Jessica Hoffman

April 2012

The Identification of *Aeromonas hydrophila* subsp. *ranae* and *Bacillus amyloliquefaciens*

Abstract:

The organism UK-JAH, which was isolated from Loyalsock Creek near Montoursville, was identified as *Aeromonas hydrophila* subsp*. ranae*, a bacterial organism that was found to be pathogenic to some cold-blooded organisms, including frogs. The identity of the organism *Bacillus amyloliquefaciens KLH* was confirmed. The identifications were done using a combination of phenotypic and biochemical tests, 16S rRNA sequencing, Biolog Gen III plates, and MIDI/FAME analysis.

Introduction:

Bacterial species are everywhere. Humans use them for many reasons, such as for food and antibiotic testing. They can be harmful, sometimes causing infectious diseases that can be debilitating or even fatal if left untreated. It is important to identify organisms so they can be utilized in the most useful and efficient manner. For infectious bacteria, identification of organisms can be especially important for the treatment of diseases and the prevention of future contraction. Many different methods of identification exist, such as API tests, Biolog tests, MIDI/FAME analysis, and 16S rRNA sequencing.

API tests are used in clinical settings for the purpose of identifying infectious organisms. To perform an API test, API strips, each containing cupules with dried medium, are inoculated with the organism to be tested. After incubation, the results are compared to a database for identification. This is a good and fairly easy test for a clinical setting, but since the database contains mostly organisms found in clinical settings, the tests are not as useful for identifying organisms from the environment (Analytical Profile Index).

Biolog tests, using Gen III plates and Omnilog software, can perform several different phenotype tests at once, including the ability to utilize different media, optimum pH growth, osmotic properties, and sensitivity to chemical agents. The results are compared to a database of known organisms. Biolog tests are good for gathering a large amount of data in a short amount of time, however they are expensive and the database is not comprehensive (Biolog).

MIDI/FAME analysis determines the fatty acid composition of an organism through gas chromatography. This is done by adding reagents to the organisms in vials to prepare them and placing them in the gas chromatograph. The results are compared to a database. While this is cheaper than the Biolog tests, the database is also not comprehensive and the tests do not tell much about the phenotypic characteristics of the organisms (Sherlock).

In 16S rRNA gene sequencing, polymerase chain reaction is used to amplify the 16S rRNA gene of the organism to be sequenced. The concentration of the PCR product is determined by gel electrophoresis, and then the product is sent out to be sequenced. The resulting sequence is then compared to a database of known 16S rRNA sequences to identify the organism based on similarity of the sequences. This method takes longer than the others, but the database is more comprehensive.

Identification can also be done using different media plates and tubes to test phenotypic characteristics. This method is not as quick and efficient and leaves much room for error.

In identifying UK-JAH and confirming the identity of *Bacillus amyloliquefaciens KLH* a combination of these methods were used. Overall it was determined that UK-JAH was the organism *Aeromonas Hydrophila* subsp. *ranae*, and *KLH* was confirmed to be the organism *B. amyloliquefaciens.*

Methods:

UK-JAH was isolated from a sediment sample from Loyalsock creek in December 2011. The organism was inoculated onto R2A media plates along with the known organism *Bacillus amyloliquefaciens KLH*, and both were incubated at 30°C. They were also inoculated as liquid cultures in R2A medium.

Wet mounts were prepared in order to view the organisms with the microscope. This was done from a liquid culture to promote motility. A gram stain was then performed to determine whether or not the cells had a thick peptidoglycan cell wall. For the gram stain, the cells were put on a slide from a liquid culture and then heat-fixed to the slide and stained.

The organisms were then streaked onto several different plates. One was put in a GasPak to determine oxygen requirements. Others were incubated at different temperatures (4°C, 20°C, 30°C, 37°C, and 44°C) to determine optimum growth temperature. The Kirby-Bauer test was used to determine antibiotic sensitivity. Filter paper disks were placed onto the plates containing the organisms, and a small amount of antibiotic was placed on each disk. After the organisms were allowed to grow at 30°C, the zones of inhibition around the filter paper disks were measured in millimeters.

The organisms were tested, using tubes of phenol red broth with durham tubes inside, for the ability to utilize carbohydrates for fermentation to produce acids and gases. The carbohydrates tested were glucose, lactose, sucrose, mannitol, galactose, and salicin. If the red media turned yellow, then acid was produced, and if there was a bubble in the durham tube, then gas was produced. Methyl Red-Vogues-Proskauer (MR-VP) tests were performed to determine the pH after fermentation and whether

alcohols were produced during fermentation. Litmus milk tests were performed to detect the products of lactose and casein digestion. The organisms were also tested to see if they had the ability to metabolize citrate, produce the enzyme urease, and reduce nitrate. A SIM test was used to see if the organisms could produce the enzymes cysteine desulfhydrase and tryptophanase, and to check for motility.

The organisms were spread onto agar plates containing different nutrients to test their ability to produce the exoenzymes required to hydrolyze those nutrients. They were tested for amylase, caseinase, DNase, gelatinase, and tween hydrolysis. For the amylase plate, to see if the organism broke down the starch, iodine, which stains starch, was poured onto the plate. For the DNase plate, the plate was flooded with HCl, which causes the medium to turn cloudy if DNA is present. The gelatinase tests were done in test tubes. After incubation at 35°C, the tubes were placed in an ice bath to see if the medium would solidify, indicating that the gelatinase is not present. The organisms were also tested on several differential and selective media. Bile esculin medium was used to test the ability of the organisms to hydrolyze esculin and for resistance to bile. Brilliant green agar was used to select for salmonella. Eosin methylene blue medium was used to detect coliform bacteria. Hektoen enteric agar was used to select for some gram-negative organisms. MacConkey Agar was used to select for gram-negative organisms. Mannitol salt agar was used to determine whether the organisms could grow in high salt concentrations. Phenylethyl alcohol agar selected for gram-positive organisms.

The Polymerase Chain Reaction (PCR) was used to amplify the 16S rRNA gene for sequencing. To prepare the PCR, the organisms were inoculated into 100 µL of

deionized water and passed through two freeze-thaw cycles. Then, one µL of the frozen and thawed cells were put into thin-wall PCR tubes and mixed with 12.5 µL of 2X ExTaq Premix, which contained taq polymerase, buffers, and dNTPs, and 11.5 µL of 2X primer which contained 27f primer, 1492r primer, and dH_2O. The 27f and 1492r primers are universal primers that are designed to attach to the beginning and end of the 16S rRNA sequence in order to amplify the entire gene. Mineral oil was added to the top to prevent evaporation. The PCR tubes were then run through the thermal cycler to complete the reaction. To quantify the PCR products, gel electrophoresis was performed. The samples of DNA were diluted to 20 ng per µL and sent to Beckman-Coulter for sequencing of the 16s rRNA gene using the Sanger method.

The sequences were analyzed using the programs, EzTaxon and BLAST. Each program compared the sequences to a database of known organisms' sequences and gave the best matches for the sequences inputted. These programs were used to help identify UK-JAH and to confirm the identity of *B. amyloliquefaciens*.

A Biolog test was also performed to help identify UK-JAH. The organism was spread onto a Biolog Universal Growth + Blood agar plate, kept at 4°C to inhibit growth, and then put in the incubator. After being incubated for one night, cells from the plate were inoculated into a screw-cap tube of inoculating fluid until the percent transmittance of the tube in the turbidometer was between 90 and 98%. Then, with a multi-channel pipettor, 100 µL of the inoculated fluid was pipetted into each of the ninety-six wells of the Gen III plate. The plates were then placed in the Omnilog to collect the data.

Fatty acid methyl ester (FAME) analysis was also used to help identify the organisms by determining what type of fatty acids they contained and then comparing

that list to a database. The instant method was used to prepare them, and then they were placed in the gas chromatograph.

Results:

On the initial streak plate, *Bacillus amyloliquefaciens KLH* was a beige color and formed flat, irregularly shaped, and dry colonies, the largest being about 6.7 mm in diameter. UK-JAH was also a beige color and formed flat, circular, and normally textured colonies, the largest being approximately 4.5 mm across. These results are shown in Figure 1.

Under the microscope, the *KLH* cells appeared as long, thin rods. Some formed chains while many were single cells. No motility was detected. After the gram stain, the cells appeared purple, which indicates that *KLH* is gram-positive and has a thick peptidoglycan cell wall. The UK-JAH cells were short rods that appeared to clump together. After the gram stain, the cells were pink, meaning that UK-JAH is gram-negative and does not have a thick peptidoglycan cell wall. Once again, no motility was detected, and the endospore stain was negative. These results are shown in Figure 2.

Neither *KLH* nor UK-JAH grew in the GasPak, meaining both are obligate aerobes. Both organisms produced bubbles when added to hydrogen peroxide, indicating the presence of the enzyme catalase. When oxidase reagent was added to the organisms, *KLH* produced a weak purple color, which is a weak positive for the enzyme oxidase, while UK-JAH produced a stronger purple color, indicating a strong positive for oxidase. These results are shown in Figure 3.

KLH showed optimum growth at 37°C. Growth also occurred at 30°C and 44°C, with weak growth at 20°C and no growth at 4°C. UK-JAH showed optimum growth at 20°C, with growth also at 30°C and 37°C. Weak growth occurred at 4°C, while no growth occurred at 44°C. These results are shown in Figure 4.

For the antibiotic testing, the zones of inhibition for *KLH* were as follows: 0.0 mm for ampicillin, 52 mm for carbenicillin, 48 mm for chloramphenicol, 60 mm for chlortetracycline, 36 mm for erythromycin, 20 mm for kanamycin, 46 mm for nalidixic acid, 50 mm for penicillin, 28 mm for rifampicin, 0.0 mm for spectinomycin, 24 mm for streptomycin, and 52 mm for tetracycline. These results show that *KLH* is sensitive to all of the antibiotics tested, except ampicillin and spectinomycin. The zones of inhibition for UK-JAH were as follows: 0.0 mm for ampicillin, 0.0 mm for carbenicillin, 42 mm for chloramphenicol, 24 mm for chlortetracycline, 14 mm for erythromycin, 18 mm for kanamycin, 0.0 mm for nalidixic acid, 0.0 mm for penicillin, 30 mm for rifampicin, 12 mm spectinomycin, 10 mm for streptomycin, and 20 mm for tetracycline. These results show that UK-JAH is sensitive to all antibiotics tested except ampicillin, carbenicillin, nalidixic acid, and penicillin. However, some of the zones of inhibition were small, showing less sensitivity to those antibiotics than if the zones of inhibition were larger. These results are shown in Figure 5.

