Characterisation of new bacteria strains isolated from Icelandic sea waters

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Raunvísindadeild
Háskóli Íslands
2014

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Lýsing nýrra bakteríustofna af íslenskum sjávarmiðum

Jónína Sæunn Guðmundsdóttir

15 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefna- og sameindalíffræði

Leiðbeinandi
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Raunvísindadeild
Verkfræði- og náttúruvísindasvið
Háskóli Íslands
Reykjavík, maí 2014

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Reykjavík, maí 2014

Statement

I hereby declare that this essay was written by me and has not been used before by part or whole to a higher degree.

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Jónína Sæunn Guðmundsdóttir

Útdráttur

Markmið þessa verkefnis var að lýsa þremur nýjum bakteríustofnum sem höfðu áður verið einangraðir, hjá Matís úr sýnum teknum á Íslenskum sjávarmiðum sumarið 2012 sem hluti af samvinnuverkefninu MaCuMBA. Þessir stofnar urðu fyrir valinu eftir að hluti 16S rRNA gens þeirra hafði verið raðgreindur og sýnt að um stofna sem ekki hefði verið lýst áður var að ræða.

Á þeim þremur mánuðum sem verkefnið varði voru stofnarnir skoðaðir í smásjá og fylgst með útliti þeirra auk þess sem svipgerð stofnanna var skoðuð með gram litun og með mælingum á vexti við mismunandi vaxtarskilyrða (hitastig, pH, seltustig) og einnig var athugaðir hvaða kolefnis og orkugjafar voru nýttir. Allir reyndust stofnarnir vaxa betur eftir því sem hitastig var hækkað, vera viðkvæmir fyrir breytingum á sýrustigi og vaxa best við hlutlaust sýrustig og vaxa best í æti með seltustig um 2% NaCl. Því miður voru niðurstöður fyrir vöxt með mismunandi kolefnis- og orkugjöfum ekki marktækar og átti það sama við um prófanir á þoli stofnanna gegn fúkkalyfjum. Upp komu vandamál með að fá bakteríurnar til að vaxa auk þess sem að allur vöxtur tók lengri tíma en áætlað hafði verið í upphafi, þannig að þegar ljóst var að þessar mælingar höfðu ekki tekist gafst því miður ekki tími til að endurtaka þær.

Í upphafi var stefnt á að heilraðgreina 16S rRNA genið fyrir alla stofnana. Þegar þessi ritgerð er skrifuð er heilraðgreiningin enn í gangi en niðurstöður fyrir hluta gensins liggja fyrir og hafa verið greindar. Þær staðfesta að um þrjá stofna sem ekki hefur verið lýst áður er að ræða en 16S rRNA gen þeirra hafði verið greint í umhverfi án þess að rækta stofnanna.

Abstract

The main objective of this research was to characterise three bacteria strains that had previously been isolated at Matís from Icelandic sea waters as a part of a collaborative project called MaCuMBA. These strains were chosen after the partial 16S rRNA was sequenced.

Over the three months period the project took morphological observations were made and the bacteria phenotypes were observed with gram staining and the effect of different growth parameters (temperature, pH and salinity concentration) and it was also observed which carbon and energy sources were used. All of the strains turned out to prefer higher temperatures than *in situ*, be sensitive to change in acidity and prefer neutral pH and grow best in medium containing around 2% NaCl. The results for growth with different carbon and energy sources were inconclusive, as were results for antibiotic susceptibility. This was due to problems encountered with getting the strains to grow and the fact the strains growth took longer than anticipated so when the growth failed for those measurement it was not possible to repeat them.

In the beginning, I set out to sequencing the whole 16S rRNA gene of each strain. The sequencing is ongoing when this thesis are being written but a part of each gene has been successfully sequenced and the results have confirmed that those are indeed three new strains that have never before described before but their 16S rRNA has been found in the nature before but not cultivated.

Acknowledgements

I want to start with thanking Matís and especially my mentor Viggó Þór Marteinsson for giving me the opportunity to work on this interesting project. I would also like to thank Viggó for all the help in the past months both in the laboratory and while writing this essay.

I particularly want to thank Pauline Vannier, my laboratory supervisor, for the endless help she has given me for the duration of my project. She has managed me for the entire duration of my project, helped me finding my way around a microbiology lab and has been there to answer my questions every step of the way. I would also like to thank both her and Viggó for taking the time to read and correct my essay.

At last I would like to thank my teacher Bjarni Ásgeirsson for taking responsibility for my project and for his endless help and guidance throughout my time at the University of Iceland.

Contents

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Abbreviations

BLAST : Basic Local Alignment Search Tool

DNA : Deoxyribonucleic acid

PCR : Polymerase Chain Reaction

rRNA : ribosomal Ribonucleic Acid

SS : Single-stranded

TAE : Tris Acetate Ethylenediaminetetraacetic acid (EDTA)

1 Introduction

1.1 Microorganisms

When the Earth was formed 4.5 billion years ago, there was no life on the planet and for the first 100 million years or so after it's formation conditions on the planet stayed far to harsh to sustain any type of life. The first direct evidence of primitive cellular life comes from microbial fossils dated at about 3.5 billion years old and for the next 1.4 billion years prokaryotes were the only form of life on Earth (Willey, Sherwood, & Woolverton, 2011).

Microorganisms can be both single cell organisms (prokaryotes) and multicellular organisms (eukaryotes). The term prokaryote covers two disparate groups of organisms, the Bacteria and the Archaea. Those three domains can be linked together in a universal phylogenetic tree (Fig. 1) via small subunit rRNA sequences defined by Carl Woese (Olsen & Woese, 1998).

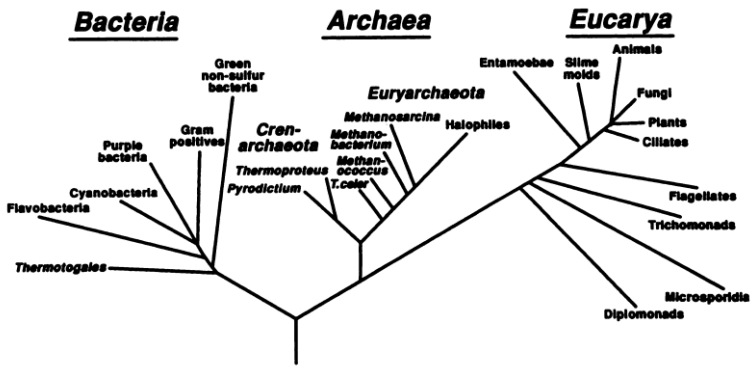

Figure 1: *Rooted universal phylogenetic tree showing the three domains based upon 16S rRNA sequences.*

1.1.1 Microbial growth

In microbiology, the term *growth* generally refers to increase in the number of cells in a population but not the growth of individual bacteria or archeal cell. Adding a small population of a bacteria (referred to as inoculum) to a suitable volume of rich medium results in a predictable increase in cell numbers (Perry, Staley, & Lory, 2002).

Growth curve for the microorganisms can be obtained by determining the number of organisms present at regular intervals throughout population growth and plotting it (either with the use of semilogarithmic paper or computer). The growth curve is divided into four phases that each has it's own slope. These phases are lag, exponentional growth, stationary and death phases. During the lag phase there is no increase in cell number as the cells are preparing themselves for growth during this phase. The length of the lag phase can vary considerably. The lag phase is followed

by the exponentional growth phase (also called log phase) where the generation time is constant. The rate of the exponentional growth itself varies between populations and also depends on ambient conditions such as the composition of the medium. When end is brought to the exponentional growth, the culture enters the stationary phase. During stationary phase the cell division and cell death are exactly balanced. If a culture is maintained in stationary phase for too long the microorganisms die. Generally the death phase is an exponential function (Perry, Staley, & Lory, 2002).

1.2 Icelandic sea waters

Iceland is situated between cold sea from the artic and warmer sea from the south.They meet here because of the Greenland-Scotland Ridge which acts as a natural barrier against the main ocean currents around the country. In the south, we have a branch of the North Atlantic Current, the Irminger Current, whith temperature around 6-8°C. In the north we have the cold East-Greenland and East Icelandic Currents, with temperature around -1-2°C (Malmberg, Mortensen, & Valdimarsson, 1999). The temperature of the ocean around Iceland is mostly determined by the spread of different types of sea waters in the North-Atlantic ocean (Jónsson, 2004)

1.3 Marine microbial biodiversity

The largest continuous habitat in the world is the ocean, covering more than 70% of the earth surface with volume of 1.5×10^9 km^3. It has been estimated that about 90% of the total biomass found in the ocean consists of prokaryotes, more specifically 1.2×10^{29} of earth's prokaryotes (Whitman, Coleman, & Wiebe, 1998). As of today, less than 10.000 prokaryotes have been described and cultivated, at the same time that it's estimated that up to one billion may be found in each litre of ocean water. The number of viruses found in the ocean is at least a factor 10 higher and eukaryotic microorganism constitute an enormous diversity although their number is thought to be 100-1000 times lower than the number of prokaryotes. In each millilitre of the ocean, thousands of different microbial species may thrive and as most of the marine diversity (99%) has not been taken into culture we see that the microbe diversity is untold and has yet to be discovered. Strains of *Bacteria* and *Archaea* that have not been taken into culture are often referred to as “unculturable”. However, this concept is likely wrong. If the organism can survive and thrive in the nature then culturing the uncultured should only be a matter of finding the right conditions and growth medium to mimic it's natural habitat. That, however, may prove extremely time consuming.

1.4 Characterisation and identification

Characterisation and identification are two concepts that often come hand in hand with each other. The aim of prokaryote characterisation is to gain as much data describing the properties of a pure culture as possible. The aim of identification is to compare the properties of a pure culture to those of an accepted species. When identification can't be accomplished by comparison it shifts to characterisation of a new species i.e. to a new description.

It is apparent that more data is needed for characterisation than for identification. It is also apparent that when the final aim of characterisation is to collect as much data about a species as possible, the goal will never truly be reached as progress in scientific and technological methods allows the study of an ever-increasing number of properties of a species (Trüper & Schleifer, 2006).

If a prokaryote strain is considered as a new species (after the sequencing of their 16S rRNA), authors should start with comparison between the "new" species and the bacterial group it showes most similarites with and rationalise why it is they belive the new strain to be a new species. Table 1, as presented by Trüper & Schleifer (2006), shows the minimum information that a description of a new species should contain.

