TTI NRI DEV LAP FVT ASY NKH RKT AEE WNI PDA ERY ANS RTI KEC YDA FQS LEY EVN TLH TAN GQT PFV TFG FGL GTS WES RLI QES ILR NRI AGL GKN RKT AVF PKL VFA IRD GLN HKK GDP NYD IKQ LAL ECA SKR MYP DIL NYD QVV KVT GSF KTP MGC RSF LGV WEN ENG EQI HDG RNN LGV ISL NLP RIA LEP KGD EAT FWK LLD ERL VLA RKA LMT RIA RLE GVK ARV API LYM EGA CGV RLN ADD DVS EIF KNG RAS ISL GYI GIH ETI NAL FGG EHV YDN EQL RAK GIA IVE RLR QAV DQW KEE TGY GFS LYS TPS ENL CDR FCR LDT AEF GVV PGV TDK

GYY TNS FHL DVE KKV NPY DKI DFE APY PPL ANG GFI CYG EYP NIQ HNL KAL EDV WDY SYQ HVP YYG TNT PID ECY ECG FTG EFE CTS KGF TCP KCG NHD ASR VSV TRR VCG YLG SPD ARP FNA GKQ EEV KRR VKH LGN GQI G

**The *Lactobacillus leichmannii* nrdJ protein sequence used was (Q59490):**

MSE EIS LSA EFI DRV KAS VKP HWG KLG WVT YKR TYA RWL PEK GRS ENW DET VKR VVE GNI NLD PRL QDS PSL ELK QSL TEE AER LYK LIY GLG ATP SGR NLW ISG TDY QRR TGD SLN NCW FVA IRP QKY GDS KIV PSY LGK QEK AVS MPF SFL FDE LMK GGG VGF SVA RSN ISQ IPR VDF AID LQL VVD ETS ESY DAS VKV GAV GKN ELV QDA DSI YYR LPD TRE GWV LAN ALL IDL HFA QTN PDR KQK LIL DLS DIR PYG AEI HGF GGT ASG PMP LIS MLL DVN EVL NNK AGG RLT AVD AAD ICN LIG KAV VAG NVR RSA ELA LGS NDD QDF ISM KQD QEK LMH HRW ASN NSV AVD SAF SGY QPI AAG IRE NGE PGI VNL DLS KNY GRI VDG YQA GID GDV EGT NPC GEI SLA NGE PCN LFE VFP LIA EEQ GWD LQE VFA LAA RYA KRV TFS PYD WEI SRE IIQ KNR RIG ISM SGI QDW LLT RLG NRV VTG FKD DFD PET HEA IKV PVY DKR AIK MVD QYK AVV KAD QDY SKT LGC NES IKH TTV KPS GTV AKL AGA SEG MHF HYG AYL IQR IRF QDS DPL LPA LKA CGY RTE ADI YTE NTT CVE FPI KAV GAD NPN FAS AGT VSI AEQ FAT QAF LQT YWS DNA VSC TIT FQD SEG DQV ESL LRQ YRF ITK STS LLP YFG GSL QQA PKE PID KET YEK RSQ EIT GNV EEV FSQ LNS DVK DLE LVD QTD CEG GAC PIK

## Results

### Anaerobic Growth with DNA

*B. subtilis* (ATCC 12332) required DNA for anaerobic growth in reduced medium E (Figure 3.1). The addition of DNA plus 10 g/l Proteose Peptone to medium E further enhanced growth but the addition of ribonucleosides plus Casamino acids to medium E without DNA did not support anaerobic growth. In contrast, *B. licheniformis* T68-25, *B. licheniformis* T68-35 and *B. sonorensis* T68-8 did not require DNA for anaerobic growth

although the addition of amino acids and ribonucleosides enhanced growth slightly (see Appendix III).

**The Addition of Deoxyribonucleosides**

After finding that deoxyribonucleosides could replace DNA in supporting anaerobic growth of JF-2 (Chapter 2), we hypothesized that DNA could be replaced by deoxyribonucleosides for anaerobic growth of *B. subtilis*. Three *B. subtilis* strains required the addition of deoxyribonucleosides to medium E for anaerobic growth (Figures 3.2 to 3.4). The addition of an increased concentration of nitrate did not replace the requirement for DNA (Figure 3.2). The DNA requirement could be replaced with 3 milliters of air (with static incubation), which may explain the numerous reports of anaerobic growth of *B. subtilis* in defined medium under microaerophilic conditions. *B. cereus* did not require the addition of deoxyribonucleosides to medium E for anaerobic growth and was in fact inhibited by deoxyribonucleosides (Figure 3.5).

*B. subtilis* JH642 was used in many previous studies mentioned in the introduction. The studies that did establish anoxic conditions by boiling off oxygen combined with a nitrogen headspace used Luria-Bertani broth. So, the ability of *B. subtilis* JH642 to grow in LB broth under four redox conditions, aerobic, microaerophilic, anaerobic-without reductant, and anaerobic-with reductant, was tested. *B. subtilis* JH642 was able to grow equally well under all four redox conditions ($A_{max}$ ~ 0.6) in Luria-Bertani broth (Figure 3.6). This confirmed that growth under strict anaerobic conditions was possible in Luria-Bertani broth. Since Tryptone supported anaerobic growth of *B. mojavensis* JF-2 (Chapter 1), it was likely that the Tryptone (10 g/l) in Luria-Bertani broth provided deoxyribonucleosides (it was possible that the 5 g/l of yeast extract also

provided a small amount). To test this hypothesis, the ability of *B. subtilis* JH642 to grow in low strength LB broth (with less Tryptone and yeast extract as described below) under these same redox conditions was also tested.

If Tryptone and yeast extract were completely removed from LB broth, then the medium would consist of little more than salt and glucose. This would most likely be too stringent; so a low strength LB broth was made which contained a reduced amount (one tenth the original amount) of Tryptone and yeast extract and included a 10 mM phosphate buffer and trace metals. In low strength LB broth, *B. subtilis* JH642 was not able to grow as well under anaerobic conditions with reductant ($A_{max} < 0.2$) as it did under aerobic conditions ($A_{max} > 0.45$) (Figure 3.7). It grew just as well under microaerophilic conditions as it did under anaerobic conditions without a reductant ($A_{max} < 0.3$ for both). Again, the introduction of small amounts of oxygen does support growth under conditions many believe are "anaerobic". Obviously, the high concentrations of Tryptone and yeast extract were necessary for strict anaerobic growth.

To test the hypothesis that high concentrations of Tryptone and yeast extract were providing DNA or deoxyribonucleosides, *B. subtilis* JH642 was grown in anaerobic low strength LB broth with and without deoxyribonucleosides. Full strength anaerobic LB broth was used as a positive control. In low strength LB with deoxyribonucleosides, growth of *B. subtilis* JH642 reached an absorbance of about 0.6 and remained at that absorbance whereas growth in low strength LB without deoxyribonucleosides reached an absorbance of only about 0.2 and rapidly declined after 2 hours (Figure 3.8).

**Minimum Concentration of Deoxyribonucleosides**

Since *B. subtilis* required deoxyribonucleosides for anaerobic growth, an experiment was performed to determine the minimal concentration of deoxyribonucleosides required. The expected minimal required deoxyribonucleoside concentration was determined from the dry weight of the culture and the literature value indicating the percentage of DNA in a cell. First, the absorbance of the culture was converted to dry weight using a standard curve determined for *B. mojavensis* JF-2. This gave an approximate relationship of dry weight to absorbance:

Dry weight $= 0.0064(A) + 0.001$ $(R^2 = 0.91)$

Where A = absorbance of a 10 ml culture

So, if a 10 ml culture grew to an approximate absorbance of 0.6, it would yield approximately 5 mg dry weight of cells. If 3.1% of total dry weight consists of DNA (28), then only 150 μg of that dry weight would consist of DNA. A very small amount (approximately 200 μg) of DNA would be required as DNA precursors. However, as seen in Figure 3.9A, *B. subtilis* 168 required about 2000 μg (total deoxyribonucleosides in 10 ml of media) to reach an absorbance of 0.6. In contrast, *B. subtilis* JH642 reached that absorbance with 500 μg of deoxyribonucleosides and possibly required even less (Figure 3.9B).

**Inhibition of Microaerophilic Growth with Hydroxyurea**

The role of ribonucleotide reductases in microbial DNA synthesis was discussed in Chapter 2. In that discussion, it was hypothesized that *B. mojavensis* JF-2 lacked a

Class III ribonucleotide reductase and thus required DNA for anaerobic growth but not for aerobic growth. *B. subtilis* most likely requires DNA for the same reason. We then hypothesized that the growth of *B. subtilis* in the presence of trace amounts of oxygen (such as that available in aerobic medium under a nitrogen headspace) was due to the activity of a Class I ribonucleotide reductase. This class of reductase is inhibited by hydroxyurea, thus the presence of hydroxyurea would inhibit microaerophilic growth and the addition of DNA or deoxyribonucleosides would overcome that inhibition. The addition of 0.1 g/l hydroxyurea to Tryptic Soy broth inhibited microaerophilic growth of *B. subtilis* 168 (final A = 0.08) and the addition of deoxyribonucleosides allowed growth in the presence of hydroxyurea (final A = 0.3) (Figure 3.10). Similar data was obtained with *B. subtilis* JH642 (data not shown). In contrast, the addition of 0.1 g/l hydroxyurea to Tryptic Soy broth did not inhibit microaerophilic growth of *B. licheniformis* or *B. sonorensis* (final A > 0.7 for both) (Figure 3.11)

**Results of Blast Search**

Due to the possible role of ribonucleotide reductase in DNA-requiring physiology (described in Chapter 2) A BLAST (NCBI) search using the protein sequence of the Class III ribonucleotide reductase, *nrd*D, from *Bacillus cereus* (AAP10536) was performed against the genomic sequences of *Bacillus anthracis* str. A2012, *Bacillus anthracis* str. Ames, *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 14579; *Bacillus halodurans*, *Bacillus subtilis* subsp. subtilis str. 168, *Lactobacillus johnsonii* NCC 533, and *Escherichia coli* K12 (Appendix IV). All the bacilli except for *Bacillus subtilis* subsp. *subtilis* strain 168 and *B. halodurans* had at least a putative *nrd*D gene (at

least 97% identical and 99% similar) and were annotated as anaerobic ribonucleotide-triphosphate reductases.

Similar results were obtained when the search was performed with the *nrd*D gene of *Escherichia coli* (AE000495) (Appendix V). All the bacilli except for *B. subtilis* subsp. *subtilis*strain 168 and *B. halodurans* had at least a putative *nrd*D (at least 32% identical and 49% similar) and were annotated as anaerobic ribonucleotide-triphosphate reductases. It is possible that *B. halodurans* also has an *nrd*D, but the match in this case was not strong enough (28% identical and 47% similar) to make a definitive conclusion and was not annotated as such.

A BLAST search was also performed using the amino acid sequence of the Class II ribonucleotide reductase (*nrdJ*) of *Lactobacillus leichmannii* against the genomic sequences of *Prochlorococcus marinus* str. MIT 9313, *Synechococcus elongatus* PCC 7942, *Bacillus anthracis* str. A2012, *Bacillus anthracis* str. Ames, *Bacillus anthracis* str. Ames 0581, *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 14579, *Bacillus cereus* G9241, *Bacillus halodurans* C-125, *Bacillus subtilis* subsp. subtilis str. 168, *Lactobacillus gasseri*, *Lactobacillus johnsonii* NCC 533, *Lactobacillus plantarum* WCFS1, and *Escherichia coli* K12 (Appendix VI). A Class II ribonucleotide reductase was found only in the genomes of *Lactobacillus gasseri*, *Prochlorococcus marinus* str. MIT 9313, *Synechococcus elongatus* PCC 7942 and *Bacillus halodurans* C-125 (at least 27% identical and 43% similar).

A BLAST search using the amino acid sequence of the *Bacillus cereus* nrdE (Class I ribonucleotide reductase) did identify the *B. subtilis* nrdE (54% identical and 75% similar) (Appendix VII) and a *B. subtilis nrdE* has been annotated in the *B. subtilis*

genome (http://genolist.pasteur.fr/SubtiList/). These BLAST searches support the hypothesis that *B. subtilis* has a Class I ribonucleotide reductase but does not have a Class III or a Class II ribonucleotide reductase. In addition, neither an *nrd*D nor an *nrdJ* were among the list of annotated genes in the genome of *B. subtilis* strain 168.

### Pyruvate Requirement

As mentioned in the introduction, Nakano *et al.*, (22) made the observation that pyruvate stimulated fermentation of *B. subtilis*; further, they noted that the requirement for pyruvate could be replaced with amino acids. They used anaerobic jars and static incubation of aerobic media when they made this observation, which created microaerophilic conditions. We tested their observation under strict anaerobic conditions to see if it would still hold true and also to test if pyruvate could replace the requirement for deoxyribonucleic acids. Pyruvate did not replace the requirement for deoxyribonucleic acids, even if added in the high concentration of nearly 100 mM as reported by Nakano *et al.*, although it decrease the lag time slightly (Figure 3.12). In contrast, amino acids were found to be required for anaerobic growth and this requirement was not replaced with the addition of pyruvate (Figure 3.12). The requirement for amino acids was not replaced by nitrate or glutamine either (data not shown). Similar results were obtained for *B. subtilis* JH642 (data not shown).

## Discussion

In 1995, Hoffman observed that a small amount of oxygen led to subsequent anaerobic growth of *B. subtilis* (15). In this work I have shown that three milliliters of air could replace the requirement for deoxyribonucleosides for three strains of *Bacillus*

*subtilis* grown in anaerobic medium E. In Luria-Bertani broth, the requirement for 10 g/l tryptone plus 5 g/l yeast extract could also be replaced by microaerophilic conditions. Although the data were shown for only four of the strains, the requirement for deoxyribonucleosides of all five of the strains could be replaced with a small amount of air, in agreement with Hoffman's observation.

Thus, both *B. mojavensis* and *B. subtilis* have been observed to exhibit enhanced "anaerobic" growth with the addition of small amounts of oxygen. Further, microaerophilic growth of both organisms is inhibited by the addition of hydroxyurea whereas microaerophilic growth of *B. licheniformis* and *B. sonorensis* is not. *Bacillus subtilis* has a Class I ribonucleotide reductase (39). This inhibition of microaerophilic growth with hydroxyurea combined with the growth due to a small addition of oxygen supports the hypothesis that microaerophilic growth is due to the activity of a Class I ribonucleotide reductase. When the activity of the Class I ribonucleotide reductase is inhibited with hydroxyurea, *B. subtilis* and *B. mojavensis* do not have an alternative for converting ribonucleotides to deoxyribonucleotides and cease to grow, whereas *B. licheniformis* and *B. sonorensis* apparently have an alternate enzyme and microaerophilic growth is not inhibited. The fact that the addition of deoxyribonucleosides overcomes hydroxyurea induced anaerobic growth inhibition further demonstrates that the anaerobic growth inhibition is due to a lack of DNA precursors and not due to some other inhibitory effect of hydroxyurea.

Potential alternatives to the Class I aerobic enzyme consist of the Class II (which functions under both aerobic or anaerobic conditions) and the Class III (which functions under only anaerobic conditions). *B. cereus* does have a Class III ribonucleotide

reductase and, as a result, does not require DNA or deoxyribonucleosides for anaerobic growth. Unfortunately neither *B. licheniformis* nor *B. sonorensis* have been sequenced so it was not possible to search their genome for ribonucleotide reductases. Apparently, no alternative ribonucleotide reductases have been identified in *B. licheniformis* or *B. sonorensis* to date; but since they are not inhibited by hydroxyurea, it can be assumed that they do have some other ribonucleotide reductase other than the Class I. Fortunately *B. subtilis* has been sequenced, and it was possible to perform an electronic search of this genome. The BLAST search failed to identify either a Class III or a Class II ribonucleotide reductase and no *nrdD* or *nrdJ* has been annotated for *B. subtilis* (http://genolist.pasteur.fr/SubtiList/).

In summary, these data support the hypothesis that *B. mojavensis* and *B. subtilis* do not have any ribonucleotide reductase other than the Class I.

1. The growth experiments demonstrating enhanced anaerobic growth under microaerophilic conditions.
2. The growth experiments demonstrating that DNA or deoxyribonucleosides are required for strict anaerobic growth.
3. The microaerophilic growth inhibition due to hydroxyurea.
4. The ability to overcome hydroxyurea-induced microaerophilic growth inhibition with the addition of deoxyribonucleosides.
5. The BLAST search which failed to identify a Class II or a Class III ribonucleotide reductase in the *Bacillus subtilis* genome.

Bacterial growth in the absence of ribonucleotide reductase activity for *B. subtilis* is not entirely unheard of. In 1978 Rima and Takahashi isolated deoxyribonucleoside-requiring mutants of *Bacillus subtilis* (35). This demonstrated that *B. subtilis* has the kinases necessary to phosphorylate deoxyribonucleosides to deoxyribonucleotides. This work is also in agreement with the hypothesis that if ribonucleotide reductase activity is lost or diminished, then *Bacillus subtilis* cannot grow without deoxyribonucleosides as DNA precursors. They also found that the addition of any deoxyribonucleoside plus the other three bases did not support growth of these mutants. This is in agreement with earlier work (1972) that suggested that *B. subtilis* does not have trans-N-deoxyribosylase (3). In light of these findings, it is not too surprising that *B. subtilis* can use deoxyribonucleosides for anaerobic growth since it has already been demonstrated that deoxyribonucleosides can be used as sole DNA precursors.

Successful anaerobic bacterial growth dependent on the presence of a ribonucleotide reductase is not unheard of either. Anaerobic growth of *Alcaligenes eutrophus* H16 is dependent on the presence of conjugative 450-kb megaplasmid (42). This megaplasmid was found to contain a Class III ribonucleotide reductase essential for anaerobic growth.

Another observation regarding anaerobic growth of *B. subtilis* concerns the amount of deoxyribonucleosides required for growth. Obviously, more deoxyribonucleosides are required than needed for DNA assembly. This could suggest that (1) the deoxyribonucleosides are used for something other than DNA production, (2) the uptake systems are not efficient (*e.g.* a high $K_m$), or (3) that a high concentration is required to turn on the genes required for growth under these conditions. Considering

that deoxyribonucleosides are not used as a sole carbon and energy source (Chapter 2), it seems unlikely that the high concentration needed is for an alternate use other than DNA synthesis. Either of the other two options is more likely and both may play a role. It is an interesting coincidence that strain JH642 requires a lower concentration of deoxyribonucleosides than strain 168 and that strain JH642 was the strain used most in past anaerobic work. It is possible that strain JH642 has a much lower $K_m$ or requires a lower concentration to turn on the genes necessary for anaerobic growth. Either of these characteristics would make it a better candidate for anaerobic growth.

Finally, an observation found in the literature about the anaerobic growth of *B. subtilis* was that pyruvate stimulated fermentation and could be replaced with amino acids (22). This observation was made with cultures grown under microaerophilic conditions. Our studies found that pyruvate slightly shortened the lag phase, but was not required for anaerobic growth when amino acids were included in the medium. The addition of pyruvate did not replace the requirement for deoxyribonucleosides. The role of pyruvate in anaerobic growth of *B. subtilis* is still unclear.

Amino acids on the other hand, were required for anaerobic growth of *B. subtilis* 168. Amino acids may indeed supply a possible pyruvate requirement, but pyruvate alone does not replace the requirement for amino acids. The amino acid requirement was not solely due to a nitrogen requirement since the amino acid requirement was not replaced with nitrate or glutamine. The amino acid requirement is curious considering that the strain of *B. subtilis* 168 has not been reported to require amino acids when grown under aerobic conditions. Possibly, the pathways of amino acid synthesis in *B. subtilis*

168 do not function under strict anaerobic conditions, or *B. subtilis* 168 requires the presence of amino acids to turn on the genes required for strict anaerobic growth.

**General Discussion**

Although DNA has not been considered a required growth factor, clearly some organisms require DNA. Hoff-Jorgensen identified a *Lactobacillus* thatrequired DNA for aerobic growth (13). In chapter one, I showed three strains of *B. mojavensis* (*B. mojavensis*$^{T}$, *B. mojavensis* ROB2 and *B. mojavensis* JF-2) required DNA for anaerobic growth. Here, I show five strains of *B. subtilis* [*B. subtilis* 168 (ATCC 23857), *B. subtilis* (ATCC 6051), *B. subtilis* 1A700 (Bacillus Genetic Stock Collection in Iowa), *B. subtilis* (ATCC 12332) and *B. subtilis* JH642] require DNA or deoxyribonucleosides for anaerobic growth.

These studies demonstrate the use of DNA or deoxyribonucleosides as a required growth factor; other currently uncultured organisms may be found which also require DNA. There is evidence of the presence of organisms in the environment that are not possible to culture using medium, growth factors and techniques known today. In 1987 Colwell introduced the notion of viable but not culturable bacterial cells (37) and a number of environmental studies have shown that organisms seen in a direct count are not recovered through culture techniques (16, 20, 30, 37). Giovannoni and Rappe (2000) (9) reported the presence of previously uncultured bacteria in the ocean and Zengler *et al*., (2002) had some success cultivating some of these using a cell encapsulation/flow cytometry procedure (47). However, not only in environmental microbiology are nonculturables important, but the possibility also exists within the study of microbial pathogenesis. David Relman (2002) (34) presents evidence for the presence of

significant microbial pathogens that are non-culturable. He states that, for most cases of aseptic meningitis, encephalitis, and cerebral vasculitis, the pathogen is not cultured or identified in spite of clear evidence of infection. Brouqui (4) describes endocarditis due to culture-negative bacteria. Non-culturable *Salmonella*, *Rhodococcus*, and some enteric bacteria have also been reported (11, 41, 43).

It has been hypothesized that some marine bacteria are not cultured due to high substrate concentration (47), but it is also possible that others may have growth requirements that are not met in common culture media. DNA may be such a requirement. It may serve as a source of nucleic acid bases for organisms that cannot uptake individual bases or as a source of deoxyribonucleosides for organisms that cannot reduce the ribonucleotide to a deoxyribonucleotide. DNA is not usually added to culture media so any organism(s) requiring it for growth would remain uncultured.

There are obvious reasons for wanting to isolate pathogens but the uncultivated environmental bacteria also represent an untapped resource of novel bacterial products and are a vital part of understanding microbial ecology. Many can be detected using molecular techniques but it is difficult to study their physiology if they are not cultured.

This work provides an explanation of the observation made in 1995 by Hoffman and for several years by others in this laboratory, that small amounts of oxygen led to apparent anaerobic growth of *Bacillus* sp. This also shows that it is important to completely remove oxygen in order to determine the true anaerobic growth requirements and to study the anaerobic physiology of an organism. This work also suggests that bacterial nutrition, in addition to bacterial energy metabolism, may determine if an organism is defined as aerobic or anaerobic.

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**Table 3.1.** Examples of how anaerobic growth conditions were established for *Bacillus subtilis* as reported in the literature.

| *Bacillus subtilis* strain | Medium | Growth conditions (solid medium/liquid medium) | How oxygen was removed | Reference |
|---|---|---|---|---|
| 10 different strains | Peptone Medium | Static incubation | Prepared medium in anaerobic chamber Initial Oxygen was estimated at ~ 2% | Rodel and Lucke 1990 (36) |
| 22 different strains | Difco Sporulation Medium | Static incubation | Flushed headspace with nitrogen for 1 minute | Nakano et al., 1995 (24) |
| JH642 | Glucose minimal medium | Not specified | Not specified | Ogawa et al., 1995 (29) |
| JH642 | Luria-Bertani | Anaerobic jar | Gas-generating system (Becton Dickinson) | Glaser et al., 1995 (10) |
| JH642 | Luria-Bertani | Static incubation | Boiled to remove oxygen, nitrogen headspace | Hoffman et al., 1995 (15) |
| JH642 | Schaeffer's sporulation | Anaerobic jar | Gas-generating system (Becton Dickinson) | Sun et al., 1996 (45) |
| JH642 | Luria-Bertani plates/broth | Anaerobic jar/static incubation | Gas-generating system (Becton Dickinson)/Removed all headspace | LaCelle et al., 1996 (17) |
| JH642 (trpC2pheA1) | Spizizen's minimal medium | Anaerobic jar/static incubation | Gas-generating system (Becton Dickinson)/Flushed headspace with nitrogen for1 minute | Nakano et al., 1997 (22) |
| JH642 | Difco sporulation medium | Static incubation | Flushed headspace with nitrogen for 1 minute | Nakano et al., 1998 (27) |

**Table 3.1 (Continued).** Examples of how anaerobic growth conditions were established for *Bacillus subtilis* as reported in the literature.

| *Bacillus subtilis* strain | Medium | Growth conditions (solid medium/liquid medium) | How oxygen was removed | Reference |
|---|---|---|---|---|
| JH642 | Luria-Bertani | Static incubation | Culture consumed residual oxygen or heated medium to remove oxygen and flushed with nitrogen | Nakano et al., 1998 (23) |
| JH642 | Luria-Bertani | Static incubation | Culture consumed residual oxygen | Hoffman et al., 1998(14) |
| 50 different strains including JH642 | DS | Static incubation | Flushed headspace with nitrogen for 1 minute | Nakano et al., 1999 (25) |
| JH642, THB2, and LAB2313 | Luria-Bertani | Static incubation | Boiled to remove oxygen, nitrogen headspace | Marino et al., 2000 (18) |
| 46 strains including 168 | Luria-Bertani | Shaking incubation, rubber stoppered flasks | Removed all headspace by filling completely and sealing | Ramos et al., 2000 (7) |
| 49 different strains | Luria-Bertani broth | Not specified | Not specified | Marino et al., 2001 (19) |
| BSR6 | Luria-Bertani broth | Stirred fermentor | Sparged nitrogen gas 5 hours prior to inoculation | Espinosa-De-Los-Monteros et al., 2001(8) |
| LCB6 (derived from 168) | Spizizen minimal medium | Stationary incubation | Removed all headspace by filling completely and sealing | Clements et al., 2002(6) |

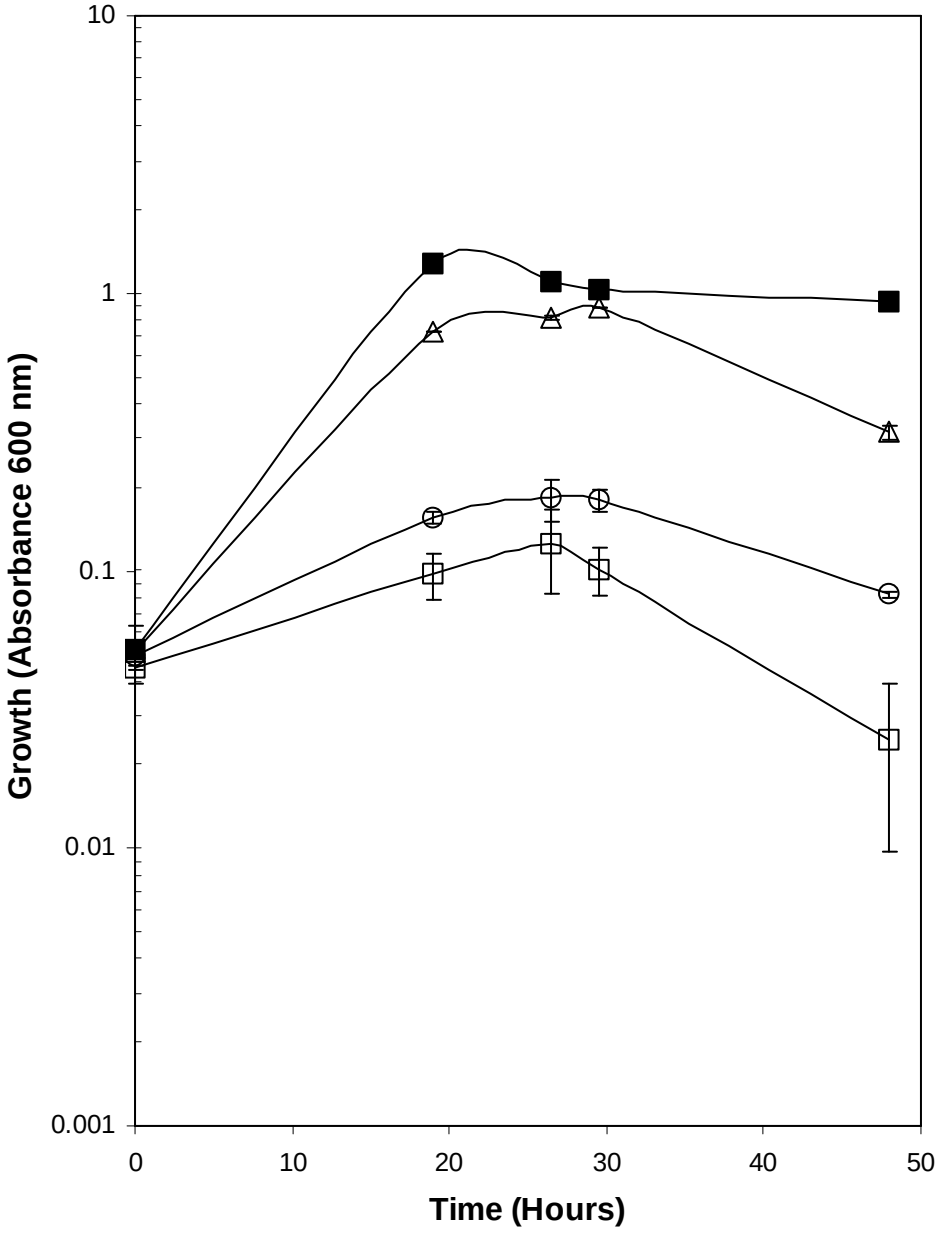


**Figure 3.1.** The effect of DNA on anaerobic growth of *Bacillus subtilis* (ATCC 12332).

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.