For the carbon metabolism tests, *KLH* was able to metabolize all carbon sources tested to produce acids, indicated by the yellow color of the phenol red broth. Lactose and galactose showed especially strong positives, while the yellow colorings for glucose, sucrose, mannitol, and salicin were slightly weaker. No gases were produced. UK-JAH only produced acid for lactose, while the rest of the tubes kept their red color, indicating a negative result for acid production. *KLH* did not turn red when methyl-red indicator was added, showing a negative result for the methyl red test, while UK-JAH did turn red, which indicates a positive result. *KLH* was positive for the Vogues-Proskauer test, as the medium turned pink, indicating the presence of

acetylmethylcarbinol after fermentation. UK-JAH did not turn pink, so the result was negative. Neither *KLH* nor UK-JAH showed change in the litmus milk medium, meaning that neither organism produced detectable acid products from lactose fermentation or alkaline products of casein digestion, and neither formed curd. For the SIM test, neither *KLH* nor UK-JAH turned black, meaning that neither organism produces the enzyme cysteine desulfhydrase. Also, neither turned red after adding Ehrlich's aldehyde, indicating that neither organism was capable of producing the enzyme tryptophanase. Motility was detected in UK-JAH, as the cloudiness in the medium was extending away from the original location of the organisms. For the urease test, both organisms remained an orange color, indicating that the enzyme urease was not produced. Both organisms showed a positive result for the nitrate reductase test, meaning both are capable of reducing nitrate to nitrite. These results are shown in Figure 6.

KLH showed a positive result for the exoenzymes amylase and caseinase, while showing negative results for DNase, gelatinase, and tween hydrolysis. UK-JAH was positive for all of the exoenzymes. These results are shown in Figure 7.

For both *KLH* and UK-JAH, there was weak growth on the EG minimal medium. UK-JAH grew on the bile esculin medium and turned the medium a black color. It also showed weak growth on the eosin methylene blue medium, but with no color changes of the colonies. UK-JAH showed growth on hektoen enteric agar with orange colonies and turned the agar bright pink. It also grew on the Macconkey agar. UK-JAH grew on neither the brilliant green agar, meaning the organism is likely not salmonella, nor the mannitol salt agar, meaning the organism is not halophilic. UK-JAH also did not show growth on the phenylethyl alcohol agar. *KLH* showed no growth on the bile esculin

medium, the brilliant green agar, esosin methylene blue medium, hektoen enteric agar, Macconkey agar, mannitol salt agar, and phenylethyl alcohol agar. These results are shown in Figure 8.

The PCR produced good results. After gel electrophoresis, the concentration of DNA for *KLH* was approximately 60 ng/µL. The concentration of UK-JAH was about 200 ng/µL. The gel electrophoresis photo is shown in Figure 9. The sequences for *KLH* and UK-JAH are shown in Figure 10 and Figure 13, respectively.

When the sequences were compared to the EzTaxon database, the best match for *KLH* in EzTaxon was *Bacillus amyloliquefaciens subspecies plantarum FZB42*, with a pairwise similarity score of 98.705%. According to BLAST, the 16S rRNA sequences of *KLH* and *B. amyloliquefaciens subsp. plantarum FZB42* were 99% similar with 687 out of 695 base pairs matching. The EzTaxon screenshot for *KLH* is shown in Figure 11 and the BLAST alignment is shown in Figure 12. The best match for UK-JAH was *Aeromonas hydrophila subspecies ranae LMG*, with a pairwise similarity score of 99.726%. According to BLAST the 16S rRNA sequences of UK-JAH and *A. hydrophila subsp. ranae LMG* are 99% similar with 728 out of 731 base pairs matching. The EzTaxon screenshot for UK-JAH is shown in Figure 14 and the BLAST alignment is shown in Figure 15. The phylogenetic tree, assembled in MEGA, containing both organisms is shown in Figure 16.

The best match for UK-JAH according to the Biolog results was *Aeromonas media-like DNA group 5A*, with a similarity score of .664 and a probability of .790. The Biolog results are shown in Figures 17 and 18.

According to the fatty acid methyl ester analysis, the best match for *KLH* was *Bacillus subtilis GC subgroup D* with a similarity of .452. The best match for UK-JAH was *Alcaligenes faecalis* with a similarity of .551, followed by *Aeromonas hydrophila GC subgroup A* with a similarity of .527. The gas chromatograms for *KLH* and UK-JAH are shown in Figure 19 and Figure 21, respectively. The results for the FAME analysis for *KLH* and UK-JAH are shown in Figure 20 and Figure 21, respectively.

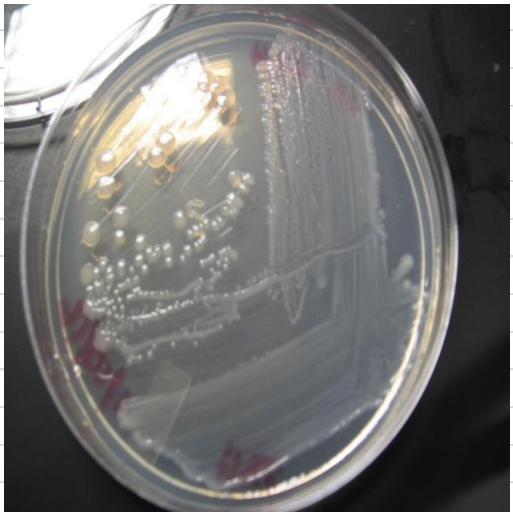
Figure 1 - Colony Morphology		
	B. Amyloliquefaciens KLH	**UK - JAH**
Color	Beige	Beige
Size	6.7 mm	4.5 mm
Shape	Irregular	Circular
Elevation	Flat	Flat
Texture	Dry	Normal
		

Figure 1 – Morphological characteristics of organisms on R2A plates, including pictures of plates

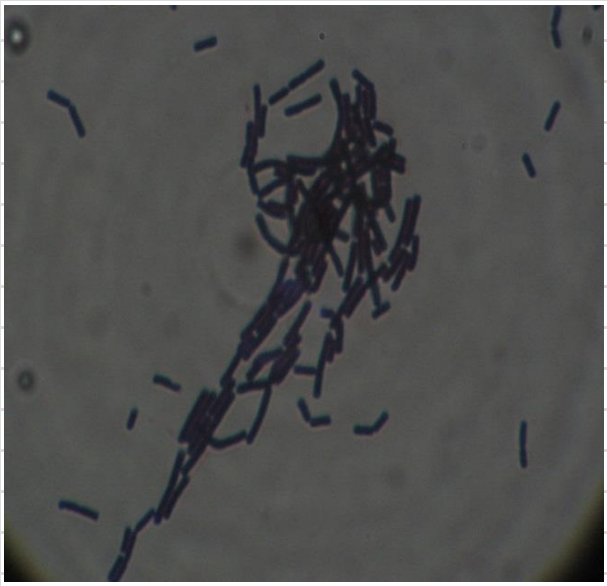
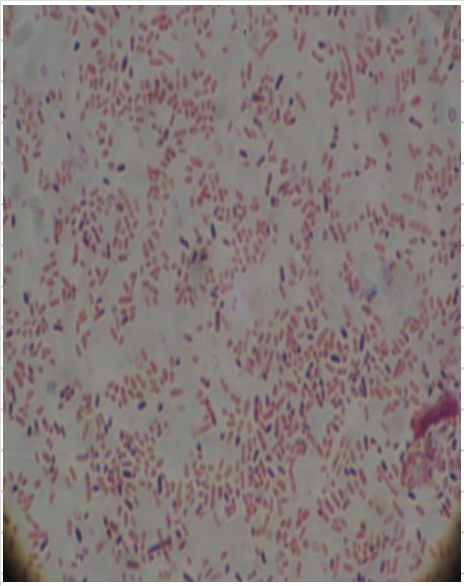
Figure 2 - Cell Morphology		
	B. amyloliquefaciens KLH	**UK - JAH**
Shape	Long Rods	Short Rods
Arrangement	Few Large Clumps	Clumps
Motility	Nonmotile	Nonmotile
Endospores	-	Nonsporeforming
Gram Stain	Positive	Negative
		

Figure 2 – Characteristics of organisms when viewed under microscope, including pictures of gram stains

Figure 3 - Oxygen Requirements		
	B. amyloliquefaciens KLH	UK - JAH
Aerobic	+	+
Anaerobic	-	-
Catalase	+	+
Oxidase	+ Weak	+

Figure 3 – Oxygen requirements of the organisms; for “aerobic” and “anaerobic”, “+” indicates growth while “-” indicates no growth; for catalase and oxidase, “+” indicates the presence of the enzyme while “-” indicates the absence of the enzyme

Figure 4 - Temperature Growth Requirements		
	B. amyloliquefaciens KLH	**UK - JAH**
4°C	-	+ Weak
20°C	+ Weak	++
30°C	+	+
37°C	++	+
44°C	+	-

Figure 4 – Temperature requirements for the organisms; "+" indicates growth while "-" indicates no growth; "++" indicates strong growth

