Identification of *Vibrionaceae* from Australian aquatic animals using phenotypic and PCR procedures

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SUMMARY

The *Vibrionaceae* is a large and complex group of marine bacteria that can have a significant impact on the health of aquatic animals. A range of pathogenicity is seen among the species but a consistent feature is the opportunistic basis of infection. Nearly all phases of farm production are affected from larval rearing to competent adult animals. Disease outbreaks may occur in disparate animal groups including marine mammals, fin fish, crustacea, molluscs and zooxanthellae of coral. Some strains, however, can act as probionts and have proved effective as a means of controlling disease caused by other species of *Vibrionaceae*.

Identification: Routine, high-volume identification is achieved by phenotyping using standardised tests. To accommodate the large number of taxa and the phenotypic diversity that exists intra-species, identification is only practicable using computer-assisted probabilistic methods. The use of molecular tools for identification remains limited but PCR for several species is useful as a means of rapid screening or confirmatory identification.

Status of Australia and New Zealand: The range of *Vibrionaceae* associated with aquatic animals in Australia is relatively small despite the diversity of habitats, geographic range and climatic variation. Major pathogens encountered are *Photobacterium damselae* ssp. *damselae*, *Vibrio anguillarum* and *Vibrio harveyi*. More unusual species isolated are *Vibrio scophthalmi* (Atlantic salmon), *Vibrio penaeicida* (southern rock lobster) and *Photobacterium damselae* ssp. *piscicida* (southern blue fin tuna). These species are rarely encountered and appear to be incidental findings not associated with disease.

Part 1. Diagnostic Overview

Introduction

The *Vibrionaceae* is an important and ubiquitous group of bacteria in marine and estuarine environments.[1] Characteristically they have a close association with aquatic animals either
35 as symbionts, normal flora or as pathogens. The ability of the group to occupy a wide diversity of habitats means that the *Vibrionaceae* have direct and indirect affects on aquaculture either as pathogens that are the cause of major disease, low grade infections that erode productivity or beneficial effects as probionts like some strains of *Vibrio alginolyticus*[2] or *V mediterranei*.[3] To varying degrees, *Vibrionaceae* have an intimate
40 relationship with all farmed marine species of aquatic animals from live feed inputs such as *Artemia* and rotifers, crustaceans, bivalved and single shelled molluscs to fin fish. The myriad of types, the range of species and their diverse role in aquaculture make the *Vibrionaceae* an important but challenging group of bacteria.

Taxonomy

45 The *Vibrionaceae* comprise six genera: *Aliivibrio, Enterovibrio, Grimontia, Photobacterium, Salinivibrio* and *Vibrio.* The genus *Listonella* has also been proposed and although a validly published name, there is now little evidence to justify its status[4], and use of the name is declining. Related to the *Vibrionaceae* is the family *Moritellaceae* and includes *M. marina* and *M. viscosa*, species pathogenic for salmonids. The taxonomy of the
50 *Vibrionaceae* is based largely on phylogenetic analysis of small subunit ribosomal DNA, in particular, the 16S rDNA gene sequence, latterly supported by fluorescent amplified fragment length polymorphism (FAFLP) data.[5,6] FAFLP has proved particularly useful as a means of species delineation because of the high level of 16S rDNA sequence similarity that exists between many species of the *Vibrionaceae*. Currently some 98 species across the five
55 genera are recognised; most of the species occur in the genus *Vibrio*. Phenotypes, when based on numerical taxonomy, are generally useful and predictive[7,8] but where kit systems have been used to define species, invariably descriptions are poor and insufficient for the purpose of identification. There is marked genomic plasticity within the *Vibrionaceae,* which is seen as pronounced phenotypic diversity between and within species.[9,10] As a
60 consequence, finding traits that differentiate species continues to be a problem, particularly those delineated by molecular taxonomy.

Disease in Aquatic Animals

Disease involving *Vibrionaceae* is characterised by the opportunistic basis of infection. The pathogen may have an intimate association with the host as normal flora and may exhibit its
65 pathogenic capacity if host defences are breached. This may occur either from stress events that lead to immunosuppression, physical damage to the integument, or the emergence of aggressive biovars within a population of aquatic animals. Severity of disease may range from fulminating septicaemias, typical of disease outbreaks, through to chronic infections that affect just a few individuals.

70 Species of *Vibrionaceae* have an obligate requirement for NaCl[11] that ranges from as little as 5-15mM for *V cholerae* and *V metschnikovii* to 300-400 mM for *V splendidus*[12,13]. A critical factor for potential pathogens is the coincidence of physiologies between host and pathogen. For osmoregulators such as fin fish, pathogens must have an optimum NaCl requirement close to physiological levels of vertebrates while for osmoconformers such as molluscs and
75 crustacea, pathogens have optimum NaCl concentrations close to that of seawater. Extracellular pathogenicity factors such as siderophores, haemolysins, cytotoxins and proteases have been described in several species of *Vibrionaceae* and appear to be important in expression of disease.[14,15,16]

Host Range

80 Development of an exhaustive list of Australian host species serves little purpose. More importantly, cognisance of the range of host types is central to obtaining an understanding of the pathogenic versatility of the *Vibrionaceae*. In nearly every group of marine species, examples can be found of *Vibrionaceae* acting as pathogens. Major groups affected are: marine mammals, teleost fish, crustacea, molluscs, both univalve and bivalve, and plants
85 represented by the zooxanthellae of coral.[17]

Species Enzootic in Australia

Listing *Vibrionaceae* known to occur in Australia provides a guide to species that have been found associated with aquatic animals, Table 1. Not all the species listed were found as pathogens.

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Table 1. *Vibrionaceae* enzootic in Australia

Aliivibrio fischeri	*Vibrio mediterranei*
Photobacterium damselae ssp. *damselae*	*Vibrio mimicus*
Photobacterium damselae ssp. *piscicida*	*Vibrio mytili*
Photobacterium iliopiscarium	*Vibrio natriegens*
Vibrio alginolyticus	*Vibrio navarrensis*
Vibrio anguillarum	*Vibrio nereis*
Vibrio chagasii	*Vibrio parahaemolyticus*
Vibrio cholerae non-O1	*Vibrio pelagius*
Vibrio cyclitrophicus	*Vibrio penaeicida*
Vibrio diazotrophicus	*Vibrio proteolyticus*
Vibrio furnissii	*Vibrio scophthalmi*
Vibrio fluvialis	*Vibrio splendidus* biovar I
Vibrio halioticoli	*Vibrio tasmaniensis*
Vibrio harveyi	*Vibrio tubiashii*
Vibrio ichthyoenteri	*Vibrio vulnificus* biovar I
Vibrio lentus	

Species Exotic to Australia

Several pathogens, as listed in Table 2, have not been detected in association with aquatic animals and are considered exotic.

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Table 2. *Vibrionaceae* exotic to Australia

Species	Primary host
Aliivibrio salmonicida	salmonids
Aliivibrio wodanis	salmonids
Moritella viscosa	salmonids
Vibrio ordalii	salmonids
Vibrio pectenicida	scallops
Vibrio tapetis	clams
Vibrio vulnificus biovar II	eels

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Zoonotic Agents

Some species of *Vibrionaceae* are the cause of zoonoses[18], Table 3. Infection invariably is the result of physical trauma arising from puncture wounds or the result of ingesting uncooked seafood. Life-threatening conditions have been reported occasionally in the
20 immunocompromised.

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Table 3. Zoonotic *Vibrionaceae*

Photobacterium damselae ssp. damselae
Vibrio alginolyticus
Vibrio cholerae non-O1
Vibrio cincinnatiensis
Vibrio fluvialis
Vibrio furnissii
Vibrio harveyi
Vibrio mimicus
Vibrio parahaemolyticus
Vibrio vulnificus

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Characteristics of the *Vibrionaceae*

Sodium chloride is critical for the growth of *Vibrionaceae*. Many species have an obligate requirement for sodium ions and growth of nearly all species is stimulated by NaCl, even those that have a low requirement for NaCl, such as *V cholerae*.[19] Species of the 40 *Vibrionaceae* do not in general have fastidious growth requirements and can be readily grown on peptone-based media as long as NaCl requirements are met. Growth of *Aliivibrio salmonicida, A wodanis* and *M viscosa,* is more reliable on media enriched with blood. In defined media some *Vibrionaceae* require supplementation with vitamins[20], while most strains, even in complex media, respond well to the addition of low levels of yeast extract.[21] 45 As the natural habitat of the *Vibrionaceae* is the marine environment, better growth is obtained at a slightly alkaline pH in the range of 7.5-7.8. Many species and strains will form distinctive curved rods but this characteristic is not diagnostic of the *Vibrionaceae*. In tissue smears, rods can appear preternaturally large or pleomorphic but on culture will assume more typical form and proportions.

50 Uniformly the *Vibrionaceae* are facultative anaerobes that ferment glucose. All species are oxidase positive with the exception of *V metschnikovii,* which is oxidase negative. Species are sensitive to the vibriostat 0/129, a pteridine derivative related to trimethoprim.[22] Some species however, such as *V lentus*, may appear resistant if inappropriate test media are used or if strains have acquired resistance to trimethoprim from the *drfA1* gene in plasmid class I 55 integrons.[23] Most species of *Vibrionaceae* will grow at 25°C except for *A salmonicida, A wodanis, M marina, M viscosa, Ph iliopiscarium* and *Ph phosphoreum,* which grow at 15°C. All zoonotic species will grow at 35-37°C.