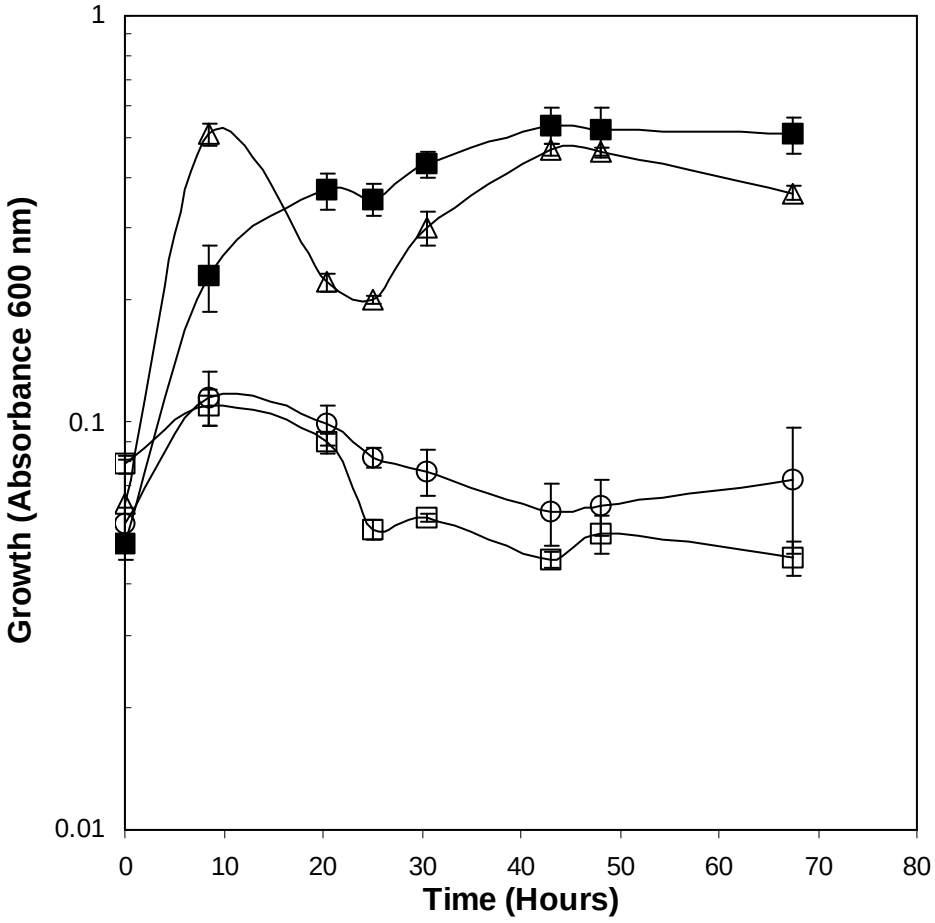


**Figure 3.2.** The effect of deoxyribonucleosides and nitrate on anaerobic growth of *Bacillus subtilis* ATCC 6051.

Medium E normally contained about 10 mM nitrate. In this case the concentration of nitrate in medium E was altered so as to determine if increased nitrate might increase anaerobic growth of *Bacillus subtilis* ATCC 6051.

Squares, medium E with 5 mM nitrate; circles, medium E plus 20 mM nitrate; triangles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) and 20 mM nitrate; filled squares, medium E plus 3 ml of air.

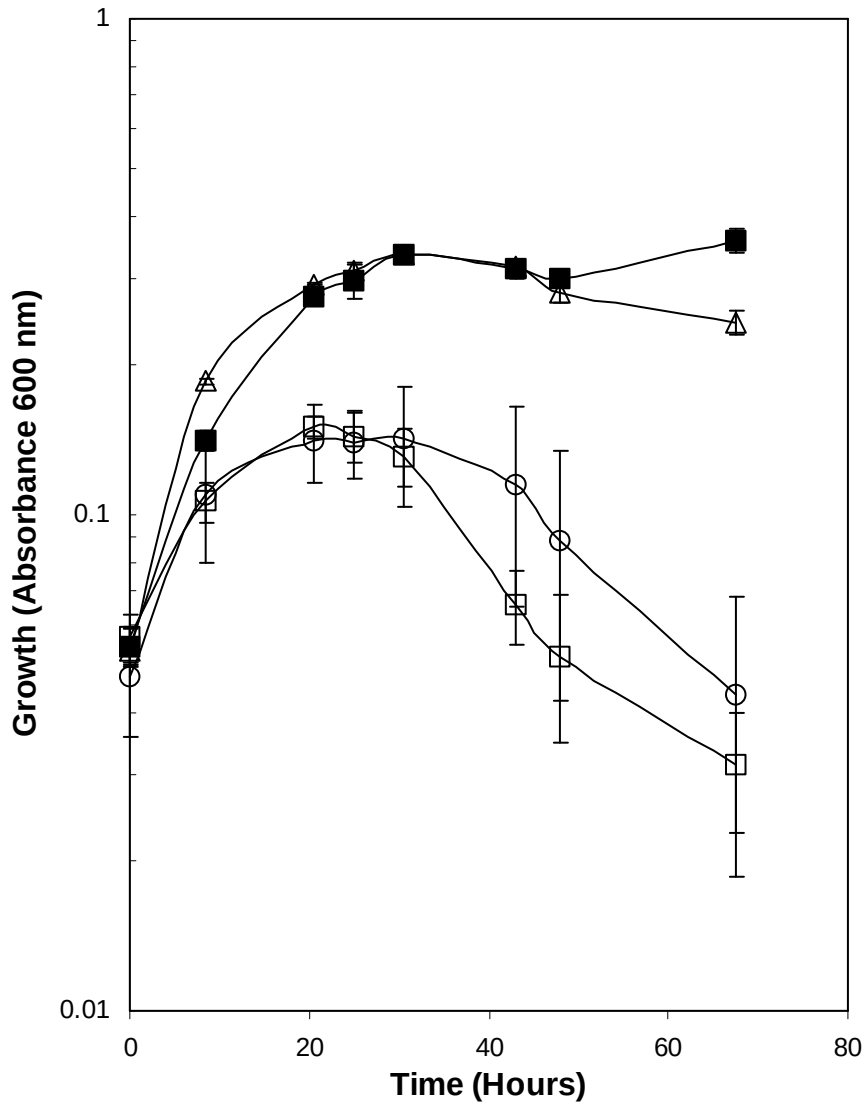


**Figure 3.3.** The effect of deoxyribonucleosides on anaerobic growth of *Bacillus subtilis* (BGSC 1A700).

Squares, medium E with 5 mM nitrate; circles, medium E plus 20 mM nitrate; triangles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) and 20 mM nitrate; filled squares, medium E plus 3 ml of air.

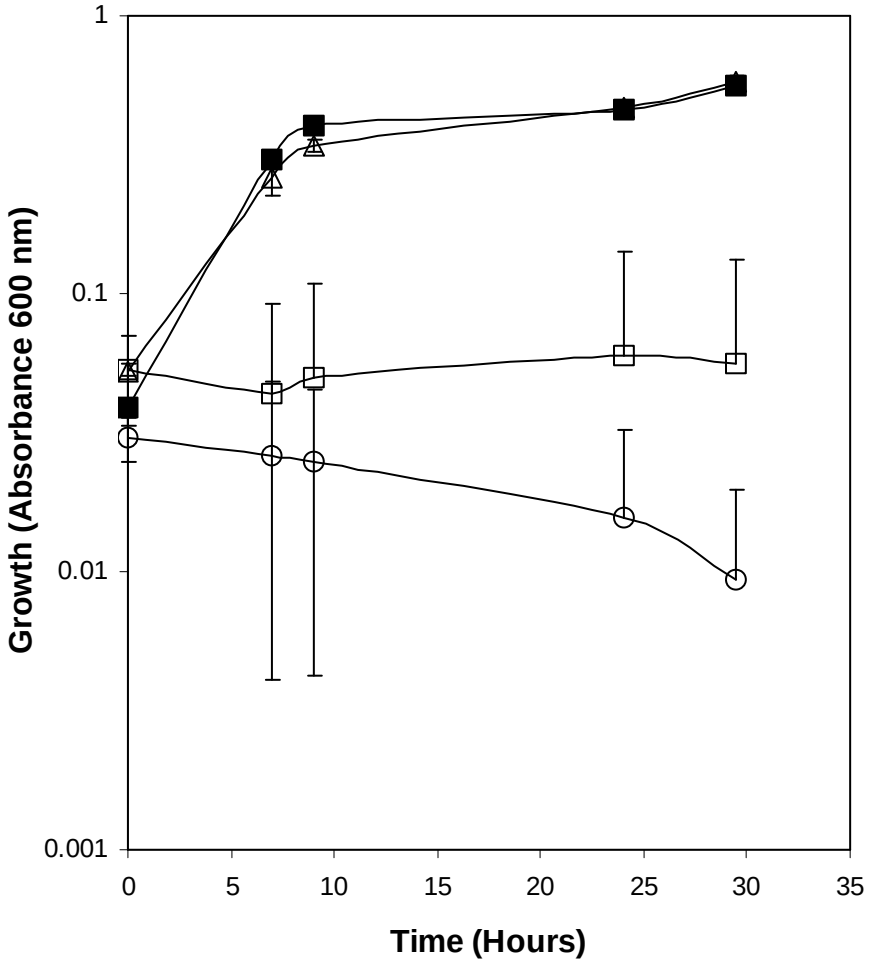


**Figure 3.4.** The effect of deoxyribonucleosides on anaerobic growth of *Bacillus subtilis* 168 (ATCC 23857).

Squares, medium E with 20 mM nitrate; circles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine); triangles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) and 20 mM nitrate; filled squares, medium E plus 3 ml of air.

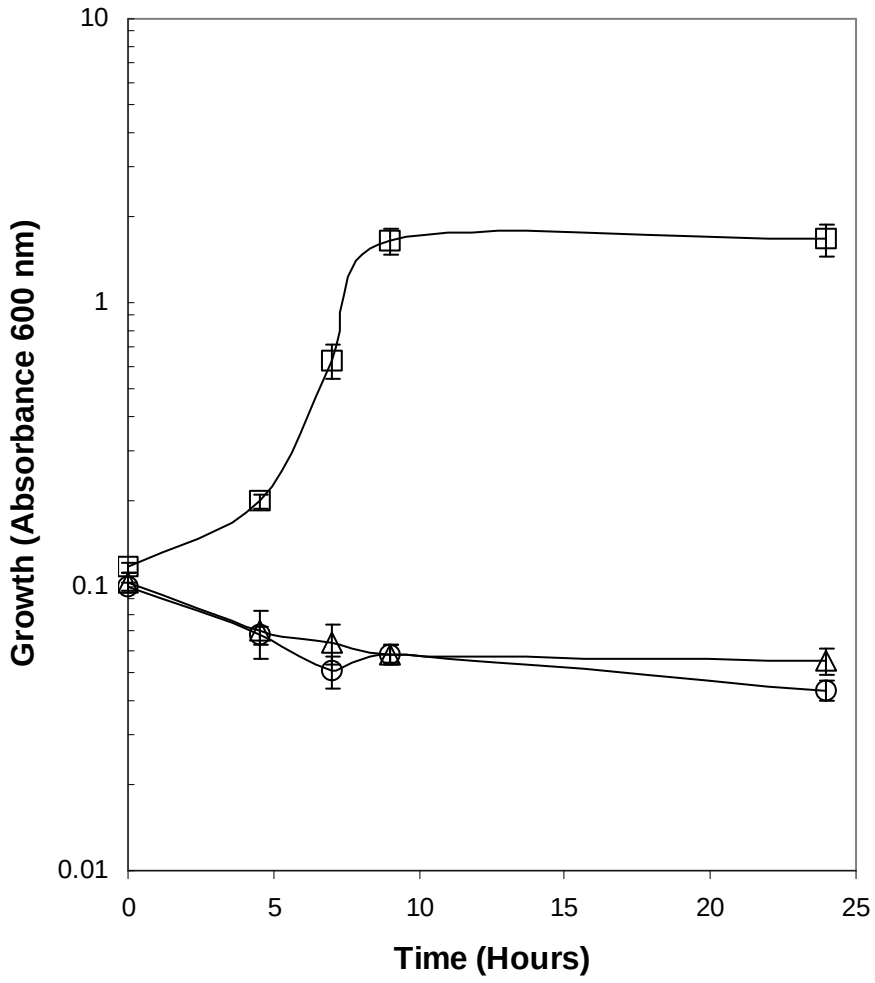


**Figure 3.5.** The effect of deoxyribonucleosides on anaerobic growth of *Bacillus cereus* ATCC 1457.

Squares, medium E with 20 mM nitrate; circles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine); triangles, medium E plus deoxyribonucleosides (0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine) and 20 mM nitrate.

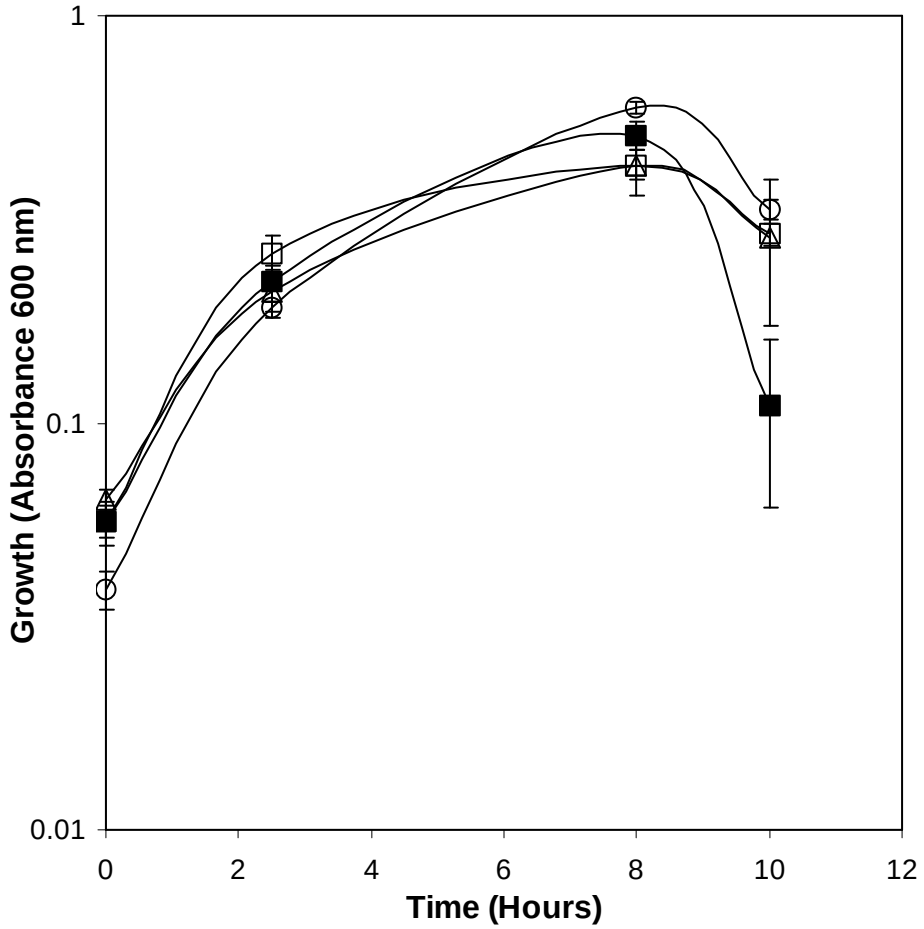


**Figure 3.6.** The growth of *Bacillus subtilis* JH642 in Luria-Bertani medium under aerobic, microaerophilic, and anaerobic (reduced and not reduced) conditions.

Squares, aerobic Luria-Bertani broth; circles, microaerophilic Luria-Bertani broth; triangles, anaerobic Luria-Bertani broth without reductant; filled squares, anaerobic Luria-Bertani broth with 0.5 g/l cysteine hydrochloride.

Microaerophilic conditions were established by flushing the headspace of aerobic medium with nitrogen (30 sec) before sealing

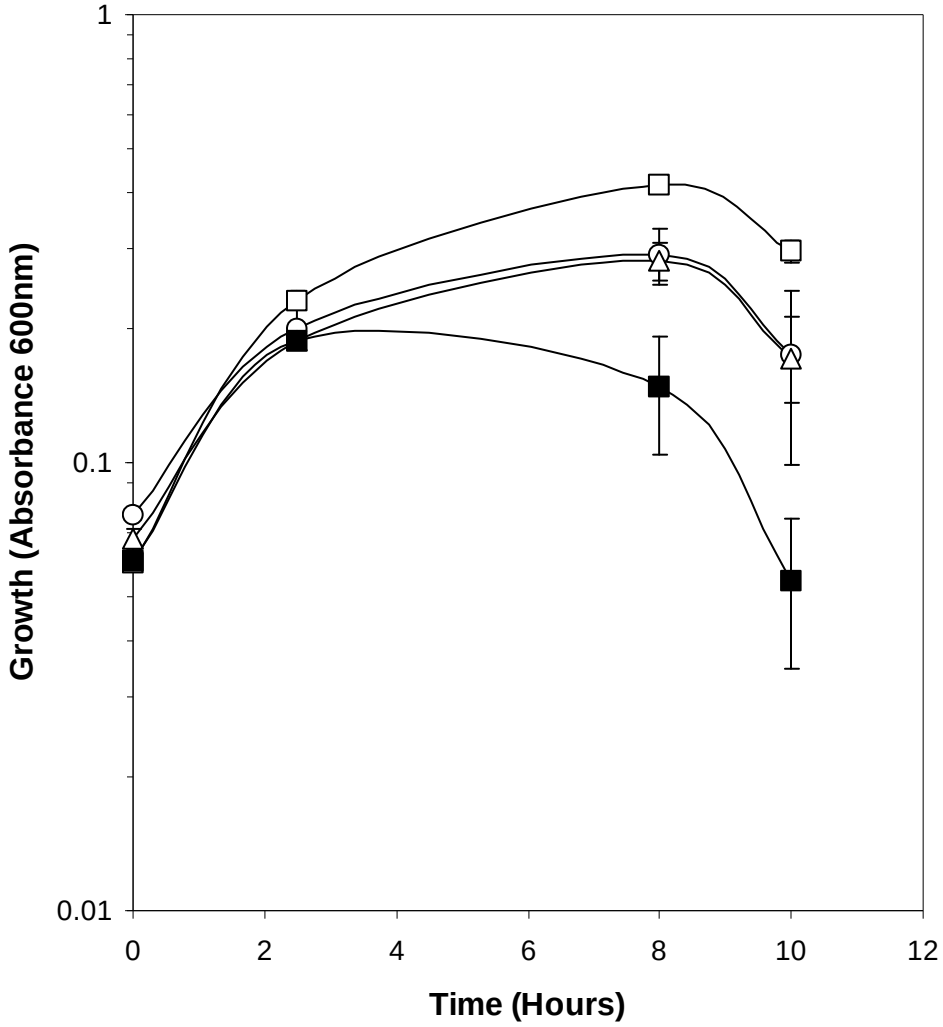


**Figure 3.7.** The growth of *Bacillus subtilis* JH642 in low strength Luria-Bertani medium under aerobic, microaerophilic, and anaerobic (reduced and not reduced) conditions.

Squares, aerobic low strength Luria-Bertani broth; circles, microaerophilic low strength Luria-Bertani broth; triangles, anaerobic low strength Luria-Bertani broth without reductant; filled squares, anaerobic low strength Luria-Bertani broth with 0.5 g/l cysteine hydrochloride.

Low strength Luria-Bertani broth has only 1g/l Tryptone and 0.5 g/l yeast extract.

Microaerophilic conditions were established by flushing the headspace of aerobic medium with nitrogen (30 sec) before sealing.

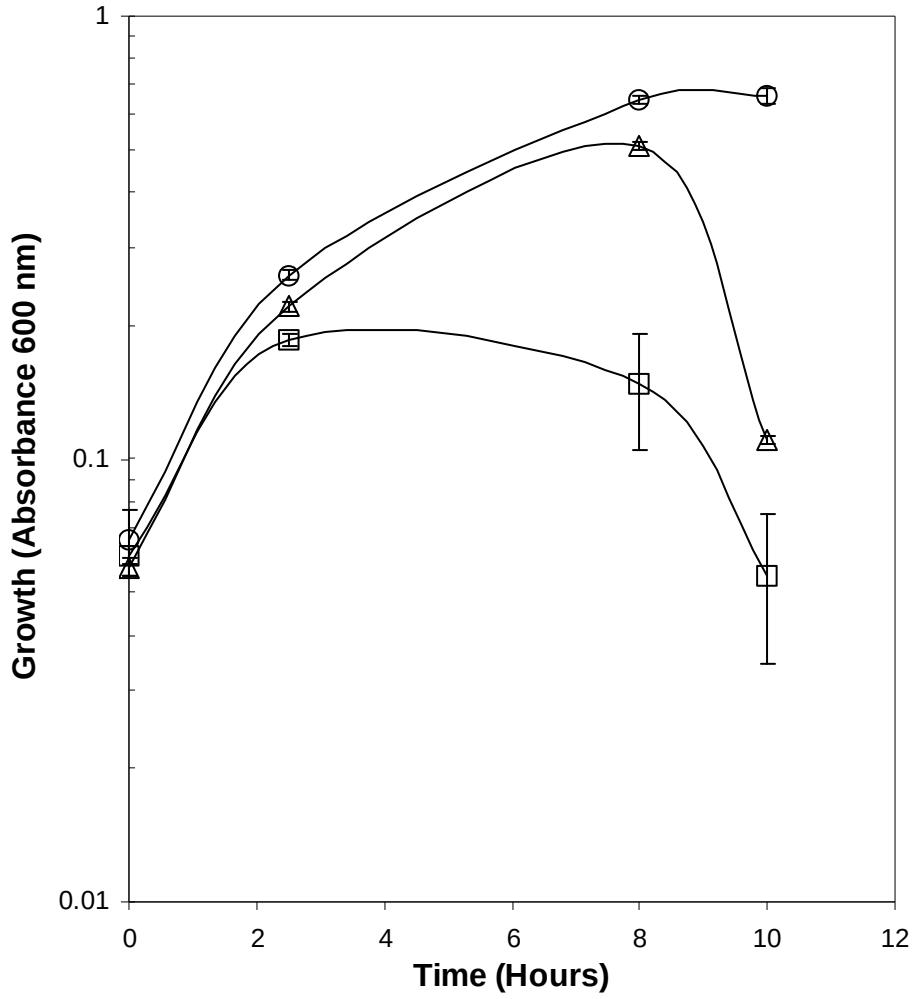


**Figure 3.8.** The growth of *Bacillus subtilis* JH642 in low strength anaerobic Luria-Bertani broth with and without deoxyribonucleosides.

Squares, anaerobic low strength Luria-Bertani broth; circles, low strength anaerobic Luria-Bertani broth and 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine; triangles, anaerobic Luria-Bertani broth.

Low strength Luria-Bertani broth has only 1g/l Tryptone and 0.5 g/l yeast extract. All media contained 0.5 g/l cysteine hydrochloride.

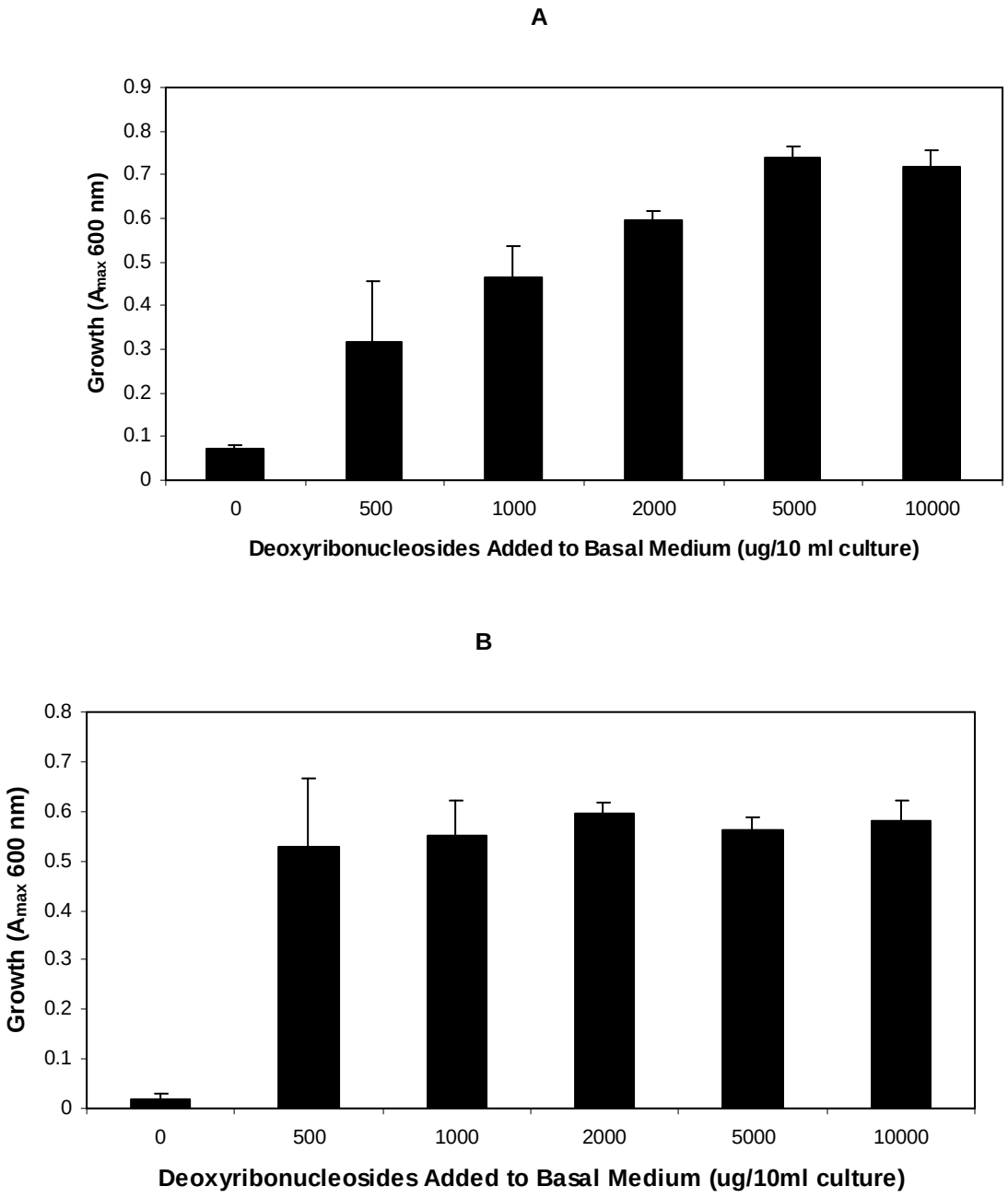


**Figure 3.9.** The effect of deoxyribonucleoside concentration on the anaerobic growth of Bacillus *subtilis* in medium F. Figure A, *Bacillus subtilis* 168; Figure B, *Bacillus subtilis* JH642.