Figure 5 - Antibiotic Resistance				
	B. amyloliquefaciens KLH	B. amyloliquefaciens	UK - JAH	A. hydrophila
Ampicillin	0.0 mm*		0.0 mm*	r
Carenicillin	52 mm		0.0 mm	
Chloramphenicol	48 mm		42 mm	
Chlortetracycline	60 mm		24 mm	
Erthromycin	36 mm		14 mm	
Kanamycin	20 mm		18 mm	s
Nalidixic Acid	46 mm		0.0 mm	
Penicillin	50 mm		0.0 mm	r
Rifampicin	28 mm		30 mm	
Spectinomycin	0.0 mm		12 mm	
Streptomycin	24 mm		10 mm	s
Tetracyline	52 mm		20 mm	s

Figure 5 – Zones of inhibition of the organisms for the antibiotic resistance tests; Available published data are indicated in shaded area; “r” indicates resistance while “n” indicates susceptibility

Figure 6 - Metabolism				
	B. amyloliquefaciens KLH	B. amyloliquefaciens	UK - JAH	A. hydrophila
Glucose	+	+	-	+
Lactose	++	+	+	-
Sucrose	+	+	-	-
Mannitol	+	+	-	+
Galactose	++	+	-	+
Salicin	+	+	-	-
Methyl Red	-		+	+
Vogues-Proskauer	+		-	d
Litmus Milk	-		-	
Simmons Citrate	-		-	d
Cysteine Desulfhydrase	-		-	
Indole	-		-	+
Motile	-		+	+
Urease	-	-	-	-
Nirate	+ Nitrate	+	+ Nitrate	+

Figure 6 – Results of metabolism tests for the organisms; "+" indicates a positive result, while "-" indicates a negative result; "++" indicates a strong positive; available published data is shown in the shaded areas; "d" indicates that results vary by strain

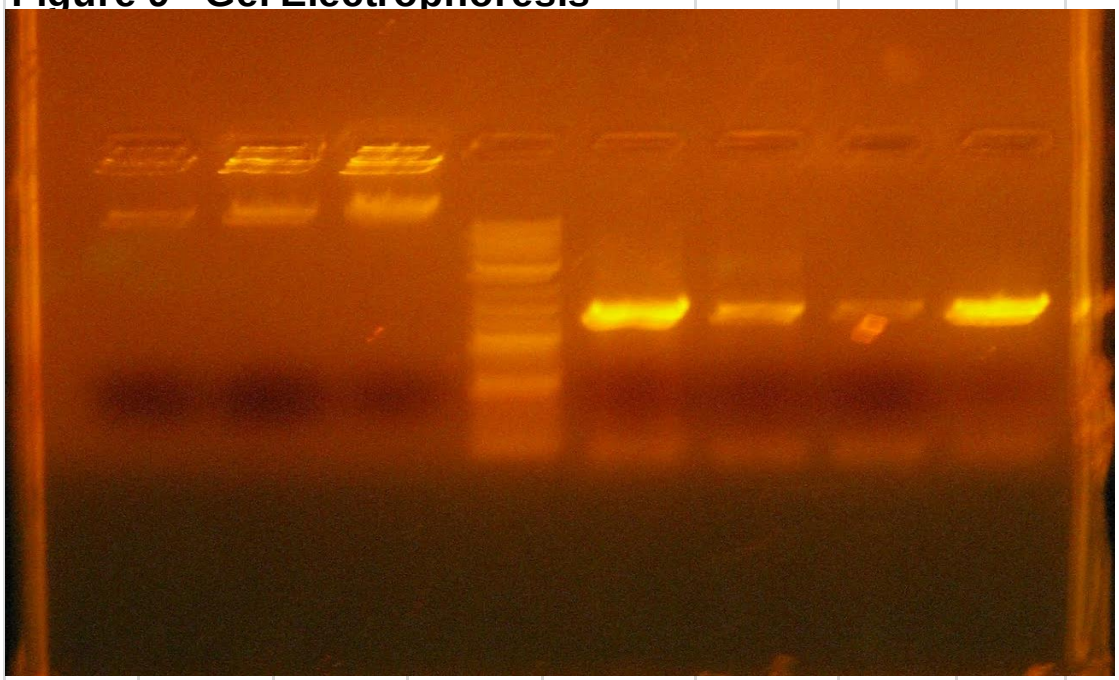
Figure 7 - Exoenzymes				
	B. amyloliquefaciens KLH	B. amyloliquefaciens	UK - JAH	A. Hydrophila
Amylase	+	+	+	
Caseinase	+	+	+	
DNase	-	-	+	+
Gelatinase	-	+	+	+
Tween Hydrolysis	-	+	+	

Figure 7 – Results for the exoenzyme tests for the organisms; "+" indicates the presence of the enzyme, while "-" indicates the absence of the enzyme; available published values are shown in the shaded

Figure 8 - Differential and Selective Media				
	B. amyloliquefaciens KLH	B. Amyloliquefaciens	UK - JAH	A. Hydrophila
EG Minimal Medium	+ Weak		+ Weak	
Bile Esculin Medium	-	-	+ Esculin	+ Esculin
Brilliant Green Augar	-		-	
Eosin Methyline Blue Medium	-		+ Weak	
Hektoen Enteric Agar	-		+ Orange	
MacConkey Agar	-	-	+	+
Mannitol Salt Agar	-		-	-
Phenylethyl Agar	-		-	

Figure 8 – Results for growth of the organisms on differential and selective media; "+" indicates growth, while "-" indicates no growth; available published data is shown in the shaded areas

Figure 9 - Gel Electrophoresis



1	2	3	4	5	6	7	8	
λ DNA	λ DNA	λ DNA	Ladder	UK PCR	K PCR			
10 ng/µL	25 ng/µL	60 ng/µL		200 ng/µL	60 ng/µL			

Figure 9 – Photo of gel with PCR products after gel electrophoresis; rows 1 – 3 contain λ DNA markers; row 4 contains the ladder; rows 5 and 6 contain the PCR products for UK-JAH and *KLH* respectively