Table 1: *Minimum data required for the description of a new species*

	Required data	Desired/required data, if applicable
Cell morphology	Cell shape[a]	Color
	Cell size (diameter, length)	Flagellation type[a]
	Motility	
	Visible internal or external structures[a]	Spores[a], appendages[a], capsules[a], sheaths[a]
	Formation of typical cellular aggregate[a]	
	Occurrence of cell differentiation[a]	Life cycle[a], heterocysts[a], hormogonia[a]
	Ultrastructure (general)[b]	Ultrastructure of flagella, envelope, cell wall[b]
Colonial morphology	Appearance of cell suspensions	Color of suspension (absorption spectra)
	Appearance of colonies	Color of colonies
		Motility of colonies
		Formation of fruiting bodies[a]
		Formation of mycelia[a]
Staining behavior	Gram stain	Acid-fast stain, spore stain, flagellum stain
Cell constituent	DNA base ratio	Nucleic acid homology; rRNA sequences
	Reserve materials	Cellular pigments
		Cell wall and membrane constituents
		Typical enzymes
Physiology	Temperature range and optimum	Salinity or osmolarity requirements
	pH range and optimum	Vitamin requirements
	Modes of energy metabolism (phototrophy, chemotrophy, lithotrophy, organotrophy)	Typical metabolic products formed (acids, osmolytes, pigments, antibiotics, toxins, antigens)
	Relation to oxygen	
	List of electron acceptors	Tolerances and susceptibilities
	List of carbon sources	
	List of nitrogen sources	
	List of sulfur sources	
Ecology	Natural habitat(s)	Pathogenicity, host range
		Antigen formation
		Serology
		Phage susceptibility
		Symbiosis

[a]To be demonstrated by light microscopy.
[b]To be demonstrated by electron microscopy.

1.5 This project

In 2012, as a part of a large EU project called MaCuMBA, sea samples were collected from 106 different stations around Iceland at different depths (from 0m to 1400m). As a result around 150 samples were collected for strain isolation and for DNA extraction for diversity analysis without cultivation. The strains characterised in this project were all isolated from these samples previously by former intern at Matís. Strain A was sampled from location 255 at 50m, strain B from location 271 at 1000m and strain C from location 235 at 30m.

The reason those three strains were chosen to be characterised and analysed better out of the number of other isolates from the sampling trip lies in their uniqueness. When a part of their 16S rRNA was sequenced and compared to data bases, it showed that those strains showed homology to 16S rRNA sequences or OTUs (operational taxonomic unit) but not from cultivated species or isolated strains, This made them perfect candidates for this characterisation project.

The sampling map of the 2012 sampling trip can be seen in figure 2. The locations from where the strains characterised in this project were collected have been labelled with blue circles.

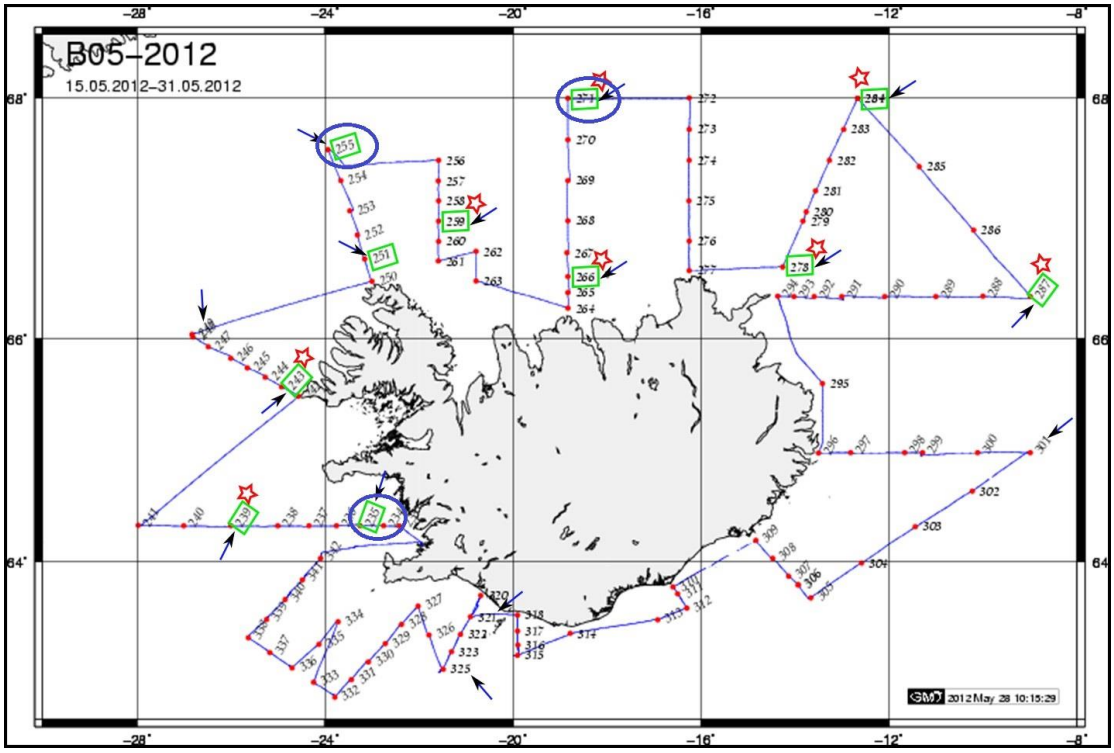

Figure 2: *Sampling map of the 2012 samling trip. The locations from where strains A, B and C were collected have been marked with blue circles.*

2 Materials and methods

All work with the bacteria strains was done under sterile conditions.

2.1 Morphological observation

Morphological observations were made using a light microscopy. An Olympus BX51 microscope was used with an oil-immersed objective (100x/1.30 Oil pH3) in phase contrast (Olympus Microscopy Resource Center). The magnification was x1000 which made it possible to observe the bacteria, their number, shape and behaviour. Attached to the microscope is an Olympus DP72 camera which makes it possible to snap high resolution digital pictures of the bacteria observed.

2.2 Gram staining

The staining method that was used on these strains was a gram staining. It is named after its inventor, Hans Christian Gram, and the method is still used although it is less important than it used to be. Gram staining is a method of separating bacterial species into two groups called gram-positive and gram-negative. Fresh cultures were made on Petri Dishes some days before the observation. Sterile loop was used to take one colony and smear it on a slide which was quickly passed through a flame 2-3 times to fix the deposit. Crystal violet solution was then poured over the fixed smear and left waiting for 2 minutes. The slide was next rinsed off with distilled water and then washed with iodine solution which was left on the slide for 1 minute. The slide was rinsed off with distilled water and gram decolorizer (mixture of alcohol and acetone). The smear was then stained with safranine for 2 minutes before rinsing with distilled water. The slide was then dried with absorbent paper and examined with microscope (Benediktsdóttir, Guðmundsson, & Alfreðsson, 2012).

2.3 Growth parameters under different conditions

Each growth parameter was studied by using the BioScreen C. The BioScreen C Analyzer System is an instrument used to perform a wide range of microbiology experiments automatically. The BioScreen monitors microorganism growth by measuring turbity of liquid growth medium in the wells (the maximum capacity is 200 different samples per run). The measurements are recorded as optical density measurements and the values are recorded by a PC connected to the analyzer system. The software used, EZExpermient stores all results from the experiment in a CSV file so all data can easily be exported to Excel for graph generation and analysis (User's manual BioScreen C, 2009).

Strains A and B were isolated in medium 172 and strain C on 514 (Appendix A). Thus, a first study has been done for each temperature to define which medium to use

2.3.1 Temperature range

Growth at different temperatures was examined by using BioScreen. To inoculate medium with each strain, 20 µL of fresh culture was added in 2000 µL of sterile medium in 2 mL microtube. After thorough mixing, 300 µL were added into each well on the plate. As control, 300 µL of sterile medium was used. Each strain growth was measured in both medium 172 and 514 and the growth experiments took 3 days with measurement interval of 4 hours and with continuous shaking.

2.3.2 pH range

Each medium (172 and 514) was prepared in flasks with 10 different pH. For medium 172 the pH tested were: 3.7, 4.9, 5.1, 6.3, 7.1, 7.8, 8.3, 8.4, 8.6 and 8.9. For medium 514 the pH tested were: 3.4, 4.5, 4.9, 5.7, 6.5, 6.8, 7.4, 7.6, 8.0 and 8.5. The pH was measured after autoclaving and adjusted when needed with HCl and NaOH.

BioScreen was used to examine the growth of strains A, B and C at different pH. Twenty µL of fresh culture of each strain was added in 2000 µL of sterile medium in 2 mL microtube to inoculate medium. After thorough mixing, 300 µL were added into each well on the plate. 300 µL of sterile medium was used as control. For strain A, the medium 172 was used but medium 514 for strains B and C. The experiment took 3 days with measurement interval of 4 hours with continuous shaking.

2.3.3 NaCl concentrations

For medium 172, the NaCl concentrations tested were: 0, 1, 2, 3, 4 and 5% (w/v). For medium 514, the NaCl concentrations tested were 0, 1, 2, 3 and 4% (w/v).

BioScreen was used to examine the growth of strains A, B and C at different NaCl concentrations. To inoculate medium with each strain, 200 µL of fresh culture was added in 2000 µL of sterile medium in 2 mL microtube. After thorough mixing 300 µL were added into each well on the plate 300 µL of sterile medium was used as control. For strain A, the medium 172 was used but medium 514 for strains B and C. The experiment took 3 days with measurement interval of 4 hours with a continuous shaking.

2.3.4 Catalase and oxidase activity

These test are still valid and are currently used for identification and characterization of strains. Catalase test is used to tell if the bacteria does contain cytochrome c oxidase or not, which means if it can use oxygen for energy production with an electron transfer chain or not.

Catalase test was done using hydrogen peroxide. Few drops of hydrogen peroxide were added on a clean slide and a colony taken with a loop and added into the liquid. If the bacteria has the catalase enzyme it will neutralize the bactericidal effects of the hydrogen peroxide by breaking it down into water and oxygen. The reaction will make bubbles appear in the hydrogen peroxide.

The BBL™ DrySlide™ was used for the oxidase test. Colony was taken with a loop and spread on the slide from the test kit. If the slide turns dark purple, the bacteria contains cytochrome c oxidase, if nothing happens it does not contain the enzyme (Product Center, 2014).

2.3.5 Carbon and energy sources

When making a list of which carbon and energy sources to test the selected trains the same sources were used as Young-Ok Kim et al. did when describing the *Colwellia meonggei*, which is a psychrophilic marine bacteria (Kim, Park, Nam, Jung, Kim, & Yoon, 2013). The complete list of carbon and energy sources tested can be found in Appendix A.

