THE PHYLOGENY OF MEMBERS OF THE FAMILY *HALOMONADACEAE*, AND OF FLAVOBACTERIA ISOLATED FROM A HYPERSALINE ANTARCTIC LAKE

by

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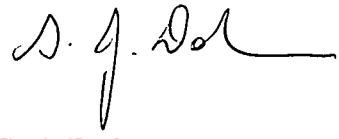

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LIST OF ABBREVIATIONS

Genus abbreviations:

A.	*Antarcticum*
Ac.	*Alcaligenes*
B.	*Bacteroides*
C.	*Cytophaga*
Cyc.	*Cyclobacterium*
D.	*Deleya*
E.	*Escherichia*
F.	*Flavobacterium*
Fc.	*Flectobacillus*
Fx.	*Flexibacter*
H.	*Halomonas*
Hv.	*Halovibrio*
L.	*Listonella*
M.	*Microscilla*
Mb.	*Marinobacter*
Mm.	*Marinomonas*
O.	*Oceanospirillum*
P.	*Pseudomonas*
Pt.	*Pasteurella*
S.	*Sphingobacterium*
Sps.	*Spirosoma*
R.	*Ruminobacter*
Rn.	*Runella*
V.	*Vibrio*
W.	*Wolbachia*

Other abbreviations:

ANGIS	Australian National Genomic Information Service
bp	base pair
ddH_2O	distilled, deionised H_2O
DNA	deoxyribonucleic acid
ssDNA	single-stranded DNA
dsDNA	double-stranded DNA

LIST OF ABBREVIATIONS

Other abbreviations (cont'd):

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RDP	ribosomal RNA database project
rRNA	ribosomal ribonucleic acid
SSU	small subunit
UV	ultraviolet

ABSTRACT

Two recently described species of the genus *Halomonas* occur in the microbiota of some Antarctic saline and hypersaline lakes. The genera *Halomonas* and *Deleya*, which comprise the family *Halomonadaceae*, are difficult to differentiate on the basis of phenotypic and chemotaxonomic attributes. rRNA:DNA hybridisation studies have indicated that some *Halomonas* spp. have the same level of relationship to the type species of the genus *Deleya*, as some *Deleya* spp. Near complete 16S rRNA sequences of 3 *Deleya* spp., 3 *Halomonas* spp., and *Halovibrio variabilis* are obtained by direct sequencing of the PCR-amplified 16S rRNA gene using α-^{35}S dATP. The members of the genera *Halomonas* and *Deleya* do not form separate monophyletic subgroups in the phylogenetic trees, derived from the sequence data, confirming the lack of any phylogenetic support for their retention as separate genera. *Halovibrio variabilis* also clusters within this group of organisms. All the members of the *Halomonadaceae* examined, and *Halovibrio variabilis*, possess a cytosine (C) residue at position 486 (*E. coli* numbering), which is an extremely rare attribute among the prokaryotes, and has been reported in only one other species, *Listonella anguillarum*. Several other signature characteristics which define this group within the gamma-subclass of the *Proteobacteria* are identified. The Jukes-Cantor distances between the members of the family *Halomonadaceae,* and including *Halovibrio variabilis*, range from 0.086 to 0.000 (the % similarities between sequences are in the range 92.6 - 100). The members of the genera *Halomonas*, *Deleya*, and *Halovibrio* form a monophyletic group and share common chemotaxonomic and phenotypic characteristics. Sub-groups among the members of the genera *Halomonas*, *Deleya* and *Halovibrio* can not be resolved on the basis of phylogenetic, chemotaxonomic or phenotypic data. It is proposed that the genera *Halomonas*, *Deleya* and *Halovibrio* be united in a single genus *Halomonas*. The new names *Halomonas aquamarina* comb. nov., *Halomonas cupida* comb. nov., *Halomonas halophila* comb. nov., *Halomonas marina* comb. nov., *Halomonas pacifica* comb. nov., *Halomonas venusta* comb. nov., *Halomonas salina* comb. nov. and *Halomonas variabilis* comb. nov. are proposed. The descriptions of the genus *Halomonas* and the family *Halomonadaceae* are emended.