The concentration is the sum of an equal concentration of all four deoxyribonucleosides, deoxyadenosine, deoxyguanosine, deoxcytidine and thymidine.

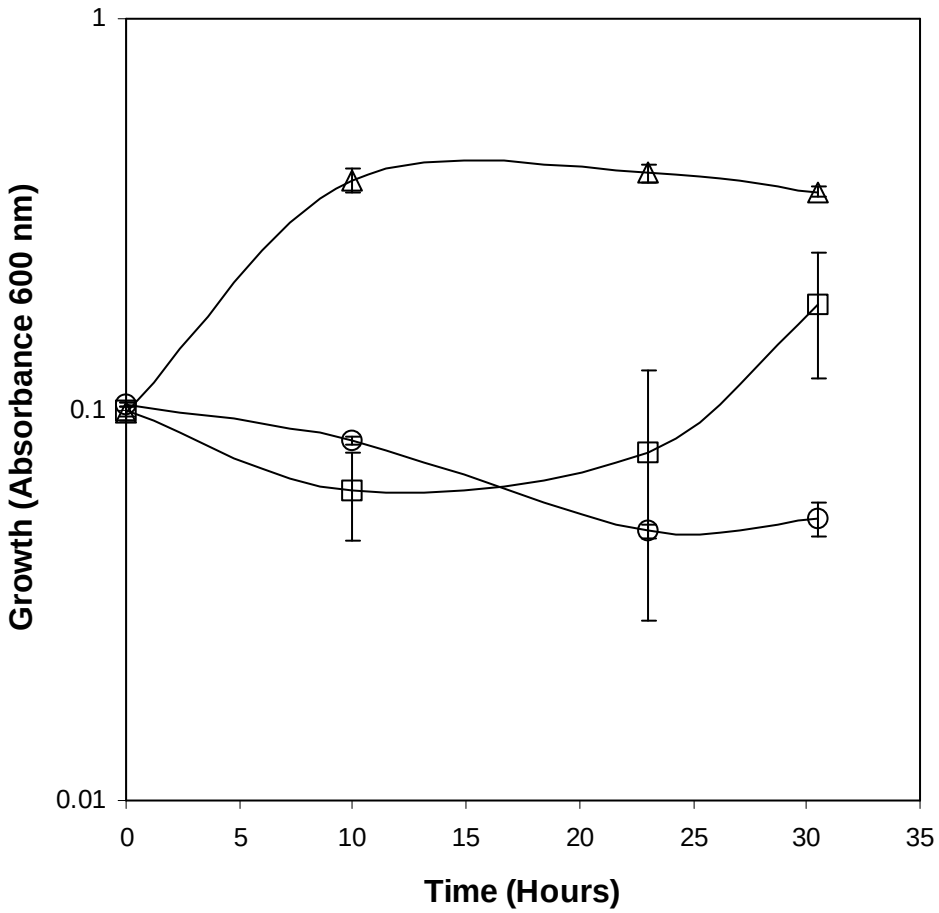


**Figure 3.10.** The effect of the addition of deoxyribonucleosides and with and without 0.1 g/l hydroxyurea to Tryptic Soy broth (with 1 g/l nitrate and 10g/l sucrose) on microaerophilic growth of *Bacillus subtilis* 168.

Squares, Tryptic Soy broth; circles, Tryptic Soy broth plus 0.1 g/l hydroxyurea; triangles, Tryptic Soy broth plus 0.1 g/l each of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine plus 0.1 g/l hydroxyurea.

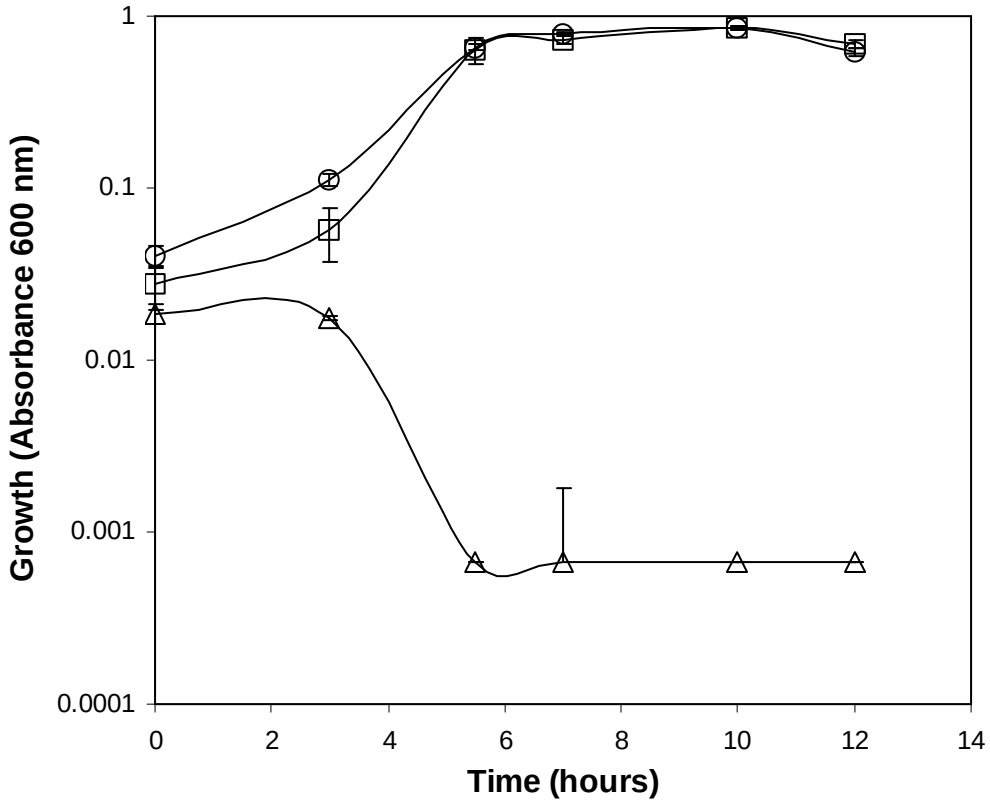


**Figure 3.11.** The effect of the addition of 0.1 g/l hydroxyurea to Tryptic Soy broth (with 1 g/l nitrate and 10g/l sucrose) on microaerophilic growth of *Bacillus licheniformis*, *Bacillus sonorensis* and *Bacillus subtilis* 168.

Squares, *Bacillus licheniformis*; circles, *Bacillus sonorensis*; triangles, *Bacillus subtilis* 168.

Microaerophilic conditions were established by flushing the headspace of aerobic medium with nitrogen (30 sec) before sealing.

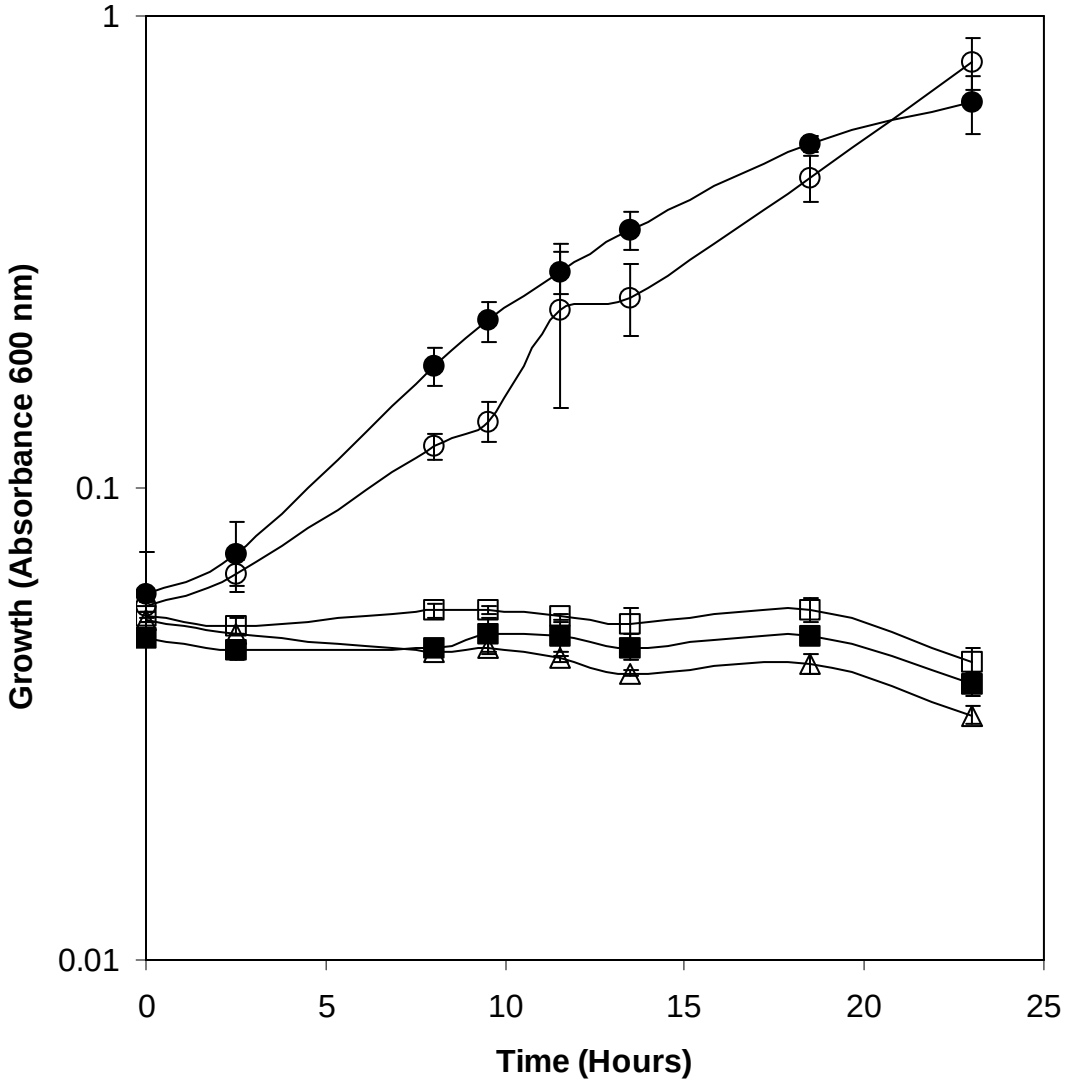


**Figure 3.12.** The effect of the addition of amino acids and pyruvate on anaerobic growth of *Bacillus subtilis* 168 in Medium F.

Squares, medium F minus amino acids; circles, medium F; triangles, medium F minus amino acids plus 10 mM pyruvate; filled squares, medium F minus amino acids plus 91 mM pyruvate; filled circles, medium F plus 10 mM pyruvate.

# Appendix I: Subsurface Hydrocarbon Mobilization Using Biosurfactants Requires Viscosity Control and a Low Molecular Weight Alcohol.

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## Abstract

Biosurfactants enhance hydrocarbon biodegradation by increasing the apparent aqueous solubility or affecting the association of the cell with the poorly soluble hydrocarbon. Here, we show that a lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 mobilized substantial amounts of residual hydrocarbon from sand-packed columns when a viscosifying agent and a low molecular weight alcohol were present. The amount of residual hydrocarbon mobilized depended on the biosurfactant concentration. One pore volume of cell-free culture fluid with 900 mg $l^{-1}$ of the biosurfactant, 10 mM 2,3-butanediol and 1000 mg $l^{-1}$ of partially hydrolyzed polyacrylamide polymer mobilized 82% of the residual hydrocarbon. Even low biosurfactant concentrations (16 mg $l^{-1}$) mobilized substantial amounts of residual hydrocarbon (29%). The lipopeptide biosurfactant system may be an effective agent in removing hydrocarbon contamination sources in soils and aquifers and for the recovery of entrapped oil from low production oil reservoirs.

## Introduction

The widespread use of petroleum hydrocarbons has resulted in the contamination of valuable groundwater resources. Petroleum hydrocarbons may exist in the vadose and saturated zones as a free liquid or ganglia of residual hydrocarbon (8, 20, 51). Even if the free liquid hydrocarbon can be removed, substantial amounts of residual hydrocarbon remain entrapped by capillary forces and represent a long-term source of contamination (20). Entrapment of petroleum hydrocarbons by capillary forces is also a major factor that limits oil recovery (4, 33, 36). Current technology recovers only one-third to one-half of the oil that is originally present in an oil reservoir (1). Since almost all regions of the world have been intensively explored for oil, the discovery of large new oil resources is unlikely and the exploitation of oil resources in existing reservoirs will be essential in the future (1).

Surfactants of synthetic or biological origin enhance hydrocarbon biodegradation by increasing the apparent aqueous solubility of the hydrocarbon (2, 14, 16-18, 23, 26, 34, 35, 37, 38, 45, 47, 48, 50, 54, 55) or by enhancing the interaction of the microbial cell with the hydrocarbon (2, 7, 37). Alternately, bulk hydrocarbon displacement can occur if capillary forces that entrap the hydrocarbon are reduced (40, 43). Interfacial tension between the hydrocarbon and aqueous phases is largely responsible for trapping the hydrocarbon in the porous matrix and ultra-low values (several orders of magnitude reduction) are needed for hydrocarbon mobilization (4, 10, 11, 36, 51). To achieve ultra-low interfacial tensions, surfactant concentrations significantly above that needed to form micelles (e.g., the critical micelle concentration) are required (8, 41). Optimized surfactant formulations recover almost all of the residual hydrocarbon present in

laboratory test systems (4, 5, 14, 40, 43) and have been shown to be effective in removing hydrocarbon contamination in aquifers (39, 40). However, the large concentrations (> g $l^{-1}$) of surfactants required for mobilization necessitate recycling and reusing the surfactants and treating only small portions of the contaminated zone at a time (27). High chemical and low crude oil prices have prevented the widespread use of surfactants for enhanced oil recovery.

Microorganisms produce a variety of biosurfactants (13), several of which generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize residual hydrocarbon (13, 19, 28). In particular, the lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the interfacial tension between oleic and aqueous phases to very low levels (<0.016 mN/m) (28, 31). The critical micelle concentration is 20 mg/l, indicating that the biosurfactant is effective even at very low concentrations (28).

The use of biosurfactants to mobilize residual hydrocarbon has met with mixed results. From 20 to 90% of hydrocarbons present in contaminated soils or oil shale cuttings were removed in the presence of biosurfactants (23, 49). The rhamnolipid biosurfactant produced by certain strains of *Pseudomonas* was 20 times more effective in solubilizing hexadecane than some synthetic surfactants (45) and mobilized up to 75% of the residual hexadecane from sand-packed columns (21, 22). However, the number of pore volumes required (40 to 70) was large. Residual oil is recovered when a biosurfactant-producing bacterium and the nutrients needed to support growth are introduced into sandstone cores (30, 46, 52), but residual hydrocarbon recoveries were often low (5 to 20%) and required multiple pore volumes of recovery fluid (30, 46).

Here, we show that substantial mobilization of residual hydrocarbon from a model porous system does occur at biosurfactant concentrations made naturally by *B. mojavensis* strain JF-2. Effective mobilization with a minimal volume of recovery fluid requires three components: a biosurfactant, a polymer and 2,3-butanediol.

## Results

### Effect of the Biosurfactant Alone

Since surfactant-enhanced remediation processes use surfactant concentrations far above the critical micelle concentration (8), we tested whether increasing the biosurfactant concentration would result in substantial mobilization of residual hydrocarbon. The amount of oil recovered when the biosurfactant-free culture fluid was used alone or with the addition of up to 1.7 g $l^{-1}$ of the partially purified biosurfactant was very low and similar to the 5% NaCl control (Table AI.1). Even at a very high biosurfactant concentration (12.3 g $l^{-1}$), similar to that used in surfactant-based enhanced oil recovery or groundwater remediation technologies (15, 40), the recovery of residual hydrocarbon was poor and not statistically different from that obtained with lower biosurfactant concentrations.

When the concentration of the biosurfactant used was 1.7 g $l^{-1}$ or less, no visible oil bank was formed. A few sand packs had places where clean sand was visible indicating that some oil had been mobilized. A visible oil bank formed when the concentration of the biosurfactant was 12.3 g $l^{-1}$. However, the oil bank dissipated by the time it reached the middle of the sand pack, suggesting that substantial oil could be produced if a mechanism to stabilize the oil bank could be developed.

**Effect of Polymer**

The addition of partially-hydrolyzed polyacrylamide (1 g $l^{-1}$) to cell-free culture fluid that contained 12.3 g $l^{-1}$ of the biosurfactant significantly improved residual hydrocarbon recovery (Table AI.1). As these polymer-biosurfactant-treated packs were eluted, an oil-bank formed that grew in thickness as it migrated through the sand pack. The same concentration of the polymer alone in 2.5% NaCl did not lead to the formation of an oil-bank, nor was residual hydrocarbon recovered (Table AI.1).

**Components Needed for Significant Residual Hydrocarbon Recovery**

Surfactant-based oil or contaminant recovery technologies use a small molecular weight alcohol in addition to the viscosifying agent and the surfactant (3, 5, 41). We tested whether the addition of 2, 3-butanediol, an alcohol commonly made by *Bacillus* species during anaerobic growth (42) along with partially-hydrolyzed polyacrylamide would recover residual hydrocarbon at biosurfactant concentrations made naturally by *B. mojavensis* strain JF-1 (i. e., at mg $l^{-1}$ rather than g $l^{-1}$ concentrations used in Table AI.1). When both partially-hydrolyzed polyacrylamide and 2,3-butanediol were added to cell-free culture fluid that contained the 16 ± 2.5 mg $l^{-1}$ (mean with the range) of the biosurfactant, 4.4 ± 3.4 ml of oil, representing about 29% of the hydrocarbon remaining in the sand pack was recovered (Table AI.2). Very little oil was recovered when partially-hydrolyzed polyacrylamide and 2,3-butanediol were added to sterile medium in the absence of the biosurfactant. Some oil was recovered when cell-free culture fluid with 16 mg $l^{-1}$ of biosurfactant was used alone or when supplemented with either partially-hydrolyzed polyacrylamide or 2,3-butanediol. However, these values were significantly less than the treatment that contained the biosurfactant, butanediol, and polymer.

Analysis of variance coupled with a Tukey test (53) showed that increasing the biosurfactant concentration significantly increased the amount of residual hydrocarbon recovered ($P<0.05$). About 15.5 ml of oil representing about 81.6% of the residual hydrocarbon was recovered when 1 g $l^{-1}$ of partially-hydrolyzed polyacrylamide and 10 mM 2,3-butanediol were added to cell-free culture fluid containing 914 mg $l^{-1}$ of the biosurfactant. The relationship between residual hydrocarbon recovery and the amount of biosurfactant was linear (Figure AI.1). About 5.3 ml of oil were produced per mg of biosurfactant injected into the sand packs, corresponding to about 290 to 300 µmoles of oil per µmole of biosurfactant present.

**Optimization of the Injection Protocol.**

Surfactant-based recovery technologies usually inject a small volume of the polymer before injection of the surfactant-alcohol-polymer mixture (4, 5). We found that there was a significant improvement in the volume of oil recovered (15.5 ml) when 5 ml of 1 g $l^{-1}$ of partially purified polyacrylamide dissolved in 2.5% NaCl was injected immediately before the biosurfactant-containing recovery fluid compared to the amount of oil recovered without the polymer pre-injection (13.1 ml). The biosurfactant and 2,3-butanediol concentrations in this experiment were 914 mg $l^{-1}$ and 10 mM, respectively. In another experiment, large amounts of oil were recovered even when less than one pore volume of the recovery fluid was injected into the sand packs (Table AI.3).

## Discussion

Our data show that the lipopeptide biosurfactant produced by *B. mojavensis* strain JF-2 mobilized large amounts of residual hydrocarbon from sand-packed columns at concentrations about 10 to 100-fold lower than typically used for surfactant-enhanced remediation process (4, 27, 41, 43, 44). Consistent with these findings, we found that the ratio of moles of oil recovered per mole of biosurfactant present was about 100 times greater than reported molar solubility ratios (MSR) for synthetic surfactants (8). The rhamnolipid biosurfactant was also shown to have a MSR 20 times greater than alkyl benzyl sulfonate surfactants (45). Thanamani and Shreve (45) argued that the rhamnolipid structure results in a large volume, low-density micelle that accommodates more hydrocarbon than alkyl benzyl sulfonate micelles. However, in our work, an oil bank formed, which suggests that once mobilized, the oil formed a separate phase that may not have required large amounts of biosurfactants to maintain.

Much anecdotal evidence implicates the need for a variety of products such as acids, gases, solvents, polymers, emulsifiers and/or biosurfactants or a combination of microorganisms that collectively make these products to recover residual oil from low production oil reservoirs (32). However, it is not clear whether these products are effective alone or if combinations of products are needed. The use of a mutant strain of *B. mojavensis* strain JF-2 defective in biosurfactant production clearly showed importance of biosurfactant production for residual oil recovery (30). Here, we found that a small molecular weight alcohol, 2,3-butanediol, and a viscosifying agent, PHPA, are also required. Thus, the belief that microbially enhanced oil recovery requires multiple microbial species or products may be due to the need to form the three components we define here as necessary for residual hydrocarbon mobilization.

We observed with biosurfactant alone that an oil-bank formed, but dissipated before it reached the effluent end of the pack. Compared to oil, water moves rapidly through the porous material. This results in an irregular front with water pushing through the oil and reaching the production well first. To prevent this, polymers such as xanthan gum and polyacrylamide are often added to chemical surfactant floods to increase the viscosity of the water phase (4). The role of 2,3-butanediol is less clear. Mobilization of residual oil requires middle-phase microemulsions where the surfactant is equally soluble in the water and oil phases and the prevention of mesophases (e.g., liquid crystals) (41). Alcohols are usually added to prevent surfactant liquid crystal formation and act to increase the effective surfactant concentration. Whether 2,3-butanediol serves such a role is unclear. In actual *in situ* applications, the addition of 2,3-butanediol may not be required since it is a common fermentative product of *Bacillus* species (42). We found that *B. mojavensis* strain JF-1 consistently produces between 5 to 10 mM 2,3-butanediol when grown anaerobically in our medium (unpublished data).

Our data indicate that the injection of biosurfactant-containing culture fluids with 2,3-butanediol and the polyacrylamide polymer will result in substantial recovery of residual hydrocarbon. The *in situ* biosurfactant production would be more difficult. The distribution of biosurfactant bacteria in aquifers and oil reservoirs is not known so it is not clear whether such organisms would have to be added. Once they are in the reservoir, a process that selectively stimulates the growth of biosurfactant-producing bacteria is needed. Preliminary results indicate that 15 to 90% of the total culturable population in groundwater samples contained the genes for the lipopeptide biosurfactant when the supplemented with proteose peptone and nitrate (unpublished data). Thus, it may be

possible to enhance the growth of biosurfactant-producing bacteria by selective nutrient additions.

## Experimental methods

### Cultivation

*Bacillus mojavensis* strain JF-2 (ATCC 39307) was grown in a phosphate-buffered, mineral salts medium (medium E) (24) with (in g $l^{-1}$) 1 g yeast extract, 1 g $NaNO_3$ and 30 g Proteose Peptone #3 (Difco Laboratories, Inc., Detroit, MI). *B. mojavensis* strain JF-2 was streaked onto agar plates of the above medium with 15 g $l^{-1}$ agar and colonies were used to inoculate 100-ml liquid cultures of the above medium. The 100-ml cultures were incubated without shaking at 37$^{o}$C for 24 hours and used to inoculate a 1-liter culture of the same medium. The 1-liter culture was incubated aerobically at room temperature until stationary phase was reached (about 48 h) with stirring provided by a magnetic stirrer and a stir bar.

Anaerobically-prepared medium contained 0.025% cysteine • HCl and was boiled and dispensed under $O_2$-free 100% nitrogen gas phase (6). Additions to and transfers from sterile, anaerobic media were done by using sterile syringes and needles degassed with $O_2$-free, 100% nitrogen prior to use (6).

### Cell-free Culture Fluid Preparation

After incubation, the cells from aerobically-grown cultures were removed by centrifugation (10,000 x *g*; 4$^{o}$C; 20 min). The cell-free culture fluid was divided into two portions and 2,3-butanediol was added to one portion to give a final concentration of 10 mM. 2,3-Butanediol was not formed in aerobically-grown cultures (data not shown). Each portion then received sufficient partially-hydrolyzed polyacrylamide to give a final concentration of 1 g $l^{-1}$. Uninoculated medium received the same concentrations of polymer and butanediol.

**Preparation of Cell-free Culture Fluid without Biosurfactant**

*B. mojavensis* strain JF-2 was grown anaerobically in the above medium in one-liter volumes using 2-liter bottles. After growth ceased, the cells were removed by centrifugation as above and the pH of the cell-free medium was reduced to less than 2 by the addition of 6 N HCl. The acidified, cell-free medium was left at 4$^{o}$C overnight to precipitate the biosurfactant (12). The precipitated material was removed by centrifugation as described above. The pH of the biosurfactant-free, cell-free medium was adjusted to 7.0 by the addition of NaOH pellets.

**Biosurfactant Preparation**

Large amounts of the biosurfactant were obtained by growing *B. mojavensis* strain JF-2 aerobically in carboys containing 8 l of medium without Proteose peptone. After growth ceased, the pH of the medium was adjusted to less than 2 by the addition of concentrated HCl. The acidified medium was kept overnight at 4$^{o}$C to precipitate the biosurfactant. The medium was centrifuged as described above. The supernatant was discarded and the pellet was dissolved in 200 ml of methanol. The methanol solution was centrifuged as above to remove particulate material. The concentration of the biosurfactant in methanol was measured by high-pressure liquid chromatography (HPLC). An appropriate volume of the biosurfactant solution was added to the neutralized, biosurfactant-free, cell-free medium to give the biosurfactant concentrations shown in Table AI.1.

**Biosurfactant Quantification**

The biosurfactant from a 20-ml sample of cell-free culture fluid was collected by acid precipitation and centrifugation as described above. The pellet containing the biosurfactant was extracted with 2 ml of methanol for 1 min with agitation. The insoluble material was removed by centrifugation as above. The biosurfactant was then quantified by a HPLC equipped with a $C_{18}$ column and an ultraviolet detector set at 210 nm(29). The mobile phase was 70% methanol and 30% of a 10 mM phosphate buffer (pH 6.8). The flow rate was 1 ml/min and the injection volume was 20 µl. Surfactin (Sigma Chemical Co. St. Louis, MO) was used as the standard. The amount of biosurfactant present in cultures was corrected for the percent recovery of known amounts of surfactin that were added to sterile medium and carried through acid precipitation and methanol extraction procedures.