A serial dilutions of the strains was performed in basal medium (Appendix A). Basal medium was used to have medium without any carbon sources. That way, carbon and energy sources can be added later on with known concentration. This shows which carbon sources the bacteria uses and which ones it cannot use. For this reason, rich medium with 172 and 514 cannot be used for this step. First 5.0 mL of basal medium was put into a tube with 0.1 mL of the strain and mixed thoroughly. Then 0.1 mL were moved to another tube with 5.0 mL of basal medium and mixed thoroughly. The dilution was performed five times to be sure not to have any carbon sources from the inoculum. The tubes were kept over two nights at 10°C and the tube with the most dilution that still had growth used for the next step. Into tube, 5 mL of basal medium the correct amount of carbon and energy source to gain final concentration of 10 mM were put along with 0.5 mL of inoculum from the correct dilution. The tubes were then incubated at 17°C. Light microscope was used to watch if growth occurred.

The BioScreen was used to examine the growth of strains A, B and C with different carbon and energy sources. Each carbon source was tested in triplicate at 20°C with positive controls (each strain on rich medium, both medium 172 and 514 was tested) and negative controls (of both rich medium without inoculum and basal medium with inoculum). First, a master mix of basal medium and each strain was made. Then 270 µL of this mixture was put in each well along with 30 µL of the correct carbon source. For each triplicate, there was a negative control of just basal medium and the correct carbon source. The experiments took 3 days with measurement interval of 4 hours with continuous shaking.

2.3.6 Antibiotic susceptibility

For each antibiotic tested asterile solution was prepared with the initial concentration of 1 mg/mL. The antibiotics tested were Streptomycin, Chloramphenicol, Kanamycin, Polymixin B and Neomycin.

The BioScreen was used to examine the antibiotic susceptibility of strains A, B and C. Each antibiotic was tested in triplicate at three different concentrations; 10 μg/mL, 30 μg/mL and 100 μg/mL at 20°C with positive controls (each strain on rich medium, both medium 172 and 514 was tested) and negative controls (of both rich medium without inoculum and basal medium with inoculum). First, a master mix of rich medium (172 for strain A and 514 for strains B and C) and each strain was made. Then, 270 μL of this mixture was put into each well along with 30 μL of the correct antibiotic solution. For each triplicate, there was a negative control of just rich medium and the correct antibiotic solution. The experiments took 3 days with measurement interval of 4 hours with continuous shaking.

2.4 16S rRNA gene sequencing

2.4.1 DNA extraction with Chelex

The first step of the DNA sequencing was extracting DNA. This was done using Chelex. The protocole I used is inspired by the protocol used by Fenicie et al (Fenicia, Anniballi, De Medici, Delibato, & Aureli, 2007) but had been altered by Floriane Briere during her internship for Matís.

A 6% Chelex® 100 solution was prepared by autoclaving 1.32g of the BioRad Biotechnology Grade Chelex® 100 resin in the sodium form and then mixing it into 22.5mL of sterile water.

Under sterile conditions, some colonies were suspended into 200 μL of 6% Chelex® solution and vortexed for 15 seconds before incubation. The tubes were first incubated during 20 minutes at 56°C to lyse cells and then incubated during 8 minutes at 100°C. The alkalinity of Chelex suspensions in association with the high temperature causes cell disruption of the cell membranes, destruction of cellular proteins and DNA is denatured to yield single-stranded (SS) DNA (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992).

The tubes were then left on ice to cool for about 10 minutes and then taken to a centrifuge during 5 minutes at 14000g and 4°C. The supernatant containing the DNA was picked up with a pipette and the pellet thrown away.

The purity of DNA extracts was assessed using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer to determine DNA concentration and purity in all the extracts. This one step method was chosen for it's rapidity, for the low consumption of sample, it's accuracy and reproducibility (NanoDrop 1000 Spectrophotometer, V3.7 User's Manual, Thermo Scientific).

2.4.2 DNA amplification by PCR

To obtain a sufficient amount of 16S rDNA for the sequencing step, the extracted DNA has to be amplified with universal 16S rRNA gene primers. Thus, the Polymerase Chain Reaction (PCR) method was performed using the One Taq® Hot Start DNA Polymerase kit from New England BioLabs©.

PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand (Joshi & Deshpande, 2011). Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. Thus, two primers (forward and reverse) were chosen in order to specifically target the desired DNA sequence. The primers used and their sequences can be seen in table 2.

Table 2: *Primers' sequences used for PCR amplification*

Forward 9 primer	**5'**GAGTTTGATCCTGGCTCAG**3'**
Reverse 1544 primer	**5'**CCCGGGATCCAAGCTTAGAAAGGA**3'**

The reaction needs other compounds (Table 3) and a specific thermal profile (Table 4) that are detailed below. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Table 3: *Composition of the reaction mix used for PCR amplification, table continued on next page*

Reagent	Final Concentration	Rule
One Taq GC Reaction Buffer	1x	Contains all the compounds that are needed for the enzymatic reaction to occur.
One Taq High GC Enhancer	2%	This solution promotes PCR amplification for difficult amplicons such as high GC content templates.
dNTPs	200 μM	Nucleotides (A, T, G, C) which are "building blocks" for the new DNA strands.
Primer F9	0.4 μM	Oligonucleotide primers are short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primers. Primer's sequences are presented in Table 1.
Primer R1544	0.4 μM	

One Taq® Hot Start DNA polymerase	25 U/mL	DNA polymerase enzyme that is going to synthesize the new DNA strands.
DNA template	0.5 ng – 0.5 µg	Sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

Table 4: *Thermal profile used for PCR amplification*

	Temperature	**Time**	**Rule**
Initial Denaturation	94°C	4 minutes	Due to high temperature, the double stranded DNA template is going to be denatured allowing the enzyme and primers to access single strand molecules. A particularly long time has been chosen to insure denaturation of difficult templates, such as GC-rich sequences.
Denaturation	94°C	40 seconds	DNA denaturation between each cycle.
Annealing	52°C	40 seconds	Allows primers to hybridize to their respective complementary template strands. The annealing temperature is based on the Tm of the primer pair.
Extension	68°C	1 minute	Extension occurs thanks to the polymerase enzyme which begins at the end of the annealed primers to create a complementary copy strand of DNA. Extension time are generally 1 minute per kb.
Final Extension	68°C	7 minute	Permits to end the DNA strand extension.
Hold	4°C	Forever	Keep the DNA at low temperature to avoid denaturation and degradation.

For each PCR performed there was a positive control made with the same reagent mix with a known DNA template from *Campylobacter* to check if the PCR amplification works and a negative control made with the same reagent mix but without DNA template to check that the DNA that is being amplified is not an extern contamination.

2.4.3 Gel electrophoresis

In order to verify the PCR reaction quality and yield, a gel electrophoresis was performed. An agarose gel was prepared of 1% with 0.003% ethidium bromide and a 1x TAE buffer. While the gel was curing samples were prepared by adding 5 μL of 6x loading dye to 5 μL of PCR product. When the gel had set, it was moved to the electrophoresis tank and oriented so that the wells were oriented toward the negative electrode. The DNA fragments will move from the wells toward the positive electrode. The tank was filled with TAE buffer to submerge the gel and one sample (10 μL) loaded into each well, molecular weight standard (1kb DNA ladder) went into the first well. The power was turned on and constant voltage of 90 V applied for 30 minutes. Observation was made with UV light (Chapter 8: Objectives Agarose gel electrophoresis, 2010).

2.4.4 EXO-SAP® purification

In order to remove unincorporated primers and unconsumed dephosphorylate dNTPs that can interfere with subsequent reactions and lead to an unreadable sequence, a PCR products clean-up was performed. The ExoSAP® protocol is a one-step enzymatic treatment that is based upon the activity of an exonuclease and a phosphatase that are added to the PCR product, was applied (Bell , 2008).

Table 5: *Composition of the reaction mix used for the ExoSAP® puricication*

Reagent	Final concentration	Role
Exonuclease I (BioLabs - 20,000 U/mL)	76.9 U/mL	Catalyses the removal of nucleotides from single-stranded DNA in the 3' to 5' direction. This enzyme degrades excess single-stranded primer oligonucleotide from a reaction mixture containing double-stranded extension.
Antartic Phosphatase (BioLabs - 5,000 U/mL)	38.5 U/mL	Removes 5' phosphates from DNA and dNTPs. This enzyme removes dNTPs and pyrophosphate from PCR reactions mixture.
Sterile, distilled H_20	NA	
PCR product	NA	

The PCR plate was then incubated with the thermal profile in table 6.

Table 6: *Thermal profile used for the ExoSAP® purification*

Temperature	Time	Rule
37°C	30 minutes	The optimal activity temperature allows the two enzymes to perform their exonuclease and phosphatase activities.
95°C	5 minutes	The two enzymes are inactivated thanks to this high temperature treatment. The duration is short enough so that double strand DNA is not affected.
4°C	Forever	Keep the DNA at low temperature to avoid denaturation and degradation.

2.4.5 DNA sequencing using Big Dye Terminator

In order to sequence the whole 16S RNA gene, a fluorescence-based cycle DNA sequencing was performed using different primers that code for different parts of the 16S RNA complex. Thus, The BigDye® Terminator v3.1 Cycle Sequencing kit was used; this kit was chosen because it is particularly adapted for GC rich sequences and difficult templates, but also because it increases the robustness and the peak heights of sequencing (Applied BioSystems).

To each ExoSAP mix, the reagents in table 7 were added. This was repeated for each of the primers in table 7.

Table 7: *Primers' sequences used for Big Dye® Terminator v3.1 protocol*

Forward 9 primer	**5'**GAGTTTGATCCTGGCTCAG**3'**
Reverse 1544 primer	**5'**CCCGGGATCCAAGCTTAGAAAGGA**3'**
Forward 515 primer	**5'**GTGCCAGCMGCCGCGGTAA**3'**
Reverse 1046 primer	**5'**CGACAGCCATGCANCACCT**3'**
Forward 805 primer	**5'**GACTACCCGGGTATCTAATCC**3'**
Reverse 805 primer	**5'**GGATTAGATACCCGGGTAGTC**3'**
Forward 228 primer	**5'**ACXCCTACGGGXGGCAGCAG**3'**

Table 8: *Composition of the reaction mix used for the Big Dye® Terminator v3.1 protocol*

Reagent	Final concentration	Role
Reverse primer	0.35 μM	Different primers were used to perform the cycling amplification, see table 7 for list of primers used
Big Dye	NA	Dye labelled terminators, the mix contain the four fluorescently labelled dNTPs that will be incorporated and whose rule is to stop extension
Sequencing buffer 3.1	1x	Contains dNTPs, the AmfiTaq® DNA Polymerase is a mutant form of the Taq DNA polymerase, it contains a point mutation in the active site, resulting in less discrimination against ddNTPs, and leads to a much more even peak intensity pattern.
DNA template	NA	PCR products which were previously checked using an electrophoresis migration

The thermal profile in table 9 was then applied to the mix.