The phylogenetic placement of pigmented bacteria isolated from Organic Lake, a hypersaline Antarctic Lake, is examined by the determination and analysis of their 16S rRNA sequences. Sequence signatures confirm that the pigmented bacteria from Organic Lake are members of the "flavobacteria-*Bacteroides*" phylum. Comparison of the sequences of the Organic Lake strains with those of a large number of sequences available for members of the "flavobacteria-*Bacteroides*" phylum show that they are phylogenetically distinct. These organisms can also be distinguished on the basis of phenotypic and chemo-taxonomic data, supporting their description as new taxa, at least at the species level. The Organic Lake flavobacteria cluster within a group of organisms that contains the type species of the genus *Flavobacterium*, *Flavobacterium aquatile*. Two new species of the genus *Flavobacterium* are described to accommodate the Organic Lake flavobacteria. The names *Flavobacterium gondwanense* and *Flavobacterium salegens* are proposed.

Potential oligonucleotide probes for the genus *Halomonas* (emended as proposed), some member-species of the genus *Halomonas*, *F. gondwanense* and *F.salegens* are identified from their 16S rRNA sequences.

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1 INTRODUCTION

The comparison of 16S rRNA sequence data is widely accepted as a means of estimating phylogenetic relationships among prokaryotes (Woese, 1987; Murray et al, 1990; Stackebrandt, 1992; Woese, 1992). For the taxonomy of the prokaryotes to be constructed on the basis of phylogenetic relationships between organisms, means not only that the taxonomic structure is informative of phylogenetic relationships but also that the structure is stable. The determination of 16S rRNA-based phylogenetic relationships has provided a means of clarifying taxonomic relationships within several groups of prokaryotic organisms which have been recalcitrant to analysis on the basis of the range of phenotypic characteristics traditionally employed to classify bacteria, chemotaxonomic data, and mol% G+C values.

During the past 10 years the taxonomy of prokaryotes isolated from lakes in the Vestfold Hills region of Antarctica, has been investigated, and several new taxa have been described (James et al, 1990; Franzmann et al, 1987; Franzmann et al, 1988; Franzmann et al, 1991; Franzmann and Rohde, 1991; Franzmann and Dobson, 1992; McGuire et al, 1987). The taxonomic placement of some of these organisms has been made difficult by the uncertain taxonomic structure of the groups to which they appear to belong. The Organic Lake flavobacteria are one such set of organisms. This set of bacterial strains have been examined phenotypically, their phospholipid fatty acids and respiratory lipoquinone profiles and their mol% G+C values have been determined (Dobson et al, 1991; Skerratt et al, 1991; P. D. Franzmann pers. comm.). These characteristics have suggested that the Organic Lake flavobacteria are members of the *Flavobacterium-Cytophaga* complex, however the many taxonomic difficulties associated with this complex and related genera have made the generic assignment of the Organic Lake flavobacteria problematic. A large database of 16S rRNA sequences of members of the *Flavobacterium-Cytophaga* complex and related genera has been assembled in the laboratory of C. R. Woese (Olsen et al,1991). A comparison of the 16S rRNA sequences of the Organic Lake flavobacteria with those of the wide range of organisms represented in this database, would allow the phylogenetic placement of the Organic Lake flavobacteria within this set of organisms to be determined.