**Preparation of Sand-packed Columns**

Plexiglas columns were approximately 3.8 cm (inside diameter) by 26 cm long and packed with quartz sand (approximately 20-40 mesh grain size). Each end had a plate had an O-ring to prevent leaks and a fitting sealed with a rubber septum. Connections to sources of vacuum, gas, and liquids were made through nylon tubing attached to a syringe needle that was inserted into this septum. The weight of the sand was calculated from the difference in weight before and after packing with sand. Air was removed from the column by placing the column under vacuum for 10 minutes. The column was then saturated with a 5% NaCl brine solution by positive displacement. Once the brine reached the top of the column, a syringe needle was inserted into the top septum to allow the solution to exit the column. After one pore volume of the solution passed through the column, the flow rate was measured with a stopwatch and a graduated cylinder. The

injection pressure was measured by using a pressure gauge attached between the fluid reservoir and the column and used to calculate the permeability of the column to the brine solution according to Darcy's law (25). The column was then weighed and the volume of brine inside the column (pore volume) was calculated from the difference in the wet and dry weight of the column and the brine density.

The column was then saturated with oil by positive displacement by keeping the oil reservoir pressurized with nitrogen gas. The displaced water was collected in a graduated cylinder to measure the volume. After only oil was displaced from the column, the flow rate and injection pressure were determined as described above. These data were used to calculate the effective permeability of the column to oil at residual water saturation. The amount of residual water present in the column was calculated from the amount of water displaced from the column during oil flooding and the amount of water present after brine saturation. The column was then flooded to residual oil saturation by again injecting the brine solution into the column until no more oil was displaced from the column. The amount of oil displaced from the column was determined volumetrically and used to calculate the residual oil saturation from difference in oil volume before and after brine flooding. After water breakthrough, the flow rate of brine and the injection pressure were determined as described above and used to calculate the effective permeability of the column to brine at residual oil saturation. At least 10-11 pore volumes of brine were injected through the column to ensure that it was at residual oil saturation.

**Biosurfactant Treatments**

The column was flooded with a biosurfactant solution as described above for brine flooding. Unless otherwise indicated, each column was flooded with 200 ml of the biosurfactant solution, approximately 2 pore volumes. Effluent from the columns was collected in 50-ml syringes held in a vertical position to allow the measurement of oil and brine volumes. Duplicate columns were used for each treatment. When the biosurfactant solution contained partially hydrolyzed polyacrylamide (PHPA) (1 g $l^{-1}$), 5 ml of the PHPA (1 g $l^{-1}$) in 2.5% NaCl was injected into the column prior to injection of biosurfactant-containing solution. After the biosurfactant-containing solution passed through the column, 25 ml of 1 g $l^{-1}$ of PHPA in 2.5% NaCl followed by 25 ml of 0.7 g $l^{-1}$ of PHPA in 2.5% NaCl were injected into the column. Each column was then flooded with 150 ml of 2.5% NaCl.

**Petrophysical Data**

The sand packs had the following properties (mean ± standard deviation): porosity, 31.9 ± 1.2 %; pore volume, 90.3 ± 6.9 ml; permeability, 2.0 ± 0.1 Darcies; and residual oil saturation, 21.9 ± 3.0 %. The crude oil had a density of 0.825 g $cm^{-3}$. An average molecular weight for crude oil of 320 to 330 g $mol^{-1}$ was estimated from the crude oil composition of an Oklahoma crude oil by using the method of Waston, Nelson and Murphy(9). The molar ratio oil recovered to biosurfactant present was estimated from the slope of the line in Figure AI.1, the crude oil density, and an assumed average molecular weight of crude oil of 320 to 330 g mol(8).

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**Table AI.1**. Oil recovery with different concentrations of the biosurfactant [a].

| Type of Fluid Injected | Polymer Present | Biosurfactant Concentration (mg $l^{-1}$) | Volume Injected (ml) | Residual Oil Saturation (%)[b] | Volume of Oil Recovered (ml) [b] | Percent Residual Oil Recovery [b] |
|---|---|---|---|---|---|---|
| 5% NaCl | - | 0 | 200 | 20 (10) | 0.2 (0) | 1.3 (0.6) |
| 2.5% NaCl | + | 0 | 150 | 8.4 (7.1) | ND [c] | ND [c] |
| Biosurfactant-free culture fluid | - | 0 | 200 | 25 (4) | 0.2 (0.5) | <0.1 |
| | - | 100 | 100 | 13 (8) | 0.1 (0) | 1.1 (0.9) |
| | - | 175 | 100 | 16 (4) | 0.4 (0.1) | 2.2 (0.5) |
| | - | 300 | 100 | 17 (10) | 0.4 (0.3) | 2.3 (1.6) |
| | - | 1700 | 200 | 27 (10) | 0.4 (0.1) | 1.6 (0.1) |
| | - | 12300 | 200 | 22 (3.4) | 0.9 (0.7) | 4.9 (3.1) |
| | + | 12300 | 60 | 13 (7.2) | 2.6 (0.1)[d] | 23.6 (13.9)[d] |

[a] Biosurfactant-free culture fluid was prepared by removing cells from an anaerobically grown culture by centrifugation and then removing any biosurfactant that may have been present in the culture by acid precipitation. The pH of the medium was then adjusted to 7.0 and the indicated concentration of the partially purified biosurfactant was added. The concentration of partially hydrolyzed polyacryalmide was 1 g $l^{-1}$.

[b] Mean of duplicate determinations with the range shown in parentheses.

[c] ND, not determined.

[d] Analysis of variance and Tukey test showed that these means were significantly different from the means of the other treatments ($P<0.05$).

**Table AI.2**. Effect of biosurfactant concentration and the addition of partially hydrolyzed polyacrylamide and 2,3-butanediol on oil recovery by cell-free spent medium of *B. mojavensis* strain JF-2.

| Injected [a] solution | Additions | Number of Replicates | Residual Oil Saturation (%)[b] | Volume of Oil Recovered (ml)[b] | Percent Residual Oil Recovery [b] |
|---|---|---|---|---|---|
| Sterile medium | None | 2 | 14.6 (2.5) | <0.1 | NA [c] |
| | Butanediol + Polymer | 2 | 24.2 (5) | 0.2 (0.1)* | 1.1 (0.2)* |
| Spent medium | None | 4 | 14.6 (6.5) | 1.8 (0.6)** | 10.6 (7)** |
| | Butanediol | 2 | 19.2 (5.7) | 1.9 (0.6)** | 15.2 (0.6)** |
| | Polymer | 2 | 16.3 (0.7) | 2.4 (0.1)** | 15.2 (0.6)** |
| | Butanediol + Polymer | 4 | 15.8 (5.6) | 4.4 (3.4)*** | 29.3 (15.6)*** |

[a] Spent medium was prepared by removing the cells by centrifugation from an aerobically grown culture of *B. mojavensis* strain JF-2 that contained the 16 ± 2.5 mg $l^{-1}$ of the biosurfactant. Sterile and spent media were amended with 1 g $l^{-1}$ of partially purified polyacrylamide and 10 mM 2,3-butanediol as indicated.

[b] Mean value with the range shown in parentheses. Means with different number of * were significantly different from each other by analysis of variance and a Tukey test ($P < 0.05$).

[c] NA, not applicable.

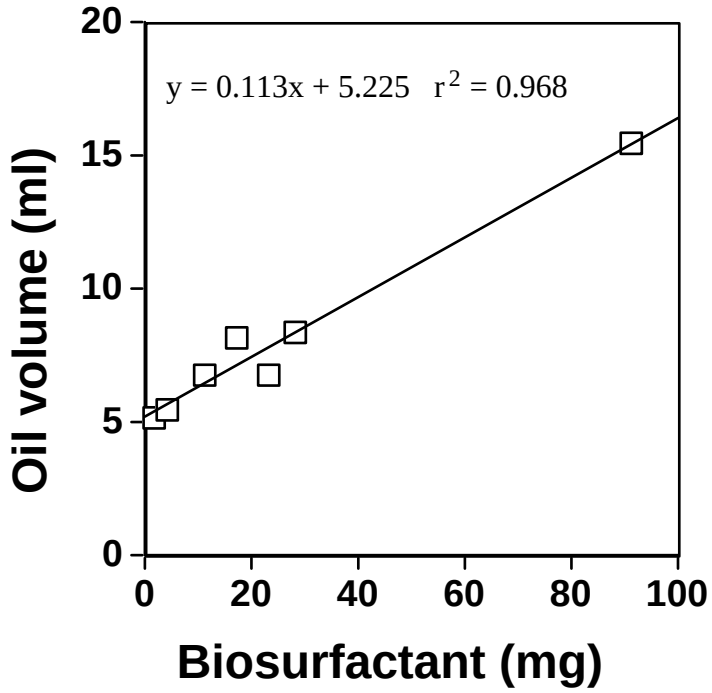
**Table AI.3**. Oil recovery when different volumes of recovery fluid were injected into sand packs.[a]

| Volume of Recovery Fluid (Pore Volume) | Residual Oil Recovery | Volume of Oil Recovered (ml)[b] | Percent Residual Oil Recovery[b] |
|---|---|---|---|
| 1.1 | 16.2 (0.9) [b] | 8.4 (0.9)[y] | 50.4 (4.6)* |
| 0.87 | 21.1 (3.4) | 6.8 (0.5)[y] | 32.9 (0.4)** |
| 0.66 | 20.8 (0.1) | 8.2 (1.4)[y] | 38.4 (4.5)** |
| 0.46 | 24.6 (3.9) | 6.8 (0.9)[y] | 30.2 (0.3)** |

[a]Cells from an aerobically grown culture of *B. mojavensis* strain JF-2 that contained the 283 mg $l^{-1}$ of the biosurfactant were removed by centrifugation and 1 g $l^{-1}$ of partially purified polyacrylamide and 10 mM 2,3-butanediol were added.

[b] Mean of duplicate sand packs with the range shown in parentheses. Means with different number of * were shown to be significantly different from each other by analysis of variance and a Tukey test ($P < 0.05$).

**Figure AI.1**. The relationship between oil recovery and the amount of biosurfactant injected into the sand packs.



# Appendix II: Other Nutritional Requirements of *Bacillus mojavensis* JF-2 for Anaerobic Growth


## Abstract

The goal of the following work was to determine other anaerobic growth factor requirements of *Bacillus mojavensis* JF-2. JF-2 requires nitrate, amino acids and vitamins in addition to DNA or deoxyribonucleosides for anaerobic growth. Nitrate can be replaced with pyruvate.


## Introduction

One of the potential methods to control biosurfactant production was to limit or vary the source of nitrogen or carbon. Many biosurfactant-producing microorganisms produce biosurfactant dependent on the available carbon source or the conditions of nitrogen metabolism (1, 2, 4-6, 8). So the first step towards improving biosurfactant production was to improve growth under anaerobic conditions and to clearly define the nutritional conditions necessary for anaerobic growth. It was hoped that this would lead to a medium that could be used to detect subtle nutritional influences on biosurfactant production. Chapter one described the requirement for DNA for anaerobic growth of JF-2. It was noted that Proteose Peptone appeared to supply nutrients in addition to DNA for anaerobic growth. Other growth factors were identified so that it might be possible to limit specific nutrients in the medium and thus control or improve biosurfactant production.

## Methods and Materials

### Cultivation

Herring sperm DNA (1g/l) was added to medium E before autoclaving. The amino acid supplement consisted of (final concentration, g/l): Casamino acids (DF 0230-15-5) (4), glutamine (Sigma G7029) (0.1), tryptophan (Sigma T0254) (0.1), asparagine (Sigma 4284) (0.02), and methionine (Sigma M9625) (0.02). Amino acids were added after autoclaving.

The vitamin solution consisted of (mg/l) (3): pyridoxine-HCl (10), thiamine-HCl (5), riboflavin (5), nicotinic acid (5), calcium pantothenate (5), PABA (5), lipoic acid (5) biotin (2), folic acid (2), vitamin $B_{12}$ (0.1). Ten milliters of this solution was added per liter of medium.

Initially, it appeared that vitamins were not required for anaerobic growth of JF-2. To test this hypothesis, JF-2 was transferred several times in vitamin-free medium. For this transfer, JF-2 was grown in vitamin and yeast extract-free medium E containing 0.5 g/l DNA and 4g/l Casamino acids. After a 1-week incubation, a 1% inoculum of this culture was transferred to two tubes of the same with and without vitamins. One tube of each was kept uninoculated. This was repeated until the vitamin-free culture failed to grow.

## Results and Discussion

The effect of nitrate on anaerobic growth of JF-2 in Proteose Peptone supplemented medium E was tested. Nitrate was required for anaerobic growth (Figure AII.1) but increasing the concentration from 6 to 60 mM increased the extent of growth

very little ($A_{max}$ of 0.5 vs. 0.6). The effect of nitrate on anaerobic growth in DNA supplemented medium E was also tested. Again, nitrate was required for anaerobic growth (Figure AII.2). One millimolar nitrate or less was growth limiting, and only 5 mM nitrate was required for complete growth ($A_{max}$ = 0.56); increasing the concentration to 15 mM nitrate did not significantly increase the extent of growth ($A_{max}$ = 0.6).

Aside from DNA and nitrate, yeast extract was also a source of nitrogen in medium E. Yeast extract could provide nucleic acid bases, amino acids, and/or vitamins. After determining that yeast extract was required for anaerobic growth of JF-2 in DNA supplemented medium E, the effect of nucleic acid bases, amino acids, and vitamins on anaerobic growth was tested. Amino acids were required for anaerobic growth of JF-2 in medium E with 0.5 g/l DNA (Figure AII.3). The addition of amino acids to DNA-supplemented medium E without yeast extract and without vitamins, supported anaerobic growth to an $A_{max}$ of about 0.4. It appeared that vitamins were not required, however, subsequent subculturing without vitamins did not result in growth after the third transfer (Table AII.1), revealing that vitamins were required for anaerobic growth. The addition of vitamins alone to DNA-supplemented medium E without yeast extract did not support anaerobic growth above that in DNA-supplemented medium E without yeast extract, but vitamins combined with amino acids did support growth up to an $A_{max}$ of 0.8. It appears that Proteose Peptone supplied DNA, amino acids and vitamins.

As described in Chapter 3, pyruvate was reported to enhance fermentative growth of *Bacillus subtilis* (7). Since the anaerobic growth requirements found to date of *B. subtilis* were similar to JF-2, we hypothesized that pyruvate might enhance fermentative growth of JF-2 also. We found that pyruvate did not replace the requirement for amino

acids and did not enhance anaerobic growth in medium F (which contains nitrate) (Figure AII.4).

However, pyruvate did replace the requirement for nitrate (Figure AII.5). Without nitrate in the medium, 1 mM pyruvate was limiting for growth. Increasing the pyruvate concentration from 5 to 10 mM did not greatly increase the extent of growth ($A_{max}$ of 0.4 to 0.49) but increasing the concentration to about 90 mM increased growth ($A_{max}$ of 0.9).

Nakano *et al*., observed that pyruvate (90 mM) and amino acids were equally effective for meeting a pyruvate requirement. They suggested that amino acids supplied pyruvate, which served to activate the genes required for fermentative growth. If this were true in the case of JF-2, then amino acids or enzymatic digests of protein should also replace the nitrate requirement (by supplying pyruvate to stimulate fermentation). Since this is not the case, it appears unlikely that pyruvate enhances fermentative growth by activating the genes required for fermentation. It seems more likely that pyruvate acts as an electron acceptor and that amino acids do not supply sufficient pyruvate for growth under strict anaerobic conditions. In this case JF-2, would not be growing fermentatively. Instead JF-2 would be respiring pyruvate; which would be unusual since pyruvate reduction has not been reported in the literature for any bacilli.

## References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2. **Akpa, E., P. Jacques, B. Wathelet, M. Paquot, R. Fuchs, H. Budzikiewicz, and P. Thonart.** 2001. Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. Appl. Biochem. Biotech. **91-93:**551-561.
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6. **Mulligan, C. N., and B. F. Gibbs.** 1989. Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **55:**3016-3019.

7. **Nakano, M. M., Y. P. Dailly, P. Zuber, and D. P. Clark.** 1997. Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. J. Bact. **179:**6749-6755.

8. **Robert, M., M. E. Mercade, M. P. Bosch, J. L. Parra, M. J. Espuny, M. A. Manresa, and J. Guinea.** 1989. The effect of carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnology Letters **11:**871-874.

**Table AII.1** The effect of serial transfer of anaerobically grown culture of *Bacillus mojavensis* strain JF-2 to Medium E without yeast extract.

| Addition to yeast extract-free Medium E with 0.5 g/l DNA and 4 g/l Casamino acids | $Absorbance_{MAX}$ after second transfer. | $Absorbance_{MAX}$ after third transfer |
|---|---|---|
| No addition | 0.37 ± 0.058 | 0.064 ± 0.023 |
| Vitamins | 0.61 ± 0.17 | 0.60 ± 0.05 |

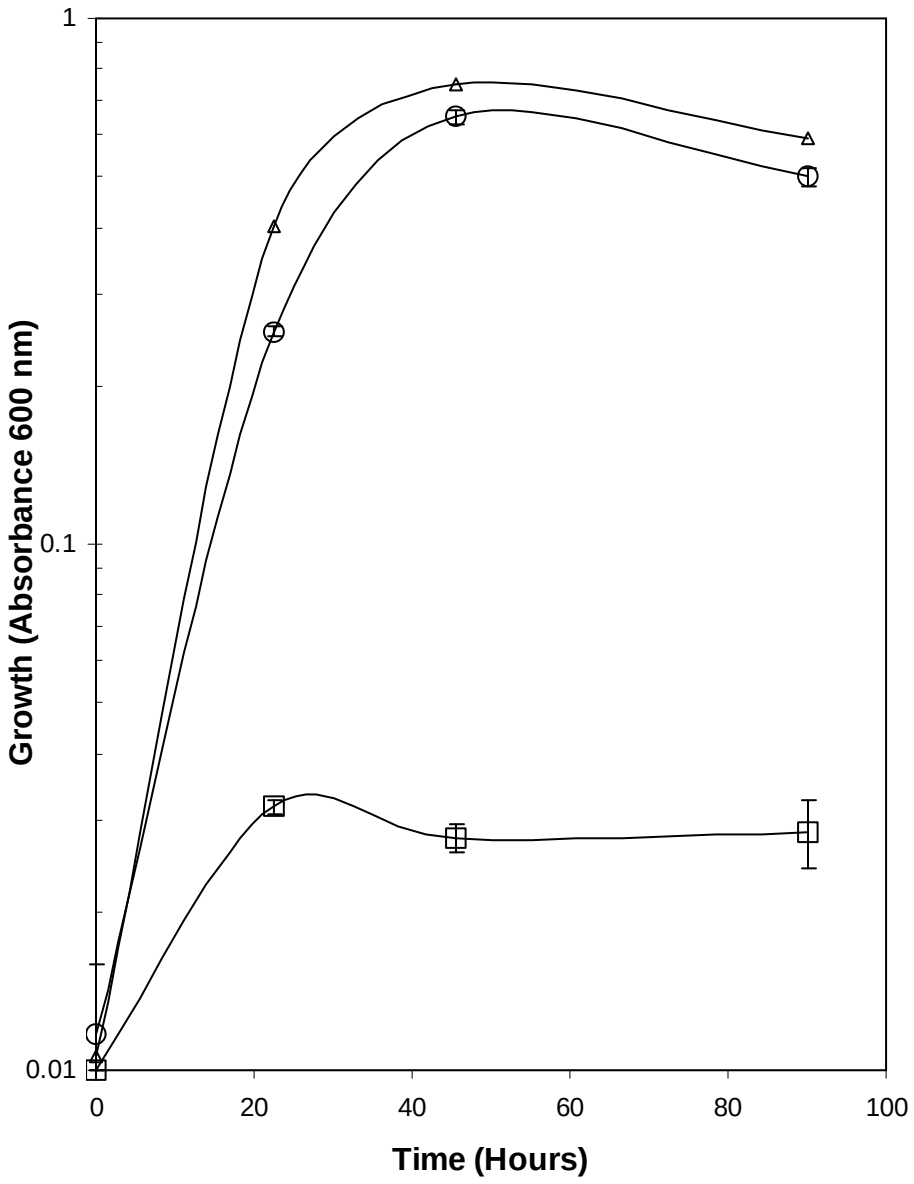


**Figure AII.1.** The effect of nitrate on anaerobic growth of *Bacillus mojavensis* JF-2 in Proteose Peptone supplemented medium E.

Squares, no nitrate; circles, 6 mM nitrate; triangles, 60 mM nitrate.

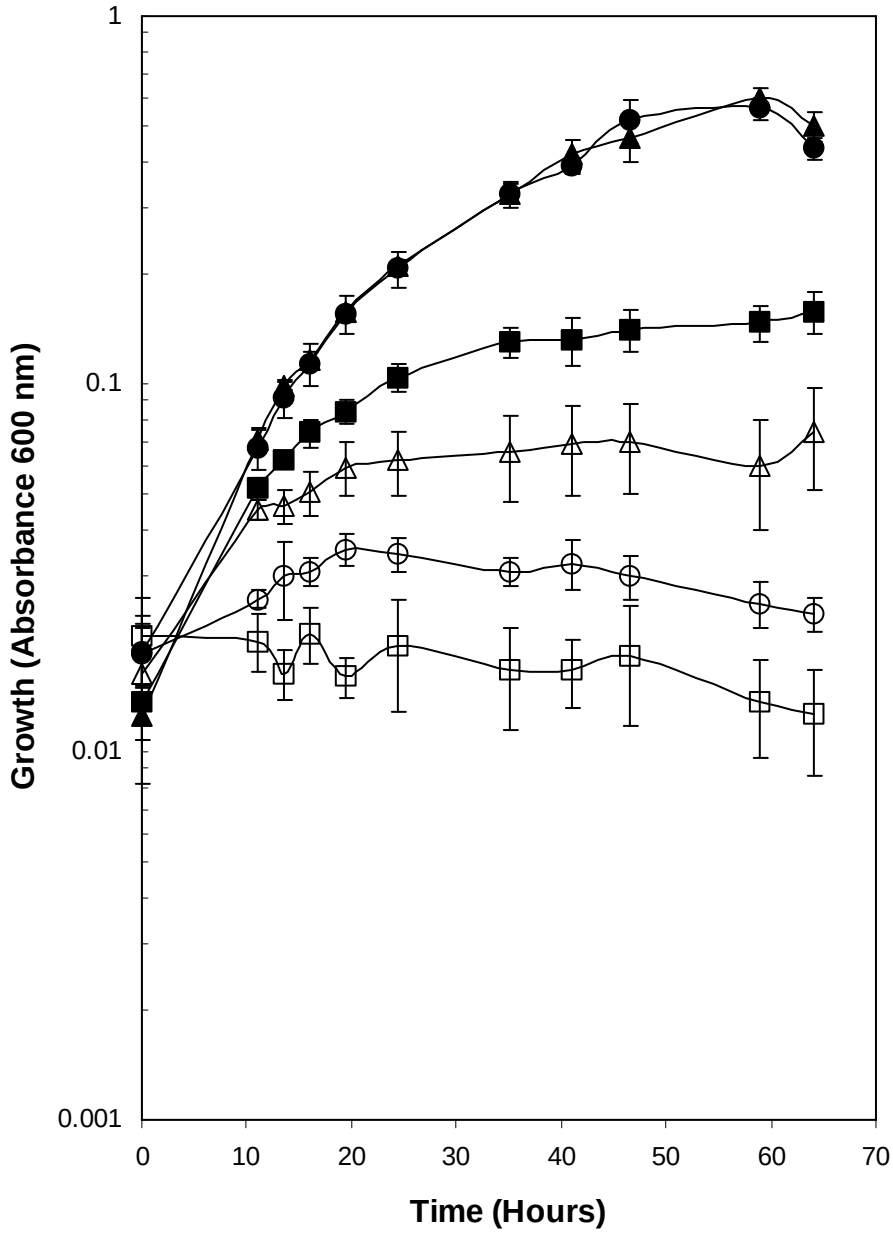


**Figure AII.2.** The effect of nitrate on anaerobic growth of *Bacillus mojavensis* JF-2 in DNA supplemented Medium E.

Squares, no nitrate; circles, 0.01 mM nitrate; triangles, 0.05 mM nitrate; filled squares, 1 mM nitrate; filled circles, 5 mM nitrate; filled triangles, 15 mM nitrate.

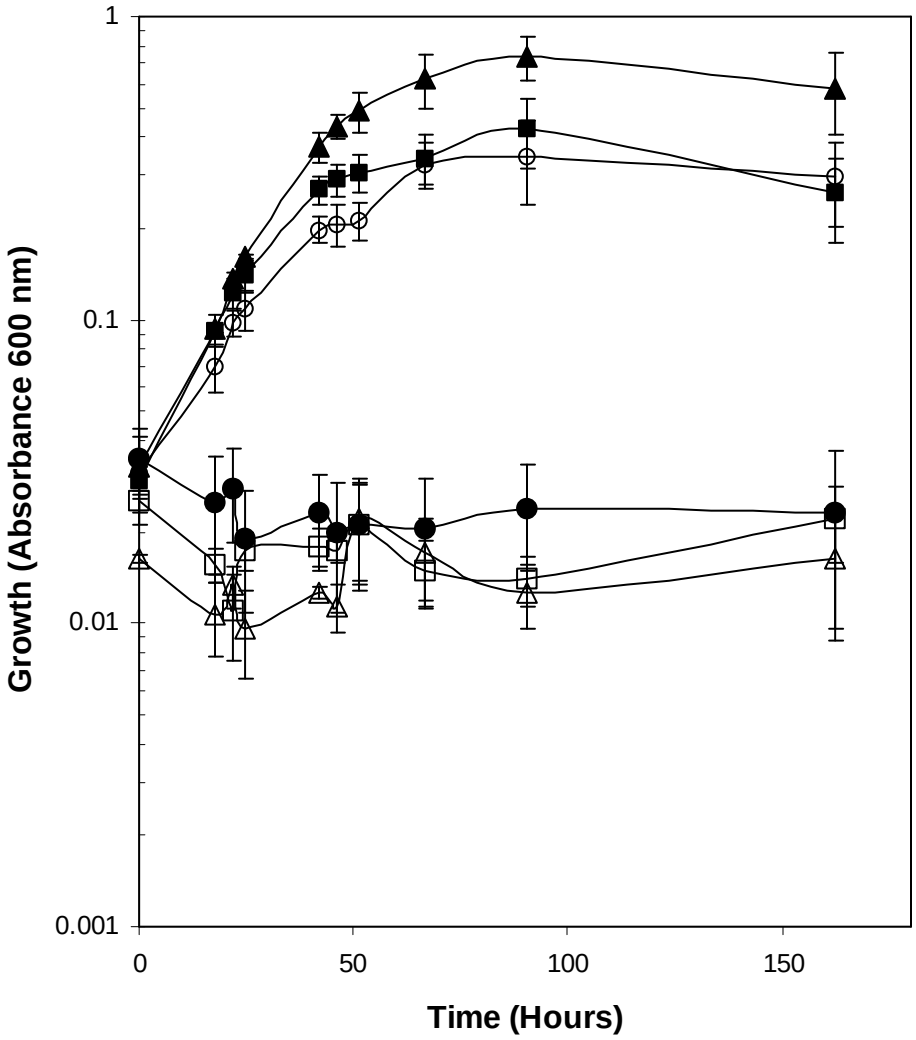


**Figure AII.3.** The effect of yeast extract, nucleic acid bases, amino acids and vitamins on anaerobic growth of *Bacillus mojavensis* JF-2 in DNA supplemented (0.5 g/l) medium E without yeast extract.

