Appendix 1

Material

Samples

Water and sediment were collected from Kuala Sepetang, Taipain, Malaysia. Water and sediment samples were collected in eight different stations from Kuala Sepetang, Kuala Sangga Besar and Kuala Selinsing river of Matang mangrove estuarine.

Media

All the media used for this study were obtained Oxoid Ltd, England and CHROMagar Ltd, France.

General media

Nutrient agar

Yeast extraction	2 g
Peptone	5g
Sodium Chloride	5g
Agar	15g
Distilled water	1000ml

(PH=7.4 ± 0.2)

The formula for preparation is 28g/l. The medium is needed to be autoclaved before using.

Luria Bertani Broth

Yeast extraction	0.5g
Sodium Chloride	0.5g
Tryptone	1g
Distilled water	100ml

PH=7.5

The ingredients were mixed and boiled till 100C˚and autoclaved.

Luria-Bertani Agar

Yeast extraction	0.5g
Sodium chloride	0.5g
Tryptone	1g
Agar	1.5g
Distilled water	100ml

PH=7.5

The ingredients were mixed and boiled till 100C˚and autoclaved.

Enrichment Media

Buffered Peptone Water (BPW)

Peptone	10g
Sodium chloride	5g
Di-Sodium phosphate	3.5g
Potassium dihydrogen phosphate	1.5g
Distilled water	1000ml

The medium was boiled and autoclaved before using

Selective and Differentiated Media

CHROM ^{TM} orientation

Agar	15g
Yeast Extract	17g
Chromogenic Mix	1g
Distilled water	1000ml

PH= 7±2

The proportion of 33g/l was prepared using distilled water. The medium was boiled and autoclaved before using.

CHROMagar^{TM} ECC

Agar	15g
Peptone and Yeast extract	8g
NaCl	5g
Chromogenic Mix	4g
Distilled water	1000ml

PH= 7.2±0.2

The proportion of 32.8g/l was prepared using distilled water. The medium was boiled and used without autoclaving

MacConky

Peptone	20g
Lactose	10g
Bile salt	5g
Sodium chloride	5g
Neutral red	0.075g
Agar	12g

Distilled water	1000ml

PH=7.5±0.2

The proportion of 52.075% g/l was prepared using distilled water. The medium was boiled and autoclaved before using.

Eosin-Methylene Blue (EMB)

Peptone	10g
Sucrose	10g
Lactose	5g
Dipotassuim phosphate	2g
Eosine Y	0.4g
Methylene Blue	0.065g
Agar	14g
Distilled water	1000ml

PH=7.1

The proportion of 36.4 g/l was prepared using distilled water. The medium was boiled and autoclaved before using

Material Biochemical Test

Gram staining

Glass slide

Reagents

Crystal Violet dye

Iodine

Ethanol 95%

Safranin

Water

Oxidase test

N, N, N’, N’ –tetra methyl-p-phenylenediamine dihydrochloride (7.95%) powder

MR-VP

Peptone	7g
Glucose	5g
Phosphate buffer	5g
Distilled water	1000ml

PH 6.9

The proportion of 17g/l was prepared using distilled water. The medium was boiled and autoclaved before using

Methyl Red reagent

Methyl Red	0.1g
95% ethyl alcohol	300 ml
Distilled water	500ml

Kovacs reagent

Isoamyl alcohol	150 ml
Concerntated Hydrochloric Acid	50 ml
ρ-dimethylaminobenzaldehyde	10 g

Alpha Napthol solution

Purified a- naphthol	5 g
Ethyl alcohol	100 ml

Sulfide Indole Motility (SIM)

Tryptone	20g

Peptone	6.1g
Ferric Ammonium Sulphate	0.2g
Sodium Chloride	0.2g
Agar	3.5g
Distilled water	1000ml

PH: 7.3 ± 0.2

The proportion of 30g/l was prepared using distilled water. The medium was boiled and autoclaved before using

Simmons Citrate

Magnesium sulfate	0.2 g
Monoammonium phosphate	1 g
Dipotassium phosphate	1g
Sodium citrate	2g
Sodium chloride	5g
Bromthymol Blue	0.08 g
Agar	15 g
Distilled water	1000ml

PH= 6.9 ± 0.2

The proportion of 24.28g/l was prepared using distilled water. The medium was boiled and autoclaved before using

H_2O_2 3%

Hydrogen peroxide	30g
Distilled water	1000ml

KOH 3%

Potassium hydroxide	30g
Distilled water	1000ml

3.1.3.11. KOH 40%

Potassium hydroxide	40 g
Distilled water	100 ml

Other reagent

Saline Buffred water (0.85%)

NaCl	0.85g
Distilled water	1000ml

Alcohol 70% for 500 mL (from 95%)

95% alcohol	395ml
Distilled water	132ml

PCR Material

Oligonucleotide primer

The oligonucleotide primers used in this study for PCR assay and the expected size for PCR product are listed in Table 1. phoA primer was used to detect E.coli housekeeping gene in monoplex PCR assay.

Chemicals and Enzymes

All the PCR enzymes and chemicals used in this were purchased from Promega Corporation, USA.

Colourless and Green GoTaq® Flexi Buffer	5x
Deoxynucleotide triphosphates (dNTPs)	10mM
Magnesium Chloride (MgCl2)	25mM

Taq polymerase (GoTaq® DNA polymerase)	5U/1µl

Material for Agarose GEL Electrophoresis

Agarose Gel (1.5%)

Agarose gel	1.5 g
0.5x TBE buffer	100ml

Gel Loading Dye, 100bp DNA ladder and 1kb DNA ladder

6x Loading Dye, 100bp DNA ladder and 1kb DNA ladder were purchased from Promega Corporation, USA.

10x Tris Borate EDTA Buffer (TBE)

Boric acid	61.8 g
Tris	121.2 g
Na2EDTA.2H2O	0.745 g
Distilled water	1000ml

The solution PH was adjusted to 8.3. 50ml of 10 x TBE was then diluted into 950ml of distilled water for preparing 0.5x TBE.

Ethidium Bromide

Ethidium Bromide	100 mg
Deionised water	10ml

The solution was stored in a dark bottle at room temperature and diluted to 0.5µg/ml with distilled water before use.