Figure 10 – 16S rRNA Sequence B. Amyloliquefaciens KLH

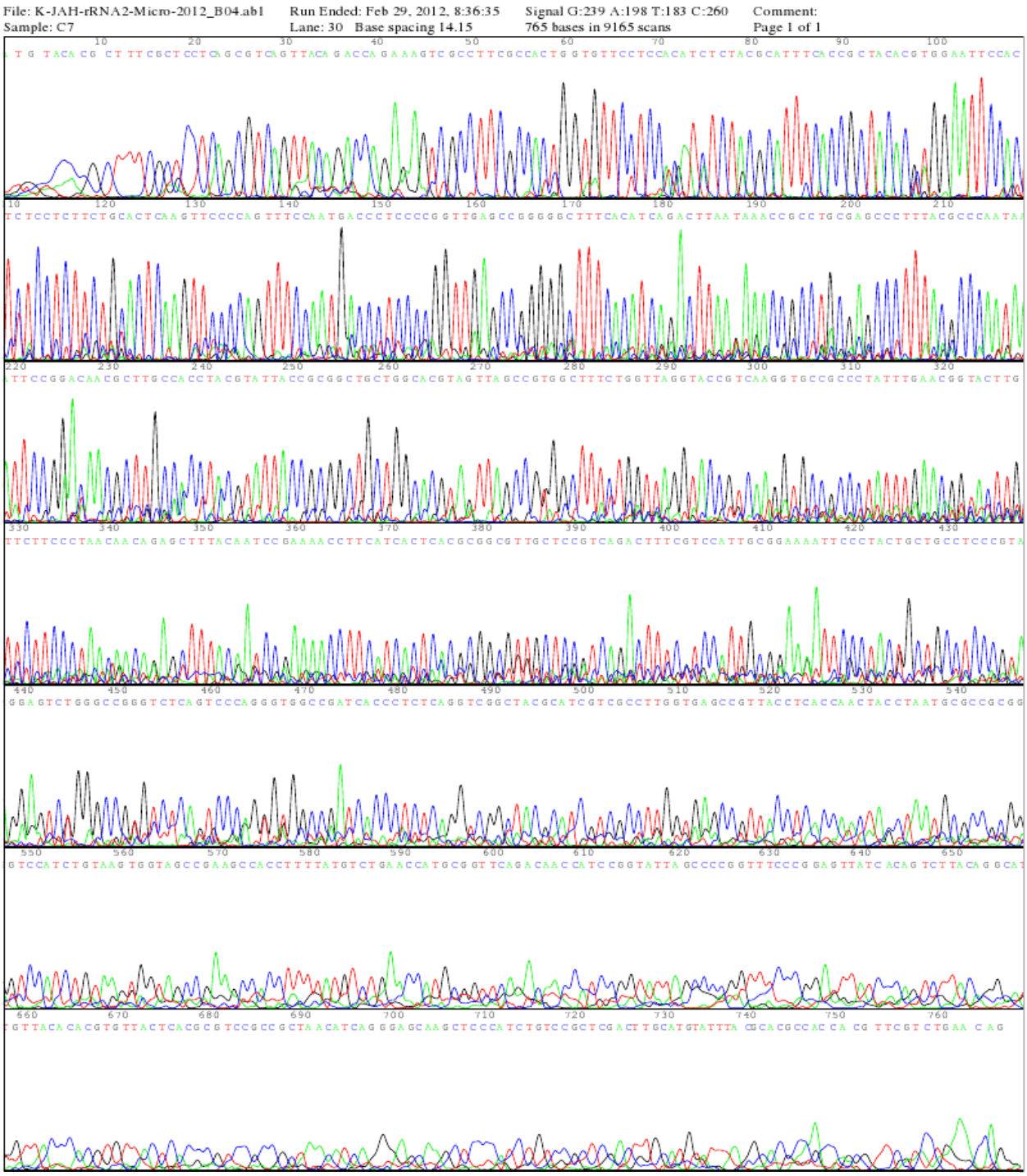


Figure 10 - 16S rRNA gene DNA sequence for *KLH*

Figure 11 – EzTaxon Results B. amyloliquefaciens KLH

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
1	Bacillus amyloliquefaciens subsp. plantarum	Borriss et al. 2011	FZB42(T)	CP000560	98.705	9/695	1306	1306
2	Bacillus methylotrophicus	Madhaiyan et al. 2010	CBMB205(T)	EU194897	98.705	9/695	1298	1292
3	Bacillus siamensis	Sumpavapol et al. 2010	PD-A10(T)	GQ281299	98.561	10/695	1298	1298
4	Bacillus amyloliquefaciens subsp. amyloliquefaciens	Borris (ex Fukumoto 1943) Priest et al. 1987	DSM 7(T)	FN597644	98.417	11/695	1290	1291
5	Bacillus subtilis subsp. subtilis	(Ehrenberg 1835) Cohn 1872	NCIB 3610(T)	ABQL01000001	98.273	12/695	1283	1283
6	Bacillus atrophaeus	Nakamura 1989	JCM 9070(T)	AB021181	98.273	12/695	1283	1283
7	Bacillus vallismortis	Roberts et al. 1996	DSM 11031(T)	AB021198	98.129	13/695	1275	1275
8	Bacillus tequilensis	Gatson et al. 2006	10b(T)	HQ223107	98.129	13/695	1275	1275
9	Bacillus mojavensis	Roberts et al. 1994	IFO 15718(T)	AB021191	97.986	14/695	1267	1267
10	Bacillus subtilis subsp. spizizenii	Nakamura et al. 1999	NRRL B-23049(T)	AF074970	97.986	14/695	1267	1267
11	Bacillus subtilis subsp. inaquosorum	Rooney et al. 2009	BGSC 3A28(T)	EU138467	97.940	13/631	1148	1148
12	Brevibacterium halotolerans	Delaporte and Sasson 1967	DSM 8802(T)	AM747812	97.842	15/695	1259	1259
13	Bacillus licheniformis	(Weigmann 1898) Chester 1901	ATCC 14580(T)	AE017333	96.691	23/695	1181	1168
14	Bacillus sonorensis	Palmisano et al. 2001	NRRL B-23154(T)	AF302118	96.403	25/695	1172	1166
15	Bacillus aerius	Shivaji et al. 2006	24K(T)	AJ831843	96.248	26/693	1126	1094
16	Bacillus stratosphericus	Shivaji et al. 2006	41KF2a(T)	AJ831841	94.957	35/694	1098	1088
17	Bacillus altitudinis	Shivaji et al. 2006	41KF2b(T)	AJ831842	94.957	35/694	1098	1088
18	Bacillus aerophilus	Shivaji et al. 2006	28K(T)	AJ831844	94.957	35/694	1098	1088
19	Bacillus safensis	Satomi et al. 2006	FO-036b(T)	AF234854	94.813	36/694	1090	1076
20	Bacillus pumilus	Meyer and Gottheil 1901	ATCC 7061(T)	ABRX01000007	94.669	37/694	1082	1068
21	Bacillus galliciensis	Balcázar et al. 2010	BFLP-1(T)	FM162181	94.532	38/695	1094	1086
22	Bacillus acidicola	Albert et al. 2005	105-2(T)	AF547209	94.236	40/694	1061	1045
23	Bacillus sporothermodurans	Petterson et al. 1996	M215(T)	U49079	93.813	43/695	1029	1023
24	Bacillus pseudofirmus	Nielsen et al. 1995	DSM 8715(T)	X76439	93.669	44/695	1029	1015
25	Bacillus carboniphilus	Fujita et al. 1996	JCM 9731(T)	AB021182	93.669	44/695	1053	1025
26	Bacillus vietnamensis	Noguchi et al. 2004	15-1(T)	AB099708	93.660	44/694	1015	1009
27	Bacillus shackletonii	Logan et al. 2004	LMG 18435(T)	AJ250318	93.651	44/693	1072	1045
28	Bacillus aquimaris	Yoon et al. 2003	TF-12(T)	AF483625	93.525	45/695	0	1007
29	Bacillus oleronius	Kuhnigk et al. 1996	DSM 9356(T)	X82492	93.516	45/694	1015	1001
30	Bacillus marisflavi	Yoon et al. 2003	TF-11(T)	AF483624	93.094	48/695	0	997
31	Bacillus seohaeanensis	Lee et al. 2006	BH724(T)	AY667495	93.084	48/694	1025	0
32	Bacillus isabeliae	Albuquerque et al. 2008	CVS-8(T)	AM503357	92.086	55/695	1015	0

0 score value means that it was not found in the initial searches

Figure 11 – Screenshot of EzTaxon database best matches for *KLH*

Figure 12 - BLAST Sequence Alignment B. amyloliquefaciens KLH

```
>gb|CP000560.1| Bacillus amyloliquefaciens FZB42, complete genome
Length=3918589

                                                    Sort alignments for this subject sequence by:
                                                      E value  Score  Percent identity
                                                      Query start position  Subject start position
 Score = 1240 bits (671),  Expect = 0.0
 Identities = 687/695 (99%), Gaps = 0/695 (0%)
 Strand=Plus/Plus

Query  1        TTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCAC  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2926870  TTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCAC  2926929

Query  61       ATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTC  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2926930  ATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTC  2926989

Query  121      CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAAC  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2926990  CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAAC  2927049

Query  181      CGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCG  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2927050  CGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCG  2927109

Query  241      CGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCT  300
                |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  2927110  CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCT  2927169

Query  301      ATTTGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACAATCCGAAAACCTTCATCA  360
                |||||||||| ||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  2927170  ATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCA  2927229

Query  361      CTCACGCGGCGTTGCTCCGTCAGACTTTCCTCCATTGCGGAAGATTCCCTACTGCTGCCT  420
                |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  2927230  CTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCT  2927289

Query  421      CCCGTAGGAGTCGGGGCCGTGTCTCAGTCCCGGGGTGGCCGATCACCCTCTCAGGTCGGC  480
                |||||||||||| |||||||||||||||||| | ||||||||||||||||||||||||||
Sbjct  2927290  CCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGC  2927349

Query  481      TACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTACCTAATGCGCCGCGGGTCCA  540
                |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  2927350  TACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCA  2927409

Query  541      TCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCAGACAACCATC  600
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2927410  TCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCAGACAACCATC  2927469

Query  601      CGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTT  660
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2927470  CGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTT  2927529

Query  661      ACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGC  695
                |||||||||||||||||||||||||||||||||||
Sbjct  2927530  ACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGC  2927564
```

Figure 12 – BLAST sequence alignment for *KLH* and *B. amyloliquefaciens subsp. plantarum FZB42*