Isolation Strategies

For osmoregulators, cultures from internal sites should be made on media enriched with blood such as Blood Agar Base No. 2 (Oxoid) with 7% defibrinated sheep's blood as a non-selective medium, and thiosulphate citrate bile sucrose (TCBS; Oxoid[24]) agar as a selective indicator medium for *Vibrionaceae*. TCBS should be used only in conjunction with a non-selective medium as not all species will grow on TCBS. For external sites on osmoregulators or any site of an osmoconformer, samples should be plated on either ZoBell's 2216E[25] or Johnson's marine agar[26] (see Appendix 1) as non-selective media and TCBS as the selective medium. For salmonids, particularly during periods of low water temperatures, samples from external sites should be plated on blood agar supplemented with 1.5% NaCl.[27] Plates should be incubated at 25°C for 2-3 days or at 15°C for up to 7 days for psychrophiles. Procedure for sampling should follow the ANZSDP guidelines for sample collection from finfish.[28]

Preservation

Cultures of *Vibrionaceae* can be held frozen at -80°C or in liquid nitrogen. A cryopreservative (see Appendix 1) based on peptone and glycerol is suitable.[29] Long-term storage based on freeze-drying is effective but the menstruum must be based on *meso*-inositol[30] so as to regulate membrane phase transition temperature effects and protein stabilisation[31] during freeze-drying and rehydration. Some species, notably *A salmonicida* and *M marina* have proved refractory to freeze-drying and are best preserved frozen. Generally, cultures recover well from preservation. ZMA or JMA are suitable recovery media but for more fastidious species particularly *A salmonicida, M marina* and *M viscosa* blood agar supplemented with 2% NaCl should be used. A prudent strategy for recovery from freeze-drying is to use *Vibrio* Recovery Medium (Appendix 1), which contains sodium pyruvate[32,33], which has been found useful in repair of damaged cells.

Identification Strategies

Phenotyping: Typically the *Vibrionaceae* exhibit a wide diversity of phenotype both between and within species. This heterogeneity in phenotype means that identification using a small number of tests either with keys or tables is unreliable. A more dependable strategy is to use a simultaneous polythetic approach[34] combined with computer-assisted probabilistic identification.[35]

An identification matrix, VibEx7 (Appendix 3), has been developed for species of *Vibrionaceae* associated with a diversity of aquatic animals in Australia from both temperate and tropical regions[8]. The matrix can identify 61 species and biovars and a further 25 as yet un-named protospecies of *Vibrionaceae* from aquatic animals. The panel of tests consists of 39 biochemical and 5 antibiotic sensitivity tests; details of the tests and formulations are given in Appendix 2. The biochemical tests are in conventional tube or plate format. Alternatively, the panel of tests is available commercially in miniaturised format as MicroSys® V36 (DPIW, Launceston, Tasmania).

Probabilistic identification is undertaken using an implementation of Bayes Theorem.[35] An identification is reached if the Willcox probability value $P \geq 0.99$ and the modal likelihood score ≥ 0.001. The Willcox probability is a measure of the most likely identification, while the modal likelihood is a measure of the goodness-of-fit of the unknown to the nominated species.[36] Probabilistic identification is undertaken using PIBWin[37], an intuitive software package, available freely from the University of Southampton, UK at: http://www.som.soton.ac.uk/staff/tnb/pib.htm

Molecular: Identification of *Vibrionaceae* using molecular methods is controversial. Amplified fragment length polymorphism (AFLP) and multilocus sequence typing[38] have proved important in establishing the taxonomic structure of the *Vibrionaceae* but these procedures are not as yet suitable for routine identification purposes. PCR amplification of

sequence motifs characteristic of species is a practical means of molecular identification but the scope of application is limited.[39] The most widely described and conserved construct is
110 the 16S rRNA gene but for the *Vibrionaceae*[40] sequence divergence is only 9% which greatly limits the possibilities of identifying motifs that are species unique. Sequences other than 16S rRNA have been identified, typically virulence factors, but the constancy of these targets across strains of a species is unknown and their suitability as constructs for identification purposes is questionable.

115 Recommended PCR primer sets relevant for *Vibrionaceae* of aquatic animals in Australia are for *Ph damselae* and *V harveyi*. Based on the 16S rRNA gene both primer sets are suitable for species identification. The PCR for *Ph damselae* is in multiplex format[41] with one primer pair specific for *Ph damselae sensu lato* and a second primer pair for the urease gene *ure*C that is specific for *Ph damselae* ssp. *damselae*. Evidence of *Ph damselae* ssp
120 *piscicida* is inferred by the absence of a *ure*C amplicon. The primer pair for *V harveyi*[42] is compromised to some extent by known cross-reaction with some, but not all, strains of *V alginolyticus*. For the primers to be truly discriminating, positive PCR reactions must be verified using at least one of the phenotypic tests listed in Table 4.

Table 4. Differential phenotypic tests for *Vibrio harveyi* and
125 *Vibrio alginolyticus* for confirming a positive PCR reaction for 16S rRNA *V harveyi* primers.

Species	PNPG*	Aesculin§	Putrescine¤
V alginolyticus	16%	10%	100%
V harveyi bv I	95%	95%	2%
V harveyi bv II	100%	100%	0%

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*PNPG: 2-nitrophenyl β-D-galactopyranoside;
§Aesculin hydrolysis;
¤Utilisation of putrescine

135 Identification by PCR should be limited to pure cultures and used as a means of confirming the identity of strains with atypical phenotypes. Performance of the primers has not been validated for the purpose of direct detection in tissues or environmental samples.

A range of primer sets for other constructs in the *Vibrionaceae* have been described, of which some have been critically evaluated. Of these, primers for the *cth*
140 cytolysin/haemolysin gene of *V vulnificus*[43] and the *vah*1 haemolysin gene of *V anguillarum*[44] appear robust. Use of these primers for identification in the absence of other species defining characteristics is not recommended since the frequency at which the targets occur intra-species is not known. The primers may have value however for screening purposes, particularly for strains where the target is known to occur or for establishing the
145 presence of virulence factors.

Quality Control

The most important factor determining success in identification is the use of standardised tests. Tests of different format should not be used unless extensive testing has been undertaken to verify test equivalence. It is important to recognise and identify sources of
150 error that, if not well controlled, can result in unreliable identification outcomes.[34] Intrinsic error is associated with some tests and some species that can result in variable test outcomes. A second form of error arises from procedural deficiencies, particularly interpretation of weak positive tests. Regular use of quality control organisms (Table 5) is recommended, together with trend analysis to identify drift in performance. With practice, intra-laboratory
155 test error of 2% is achievable.[8,45,46]

Table 5. *Vibrio* Quality Control Strains

Vibrio anguillarum ATCC 19264^T

Vibrio fluvialis NCTC 11327^T
Vibrio mediterranei CIP 103203^T
Vibrio parahaemolyticus ATCC 17802^T
Vibrio tubiashii NCIMB 1340^T

Limitations

The identification matrix VibEx7 reflects the diversity of *Vibrionaceae* associated with aquatic animals in Australia. It does not however include all the validly published species of *Vibrionaceae* as listed in Table 6 and these species will not be identified using the matrix.

Data for new species as they occur can be readily added to the VibEx7 matrix. The ability to differentiate the new species from those already in the matrix can be assessed using the IDSC tool in the PIBWin software.

Table 6. Species not in the VibEx7 matrix

Species	Host	Species	Host
Enterovibrio coralii	Plant	*Vibrio diabolicus*	Environment
Enterovibrio norvegicus	Animal	*Vibrio ezurae*	Animal
Moritella abyssi	Environment	*Vibrio fortis*	Animal
Moritella japonica	Environment	*Vibrio gallicus*	Animal
Moritella profunda	Environment	*Vibrio gigantis*	Animal
Moritella yayanosii	Environment	*Vibrio hepatarius*	Animal
Photobacterium aplysiae	Animal	*Vibrio hispanicus*	Animal
Photobacterium frigidiphilum	Environment	*Vibrio kanaloae*	Animal
Photobacterium ganghwense	Environment	*Vibrio neonatus*	Animal
Photobacterium halotolerans	Environment	*Vibrio neptunius*	Animal
Photobacterium indicum	Environment	*Vibrio pacinii*	Environment
Photobacterium lipolyticum	Environment	*Vibrio pomeroyi*	Animal
Photobacterium profundum	Environment	*Vibrio ponticus*	Animal
Photobacterium rosenbergii	Plant	*Vibrio rotiferianus*	Animal
Vibrio aerogenes	Environment	*Vibrio ruber*	Environment
Vibrio brasiliensis	Animal	*Vibrio superstes*	Animal
Vibrio coralliilyticus	Plant	*Vibrio xuii*	Animal
Vibrio crassostreae	Animal		

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Part 2 – Test Methods

Identification by Phenotype

Principle

Identification of *Vibrionaceae* is made using the entire panel of tests listed in Table 7 using
350 the standardised test formulations given in Appendix 2. An identification is made by matching the phenotype of an unknown against the probability data matrix VibEx7 and determining the most likely identification using the computer software package PIBWin.

Table 7. Panel of tests for the identification of *Vibrionaceae*

Arginine dihydrolase
355 Lysine decarboxylase
Ornithine decarboxylase
Acid (fermentation)
- Arbutin
- Mannitol
360 - Salicin
- Sucrose
- Gentiobiose

Growth
- 7% NaCl
365 - 10% NaCl

Acetoin
Indole
Alkaline phosphatase, pH 8.0
Oxidase
370 Hydrolysis
- 2-nitrophenyl β-D-galactopyranoside
- L-glutamic acid 5-(4-nitroanilide)
- 4-nitrophenyl sulfate
- Aesculin
375 - Agar
- Gelatin
- Starch

Sole carbon utilisation:
- α-ketoglutarate
380 - Acetate
- D-alanine
- Citrate
- L-citrulline
- D-galactose
385 - D-gluconate
- D-glucosamine
- D-glucose
- D-glucuronate
- Glycerol
390 - L-histidine
- DL-3-hydroxybutyrate
- trans-4-hydroxy-L-proline
- DL-lactate
- D-lactose
395 - Propionate

Table 7. Panel of tests for the identification of *Vibrionaceae* (cont.)

Putrescine
Succinate
Sucrose
Resistance:
0/129 10 µg
0/129 150 µg
Ampicillin 10 µg
Carbenicillin 100 µg
Novobiocin 5 µg

Sample Requirements

Pure cultures less than 48 hours old should be used for the inoculum. Cultures recovered from preservation by freezing or drying must be subcultured at least twice before commencing identification.