Other reagent

Phosphate buffered saline (PBS)

PBS tablets	10
Distilled water	1000ml

The solution pH was adjusted to 8.3.

Tris-EDTA (TE) buffer

1M Tris	10 ml
0.5M EDTA	2 ml
Distilled water	1000 ml

The solution pH was adjusted to 8

Mater mixture for PCR experiment

Table 1.1. The PCR conditions for monoplex PCR

Material	Stock concentration	Working concentration	Volume (µl)
Green buffer	5x	1x	5.00
$MgCl_2$	25mM	1mM	1.00
dNTPs	10mM	140 µM	0.35
PhoA F	10mM	0.1 µM	0.25
PhoA R	10mM	0.1 µM	0.25
Taq polymerase	5U/ µl	0.5U	0.10
Template			5.00
ddH_2O			11.925
Total volume (µl)			25.00

Table 1.2. PEP-PCR Mater mixture

Material	Stock concentration	Working concentration	Volume (µl)
Colorless buffer	5x	1x	5.00
$MgCl_2$	25mM	2.5mM	2.5
dNTPs	10mM	200 µM	0.5
REP-Primer	10mM	0.5 µM	1.25
Taq polymerase	5U/ µl	1U	0.20
Template			5.00
ddH_2O			10.55
Total volume (µl)			25.00

Table 1.2. Mater mixture condition for 16S rDNA

Material	Stock concentration	Working concentration	Volume (µl)
Green buffer	5x	1x	5.00
$MgCl_2$	25mM	1mM	1.00
dNTPs	10mM	140 µM	0.35
PhoA F	10mM	0. 08 µM	0.2
PhoA R	10mM	0.08 µM	0.2
Taq polymerase	5U/ µl	0.5U	0.10
Template			3.00
ddH_2O			15.15
Total volume (µl)			25.00

Appendix 2

REP-PCR gel using REP-PCR primer

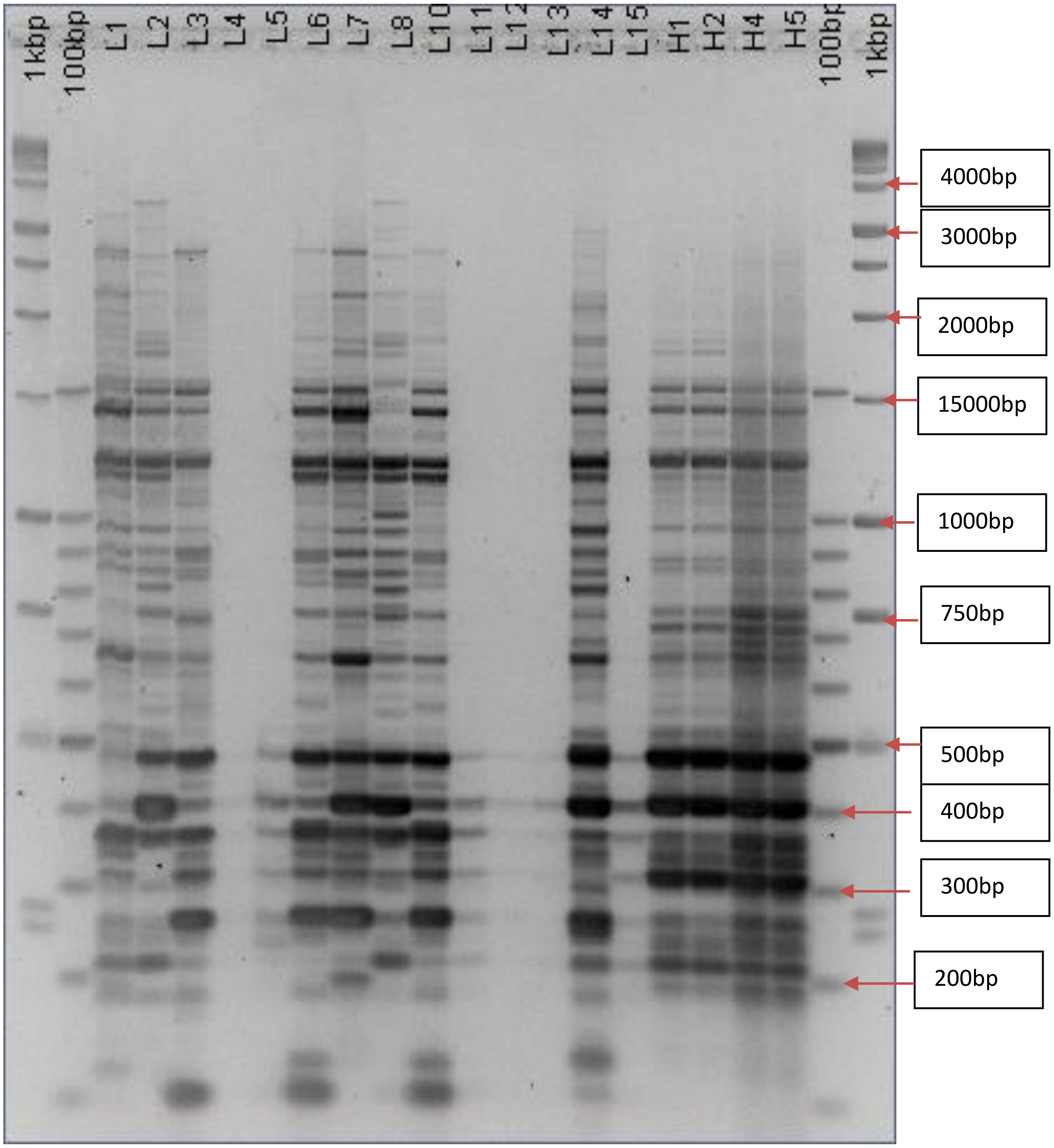


Fig1. Rep-PCR gel, L1, L2, L3, L4, L5, L6, L7, L8, L10, L11, L12, L13, and L15 strains were isolated from station H; H1, H2, H4 and H5 were isolated from station E.