Squares, no addition; circles, plus 1 g/l yeast extract; triangles, plus nucleic acid bases (0.1 g/l each adenine, guanine, cytosine and thymine); filled squares, plus amino acids; filled circles, plus vitamins; filled triangles, plus amino acids and vitamins.

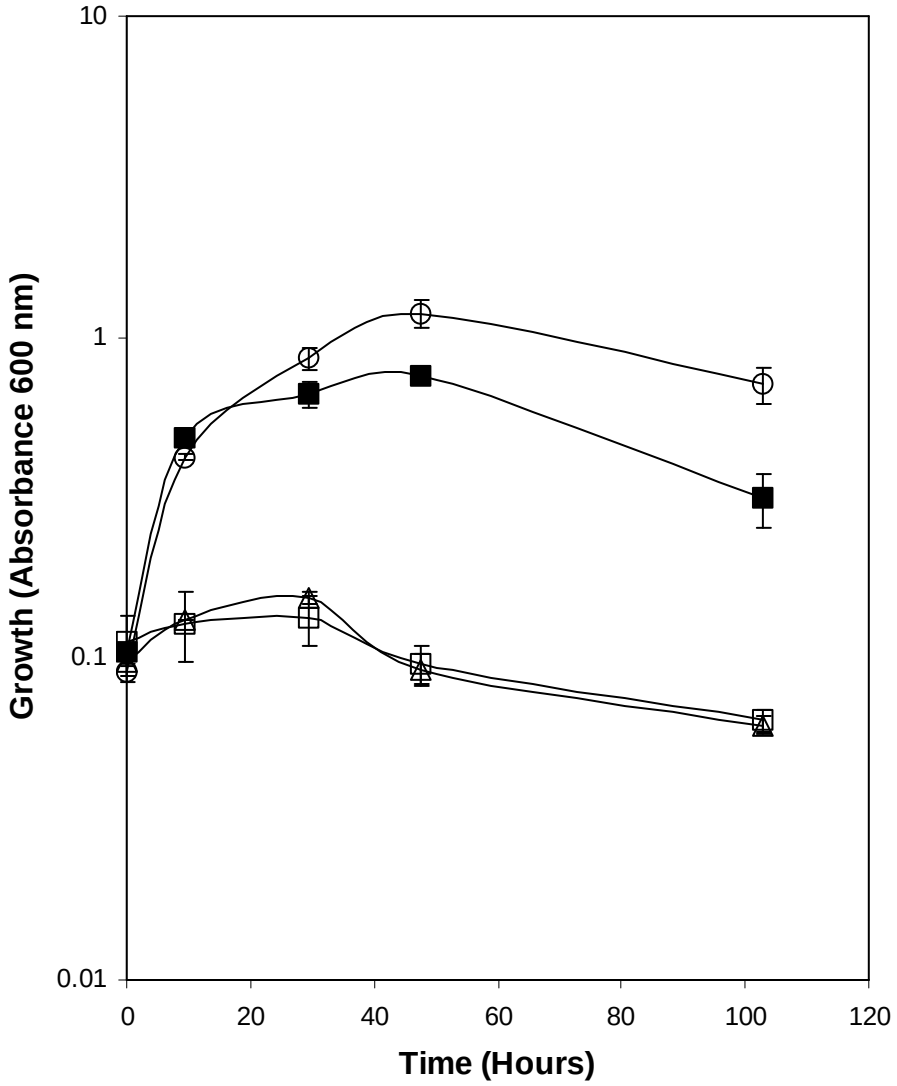


**Figure AII.4.** The effect of the addition of pyruvate with or without amino acids to medium F on anaerobic growth of *Bacillus mojavensis* JF-2.

Open squares, medium F minus amino acids; open circles, medium F; open triangles, medium F minus amino acids plus 10 mM pyruvate; closed squares, medium F plus 10 mM pyruvate.

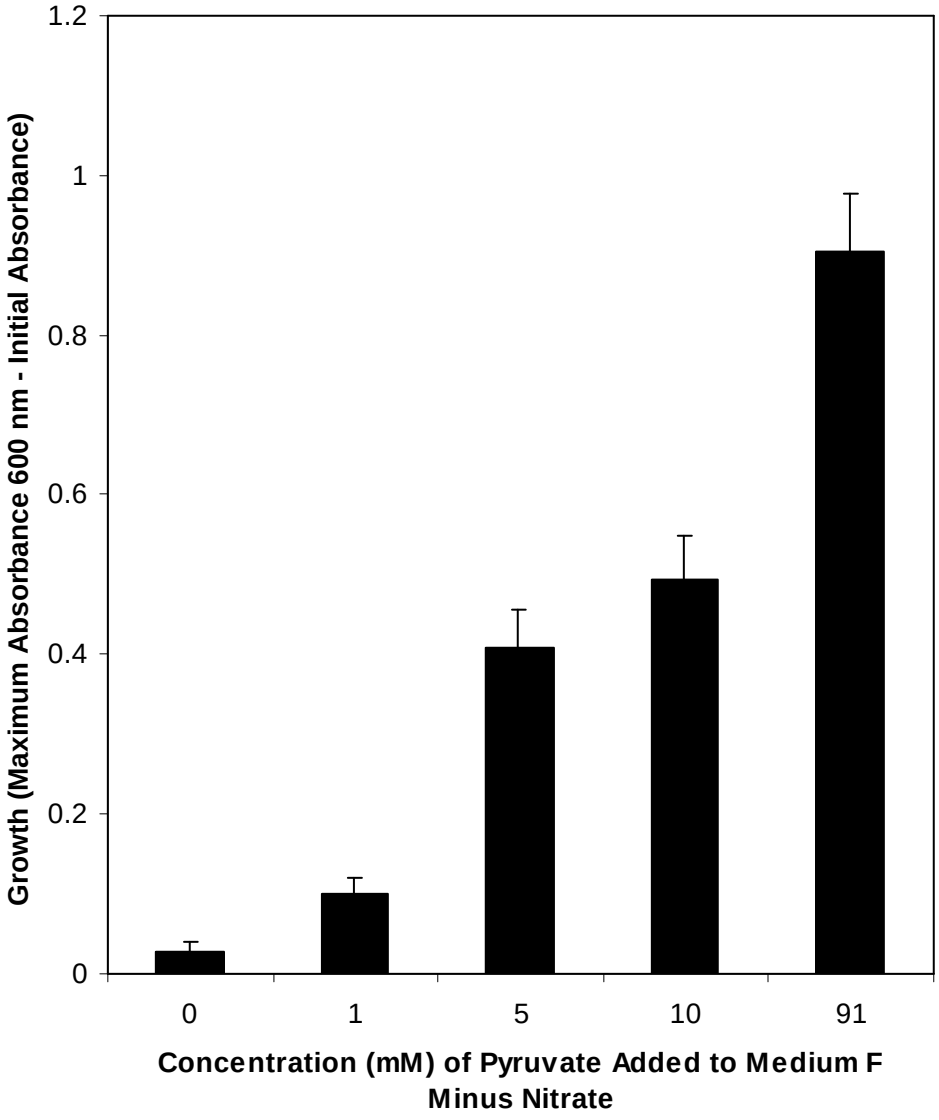


**Figure AII.5.** The effect of pyruvate concentration on anaerobic growth of *Bacillus mojavensis* JF-2 in Medium F without nitrate.

# Appendix III: The Effect of the Addition of DNA to Medium E on Anaerobic Growth of *Bacillus licheniformis* T68-25, *Bacillus licheniformis* T68-35and *Bacillus sonorensis* T68-8.

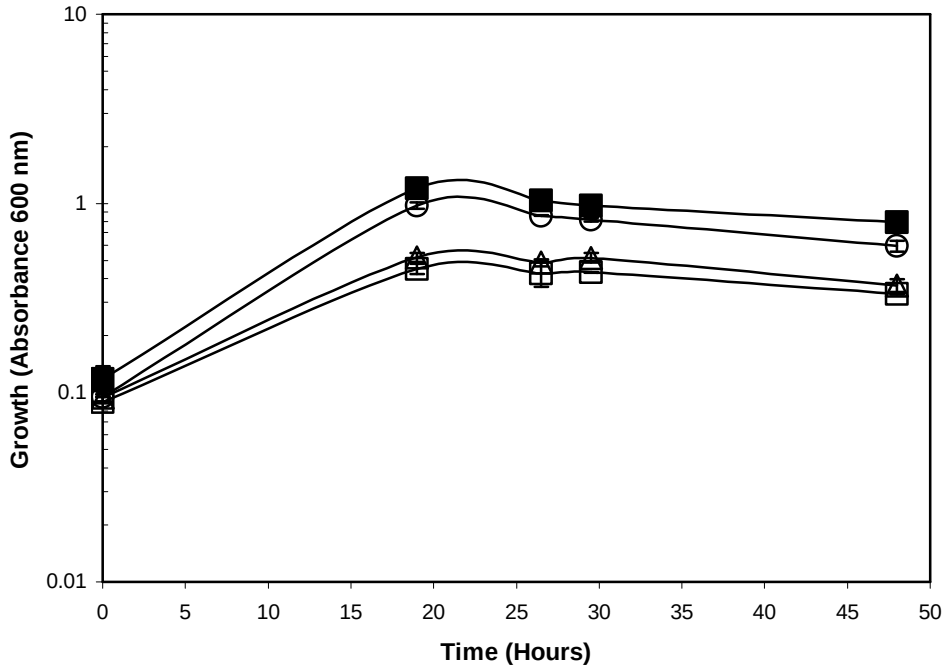


**Figure AIII.1.**The effect of the addition of DNA to medium E on anaerobic growth of *Bacillus licheniformis* T68-25.

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.

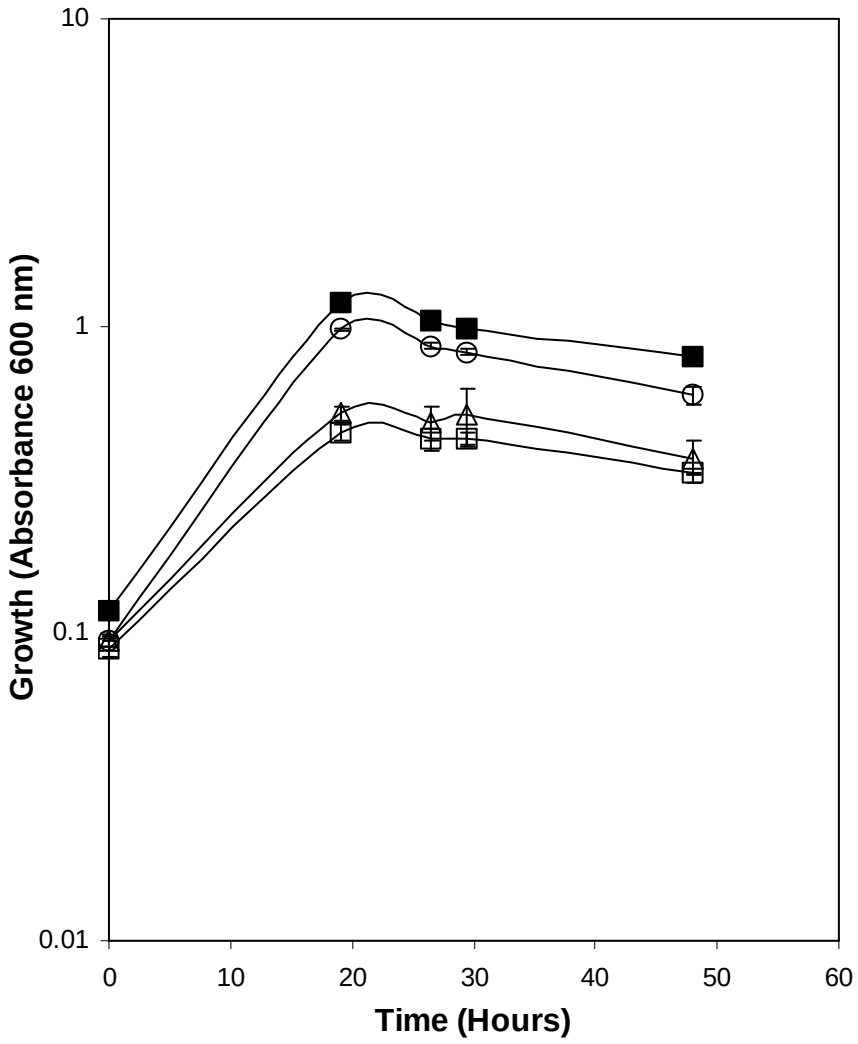


**Figure AIII.2.**The effect the addition of DNA to medium E on anaerobic growth of *Bacillus licheniformis* T68-35.

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.

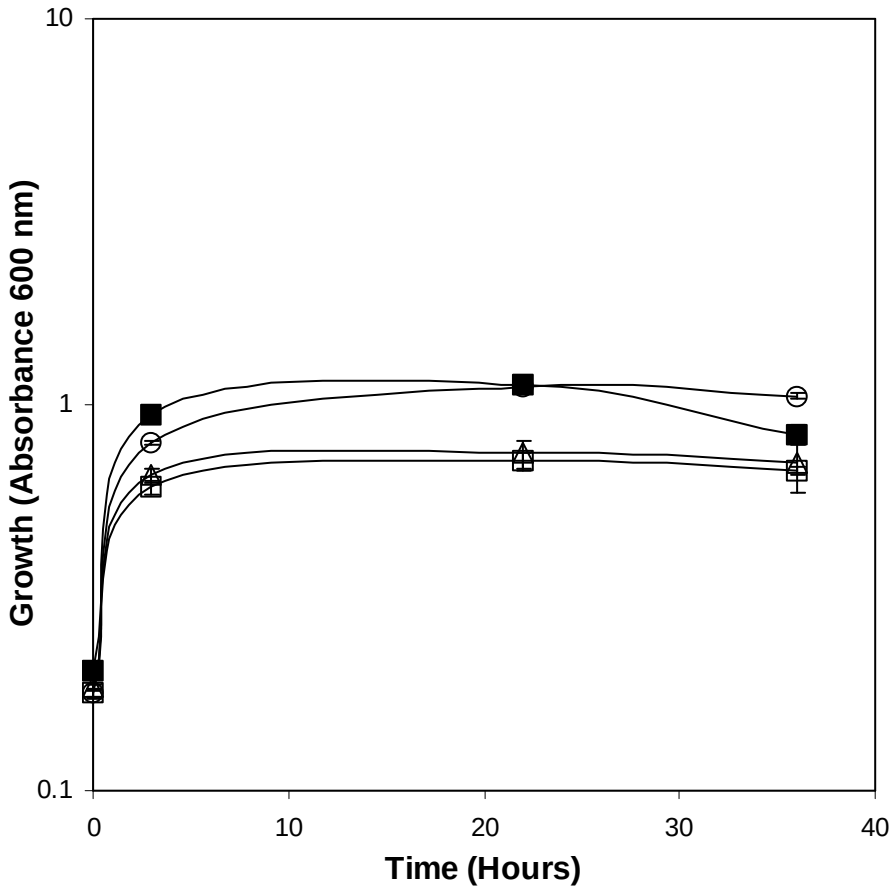


**Figure AIII.3.**The effect the addition of DNA to medium E on anaerobic growth of *Bacillus sonorensis* T68-8.

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.

# Appendix IV: The results of the BLAST search using the amino acid sequence of the Class III ribonucleotide reductase gene from *Bacillus cereus*

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= (618 letters)

**Database:**

Completed *Bacillus anthracis* str. A2012;
Completed *Bacillus anthracis* str. Ames;
Completed *Bacillus cereus* ATCC 10987;
Completed *Bacillus cereus* ATCC 14579;
Completed *Bacillus halodurans*;
Completed *Bacillus subtilis* subsp. subtilis str. 168;
Completed *Lactobacillus johnsonii* NCC 533;
Completed *Escherichia coli* K12;

36,023 sequences; 10,083,375 total letters

**Alignments:**

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Score = 1261 bits (3264), Expect = 0.0
Identities = 618/618 (100%), Positives = 618/618 (100%)

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           DTYQACEAF+HNSNSMHSRGGGQVPFISINYGTDTSKEGR+LIKQLLKATQAGLGKGETP
Sbjct: 181 DTYQACEAFIHNSNSMHSRGGGQVPFISINYGTDTSKEGRLLIKQLLKATQAGLGKGETP 240

Query: 240 IFPIQIFKMKKGVNFEESDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV 299
           IFPIQIFKMKKGVNFEE DPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV
Sbjct: 241 IFPIQIFKMKKGVNFEECDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV 300

Query: 300 CYMGCRTRVMSNIHGEETAIGRGNLSFTSINLVKLALISGSKEAFFEALNYYLDLGIKQL 359
           CYMGCRTRVMSNIHGEETAIGRGNLSFTSINLVKLALISGS+EAFFEALNYYLD+GI+QL
Sbjct: 301 CYMGCRTRVMSNIHGEETAIGRGNLSFTSINLVKLALISGSQEAFFEALNYYLDVGIQQL 360

Query: 360 LERYAYQCTKRARDFQFLYSQGVWRGGETLQPEDSVASILKQGTLSIGFIGLAECLVALT 419
           LER+ YQCTKRARDF+FLYSQGVWRGGE LQPEDSVASILKQGTLS+GFIGLAECLVALZ
Sbjct: 361 LERFEYQCTKRARDFRFLYSQGVWRGGEKLQPEDSVASILKQGTLSLGFIGLAECLVALT 420

Query: 420 GKHHGEDEESWKLGYEIISFMRDRMDKATEEHQLNFSVIATPAEGLSGKFVKKDRKEFGV 479
           GKHHGEDEESWKLGYEIISFMRDRMDKATEEH+LNFSVIATPAEGLSGKFVKKDR+EFGV
Sbjct: 421 GKHHGEDEESWKLGYEIISFMRDRMDKATEEHELNFSVIATPAEGLSGKFVKKDREEFGV 480

Query: 480 ISGITNHNYYTNSFHIPVYYNIQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALK 539
           ISGITNHNYYTNSFHIPVYY+IQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALK
Sbjct: 481 ISGITNHNYYTNSFHIPVYYDIQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALK 540

Query: 540 QIVQAMAEHGVGYGSINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGD 599
           QIVQAMAE+GVGYGSINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGD
Sbjct: 541 QIVQAMAENGVGYGSINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGD 600

Query: 600 MSKWNSAKRSEEIDRVKHK 618
           MSKWNSAKRSEE+DRVKHK
Sbjct: 601 MSKWNSAKRSEEMDRVKHK 619
```

**>ref|NP_657512.1|** Gly_radical, G radical [***Bacillus anthracis*** A2012] Length = 605

Score = 1211 bits (3133), Expect = 0.0

Identities = 587/605 (97%), Positives = 600/605 (99%)

```
Query: 14  VFETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSDQVKKAINENILY 73
           +FETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVE LLSDQVKKAINENILY
Sbjct: 1   MFETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVEKLLSDQVKKAINENILY 60

Query: 74  PHDLDFYATGTTTCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHG 133
           PHDLDFYATGTTTCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHG
Sbjct: 61  PHDLDFYATGTTTCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHG 120

Query: 134 GQSFALFDIDLAPYVRKTIERHKKRLQSYPLKKEQIEEFAWKETENDTYQACEAFVHNSN 193
           GQSFALFD+DLAPYVRKT+ERHKKRLQSYPL KEQIEEFAWKETENDTYQACEAF+HNSN
Sbjct: 121 GQSFALFDVDLAPYVRKTVERHKKRLQSYPLTKEQIEEFAWKETENDTYQACEAFIHNSN 180

Query: 194 SMHSRGGGQVPFISINYGTDTSKEGRMLIKQLLKATQAGLGKGETPIFPIQIFKMKKGVN 253
           SMHSRGGGQVPFISINYGTDTSKEGR+LIKQLLKATQAGLGKGETPIFPIQIFKMKKGVN
Sbjct: 181 SMHSRGGGQVPFISINYGTDTSKEGRLLIKQLLKATQAGLGKGETPIFPIQIFKMKKGVN 240

Query: 254 FEESDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRTRVMSNIH 313
           FE SDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRTRVMSNIH
Sbjct: 241 FEGSDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRTRVMSNIH 300

Query: 314 GEETAIGRGNLSFTSINLVKLALISGSKEAFFEALNYYLDLGIKQLLERYAYQCTKRARD 373
           GEETAIGRGNLSFTSINLVKLALISGSKEAF+EALNYYLDLGIKQLLER+ YQCTKRARD
Sbjct: 301 GEETAIGRGNLSFTSINLVKLALISGSKEAFYEALNYYLDLGIKQLLERFEYQCTKRARD 360

Query: 374 FQFLYSQGVWRGGETLQPEDSVASILKQGTLSIGFIGLAECLVALTGKHHGEDEESWKLG 433
           F+FLYSQGVWRGGE LQPEDSVASILKQGTLS+GFIGLAECLVALTGKHHGEDEESWKLG
Sbjct: 361 FRFLYSQGVWRGGEKLQPEDSVASILKQGTLSLGFIGLAECLVALTGKHHGEDEESWKLG 420

Query: 434 YEIISFMRDRMDKATEEHQLNFSVIATPAEGLSGKFVKKDRKEFGVISGITNHNYYTNSF 493
           YEIISFMRDRMDKATEEH+LNFSVIATPAEGLSGKFVKKDR+EFGVISGITNHNYYTNSF
Sbjct: 421 YEIISFMRDRMDKATEEHELNFSVIATPAEGLSGKFVKKDREEFGVISGITNHNYYTNSF 480

Query: 494 HIPVYYNIQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMAEHGVGYG 553
           HIPVYYN+QAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMAEHGVGYG
Sbjct: 481 HIPVYYNMQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMAEHGVGYG 540

Query: 554 SINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGDMSKWNSAKRSEEID 613
           SINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGDMSKWNSAKRSEE+D
Sbjct: 541 SINHPVDRCKCCSYHGVIGNECPSCGNEDEANIERIRRITGYLVGDMSKWNSAKRSEEMD 600

Query: 614 RVKHK 618
           RVKHK
Sbjct: 601 RVKHK 605
```

**>ref|NP_418659.1|** anaerobic ribonucleoside-triphosphate reductase [***Escherichia coli*** K12]Length = 712

Score = 309 bits (792), Expect = 2e-84
Identities = 208/630 (33%), Positives = 319/630 (50%), Gaps = 28/630 (4%)

```
Query: 2   LQNNTRGEELMKVFETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSD 61
           ++   RG  L +    +V      L+ ENAN D +         A   AK+YA ++LL
Sbjct: 90  IEREKRGR-LNQEIRGLVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPR 148

Query: 62  QVKKAINENILYPHDLDFYATGTT-TCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALS 120
            V +A     ++ HDLD+        C  I L  ML  GF  G+  +  P+ I  +A A++
Sbjct: 149 DVVQAHERGDIHYHDLDYSPFFPMFNCMLIDLKGMLTQGFKMGNAEIEPPKSISTATAVT 208

Query: 121 SIIFQANQNMQHGGQSFALFDIDLAPYVRKTIERHKKRLQSYPLKKEQIEEFAWKETEND 180
           + I     +  +GG +    D  LAP+V  +  +H+K  + + +     E +A   T  +
Sbjct: 209 AQIIAQVASHIYGGTTINRIDEVLAPFVTASYNKHRKTAEEWNIP--DAEGYANSRTIKE 266

Query: 181 TYQACEAFVHNSNSMHSRGGGQVPFISINYGTDTSKEGRMLIKQLLKATQAGLGKG-ETP 239
```

```
            Y A ++  +  N++H+   GQ PF++  +G  TS E R++ + +L+   AGLGK  +T
Sbjct: 267 CYDAFQSLEYEVNTLHT-ANGQTPFVTFGFGLGTSWESRLIQESILRNRIAGLGKNRKTA 325

Query: 240 IFPIQIFKMKKGVNFEESDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV 299
           +FP  +F ++ G+N ++ DPNYD+ +LALE  ++R++P+    D           P
Sbjct: 326 VFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGSFKTP---- 381

Query: 300 CYMGCRT--RVMSNIHGEETAIGRGNLSFTSINLVKLAL-ISGSKEAFFEALNYYLDLGI 356
             MGCR+   V  N +GE+   GR NL   S+NL ++AL   G +  F++ L+  L L
Sbjct: 382 --MGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEAKGDEATFWKLLDERLVLAR 439

Query: 357 KQLLERYAYQCTKRARDFQFLYSQGVWRGGETLQPEDSVASILKQG--TLSIGFIGLAEC 414
           K L+ R A     +AR    LY +G    G  L  +D V+ I K G  ++S+G+IG+ E
Sbjct: 440 KALMTRIARLEGVKARVAPILYMEGAC--GVRLNADDDVSEIFKNGRASISLGYIGIHET 497

Query: 415 LVALTGKHHGEDEESWKL-GYEIISFMRDRMDKATEEHQLNFSVIATPAEGLSGKFVKKD 473
           + AL G  H  D E  +  G  I+  +R  +D+  EE    FS+ +TP+E L  +F + D
Sbjct: 498 INALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLD 557

Query: 474 RKEFGVISGITNHNYYTNSFHIPVYYNIQAINKIRLEGPFHALCNGGHITYIELDGAAMH 533
             EFGV+ G+T+  YYTNSFH+ V   +   +KI  E P+  L NGG I Y E      H
Sbjct: 558 TAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPLANGGFICYGEYPN-IQH 616

Query: 534 NKKALKQIVQAMAEHGVGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIER 588
           N KAL+ +     +H   YG+ N P+D C  C + G       G  CP CGN D + +
Sbjct: 617 NLKALEDVWDYSYQHVPYYGT-NTPIDECYECGFTGEFECTSKGFTCPKCGNHDASRVSV 675

Query: 589 IRRITGYLVG-DMSKWNSAKRSEEIDRVKH 617
            RR+ GYL   D   +N+ K+ E   RVKH
Sbjct: 676 TRRVCGYLGSPDARPFNAGKQEEVKRRVKH 705
```

**>ref|NP_964153.1|** anaerobic ribonucleoside-triphosphate reductase [***Lactobacillus johnsonii*** NCC 533]Length = 736

Score = 270 bits (691), Expect = 9e-73
Identities = 200/617 (32%), Positives = 301/617 (48%), Gaps = 31/617 (5%)

```
Query: 18  IVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSDQVKKAINENILYPHDL 77
           ++H ++  ++ ENAN D R         A    K   +  ++ + + KA     ++ HDL
Sbjct: 114 LIH-HDPTIVNENANKDSRVFSTQRDLTAGVVGKTIGL-TMMPEHIAKAHLRGDIHWHDL 171

Query: 78  DFYATGT-TTCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHGGQS 136
           D+      T C  I   +ML +GF  G+ ++  P  I++A A  S I     + Q+GG S
Sbjct: 172 DYTPLSPLTNCCLIDFKEMLGHGFTIGNANIESPHSIETATAQMSQIIANVASSQYGGCS 231

Query: 137 FALFDIDLAPYVRKTIERHKKRLQSYPLKKEQIEEFAWKETENDTYQACEAFVHNSNSMH 196
               D  LAPY  K   ++ K    +   +E+I+ FA K T+ D Y A +A  +  N++
Sbjct: 232 ADRVDEVLAPYAEKNYHKNIKEASEFFDDEEKIKAFAVKRTKKDIYDAMQALEYEINTLF 291

Query: 197 SRGGGQVPFISINYGTDTSKEGRMLIKQLLKATQAGLGKGE-TPIFPIQIFKMKKGVNFE 255
           S   GQ PF ++ +G  TS   R + K +LK    GLGK + T IFP  +F +KKG+N
Sbjct: 292 S-SQGQTPFTTLGFGLGTSWIEREIQKAILKIRIEGLGKDKRTAIFPKLVFTLKKGLNLH 350