Test Procedures

Perform an oxidase test by a preferred method. Observe agarolytic activity as pitting of colonies on maintenance agar such as JMA or ZMA.

Inocula: Prepare two inocula in 3 mL volumes of 2% saline: one to a density equal to McFarland 0.5, the other to McFarland 2.

Inoculation of media: Inoculate the decarboxylase test media with 100 µL of McFarland 2 density cell suspension. Inoculate the remaining liquid media with 100 µL of McFarland 0.5 density cell suspension. For the acetoin and arginine dihydrolase tests, inoculate the semi-solid media with a straight wire.

Sole carbon source media are spot inoculated with 2 µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 30.

Gelatin and starch plates are spot inoculated with 2 µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 6, well separated inoculum points.

Tests for arginine dihydrolase and decarboxylases are overlayed with 20-25 mm of sterile liquid paraffin.

Sensitivity tests are undertaken on Mueller-Hinton agar supplemented with 2% w/v NaCl. The medium is inoculated with the McFarland 0.5 suspension.

Incubation: Tests are incubated at 25°C for 48 hours. Observe tests daily and record changes. Sensitivity tests are incubated for 24 hours and the diameter of the zone of inhibition measured. Psychrophilic species are tested at 15°C for 8 days; sensitivity tests for 3-4 days. Known psychrophiles are listed in Table 8.

Table 8. Species requiring incubation at 15°C

Aliivibrio logei
Aliivibrio wodanis
Aliivibrio salmonicida
Moritella marina
Moritella viscosa
Photobacterium iliopiscarium
Photobacterium phosphoreum

Test Interpretation

445 **Arginine dihydrolase**: A positive reaction is a pink/red colour; yellow to orange is negative. See Figure 1.

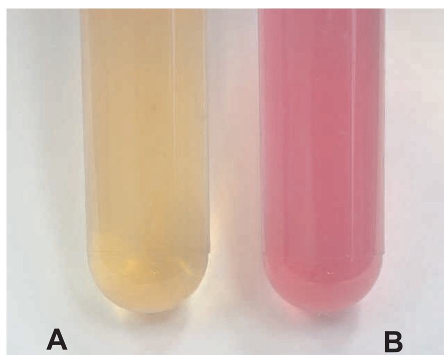


Figure 1. Arginine dihydrolase. **A**: negative; **B**: positive

450 **Decarboxylase tests**: The negative control should be yellow for the test to be valid; a positive test for ornithine or lysine decarboxylase is purple, See Figure 2.

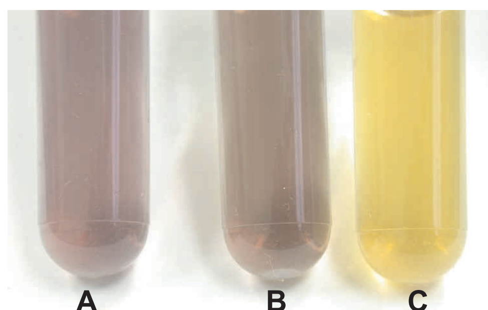


Figure 2. Decarboxylase test. **A**: positive ornithine; **B**: positive lysine; **C**: negative control

455

Acid from carbohydrates: A dirty yellow to bright yellow colour is positive; pale purple to deep purple is negative. See Figure 3. Protein deamination may occur with prolonged incubation and may cause positive tests to appear negative due to alkaline pH shifts; ignore reversions.

460

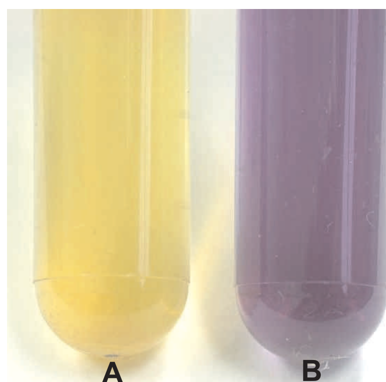
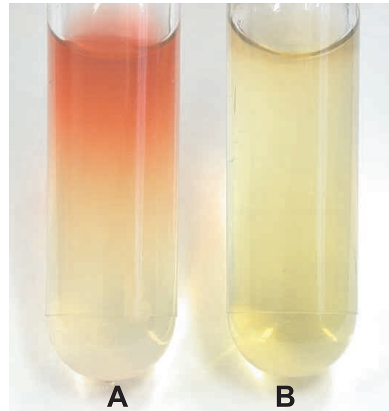


Figure 3. Fermentation test. **A**: positive; **B**: negative

NaCl tolerance tests: Any signs of growth is a positive reaction.

465

Acetoin: Overlay semi-solid medium with 200µL of α-naphthol followed by 100µL of KOH/creatine. Leave at room temperature for up to 30 minutes. Pale pink to red layer is positive; yellow to tan is negative. See Figure 4.



470 **Figure 4**. Acetoin (Voges-Proskauer) test. **A**: positive; **B**: negative

Indole: In a microtitre tray well add 100 µL of Kovács' indole reagent to an equal volume of culture. Mix the contents of the well by careful aspiration with a pipette. If a pink/red colour is visible record as positive. See Figure 5. If in doubt, remove contents with a glass Pasteur pipette and allow the phases to separate in the pipette body; record the reaction based on the
475 top phase only.

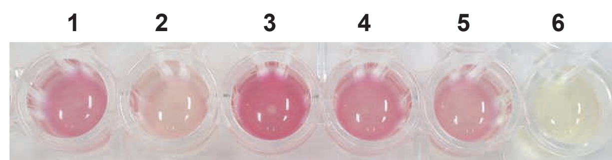


Figure 5. Indole test. Wells 1-5 are a range of positive reactions; well 6 is negative.

Gelatin hydrolysis: Flood plate with saturated ammonium sulphate. Any zone of clearing
480 around inocula is positive.

Starch hydrolysis: Flood plate with Gram's iodine. Zones of yellow to light tan colour around inocula is positive; black to dark blue is negative.

485 **Urease**: A pink/red colour is positive; yellow is negative. See Figure 6.

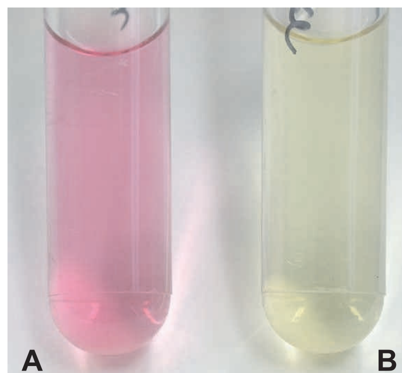


Figure 6. Urease test. **A**: positive; **B**: negative

Chromogens:

490 *Indoxyl alkaline phosphatase*: Any blue/black colour is positive; no colour is negative. See Figure 7.

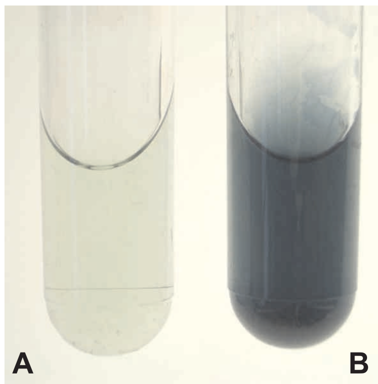


Figure 7. Indoxyl phosphate for alkaline phosphatase test, pH 8.0. **A**: negative; **B**: positive.

495 *Nitrophenol & nitroaniline chromogens*: Any yellow colour is positive; no colour is negative. See Figure 8.

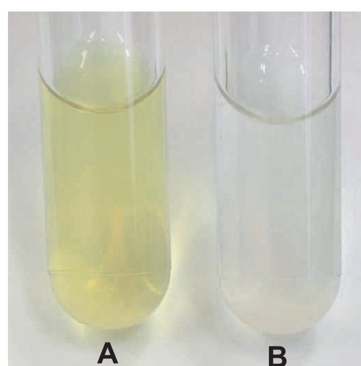


Figure 8. Nitrophenol and nitroanilide chromogen test. Substrate: 2-nitrophenyl β-D-galactopyranoside (PNPG). **A**: positive; **B**: negative.

500

Aesculin: A brown to black colour is positive for hydrolysis; a very light tan to colourless is negative. See Figure 9.

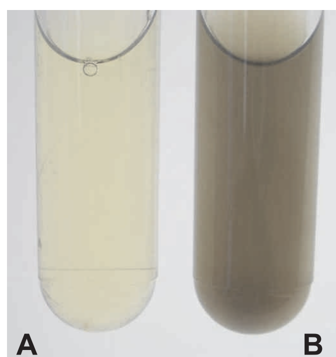
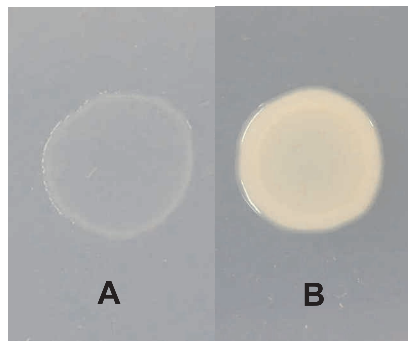


Figure 9. Aesculin test. **A**: negative; **B**: positive

505

Sole carbon source tests: Examine the negative control plate; some strains and some species may show very slight growth even when using purified agar. See Figure 10.



510 **Figure 10**. Sole carbon source test. **A**: negative control; **B**: glucose. Test organism *Vibrio fluvialis* NCTC 11327^{T}. Differentiation between positive and negative tests.

With reference to the control plate, examine growth on the remaining test plates. Growth in excess of the negative control is read as positive. If there is no growth with glucose the 515 strain may be nutritionally fastidious. Retest with sole carbon source media containing 0.015g/L Casamino acids.

Antibiotic sensitivity test: The diameter of the zone of inhibition is interpreted using the data in Table 9; disc size is 6mm.