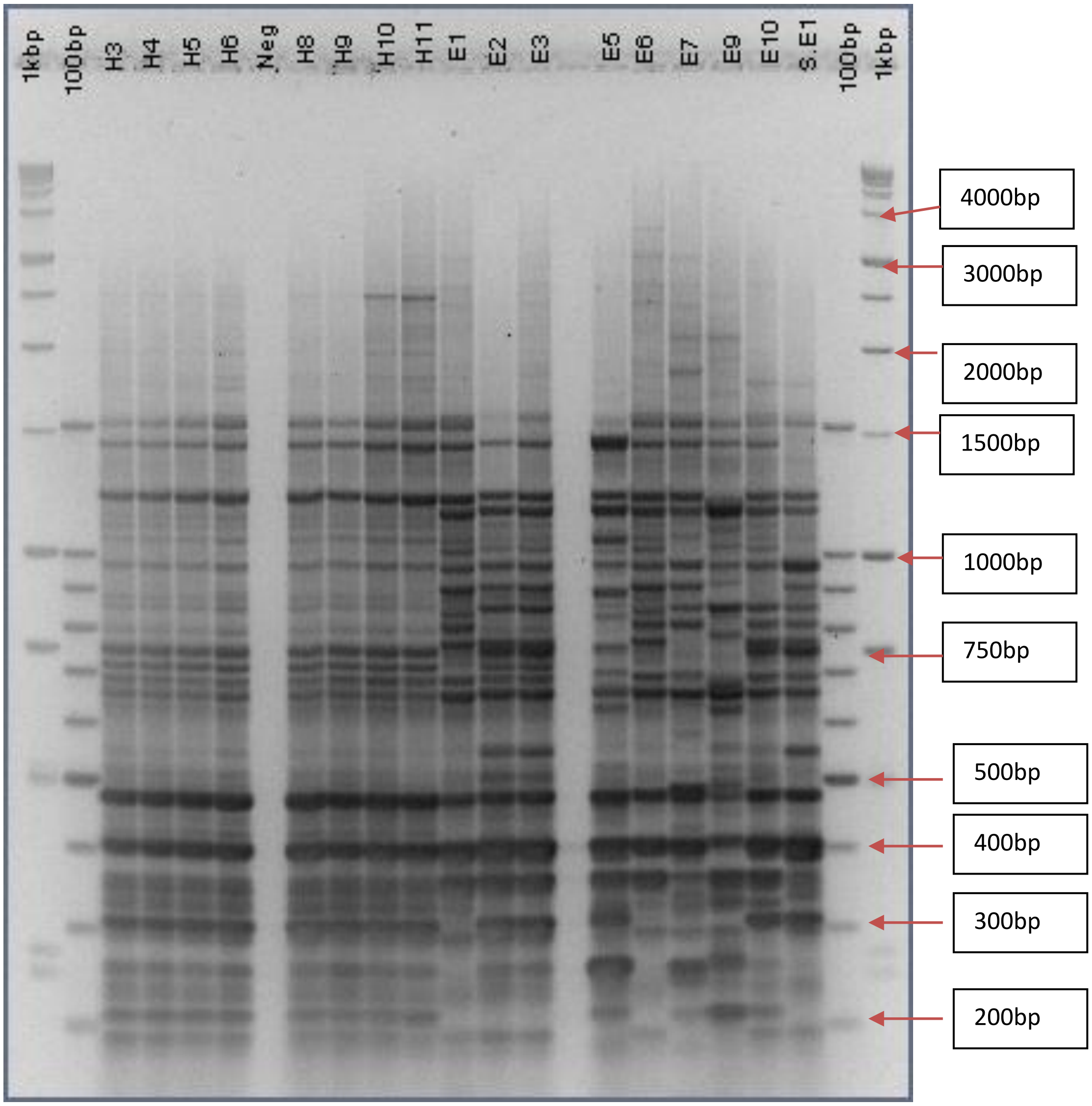
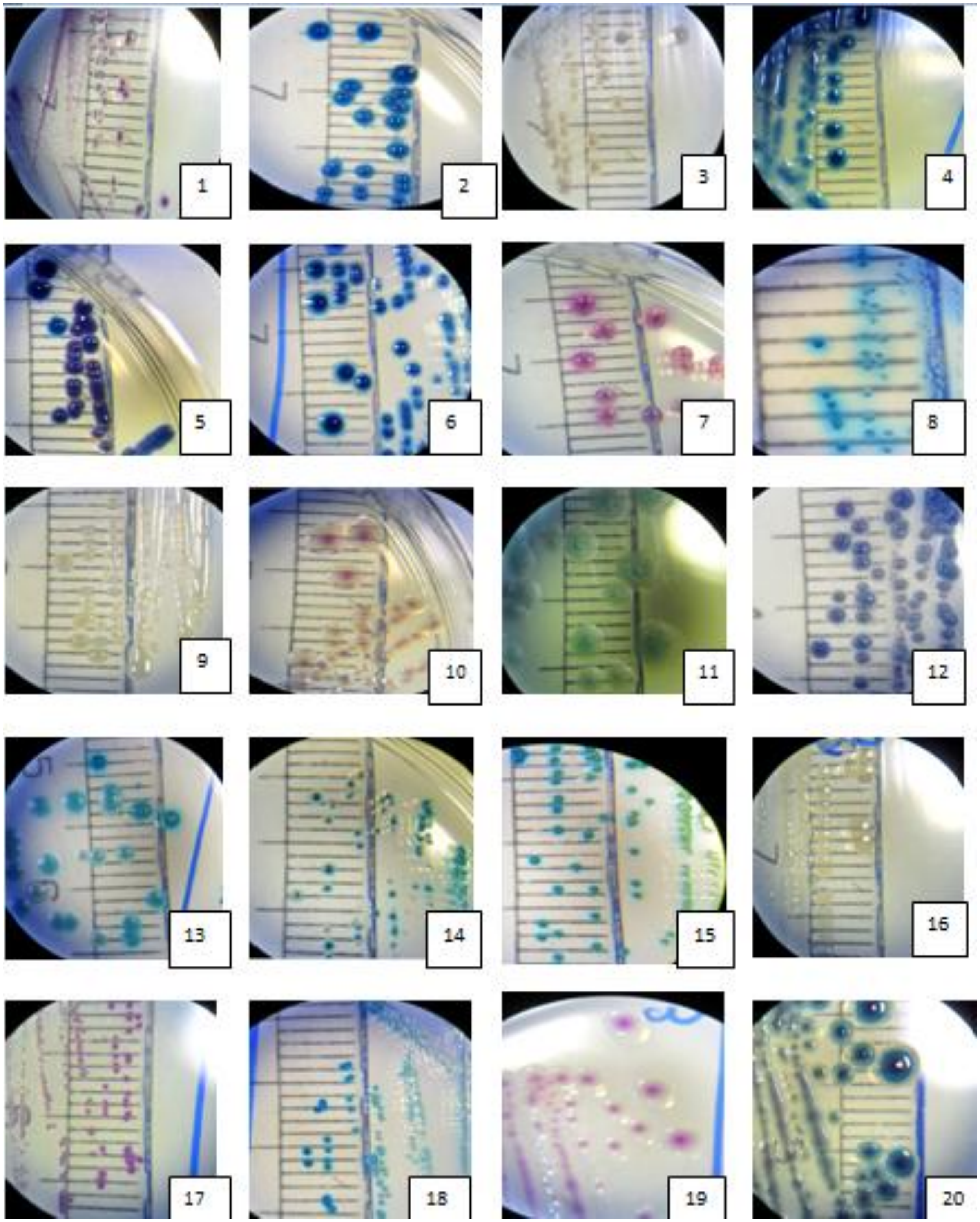