The species *Halomonas subglaciescola* and *Halomonas meridiana* have been described to accommodate strains isolated from some hypersaline lakes of the Vestfold Hills region (Franzmann et al, 1987a; James et al, 1990). Phenotypic and chemotaxonomic differentiation of the genera *Halomonas* and *Deleya* is difficult, and has suggested that the members of these two genera could be placed in a single genus. An examination of 16S rRNA-based phylogenetic relationships among members of these genera, representative of the diversity within the group, would indicate whether such a proposal was valid. Chemotaxonomic data have suggested a close taxonomic relationship between *Halovibrio variabilis* and members of the genera *Deleya* and *Halomonas*. To clarify the phylogenetic relationship of *Halovibrio variabilis* to members of the genera *Deleya* and *Halomonas* this organism is included in the comparison of 16S rRNA sequences.

The introduction of the polymerase chain reaction (PCR) in recent years has made available an alternative and potentially more efficient means of obtaining 16S rRNA gene sequence data (Saiki et al, 1988). Difficulties are encountered in generating sequence data from PCR-generated dsDNA using ^{35}S-labelled nucleotides and the standard protocol. A modification of the standard protocol proposed by Bachmann et al (1990) suggests that inclusion of the detergent non-idet P40 in the reaction mixture, and snap-freezing of the boiled annealing mixture in the first stage of the sequencing reaction, significantly improves the quality of the sequence data. This method is used to obtain 16S rRNA sequence data in this study.

Descriptions of genera and species are most important for the identification of isolates. At these taxonomic levels it is considered by, the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne et al, 1987) and the Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria* (Murray et al, 1990), that DNA:DNA hybridisation and rRNA sequence data are not sufficient in themselves for the description of taxa. Taxonomic descriptions at these levels must include phenotypic data. A taxonomic approach which integrates the phenotypic, chemotaxonomic and phylogenetic characteristics of a group of organisms has been termed "polyphasic" taxonomy (Colwell,

1970; Murray et al, 1990). The examination of the Organic Lake flavobacteria, and the members of the genera *Deleya*, *Halomonas* and *Halovibrio*, in this study, is based on a polyphasic approach.

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2 LITERATURE REVIEW

2.1 Phylogeny in bacterial classification

2.1.1 An overview

Bacterial phylogenies are based on the comparison of organisms by, comparison of the primary structure of homologous sections of their DNA, or by comparison of the transcribed or translated products of homologous genes. Differences in the structures of these homologous moieties indicate the number of mutations that have become fixed in the DNA of the organisms during their evolutionary history from a hypothetical common ancestor. Where there is evidence that mutations have occurred randomly, as is assumed for mutations that do not result in a functional change, the section of DNA, the RNA or the protein, acts as a molecular or evolutionary clock (Woese, 1987; Zuckerland and Pauling, 1965).

Prior to molecular data becoming readily accessible for the taxonomy of prokaryotes, the means for testing hypotheses about prokaryotic phylogeny were not available. The fossil record is extremely limited. Speculation on the order of appearance of physiological groups could only be considered in relation to information about the earth's environmental conditions during its history.

Prior to the determination of phylogenetic relationships, the details of bacterial taxonomy were derived from a comparison of the morphological and physiological data for extant organisms. It was recognised that the selection of characters that defined taxonomic groups at the higher levels was a largely intuitive process. Prokaryotic taxonomy was inherently unstable and primarily a determinative structure for the identification of organisms. While genera and higher taxonomic levels were defined in terms of Gram reaction, morphology, motility and major physiological features, the difficulty of selecting significant identifying features at the species level was so apparent that it became a standard practice to assess the overall similarity of organisms across a wide range of characteristics and give

all characteristics an equal weighting (Sneath and Sokal, 1973). This approach was termed Adansonian numerical taxonomy. Characteristics, or combinations of characteristics associated with the groups of organisms which resulted from the numerical analysis, were identified, and used to define species.

Genotypic data in the form of mol% G+C in the genome, and chemotaxonomic data were new sources of information, utilised to assess the validity of taxa defined on the basis of selected morphological and physiological characteristics. Mol% G+C data indicated where organisms which might appear to be similar, based on the range of morphological and physiological characteristics examined, were clearly not because of disparate mol% G+C values (Mamur and Doty 1962). Groups of organisms, formed on the basis of common chemotaxonomic features, particularly those features that were relatively rare and therefore distinctive, could be assessed against the existent taxa. The majority of chemotaxonomically informative moieties occurred in the membrane and wall of the cell envelope (Goodfellow and Minnikin, 1985).