Table 9. Zone size interpretation for diagnostic antibiotics

520

Test	Resistant	Sensitive
0/129 10 µg	≤15 mm	≥16 mm
0/129 150 µg	No zone	Any zone
Ampicillin 10 µg	≤13 mm	≥14 mm
Novobiocin 5 µg	≤16 mm	≥17 mm
Carbenicillin 100 µg	≤22 mm	≥23 mm

525

Probabilistic identification

An identification is obtained using the software PIBWin and the probability matrix VibEx7. An identification is accepted if the Willcox probability score P is ≥0.99 and the modal likelihood score (MLS) is ≥0.001.[8]

530 If P is ≥0.99 but the MLS is <0.001, the unknown may represent an outlier of the nominated species. Differences between expected and observed reactions should be inspected to determine whether the suggested identification can be accepted. If test variance for unexpected results is between 30-70% then the identification can be accepted but should be reported as a poor fit that represents a species outlier. If observed and expected reactions for 535 two or more tests are in complete disagreement then the suggested identification is erroneous and the unknown should be reported as unidentifiable.

Each species in the data matrix is proceeded by the number of strains used to establish the phenotypic range. For taxa based on only a few strains an acceptable match will occur only if the unknown is very similar to the species description. Since the estimate of diversity is 540 narrow, it is likely that strains of the species may appear as outliers.

In some instances, an unknown may appear to span two near related taxa such as biovars of a species. If the first and second most likely identifications are for related taxa then the probability values can be summed[47], and if P_{sum} is ≥0.99, the unknown can be assigned to the level of taxon complex or group.

545 Care must be exercised when accepting a computer-assisted identification. Ultimately professional judgement and microbiological sense must be used for interpretation.

Identification by PCR

Introduction

The primer sets have been evaluated and optimised for the purpose of culture identification. 550 The protocols represent optimal conditions but minor refinements may be required to account for variation in the characteristics of different thermal cyclers.

Extraction of DNA

Reference DNA: Extract and purify DNA from control strains using a QIAamp DNA mini-kit (cat. no. 51304, QIAGEN). Purified control DNA is used at a concentration of 50pg μL^{-1}.

555 *Sample DNA*: To 100 µL of PCR grade 18MΩ water in a 1.5 mL microfuge tube, suspend sufficient cells to a density equivalent to McFarland 1. Hold the tube at 100°C in a dry-heat block for 10 minutes and then cool rapidly in a cool block for 5 minutes. Pellet the cells at 10,000 rcf for 5 minutes and collect the supernatant containing liberated DNA. The extracted DNA is suitable for amplification without purification.

560 **PCR reaction volume**

All PCR reactions are as 25 µL volumes in 200 µL thin-walled tubes.

Standard PCR reagents

Standard reagents for the PCR primer sets are listed in Table 10.

Table 10. Standard PCR reagents

565 PCR grade water, 18MΩ
50mM magnesium chloride
10x Invitrogen Platinum® Taq buffer
16mM dNTP stock (4mM each dNTP)
Invitrogen Platinum® Taq DNA polymerase
570 Primers (20µM stock)

Electrophoresis of amplicon

Amplicon is visualised by electrophoresis using 2% agarose gel containing 0.5 $\mu g\ mL^{-1}$ ethidium bromide and 1xTBE buffer, Table 11. Use a 100bp ladder as a comparative index 575 of amplicon size. Gels should be run at 7 volts cm^{-1} constant voltage.

Table 11. 10x Tris-Boric-EDTA buffer

Tris (base)	108.0 g
Boric acid	55.0 g
EDTA	8.3 g
580 pH	8.0

Photobacterium damselae[41]

Targets

16S rRNA gene for species identification and *ure*C gene for subspecies identification.

Primers

Primer	Sequence 5' → 3'
Car1	gcttgaagagattcgagt
Car2	cacctcgcggtcttgctg
Ure-5'	tccggaataggtaaagcggg
Ure-3'	cttgaatatccatctcatctgc

585 *DNA controls*

Ph damselae ssp *damselae* NCIMB 2184[T]
Ph damselae ssp *piscicida* NCIMB 2058[T]
Ph iliopiscarium ATCC 51760[T]

Master mix

Component	Volume
Water	11.15 µL
50mM $MgCl_2$	1 µL
10x reaction buffer	2.5 µL
16mM dNTPs	1.25 µL
20µM Car1	2 µL
20µM Car2	2 µL
20µM Ure-5'	2 µL
20µM Ure-3'	2 µL
5U μL^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Volume	25 µL

590

Cycle parameters

Cycle parameters
95°C 4min.
30 cycles
95°C 1min.
60°C 1min.
72°C 40sec.
72°C 5min final extension

Interpretation

Ph damselae ssp *damselae*. Species specific amplicon at 267bp for 16S rDNA with an
595 additional amplicon at 448bp for the *ure*C gene that is diagnostic for the subspecies *damselae*.

Ph damselae ssp piscicida. A single amplicon at 267bp for 16S rDNA. Absence of an amplicon for *ure*C is diagnostic for the subspecies *piscicida*.

Limitations

600 A single band of 267bp is diagnostic of *Ph. damselae sensu lato*. An identification of *Ph damselae* ssp *piscicida* is inferred if only this band is present but some caution needs to be used where an identification is reached on the basis of absence. Corroborating phenotypic evidence and complete 16S rRNA gene sequence should be obtained if the identification represents a new finding for a region or host not previously associated with *Ph damselae* ssp
605 *piscicida*.

Vibrio harveyi[42]

Target

16S rRNA gene

Primers

Primer	Sequence 5' → 3'
VH-1	AACgAgTTATCTgAACCTTC
VH-2	gCAgCTATTAACTACACTACC

610 *DNA controls*

V harveyi ATCC 14126[T]

V alginolyticus ATCC 17749[T]

Master mix

Component	Volume
Water	18.15 µL
50mM $MgCl_2$	1.5 µL
10x reaction buffer	2.5 µL
16mM dNTPs	1.25 µL
20µM VH-1	0.25 µL
20µM VH-2	0.25 µL
5U μL^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Volume	25 µL

Cycle parameters

Cycle parameters
94°C 2min.
40 cycles
94°C 1min.
65°C 1min.
72°C 2min.
72°C 5min. final extension

615 *Interpretation*
A single band of 413bp is characteristic of *V harveyi*.

Limitations
On the basis of testing undertaken, the primers are specific for strains of both *V harveyi* biovar I and II associated with blister disease in abalone. Some strains of *V alginolyticus* are
620 known to cross-react because of sequence similarity with the primer regions. A positive finding must be corroborated by phenotype using the tests given in Table 4.

Vibrio vulnificus [43]

Target
cth cytolysin/haemolysin gene.

625 *Primers*

Primer	Sequence 5' → 3'
L-CTH	ttccaacttcaaaccgaactatgac
R-CTH	gctactttctagcattttctctgc

DNA controls

V vulnificus ATCC 27562^T
V parahaemolyticus ATCC 17802^T

Master mix

Component	Volume
Water	16.375 µL
50mM $MgCl_2$	1.25 µL
10x reaction buffer	2.5 µL
16mM dNTPs	1.25 µL
20µM L-CTH	1.25 µL
20µM R-CTH	1.25 µL
5U μL^{-1} Taq polymerase	0.125 µL
Template DNA	1 µL
Volume	25 µL

630 *Cycle parameters*

Cycle parameters	
94°C	3min.
30 cycles	
94°C	1min.
57°C	1min.
72°C	3min.
72°C	5min. final extension

Interpretation
A single band of 205bp is characteristic of the cytolysin/haemolysin gene in *V vulnificus*.

Limitations
An identification of *V vulnificus* is inferred by the presence of the *cth* gene which appears to
635 be specific for *V vulnificus*. It is noteworthy that the forward primer L-CTH is unique to *V vulnificus* while R-CTH is homologous with the thermolabile haemolysin gene of *V parahaemolyticus.*[48] Based on limited testing the primers appear specific for *V vulnificus*, however the frequency with which the gene occurs within the species is not known and may not be sufficiently reliable for the purpose of identification in the absence of *a priori*
640 information.

Vibrio anguillarum[44]

Target
*vah*1 haemolysin gene.

645 *Primers*

Primer	Sequence 5' → 3'
VaH1-P1	accgatgccatcgctcaaga
VaH1-P2	ggatattgaccgaagagtca

DNA controls
V anguillarum ATCC 19264[T]
V parahaemolyticus ATCC 17802[T]

Master mix

Component	Volume
Water	14.4 µL
50mM $MgCl_2$	0.75 µL
10x reaction buffer	2.5 µL
16mM dNTPs	1.25 µL
20µM VaH1-P1	2.5 µL
20µM VaH1-P2	2.5 µL
5U μl^{-1} Taq polymerase	0.1 µL
Template DNA	1 µL
Volume	25 µL

650 *Cycle parameters*

Cycle parameters	
94°C	4min.
30 cycles	
94°C	30sec.
55°C	30sec.
72°C	60sec.
72°C	5min. final extension

Interpretation

A single band of 490bp is characteristic of the haemolysin gene of *V anguillarum*.

Limitations

The *vah*1 gene appears to be specific to *V anguillarum* despite having a common ancestry with haemolysins from other species of *Vibrionaceae*. From limited testing however it appears that not all strains of *V anguillarum* possess the *vah*1 gene and its use as a primary means of identification is limited. Primers for the *vah*1 gene should be limited to determining the presence of the haemolysin gene in strains or as a supporting test.

APPENDICES

Appendix 1

Maintenance Media

Johnson's Marine Agar[26]

Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous (II) sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g
Agar	12.0 g
Aged seawater	900 mL
Distilled water	100 mL
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

Sheep Blood Agar +2% NaCl

Blood agar base no.2 (Oxoid CM0271)	40.0 g
NaCl	15.0 g
Distilled water	1000 mL
pH	7.4±0.2

Autoclave at 121°C for 15 minutes and cool to 50°C; aseptically add 70 mL of sterile defibrinated sheep's blood, mix gently and pour as plates.