Fig 2. Rep-PCR gel, H3, H4, H5, H6, H8, H9, H10, and H11 were isolated from station E; E1, E2, E3, E5, E6, E7, E9, E,10 and S.E1 strains were isolated from station D.

Appendix 3

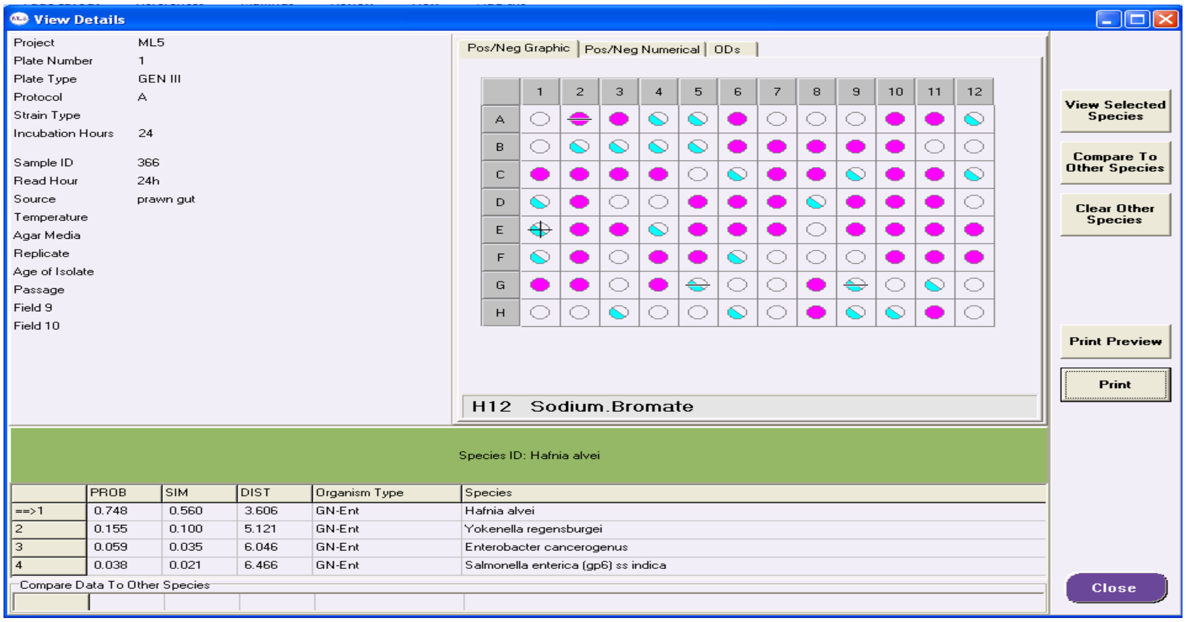
Some pictures that have been taken during sampling time in Kula Sepetang estuary

Appendix 4



1,*C. Koseri*; 2,*E. aerogenes*; 3,*H.alive*; 4,*K. Oxytoca*; 5, *S. marcescens*; 6,*R. Planticola*; 7,*E.coli*; 8,*Entrococcus casseliflavus*; 9,*Acinetobactor johansonii*; 10,*Aeromonas sp*; 11,12,13,*Bacillis sp*; 14,*Exigubacteria sp*; 15,*Paenibacillus papillae*; 16,*Pesudomonase sp*, 17,*Staphylococcus sciuris* and ,S. *hominis ss hominis* and *S.saprophyticus*; 18, *Stenotrophomonas rhizophila*; 19,*Vibrio sp*; 20, *Vibrio sp* at room temperature

The color wheel



Project ML5
Plate Number 1
Plate Type GEN III
Protocol A
Strain Type
Incubation Hours 24

Sample ID 366
Read Hour 24h
Source prawn gut
Temperature
Agar Media
Replicate
Age of Isolate
Passage
Field 9
Field 10

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	●	●	◐	◐	●	○	○	○	●	●	◐
B	○	◐	◐	◐	◐	●	●	●	●	●	○	○
C	●	●	●	●	○	◐	●	●	◐	●	●	◐
D	◐	●	○	○	●	●	●	◐	●	●	●	○
E	◐	●	●	◐	●	●	●	○	●	●	●	●
F	◐	●	○	●	●	◐	○	○	○	●	●	●
G	●	●	○	●	◐	○	○	●	◐	○	◐	○
H	○	○	◐	○	○	◐	○	●	◐	◐	●	○