Table 9: *Thermal profile used for the Big Dye® Terminator v3.1 protocol*

Temperature	Time	Role
96°C	30 seconds	Due to high temperature, the double stranded DNA template is going to be denatured, allowing the enzyme and primers to access single strand molecules.
50°C	15 seconds	This allows the primer to hybridise to it's complementary template strands
60°C	4 minutes	Extension occurs thanks to the polymerase enzyme which begins at the end of the annealed primers to create a complementary copy strand of DNA. It stops as soon as a dye-labeled terminator is incorporated.
4°C	Forever	To keep the DNA at low temperature to avoid denaturation and degradation. Protects from light to keep fluorescing properties of the dye-labeled dNTPs

Prior to injection into the sequencing machine, the mixture that resulted from the BigDye® Termintor v3.1 Cycle Sequencing protocol was cleaned using the Agencourt CleanSEQ method. The Agencourt CleanSEQ method contains magnetic particles in an optimized binding buffer to selectively capture sequencing extension products. Unincorporated dyes, nucleotides, salts and contaminants are removed using a simple washing procedure.

First, homogeneised Agencourt CleanSEQ solution was added to the reaction mixture to achieve a 35% final concentration. After vortexing, 85% ethanol solution was added and incubation performed at room temperature for 3 minutes. After incubation, the plate was put onto the Agencourt SPRI magnetic plate and left there for 3 minutes. Magnetic beads bound to DNA are going to be attracted by the magnetic field and bind to the sides of the plate. The supernatant is then removed and discarded, leaving only the beads and DNA in the tube. An ethanol wash is performed next by adding 85% ethanol and incubation at room temperature for 2 minutes on the magnetic plate. This wash will insure that all unwanted molecules have been removed. The reaction plate is then air-dried for 10 minutes at room temperature. To finish, water is added to the dried bead to elute purified sequencing product from the magnetic beads (Beckman Coulter).

DNA of each well was analysed by the 3730 DNA analyser from Applied Biosystem. The products are injected into a capillary. Fluorescence from four different dyes that are used to identify the A, C, G and T extension reactions is then detected. Each dye emits light at a different wavelength when excited by an argon ion laser. All four colours and therefore all four bases can be detected and distinguished in a single capillary injection.

2.4.6 Data analysis

The DNA sequencing will give us chromatograms from which the bases in the DNA sequences can be read. The beginning and end of sequences are generally of poor quality and for that reason the data needs to be cleaned prior to analysis. The Sequencer software v5.2.2 was used to clean the obtained chromatograms, using the Trim Ends tool. The sequences generated after this process should have quality rate superior to 80%. The software was then used to align the sequences in order to create contigs, which are groups of overlapping DNA segments that represent a consensus region of DNA.

When the contigs had been created, consensus sequences were generated and entered into the Basic Local Alignment Search Tool (BLAST) in order to find regions of local similarity between sequences. The program compares the nucleotides of the sequences entered to sequence databases and calculates the statistical significance of matches made.

3 Results

3.1 Strain A

3.1.1 Morphological observation

Microscope observations of this strain over the weeks the project took showed it to be coccobacilli. When only few bacteria were present they would be bacilli, but then draw in when more bacteria was present. It was also observed that they tended to stick together and form chains.

3.1.2 Gram staining

Gram staining coloured the bacteria pink which tells us that the bacteria is gram-negative (Fig. 3).

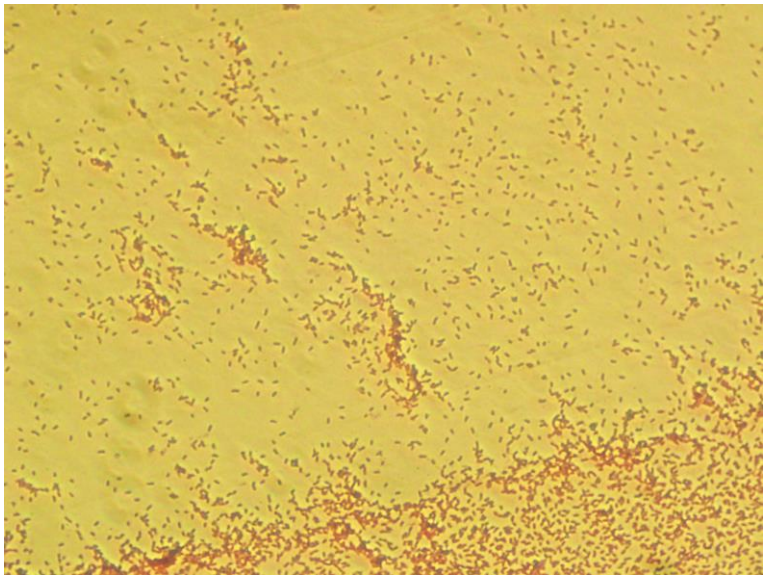

Figure 3: *Gram observation of strain A*

3.1.3 Growth parameters

Cultures of strain A were made in both liquid medium 172 and 514 and tested at 4°C, 10°C and 17°C. The bacteria showed growth at all temperatures after 6 days, but the growth was very weak at 4°C, and better at 10°C and good at 17°C.

The results of temperature measurements done with the BioScreen at 15°C and 20°C can be seen in figure 4. Growth at 20°C in medium 172 was very good, but this strain proved to grow better in medium 172 than in medium 514.

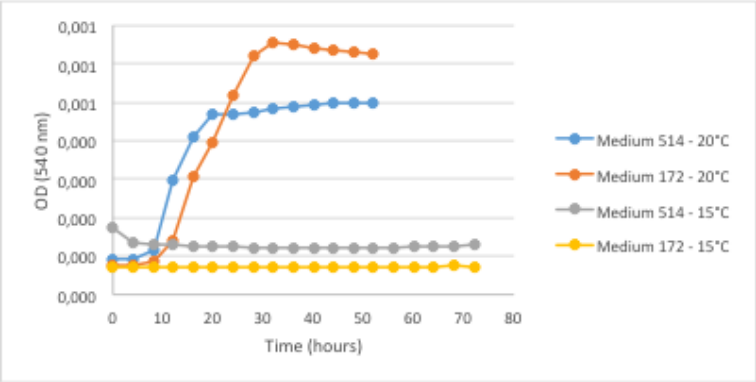

Figure 4: *Growth curves for strain A at different temperatures*

Better growth at 20°C with the medium 172, seems to be faster and better growth in medium 514 but less growth was observed inmedium 172 althought the growth rate was similar to medium 514.

The growth curves of strain A at different pH and 20°C can be seen in figure 5. Growth occurred at all pH except 3.7 but took longer at pH 4.9 and 5,1 than for the higher pH. The optimum pH seems to be around 6.

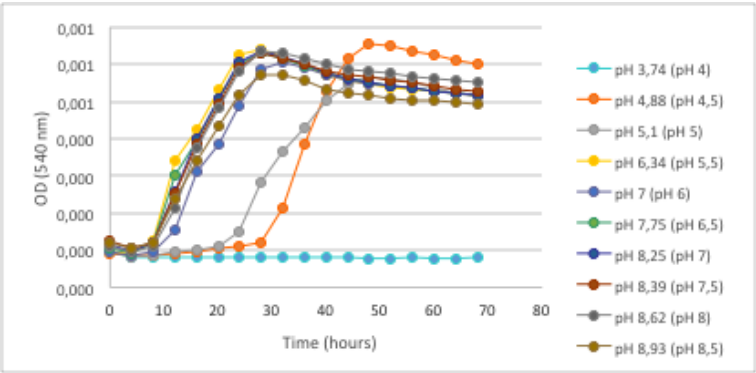

Figure 5: *Growth curves for strain A at different pH and 20°C*

The growth curves of strain A at different NaCl and 20°C can be seen in figure 6. Growth occurred at all concentrations, slower and not as much at 4 and 5% and with an optimum between 0 and 1.

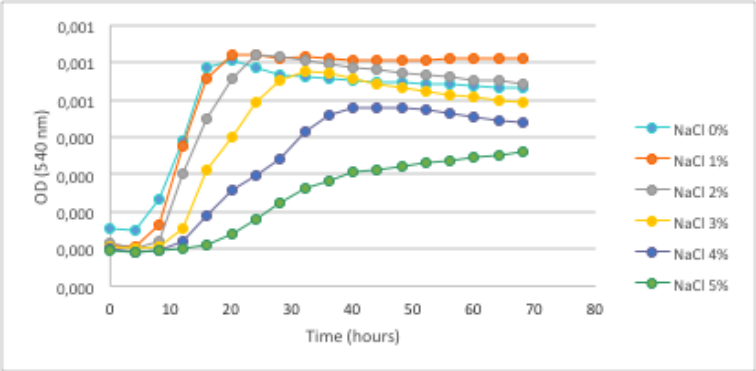

Figure 6: *Growth curves for strain A at different concentrations of NaCl*

Results for carbon and energy sources can be found in Appendix B. Growth in tubes showed only growth with maltose. The BioScreen measurement had to be divided into three parts, due to the number of samples. The results were that strain A showed growth for glycerol, succinic acid, histidine, proline and tyrosine. Antibiotic susceptibility was tested using the BioScreen. Results can be found in figure 7. This strain showed resistance against polymyxin B and kanamycin. The results for kanamycin were however questionable, no growth at 100 µg/mL and 10 µg/mL but growth at 30 µg/mL.

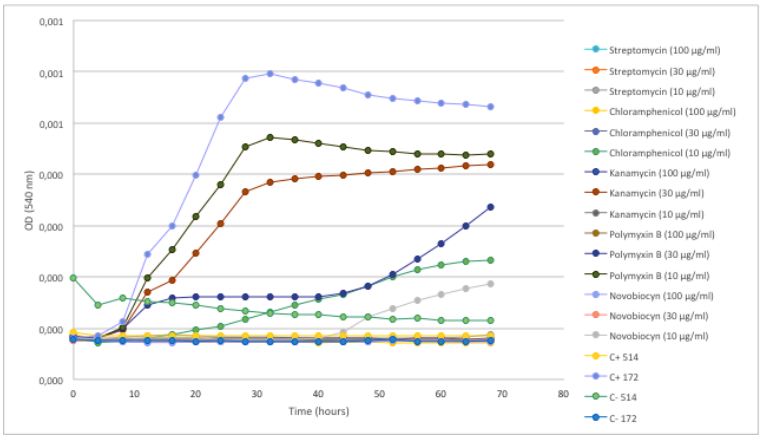

Figure 7: *Antibiotic susceptibilty of strain A measured at 20°C*

The strain showed negative oxidase activity. This tells us that the bacteria do not contain cytochrome c oxidase which means that it either can't use oxygen for energy production with an electron transfer chain or that it employs a different cytochrome for transferring electrons to oxygen.