Figure 13 – 16S rRNA Sequence UK-JAH

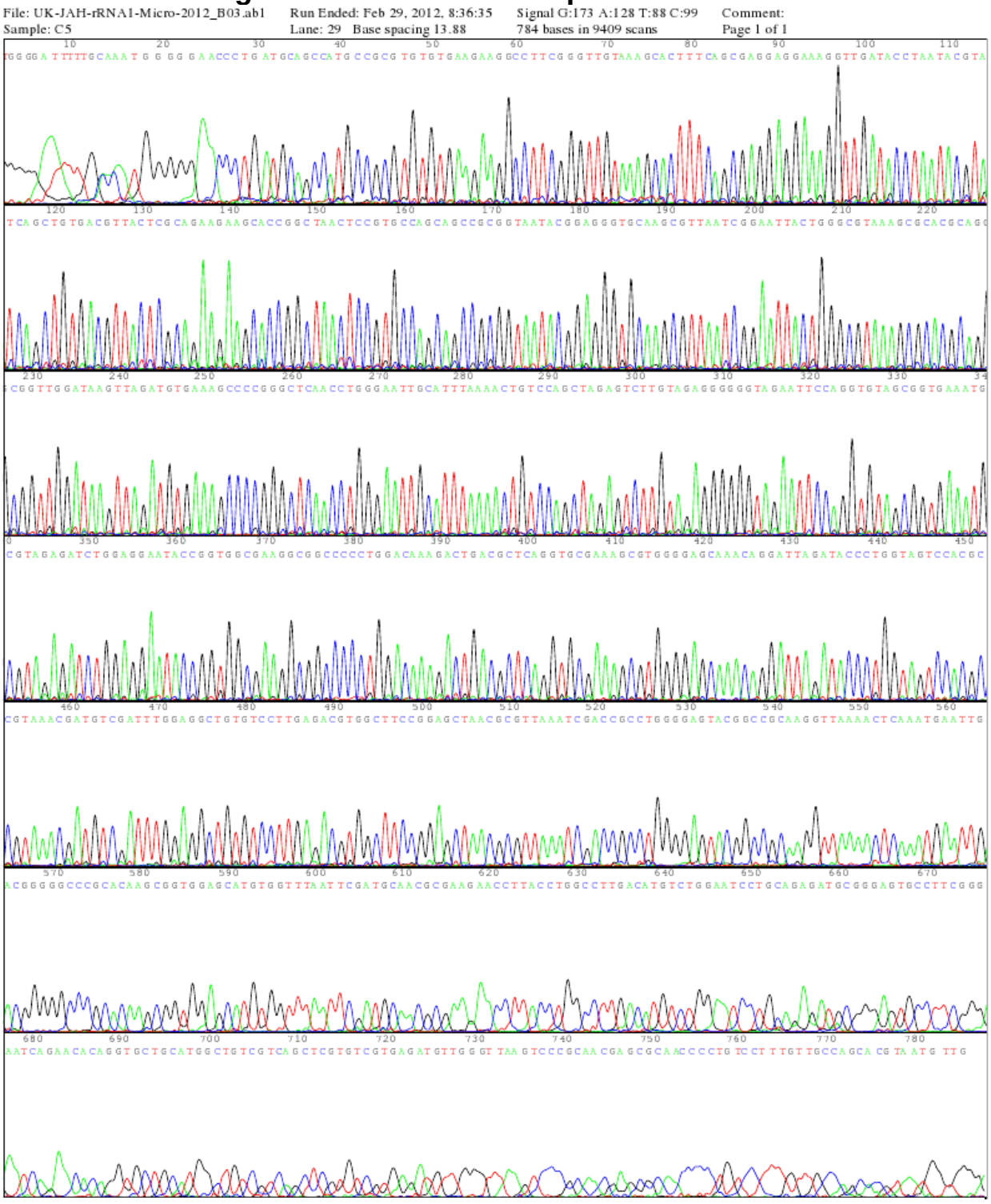


Figure 13 - 16S rRNA gene DNA sequence for UK-JAH

Figure 14 – EzTaxon Results UK-JAH

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
1	Aeromonas hydrophila subsp. ranae	Huys et al. 2003	LMG 19707(T)	AJ508766	99.726	2/729	1429	1429
2	Aeromonas media	Allen et al. 1983	ATCC 33907(T)	X74679	99.589	3/730	1425	1425
3	Aeromonas hydrophila subsp. hydrophila	(Chester 1901) Stanier 1943	ATCC 7966(T)	CP000462	99.589	3/730	1425	1425
4	Aeromonas tecta	Demarta et al. 2010	F518(T)	AJ458402	99.315	5/730	1409	1409
5	Aeromonas molluscorum	Miñana-Galbis et al. 2004	848(T)	AY532690	99.315	5/730	1409	1409
6	Aeromonas encheleia	Esteve et al. 1995	LMG 16331(T)	AJ458409	99.315	5/730	1409	1409
7	Aeromonas eucrenophila	Schubert and Hegazi 1988	NCIMB 74(T)	X60411	99.178	6/730	1401	1402
8	Aeromonas piscicola	Beaz-Hidalgo et al. 2010	S1.2(T)	FM999971	99.178	6/730	1401	1402
9	Aeromonas salmonicida subsp. achromogenes	(Smith 1963) Schubert 1967	NCIMB 1110(T)	X60407	99.178	6/730	1401	1402
10	Aeromonas salmonicida subsp. masoucida	Kimura 1969	ACC 27013(T)	X74680	99.178	6/730	1401	1402
11	Haemophilus piscium	Snieszko et al. 1950	NCIMB 1952	AJ009860	99.041	7/730	1394	1394
12	Aeromonas salmonicida subsp. smithia	Austin et al. 1989	CCM 4103(T)	AJ009859	98.904	8/730	1386	1386
13	Aeromonas salmonicida subsp. salmonicida	(Lehmann and Neumann 1896) Griffin et al. 1953	ACC 33658(T)	X74681	98.904	8/730	1386	1386
14	Aeromonas popoffii	Huys et al. 1997	LMG 17541(T)	AJ224308	98.904	8/730	1386	1386
15	Aeromonas bestiarum	Ali et al. 1996	CIP 7430(T)	X60406	98.904	8/730	1386	1386
16	Aeromonas salmonicida subsp. pectinolytica	Pavan et al. 2000	34mel(T)	AF134065	98.903	8/729	1376	1370
17	Aeromonas veronii	Hickman-Brenner et al. 1988	ATCC 35624(T)	X60414	98.767	9/730	1378	1378
18	Aeromonas ichthiosmia	Schubert et al. 1991	DSM 6393(T)	X71120	98.765	9/729	1368	1362
19	Aeromonas sobria	Popoff and Veron 1981	ACC 43979(T)	X74683	98.630	10/730	1370	1370
20	Aeromonas rivuli	Figueras et al. 2011	WB4.1-19(T)	FJ976900	98.630	10/730	1370	1370
21	Aeromonas fluvialis	Alperi et al. 2010	717(T)	FJ230078	98.493	11/730	1362	1362
22	Aeromonas jandaei	Carnahan et al. 1992	ATCC 49568(T)	X60413	98.493	11/730	1362	1362
23	Aeromonas punctata subsp. punctata	(Zimmermann 1890) Snieszko 1957	NCIMB 13016(T)	X60408	98.493	11/730	1362	1362
24	Aeromonas sanarellii	Alperi et al. 2010	A2-67(T)	FJ230076	98.493	11/730	1362	1362
25	Aeromonas punctata subsp. caviae	(Scherago 1936) Schubert 1964	ATCC 15468(T)	X74674	98.489	11/728	1350	1350
26	Aeromonas taiwanensis	Alperi et al. 2010	A2-50(T)	FJ230077	98.356	12/730	1362	1354
27	Aeromonas aquariorum	Martínez-Murcia et al. 2008	MDC47(T)	EU085557	98.356	12/730	1362	1354
28	Aeromonas enteropelogenes	Schubert et al. 1991	ATCC 49657(T)	X60415	98.356	12/730	1354	1354
29	Aeromonas diversa	Miñana-Galbis et al. 2010	ATCC 43946(T)	GQ365710	98.356	12/730	1354	1354
30	Aeromonas bivalvium	Miñana-Galbis et al. 2007	868E(T)	DQ504429	98.356	12/730	1354	1354

Figure 14 – Screenshot of EzTaxon database best matches for UK-JAH

Figure 15 – BLAST Sequence Alignment UK-JAH

```
>emb|AJ508766.1|  Aeromonas hydrophila subsp. ranae 16S rRNA gene, type strain
LMG 19707T
Length=1497

 Score = 1334 bits (722),  Expect = 0.0
 Identities = 728/731 (99%), Gaps = 1/731 (0%)
 Strand=Plus/Plus

Query  1     GGG-AACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT  59
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  353   GGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT  412

Query  60    TTCAGCGAGGAGGAAAGGTTGATACCTAATACGTATCAGCTGTGACGTTACTCGCAGAAG  119
             || |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  413   TTMAGCGAGGAGGAAAGGTTGATACCTAATACGTATCAACTGTGACGTTACTCGCAGAAG  472

Query  120   AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473   AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG  532

Query  180   GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGG  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  533   GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGG  592

Query  240   CTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAAT  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  593   CTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAAT  652

Query  300   TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  653   TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC  712

Query  360   CCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713   CCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC  772

Query  420   TGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCC  479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773   TGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCC  832

Query  480   GGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG  539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833   GGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG  892

Query  540   AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA  599
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  893   AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA  952

Query  600   ACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAAT  659
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  953   ACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAAT  1012

Query  660   CAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC  719
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1013  CAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC  1072

Query  720   CGCAACGAGCG  730
             ||||||||||| 
Sbjct  1073  CGCAACGAGCG  1083
```

Figure 15 – BLAST sequence alignment for UK-JAH and *A. hydrophila subsp. ranae LMG*

Figure 16 – Phylogenetic Tree

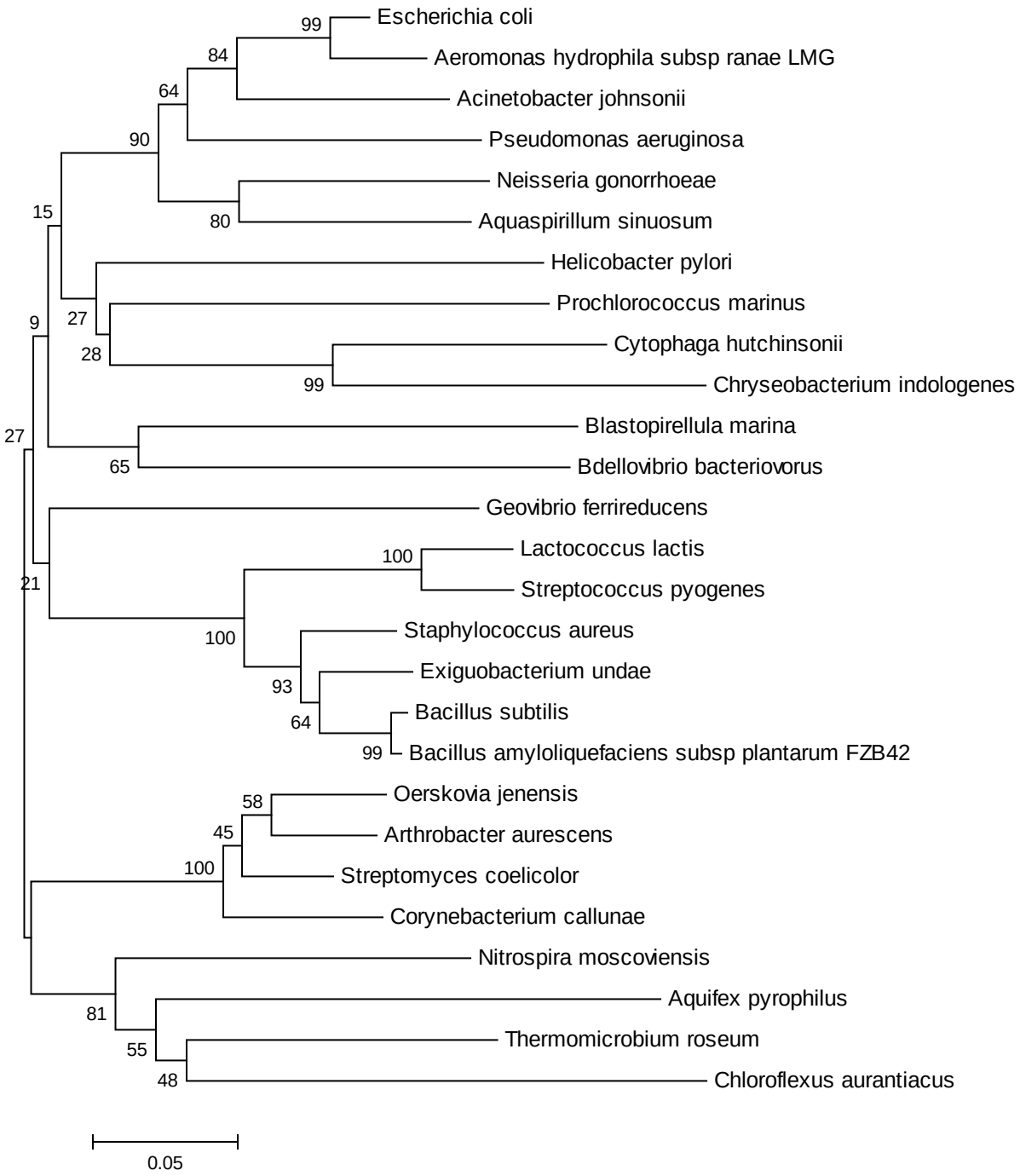


Figure 16 – Phylogenetic tree, composed in MEGA, containing *both B. amyloliquefaciens subsp. plantarum FZB42* and *A. hydrophila subsp. ranae LMG*