H12 Sodium.Bromate

Species ID: Hafnia alvei

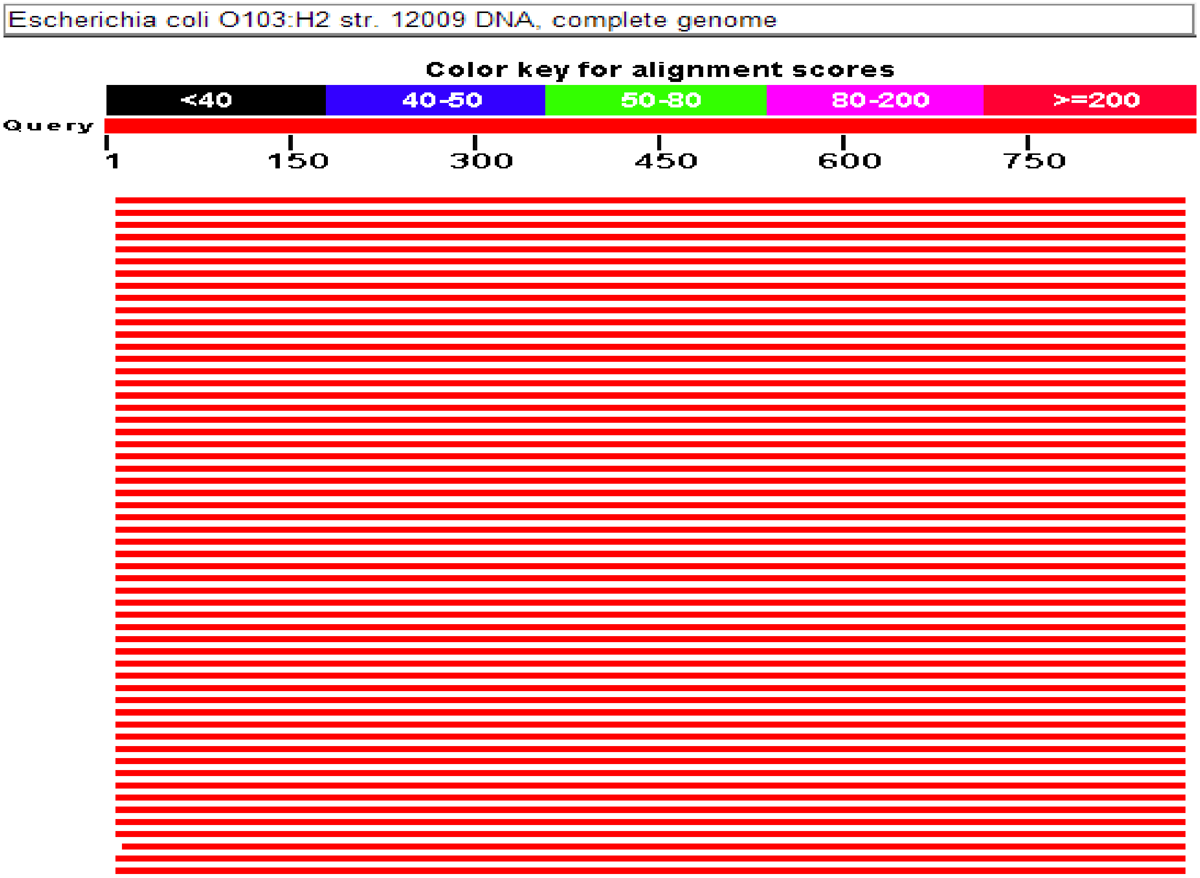
	PROB	SIM	DIST	Organism Type	Species
==>1	0.748	0.560	3.606	GN-Ent	Hafnia alvei
2	0.155	0.100	5.121	GN-Ent	Yokenella regensburgei
3	0.059	0.035	6.046	GN-Ent	Enterobacter cancerogenus
4	0.038	0.021	6.466	GN-Ent	Salmonella enterica (gp6) ss indica

Compare Data To Other Species

Biochemical profile using Biolog Gen III to identify *Hafnia alvei*

Appendix 5

E. coli; phoA BLAS sequencing result (*phoA* chromatogram result)



E. coli; phoA BLAS sequencing result

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Sequences producing significant alignments:						
Description	Max score	Total score	Query cover	E value	Max ident	Accession
Escherichia coli O103:H2 str. 12009 DNA, complete genome	1568	1568	97%	0	99%	AP010958.1
Escherichia coli HS, complete genome	1568	1568	97%	0	99%	CP000802.1
Escherichia coli W, complete genome	1563	1563	97%	0	99%	CP002967.1
Escherichia coli KO11, complete genome	1563	1563	97%	0	99%	CP002516.1
Escherichia coli W, complete genome	1563	1563	97%	0	99%	CP002185.1