Vibrio Recovery Medium[8]

Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g
Sodium pyruvate	1.0 g
Bacteriological charcoal	2.0 g
Aged seawater	900 mL
Distilled water	100 mL
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

ZoBell's Marine Agar 2216E[25]

695 Peptone (Oxoid LP0037) 5.0 g
Yeast extract (Oxoid LP0021) 1.0 g
Ferric (III) phosphate ($FePO_4 \cdot 4H_2O$) 0.1 g
Aged seawater 900 mL
Distilled water 100 mL
700 Agar 12.0 g
pH 7.5-7.6
Autoclave at 121°C for 15 minutes

Cryopreservative (freezing)[29]

705 Proteose peptone no.3 (Difco) 1.0 g
Glycerol 8 mL
Distilled water 92 mL

Dispense as 5 mL volumes and autoclave at 121°C for 15 minutes.

710 *Cryopreservative (freeze-drying)*[49]

Nutrient Broth No.2 (Oxoid CM067B) 16 mL
meso-inositol 2.4 g

Filter sterilise and add aseptically to 32 mL of sterile inactivated horse serum. Mix and divide into 3 mL volumes. Store at -20°C.

715

Appendix 2

Culture Media for Identification

Note: Media formulations are based on the cited source references but have been modified
720 in some instances for use with the *Vibrionaceae*. In most cases the modification relates to the addition of NaCl and supplementation with yeast extract.

The tests as formulated should not be substituted with variants. The media as described are standardised for the identification of *Vibrionaceae* using the identification matrix VibEx7.

Arginine Dihydrolase[50,51]

725 Peptone (Oxoid LP0037) 0.1 g
Yeast extract (Oxoid LP0021) 0.1 g
NaCl 1.5 g
K_2HPO_4 0.03 g
Phenol red (1% *aq*) 0.1 mL
730 L-arginine hydrochloride 1.0 g
Agar 0.3 g
Distilled water 100 mL
pH 6.8

Dispense as 4mL volumes in 12x90 mm culture tubes. Autoclave at 121°C for 15
735 minutes.

Fermentation Test Medium[52]

Peptone (Oxoid LP0037)	10.0 g
Yeast extract (Oxoid LP0021)	1.0 g
Lab Lemco (Oxoid LP0029)	3.0 g
740 NaCl	15.0 g
Bromocresol purple 0.5%*	8 mL
Distilled water	900 mL
pH	7.2

*0.5 g bromocresol purple in 100 mL 50:50 v/v ethanol/distilled water

745 Dissolve the ingredients, check and adjust pH and dispense as 90 mL volumes in screw cap bottles. Sterilise by autoclaving at 121°C for 15 minutes.

Prepare filter sterilised stocks of the following sugars as 10% stocks in distilled water: arbutin, mannitol, salicin, sucrose and β-gentiobiose.

To 90 mL of sterile base add 10 mL of 10% sugar stock to give a final concentration 750 of 1%. Dispense medium as 3 mL volumes in 12 x 90 mm diameter sterile tubes.

Tolerance to NaCl[51]

7% NaCl

Tryptone (Oxoid L0042)	1.0 g
755 Yeast extract (Oxoid LP0021)	0.1 g
NaCl	7.0 g
Distilled water	100 mL
pH	7.2

10% NaCl

760 Tryptone (Oxoid L0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	10.0 g
Distilled water	100 mL
pH	7.2

765 Autoclave at 121°C for 15 min as 100 mL volumes in sealed bottles to prevent evaporation. Dispense as 3mL volumes in 12x90 mm sterile tubes.

Amylase[51]

Nutrient broth No. 2 (Oxoid CM0067)	2.5 g
770 NaCl	1.0 g
Soluble starch	0.1 g
Agar	1.5 g
Distilled water	100 mL
pH	7.5

775 Dissolve all the ingredients except for the agar. Warm to assist solution of the starch if required. Add the agar. Autoclave at 121°C for 10 minutes. Pour as plates.

Acetoin (Voges-Proskauer) Test[49]

Tryptone (Oxoid LP0042)	0.7 g
Soya peptone (Oxoid LP0044)	0.5 g
Yeast extract (Oxoid LP0021)	0.1 g
Glucose	1.0 g
NaCl	1.5 g
Agar	0.3 g
Distilled water	100 mL
pH	7.0

Dissolve all the ingredients including the agar. Dispense as 3 mL volumes in 12x90 mm tubes. Autoclave at 115°C for 10 min. Cool as butts.

Gelatin[51]

Nutrient broth No. 2 (Oxoid CM0067)	2.5 g
NaCl	1.0 g
Gelatin	0.5 g
Agar	1.5 g
Distilled water	100 mL
pH	7.5

Dissolve all the ingredients except for the agar. Warm to assist solution of the gelatin. Add the agar.
Autoclave at 115°C for 20 min. Pour as plates.

Indole[51,53]

Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	1.5 g
L-tryptophan	0.04 g
Distilled water	100 mL
pH	7.5

Dissolve ingredients. Dispense as 3 ml volumes in 12x90 mm tubes. Autoclave at 121°C for 15min.

Chromogen Tests[8,54]

Nutrient Base

Tryptone (Oxoid LP0042)	4.0 g
Yeast extract (Oxoid LP0021)	0.4 g
NaCl	6.0 g
Distilled water	300 mL
pH	7.5

Dissolve ingredients and dispense as four volumes of 75 mL. Autoclave at 121°C for 15 min.

Phosphate buffer: 0.01M, pH 7.5

Stock 0.1M buffer

$NaH_2PO_4 \cdot 2H_2O$	0.245 g
$Na_2HPO_4 \cdot 12H_2O$	3.022 g
Distilled water, made up to	100 mL
pH	7.5

Prepare a stock of 0.01M buffer, pH 8.0

0.1M phosphate buffer	10 mL
Distilled water	90 mL

Phosphate buffer: 0.01M, pH 8

830 Prepare a x10 stock of 0.1M buffer

$NaH_2PO_4 \cdot 2H_2O$	0.083 g
$Na_2HPO_4 \cdot 12H_2O$	3.395 g
Distilled water, made up to	100 mL
pH	8.0

835 Prepare a stock of 0.01M buffer, pH 8

0.1M phosphate buffer	10 mL
Distilled water	90 mL

Chromogen stocks

IXP medium

840 3-indoxyl phosphate	0.08 g
0.01M phosphate buffer, pH 8	25 mL

Filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12x90 mm sterile tubes.

PNPG medium

845 4-nitrophenyl α-D-galactopyranoside	0.06 g
0.01M phosphate buffer, pH 7.5	25 mL

Filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12x90 mm sterile tubes.

LGN medium

850 L-Glutamic acid 5-(4-nitroanilide)	0.06 g
0.01M phosphate buffer, pH 7.5	25 mL

Filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12x90 mm sterile tubes.

NPS medium

855 4-nitrophenyl sulphate	0.04 g
0.01M phosphate buffer, pH 7.5	25 mL

Filter to sterilise. Aseptically add to 75 mL of nutrient base. Aseptically dispense as 2 mL volumes in 12x90 mm sterile tubes.

Protect media from light.

860

Aesculin Hydrolysis[55]

Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	1.5 g
865 Aesculin	0.1 g
Ferric citrate	0.05 g
Distilled water	100 mL
pH	7.5

Dissolve ingredients and dispense as 3 mL volumes in 12x90 mm tubes. Autoclave at
870 115°C for 10min. Protect medium from light.

Carbon Source Utilisation Tests[13]

Inorganic nitrogenous base

Buffer base

875

TRIS (basic)	6.1 g
Distilled water	500 mL
pH	7.5

Adjust pH with concentrated HCl

Salts solution

880, 885

NH_4Cl	1.0 g
$K_2HPO_4 \cdot 3H_2O$	0.075 g
$FeSO_4 \cdot 7H_2O$	0.028 g
NaCl	11.7 g
$MgSO_4 \cdot 7H_2O$	12.3 g
KCl	0.75 g
Yeast extract (Oxoid LP0021)	0.015 g
$CaCl_2 \cdot H_2O$	1.45 g
Distilled water	400 mL

Weight out all the salts and combine except for the calcium chloride. Add
890 the distilled water; once the salts are fully dissolved add the calcium chloride.

Combine the buffer base and salts solution.

Divide the medium into 10 x 90 mL volumes. To each volume add 1.2 g of *purified* agar (Oxoid LP0028) and autoclave at 121°C for 15 min; cool to 55°C.

895 Carbon sources

Prepare 2% w/v or v/v concentrations in distilled water, of the carbon substrates listed; filter to sterilise.

α-ketoglutarate	L-histidine
Acetate	DL-3-hydroxybutyrate
D-alanine	trans-4-hydroxy-L-proline
Citrate	DL-lactate
L-citrulline	D-lactose
D-galactose	Propionate
D-gluconate	Putrescine
D-glucosamine	Succinate
D-glucose	Sucrose
D-glucuronate	Water (control)
Glycerol	

Complete medium

To a 90 mL volume of cooled molten inorganic nitrogenous base, add 10 mL of carbon
900 source. Mix well and pour as plates.

Supplement for nutritionally fastidious strains

To the nitrogenous base add 0.015 g/L Casamino Acids (Difco, 0230-15).

Decarboxylase Test[51,56,57]

905

Difco Decarboxylase broth base (Møller)	300 mL
Yeast extract (Oxoid LP0021)	0.3 g
NaCl	4.5 g
pH	6.5

Divide the base into three volumes of 100 mL each. To one volume add 1.0 g of L-lysine and to the second volume 1.0 g of L-ornithine; the third volume is a control. Check and adjust the pH to 6.5 if required. Autoclave at 121°C for 10 min. Check the pH of the media and, if required, adjust aseptically with 1N NaOH or HCl. Aseptically dispense the media as 3 mL volumes into 12x90 mm sterile tubes.