Catalase test was positive which tells us that the strain has the catalase enzyme.

3.1.4 16S rRNA gene sequencing

The first step of the 16S rRNA sequencing was, as described in chapter 2.4, DNA extraction with Chelex. The purity and concentration of the DNA extract was assessed using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer, the results can be seen in Appendix B. To obtain a sufficient amount of molecules to allow for the sequencing step, the extracted DNA had to be amplified using PCR. The PCR quality was then verified using gel electrophoresis. A picture of the agarose gel after electrophoresis, observed with UV light, can be seen in figure 8.

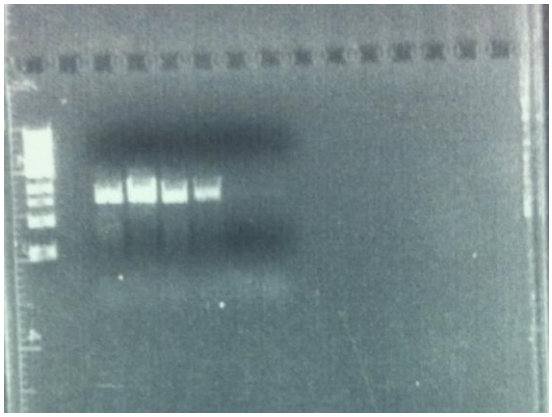

Figure 8 : *Picture of the agarose gel after electrophoresis. The ladder was in well number 1, well number 2 was kept empty to preemt contamination, strain A in well 3, strain B in well 4, strain C in well 5, positive control with DNA template from Campylobacter in well 6 and in wells 7 and 8 negative controls without DNA*

About 493 basepairs of 16S rRNA gen was sequenced. The sequence can be found in Appendix B. Running the sequence through BLAST gave 100% query coverage and 100% identity with the uncultured bacterium clone DFCb31 extracted from artic snow in 2011 (Harding, Jungblut, Lovejoy, & Vincent, 2011).

3.2 Strain B

3.2.1 Morphological observation

Microscope observations of this strain over the weeks the project took showed it to be very thin spirilla that move very fast spinning. Some very long bacteria were observed that might have been a couple of them attaching to each other on the end.

3.2.2 Gram staining

Gram staining coloured the bacteria blue which tells us that the bacteria is gram-positive (Fig. 9).

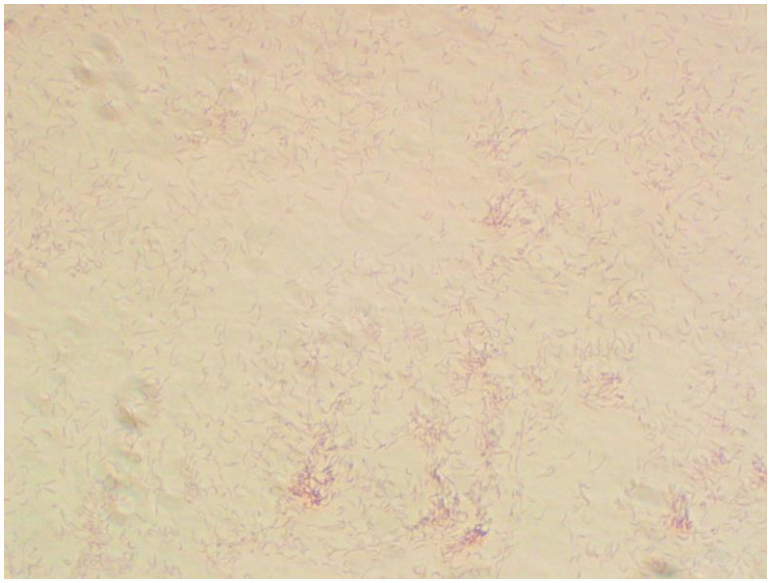

Figure 9: *Gram observation of strain B*

3.2.3 Growth parameters

Cultures of the strain were made in both liquid medium 172 and 514 and tested at both 4°C and 10°C. The bacteria showed growth at both temperatures after 5 days. The growth was very weak at 4°C and better at 10°C. Growth was good at 17°C took less than 3 days.

The results of temperature measurements done with the BioScreen at 15°C and 20°C can be seen in figure 10. Growth at 20°C in medium 514 was very good, but this strain proved to grow better in medium 514 than in medium 172.

Better growth at 20°C with the medium 514, seems to be both faster and better growth in medium 514 than medium 172.

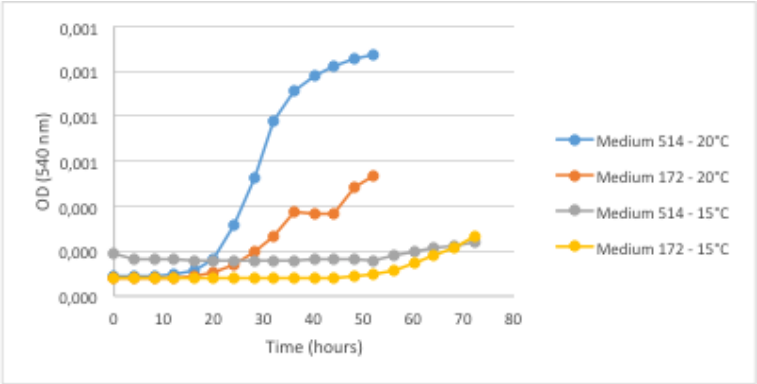

Figure 10: *Growth curves for strain B at different temperatures*

The growth curves of strain B at different pH and 20°C can be seen in figure 11. Growth occurred at pH 6.5 – 8.5 but was not observed at lower pH. The optimum pH seems to be at pH 6.5.

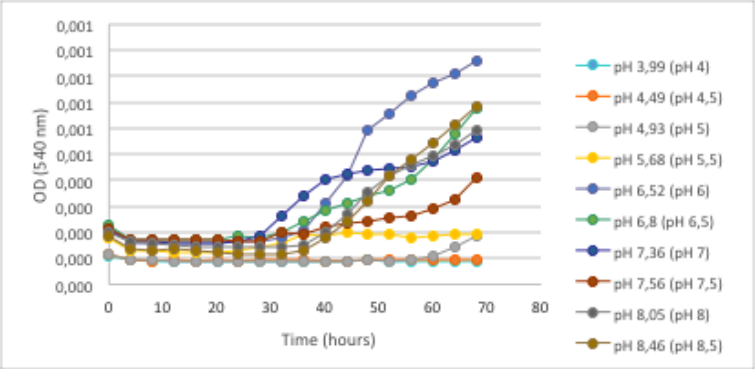

Figure 11: *Growth curves for strain B at different pH and 20°C*

The growth curves of strain B at different NaCl and 20°C can be seen in figure 12. Growth occurred only at 1 and 2% NaCl and was both quicker and with higher rate at 1% than 2%.

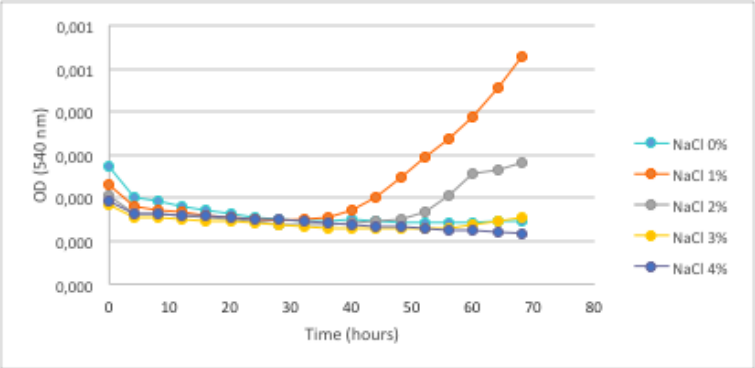

Figure 12: *Growth curves for strain B at different concentrations of NaCl and 20°C*

Results for carbon and energy sources can be found in Appendix B. The BioScreen measurement for carbon and energy sources had to be divided into three parts, due to the number of samples. The results were that strain B showed growth for lactic acid, succinic acid, mannitol and arginine.

Antibiotic susceptibility was tested using the BioScreen. Results can be found in figure 13. No growth was observed, not even for the positive controls.

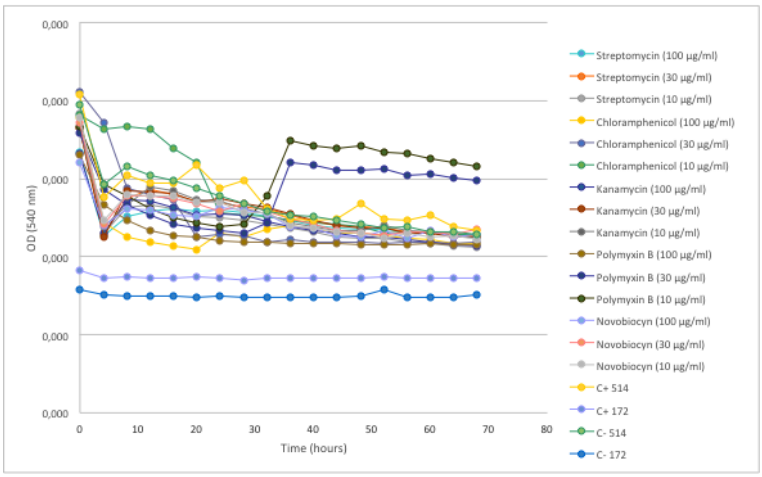

Figure 13: *Antibiotic susceptibilty of strain B measured at 20°C*

The strain showed positive oxidase activity. This tells us that the bacteria contains cytochrome c oxidase and can therefore use oxygen for energy production with an electron transfer chain.

Catalase test was negative which tells us that the strain does not have the catalase enzyme.

3.2.4 16S rRNA gene sequencing

The first step of the 16S rRNA sequencing was, as described in chapter 2.4, DNA extraction with Chelex. The purity and concentration of the DNA extract was assessed using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The results can be seen in Appendix B. To obtain a sufficient amount of molecules to allow for the sequencing step, the extracted DNA had to be amplified using PCR. The PCR quality was then verified using gel electrophoresis. A picture of the agarose gel after electrophoresis, observed with UV light, can be seen in figure 8.

About 580 basepairs of 16S rRNA gen was sequenced.. The sequence can be found in Appendix B. Running the sequence through BLAST gave 100% query coverage and 99% identity to gamma proteobacterium O-011 that has not been published.