Figure 17 – Biolog Plate

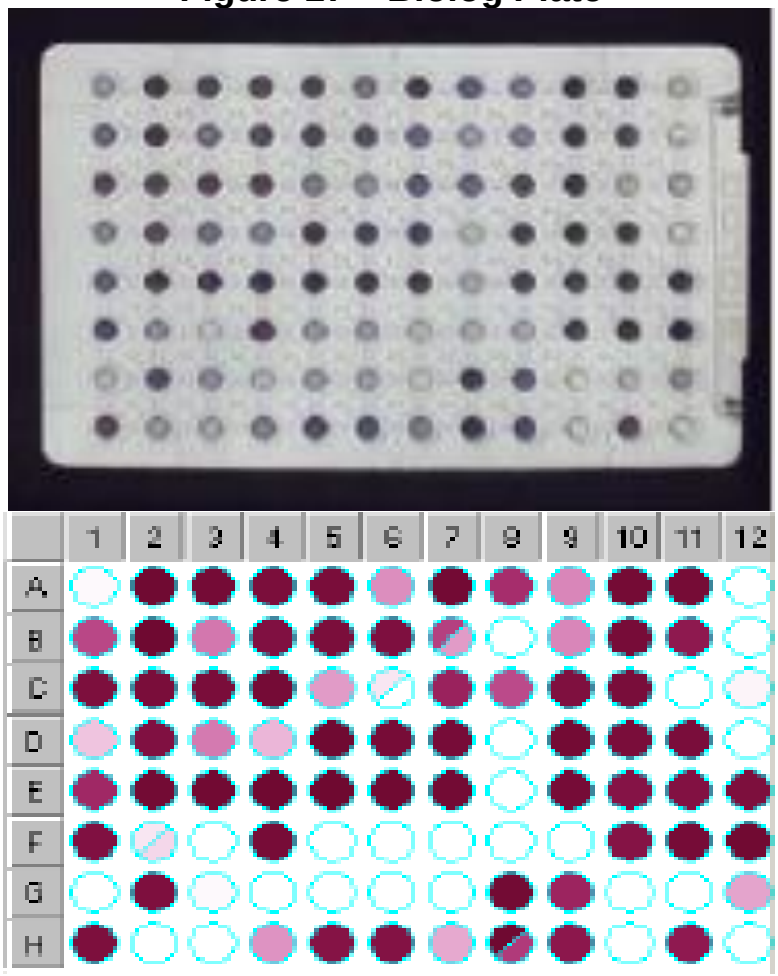


Figure 17 – On top: picture of Biolog Gen III plate for UK-JAH; on bottom: results of Biolog test; purple indicates a positive result for that well, while lighter purple indicates a weaker positive, and white indicates a negative

Figure 18 – Biolog Matches

	PROB	SIM	DIST	Organism Ty	Species
==>1	0.790	0.664	2.576	GN-NENT	Aeromonas media-like DNA group 5A
2	0.168	0.129	3.554	GN-NENT	Aeromonas salmonicida ss pectinolytica
3	0.034	0.024	4.898	GN-NENT	Aeromonas archiebiia
4	0.007	0.004	5.534	GN-NENT	Aeromonas hydrophila-like DNA group 3

Figure 18 – Biolog database best matches for UK-JAH with probability and similarity index

Figure 19 - MIDI Chromatogram B. amyloliquefaciens KLH

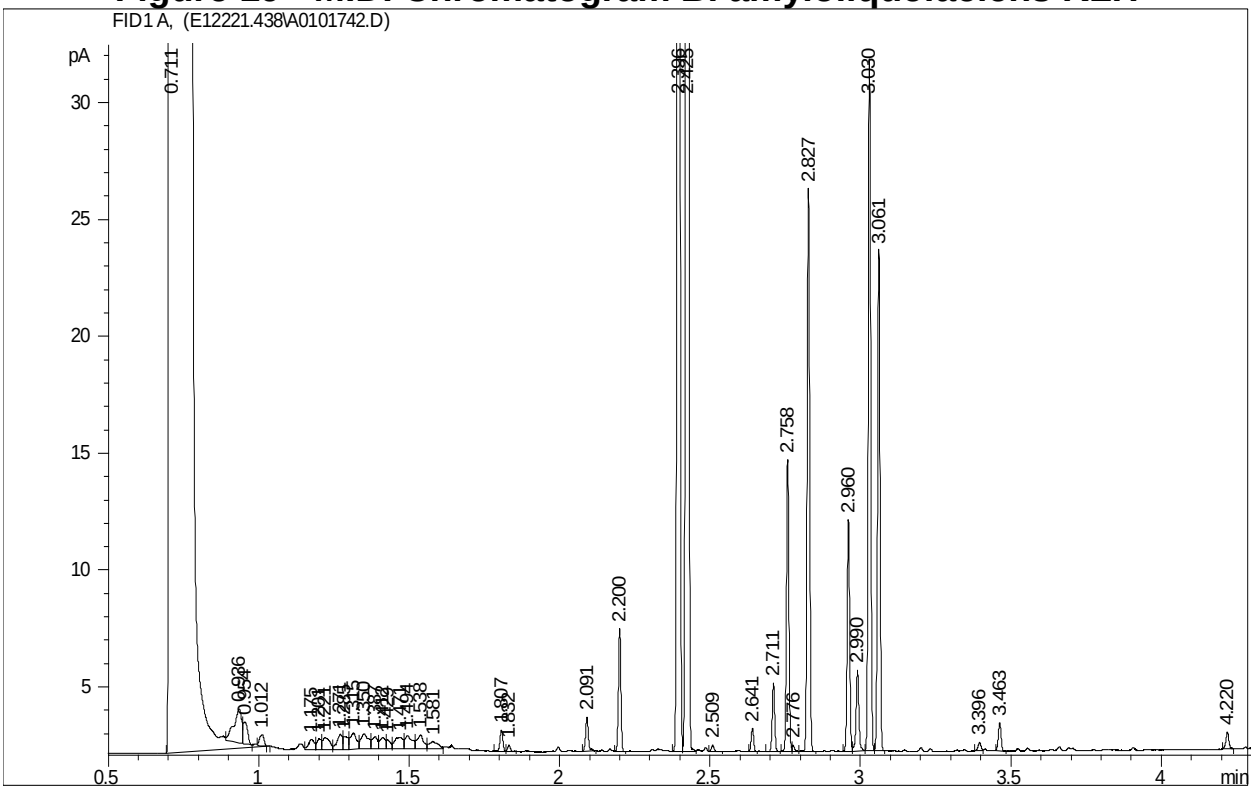


Figure 19 – MIDI chromatogram of *KLH* after FAME analysis

Figure 20 – MIDI/FAME Results for *B. amyloliquefaciens KLH*

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.7113	2.326E+9	0.018	----	6.6138	SOLVENT PEAK	----	< min rt	
0.9359	5309	0.023	----	8.1016		----	< min rt	
0.9541	1877	0.015	----	8.2224		----	< min rt	
1.0120	963	0.014	----	8.6083		----	< min rt	
1.1753	1111	0.017	----	9.6960		----		
1.2011	954	0.014	----	9.8682		----		
1.2211	1628	0.020	1.165	10.0002	10:0	0.55	ECL deviates 0.000	Reference 0.003
1.2710	1492	0.015	----	10.2625		----		
1.2853	1314	0.014	----	10.3371		----		
1.3150	2059	0.020	----	10.4930		----		
1.3500	2420	0.022	----	10.6770		----		
1.3873	1383	0.017	----	10.8726		----		
1.4117	1225	0.016	1.098	11.0008	11:0	0.39	ECL deviates 0.001	Reference 0.001
1.4288	744	0.012	----	11.0744		----		
1.4707	1977	0.024	----	11.2558		----	> max ar/ht	
1.4942	1871	0.021	----	11.3575		----		
1.5382	1847	0.020	----	11.5478		----		
1.5809	1343	0.027	----	11.7328		----	> max ar/ht	
1.8074	1180	0.010	1.020	12.6211	13:0 iso	0.35	ECL deviates -0.002	Reference -0.005
1.8321	344	0.010	1.016	12.7143	13:0 anteiso	0.10	ECL deviates 0.000	Reference -0.003
2.0914	1860	0.010	0.987	13.6274	14:0 iso	0.54	ECL deviates -0.001	Reference -0.006
2.2001	5859	0.009	0.978	13.9985	14:0	1.67	ECL deviates -0.002	Reference -0.007
2.3958	99226	0.009	0.964	14.6328	15:0 iso	27.91	ECL deviates 0.001	Reference -0.006
2.4246	124498	0.009	0.962	14.7262	15:0 anteiso	34.95	ECL deviates 0.001	Reference -0.005
2.5092	311	0.009	----	15.0001	15:0	----	ECL deviates 0.000	
2.6412	1145	0.009	0.951	15.4151	16:1 w7c alcohol	0.32	ECL deviates 0.001	
2.7111	3326	0.009	0.948	15.6346	16:0 iso	0.92	ECL deviates 0.002	Reference -0.006
2.7583	14150	0.009	0.946	15.7828	16:1 w11c	3.91	ECL deviates 0.001	
2.7763	316	0.009	0.946	15.8393	Sum In Feature 3	0.09	ECL deviates -0.001	16:1 w7c/16:1 w6c
2.8272	27787	0.009	0.944	15.9994	16:0	7.65	ECL deviates -0.001	Reference -0.008
2.9601	11358	0.009	0.940	16.4166	17:1 iso w10c	3.11	ECL deviates 0.003	
2.9903	4374	0.009	0.939	16.5114	Sum In Feature 4	1.20	ECL deviates -0.001	17:1 anteiso B/iso I
3.0300	33339	0.009	0.938	16.6360	17:0 iso	9.12	ECL deviates -0.001	Reference -0.009
3.0614	24371	0.009	0.937	16.7346	17:0 anteiso	6.66	ECL deviates 0.002	Reference -0.006
3.3963	516	0.010	0.930	17.7878	18:1 w9c	0.14	ECL deviates -0.006	
3.4634	1549	0.010	0.928	17.9991	18:0	0.42	ECL deviates -0.001	Reference -0.009
4.2197	985	0.010	----	20.4731		----	> max rt	
----	316	---	----	----	Summed Feature 3	0.09	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	-----	---	----	----		----	17:0 cyclo	
----	4374	---	----	----	Summed Feature 4	1.20	17:1 iso I/anteiso B	17:1 anteiso B/iso I

ECL Deviation: 0.002 Reference ECL Shift: 0.006 Number Reference Peaks: 13
Total Response: 376566 Total Named: 358051
Percent Named: 95.08% Total Amount: 342724

Matches:

Library	Sim Index	Entry Name
ITSA1 1.10	0.452	Bacillus-subtilis-GC subgroup D
	0.439	Bacillus-subtilis-GC subgroup C

Figure 22 – FAME results for *B. amyloliquefaciens* with best matches including similarity index.