Urease[58]

Broth base

Peptone (Oxoid LP0037)	0.1 g
Glucose	0.1 g
Sodium chloride	1.5 g
Na_2HPO_4 (anhydrous)	0.12 g
KH_2PO_4 (anhydrous)	0.08 g
Phenol red (0.01%)	4 mL
Distilled water	95 mL
pH	6.8

Dissolve the ingredients and autoclave as a single volume at 115°C for 20 min.

Urea stock (40% w/v)

Urea	8 g
Distilled water	20 mL

Dissolve the urea and filter to sterilise.

Complete medium

To the cool sterile base add 5 mL of sterile urea stock; mix. Dispense aseptically as 2 mL volumes in 12x90 mm sterile tubes.

Shelf Life of Media

Agar plates have a shelf life of 4 weeks and liquid media have a shelf life of 8 weeks when stored at 2-8°C.

Saturated Ammonium Sulphate (Gelatin test)[59,60]

Ammonium sulphate	10 g
Distilled water	10 mL

Store at room temperature.

Coblentz Reagents (Acetoin test)[61]

Reagent 1

α-naphthol	0.5 g
Ethanol	10 mL

Store refrigerated in a dark bottle.

Reagent 2

Creatine	0.03 g
KOH	4.0 g
Distilled water	10 mL

Store refrigerated.

Iodine (Starch test)[62]

Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

Dissolve the potassium iodide in 20 mL of water and then add the iodine. Once dissolved, make up to 300 mL with water.

960 Store at room temperature in a dark bottle.

Kovács' Reagent (Indole test)[63]

p-dimethylaminobenzaldehyde	2 g
pentan-1-ol (*n* amyl alcohol)	30 mL
965 Concentrated HCl	10 mL

Dissolve the aldehyde in the alcohol by gently warming at 50-55°C. Cool and slowly add the acid. Protect from light and store at 4°C.

Note: *iso* amyl alcohol is not the same as *n* amyl alcohol. The *iso*- form of pentanol cannot be used for Kovács' reagent.

970

Appendix 3: VibEx7 probability matrix for the identification of *Vibrionaceae*

Data as % strains positive

Test	*No. strains*	***Aeromonas . sobria HG7***	***G. hollisae***	***M. marina***	***M. viscosa***	***A. fischeri* biovar I**	***A. fischeri* biovar II**	***A. logei***	***A. salmonicida***	***A. wodanis***	***Ph. angustum***	***Ph. damselae* ssp. *damselae* biovar I**	***Ph. damselae* ssp. *damselae* biovar II**	***Ph. damselae* ssp. *piscicida***	***Ph. iliopiscarium***	***Ph. leiognathi***	***Ph. phosphoreum***
		7	4	1	8	5	10	2	1	1	1	29	7	5	3	4	1
Arginine dihydrolase		86	1	1	57	1	1	1	1	1	1	99	99	99	99	75	99
Acid:	Arbutin	14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Mannitol	99	1	1	1	40	87	99	99	1	99	1	1	1	33	1	1
	Salicin	14	1	1	1	40	40	1	1	1	1	1	1	1	1	1	1
	Sucrose	57	1	1	1	20	1	1	1	99	1	1	1	1	33	50	1
	Gentiobiose	14	1	1	1	60	90	1	1	1	99	1	1	1	1	1	1
Growth:	7% NaCl	1	99	99	1	1	70	1	99	1	99	86	99	1	99	50	99
	10% NaCl	1	25	99	1	1	1	1	1	1	1	1	1	1	1	1	1
Amylase		99	1	1	99	40	55	1	1	99	1	27	14	20	99	1	1
Voges Proskauer (Acetoin)		86	1	1	1	1	1	1	1	1	1	99	99	99	99	99	99
Gelatinase		71	1	1	99	20	1	1	1	1	1	24	33	20	1	25	1
Indole		86	75	1	1	20	1	1	1	99	1	3	1	1	1	1	1
IXP alkaline phosphatase		86	50	1	13	80	99	99	1	1	99	99	85	20	33	25	99
PNPG α-D-galactosidase		29	1	99	1	1	20	1	1	1	1	3	1	20	1	1	99
LGN γ-glutamyl transpeptidase		71	1	99	99	1	50	99	1	1	1	48	14	20	1	75	1
NPS sulphatase		14	1	99	1	60	99	99	1	1	99	1	1	1	1	25	1
Aesculin hydrolysis		71	1	1	20	80	99	99	1	1	50	31	1	1	1	25	1
Utilisation:	α-ketoglutarate	14	50	99	1	1	1	99	1	1	99	3	1	20	1	1	99
	Acetate	29	99	99	88	80	1	50	99	99	99	93	1	60	99	50	1
	Alanine	1	99	99	25	20	1	1	1	1	1	10	1	99	1	1	1
	Citrate	43	99	1	1	1	60	99	99	99	99	1	1	1	99	1	1
	Citrulline	1	1	1	38	1	1	1	1	1	1	1	1	1	1	1	1
	Galactose	57	99	1	1	40	99	99	99	1	99	93	28	60	99	25	99
	Gluconate	99	75	99	1	1	20	99	99	1	1	3	14	20	99	99	1
	Glucosamine	99	25	1	50	20	99	99	1	1	99	99	42	60	99	99	99
	Glucuronate	1	1	1	1	1	1	1	1	1	1	1	1	1	33	1	1
	Glycerol	99	75	99	99	40	90	99	99	99	99	99	42	60	99	75	99
	Histidine	29	50	1	88	1	1	1	1	1	1	1	1	1	1	1	1
	DL-3-hydroxybutyrate	1	1	1	1	40	1	1	99	1	1	1	1	1	1	25	1
	Hydroxyproline	1	1	1	1	1	1	1	1	1	99	1	1	1	1	1	1
	DL-lactate	14	99	99	99	1	1	1	1	1	1	79	1	1	1	99	99
	Lactose	1	1	1	1	1	1	1	1	1	1	1	1	20	1	1	99
	Propionate	14	99	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Putrescine	14	1	1	1	1	1	1	99	1	1	1	1	1	99	1	1
	Succinate	86	75	99	99	40	90	99	99	99	99	89	42	40	99	75	99
	Sucrose	57	1	1	1	60	1	1	99	99	1	1	1	1	33	50	1
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	1
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10 µg	99	1	99	38	1	1	1	1	1	1	1	14	1	1	1	1
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Data as % strains positive	***Aeromonas . sobria HG7***	***G. hollisae***	***M. marina***	***M. viscosa***	***A. fischeri* biovar I**	***A. fischeri* biovar II**	***A. logei***	***A. salmonicida***	***A. wodanis***	***Ph. angustum***	***Ph. damselae* ssp. *damselae* biovar I**	***Ph. damselae* ssp. *damselae* biovar II**	***Ph. damselae* ssp. *piscicida***	***Ph. iliopiscarium***	***Ph. leiognathi***	***Ph. phosphoreum***
Test *No. strains*	7	4	1	8	5	10	2	1	1	1	29	7	5	3	4	1
Ampicillin 10 µg	57	1	1	1	60	80	99	99	1	99	65	14	20	1	75	1
Novobiocin 5 µg	86	25	1	1	1	10	1	1	1	50	27	14	20	33	50	1
Carbenicillin 100 µg	17	1	1	1	99	80	99	99	1	99	89	42	20	33	75	1
Lysine decarboxylase	99	1	1	38	20	55	99	1	1	99	62	57	1	99	1	1
Ornithine decarboxylase	29	1	1	1	20	33	99	1	1	1	1	1	1	1	1	1
Urease	14	1	1	1	99	99	1	1	1	99	99	99	1	1	25	1

Data as % strains positive	***V. aestuarianus***	***V. agarivorans***	***V. alginolyticus***	***V. anguillarum***	***V. calviensis***	***V. campbellii***	***V. chagasii***	***V. cholerae***	***V. cincinnatiensis***	***V. cyclitrophicus***	***V. diazotrophicus***	***V. fluvialis***	***V. furnissii***	***V. gazogenes***	***V. halioticoli***	***V. harveyi* biovar I**
Test No. strains	3	7	30	60	4	4	21	12	3	7	4	9	10	3	3	62
Arginine dihydrolase	99	1	3	96	99	1	95	1	1	99	99	99	99	1	1	15
Acid: Arbutin	1	14	3	1	67	1	1	1	66	1	99	99	1	99	1	29
Mannitol	99	99	99	98	50	50	99	99	99	85	99	99	99	33	99	94
Salicin	1	57	20	1	75	75	5	1	66	1	99	99	1	99	1	79
Sucrose	99	1	99	98	75	1	43	99	66	99	99	99	99	99	67	65
Gentiobiose	1	99	1	1	75	50	1	1	33	14	1	11	1	1	1	95
Growth: 7% NaCl	67	29	99	93	25	99	95	58	99	99	99	99	99	99	67	99
10% NaCl	1	1	99	3	1	1	10	1	66	1	50	56	90	66	33	5
Amylase	99	43	99	96	99	75	99	75	66	99	25	99	70	99	1	99
Voges Proskauer (Acetoin)	1	14	99	94	1	1	5	58	33	1	1	11	1	99	1	2
Gelatinase	67	1	99	77	50	99	99	67	33	99	25	44	30	99	33	97
Indole	99	1	99	93	99	99	95	99	1	85	99	99	99	1	33	97
IXP alkaline phosphatase	99	43	99	88	50	99	99	92	99	85	99	99	99	50	33	99
PNPG α-D-galactosidase	99	99	16	53	99	1	76	75	99	99	99	33	44	99	99	95
LGN γ-glutamyl transpeptidase	1	29	96	15	50	99	86	99	1	71	99	99	99	1	1	94
NPS sulphatase	1	14	40	1	1	1	99	8	1	71	25	11	1	1	1	60
Aesculin hydrolysis	33	99	10	63	99	99	99	22	99	99	99	88	10	99	33	95
Utilisation: α-ketoglutarate	67	1	99	36	25	99	99	99	1	99	1	99	99	1	1	97
Acetate	67	43	96	51	99	25	99	99	33	99	99	99	90	99	33	89
Alanine	99	14	99	76	99	50	99	67	1	99	99	99	99	33	1	99
Citrate	99	1	96	95	99	99	95	92	99	99	99	99	99	66	1	95
Citrulline	1	1	20	16	99	25	62	1	1	85	1	56	10	1	1	6