Query: 256 ESDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRTRVM---SNI 312
             DPNYD+ ELALE + +R++P+    D           P       MGCR+ +
Sbjct: 351 PGDPNYDIKELALECSTKRMYPDIVSYDMIKKITGSFKAP------MGCRSFLQGWKDPE 404

Query: 313 HGEETAIGRGNLSFTSINLVKLALIS-GSKEAFFEALNYYLDLGIKQLLERYAYQCTKRA 371
            G+E   GR NL   ++NL ++A+ S G K+ F++     +     + L+  A +CT
Sbjct: 405 TGKEVNSGRMNLGVVTLNLPRIAMESKGDKDLFWQIFREKVQTA-HEALQIKAKRCTDAV 463

Query: 372 RD-FQFLYSQGVWRGGETLQPEDSVASILKQG--TLSIGFIGLAECLVALTGKHHGEDEE 428
            D    LY  G +  G+ L  ED+V  + K G  T+S+G+IGL E      G +   +EE
Sbjct: 464 PDNAPILYEYGAF--GKRLNAEDNVNDLFKNGRCTVSLGYIGLYEVGTVFYGPNWEHNEE 521
```

```
Query: 429 SWKLGYEIISFMRDRMDKATEE--HQLNFSVIATPAEGLSGKFVKKDRKEFGVISGITNH 486
          + +    I+  + D   K  +E  +  ++SV +TP+E L+ +F + D K+FG +  IT+
Sbjct: 522 AHQFTINIVKELHDYCAKWEKEDPNHYHYSVYSTPSESLTDRFCRLDTKKFGKVKDITDK 581

Query: 487 NYYTNSFHIPVYYNIQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMA 546
           YYTNSFH  V  +     K+  E P+     GG I Y E       N KAL + V   A
Sbjct: 582 EYYTNSFHYDVRKHPTPFEKLAFEAPYPFYAAGGFIHYCEYPN-LKQNPKAL-EAVWDWA 639

Query: 547 EHGVGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIERIRRITGYLVGDMS 601
             VGY   N P+D+C  C + G       G ECP CGN D    + ++R  GYL   +
Sbjct: 640 YDKVGYLGTNTPIDQCYKCGFKGEFKATAKGFECPQCGNHDPETCDCVKRTCGYLGNPLK 699

Query: 602 KWNSAKRSEEI-DRVKH 617
           +     R EEI  RVKH
Sbjct: 700 RPMVHGRHEEIVHRVKH 716
```

**>ref|NP_833941.1|** Superfamily II DNA/RNA helicases, SNF2 family [***Bacillus cereus*** ATCC 14579]Length = 560

Score = 31.6 bits (70), Expect = 0.93
Identities = 15/36 (41%), Positives = 19/36 (52%)

```
Query: 260 NYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRP 295
           N+DL+ LA ET    L P F  L AP +  H+   P
Sbjct: 27  NWDLYHLAYETEKSLLVPTFDGLQAPKHLSHFTPLP 62
```

**>ref|NP_980819.1|** ABC transporter, permease protein [**Bacillus cereus** ATCC 10987]Length = 637

Score = 31.6 bits (70), Expect = 0.93
Identities = 17/46 (36%), Positives = 26/46 (56%), Gaps = 6/46 (13%)

```
Query: 356 IKQLLERYAYQCTKRARDFQFLYSQGVWRGGETLQPEDSVASILKQ 401
           ++QLL +Y Y       DFQF+   GV+   +T + E  VA ++KQ
Sbjct: 337 VEQLLHQYGYD------DFQFMSFVGVYASFQTNKGETEVAPLIKQ 376
```

**>ref|NP_980599.1|** helicase, putative [***Bacillus cereus*** ATCC 10987]Length = 560

Score = 31.6 bits (70), Expect = 0.93
Identities = 15/36 (41%), Positives = 19/36 (52%)

```
Query: 260 NYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRP 295
           N+DL+ LA ET    L P F  L AP +  H+   P
Sbjct: 27  NWDLYHLAYETEKSLLVPTFDGLQAPKHLSHFTPLP 62
```

**>ref|NP_846678.1|** helicase, putative [***Bacillus anthracis*** str. Ames]Length = 560

Score = 31.6 bits (70), Expect = 0.93
Identities = 15/36 (41%), Positives = 19/36 (52%)

```
Query: 260 NYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRP 295
           N+DL+ LA ET    L P F  L AP +  H+   P
```

```
Sbjct: 27  NWDLYHLAYETEKSLLVPTFDGLQAPKHLSHFTPLP 62
```

**>ref|NP_658264.1|** SNF2_N, SNF2 and others N-terminal domain [***Bacillus anthracis*** A2012]Length = 464

Score = 31.6 bits (70), Expect = 0.93
Identities = 15/36 (41%), Positives = 19/36 (52%)

```
Query: 260 NYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRP 295
           N+DL+ LA ET    L P F  L AP +  H+   P
Sbjct: 27  NWDLYHLAYETEKSLLVPTFDGLQAPKHLSHFTPLP 62
```

**>ref|NP_388288.1|** alternate gene name: ycsH~similar to branched chain amino acids transporter [***Bacillus subtilis***] Length = 386

Score = 29.6 bits (65), Expect = 3.5
Identities = 21/82 (25%), Positives = 40/82 (48%), Gaps = 7/82 (8%)

```
Query: 200 GGQVPFISINYGTDTSKEGRMLIKQLLKATQAGLGKGETPIFPIQIF-----KMKKGVNF 254
           GG + F    +   D   +G+  I Q+ K++  G+    T +  I +F      + KG++
Sbjct: 183 GGYITFAGGHRLLDAGIKGKESIPQVTKSSVVGILI--TSVMRIALFFAVLGVVSKGLHI 240

Query: 255 EESDPNYDLFELALETTAERLF 276
           +ES+P   +F+LA      ++F
Sbjct: 241 DESNPAASVFKLAAGNVGYKIF 262
```

**>ref|NP_390796.1|** pyruvate kinase [***Bacillus subtilis***] Length = 585

Score = 28.5 bits (62), Expect = 7.9
Identities = 14/44 (31%), Positives = 21/44 (47%)

```
Query: 41  VMGTFASESAKYYAVENLLSDQVKKAINENILYPHDLDFYATGT 84
           V G FA       + + +L D V+K++N  I+   DL     GT
Sbjct: 414 VSGVFAESGQNASSTDEMLEDAVQKSLNSGIVKHGDLIVITAGT 457
```

**>ref|NP_655608.1|** SBP_bacterial_1, Bacterial extracellular solute-binding protein [***Bacillus anthracis*** A2012]
**ref|NP_844170.1|** ABC transporter, substrate-binding protein, putative [***Bacillus anthracis*** str. Ames] Length = 341

Score = 28.5 bits (62), Expect = 7.9
Identities = 20/76 (26%), Positives = 35/76 (46%), Gaps = 15/76 (19%)

```
Query: 17  TIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSDQVKKAINENILYPHD 76
           T++      +M+++ NV+G              AK + ++ LLSD V+K I++  L P
```

```
Sbjct: 257 TVISPRAAGIMKDSKNVEG--------------AKEF-IDYLLSDDVQKQISKAYLLPGR 301

Query: 77  LDFYATGTTTCSQIPL 92
           D  A       +IP+
Sbjct: 302 TDIKAENRPNVEEIPV 317
```

**>ref|NP_979741.1|** conserved hypothetical protein [***Bacillus cereus*** ATCC 10987]Length = 235

Score = 28.5 bits (62), Expect = 7.9
Identities = 23/97 (23%), Positives = 46/97 (47%), Gaps = 8/97 (8%)

```
Query: 129 NMQHGGQSFALFDIDLAPYVRKTIERHKKRLQSYPLKKEQIEEFAWKET-ENDTYQACEA 187
           N+Q+ G+    F +   P++RK+I    +R  +    +  I  FA+ E  E  +Y+  ++
Sbjct: 15  NIQNNGEDKEAFIVQYQPFIRKSISSVCRRYITEQDDEYSIGLFAFNEAIEQYSYKKGKS 74

Query: 188 FVHNSNSMHSRGGGQVPFISINYGTDTSKEGRMLIKQ 224
           F+  ++ +  R         I+Y    SK  R+ +K+
Sbjct: 75  FLAFADLLIKRD-------VIDYIRKESKHNRVFLKE 104
```

**>ref|NP_964532.1|** leucyl-tRNA synthetase [***Lactobacillus johnsonii*** NCC 533]Length = 804

Score = 28.5 bits (62), Expect = 7.9
Identities = 13/40 (32%), Positives = 26/40 (65%), Gaps = 2/40 (5%)

```
Query: 518 NGGHITYIELDGAAMHNKKALKQIVQAMAEHGVGYGSINH 557
           +G HI    LDG  ++N++A K++++ + +H VG   +N+
Sbjct: 372 DGKHINSEFLDG--LNNEEAKKRMIEWLEDHNVGEKKVNY 409
```

# Appendix V: The results of the BLAST search using the amino acid sequence of the Class III ribonucleotide reductase gene from *Echerichia coli*

Query= (712 letters)

**Database:**

Completed *Bacillus anthracis* str. A2012;
Completed *Bacillus anthracis* str. Ames;
Completed *Bacillus cereus* ATCC 10987;
Completed *Bacillus cereus* ATCC 14579;
Completed *Bacillus halodurans*;
Completed *Bacillus subtilis* subsp. subtilis str. 168;
Completed *Lactobacillus johnsonii* NCC 533;
Completed *Escherichia coli* K12;

36,023 sequences; 10,083,375 total letters

**Alignments:**

**>ref|NP_418659.1|** anaerobic ribonucleoside-triphosphate reductase [***Escherichia coli* K12**]
Length = 712

Score = 1425 bits (3690), Expect = 0.0
Identities = 694/712 (97%), Positives = 694/712 (97%)

```
Query: 1   MTPHVMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSEQMQGRNQVDINE 60
           MTPHVMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSEQMQGRNQVDINE
Sbjct: 1   MTPHVMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSEQMQGRNQVDINE 60

Query: 61  IQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANK 120
           IQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANK
Sbjct: 61  IQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANK 120

Query: 121 DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL 180
           DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL
Sbjct: 121 DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL 180

Query: 181 KGMLTQGFKMGNAEIEPPKXXXXXXXXXXXXXXXXXXHIYGGTTINRIDEVLAPFVTASY 240
           KGMLTQGFKMGNAEIEPPK                  HIYGGTTINRIDEVLAPFVTASY
Sbjct: 181 KGMLTQGFKMGNAEIEPPKSISTATAVTAQIIAQVASHIYGGTTINRIDEVLAPFVTASY 240

Query: 241 NKHRKTAEEWNIPDAEGYANSRTIKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS 300
           NKHRKTAEEWNIPDAEGYANSRTIKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS
Sbjct: 241 NKHRKTAEEWNIPDAEGYANSRTIKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS 300

Query: 301 WESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKR 360
           WESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKR
Sbjct: 301 WESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKR 360

Query: 361 MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA 420
           MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA
```

```
Sbjct: 361 MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA 420

Query: 421 KGDEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIF 480
           KGDEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIF
Sbjct: 421 KGDEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIF 480

Query: 481 KNGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFS 540
           KNGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFS
Sbjct: 481 KNGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFS 540

Query: 541 LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL 600
           LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL
Sbjct: 541 LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL 600

Query: 601 ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF 660
           ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF
Sbjct: 601 ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF 660

Query: 661 TCPKCGNHDASRVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG 712
           TCPKCGNHDASRVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG
Sbjct: 661 TCPKCGNHDASRVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG 712
```

**>ref|NP_964153.1|** anaerobic ribonucleoside-triphosphate reductase [***Lactobacillus johnsonii*** NCC 533]Length = 736

Score = 677 bits (1748), Expect = 0.0
Identities = 345/710 (48%), Positives = 465/710 (65%), Gaps = 16/710 (2%)

```
Query: 6   MKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSEQMQGRNQVDINEIQTAV 65
+KRDG            PF   +I E +L +    E +     A +   + +++ G  Q    +I  A
Sbjct: 21  IKRDGSIYPFALYKI-EMVLDSLHLTEHE-----ADILPAILQKLNGAKQTSTEKIADAF 74

Query: 66  ENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANKDSKVI 125
              L+   +   A AY++YR + +   +K+      +  L+   + +++NENANKDS+V
Sbjct: 75  VQTLIELGFSDEANAYVDYRREDEANFKKQTETTNRLGRLIHH-DPTIVNENANKDSRVF 133

Query: 126 PTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDLKGMLT 185
            TQRDL AG+V K      ++P  + +AH RGDIH+HDLDY+P  P+ NC LID K ML
Sbjct: 134 STQRDLTAGVVGKTIGLT-MMPEHIAKAHLRGDIHWHDLDYTPLSPLTNCCLIDFKEMLG 192

Query: 186 QGFKMGNAEIEPPKXXXXXXXXXXXXXXXXXHIYGGTTINRIDEVLAPFVTASYNKHRK 245
            GF +GNA IE P                    YGG + +R+DEVLAP+   +Y+K+ K
Sbjct: 193 HGFTIGNANIESPHSIETATAQMSQIIANVASSQYGGCSADRVDEVLAPYAEKNYHKNIK 252

Query: 246 TAEEWNIPDAE---GYANSRTIKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTSWE 302
            A E+   D E    +A  RT K+ YDA Q+LEYE+NTL  ++ GQTPF T GFGLGTSW
Sbjct: 253 EASEF-FDDEEKIKAFAVKRTKKDIYDAMQALEYEINTLFSSQGQTPFTTLGFGLGTSWI 311

Query: 303 SRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMY 362
            R IQ++IL+ RI GLGK+++TA+FPKLVF ++ GLN   GDPNYDIK+LALEC++KRMY
Sbjct: 312 EREIQKAILKIRIEGLGKDKRTAIFPKLVFTLKKGLNLHPGDPNYDIKELALECSTKRMY 371

Query: 363 PDILNYDQVVKVTGSFKTPMGCRSFLGVWEN-ENGEQIHDGRNNLGVISLNLPRIALEAK 421
           PDI++YD + K+TGSFK PMGCRSFL  W++ E G++++ GR NLGV++LNLPRIA+E+K
Sbjct: 372 PDIVSYDMIKKITGSFKAPMGCRSFLQGWKDPETGKEVNSGRMNLGVVTLNLPRIAMESK 431

Query: 422 GDEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIFK 481
           GD+  FW++  E++  A +AL  +  R        APILY  GA G RLNA+D+V+++FK
Sbjct: 432 GDKDLFWQIFREKVQTAHEALQIKAKRCTDAVPDNAPILYEYGAFGKRLNAEDNVNDLFK 491

Query: 482 NGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEE--TGYGF 539
           NGR ++SLGYIG++E +  +F G +   NE+     I IV+ L     +W++E    Y +
Sbjct: 492 NGRCTVSLGYIGLYE-VGTVFYGPNWEHNEEAHQFTINIVKELHDYCAKWEKEDPNHYHY 550
```

```
Query: 540 SLYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPP 599
           S+YSTPSE+L  DRFCRLDT +FG V  +TDK YYTNSFH DV K   P++K+ FEAPYP
Sbjct: 551 SVYSTPSESLTDRFCRLDTKKFGKVKDITDKEYYTNSFHYDVRKHPTPFEKLAFEAPYPF 610

Query: 600 LANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKG 659
            A GGFI Y EYPN++ N KALE VWD++Y  V Y GTNTPID+CY+CGF GEF+ T+KG
Sbjct: 611 YAAGGFIHYCEYPNLKQNPKALEAVWDWAYDKVGYLGTNTPIDQCYKCGFKGEFKATAKG 670

Query: 660 FTCPKCGNHDASRVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNG 709
           F CP+CGNHD       +R CGYLG+P   RP    G+ EE+  RVKHL  G
Sbjct: 671 FECPQCGNHDPETCDCVKRTCGYLGNPLKRPMVHGRHEEIVHRVKHLNMG 720
```

**>ref|NP_845927.1|** anaerobic ribonucleoside-triphosphate reductase, putative [***Bacillus anthracis*** str. Ames]Length = 618

Score = 300 bits (769), Expect = 1e-81
Identities = 205/630 (32%), Positives = 309/630 (49%), Gaps = 28/630 (4%)

```
Query: 90  IEREKRGR-LNQEIRGLVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPR 148
           +++  RG  L +    +V      L+ ENAN D +         A   AK+YA + LL
Sbjct: 2   LQKNTRGEGLMRVFETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVEKLLSD 61

Query: 149 DVVQAHERGDIHYHDLDYSPFFPMFNCMLIDLKGMLTQGFKMGNAEIEPPKXXXXXXXXX 208
            V +A     ++ HDLD+        C  I L  ML  GF  G+  +  P+
Sbjct: 62  QVKKAINENILYPHDLDFYATGTT-TCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALS 120

Query: 209 XXXXXXXXXHIYGGTTINRIDEVLAPFVTASYNKHRKTAEEWNIP--DAEGYANSRTIKE 266
                    +GG +    D  LAP+V  +  +H+K  + + +     E +A   T  +
Sbjct: 121 SIIFQANQNMQHGGQSFALFDVDLAPYVRKTVERHKKRLQSYPLTKEQIEEFAWKETEND 180

Query: 267 CYDAFQSLEYEVNTLHT-ANGQTPFVTFGFGLGTSWESRLIQESILRNRIAGLGKNRKTA 325
            Y A ++  +  N++H+   GQ PF++  +G  TS E RL+ + +L+   AGLGK  +T
Sbjct: 181 TYQACEAFIHNSNSMHSRGGGQVPFISINYGTDTSKEGRLLIKQLLKATQAGLGKG-ETP 239

Query: 326 VFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGSFKTP---- 381
           +FP  +F ++ G+N +  DPNYD+ +LALE  ++R++P+    D           P
Sbjct: 240 IFPIQIFKMKKGVNFEGSDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV 299

Query: 382 --MGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEAKGDEATFWKLLDERLVLAR 439
             MGCR+   V  N +GE+   GR NL    S+NL ++AL   G +  F++ L+  L L
Sbjct: 300 CYMGCRT--RVMSNIHGEETAIGRGNLSFTSINLVKLALISGSKEAFYEALNYYLDLGI 356

Query: 440 KALMTRIARLEGVKARVAPILYMEGAC--GVRLNADDDVSEIFKNGRASISLGYIGIHET 497
           K L+ R       +AR    LY +G    G +L  +D V+ I K G  ++SLG+IG+ E
Sbjct: 357 KQLLERFEYQCTKRARDFRFLYSQGVWRGGEKLQPEDSVASILKQG--TLSLGFIGLAEC 414

Query: 498 INALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLD 557
           + AL G  H  D E  +  G  I+  +R  +D+  EE    FS+ +TP+E L  +F + D
Sbjct: 415 LVALTGKHHGEDEESWKL-GYEIISFMRDRMDKATEEHELNFSVIATPAEGLSGKFVKKD 473

Query: 558 TAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPLANGGFICYGEYPN-IQH 616
             EFGV+ G+T+  YYTNSFH+ V   +   +KI  E P+  L NGG I Y E      H
Sbjct: 474 REEFGVISGITNHNYYTNSFHIPVYYNMQAINKIRLEGPFHALCNGGHITYIELDGAAMH 533

Query: 617 NLKALEDVWDYSYQHVPYYGT-NTPIDECYECGFTGEFECTSKGFTCPKCGNHDASRVSV 675
           N KAL+ +     +H    YG+ N P+D C  C + G       G  CP CGN D + +
Sbjct: 534 NKKALKQIVQAMAEHGVGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIER 588

Query: 676 TRRVCGYLGSPDARPFNAGKQEEVKRRVKH 705
            RR+ GYL   D   +N+ K+ E   RVKH
Sbjct: 589 IRRITGYLVG-DMSKWNSAKRSEEMDRVKH 617
```

**>ref|NP_833335.1|** Anaerobic ribonucleoside-triphosphate reductase [***Bacillus cereus* ATCC 14579**]
Length = 618

Score = 300 bits (769), Expect = 1e-81
Identities = 204/630 (32%), Positives = 310/630 (49%), Gaps = 28/630 (4%)

```
Query: 90  IEREKRGR-LNQEIRGLVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPR 148
           ++   RG  L +    +V      L+ ENAN D +         A   AK+YA ++LL
Sbjct: 2   LQNNTRGEELMKVFETIVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSD 61

Query: 149 DVVQAHERGDIHYHDLDYSPFFPMFNCMLIDLKGMLTQGFKMGNAEIEPPKXXXXXXXXX 208
            V +A     ++ HDLD+        C  I L  ML  GF  G+  +  P+
Sbjct: 62  QVKKAINENILYPHDLDFYATGTT-TCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALS 120

Query: 209 XXXXXXXXXHIYGGTTINRIDEVLAPFVTASYNKHRKTAEEWNIP--DAEGYANSRTIKE 266
                    +GG +    D  LAP+V  +  +H+K  + + +     E +A   T  +
Sbjct: 121 SIIFQANQNMQHGGQSFALFDIDLAPYVRKTIERHKKRLQSYPLKKEQIEEFAWKETEND 180

Query: 267 CYDAFQSLEYEVNTLHT-ANGQTPFVTFGFGLGTSWESRLIQESILRNRIAGLGKNRKTA 325
            Y A ++  +  N++H+   GQ PF++  +G  TS E R++ + +L+   AGLGK  +T
Sbjct: 181 TYQACEAFVHNSNSMHSRGGGQVPFISINYGTDTSKEGRMLIKQLLKATQAGLGKG-ETP 239

Query: 326 VFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGSFKTP---- 381
           +FP  +F ++ G+N ++ DPNYD+ +LALE  ++R++P+    D           P
Sbjct: 240 IFPIQIFKMKKGVNFEESDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEV 299

Query: 382 --MGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEAKGDEATFWKLLDERLVLAR 439
             MGCR+   V  N +GE+   GR NL   S+NL ++AL   G +  F++ L+  L L
Sbjct: 300 CYMGCRT--RVMSNIHGEETAIGRGNLSFTSINLVKLAL-ISGSKEAFFEALNYYLDLGI 356

Query: 440 KALMTRIARLEGVKARVAPILYMEGAC--GVRLNADDDVSEIFKNGRASISLGYIGIHET 497
           K L+ R A     +AR    LY +G    G  L  +D V+ I K G  ++S+G+IG+ E
Sbjct: 357 KQLLERYAYQCTKRARDFQFLYSQGVWRGGETLQPEDSVASILKQG--TLSIGFIGLAEC 414

Query: 498 INALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLD 557
           + AL G  H  D E  +  G  I+  +R  +D+  EE    FS+ +TP+E L  +F + D
Sbjct: 415 LVALTGKHHGEDEESWKL-GYEIISFMRDRMDKATEEHQLNFSVIATPAEGLSGKFVKKD 473

Query: 558 TAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPLANGGFICYGEYPN-IQH 616
             EFGV+ G+T+  YYTNSFH+ V   +   +KI  E P+  L NGG I Y E      H
Sbjct: 474 RKEFGVISGITNHNYYTNSFHIPVYYNIQAINKIRLEGPFHALCNGGHITYIELDGAAMH 533

Query: 617 NLKALEDVWDYSYQHVPYYGT-NTPIDECYECGFTGEFECTSKGFTCPKCGNHDASRVSV 675
           N KAL+ +     +H   YG+ N P+D C  C + G       G  CP CGN D + +
Sbjct: 534 NKKALKQIVQAMAEHGVGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIER 588

Query: 676 TRRVCGYLGSPDARPFNAGKQEEVKRRVKH 705
            RR+ GYL   D   +N+ K+ E   RVKH
Sbjct: 589 IRRITGYLVG-DMSKWNSAKRSEEIDRVKH 617
```

**>ref|NP_657512.1|** Gly_radical, G radical [***Bacillus anthracis*** A2012]Length = 605

Score = 299 bits (766), Expect = 2e-81
Identities = 202/614 (32%), Positives = 302/614 (49%), Gaps = 27/614 (4%)

```
Query: 105 LVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDL 164
           +V      L+ ENAN D +         A   AK+YA + LL   V +A     ++ HDL
Sbjct: 5   IVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVEKLLSDQVKKAINENILYPHDL 64
```

```
Query: 165 DYSPFFPMFNCMLIDLKGMLTQGFKMGNAEIEPPKXXXXXXXXXXXXXXXXXXHIYGGTT 224
           D+        C  I L  ML  GF  G+  +  P+                    +GG +
Sbjct: 65  DFYATGTT-TCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHGGQS 123

Query: 225 INRIDEVLAPFVTASYNKHRKTAEEWNIP--DAEGYANSRTIKECYDAFQSLEYEVNTLH 282
               D  LAP+V  +  +H+K  + + +     E +A   T  + Y A ++  +  N++H
Sbjct: 124 FALFDVDLAPYVRKTVERHKKRLQSYPLTKEQIEEFAWKETENDTYQACEAFIHNSNSMH 183

Query: 283 T-ANGQTPFVTFGFGLGTSWESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHK 341
           +   GQ PF++  +G  TS E RL+ + +L+   AGLGK  +T +FP  +F ++ G+N +
Sbjct: 184 SRGGGQVPFISINYGTDTSKEGRLLIKQLLKATQAGLGKG-ETPIFPIQIFKMKKGVNFE 242

Query: 342 KGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGSFKTP------MGCRSFLGVWENEN 395
             DPNYD+ +LALE  ++R++P+    D           P      MGCR+   V  N +
Sbjct: 243 GSDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRT--RVMSNIH 300

Query: 396 GEQIHDGRNNLGVISLNLPRIALEAKGDEATFWKLLDERLVLARKALMTRIARLEGVKAR 455
           GE+   GR NL   S+NL ++AL   G +  F++ L+  L L  K L+ R       +AR
Sbjct: 301 GEETAIGRGNLSFTSINLVKLAL-ISGSKEAFYEALNYYLDLGIKQLLERFEYQCTKRAR 359

Query: 456 VAPILYMEGAC--GVRLNADDDVSEIFKNGRASISLGYIGIHETINALFGGEHVYDNEQL 513
               LY +G    G +L  +D V+ I K G  ++SLG+IG+ E + AL G  H  D E
Sbjct: 360 DFRFLYSQGVWRGGEKLQPEDSVASILKQG--TLSLGFIGLAECLVALTGKHHGEDEESW 417

Query: 514 RAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYY 573
           +  G  I+  +R  +D+  EE    FS+ +TP+E L  +F + D  EFGV+ G+T+  YY
Sbjct: 418 KL-GYEIISFMRDRMDKATEEHELNFSVIATPAEGLSGKFVKKDREEFGVISGITNHNYY 476

Query: 574 TNSFHLDVEKKVNPYDKIDFEAPYPPLANGGFICYGEYPN-IQHNLKALEDVWDYSYQHV 632
            NSFH+ V   +   +KI  E P+  L NGG I Y E      HN KAL+ +     +H
Sbjct: 477 TNSFHIPVYYNMQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMAEHG 536

Query: 633 PYYGT-NTPIDECYECGFTGEFECTSKGFTCPKCGNHDASRVSVTRRVCGYLGSPDARPF 691
             YG+ N P+D C  C + G       G  CP CGN D + +   RR+ GYL   D   +
Sbjct: 537 VGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIERIRRITGYLVG-DMSKW 590

Query: 692 NAGKQEEVKRRVKH 705
           N+ K+ E   RVKH
Sbjct: 591 NSAKRSEEMDRVKH 604
```

**>ref|NP_979919.1|** anaerobic ribonucleoside-triphosphate reductase, putative [***Bacillus cereus*** ATCC 10987]Length = 619

Score = 295 bits (755), Expect = 4e-80
Identities = 199/614 (32%), Positives = 304/614 (49%), Gaps = 27/614 (4%)