3.3 Strain C

3.3.1 Morphological observation

Microscope observations of this strain over the weeks the project took showed it to be cocci with a tendensy to form diplococci. Streptococci, staphylococci and tetrads were also observed.

3.3.2 Gram staining

Gram staining coloured the bacteria pink which tells us that the bacteria is gram-negative (Fig. 14).

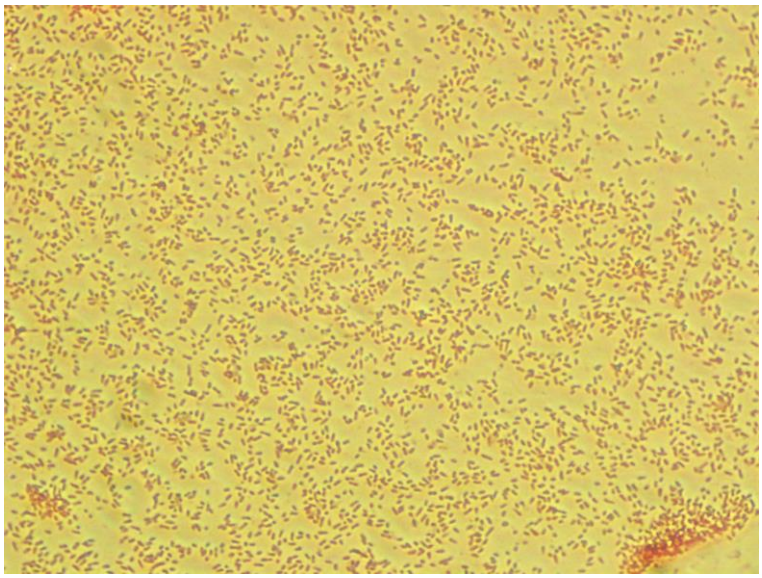

Figure 14 : Gram observation of strain C

3.3.3 Growth parameters

Cultures of the strain were made in both liquid medium 172 and 514 and tested at both 4°C and 10°C. The bacteria showed growth at both temperatures after 5 days but the growth was very weak at 4°C and better at 10°C. Growth at 17°C was good and took less than 3 days.

The results of temperature measurements done with the BioScreen at 15°C and 20°C can be seen in figure 15. This strain had much better growth in medium 514 but less growth was observed in medium 172. Growth was faster at 20°C than at 15°C.

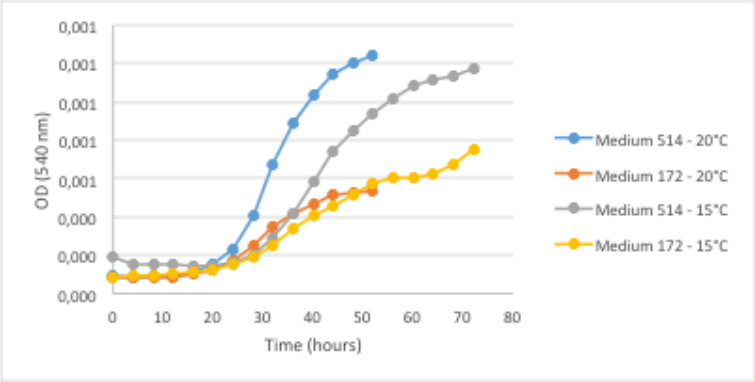

Figure 15: *Growth curves for strain C at different temperatures*

The growth curves of strain C at different pH and 20°C can be seen in figure 16. Growth occurred at pH 6.5 – 8.5 but was not observed at lower pH. The optimum pH seems to be around 7.5. No growth was observed at lower pH.

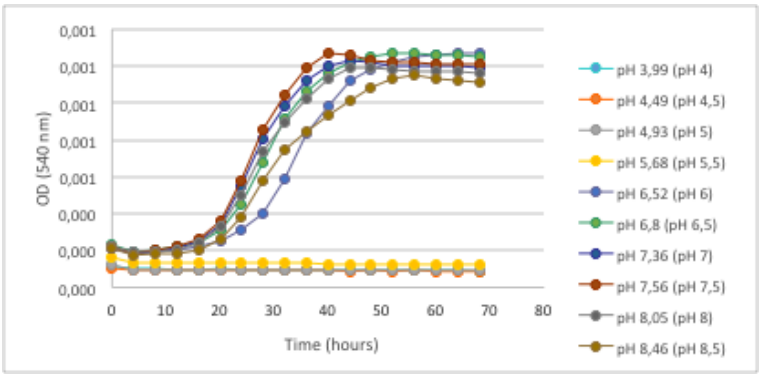

Figure 16: *Growth curves for strain C at different pH and 20°C*

When strain C was incubated at 17°C in medium 514 with 0 and 1% NaCl, and then examined using a microscope, very large, dead cocci were observed. This indicates that the strain needs more salt than 1% to survive and that the cells from the inoculum burst and died due to cytolysis at the lower pH.

The growth curves of strain C at different NaCl and 20°C can be seen in figure 17. Growth occurred at 2, 3 and 4%, slower as the concentration increases, but no growth occurred at lower concentrations.

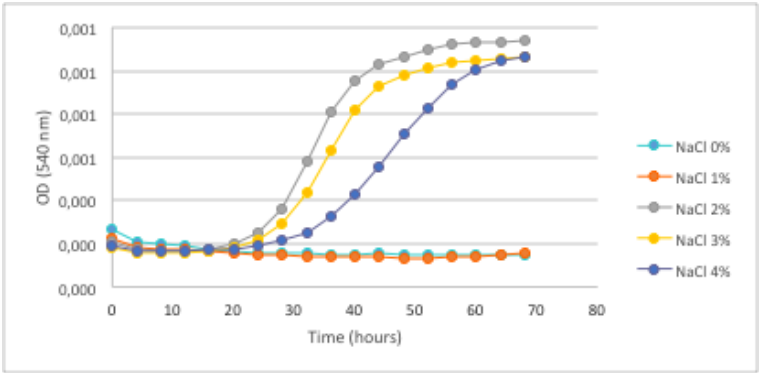

Figure 17*: Growth curves for strain C at different concentrations of NaCl and 20°C*

Results for carbon and energy sources can be found in Appendix B. The BioScreen measurement had to be divided into three parts, due to number of samples. No growth was measured in any of them, except for the positive controls.

Antibiotic susceptibility was tested using the BioScreen. Results can be found in figure 18. The only growth observed was with one of the positive controls.

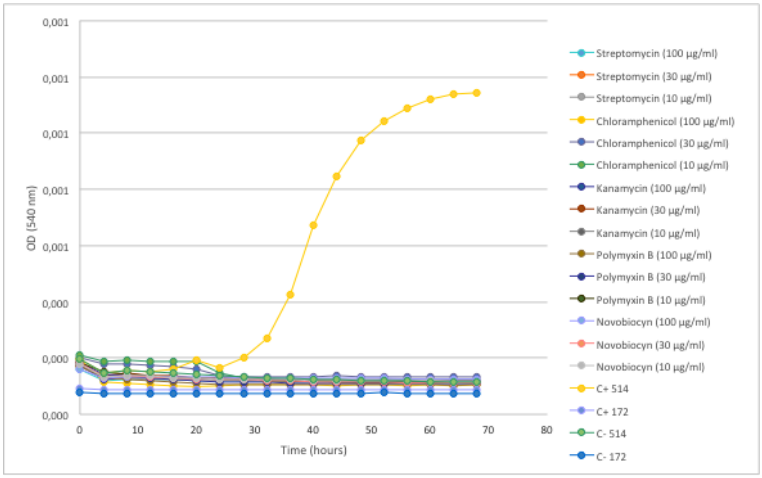

Figure 18: *Antibiotic susceptibilty of strain C measured at 20°C*

The strain showed positive oxidase activity. This tells us that the bacteria contains cytochrome c oxidase and can, therefore, use oxygen for energy production with an electron transfer chain.

Catalase test was negative which tells us that the strain does not have the catalase enzyme.

3.3.4 16S rRNA gene sequencing

The first step of the 16S rRNA sequencing was, as described in chapter 2.4, DNA extraction with Chelex. The purity and concentration of the DNA extract was assessed using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer, the results can be seen in Appendix B. To obtain a sufficient amount of molecules to allow for the sequencing step the extracted DNA had to be amplified using PCR. The PCR quality was then verified using gel electrophoresis. A picture of the agarose gel after electrophoresis, observed with UV light, can be seen in figure 8.

About 420 basepairs of 16S rRNA gen was sequenced. The sequence can be found in Appendix B. Running the sequence through BLAST gave 100% query coverage and 99% identity to marine bacterium AK6_058 that has not been published.

4 Discussions

The aim of this project was to cultivate and characterise three different bacteria strains from Icelandic seawaters that have not been cultivated or characterised before.

From the start, there were troubles with getting the strains to grow and to get them to keep on growing, for example strain A had a tendency not to make it to the stationary phase at all, growth stopped abruptly during the exponential phase. This sudden death of strain A indicates that the strain may be infected with a virus that kills the strain before it reaches stationary phase. The infection has made this strain very hard to cultivate. It has been estimated that viruses are responsible for the death of approximately 20% of the prokaryotic biomass in marine ecosystems per day (Bonin & Faure, 2011) so the fact that one of the strain seems to be infected with a virus is something that could be expected.

Another problem encountered was that strain B turned out to have longer growth time than predicted so in the end there was not enough time to do all the measurements that was foreseen in the beginning. This long growth time is possible connected to the fact that strain B was isolated from a sample collected at 1000 m depth at a location far in the north (fig. 2). The strain that showed the best growth and least troubles while growing was strain C which was collected at 30 m.

The part of the project that was less tested was growth measurements at different temperatures. In all steps when the BioScreen was used, all measurements turned out to take longer time than excepted and the measurements stood over much longer time than expected. That is the main reason for why we only have growth data at 15 and 20°C but not at other temperatures. Growth in tubes at 4, 10, 17 and 22°C was observed successfully and the growth seemed to be faster at higher temperatures. The fact that all of the strains seem to prefer high temperature came as a surprise given the fact that Icelandic sea water has a temperature between 4 and 10°C. Further measurements are needed to find the complete temperature range growth can take place at.

The results of measurements at different pH showed that the optimum for all strains was around 6.5-7.5. They all shared the trait to be able to adapt to higher pH rather than lower but out of the three strains it was strain B that was most sensitive to changes in acidity. This could suggest that the acidification of the ocean and global warming could bring some threats towards marine microbial biodiversity. The acidification of the ocean could possibly cause the death of a lot of prokaryotic strains not able to adapt to lower pH.