Molecular data which reflected the degree of nucleotide sequence similarity between a pair of organisms was first obtained from DNA:DNA and DNA:rRNA hybridisation experiments. The level of hybridisation indicated the extent of sequence similarity between two strains, and therefore provided proof of a specific relationship at the molecular level, or lack thereof (Moore and McCarthy, 1967).

The Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics has defined the bacterial species in terms of DNA:DNA hybridisation values as follows:

"The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m. Both values must be considered." (Wayne et al, 1987)

This level of DNA:DNA hybridisation suggests a high level of sequence similarity between the two organisms. It has been estimated that a 1% sequence mismatch results in a ΔT_m of 1 to 2.2 °C (Bonner

et al, 1973, Ullman and McCarthy, 1973). A ΔT_m of 5°C or less therefore implies a sequence mismatch of 5% or less between organisms belonging to the same species. DNA:DNA hybridisation will not detect similarity between organisms with greater than at most 15% sequence mismatch between their genomes (Johnson, 1991). It is therefore limited to the comparison of closely related organisms. The significant extent of sequence conservation in the rRNAs allowed similarities between more distantly related organisms to be examined by DNA:rRNA hybridisation.

More detailed and extensive use of the rRNAs was made by comparisons of sequence data (Fox et al, 1977). The limitation of the extent of mismatch which could be accommodated by hybridisation experiments did not apply. Neither was it required that pairwise data for all combinations of organisms be determined. Sequence data was determined independently for each organism, and could be compared to the sequence of the homologous moiety from any organism which had been previously determined and held in accessible databases.

The majority of studies examining bacterial phylogeny above the level of intra-species association use a comparison of 16S rRNA sequences or DNA:rRNA hybridisation using 16S rRNA or 23S rRNA. Homologs of the small-subunit (SSU) rRNA (16S rRNA in prokaryotes, 18S rRNA in eukaryotes) occur in all living organisms in the ribosomes, the site of protein synthesis in the cell. Regions of the primary and secondary structure of SSU rRNA are identical in all organisms from all the domains, thus far examined (Neefs et al, 1990). Nomura et al (1968) demonstrated the functional constancy of the 16S rRNA and protein components of the 30S ribosomal particle by forming functionally active hybrid 30S particles from 16S rRNA and protein components derived from distantly related bacteria. The functional constancy of the 16S rRNA suggested that it had not been subjected to overbearing selective forces. For this reason it was considered that the 16S rRNA was an effective molecular clock (Woese, 1987).

Different degrees of conservation occurred in different regions of the SSU rRNA. It was suggested that the less conserved regions are less functionally important (Nomura et al, 1968). The variation in the degree of conservation meant that the "speed" of this

molecular clock varied such that relationships at levels encompassing the highly unrelated (inter-domain) to the highly related (intra-generic) have been examined (Woese, 1987).

Another advantageous aspect of the SSU rRNA is the occurrence of signature nucleotides associated with monophyletic groups. These signature nucleotides occur rarely, or not at all, outside the particular group, and act in a definitive manner for the group in a way that phenotypic and chemotaxonomic characteristics have been used previously (Woese, 1987). Signature nucleotides can be used to identify membership of a group where that affiliation may be uncertain from overall sequence similarity.