Figure 21 - MIDI Chromatogram UK-JAH

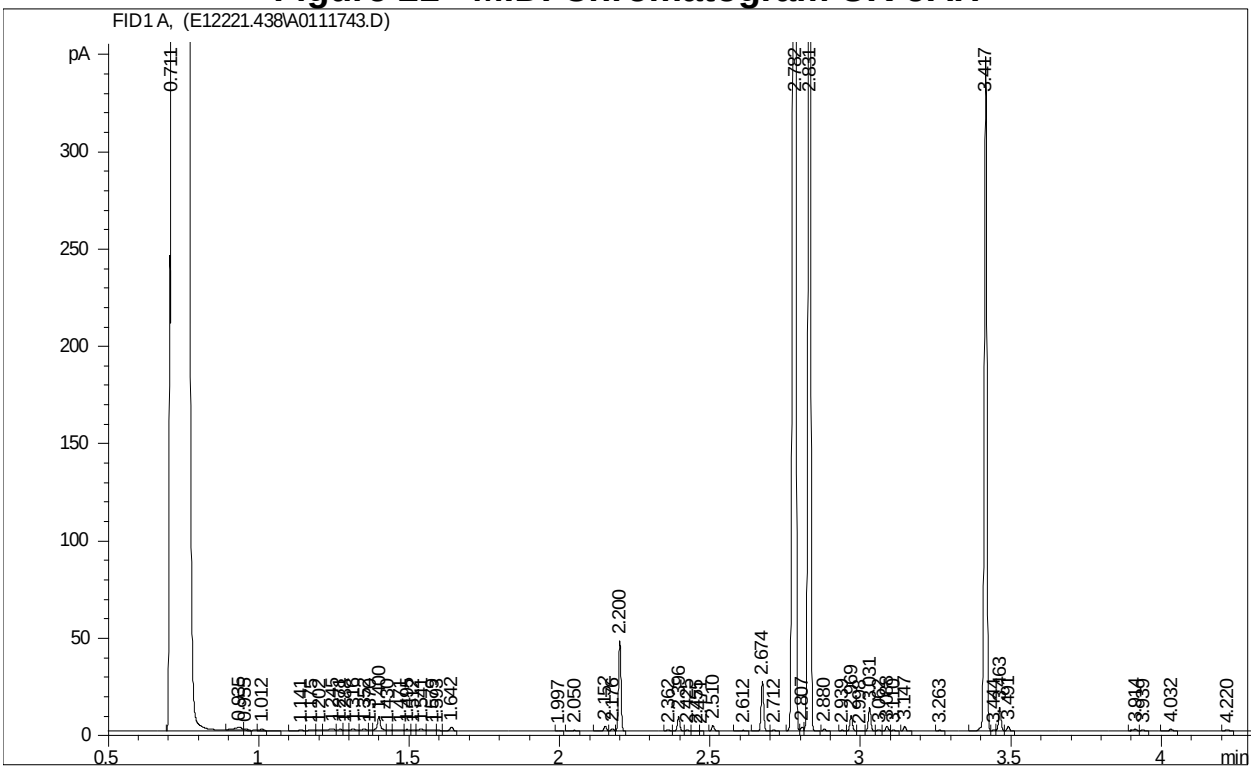


Figure 21 – MIDI chromatogram of UK-JAH after FAME analysis

Figure 22 – MIDI/FAME Results for UK-JAH

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.7105	2.349E+9	0.016	----	6.6198	SOLVENT PEAK	----	< min rt	
0.9353	6166	0.025	----	8.1121		----	< min rt	
0.9549	1618	0.016	----	8.2428		----	< min rt	
1.0117	1232	0.014	----	8.6193		----	< min rt	
1.1410	932	0.018	----	9.4782		----		
1.1755	1445	0.020	----	9.7072		----		
1.2021	1229	0.013	----	9.8836		----		
1.2448	3832	0.023	----	10.1330		----		
1.2714	1630	0.014	----	10.2725		----		
1.2882	1675	0.016	----	10.3604		----		
1.3157	2538	0.019	----	10.5091		----		
1.3516	2469	0.018	----	10.6964		----		
1.3700	1068	0.010	----	10.7928		----		
1.3997	11809	0.011	----	10.9477	unknown 10.9525	----	ECL deviates -0.005	
1.4299	1062	0.013	----	11.0889		----		
1.4710	2694	0.026	----	11.2656		----	> max ar/ht	
1.4946	1772	0.016	----	11.3672		----		
1.5124	929	0.011	1.073	11.4439	10:0 3OH	0.04	ECL deviates -0.004	
1.5406	2828	0.018	----	11.5656		----		
1.5793	1434	0.020	----	11.7324		----		
1.5932	782	0.012	----	11.7923		----		
1.6415	3258	0.011	1.046	12.0004	12:0	0.14	ECL deviates 0.000	Reference -0.007
1.9968	346	0.010	----	13.3052		----		
2.0496	639	0.010	----	13.4854	12:0 3OH	----	ECL deviates 0.002	
2.1520	3030	0.009	----	13.8343		----		
2.1762	1416	0.010	0.980	13.9168	14:1 w5c	0.06	ECL deviates 0.001	
2.2005	50849	0.009	0.978	13.9995	14:0	2.10	ECL deviates -0.001	Reference -0.006
2.3615	812	0.009	----	14.5205	unknown 14.502	----	ECL deviates 0.005	
2.3958	8286	0.009	0.964	14.6314	15:0 iso	0.34	ECL deviates -0.001	Reference -0.006
2.4246	613	0.010	0.962	14.7244	15:0 anteiso	0.02	ECL deviates -0.001	Reference -0.006
2.4547	456	0.010	0.961	14.8219	15:1 w8c	0.02	ECL deviates 0.008	
2.4712	309	0.009	0.959	14.8753	15:1 w6c	0.01	ECL deviates 0.000	
2.5100	3254	0.009	----	15.0006	15:0	----	ECL deviates 0.001	
2.6116	549	0.011	----	15.3200		----		
2.6745	29548	0.009	0.950	15.5172	Sum In Feature 2	1.19	ECL deviates 0.002	14:0 3OH/16:1 iso I
2.7118	859	0.010	0.948	15.6344	16:0 iso	0.03	ECL deviates 0.001	Reference -0.004
2.7821	1.291E+6	0.010	0.946	15.8554	Sum In Feature 3	51.64	Column Overload	16:1 w7c/16:1 w6c
2.8069	3007	0.010	0.945	15.9333	16:1 w5c	0.12	ECL deviates 0.005	
2.8314	650257	0.009	0.944	16.0100	16:0	25.96	Column Overload	
2.8802	1290	0.010	0.942	16.1632	15:0 iso 3OH	0.05	ECL deviates 0.001	
2.9394	689	0.011	----	16.3492		----		
2.9692	9517	0.009	0.939	16.4429	Sum In Feature 9	0.38	ECL deviates -0.004	17:1 iso w9c
2.9982	588	0.013	----	16.5341	15:0 3OH	----	ECL deviates 0.001	
3.0306	13913	0.009	0.938	16.6357	17:0 iso	0.55	ECL deviates -0.001	Reference -0.007
3.0616	988	0.010	0.937	16.7332	17:0 anteiso	0.04	ECL deviates 0.000	Reference -0.006
3.0883	3040	0.009	0.936	16.8170	17:1 w8c	0.12	ECL deviates 0.002	
3.1098	696	0.010	0.936	16.8847	17:1 w6c	0.03	ECL deviates 0.004	
3.1471	2666	0.009	0.935	17.0020	17:0	0.11	ECL deviates 0.002	Reference -0.004
3.2635	547	0.010	----	17.3685		----		
3.4168	412750	0.009	0.929	17.8518	Sum In Feature 8	16.23	ECL deviates 0.004	18:1 w7c
3.4439	829	0.010	0.929	17.9374	18:1 w5c	0.03	ECL deviates 0.000	
3.4635	14462	0.009	0.928	17.9990	18:0	0.57	ECL deviates -0.001	Reference -0.009
3.4913	2646	0.010	0.928	18.0889	18:1 w7c 11-methyl	0.10	ECL deviates 0.003	
3.9138	1573	0.012	0.920	19.4666	20:4 w6,9,12,15c	0.06	ECL deviates 0.001	
3.9389	878	0.010	----	19.5445		----		
4.0322	1109	0.010	0.917	19.8503	20:1 w7c	0.04	ECL deviates 0.000	
4.2198	962	0.011	----	20.4659		----	> max rt	
----	29548	---	----	----	Summed Feature 2	1.19	16:1 iso I/14:0 3OH	14:0 3OH/16:1 iso I
----	1.291E+6	---	----	----	Summed Feature 3	51.64	Column Overload	16:1 w7c/16:1 w6c
----	-----	---	----	----		----	16:1 w6c/16:1 w7c	17:0 cyclo
----	412750	---	----	----	Summed Feature 8	16.23	18:1 w7c	18:1 w6c
----	-----	---	----	----		----	19:0 cyclo w8c	
----	9517	---	----	----	Summed Feature 9	0.38	16:0 10-methyl	17:1 iso w9c

ECL Deviation: 0.004 Reference ECL Shift: 0.006 Number Reference Peaks: 9
Total Response: 2539610 Total Named: 2506180
Percent Named: 98.68% Total Amount: 2363612
Profile Comment: Column Overload: A peak's response is greater than 500000.0. Dilute and re-run.