```
Query: 105 LVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDL 164
           +V      L+ ENAN D +         A   AK+YA ++LL   V +A     ++ HDL
Sbjct: 19  IVHGNEQDLMQENANVDGRSPMGVMGTFASESAKYYAVENLLSDQVKKAINENILYPHDL 78

Query: 165 DYSPFFPMFNCMLIDLKGMLTQGFKMGNAEIEPPKXXXXXXXXXXXXXXXXXXHIYGGTT 224
           D+        C  I L  ML  GF  G+  +  P+                    +GG +
Sbjct: 79  DFYATGTT-TCSQIPLAQMLANGFHTGHGHMRQPQDIKSALALSSIIFQANQNMQHGGQS 137

Query: 225 INRIDEVLAPFVTASYNKHRKTAEEWNIP--DAEGYANSRTIKECYDAFQSLEYEVNTLH 282
               D  LAP+V  +  +H+K  + + +     E +A   T  + Y A ++  +  N++H
Sbjct: 138 FALFDVDLAPYVRKTVERHKKRLQSYPLTKEQIEEFAWKETENDTYQACEAFIHNSNSMH 197

Query: 283 T-ANGQTPFVTFGFGLGTSWESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHK 341
           +   GQ PF++  +G  TS E RL+ + +L+   AGLGK  +T +FP  +F ++ G+N +
Sbjct: 198 SRGGGQVPFISINYGTDTSKEGRLLIKQLLKATQAGLGKG-ETPIFPIQIFKMKKGVNFE 256

Query: 342 KGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGSFKTP------MGCRSFLGVWENEN 395
           + DPNYD+ +LALE  ++R++P+    D           P      MGCR+   V  N +
Sbjct: 257 GSDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRTRVMSNIHGE 316
```

```
Sbjct: 257 ECDPNYDLFELALETTAERLFPNFSFLDAPFNAVHYDGRPESEVCYMGCRT--RVMSNIH 314

Query: 396 GEQIHDGRNNLGVISLNLPRIALEAKGDEATFWKLLDERLVLARKALMTRIARLEGVKAR 455
           GE+   GR NL   S+NL ++AL   G +  F++ L+  L +  + L+ R       +AR
Sbjct: 315 GEETAIGRGNLSFTSINLVKLAL-ISGSQEAFFEALNYYLDVGIQQLLERFEYQCTKRAR 373

Query: 456 VAPILYMEGAC--GVRLNADDDVSEIFKNGRASISLGYIGIHETINALFGGEHVYDNEQL 513
               LY +G    G +L  +D V+ I K G  ++SLG+IG+ E + AL G  H  D E
Sbjct: 374 DFRFLYSQGVWRGGEKLQPEDSVASILKQG--TLSLGFIGLAECLVALTGKHHGEDEESW 431

Query: 514 RAKGIAIVERLRQAVDQWKEETGYGFSLYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYY 573
           +  G  I+  +R  +D+  EE    FS+ +TP+E L  +F + D  EFGV+ G+T+  YY
Sbjct: 432 KL-GYEIISFMRDRMDKATEEHELNFSVIATPAEGLSGKFVKKDREEFGVISGITNHNYY 490

Query: 574 TNSFHLDVEKKVNPYDKIDFEAPYPPLANGGFICYGEYPN-IQHNLKALEDVWDYSYQHV 632
           TNSFH+ V   +   +KI  E P+  L NGG I Y E      HN KAL+ +     ++
Sbjct: 491 TNSFHIPVYYDIQAINKIRLEGPFHALCNGGHITYIELDGAAMHNKKALKQIVQAMAENG 550

Query: 633 PYYGT-NTPIDECYECGFTGEFECTSKGFTCPKCGNHDASRVSVTRRVCGYLGSPDARPF 691
             YG+ N P+D C  C + G       G  CP CGN D + +   RR+ GYL   D   +
Sbjct: 551 VGYGSINHPVDRCKCCSYHGVI-----GNECPSCGNEDEANIERIRRITGYLVG-DMSKW 604

Query: 692 NAGKQEEVKRRVKH 705
           N+ K+ E   RVKH
Sbjct: 605 NSAKRSEEMDRVKH 618
```

**>ref|NP_244012.1|** BH3146~unknown conserved protein [***Bacillus halodurans***]Length = 153

Score = 37.0 bits (84), Expect = 0.026
Identities = 31/109 (28%), Positives = 52/109 (47%), Gaps = 14/109 (12%)

```
Query: 5   VMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSE-----QMQGRNQVDIN 59
           V+K+DG +  F S++I   ++RA +   V       T+  +V+E     + QG+N+VD
Sbjct: 51  VVKKDGTRQEFSSDKILRGLIRACEKRPVP----LETLEGIVNEVERELRGQGKNEVDSK 106

Query: 60  EIQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQ 108
EI            V  +L +       R    YR  +DI         QE++ L+E+
Sbjct: 107 EIGELVMERLANVDDVAYVRFASVYRQFKDI-----NVFIQELKELMER 150
```

**>ref|NP_414947.1|** hypothetical protein b0413 [***Escherichia coli*** K12]Length = 149

Score = 30.4 bits (67), Expect = 2.4
Identities = 23/90 (25%), Positives = 37/90 (41%), Gaps = 1/90 (1%)

```
Query: 3   PHVMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSE-QMQGRNQVDINEI 61
           P V+K +  + PF   E+++  +LRA +   V   D    +  + S+ +  G  +V    I
Sbjct: 49  PRVVKSNDVREPFNEEKLRSGMLRALEKRPVSSDDVEMAINHIKSQLRATGEREVPSKMI 108

Query: 62  QTAVENQLMSGPYKQLARAYIEYRHDRDIE 91
              V  QL         R    YR   DI+
Sbjct: 109 GNLVMEQLKKLDKVAYIRFASVYRSFEDIK 138
```

**>ref|NP_978932.1|** homoserine dehydrogenase, putative [***Bacillus cereus*** ATCC 10987]Length = 346

Score = 30.4 bits (67), Expect = 2.4
Identities = 27/84 (32%), Positives = 35/84 (41%),
Gaps = 8/84 (9%)

```
Query: 52  GRNQVDINEIQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNA 111
           GRN    NE   ++ N LM G        YIEY        E+R   N     LVE T
Sbjct: 43  GRNVAIHNEDGLSIHNLLMYGSSSAAIEKYIEY------HPEERATDNISGTVLVESTVT 96

Query: 112 SLLNENANKD--SKVIPTQRDLLA 133
           +L + N  K    + I  Q D++A
Sbjct: 97  NLKDGNPGKQYIKQAIEKQMDIVA 120
```

**>ref|NP_655005.1|** CBD_6, Cellulose binding domain [***Bacillus anthracis*** A2012]Length = 2168

Score = 30.0 bits (66), Expect = 3.2
Identities = 28/120 (23%), Positives = 46/120 (38%),
Gaps = 17/120 (14%)

```
Query: 54  NQVDINEIQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASL 113
           N  +I E +T  E    +       R Y E      I  EK G+L +    LVE  N  +
Sbjct: 73  NPTEIVEERTETEKVFDNNDGTYTKRVYTE-----PIHIEKDGKLEEVSPKLVEAPNEKI 127

Query: 114 LNEN---------ANKDSKVIP---TQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHY 161
           + EN         A +D K +       ++   +++ +  +  L P  V   HE+  + Y
Sbjct: 128 ITENTTLEPEFEKATQDGKYVQFKVKNHNIQYKLISANGEKGELKPTPVTATHEKNTVWY 187
```

**>ref|NP_977523.1|** wall associated protein, putative [***Bacillus cereus*** ATCC 10987]Length = 2246

Score = 30.0 bits (66), Expect = 3.2
Identities = 20/80 (25%), Positives = 38/80 (47%),
Gaps = 4/80 (5%)

```
Query: 81  YIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANKDSKVIPTQRDLLAGIVAKHY 140
           Y +  +   I  EK G+L +    LVE  NA ++ EN   + +   T +D   G   +
Sbjct: 95  YTKKVYTEPIHIEKNGKLEEVSPKLVEAPNAKIVTENTTLEPEFEKTTQD---GKYVQFK 151

Query: 141 ARQHLLPRDVVQAH-ERGDI 159
            + H +   ++ A+ E+G++
Sbjct: 152 VKDHTIKYKLMSANGEKGEV 171
```

**>ref|NP_843587.1|** wall-associated protein, putative [***Bacillus anthracis*** str. Ames]Length = 2224

Score = 30.0 bits (66), Expect = 3.2
Identities = 28/120 (23%), Positives = 46/120 (38%),
Gaps = 17/120 (14%)

```
Query: 54  NQVDINEIQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASL 113
           N  +I E +T  E    +       R Y E      I  EK G+L +    LVE  N  +
Sbjct: 73  NPTEIVEERTETEKVFDNNDGTYTKRVYTE-----PIHIEKDGKLEEVSPKLVEAPNEKI 127

Query: 114 LNEN---------ANKDSKVIP---TQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHY 161
           + EN         A +D K +       ++   +++ +  +  L P  V   HE+  + Y
```

```
Sbjct: 128 ITENTTLEPEFEKATQDGKYVQFKVKNHNIQYKLISANGEKGELKPTPVTATHEKNTVWY 187
```

**>ref|NP_390562.1|** similar to sugar-phosphate dehydrogenase [***Bacillus subtilis***]Length = 316

Score = 29.6 bits (65), Expect = 4.2
Identities = 29/82 (35%), Positives = 37/82 (45%), Gaps = 10/82 (12%)

```
Query: 59  NEIQTAVENQLMSGPYKQLARAYIEYRH--DRDIEREKRGRLNQEIRGLV-EQTNASLLN 115
           +EI  A E Q+ SG    +  +     H      E EKR        GLV EQ   SLL
Sbjct: 129 DEIWEAFETQVRSGKVDYIGSSNFAGWHLVKAQAEAEKR-----RFMGLVTEQHKYSLLE 183

Query: 116 ENANKDSKVIPTQRDLLAGIVA 137
             A  + +V+P  RDL  G+VA
Sbjct: 184 RTA--EMEVLPAARDLGLGVVA 203
```

**>ref|NP_244904.1|** cadmium-transporting ATPase [***Bacillus halodurans***]Length = 707

Score = 29.3 bits (64), Expect = 5.4
Identities = 14/41 (34%), Positives = 22/41 (53%)

```
Query: 129 RDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPF 169
           R+LLA + A  Y  QH L   V++  +   + Y D++ S F
Sbjct: 425 RELLAAVAALEYRSQHPLAAAVMKKADDEQLSYTDMEVSEF 465
```

**>ref|NP_242671.1|** BH1805~unknown conserved protein in others [***Bacillus halodurans***]Length = 433

Score = 29.3 bits (64), Expect = 5.4
Identities = 32/116 (27%), Positives = 50/116 (43%), Gaps = 28/116 (24%)

```
Query: 467 GVRLNADDDVSEIFKNGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQ 526
           GV++   D  ++ FK   A++S G I            HVY N+ +      +V+   +
Sbjct: 37  GVKVRPKDRRNQNFKGTEATVSNGSI-----------IHVYPNQFMM-----LVDG-GE 78

Query: 527 AVDQWKEETGYGFSLYSTPS-------ENLCDRFCRLDTAEFGVVPGVTDKGYYTN 575
            VD   EE  Y    +  PS       ++L + F R+   +FG VP  T K Y+ N
Sbjct: 79  IVDYTAEEGYYEVDFFGMPSMFNGEFGDSLKETFNRV---KFGGVPSKTQKVYFIN 131
```

**>ref|NP_978881.1|** fmtA-like protein, putative [***Bacillus cereus*** ATCC 10987]Length = 482

Score = 28.9 bits (63), Expect = 7.1
Identities = 20/63 (31%), Positives = 32/63 (50%), Gaps = 3/63 (4%)

```
Query: 318 LGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGS 377
           L K  +T V  +L F+  +  N+  G  NYD+  L +E  S++ Y D +  +QV    G
Sbjct: 161 LQKTVETLVDAELAFSPGEQYNY--GTVNYDVLGLVIEIVSRQSYEDFMK-EQVFLPLGL 217
```

```
Query: 378 FKT 380
           +T
Sbjct: 218 HQT 220
```

**>ref|NP_977596.1|** wall-associated protein, putative [***Bacillus cereus*** ATCC 10987]Length = 2221

Score = 28.9 bits (63), Expect = 7.1
Identities = 25/108 (23%), Positives = 46/108 (42%), Gaps = 9/108 (8%)

```
Query: 53  RNQVDINEIQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNAS 112
           +N  +I E +T  E    +       + Y E      I  EK G+L +    LVE  N
Sbjct: 77  KNPTEIIEERTETEKVFDNNDGTYTKKVYTE-----PIHVEKDGKLEEVSLKLVEAPNEK 131

Query: 113 LLNENANKDSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAH-ERGDI 159
           ++ EN   + +   T +D   G   +   + H +   ++ A+ E+G++
Sbjct: 132 IVTENTTLEPEFEKTTQD---GKYVQFKVKDHTIQYKLMSANGEKGEV 176
```

**>ref|NP_832482.1|** Penicillin-binding protein [***Bacillus cereus*** ATCC 14579]Length = 346

Score = 28.9 bits (63), Expect = 7.1
Identities = 20/63 (31%), Positives = 32/63 (50%), Gaps = 3/63 (4%)

```
Query: 318 LGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKRMYPDILNYDQVVKVTGS 377
           L K  +T V  +L F+  +  N+  G  NYD+  L +E  S++ Y D +  +QV    G
Sbjct: 161 LQKTVETLVDAELAFSPGEQYNY--GTVNYDVLGLVIEIVSRQSYEDFMK-EQVFLPLGL 217

Query: 378 FKT 380
           +T
Sbjct: 218 HQT 220
```

**>ref|NP_832878.1|** Deoxyribodipyrimidine photolyase [***Bacillus cereus*** ATCC 14579]Length = 476

Score = 28.9 bits (63), Expect = 7.1
Identities = 22/105 (20%), Positives = 45/105 (42%), Gaps = 9/105 (8%)

```
Query: 94  KRGRLNQEIRGLVEQTNASLLNENANKDSKVIPTQRDLL-----AGIVAKHYARQHLLPR 148
           ++GR  +EI  L+EQ + + +  N   D   + + + +       GI+ K +    LL
Sbjct: 71  RKGRTEEEILSLIEQLDITAVYWNICYDPDRLQSNQKMKMMLEDKGIICKEFNSHLLLEP 130

Query: 149 DVVQAHERGDIHYHDLDYSPFFPMFNCMLIDLKGMLTQGFKMGNA 193
            +++  +  +       ++PF+  F   +I       Q  K GN+
Sbjct: 131 WIIKKKDNTEYKV----FTPFYNAFQKQVIPKPFSRVQSIKWGNS 171
```

**>ref|NP_389350.1|** similar to myo-inositol-1(or 4)-monophosphatase [***Bacillus subtilis***]Length = 265

Score = 28.5 bits (62), Expect = 9.3
Identities = 13/33 (39%), Positives = 17/33 (51%)

```
Query: 479 IFKNGRASISLGYIGIHETINALFGGEHVYDNE 511
          IF+NG    I L Y  +H+ +   F G   Y NE
Sbjct: 108 IFENGEGKIGLIYDVVHDELYHAFSGRGAYMNE 140
```

# Appendix VI: The results of the BLAST search using the amino acid sequence of the Class II ribonucleotide reductase gene from *Lactobacillus leichmannii*

Query= (739 letters)

### Database

Completed *Prochlorococcus marinus* str. MIT 9313;
Unfinished *Synechococcus elongatus* PCC 7942;
Completed *Bacillus anthracis* str. A2012;
Completed *Bacillus anthracis* str. Ames;
Completed *Bacillus anthracis* str. Ames 0581;
Completed *Bacillus cereus* ATCC 10987;
Completed *Bacillus cereus* ATCC 14579;
Unfinished *Bacillus cereus* G9241;
Completed *Bacillus halodurans* C-125;
Completed *Bacillus subtilis* subsp. subtilis str. 168;
Unfinished *Lactobacillus gasseri*;
Completed *Lactobacillus johnsonii*NCC 533;
Completed *Lactobacillus plantarum* WCFS1;
Completed *Escherichia coli* K12;

63,210 sequences; 17,501,187 total letters

Note: *Lactobacillus leichmanniii* was not included in the list of genomes searched because the *Lactobacillus leichmanniii* genome was not available at the time.

### Alignments

**>ref|ZP_00046381.1|** COG0209: Ribonucleotide reductase, alpha subunit [***Lactobacillus gasseri***]Length = 757

Score = 1131 bits (2926), Expect = 0.0
Identities = 543/739 (73%), Positives = 626/739 (84%), Gaps = 4/739 (0%)

```
Query: 5   ISLSAEFIDRVKASVKPHWGKLGWVTYKRTYARWLPEKGRSENWDETVKRVVEGNINLDP 64
           I+L  +FI + K  V PHWG+LGWVTYKRTYARWL +K RSENWDETVKRV+EGNINLDP
Sbjct: 19  ITLDPDFIAQTKKEVTPHWGELGWVTYKRTYARWLDDKNRSENWDETVKRVIEGNINLDP 78

Query: 65  RLQDSPSLELKQSLTEEAERLYKLIYGLGATPSGRNLWISGTDYQRRTGDSLNNCWFVAI 124
           RL+++PS +    LT EA++L++L+YGL ATPSGRNLWISGTDYQ+R GDSLNNCWF++I
Sbjct: 79  RLKNNPSKKTIHELTAEAKQLFRLVYGLAATPSGRNLWISGTDYQKRNGDSLNNCWFISI 138

Query: 125 RPQKYGDSKIVPSYLGKQEKAVSMPFSFLFDELMKGGGVGFSVARSNISQIPRVDFAIDL 184
           RPQKYG+S IVP+YL + + A SMPFSFLFD+LMKGGGVGFSV   NI+QIP++D  +DL
Sbjct: 139 RPQKYGNSHIVPAYLTQDQVAPSMPFSFLFDQLMKGGGVGFSVVDENINQIPKLDQKVDL 198

Query: 185 QLVVDETSESYDASVKVGAVGKNELV----QDADSIYYRLPDTREGWVLANALLIDLHFA 240
            +V+D+ S+SYDAS+K+GA   +E      +  D IYY+LPDTREGWVLANA LID+HF
Sbjct: 199 TIVIDKQSKSYDASLKLGATDLDEWKKTNQEKEDYIYYKLPDTREGWVLANARLIDMHFN 258
```

```
Query: 241 QTNPDRKQKLILDLSDIRPYGAEIHGFGGTASGPMPLISMLLDVNEVLNNKAGGRLTAVD 300
           TNP+ K+KL+LD+SDIRPYGA+IHGFGGTASGPMPLI ML D+N++LN +AG +LTAVD
Sbjct: 259 STNPENKKKLVLDISDIRPYGAKIHGFGGTASGPMPLIEMLFDINQILNERAGQKLTAVD 318

Query: 301 AADICNLIGKAVVAGNVRRSAELALGSNDDQDFISMKQDQEKLMHHRWASNNSVAVDSAF 360
           A DICNLIGK VVAGNVRRSAELALGS+++QDFI+MKQD++KL HHRWASNNSVA++S F
Sbjct: 319 ATDICNLIGKTVVAGNVRRSAELALGSSNNQDFITMKQDKKKLYHHRWASNNSVAINSEF 378

Query: 361 SGYQPIAAGIRENGEPGIVNLDLSKNYGRIVDGYQAGIDGDVEGTNPCGEISLANGEPCN 420
             YQPIA  I  NGEPG+VNL+LS+NYGRI DGYQAGIDG+VEGTNPCGEISLANGEPCN
Sbjct: 379 DNYQPIADSILHNGEPGVVNLELSRNYGRIKDGYQAGIDGEVEGTNPCGEISLANGEPCN 438

Query: 421 LFEVFPLIAEEQGWDLQEVFALAARYAKRVTFSPYDWEISREIIQKNRRIGISMSGIQDW 480
           LFEVFP IA++QGWDL+E F LAARY KRVTFSPYDWE+SR+II KNRRIG+SMSGIQDW
Sbjct: 439 LFEVFPFIAQKQGWDLKEAFKLAARYTKRVTFSPYDWEVSRKIINKNRRIGVSMSGIQDW 498

Query: 481 LLTRLGNRVVTGFKDDFDPETHEAIKVPVYDKRAIKMVDQLYKAVVKADQDYSKTLGCNE 540
           +L+  G+RVVTGFK   D ET + IK PVYD   IK VD LY+AVV AD+DYS+ L CN
Sbjct: 499 ILSTFGHRVVTGFKTATDSETGKEIKDPVYDPEIIKTVDGLYQAVVDADKDYSQELNCNT 558

Query: 541 SIKHTTVKPSGTVAKLAGASEGMHFHYGAYLIQRIRFQDSDPLLPALKACGYRTEADIYT 600
           SIKHTTVKPSGTVAKLAG SEGMHFHY  YLIQRIRFQ++DPLLPALK CGYRTE DIYT
Sbjct: 559 SIKHTTVKPSGTVAKLAGVSEGMHFHYSGYLIQRIRFQETDPLLPALKDCGYRTEPDIYT 618

Query: 601 ENTTCVEFPIKAVGADNPNFASAGTVSIAEQFATQAFLQTYWSDNAVSCTITFQDSEGDQ 660
            +T CVEFPIKA  AD+ NFASAGTVSIAEQFATQAFLQTYWSDNAVSCTITFQ+ E DQ
Sbjct: 619 PHTICVEFPIKAANADSDNFASAGTVSIAEQFATQAFLQTYWSDNAVSCTITFQNDESDQ 678

Query: 661 VESLLRQYRFITKSTSLLPYFGGSLQQAPKEPIDKETYEKRSQEITGNVEEVFSQLNSDV 720
           +  LL QYR+  KSTSLLPY+GGSL+QAPKEPI KE YEK    ITGNVE VF Q N D
Sbjct: 679 IAPLLHQYRYAIKSTSLLPYYGGSLKQAPKEPISKEKYEKADNHITGNVEIVFEQTNEDQ 738

Query: 721 KDLELVDQTDCEGGACPIK 739
           K LELVDQ+DC+ GACPIK
Sbjct: 739 KGLELVDQSDCDNGACPIK 757
```

**>ref|NP_894625.1|** ribonucleotide reductase (Class II) [***Prochlorococcus marinus*** str. MIT 9313] Length = 804

Score = 130 bits (326), Expect = 3e-30
Identities = 189/788 (23%), Positives = 314/788 (39%), Gaps = 133/788 (16%)

```
Query: 31  YKRTYARWLPEKGRSENWDETVKRVVEGNINLDPRLQDSPSLELKQSLTEEAERLYKLIY  90
           + RTY+R     GR E+W+E   R ++G       LQ     +L +   EE   + ++
Sbjct: 59  FYRTYSR-RTSSGR-ESWNEVSTRNLQG-------LQ-----QLGKLRAEEVALMSRMQA 104

Query: 91  GLGATPSGRNLWISGTDYQRRTGD—SLNNCWFVAIRPQKYGDSKIVPSYLGKQEKAVSM  148
              A PSGR LWI GTD+  R  +     NC    +   +
Sbjct: 105 EKKALPSGRWLWIGGTDWIERAENFSGAYNCTSTNLVDWE-------------------- 144

Query: 149 PFSFLFDELMKGGGVGFSVARSNISQIPRVDFAIDLQLVVDETSESYDASVKVGAVGKNE 208
            F  + D  M G G G  +    I Q+P V   I +  V D           +G    ++
Sbjct: 145 AFGLMMDLAMMGCGTGAVIEPHLIDQLPVVRNPIKIVGVTD-----------IGLTAAHQ 193

Query: 209 LVQDA------DSIYYRLPDTREGWVLANALLIDLHFAQTNPDRKQKLILDLSDIRPYGA 262
             +        D++  ++ DTR GWV +  LL++L   +    R   + +DLSD+RP G
Sbjct: 194 RQEQTTHSIENDNVLIKVGDTRRGWVDSYQLLLELCSDERFAGRTINVSIDLSDVRPVGE 253

Query: 263 EIHGFGGTASGPMPLISMLLDVNEVLNNKAGGRLTAVDAADICNLIGKAVVAGNVRRSAE 322
            + GFGG A+ P+ L  +   V  +L    G RLT+V+   + +     +VAGN+RRSA
Sbjct: 254 TLKGFGGMAN-PVKLKDLYTRVARLLGKAVGRRLTSVECCLLIDEAAVTIVAGNIRRSAG 312

Query: 323 LALGSNDD------------QDF-----ISMKQDQEKLMHHRWASNN----SVAVD---- 357
           +   S +D            QD      I  ++D  ++ +H    +N    S+ V+
Sbjct: 313 MRQFSAEDLAAAEAKENLWQQDAEGNWRIDPEKDALRMANHTRVYHNRPSRSIVVEAVRK 372
```

```
Query: 358 ---SAFSGYQPIAAGIRENGEPGIVNLDLSKNY-----------GRIVDGYQAGID-GD 401
           S     Q     I  +    +   +L + +            GR +      ID  +
Sbjct: 373 QFHSGEGAIQFAPEAIARSNADLLTTQELRQEFIGIYCDQGKDEAGRWLQTNHGPIDASE 432

Query: 402 VE------GTNPCGEISLANGEPCNLFEVFPLIAEEQGWDLQ-EVFALAARYAKRVTFSP 454
           +E      G NPCGEI L     CNL E+     +    D Q + F  AA     +
Sbjct: 433 LEHRLGRYGLNPCGEI-LGADFHCNLAEIHLNQIDPCDEDGQADAFKAAALSVACLLNHR 491

Query: 455 YDWEISREIIQKNRRIGISMSGIQDWLLTRLGNRVVTGFKDDFDPETHEAIKVPVYDKRA 514
           ++    R+    +  +G+S +G+ D+ +   G   +  +++   P+T E  +   + ++
Sbjct: 492 FEVPRYRQSRAWDPIVGVSFTGLFDFFVHAFGTAWLRWWENG-RPDTDEGRQ---FKQKE 547

Query: 515 IKMVDQLYKAVVKADQDYSKTLGCNESIKHTTVKPSGTVAKLAGASEGMHFHYGAYLIQR 574
              + +  K V +   DY          + TTV+P+GT + L GA+ G H       I+R
Sbjct: 548 ASFLIRWKKIVNETVWDYCDRHELRRPSRCTTVQPAGTKSLLTGAAPGWHPPKAQRFIRR 607

Query: 575 IRFQDSDPLLPALKACGY------------RTEADIYTENTT--CVEFPIKAVGADNP- 618
           I F+ +DP+  A    GY              R   D +    +   VE P +   A+ P
Sbjct: 608 ITFRKNDPVAMACMDYGYTVVPSQSDKDEQGRLLDDPFDPRCSEWLVEIPTEVSWANLPG 667

Query: 619 -NFASAGTVSIAEQFATQAFLQTYWSDNAVSCTITFQDSEGDQVESLLRQYRFITK---S 674
            +  +    S   QF     +Q++++ +  S TI  ++ E + +   L Q    ++   S
Sbjct: 668 ADAVNINNFSALAQFDFYMQVQSHYTAHNTSATIELREDEIESLGEALHQAIDNSEGYIS 727

Query: 675 TSLLPYF---GGSLQQAPKEPIDKETYEKRSQEITGN--VEEVFSQLNS-DVKDLELVDQ 728
            +LL  F     +  + P EPID  T+E+   E+         F  L+S D  +L
Sbjct: 728 AALLARFDAHNATFPRLPFEPIDANTFERLQAEVVNRRVRSNFFEALHSYDQGELMEAGP 787

Query: 729 TDCEGGAC 736
             C+   C
Sbjct: 788 AGCDSDKC 795
```

**>ref|ZP_00164662.1|** COG1372: Intein/homing endonuclease [***Synechococcus elongatus*** PCC 7942] Length = 1137

Score = 90.9 bits (224), Expect = 2e-18
Identities = 85/297 (28%), Positives = 135/297 (45%), Gaps = 51/297 (17%)