The advantages listed above for the use of the 16S rRNA as a means for establishing bacterial phylogeny have resulted in the general acceptance of this approach. The inherent limitations of the approach have also been recognised. Were the molecular clock to behave perfectly, the resultant data over a large sample size would behave in a perfectly ultrametric manner and the derivation of phylogenetic relationships would be straightforward and precise. Alternatively, to allow for differences in rates of mutation, the data may be considered additive. Similarly if data were perfectly additive a precise solution would be achievable (Swofford and Olsen, 1990). The fact that real data sets do not behave in a perfectly ultrametric or additive manner suggests that some phenomena which weaken the molecular clock premise have operated. Therefore consideration must be given to these phenomena, and the phylogenetic relationships which are derived from a comparison of molecular data need to be regarded as an estimate of the true phylogeny.

One limitation is sampling error. Sampling of a single gene to construct an evolutionary history of the entire genome has statistical limitations. Sneath (1989) calculated the standard sampling error for a tree from the lengths of the internodes incident to a node, and the associated probabilities for the location of nodes. This analysis showed that there was a high degree of uncertainty about the placement of nodes when the number of nucleotide changes, reflected in the length of the incident internodes, was small. An example given was the radiation of several of the phyla belonging to the *Bacteria* where the internodal distances were relatively small, and consequently

the ordering of these branchings was uncertain. This type of uncertainty may also be reflected by application of bootstrap analysis to phylogenetic constructions (Felsenstein, 1985). Bootstrap analysis of sequence data involves random resampling with replacement, so that in effect some characters have a zero weight, and others have a weight of one, two or more. The phylogenetic tree is then determined from this data, and the process of resampling and tree determination repeated many times. A majority rule consensus tree is obtained from these trees which indicates the frequency that particular groupings occur within the tree. Blair-Hedges (1992) determined the relationship between the number of bootstrap replications and the accuracy of the bootstrap P values associated with groupings within a tree. It was found that 2000 replications were required for a confidence limit of 95% to have an accuracy of ± 1, and for confidence limits greater than 75% to have an accuracy of at least 2%.

Limitations more difficult to quantify than sampling error, but of possibly greater significance are those which reflect limitations in knowledge of the evolutionary process. The premise that sequences diverge constantly over time due to the fixation of randomly occurring mutations, which is the basis of the molecular clock concept, may be weakened by a variety of phenomena.

As mutations accumulate there is a probability that more than one mutation will occur at a particular site. This effect diminishes the additivity of raw data. The superimposed mutation eradicates any history of the previous mutation and possibly results in a mutation to the original identity of the nucleotide at that site, thus appearing as if no mutational events have occurred. Additionally parallel and convergent mutations may arise. These masking mutations, termed homoplasies, are corrected for in pairwise distance forms of analysis by the application of the Jukes-Cantor equation or related equations (Swofford and Olsen, 1990).

Of potentially greater significance than the occurrence of homoplasies is the evolutionary theory of punctuated equilibrium. This theory proposes that evolutionary history is made up of periods of very rapid rates of change and periods of slow rates of change, the former being associated with changes in environmental conditions (Eldredge and Gould, 1972). The historical flux of environmental

conditions, as they pertain to different groups of organisms, mitigates against the development of a simple mathematical model to define phylogenetic relationships from the molecular sequences of extant organisms.

There is evidence from the data that lineages have evolved at different rates (Woese, 1987). Woese (1987) cites the example of the mycoplasmas, where a high rate of evolution is suggested by an unusually high number of mutations in conserved regions of the 16S rRNA and which is correlated with an unusual phenotype. Variation in evolutionary rates of different lineages is suggested by the location and length of branches, particularly when the effect is pronounced (very short, or very long branches). The variation in evolutionary rates of different lineages overlays that which exists within the SSU rRNA itself in terms of the variation of the degree of conservation in different regions of its structure. In general, these possible and actual sources of variation are not accounted for in the estimation of phylogenetic relationships. Sneath (1989), noted that while there is often good correlation in the phylogenies derived from different molecules, there are examples of trees derived from 16S rRNA and 5S rRNA data respectively where discrepancies in branch length are larger than those predicted by sampling error. One explanation for this is that there are different rates of mutation in different genes and different lineages. Given the types of variation in evolutionary rates that do occur, or appear to occur, the determination and application of a single rate of mutation for the SSU rRNA gene for the prediction of the age of lineages, such as undertaken by Ochman and Wilson, (1987), should be treated with caution.