Enterococci 16S r RNA BLAST sequencing result

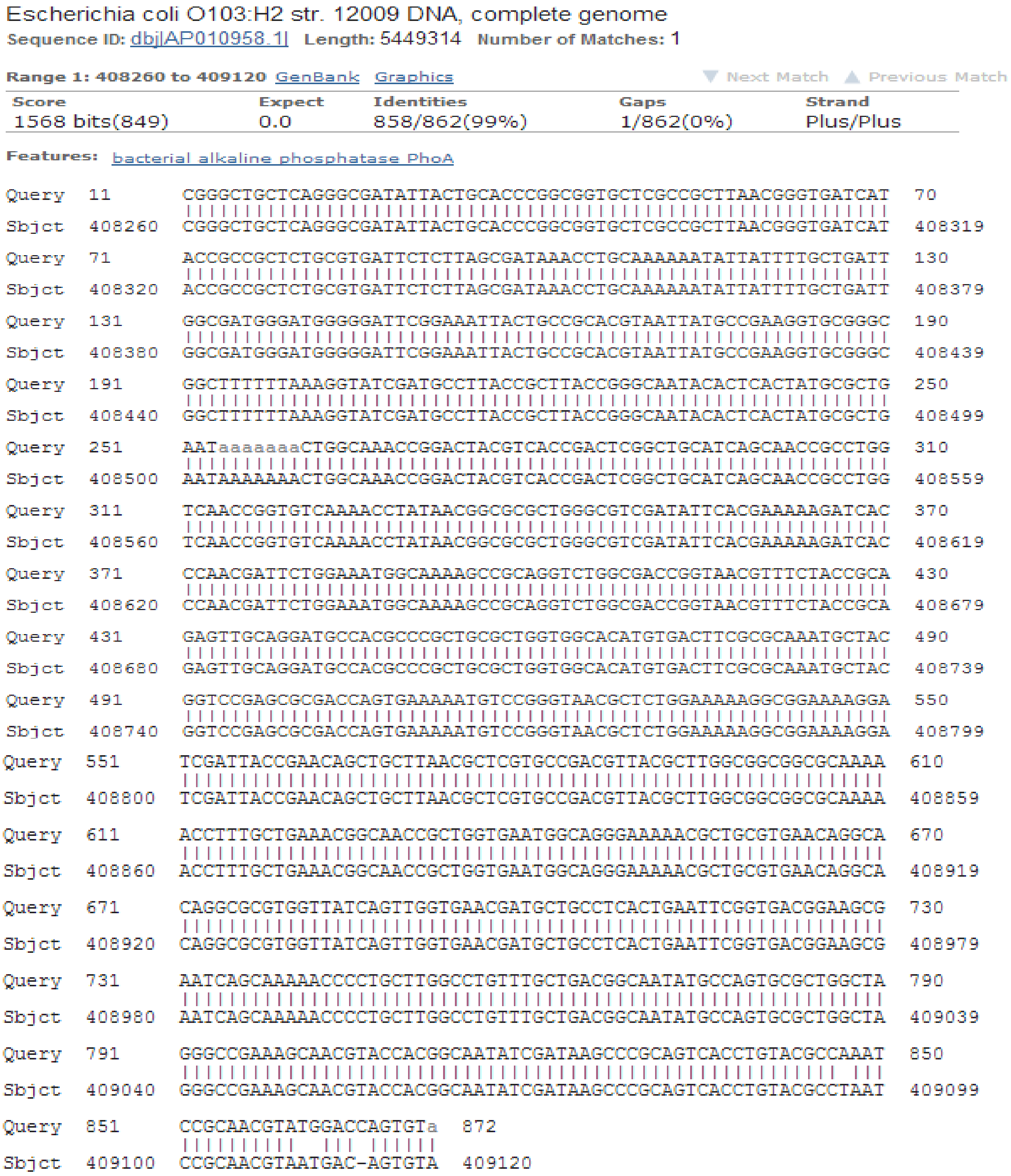
Sequences producing significant alignments:						
Sequences producing significant alignments:						
Description	Max score	Total score	Query cover	E value	Max ident	Accession
Enterococcus casseliflavus gene for 16S ribosomal RNA, partial sequence, strain: CN1	990	990	97%	0	99%	AB699730.1
Enterococcus casseliflavus gene for 16S rRNA, partial sequence, strain: C507	990	990	97%	0	99%	AB618809.1
Enterococcus casseliflavus gene for 16S rRNA, partial sequence, strain: RZC110	989	989	97%	0	99%	AB671565.1
Enterococcus casseliflavus EC20, complete genome	987	4925	98%	0	99%	CP004856.1
Enterococcus casseliflavus strain Z6006 16S ribosomal RNA gene, partial sequence	987	987	98%	0	99%	KC212047.1

Klebsiella; 16 S r RNA BLAST sequencing result

Sequences producing significant alignments:						
Sequences producing significant alignments:						
Description	Max score	Total score	Query cover	E value	Max indent	Accession
Klebsiella sp. SR1.6 16S ribosomal RNA gene, partial sequence	1000	1000	99%	0	99%	JQ912555.1
Bacterium 5-2(2013) 16S ribosomal RNA gene, partial sequence	1000	1000	99%	0	99%	KC753506.1
*Klebsiell*a sp. SR-143 16S ribosomal RNA gene, partial sequence	1000	1000	99%	0	99%	KC455430.1
Klebsiella sp. ZH-08 16S ribosomal RNA gene, partial sequence	1000	1000	99%	0	99%	KC166142.1
Klebsiella sp. T-4 16S ribosomal RNA gene, partial sequence	1000	1000	99%	0	99%	KC109001.1

Enterobacter BLAST 16S r RNA sequencing result

Sequences producing significant alignments:						
Sequences producing significant alignments:						
Description	Max score	Total score	Query cover	E value	Max ident	Accession
Enterobacter aerogenes KCTC 2190 strain KCTC 2190 16S ribosomal RNA, complete sequence	990	990	97%	0	99%	AB699730.1
Enterobacter aerogenes strain RB21 16S ribosomal RNA gene, partial sequence	990	990	96%	0	99%	KC431799.1
Enterobacter aerogenes strain ATCC 13048 16S ribosomal RNA gene, partial sequence	990	990	96%	0	99%	KC429778.1
Enterobacter aerogenes EA1509E complete genome	990	7842	96%	0	99%	FO203355.1
*Enterobacte*r sp. B1(2012) 16S ribosomal RNA gene, partial sequence	990	990	96%	0	99%	JQ886667.1
Enterobacter aerogenes strain KNUC5009 16S ribosomal RNA gene, partial sequence	990	990	96%	0	99%	JQ682634.1



Escherichia coli O103:H2 str. 12009 DNA, complete genome

Sequence ID: dbj|AP010958.1| Length: 5449314 Number of Matches: 1

Range 1: 408260 to 409120 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1568 bits(849)	0.0	858/862(99%)	1/862(0%)	Plus/Plus

Features: bacterial alkaline phosphatase PhoA

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Query  71      ACCGCCGCTCTGCGTGATTCTCTTAGCGATAAACCTGCAAAAAATATTATTTTGCTGATT  130
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Query  191     GGCTTTTTTAAAGGTATCGATGCCTTACCGCTTACCGGGCAATACACTCACTATGCGCTG  250
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Sbjct  408440  GGCTTTTTTAAAGGTATCGATGCCTTACCGCTTACCGGGCAATACACTCACTATGCGCTG  408499