The optimum salinity of the strains varied. Strain A had optimum between 0 and 1% and could grow at higher concentrations, although slower and not as well. Strain B grew very well at 1% NaCl, a little bit and slow at 2% but did not grow at all at other concentrations. Strain C did not grow at concentration lower than 2% where it has it's optimum. It's growth went down with increase in concentration. Those findings are a

little surprising given that the salinity of sea water is usually around 3.5% so it was anticipated that the strains would like higher salinity.

The results for growth with different carbon and energy sources were inconclusive. There were troubles with the growth and the measurements turned out to take longer than expected so there was not time to repeat the trial when it was evident that growth had not occurred.

The results for antibiotic susceptibility were inconclusive. There were problems with growth of the positive controls but due to lack of time they have yet to be replicated and confirmed.

When the DNA was sequenced it was first done using only primers 9F and R1544. The results of the first sequencing were inconclusive, most likely due to the age of the primers. The sequencing was repeated with new primers and the results described in chapters 3.1.4, 3.2.4 and 3.3.4 were obtained in that step. Those results are only for a part of the 16S rRNA, not the whole gene. To get the whole sequence, 9 different primers, including the two mentioned earlier were used, but due to technical problems and lack of time the samples were sent for sequencing too late and the results did not make it back in time before this thesis was written. We found that strain A is closely related to uncultured bacterium clone DFCb31, strain B to gamma proteobaceterium O-011 and strain C to marine bacteriuem AK6_058. These OTUs have never been described before as strains and exist only as clones or uncultured bacterium in the data banks. Their DNA have been found in cold sea waters, so to find them in Icelandic sea waters is not surprising (Harding, Jungblut, Lovejoy, & Vincent, 2011).

Studies on those strains need to be continued to confirm the results and to finish both the characterisation and identification. The DNA sequencing is ongoing and although the results were not finalised before the writing of this thesis this study will be continued as it is one of the first studies about the microbial diversity in Icelandic sea waters.

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Appendix A

Medium 172: *Cytophaga* marine medium

Yeast extract (Difco)	1.0g	Yeast Extract is the water soluble portion of autolyzed yeast containing vitamin B complex. Yeast Extract is an excellent stimulator of bacterial growth and used in culture media. Yeast Extract also provides vitamins, nitrogen, amino acids, and carbon.
Tryptone (Difco)	1.0g	Tryptone is an enzymatic digest of casein used as a nitrogen source in culture media. Casein is the main protein of milk, and a rich source of amino-acid nitrogen.
NaCl	24.7g	Provides salty environments in case there are halophiles bacteria
KCl	0.7g	Source of potassium
$MgSO_4$ x 7 H_2O	6.3g	Source of magnesium and sulphur
$CaCl_2$ x 2 H_2O	1.2g	Source of calcium
$MgCl_2$ x 6 H_2O	4.6g	Source of magnesium and chlorure
$NaHCO_3$ (sodium bicarbonate)	0.2g	Source of sodium and carbonate
This is a media culture for *Cytophaga* bacteria means which are extremely heterotrophic. These bacteria are able to use agarose, cellulose and also chitine as carbon source.		

Medium 514: Bacto marine broth

Peptone	5.0g	Pancreatic Digest of Casein is enzymatically hydrolysed casein that provides a complex nitrogen source in microbiological culture media. Casein is a rich source of amino acid nitrogen. This product is used to support the growth of fastidious microorganisms, and has a high tryptophan content
Yeast extract (Difco)	1.0g	Yeast Extract is the water soluble portion of autolysed yeast containing vitamin B complex. Yeast Extract is an excellent stimulator of bacterial growth and used in culture media. Yeast Extract also provides vitamins, nitrogen, amino acids, and carbon
Fe(III)citrate	0.10g	Iron source
NaCl	19.45g	Provides salty environments in case there are halophiles bacteria
$MgCl_2$	5.90g	Magnesium source
Na_2SO_4	3.24g	Sodium and Sulfur source
$CaCl_2$	1.80g	Calcium and Chlorine source
KCl	0.55g	Potassium and Chlorine source
Na_2CO_3	0.16g	Sodium and Carbonate source
NaBr	0.08g	Sodium and Bromine source
Na_2HPO_4	8.0mg	Sodium and Phosphate source
Distilled Water	1000.0mL	Liquid used to mix all components and also as life liquid

Artificial seawater

NaCl	23.6 g
KCl	0.64 g
$MgCl_2 \bullet 6H_2O$	4.53 g
$MgSO_4 \bullet 7H_2O$	5.94 g
$CaCl_2 \bullet 2H_2O$	1.3 g
In 1 liter of distilled water	

Basal medium

Tris/HCl 1M (ph 7,5)	50 mL
NH_4Cl	1 g
$K_2HPO_4 \bullet 3H_2O$	0.075 g
$FeSO_4 \bullet 7H_2O$	0.028 g
$NaNO_3$	0.02 g
Yeast extract	0.002 g
In 950 mL of artificial seawater	

Add after steriliztaion:

2 mL of Hutner's mineral base (Cohen-Bazire, Sistrom, & Stanier, 1957)

1 mL of vitamin solution (Staley, 1968)

Sole carbon and energy sources

Complete list of carbon and energy source solutions made. 10 mL were made of each solution, all solutions sterilized with filtration.

Sugars	**Ci**
L-arabinose	100 mM
D-cellobiose	10 g/L
Cellulose	10 g/L
D-fructose	10 g/L
D-glucose	10 g/L
D-lactose, monohydrate	10 g/L
Maltose, monohydrat	100 mM
D-mannose	100 mM
Melibiose	10 g/L
L-rhamnose, monohydrate	100 mM
D-ribose, minimum	100 mM
Sucrose	100 mM
D-trehalose, dihydrate	100 mM
D-xylose	100 mM

Organic acids:	**Ci**
Acetic acid	100 mM
Citric acid, monohydrate	10 mM
L-lactic acid, sodium salt	10 mM
Maleic acid	10 mM
Succinic acid, calcium salt,	10 mM

monohydrate	
L-tartaric acid, disodium salt, dihydrate	10 mM

Alcohols:	**Ci**
Ethanol	10% w:v
Glycerol	10% w:v
D-mannitol	10% w:v
2-propanol	10% w:v
D-sorbitol	10% w:v

Amino acids:	**Ci**
DL-alanine	10 mM
L-arginine	10 mM
L-cysteine, hydrocloride	10 mM
L-glutamine	10 mM
Glycine	10 mM
L-histidine	10 mM
L-isoleucine	10 mM
L-leucine	10 mM
DL-lysine, monohydrochloride	10 mM
L-phenylalanine	10 mM
L-proline	10 mM
L-serine	10 mM
L-Threonine	10 mM
L-tryptophan	10 mM
L-tyrosine	10 mM
L-valine	10 mM

Proteins:	**Ci**
Starch	10 g/L

Appendix B

Growth parameters

Table 10: *Results for growth with different carbon and energy sources in tubes*

Sugars	**Ci**	**Strain A**	**Strain B**	**Strain C**
L-arabinose	100 mM	-	++	+++
D-cellobiose	10 g/L	-	+++	+++
Cellulose	10 g/L	-	-	+++
D-fructose	10 g/L	-	++	+++
D-glucose	10 g/L	-	+++	+++
D-lactose, monohydrate	10 g/L	-	+++	+++
Maltose, monohydrat	100 mM		++	+++
D-mannose	100 mM	-	-	++
Melibiose	10 g/L	-	+	++
L-rhamnose, monohydrate	100 mM	-	++	+++
D-ribose, minimum	100 mM	-	+	++
Sucrose	100 mM	-	+++	+++
D-trehalose, dihydrate	100 mM	-	+	++
D-xylose	100 mM	-	+++	+++

Organic acids:	**Ci**	**Strain A**	**Strain B**	**Strain C**
Acetic acid	100 mM	-	-	++
Citric acid, monohydrate	10 mM	-	-	-
L-lactic acid, sodium salt	10 mM	-	++	+++
Maleic acid	10 mM	-	-	-
Succinic acid, calcium salt, monohydrate	10 mM	-	+	++
L-tartaric acid, disodium salt, dihydrate	10 mM	-	++	+

Alcohols:	**Ci**	**Strain A**	**Strain B**	**Strain C**
Ethanol	10% w:v	-	-	+
Glycerol	10% w:v	-	+	++
D-mannitol	10% w:v	-	+	++
2-propanol	10% w:v	-	++	+
D-sorbitol	10% w:v	-	+++	++

Amino acids:	**Ci**	**Strain A**	**Strain B**	**Strain C**
DL-alanine	10 mM	-	+	++
L-arginine	10 mM	-	+	+++
L-cysteine, hydrocloride	10 mM	-	-	+
L-glutamine	10 mM	-	+	++
Glycine	10 mM	-	+	++
L-histidine	10 mM	-	-	+++
L-isoleucine	10 mM	-	+	++

L-leucine	10 mM	-	++	++
DL-lysine, monohydrochloride	10 mM	-	+	+
L-phenylalanine	10 mM	-	++	+
L-proline	10 mM	-	++	+++
L-serine	10 mM	-	+	++
L-Threonine	10 mM	-	-	+
L-tryptophan	10 mM	-	+	+
L-tyrosine	10 mM	-	++	++
L-valine	10 mM	-		+