Generally, the characters which make up the SSU rRNA sequence data are treated as independent and of equal weight. Corrections which sometimes have been applied are: various weighting of gaps (Swofford and Olsen, 1990), giving paired bases a 1/2 weight (Stahl et al, 1984), and eliminating positions which demonstrate hypervariability (Weizenegger et al, 1992). As a rule positions where homology is uncertain are eliminated from analyses (Swofford and Olsen, 1990). Other types of modifications which are sometimes applied are the differential weighting of transition and transversion mutations (Swofford and Olsen, 1990), and

accommodation of the actual ratio of nucleotide types observed in the calculation of evolutionary distances rather than assuming all nucleotide types occur in equal proportions (Woese et al, 1990b).

A further consideration is the extent to which transfer of genetic material plays a role in bacterial evolution. It has been suggested that the 16S rRNA gene has not been involved in lateral transfer because it is integral to cell survival. Evidence against lateral transfer of this gene has come from a comparison of the phylogenetic relationships among some purple photosynthetic bacteria derived from cytochrome c amino acid sequences and 16S rRNA sequences (Woese et al, 1980). The relationships were similar indicating that lateral gene transfer of either gene had not occurred.

The question of transfer of genetic material, remains however, because if a large proportion of the genome has been involved in lateral transfer, then the likelihood of deriving a meaningful "natural" classification from a small number of conserved genes that have not been transferred, is diminished, as is the likelihood of locating phenotypic characters to identify with such genotypically defined groups. Evidence for the non-involvement of genes in lateral transfer comes from the positive correlation of phylogenetic relationships derived from different genes as illustrated in the example above. These correlations derive not only from the small number of different genes and proteins which have been sequenced, but also from correlations between monophyletic groupings derived from molecular data and groupings derived from chemotaxonomically informative components of the cell such as the lipids. At the intra-species level patterns of multilocus enzyme polymorphisms in *E. coli* indicated coherent genetic groups as did strong linkage disequilibria (Young, 1989).

Like many aspects of bacterial diversity the available data represents only a small sampling, so that the proportion of bacterial genomes involved in lateral transfer and the range of organisms that will accept genetic material from a donor organism have not been quantified. The accumulated evidence thus far suggests that the extent of lateral transfer of genetic material does not undermine the derivation of bacterial phylogenetic relationships from the SSU rRNA

or from other semantides which may be considered to behave as molecular clocks (Young, 1989).

2.1.2 Derivation of phylogenetic trees from 16S rRNA sequences

Several methods have been developed to determine phylogenetic trees from sequence or other data. The majority of workers who have derived phylogenetic trees from 16S rRNA sequence data have employed least-squares additive methods (Embley et al, 1988a; Fryer et al, 1992; Nazaret et al, 1991; Rössler et al, 1991; Stackebrandt and Charfreitag, 1990; Stahl et al, 1992; Tsuji, K. et al, 1990; Unterman, et al, 1989; Woese et al, 1990). The input data for these methods are a matrix of pairwise distance values. These distance values are calculated from the sequence similarity values using a formula such as the Jukes-Cantor equation to correct for homoplasies. For a given tree topology, the error is calculated as a sum of squares, and the branch lengths which minimise the error are determined. There are several variations in the manner in which the error is calculated (Felsenstein, 1982). Fitch and Margoliash (1967) calculated the error, or quantity to be minimised, as the following sum of squares,

$$\frac{\Sigma_i \Sigma_j (D_{ij} - d_{ij})^2}{D_{ij}^2}$$

where D_{ij} is the observed pairwise distance between taxa i and j and d_{ij} is the distance between taxa i and j in the tree topology under examination (Felsenstein, 1982). The formula of Olsen (1988) to calculate the error replaces the D_{ij}^2 term in the denominator of the sum of squares, with the variance for the observed distance, which allows the effective sequence length to impact on the error estimation.