Query  251     AATaaaaaaaCTGGCAAACCGGACTACGTCACCGACTCGGCTGCATCAGCAACCGCCTGG  310
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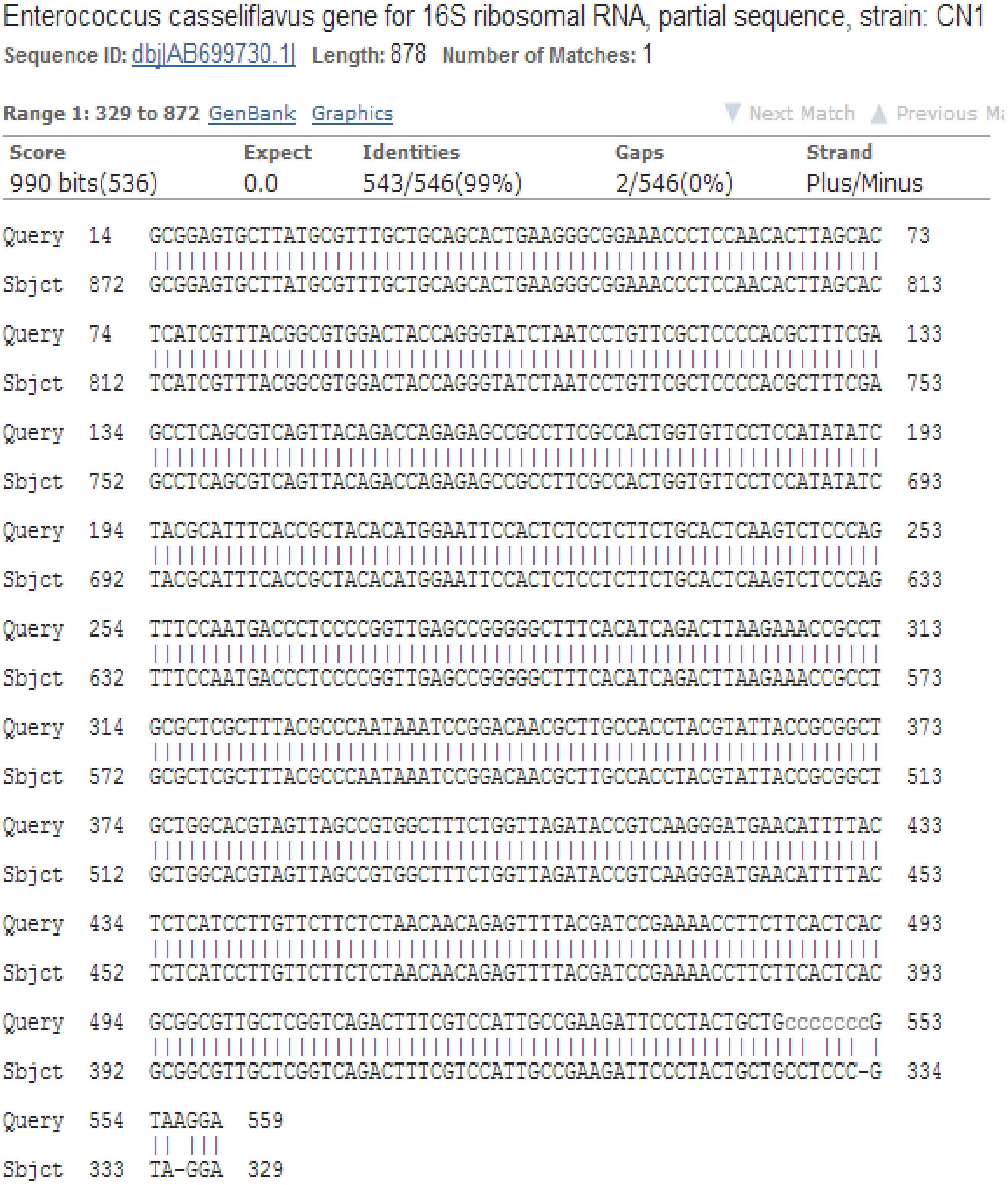
Alignment of *Escherichia coli O103:H2* str. 12009 DNA, complete genome



Enterococcus casseliflavus gene for 16S ribosomal RNA, partial sequence, strain: CN1

Sequence ID: dbj|AB699730.1| Length: 878 Number of Matches: 1

Range 1: 329 to 872 GenBank Graphics

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Alignment of *Enterococcus casseliflavus* gene for 16S ribosomal RNA, partial sequence, strain: CN1

Klebsiella sp. SR-143 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC455430.1| Length: 1440 Number of Matches: 1

Range 1: 316 to 852 GenBank Graphics

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Query  364  ACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACAA  423
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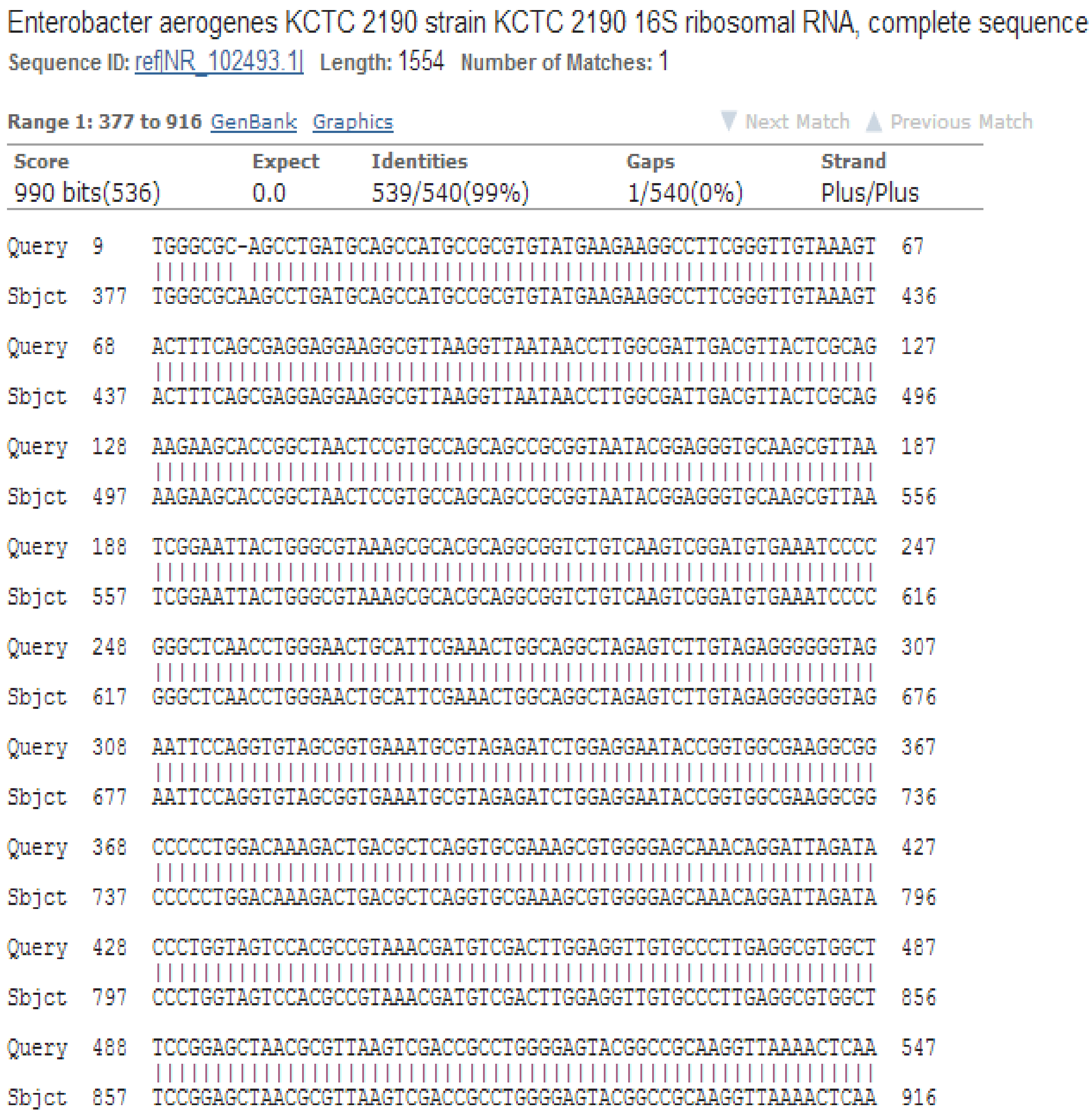
Alignment of *Klebsiella sp*. SR1.6 16S ribosomal RNA gene, partial sequence