Data as % strains positive		***V. aestuarianus***	***V. agarivorans***	***V. alginolyticus***	***V. anguillarum***	***V. calviensis***	***V. campbellii***	***V. chagasii***	***V. cholerae***	***V. cincinnatiensis***	***V. cyclitrophicus***	***V. diazotrophicus***	***V. fluvialis***	***V. furnissii***	***V. gazogenes***	***V. halioticoli***	***V. harveyi* biovar I**
Test	No. strains	3	7	30	60	4	4	21	12	3	7	4	9	10	3	3	62
	Galactose	99	71	13	71	99	1	99	83	66	85	99	99	99	1	99	73
	Gluconate	99	14	99	96	75	1	67	99	66	99	99	99	99	33	1	99
	Glucosamine	99	86	99	95	99	1	99	99	99	99	75	99	99	33	99	84
	Glucuronate	1	1	1	1	1	1	14	25	1	1	25	78	10	99	1	61
	Glycerol	99	1	99	95	99	99	99	99	99	99	1	99	99	99	1	85
	Histidine	1	1	99	93	50	1	10	67	1	71	99	89	99	1	1	8
	DL-3-hydroxybutyrate	1	1	1	1	99	1	1	1	1	14	1	78	80	1	1	1
	Hydroxyproline	1	1	99	1	75	1	10	1	1	71	1	11	1	1	1	50
	DL-lactate	99	29	99	91	99	75	99	99	66	99	99	99	99	66	99	99
	Lactose	1	1	1	1	1	1	1	1	1	28	50	1	1	1	1	1
	Propionate	67	1	99	6	99	1	95	58	1	99	75	99	99	66	1	94
	Putrescine	1	1	99	1	50	1	5	1	1	1	99	33	90	1	1	2
	Succinate	99	29	99	93	75	99	99	75	99	99	75	99	90	99	1	97
	Sucrose	99	1	99	96	75	1	43	99	66	99	99	89	99	99	67	65
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	99	1	1	1	1	1	1	33	1	1	1	1	1	1	1
Resistance:	0/129 10 µg	1	1	70	3	1	25	19	8	66	28	25	44	60	99	1	82
	0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	10	1	1	1
	Ampicillin 10 µg	1	1	99	91	1	99	99	25	1	1	1	33	80	1	33	97
	Novobiocin 5 µg	67	14	90	11	1	99	19	17	50	71	75	99	99	99	33	94
	Carbenicillin 100 µg	33	1	99	90	1	99	99	33	1	1	1	11	80	1	33	95
Lysine decarboxylase		1	1	99	1	25	50	5	99	1	28	1	1	1	1	1	98
Ornithine decarboxylase		1	1	66	1	1	1	1	99	1	28	1	11	1	1	1	99
Urease		1	1	1	1	1	1	10	1	1	1	1	1	1	1	1	63

Data as % strains positive		***V. harveyi* biovar II**	***V. ichthyoenteri* biovar I**	***V. ichthyoenteri* biovar II**	***V. lentus***	***V. mediterranei***	***V. metschnikovii***	***V. mimicus***	***V. mytili***	***V. natriegens***	***V. navarrensis***	***V. nereis***	***V. nigripulchritudo***	***V. ordalii***	***V. orientalis***	***V. parahaemolyticus***	***V. pectenicida***
Test	*No. strains*	23	6	3	9	19	5	7	5	12	4	6	3	6	6	18	2
Arginine dihydrolase		1	1	1	99	84	99	1	99	1	1	99	1	1	99	6	99
Acid:	Arbutin	1	1	1	1	1	1	1	25	83	75	1	1	1	1	1	1
	Mannitol	83	60	99	75	99	99	86	99	99	99	67	1	1	99	99	1
	Salicin	4	1	1	1	80	1	1	99	92	50	1	99	1	17	18	1
	Sucrose	1	66	99	13	95	99	1	99	99	99	99	1	99	99	11	1
	Gentiobiose	9	1	1	1	21	1	1	99	99	25	1	50	1	1	1	1
Growth:	7% NaCl	99	33	67	38	74	80	86	99	99	99	99	1	1	67	99	1
	10% NaCl	4	16	1	1	5	40	14	99	75	1	67	1	1	1	89	1
Amylase		99	1	1	38	84	99	1	99	92	99	67	67	50	99	99	99

Data as % strains positive **Test** *No. strains*	***V. harveyi* biovar II**	***V. ichthyoenteri* biovar I**	***V. ichthyoenteri* biovar II**	***V. lentus***	***V. mediterranei***	***V. metschnikovii***	***V. mimicus***	***V. mytili***	***V. natriegens***	***V. navarrensis***	***V. nereis***	***V. nigripulchritudo***	***V. ordalii***	***V. orientalis***	***V. parahaemolyticus***	***V. pectenicida***
	23	6	3	9	19	5	7	5	12	4	6	3	6	6	18	2
Voges Proskauer (Acetoin)	75	1	1	1	1	99	1	1	1	1	17	1	1	1	1	1
Gelatinase	83	16	1	63	32	60	71	1	42	75	17	99	99	67	99	1
Indole	99	1	1	63	95	60	99	1	27	99	83	67	1	99	99	1
IXP alkaline phosphatase	99	99	99	75	89	40	86	99	75	99	67	67	40	83	99	99
PNPG α-D-galactosidase	99	1	1	50	99	99	43	75	75	1	1	99	1	67	17	1
LGN γ-glutamyl transpeptidase	87	1	1	38	99	1	99	60	67	50	99	67	99	83	99	1
NPS sulphatase	61	16	33	25	79	1	33	1	58	1	1	1	1	1	56	1
Aesculin hydrolysis	99	66	1	71	95	99	1	99	99	75	1	50	1	20	11	1
Utilisation: α-ketoglutarate	1	1	1	50	37	1	99	20	67	99	99	99	17	1	89	1
Acetate	99	83	99	88	99	60	99	99	92	75	99	67	1	67	99	1
Alanine	99	33	1	75	99	60	99	99	99	75	99	99	67	99	99	99
Citrate	99	16	1	13	99	40	99	99	92	99	99	99	99	99	94	1
Citrulline	1	1	1	1	11	1	1	20	83	1	83	1	1	1	17	1
Galactose	74	1	1	25	99	40	86	80	83	1	33	99	1	67	89	1
Gluconate	99	99	1	25	5	99	99	99	92	99	99	33	1	99	99	1
Glucosamine	96	99	99	13	99	20	99	99	99	99	99	99	50	83	99	50
Glucuronate	13	99	1	1	58	1	86	1	33	25	1	33	1	1	17	1
Glycerol	83	1	1	38	99	99	99	99	92	99	99	99	33	83	99	99
Histidine	1	1	1	1	89	1	99	99	99	99	99	99	33	17	99	1
DL-3-hydroxybutyrate	1	1	1	1	16	1	1	1	83	1	99	99	1	50	6	1
Hydroxyproline	1	1	1	1	1	1	1	1	25	1	1	1	1	83	83	1
DL-lactate	99	66	99	63	99	40	99	99	99	99	99	99	17	99	99	99
Lactose	1	1	1	1	99	40	1	20	8	1	1	99	1	1	1	1
Propionate	78	16	1	13	99	1	50	99	92	99	99	67	1	67	89	1
Putrescine	1	1	1	13	99	1	1	1	83	1	83	1	1	83	89	1
Succinate	99	1	99	50	95	60	99	80	99	99	83	99	1	99	89	50
Sucrose	4	66	99	1	99	99	1	99	99	99	99	1	83	99	11	1
Oxidase	99	99	99	99	99	1	99	80	99	99	99	99	99	99	99	99
Agarolysis	1	1	1	1	5	1	1	1	8	1	1	1	1	1	1	1
Resistance: 0/129 10 µg	91	16	67	99	1	1	1	99	99	25	33	1	1	1	61	1
0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	6	1
Ampicillin 10 µg	1	33	1	13	1	40	14	1	8	50	1	1	50	1	94	50
Novobiocin 5 µg	74	66	1	1	11	40	57	60	99	75	80	1	1	17	94	1
Carbenicillin 100 µg	1	33	1	25	42	40	43	1	8	75	1	1	17	1	94	99
Lysine decarboxylase	99	1	1	1	22	60	99	1	1	1	1	1	1	1	99	1
Ornithine decarboxylase	1	1	1	13	1	1	99	1	1	1	1	1	1	1	99	1
Urease	1	1	1	1	1	1	1	1	1	1	1	1	1	1	17	1