Except for small numbers of taxa, the number of possible tree topologies is too large for a comprehensive examination. In effect only a small subset of trees is examined (Olsen, 1988). Branch rearrangement methods are widely used to test whether any improvements can be found (Olsen, 1988; Felsenstein, 1989). The range of tree topologies examined is dependent on the method by

which the original trees are constructed. Where this construction method involves adding taxa to the tree in the order in which they are listed in the matrix, different random orders may be used to extend the search and indicate whether the topology of the best tree found is sensitive to such changes (Felsenstein, 1989).

Fitch (1984) has noted that where there is an implicit assumption that the rate of evolution is approximately equal among the group of taxa under examination, e.g. if the decision to join taxa and the order in which taxa are joined in tree construction is made on the basis of distance values, then a misleading result may be obtained when taxa evolving at very different rates of evolution are being studied.

Olsen (1987) demonstrated that the branching point of a ciliate mitochondrial 16S rRNA sequence, a fast evolving lineage, was incorrectly located within the *Bacteria* when very distantly related organisms, from the *Archae* and *Eukaryae* were included in the tree. This effect was ameliorated by adding close relatives of the mitochondrial sequence to the tree. Olsen (1987) suggested that inadequacies of the Jukes-Cantor equation are responsible for errors in the branching order when lineages evolving at very different rates are involved. He suggested that the Jukes-Cantor equation underestimates evolutionary distances, because the assumptions which underlie it do not hold. Consequently a systematic error is introduced which will be most apparent when lineages evolving at very different rates are involved. One of the underlying assumptions of the Jukes-Cantor equation is that all sequence positions are equally subject to change. Olsen determined evolutionary distances on the basis that 95% of sequence positions have nucleotide substitution rates in the range 1/8 to 8 times that of the median, and found that the branching order anomalies observed under the use of the Jukes-Cantor equation did not occur.

Generally, it is not the case that a very fast evolving lineage with few specific relatives and very distantly related organisms are being compared, so that the effects that Olsen (1987) examined would not be expected to be significant. The branching order of a tree should, however, be tested for robustness or stability to changes in taxa composition. This can be tested by examining the effect of the

addition or deletion of taxa on the branching order and by examining the contribution that the presence of individual taxa have to the error value (Swofford and Olsen, 1990). As systematic errors cannot be precisely defined, the proviso generally remains, that for any phylogenetic tree the possibility exists that the addition of new taxa, as data becomes available, may affect the branching arrangement.

Parsimony methods have sometimes been used in addition to additive methods for the derivation of phylogenetic relationships from 16S rRNA sequences (Dame et al, 1992; Woese et al, 1990; Weizenegger et al, 1992) and represent a quite different treatment of the data. Parsimony analysis determines the minimum number of character state changes that would be required at each sequence position to result in the set of extant sequences for a given tree topology. The number of character state changes across all sequence positions is totalled for the given tree topology, and compared with the values obtained for other tree topologies. The tree topology/ topologies having the smallest number of character state changes is/are selected as being the best estimation of the phylogeny of that group of taxa (Swofford and Olsen, 1990). Analyses which guarantee finding the most parsimonious tree(s) are limited to small numbers of taxa, because the number of possible tree topologies increases dramatically as the number of taxa increases.

The most parsimonious tree minimises the number of homoplasies required to derive phylogenetic relationships among a group of taxa. As the relative rate of nucleotide substitution at a position increases, that position becomes less phylogenetically informative. The probability of obtaining the wrong branching order has been shown to increase with the relative rate of nucleotide substitution, where peripheral branches are much longer than internal ones (Felsenstein, 1978; Olsen 1987). Olsen (1987) limited inter-domain (inter-"urkingdom") comparison of groups of taxa to 16S rRNA sequence positions where there was > 93% conservation among members of a domain ("urkingdom").