Enterobacter aerogenes KCTC 2190 strain KCTC 2190 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_102493.1| Length: 1554 Number of Matches: 1

Range 1: 377 to 916 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
990 bits(536)	0.0	539/540(99%)	1/540(0%)	Plus/Plus

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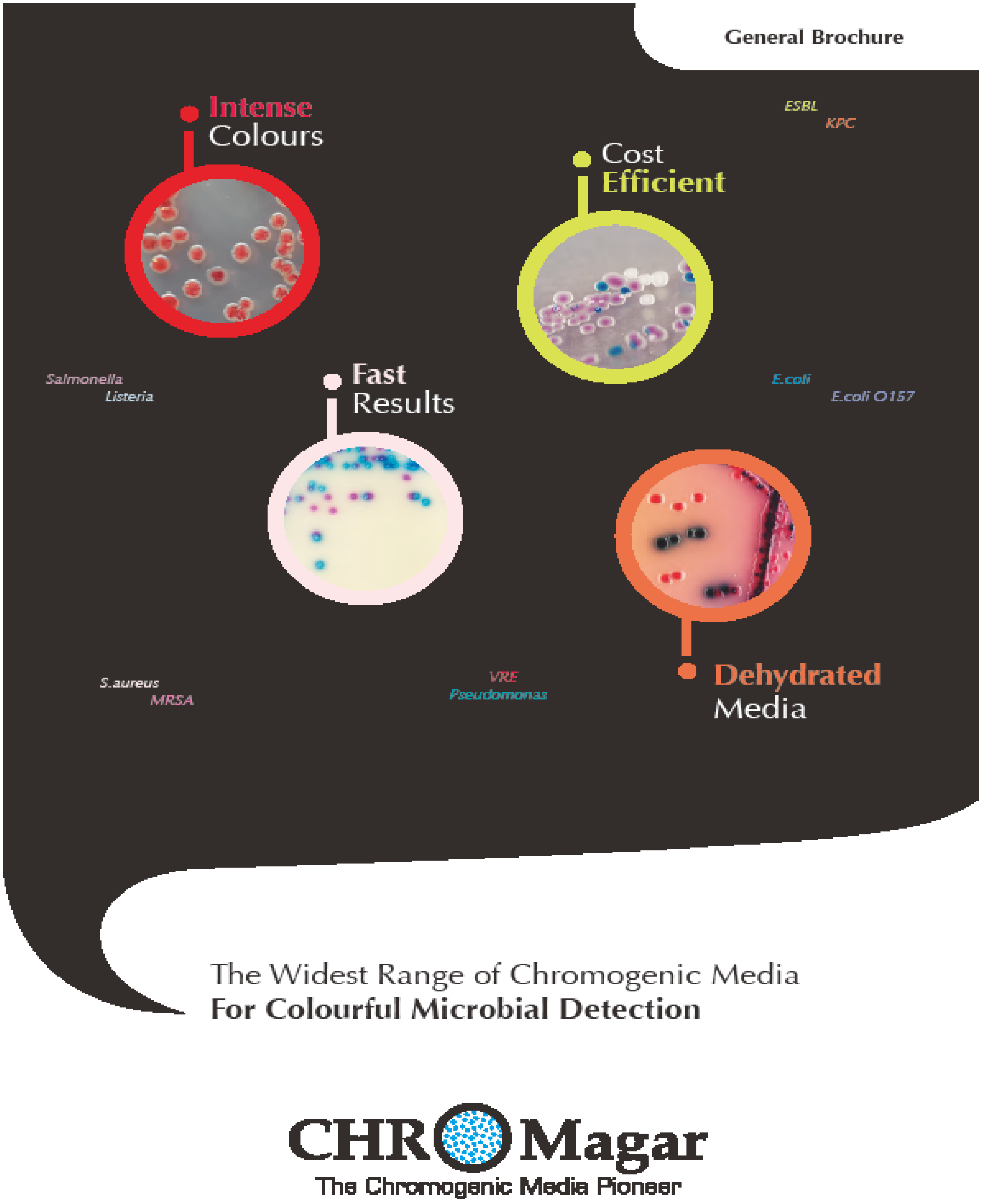
Alignment of *Enterobacter aerogenes* KCTC 2190 strain KCTC 2190 16S ribosomal RNA, complete sequence

Appendix 6

CHROMagar brochure



General Brochure

Intense Colours

Cost Efficient

Fast Results

Dehydrated Media

ESBL
KPC

Salmonella
Listeria

E.coli
E.coli O157

S.aureus
MRSA

VRE
Pseudomonas

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For Colourful Microbial Detection

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The Chromogenic Media Pioneer