Proteins:	**Ci**	**Strain A**	**Strain B**	**Strain C**
Starch	10 g/L	-	+++	++

Growth with different carbon and energy sources, BioScreen results, strain A

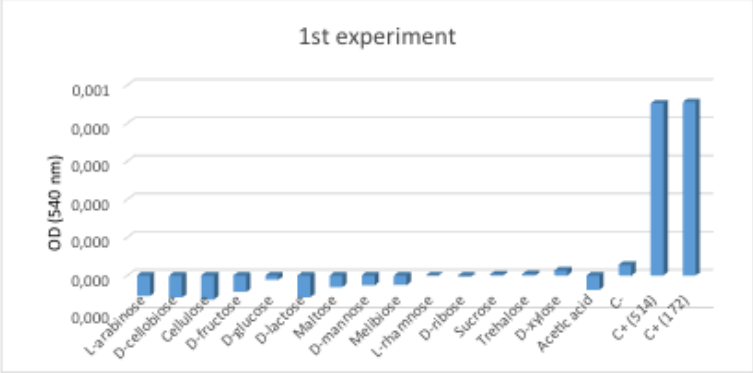

Figure 19: *Growth with different carbon and energy sources for strain A*

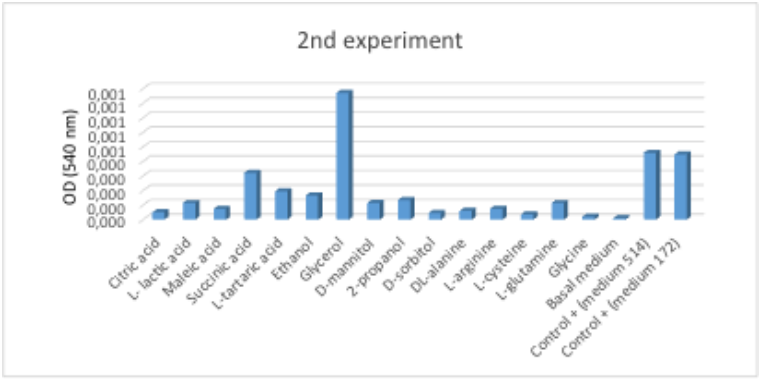

Figure 20: *Growth with different carbon and energy sources for strain A*

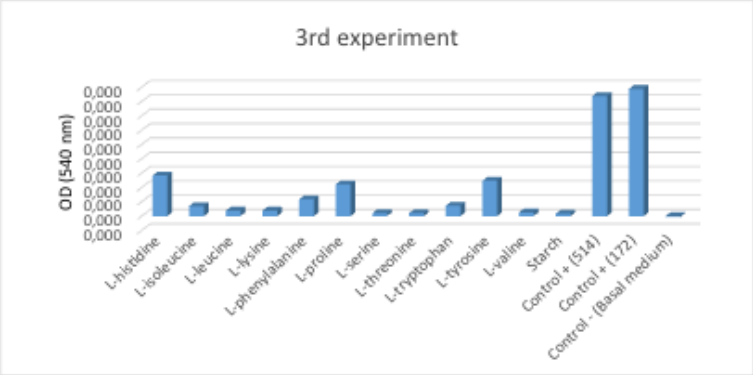

Figure 21: *Growth with different carbon and energy sources for strain A*

Growth with different carbon and energy sources, BioScreen results, strain B

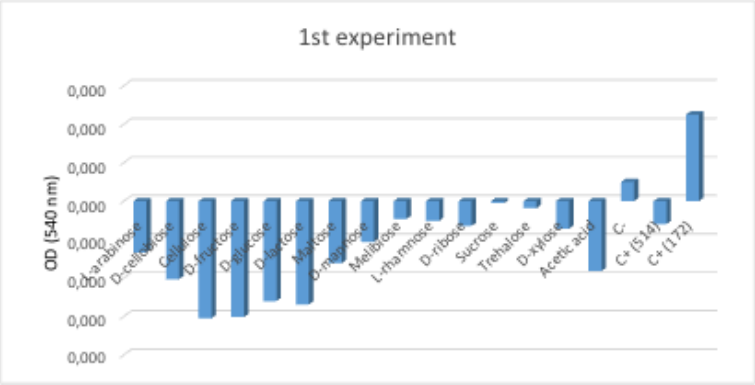

Figure 22: *Growth with different carbon and energy sources for strain B*

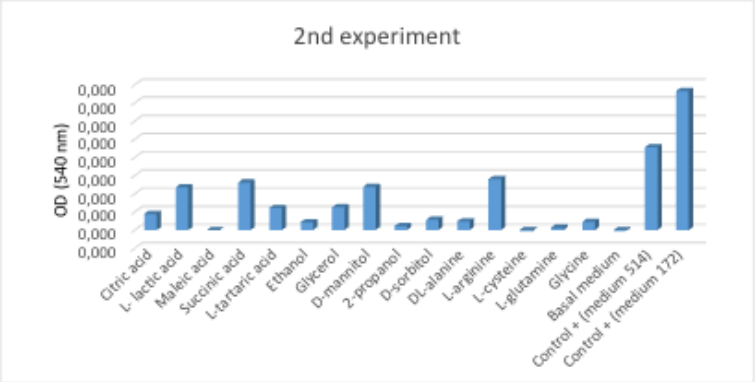

Figure 23: *Growth with different carbon and energy sources for strain B*

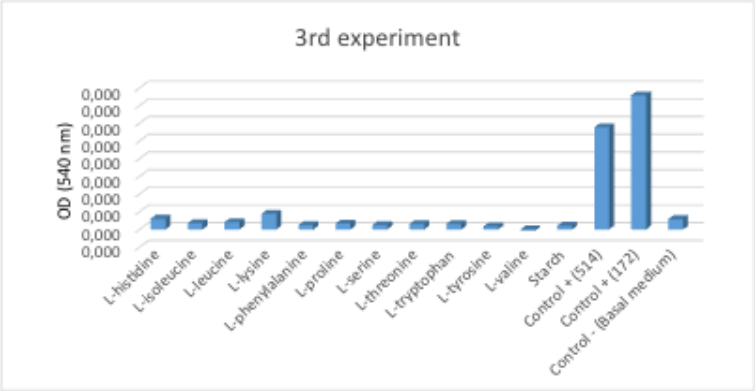

Figure 24: *Growth with different carbon and energy sources for strain B*

Growth with different carbon and energy sources, BioScreen results, strain C

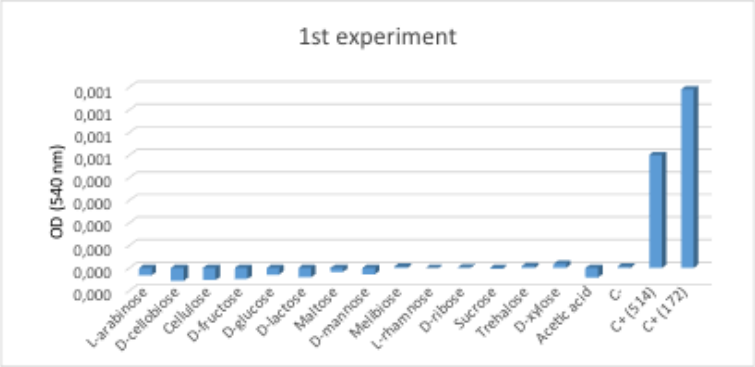

Figure 25: *Growth with different carbon and energy sources for strain C*

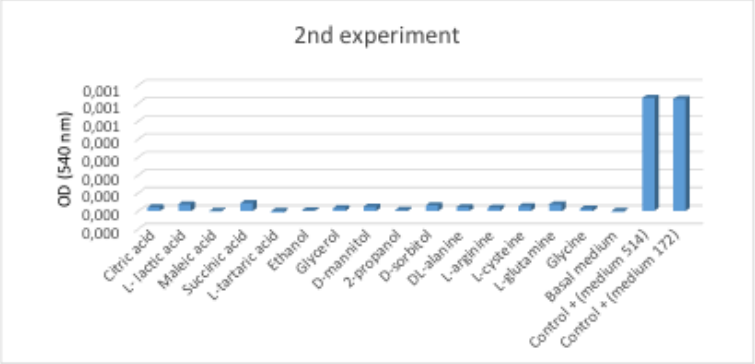

Figure 26: *Growth with different carbon and energy sources for strain C*

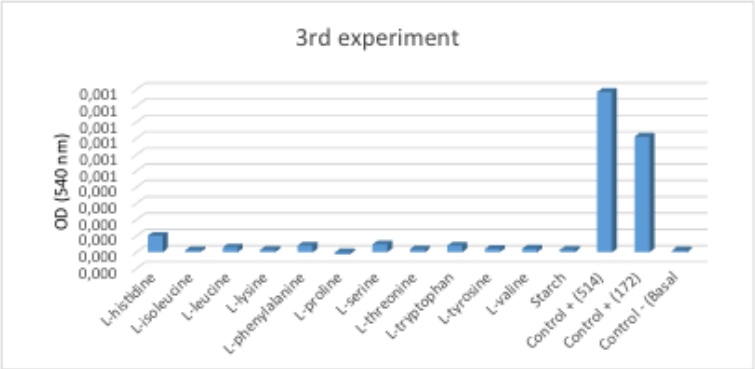

Figure 27: *Growth with different carbon and energy sources for strain C*

Nano Drop

Sample ID	ng/µL	A260	A280	260/280	260/230	Cursor abs.
Control	-1,38	-0,028	-0,020	1,35	0,57	-0,049
Strain A	36,74	0,735	0,346	2,12	0,86	0,858
Strain B	41,36	0,827	0,428	1,93	0,76	1,082
Strain C	23,53	0,471	0,233	2,02	0,99	0,473

DNA sequences

Strain A

CTGATGTTCCTTCCAATCTCTACGCATTTCACCGCTACACTGGAAATTCCA
CTACCCTCTACAAAACTCTAGTTTGCCAGTTCAAAATGCAGTTCCCAGGTT
GAGCCCAGGGCTTTCACATCTTGCTTAACAAACCACCTACGCACGCTTTAC
GCCCAGTAATTCCGATTAACGCTTGCACCCCTCGTATTACCGCGGCTGCTG
GCACGAAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCACAGCTAGTGGC
TATTAACCACTAACCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGG
CCTTCTTCACACACGCGGCATGGCTGCATCAGGCTTTCGCCCATTGTGCAA
TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAG
TGTGGCTGATCATCCTCTCAAACCAGCTAGAGATCGTCGCCTAGGTGAGC
TCTTACCTCACCTACTAGCTAATCTCACTTGGGCTA

Strain B

TCAGCGTCAGTATCAGCCCAGCAAGTCGCCTTCGCCACTGGTGTTCCTTCA
GATCTCTACGCATTTCACCGCTACACCTGAAATTCCACTTGCCTCTGCTGT
ACTCTAGTTCACCAGTTTTGTATGCAGTTCCCAGGTTGAGCCCAGGGCTTT
CACATCCAACTTAACAAACCGCCTACGCGCGCTTTACGCCCAGTAATTCC
GATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG

CCGGTGCTTCTTCTGCGAGTAACGTCACAGCTGATGGGTATTAACCATCAA
CCTTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACA
CGCGGTATGGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCCCCACTGC
TGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCAT
CCTCTCAGACCAGCTATGGATCGTCGCCTTGGTGAGCTCTTACCTCACCAA
CTAGCTAATCCAACGCAGGCTCATCCTGTAGCGTGAGCCGAAGCCCACTT
TCCTCCGTAGAGATTATTCGGT

Strain C

CATATCTACGCATTTCACTGCTACACGCGGAATTCCATCCCCCTCTACCGT
ACTCTAGCTATGCAGTCACAAATGCAGTTCCCAGGTTGAGCCCGGGGATT
TCACATCTGTCTTACATAACCGCCTGCGCACGCTTTACGCCCAGTAATTCC
GATTAACGCTTGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGGTGCTTATTCTTACGGTACCGTCATGAGACTCTCGTATTAGGAAAAGCT
TTTTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCATCCTGCAC
GCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCACTGCT
GCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGGTCGTC
CTCTCAGACCAGC