Data as % strains positive		*V. pelagius* biovar I	*V. pelagius* biovar II	*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	10	5	4	12	3	11	37	3	1	8	7	7	5	7	7	6
Arginine dihydrolase		1	1	1	99	99	9	99	99	1	99	99	1	1	99	99	17
Acid:	Arbutin	1	1	1	1	1	1	1	1	1	1	1	71	99	99	1	1
	Mannitol	99	99	1	99	33	10	97	99	1	71	99	71	1	99	99	99
	Salicin	1	1	1	1	1	1	8	1	1	25	99	57	99	99	1	33
	Sucrose	99	80	1	1	99	99	8	1	1	25	99	1	1	86	99	83
	Gentiobiose	1	40	1	1	1	1	1	1	1	1	99	28	40	40	1	1
Growth:	7% NaCl	80	80	1	99	67	36	71	33	1	75	57	85	20	99	99	99
	10% NaCl	1	1	1	99	33	1	11	1	1	1	1	1	1	99	99	83
Amylase		20	80	33	99	67	1	84	99	99	13	99	99	99	99	71	83
Voges Proskauer (Acetoin)		1	1	1	99	1	1	1	1	1	1	1	1	1	14	1	67
Gelatinase		20	80	1	99	67	1	89	99	1	13	99	99	80	14	99	99
Indole		1	99	1	75	1	1	95	66	1	63	99	99	1	99	86	83
IXP alkaline phosphatase		99	99	50	92	99	82	92	99	1	99	86	85	99	99	99	99
PNPG α-D-galactosidase		99	99	99	1	67	9	92	1	1	1	99	99	80	14	29	50
LGN γ-glutamyl transpeptidase		80	99	1	75	99	18	50	99	1	13	99	1	1	99	86	67
NPS sulphatase		70	60	1	1	1	1	79	1	1	99	1	1	1	86	86	50
Aesculin hydrolysis		99	99	1	1	33	99	97	99	1	99	99	42	25	99	1	83
Utilisation:	α-ketoglutarate	20	20	99	99	67	9	99	99	99	38	1	99	80	99	99	99
	Acetate	90	80	99	99	67	45	76	99	1	99	86	85	99	99	99	99
	Alanine	99	99	99	99	99	1	97	99	1	99	99	99	99	99	99	99
	Citrate	80	99	99	99	99	91	97	99	99	99	99	99	99	99	99	99
	Citrulline	60	99	1	25	1	1	24	1	1	1	99	1	1	71	14	17
	Galactose	99	99	99	1	99	36	92	1	1	1	99	99	99	1	43	67
	Gluconate	99	99	99	99	67	82	84	1	1	99	99	99	99	99	99	99
	Glucosamine	70	99	99	99	99	99	89	66	99	99	99	99	80	99	86	99
	Glucuronate	1	1	25	1	1	64	21	1	1	1	86	85	80	1	1	17
	Glycerol	99	99	99	99	99	1	99	99	99	99	99	85	99	99	99	99
	Histidine	80	1	99	99	33	1	3	1	1	1	99	28	1	86	99	83
	DL-3-hydroxybutyrate	20	1	25	1	1	18	1	1	1	1	99	1	1	99	1	1
	Hydroxyproline	1	1	1	99	1	1	1	1	1	1	86	1	1	1	1	99
	DL-lactate	99	99	99	99	67	99	97	66	99	99	99	99	99	99	99	99
	Lactose	10	20	99	1	33	1	21	1	1	1	99	1	1	1	1	1
	Propionate	60	80	75	99	33	18	39	33	1	13	86	99	80	99	86	99
	Putrescine	99	99	1	99	1	1	5	1	1	1	29	1	1	99	14	17
	Succinate	99	99	99	99	99	91	99	99	99	99	86	99	99	99	86	99
	Sucrose	99	80	1	8	99	99	3	33	1	25	99	1	1	86	99	83
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	60	1	25	67	1	9	29	99	1	63	14	1	1	1	57	99
	0/129 150µg	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1
	Ampicillin 10µg	10	1	1	58	1	1	3	99	1	1	1	1	1	1	57	99
	Novobiocin 5µg	70	80	1	99	1	27	3	33	1	1	57	57	20	99	57	99
	Carbenicillin 100µg	20	1	1	1	33	1	18	99	1	1	1	1	1	1	71	99
Lysine decarboxylase		1	1	1	8	33	1	1	1	1	1	1	99	60	29	1	83
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	1	99	1	29	1	99
Urease		1	1	1	1	33	1	1	1	1	1	1	1	1	14	1	1

Data as % strains positive	Phenon 52	Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46
Test *No. strains*	3	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6
Arginine dihydrolase	33	99	99	83	88	99	99	99	99	99	50	99	99	99	99	99
Acid: Arbutin	1	99	1	83	99	1	21	1	1	1	1	1	1	9	1	1
Mannitol	1	99	99	67	99	99	77	99	99	88	50	1	50	91	25	99
Salicin	1	1	1	99	99	1	57	1	1	99	1	1	1	27	1	1
Sucrose	1	99	99	99	99	99	99	99	99	12	1	99	99	82	25	1
Gentiobiose	1	86	1	83	99	25	57	1	1	1	1	1	1	9	1	1
Growth: 7% NaCl	99	63	25	83	99	99	43	99	88	88	50	83	75	91	25	99
10% NaCl	67	1	1	1	1	1	1	31	1	1	17	1	1	55	1	1
Amylase	67	63	99	99	99	99	99	93	94	99	67	99	99	73	50	99
Voges Proskauer (Acetoin)	67	25	1	1	1	1	1	1	1	1	1	17	99	9	1	1
Gelatinase	99	13	1	99	99	75	99	99	99	94	1	67	50	55	25	99
Indole	1	99	99	99	88	1	93	99	99	99	50	33	1	91	25	99
IXP alkaline phosphatase	67	99	25	83	99	99	99	99	99	99	1	83	99	99	99	99
PNPG α-D-galactosidase	33	99	99	17	99	99	99	99	88	99	99	99	1	1	1	1
LGN γ-glutamyl transpeptidase	1	50	99	50	99	1	14	99	82	94	67	99	99	55	25	17
NPS sulphatase	1	88	1	17	88	99	93	99	99	99	1	1	1	55	50	50
Aesculin hydrolysis	99	99	99	67	99	1	99	99	99	99	40	50	1	99	1	99
Utilisation: α-ketoglutarate	1	38	1	99	99	25	99	99	69	99	99	1	1	9	25	99
Acetate	67	38	75	99	88	99	57	99	88	99	99	99	50	73	99	99
Alanine	33	13	99	83	99	50	99	99	94	99	83	99	99	99	99	1
Citrate	33	88	99	83	99	50	99	99	99	94	99	67	75	91	99	1
Citrulline	1	1	25	40	88	1	1	99	1	99	50	67	25	55	1	1
Galactose	1	99	99	83	99	99	99	99	88	99	99	99	1	9	50	99
Gluconate	33	50	1	83	99	99	79	99	94	99	33	83	25	99	75	99
Glucosamine	67	99	99	99	99	99	99	99	94	99	99	1	99	99	99	99
Glucuronate	1	13	1	1	1	99	1	6	53	99	17	1	1	1	1	1
Glycerol	67	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Histidine	99	1	1	33	13	1	1	18	12	1	67	17	1	9	1	1
DL-3-hydroxybutyrate	33	1	75	67	1	1	1	1	6	6	33	1	1	1	1	1
Hydroxyproline	33	1	1	99	1	1	1	1	1	1	1	83	1	18	1	1
DL-lactate	33	99	99	99	99	25	99	99	94	99	99	99	99	99	75	99
Lactose	1	1	25	1	1	1	1	1	6	6	1	1	1	1	1	1
Propionate	67	1	99	67	99	1	7	68	88	94	83	99	99	91	75	1
Putrescine	1	99	1	17	1	1	1	6	1	1	33	1	1	9	1	1
Succinate	1	99	99	83	99	99	93	99	99	99	99	99	99	91	99	99
Sucrose	99	99	99	99	99	99	99	99	99	18	17	99	99	82	25	1
Oxidase	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis	1	13	1	1	1	1	1	1	1	6	1	1	1	9	1	1
Resistance: 0/129 10 µg	67	1	1	1	1	1	14	1	12	1	1	1	1	1	1	33
0/129 150 µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ampicillin 10 µg	33	1	1	1	1	1	1	1	1	1	1	99	75	9	25	1
Novobiocin 5 µg	99	1	50	17	1	1	1	18	1	1	1	67	99	18	1	1
Carbenicillin 100 µg	1	1	1	1	13	1	21	6	1	65	1	99	99	9	25	1
Lysine decarboxylase	1	25	1	1	1	1	1	1	1	1	1	1	1	9	1	1
Ornithine decarboxylase	1	1	1	1	1	1	1	1	1	1	1	1	1	9	1	1
Urease	1	1	1	50	1	1	1	6	1	1	1	1	1	1	1	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Arginine dihydrolase		11	99	25	1	80	99
Acid:	Arbutin	1	1	1	1	1	1
	Mannitol	99	99	99	1	99	1
	Salicin	13	1	1	7	20	1
	Sucrose	99	1	1	14	99	1
	Gentiobiose	1	25	1	93	1	1
Growth:	7% NaCl	89	75	25	21	20	1
	10% NaCl	11	1	1	1	1	1
Amylase		1	99	1	1	80	99
Voges Proskauer (Acetoin)		1	1	1	1	99	1
Gelatinase		11	75	1	21	1	99
Indole		99	75	1	7	99	1
IXP alkaline phosphatase		99	99	99	93	1	33
PNPG α-D-galactosidase		1	99	99	99	80	99
LGN γ-glutamyl transpeptidase		33	1	1	1	1	66
NPS sulphatase		44	99	50	99	20	16
Aesculin hydrolysis		56	50	1	93	1	1
Utilisation:	α-ketoglutarate	1	25	1	1	80	33
	Acetate	99	50	50	7	99	99
	Alanine	1	99	1	1	80	1
	Citrate	89	75	99	99	99	50
	Citrulline	1	1	1	1	1	1
	Galactose	1	99	99	99	60	83
	Gluconate	1	75	25	7	99	1
	Glucosamine	99	99	99	99	20	99
	Glucuronate	44	75	1	1	1	1
	Glycerol	44	99	99	99	99	99
	Histidine	1	1	1	1	60	50
	DL-3-hydroxybutyrate	89	1	1	1	60	1
	Hydroxyproline	11	1	1	1	1	1
	DL-lactate	99	75	1	1	60	99
	Lactose	1	75	99	99	20	1
	Propionate	99	1	1	1	40	1
	Putrescine	1	1	1	1	99	1
	Succinate	78	99	99	93	99	99
	Sucrose	99	1	1	7	80	1
Oxidase		99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1
Resistance:	0/129 10 µg	89	1	1	1	40	1
	0/129 150 µg	1	1	1	1	1	1
	Ampicillin 10 µg	11	75	50	14	1	1
	Novobiocin 5 µg	89	1	1	1	50	1
	Carbenicillin 100 µg	11	99	99	71	1	1
Lysine decarboxylase		1	99	99	93	1	1
Ornithine decarboxylase		1	1	1	1	1	1
Urease		1	75	99	99	80	1