```
Query: 31  YKRTYARWLPEKGRSENWDETVKRVVEGNINLDPRLQDSPSLELKQSLTEEAERLYKLIY  90
           + RTY+R     GR E+W E   R ++G   L    Q    +EL Q   E+ + L
Sbjct: 25  FYRTYSR-KTATGR-ESWQEVSDRTIQGLAELGKLTQ--AEVELLQRYQEKQQSL-----  75

Query: 91  GLGATPSGRNLWISGTDYQRRTGDSLNNCWFVAIRPQKYGDSKIVPSYLGKQEKAVSMPF 150
               PSGR LW+ GTD+               +P+ Y       S      +A    F
Sbjct: 76  -----PSGRWLWVGGTDW--------------IAKPENYNGGYNCTSTNVIDWRA----F 112

Query: 151 SFLFDELMKGGGVGFSVARSNISQIPRVDFAIDLQLV--VDET-SESYDASVKVGAVGKN 207
             + D  M G G G  +    I+Q+P +   + + +V  + +T +E      +V   G
Sbjct: 113 GLMMDLAMMGCGTGAILEPKYINQLPAIRNELQVSIVGALGQTPAEERQEQTRVAIEG-- 170

Query: 208 ELVQDADSIYYRLPDTREGWVLANALLIDLHFAQTNPDR---KQKLILDLSDIRPYGAEI 264
               +S+  R+ D+R GWV +   L++L    ++ DR     ++ +DLSD+RP G  +
Sbjct: 171 ------NSVTIRVGDSRAGWVKSYQTLLEL----SSDDRFTGPVQVAIDLSDVRPAGERL 220

Query: 265 HGFGGTASGPMPLISMLLDVNEVLNNKAGGRLTAVDAADICNLIGKAVVAGNVRRSA 321
            GFGG A+ P+ L   +   V ++LN   G +L +++   + +     VVAGNVRRSA
Sbjct: 221 KGFGGVAN-PVKLPELYDRVAQILNKAVGRQLDSIECCLLIDQAAVVVVAGNVRRSA 276
```

Score = 71.6 bits (174), Expect = 1e-12

Identities = 84/326 (25%), Positives = 141/326 (43%), Gaps = 38/326 (11%)

```
Query: 408 CGEISLANGEPCNLFEVFPLIAEEQGWDLQE----VFALAARYAKRVTFSPYDWEISREI  463
           CGEI  A+   CNL EV     + Q    QE      A++        FS   ++ SRE+
Sbjct: 780 CGEILGADFH-CNLAEVHLNQIDPQDTKTQEDAFRAGAISVAALLNHQFSEERYQFSREV  838

Query: 464 IQKNRRIGISMSGIQDWLLTRLGNRVVTGFKDDFDPETHEAIKVPVYDKRAIKMVDQLYK  523
              +  +G+S +G+ D+ +   G   +  + +   PET + ++   + ++    +++
Sbjct: 839 ---DPIVGVSFTGLFDFFVHAFGTPWLEWWTEG-RPETEQGLE---FKRQEAAYLNRWRD  891

Query: 524 AVVKADQDYSKTLGCNESIKHTTVKPSGTVAKLAGASEGMHFHYGAYLIQRIRFQDSDPL  583
            V  A  DY          + TTV+P+GT  + L GAS G H       ++RI  F+ +DP+
Sbjct: 892 VVHAAVWDYCDRHDLRRPNRCTTVQPAGTKSLLTGASPGWHPPKAQRFLRRITFRKNDPV  951

Query:584 LPALKACGYRTEA------------DIYTENTT--CVEFPIKAVGADNP—NFASAGTV    626
           A    GY                 D +    T   VE P +   A+ P  +
Sbjct:952 AMACLDYGYSIVPSQSDKDENGNLLNDPFDPRCTEWLVEIPTEVSWANLPGADQVDISQF  1011

Query:627  SIAEQFATQAFLQTYWSDNAVSCTITFQDSE----GDQV-ESLLRQYRFITKSTSLLPYF  681
           S   QF     +QT+++ +  S TI F++ E     D++ E++     ++  S +LL  F
Sbjct:1012 SALAQFDFYMQVQTHYTTHNTSATIEFREPEIQPLADRIYEAIANNQGYM— SAALLARF 1069

Query: 682  --GGSLQQAPKEPIDKETYEKRSQEI 705
               +  + P EPIDK TYE+   E+
Sbjct: 1070 DANATFPRLPFEPIDKATYERLEAEV 1095
```

**>ref|NP_243676.1|** ribonucleoside-diphosphate reductase (major subunit) [***Bacillus halodurans*** C-125] Length = 852

Score = 33.5 bits (75), Expect = 0.44
Identities = 34/123 (27%), Positives = 53/123 (43%), Gaps = 24/123 (19%)

```
Query: 373 NGEPGIVNLDLS------KNYGRIVDGYQAGIDGDVEGTNPCGEISLANGEPCNLFEV-F 425
           + EPGI  +D +      K YG+            V  TNPCGE  LA    CNL  +
Sbjct: 448 SAEPGIFFIDNANDMTNAKAYGQ-----------KVVATNPCGEQPLAPYSVCNLAAINL 496

Query: 426 PLIAEEQG-----WDLQEVFALAARYAKRV-TFSPYDWEISREIIQKNRRIGISMSGIQD 479
             +A +Q        L++   +  R    V   +PY  E + +  +  RR+G+ + G+ D
Sbjct: 497 AEMANKQEKTVDFKKLKQTVEVGVRMQDNVIDATPYFLEENTKQAKGERRVGLGVMGLHD 556

Query: 480 WLL 482
            L+
Sbjct: 557 LLI 559
```

**>ref|NP_834294.1|** Phosphate regulon sensor protein phoR [***Bacillus cereus*** ATCC 14579] Length = 587

Score = 32.0 bits (71), Expect = 1.3
Identities = 26/90 (28%), Positives = 45/90 (50%), Gaps = 13/90 (14%)

```
Query: 237 LHFAQTNPDRKQKLI---LDLSDIRPYGAEIHGFGGTASGPMPLISMLLDVNEVLNNKAG 293
           LH      +R Q LI   LDLS I   G +++       G + +  +L D++ VL+NKAG
Sbjct: 401 LHIILKESERMQGLIEDLLDLSKIEQQGFKLN------MGTVDMKGLLEDIHMVLDNKAG 454

Query: 294 GRLTAVDAADICNLIGKAVVAGNVRRSAEL 323
            +  ++      N++ +A V G+  R  ++
Sbjct: 455 EKEISLQV----NVLKRASVIGDPSRLKQI 480
```

**>ref|NP_242642.1|** glycerophosphoryl diester phosphodiesterase [***Bacillus halodurans*** C-125]
Length = 249

Score = 31.6 bits (70), Expect = 1.7
Identities = 21/56 (37%), Positives = 26/56 (46%), Gaps = 4/56 (7%)

```
Query: 406 NPCGEISLANGEPCNLFEVFPLI--AEEQGWDLQEVFALAARYAKRVTFSPYDWEI 459
           N C EI LAN EP ++    P I  A E G ++ EV     R    V F   DW +
Sbjct: 60  NGCSEIQLANTEPASIENTIPAIRAAYEAGAEIVEVDLQQTRDGNFVAFR--DWNL 113
```

**>ref|NP_658616.1|** HATPase_c, Histidine kinase-like ATPases [***Bacillus anthracis*** A2012]
ref|NP_847035.1| sensory box histidine kinase PhoR [***Bacillus anthracis*** str. Ames]
ref|YP_021477.1| sensory box histidine kinase PhoR [***Bacillus anthracis*** str. Ames 0581] Length = 587

Score = 30.0 bits (66), Expect = 4.9
Identities = 25/87 (28%), Positives = 42/87 (48%), Gaps = 7/87 (8%)

```
Query: 237 LHFAQTNPDRKQKLILDLSDIRPYGAEIHGFGGTASGPMPLISMLLDVNEVLNNKAGGRL 296
           LH      +R Q LI DL D+     E  GF   + G + +  +L D++ VL+NKAG +
Sbjct: 401 LHIILKESERMQGLIEDLLDLSKI--EQQGFK-LSMGTVDMKGLLEDIHMVLDNKAGEKE 457

Query: 297 TAVDAADICNLIGKAVVAGNVRRSAEL 323
            ++      N + +  V G+  R  ++
Sbjct: 458 ISLQV----NTLKRVSVIGDPSRLQQI 480
```

**>ref|ZP_00236041.1|** sensory box histidine kinase PhoR [***Bacillus cereus*** G9241]
gb|EAL16109.1| sensory box histidine kinase PhoR [***Bacillus cereus*** G9241] Length = 587

Score = 29.6 bits (65), Expect = 6.3
Identities = 25/90 (27%), Positives = 43/90 (47%), Gaps = 13/90 (14%)

```
Query: 237 LHFAQTNPDRKQKLI---LDLSDIRPYGAEIHGFGGTASGPMPLISMLLDVNEVLNNKAG 293
           LH      +R Q LI   LDLS I   G +++       G + +  +L D++ VL+NKAG
Sbjct: 401 LHIILKESERMQGLIEDLLDLSKIEQQGFKLN------MGTVDMKGLLEDIHMVLDNKAG 454

Query: 294 GRLTAVDAADICNLIGKAVVAGNVRRSAEL 323
            +  ++      N + +  V G+  R  ++
Sbjct: 455 EKEISLQV----NALKRVSVIGDPSRLQQI 480
```

**>ref|NP_981012.1|** sensory box histidine kinase PhoR [***Bacillus cereus*** ATCC 10987] Length = 587

Score = 29.6 bits (65), Expect = 6.3
Identities = 21/57 (36%), Positives = 30/57 (52%), Gaps = 3/57 (5%)

```
Query: 237 LHFAQTNPDRKQKLILDLSDIRPYGAEIHGFGGTASGPMPLISMLLDVNEVLNNKAG 293
           LH      +R Q LI DL D+     E  GF   + G + +  +L D++ VL+NKAG
Sbjct: 401 LHIILKESERMQGLIEDLLDLSKI--EQQGFK-LSMGTVDMKGLLEDIHMVLDNKAG 454
```

**>ref|NP_965593.1|** protease synthase and sporulation negative regulatory protein PAI 1 [***Lactobacillus johnsonii*** NCC 533] Length = 174

Score = 29.3 bits (64), Expect = 8.3
Identities = 22/81 (27%), Positives = 36/81 (44%), Gaps = 14/81 (17%)

```
Query: 606 VEFPIKAVGADNPNFASAGTVSIAEQFATQAFLQTYWSDNAVSCTITFQDSE------GD 659
           V++ IK V ADN        +   +Q + Q FL+T+ S N+      F D+        D
Sbjct: 3   VKYEIKEVLADN--------IRELQQISRQTFLETFGSQNSAEDMREFLDTAYAEEKLKD 54

Query: 660 QVESLLRQYRFITKSTSLLPY 680
           +VE+    + F+T    +  Y
Sbjct: 55  EVENANSSFYFLTVDNKVAGY 75
```

# Appendix VII: The results of the BLAST search using the amino acid sequence of the Class I ribonucleotide reductase gene from *Bacillus cereus*

Query=(694 letters)

### Database

Completed *Bacillus cereus* ATCC 10987;
Completed *Bacillus subtilis* subsp. subtilis str. 168;
Completed *Escherichia coli* K12;

14,266 sequences; 4,102,393 total letters

### Alignments

**>ref|NP_977791.1|** ribonucleoside-diphosphate reductase, alpha subunit, group I intron-containing [***Bacillus cereus*** ATCC 10987]Length = 694

Score = 1346 bits (3483), Expect = 0.0
Identities = 678/694 (97%), Positives = 678/694 (97%)

```
Query: 1   MRHIELNNEITQMQDGFYQLHKDKEALEVFMEEARENTVHFNSVAERMEYMKEHDYYYXX 60
           MRHIELNNEITQMQDGFYQLHKDKEALEVFMEEARENTVHFNSVAERMEYMKEHDYYY
Sbjct: 1   MRHIELNNEITQMQDGFYQLHKDKEALEVFMEEARENTVHFNSVAERMEYMKEHDYYYNV 60

Query: 61  XXXXXXXXXXXXXXIAYGENFEFQSYMAASKFYKDYALKTNDQKQYLESYEDRVAIVSLY 120
                         IAYGENFEFQSYMAASKFYKDYALKTNDQKQYLESYEDRVAIVSLY
Sbjct: 61  LDEYNLEEVEEVYNIAYGENFEFQSYMAASKFYKDYALKTNDQKQYLESYEDRVAIVSLY 120

Query: 121 LGRGDVAKAKQFASMIVKQNYQPATPTFLNAGRSRRGEMVSCFLLEMDDSLNSIGFNINT 180
           LGRGDVAKAKQFASMIVKQNYQPATPTFLNAGRSRRGEMVSCFLLEMDDSLNSIGFNINT
Sbjct: 121 LGRGDVAKAKQFASMIVKQNYQPATPTFLNAGRSRRGEMVSCFLLEMDDSLNSIGFNINT 180

Query: 181 AMQLSKIGGGVALNLSKLRARGEQIKGIDNAASGVVPVMKLLEDSFSYANQLGQRKGAGA 240
           AMQLSKIGGGVALNLSKLRARGEQIKGIDNAASGVVPVMKLLEDSFSYANQLGQRKGAGA
Sbjct: 181 AMQLSKIGGGVALNLSKLRARGEQIKGIDNAASGVVPVMKLLEDSFSYANQLGQRKGAGA 240

Query: 241 VYLNIFHWDIIEFLDTKKINADEKSRIQSLSIGIIVPSKFFELAEKNEPFHVFAPYTVYK 300
           VYLNIFHWDIIEFLDTKKINADEKSRIQSLSIGIIVPSKFFELAEKNEPFHVFAPYTVYK
Sbjct: 241 VYLNIFHWDIIEFLDTKKINADEKSRIQSLSIGIIVPSKFFELAEKNEPFHVFAPYTVYK 300

Query: 301 EYGKHLDDIDIDEMYDELMSNPKVKKKPLDISARDMLIKIAMIQLESGYPYLMFKSNANN 360
           EYGKHLDDIDIDEMYDELMSNPKVKKKPLDISARDMLIKIAMIQLESGYPYLMFKSNANN
Sbjct: 301 EYGKHLDDIDIDEMYDELMSNPKVKKKPLDISARDMLIKIAMIQLESGYPYLMFKSNANN 360

Query: 361 QHPLKDIGTVKMSNLCTEIFQLQETSEINDYGTDDIIRRDINCNLGSLNIVNVMENKEIR 420
           QHPLKDIGTVKMSNLCTEIFQLQETSEINDYGTDDIIRRDINCNLGSLNIVNVMENKEIR
Sbjct: 361 QHPLKDIGTVKMSNLCTEIFQLQETSEINDYGTDDIIRRDINCNLGSLNIVNVMENKEIR 420
```

```
Query: 421 EAVHAGMEALTAVSDMTIIPNAPTVKKANDELHSVGLGAMNLHGYLAKNKIAYESAEAKE 480
           EAVHAGMEALTAVSDMTIIPNAPTVKKANDELHSVGLGAMNLHGYLAKNKIAYESAEAKE
Sbjct: 421 EAVHAGMEALTAVSDMTIIPNAPTVKKANDELHSVGLGAMNLHGYLAKNKIAYESAEAKE 480

Query: 481 FARTFFMMLNYYSIEKSMEIAKEKGETFKDFDKSDYASGTYFEKYETTDYSPVTEKVQQL 540
           FARTFFMMLNYYSIEKSMEIAKEKGETFKDFDKSDYASGTYFEKYETTDYSPVTEKVQQL
Sbjct: 481 FARTFFMMLNYYSIEKSMEIAKEKGETFKDFDKSDYASGTYFEKYETTDYSPVTEKVQQL 540

Query: 541 FEGIHIPTKEDWTSLKEQVQKNGLYNSYRLAIAPTQSISYVQNATSSVMPIVSQIESRTY 600
           FEGIHIPTKEDWTSLKEQVQKNGLYNSYRLAIAPTQSISYVQNATSSVMPIVSQIESRTY
Sbjct: 541 FEGIHIPTKEDWTSLKEQVQKNGLYNSYRLAIAPTQSISYVQNATSSVMPIVSQIESRTY 600

Query: 601 ANATTYYPMPYLSKDTFWYYKSSYDMNQFKLIDLIAEIQEHIDQGISTILYVNSDISTRE 660
           ANATTYYPMPYLSKDTFWYYKSSYDMNQFKLIDLIAEIQEHIDQGISTILYVNSDISTRE
Sbjct: 601 ANATTYYPMPYLSKDTFWYYKSSYDMNQFKLIDLIAEIQEHIDQGISTILYVNSDISTRE 660

Query: 661 LARYYIYAHKKGLKSLYYTRTRKLSVEECVACTV 694
           LARYYIYAHKKGLKSLYYTRTRKLSVEECVACTV
Sbjct: 661 LARYYIYAHKKGLKSLYYTRTRKLSVEECVACTV 694
```

**>ref|NP_389620.1|** ribonucleoside-diphosphate reductase (major subunit) [***Bacillus subtilis***]Length = 700

Score = 793 bits (2047), Expect = 0.0
Identities = 380/693 (54%), Positives = 524/693 (75%), Gaps = 4/693 (0%)

```
Query: 4   IELNNEITQMQDGFYQLHKDKEALE-VFMEEARENTVHFNSVAERMEYMKEHDYYYXXXX 62
           I+LNNEI   +DG +Q  KDKEA+   F++   +NTV F+++ E+++Y+ E+ YY
Sbjct: 10  IQLNNEIMIQKDGKFQFDKDKEAVHSYFVDYINQNTVFFHNLKEKLDYLVENQYYEEEFL 69

Query: 63  XXXXXXXXXXXX-IAYGENFEFQSYMAASKFYKDYALKTNDQKQYLESYEDRVAIVSLYL 121
                        AY + F F S+M+A KFY DYALKTND+K+ LE YEDR++IV+L+
Sbjct: 70  SLYSFEDIKEVFKTAYAKKFRFPSFMSAFKFYNDYALKTNDKKKILERYEDRISIVALFF 129

Query: 122 GRGDVAKAKQFASMIVKQNYQPATPTFLNAGRSRRGEMVSCFLLEMDDSLNSIGFNINTA 181
             GD  KAK++ ++++ Q YQP+TPTFLNAGR RRGE+VSCFLLE++DSLN I   I+ +
Sbjct: 130 ANGDTEKAKEYVNLMINQEYQPSTPTFLNAGRKRRGELVSCFLLEVNDSLNDISRAIDIS 189

Query: 182 MQLSKIGGGVALNLSKLRARGEQIKGIDNAASGVVPVMKLLEDSFSYANQLGQRKGAGAV 241
           MQLSK+GGGV+LNLSKLRA+GE IK ++NA  GVV VMKLL+++F YA+Q+GQR+G+GA
Sbjct: 190 MQLSKLGGGVSLNLSKLRAKGEAIKDVENATKGVVGVMKLLDNAFRYADQMGQRQGSGAA 249

Query: 242 YLNIFHWDIIEFLDTKKINADEKSRIQSLSIGIIVPSKFFELAEKNEPFHVFAPYTVYKE 301
           YLNIFH DI +FLDTKKI+ADE  R+++LSIG+++P KF  ELA +++  +VF P+T+YKE
Sbjct: 250 YLNIFHRDINDFLDTKKISADEDVRVKTLSIGVVIPDKFVELAREDKAAYVFYPHTIYKE 309

Query: 302 YGKHLDDIDIDEMYDELMSNPKVKKKPLDISARDMLIKIAMIQLESGYPYLMFKSNANNQ 361
           YG+H+D++D++EMYD+ + NP+VKK+   I+ R +L K+AM++ ESGYPY+MF+ N N
Sbjct: 310 YGQHMDEMDMNEMYDKFVDNPRVKKEK--INPRKLLEKLAMLRSESGYPYIMFQDNVNKV 367

Query: 362 HPLKDIGTVKMSNLCTEIFQLQETSEINDYGTDDIIRRDINCNLGSLNIVNVMENKEIRE 421
           H    I  VK SNLC+E+ Q  + S   DY  +D I  DI+CNLGSLNI+NVME+K I +
Sbjct: 368 HANNHISKVKFSNLCSEVLQASQVSSYTDYDEEDEIGLDISCNLGSLNILNVMEHKSIEK 427

Query: 422 AVHAGMEALTAVSDMTIIPNAPTVKKANDELHSVGLGAMNLHGYLAKNKIAYESAEAKEF 481
            V    ++LT VS+ T I NAP V++AN  + S+GLGAMNLHGYLA+N IAYES EA++F
Sbjct: 428 TVKLATDSLTHVSETTDIRNAPAVRRANKAMKSIGLGAMNLHGYLAQNGIAYESPEARDF 487

Query: 482 ARTFFMMLNYYSIEKSMEIAKEKGETFKDFDKSDYASGTYFEKYETTDYSPVTEKVQQLF 541
           A TFFMM+N+YSI++S EIAKEKGETF  ++ S YA+G YF+KY +TD+SP  EK+  LF
Sbjct: 488 ANTFFMMVNFYSIQRSAEIAKEKGETFDQYEGSTYATGEYFDKYVSTDFSPKYEKIANLF 547

Query: 542 EGIHIPTKEDWTSLKEQVQKNGLYNSYRLAIAPTQSISYVQNATSSVMPIVSQIESRTYA 601
           EG+HIPT EDW  LK  V ++G+Y+SYRL IAPT SISYVQ++T+SVMPI+ +IE RTY
```

```
Sbjct: 548 EGMHIPTTEDWKKLKAFVAEHGMYHSYRLCIAPTGSISYVQSSTASVMPIMERIEERTYG 607

Query: 602 NATTYYPMPYLSKDTFWYYKSSYDMNQFKLIDLIAEIQEHIDQGISTILYVNSDISTREL 661
           N+ TYYPMP L+ + +++YK +YDM+ FK++D+IA IQ+HIDQGIS  L++   ++TR+L
Sbjct: 608 NSKTYYPMPGLASNNWFFYKEAYDMDMFKVVDMIATIQQHIDQGISFTLFLKDTMTTRDL 667

Query: 662 ARYYIYAHKKGLKSLYYTRTRKLSVEECVACTV 694
            R  +YAH +G+K++YY RT+    + C++C V
Sbjct: 668 NRIDLYAHHRGIKTIYYARTKDTGQDSCLSCVV 700
```

**>ref|NP_417161.1|** ribonucleoside-diphosphate reductase 2 alpha subunit [***Escherichia coli*** K12]Length = 714

Score = 584 bits (1506), Expect = e-167
Identities = 301/689 (43%), Positives = 442/689 (64%), Gaps = 14/689 (2%)

```
Query: 16  GFYQLHKDKEALEVFME-EARENTVHFNSVAERMEYMKEHDYYYXXXXXXXXXXXXXXXX 74
           G  Q  KD++A++ F+    R N+V F+S  +R+ ++    YY
Sbjct: 30  GRIQFDKDRQAVDAFIATHVRPNSVTFSSQQQRLNWLVNEGYYDESVLNRYSRDFVITLF 89

Query: 75  I-AYGENFEFQSYMAASKFYKDYALKTNDQKQYLESYEDRVAIVSLYLGRGDVAKAKQFA 133
             A+   F FQ+++ A KFY  Y LKT D K+YLE + DRV +V+L L +GD   A Q
Sbjct: 90  THAHTSGFRFQTFLGAWKFYTSYTLKTFDGKRYLEDFADRVTMVALTLAQGDETLALQLT 149

Query: 134 SMIVKQNYQPATPTFLNAGRSRRGEMVSCFLLEMDDSLNSIGFNINTAMQLSKIGGGVAL 193
             ++   +QPATPTFLN G+ +RGE+VSCFLL ++D++ SIG  +N+A+QLSK GGGVA
Sbjct: 150 DEMLSGRFQPATPTFLNCGKQQRGELVSCFLLRIEDNMESIGRAVNSALQLSKRGGGVAF 209

Query: 194 NLSKLRARGEQIKGIDNAASGVVPVMKLLEDSFSYANQLGQRKGAGAVYLNIFHWDIIEF 253
            LS LR  G  IK I+N +SGV+PVMK+LED+FSYANQLG R+GAGAVYL+  H DI+ F
Sbjct: 210 LLSNLREAGAPIKRIENQSSGVIPVMKMLEDAFSYANQLGARQGAGAVYLHAHHPDILRF 269

Query: 254 LDTKKINADEKSRIQSLSIGIIVPSKFFELAEKNEPFHVFAPYTVYKEYGKHLDDIDIDE 313
           LDTK+ NADEK RI++LS+G+++P   F LA++N    +F+PY V + YGK   D+ I +
Sbjct: 270 LDTKRENADEKIRIKTLSLGVVIPDITFHLAKENAQMALFSPYDVERVYGKPFADVAISQ 329

Query: 314 MYDELMSNPKVKKKPLDISARDMLIKIAMIQLESGYPYLMFKSNANNQHPLKDIGTVKMS 373
            YDEL+++ +++KK L  +ARD   ++A IQ ESGYPY+M++   N  +P+   G + MS
Sbjct: 330 HYDELVADERIRKKYL--NARDFFQRLAEIQFESGYPYIMYEDTVNRANPI--AGRINMS 385

Query: 374 NLCTEIFQLQETSEINDYGTDDIIRRDINCNLGSLNIVNVMENKEIREAVHAGMEALTAV 433
           NLC+EI Q+   SE ++         DI+CNLGSLNI + M++ +    V   +  LTAV
Sbjct: 386 NLCSEILQVNSASEYDENLDYTRTGHDISCNLGSLNIAHTMDSPDFARTVETAVRGLTAV 445

Query: 434 SDMTIIPNAPTVKKANDELHSVGLGAMNLHGYLAKNKIAYESAEAKEFARTFFMMLNYYS 493
           SDM+ I + P+++  N   H++GLG MNLHGYLA+  IAY S EA +F   +F  + +++
Sbjct: 446 SDMSHIRSVPSIEAGNAASHAIGLGQMNLHGYLAREGIAYGSPEALDFTNLYFYAITWHA 505

Query: 494 IEKSMEIAKEKGETFKDFDKSDYASGTYFEKYETTDYSPVTEKVQQLF--EGIHIPTKED 551
           +  SM +A+E+GETF  F +S YASG YF +Y   ++ P T KV +LF   GI +PT+E
Sbjct: 506 LRTSMLLARERGETFAGFKQSRYASGEYFSQYLQGNWQPKTAKVGELFTRSGITLPTREM 565

Query: 552 WTSLKEQVQKNGLYNSYRLAIAPTQSISYVQNATSSVMPIVSQIESRTYA-NATTYYPMP 610
           W  L++ V + G+YN    A+ PT SISY+ +ATSS+ PIV+++E R        YYP P
Sbjct: 566 WAQLRDDVMRYGIYNQNLQAVPPTGSISYINHATSSIHPIVAKVEIRKEGKTGRVYYPAP 625

Query: 611 YLSKDTFWYYKSSYDMNQFKLIDLIAEIQEHIDQGISTILYVNSDISTRELARYYIYAHK 670
           +++ +    Y+ +Y++   K+ID  AE   H+DQG+S  L+     +TR++ +  IYA +
Sbjct: 626 FMTNENLALYQDAYEIGAEKIIDTYAEATRHVDQGLSLTLFFPDTATTRDINKAQIYAWR 685

Query: 671 KGLKSLYYTRTRKLS-----VEECVACTV 694
           KG+K+LYY R R+++     +E CV+C +
Sbjct: 686 KGIKTLYYIRLRQMALEGTEIEGCVSCAL 71
```