Matches:

Library	Sim Index	Entry Name
ITSA1 1.10	0.551	Alcaligenes-faecalis
	0.527	Aeromonas-hydrophila-GC subgroup A
	0.510	Hydrogenophaga-pseudoflava
	0.510	Chromobacterium-violaceum
	0.464	Aeromonas-caviae
	0.449	Aeromonas-salmonicida-achromogenes
	0.356	Yersinia-intermedia
	0.346	Plesiomonas-shigelloides (confirm with other tests)
	0.326	Acidovorax-facilis
	0.308	Neisseria-sicca

Figure 22 – FAME results for UK-JAH with best matches including similarity index.

Discussion:

Before the rRNA sequence analysis and the biolog and FAME tests, the hypothesis was that UK-JAH was of the genus *Arthrobacter*. This is because the original gram stain came out positive. The top matches for both the rRNA sequence analysis and the biolog tests were of the genus *Aeromonas*, and the second match for the FAME test was *Aeromonas*, so it seemed likely that UK-JAH was actually of the genus *Aeromonas*. Since *Aeromonas* is a gram-negative rod, the gram-stain was redone and the second stain was negative. Since *Aeromonas hydrophila subsp. ranae* was the top match for the 16S rRNA analysis, and *Aeromonas hydrophila* was a match for both the biolog and FAME tests, *Aeromonas hydrophila* seemed like the most likely identity of the organism UK-JAH. This is confirmed by the fact that *Aeromonas hydrophila* is also a gram-negative rod that is resistant to penicillin, grows best at around 25°C, reduces nitrate to nitrite, and hydrolyses esculin, DNA, and gelatin. Also, the organism fits in the subspecies *ranae*, as neither produces acid from sucrose or salicin, unlike most *Aeromonas hydrophila* strains.

The 16S rRNA sequencing seems to be the most accurate method of species identification. The EzTaxon database is much more comprehensive than the Biolog and FAME analysis databases. Also, because phenotypic and biochemical characteristics can vary for different strains of a species, the 16S rRNA gene sequence seems like a more reliable method.

Originally, the gram stain for *A. hydrophila JAH* did not come out as expected, as it was gram positive, while the results for the gene sequencing, Biolog, and FAME analysis were all gram negative. This is likely due to human error, either staining the

wrong organism or allowing the stains to react for too long, as a second gram stain came out negative. Also, some of the biochemical results for both organisms did not match those predicted by the literature. It is possible that the particular strains *KLH and JAH* have different metabolic capabilities than the strains that were published.

Both organisms were positive for the enzymes oxidase and catalase. This means that the organisms are able to hydrolyze toxic oxygen reactive species, which is why the organisms are able to grow in aerobic environments.

A. hydrophila JAH showed resistance to penicillin, a β-lactam antibiotic that blocks transpeptidation during peptidoglycan synthesis. This could indicate the presence of β-lactamase, an enzyme that hydrolyzes β-lactam antibiotics. More likely, though, it could be due to the fact that *A. hydrophila JAH* is gram-negative and penicillin is more effective against gram-positive organisms. To be sure, more tests would have to be done with derivatives of penicillin that have a wider spectrum of effectiveness, such as Carbonicillin, which affects mainly gram-negative organisms. Sensitivity to ampicillin, a β-lactam antibiotic that affects gram-negative organisms, was tested, but all organisms showed complete resistance to it, so the sample was probably ineffective.

The positive results for *B. amyloliquefaciens KLH* for the exoenzymes amylase and caseinase indicate that the organism is capable of hydrolyzing starch and casein, which is commonly found in milk. The negative results for DNase, gelatinase, and tween hydrolysis indicate that the organism cannot hydrolyze DNA, gelatin, or tween. Since *A. hydrophila JAH* showed positive results for all of the exoenzyme tests, the organism is capable of hydrolyzing starch, casein, DNA, gelatin, and tween.

The growth of *A. hydrophila JAH* on the bile esculin plate indicates that the organism is resistant to bile, while the black color indicates that it can hydrolyze esculin to produce iron salts. The growth on the Macconkey agar supports that the organism is gram-negative. This is also supported by the fact that the organism did not grow on the phenylethyl alcohol agar, which selects for gram positive organisms.

According to the Biolog results, *A. hydrophila JAH* utilizes glucose, mannitol, and galactose. All of these tested negative for acid or gas production in the metabolism tests of *A. hydrophila JAH*, but it is possible that the organism is capable of utilizing these carbohydrate sources without producing acid or gas. In other words, the organism probably utilizes these sources in a way other than fermentation, such as respiration. This is supported by the fact that most *A. hydrophila* strains are capable of utilizing glucose, mannitol, and galactose. The Biolog results also show sensitivity to the antibiotic nalidixic acid, which is inconsistent with the results of the Kirby Bauer test. In the Kirby Bauer test, the zone of inhibition for nalidixic acid was 0.0 mm, indicating resistance.

Also according to the Biolog results, *A. hydrophila JAH* is capable of growing in 1% and 4% NaCl concentrations, but not 8%. This, along with the fact that the organism did not grow on the mannitol salt agar, means that the organism thrives most at low concentrations of salt. *A. hydrophila JAH* grew in the well that was pH 6, but not pH 5, meaning the organism probably grows best at a neutral pH, and growth decreases with acidity.

B. amyloliquefaciens is significant because it is capable of producing Poly-γ-glutamic acid, an extracellular secretion often used in medicine, cosmetics, food, and

wastewater treatment. Most organisms that produce Poly-γ-glutamic acid are dependent on glutamic acid. However, *B. amyloliquefaciens* is not, which can lead to lower production costs of Poly-γ-glutamic acid. *B. amyloliquefaciens* also produces a well-known restriction enzyme, BamHI. While *B. amyloliquefaciens KLH* has not been sequenced, other strains of the same species, such as *B. amyloliquefaciens LLC*, have been sequenced. These sequences show that *B. amyloliquefaciens* organisms generally do not have many metabolic genes, which may help to explain why *B. amyloliquefaciens KLH* did not show much growth on the selective media plates (Weito *et al*., 2011).

A. hydrophila subsp. *ranae* was isolated from septicaemic frogs in Thailand, and was found to be the cause of infection in these frogs. All the isolates had the ASH1 gene, which causes the organisms to display hemolytic properties. Also of note, while *A. hydrophila* subsp. *ranae* was found to be destructive to fish cells, it appeared to have little effect on mammalian cells. This makes sense as the organism *A. hydrophila JAH* came from a creek where fish, frogs, and other cold-blooded organisms live (Huys *et al.,* 2003). The genome sequence for organism *Aeromonas hydrophila ATCC 7966* is the only complete sequence for an *A. hydrophila* strain available on JGI. Some notable genes are those that provide resistance to toxic substances that may be found in polluted waters (Seshadri *et al.,* 2006). This allows for better survival for these organisms as they are mainly found in water or in animals that live in water.

References:

Abbott, S. L., W. K. W. Cheung, and J. M. Janda. (2003). The genus *Aeromonas*: Biochemical Characteristics, Atypical Reactions, and Phenotypic Identification Schemes. ***Journal of Clinical Microbiology.*** 41(6): 2348.

"Analytical Profile Index." *http://www.biotech.univ.gda.pl/odl/biochem/api.html.* 19 Apr. 2012. Intercollegiate Faculty of Biotechnology for University of Gdańsk - Medical University of Gdańsk.

Arcos, M. L., A. deVicente, M. A. Morinigo, et al. (1988). Evaluation of Several Selective Media for Recovery of *Aeromonas hydrophila* from Polluted Waters. ***Applied and Environmental Microbiology.*** 54(11): 2786-2792.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259-2261.

Huys, G, M. Pearson, P. Kämpher, *et al.* (2003). *Aeromonas hydrophila* subsp. *Ranae* subsp. nov. Isolated from Septicaemic Farmed Frogs in Tailand. ***International Journal of Systematic and Evolutionary Microbiology.*** 53: 885-891.

"Phenotype Microarray Services: A Breakthrough Technology." *http://www.biolog.com/products/?product=Phenotype%20MicroArray%20Services.* 19 Apr. 2012. Biolog: Manufacturer of the software.

Priest, F. G., M. Goodfellow, L. A. Shute, and R.C.W. Berkeley. (1987). *Bacillus amyloliquefaciens* sp. nov., nom. rev. ***International Journal of Systematic Bacteriology.*** 37(1): 69-71.

Seshadri, R, S. W. Joseph, A. K. Chopra, *et al.* (2006). Genome Sequence of *Aeromonas hydrophila* ATCC 7966: Jack of All Trades. ***Journal of Bacteriology.*** 188 (23): 8272-8282.

"Sherlock Microbial ID System." *http://www.midi-inc.com/pages/microbial_id.html.* 19 Apr. 2012. MIDI: Manufacturer of the software.

Weitao, G., M. Cao, C. Song, H. Xie, L. Liu. (2011). Complete Genome Sequence *of Bacillus amyloliquefaciens* LL3, Which Exhibits Glutamic Acid-Independent Production of Poly-γ-Glutamic Acid. ***Journal of Bacteriology***. 193(13): 3393–3394.

Willey, J. M., L. M. Sherwood, and C. J. Woolverton. "**Prescott's Microbiology – 8th ed."** McGraw-Hill, New York, NY, 2011.

Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.