2.1.3 The conciliation of phenotypic and phylogenetic taxonomies

Since the introduction of techniques to determine mol % G+C of DNA a great deal of confidence has been placed in molecular

data for bacterial classification, because of the stability of the data and the phylogenetic implications which can be drawn. Notwithstanding the limitations in deriving phylogenetic relationships from molecular data, which are summarised above, such phylogenetic classifications are the basis for a stable framework for the construction of a bacterial taxonomy and provide the means for deciding which phenotypic characteristics are taxonomically informative and at what rank they are informative.

The phylogenetic framework which has emerged from molecular studies, primarily from rRNA-based studies, has disturbed aspects of previous taxonomic or determinative groupings: by splitting some physiological groups, such as the placement of photosynthetic organisms in different subclasses of the class *Proteobacteria* along with non-photosynthetic organisms; by identifying relationships between physiologically distinct organisms, such as members of the genera *Bacteroides* and *Flavobacterium*; and by identifying organisms with an unusual phenotype which are evolving at a faster rate, such as the mycoplasmas which previously had been considered to represent a separate phylum, but which are now considered a fast evolving lineage within the gram-positive phylum (Woese, 1987).

Molecular data indicates where taxonomic groupings include highly unrelated organisms because of the choice of inappropriate phenotypic characteristics to define certain groups. There are also several examples, such as the genus *Bacillus*, where the traditional grouping is monophyletic but where the presence of phylogenetic sub-groups suggest the circumscription of several genera rather than a single genus (Rössler et al, 1991; Ash et al, 1991). The task in these circumstances is to identify phenotypic characteristics to associate with the underlying phylogenetic groups.

The potential for conflict between phylogenetic and phenotypic classifications arises from the possibility of disjuncture between phylogenetic groups and determinable phenotypic characters, or the apparent phenotypic incoherence of phylogenetic groups. The outcome of these possibilities would be an inability to identify new isolates within a phylogenetic scheme. Such a worst case scenario has not arisen. Phenotypic coherence is most important at the species and

genus ranks, as these are the most relevant ranks for the identification of isolates (Stackebrandt, 1992). It is usually the case that phenotypic coherency is evident at these taxonomic ranks.

Where phenotypic characteristics have not been identified to define phylogenetic groups at the species and genus levels, it is generally considered that those phylogenetic groups should not be described as new taxa until distinguishing phenotypic characteristics can be identified (Wayne et al, 1987; Stackebrandt, 1992; Murray et al, 1990). Enmeshing phylogenetic and phenotypic descriptions in this way, ensures that the application of phylogenetic groupings will not outstrip the available knowledge of phenotype, and that the classificatory structure remains useful for identification.

Taxonomic ranks above genus are often not important for the identification of isolates, and while coherent phenotypic characteristics may already be associated with taxa at the higher levels, the identification of such characteristics are not essential for the description of taxa at these levels (Stackebrandt, 1992). The class *Proteobacteria* is an example of a taxon without identifying phenotypic characteristics (Stackebrandt et al, 1988). The existence of coherent phylogenetic groups at lower taxonomic ranks which cannot be separated phenotypically, and coherent phylogenetic groups at higher taxonomic ranks which cannot be adequately described phenotypically, has promoted a search for phenotypic characteristics which will define these groups. Above the species level, such characteristics have often been located in chemotaxonomic data, rather than the range of morphological and physiological attributes traditionally employed in phenotypic descriptions of taxa. The exploration for definitive characteristics at the highest taxonomic level is exemplified by work on the *Archaea*, which has confirmed the significance of distinctive chemotaxonomic features such as the presence of ether-linked lipids and identified numerous distinctive features in the molecular biology of this group (Kandler, 1982).

2.1.4 Interpretation of taxonomic rank among the prokaryotes

The taxonomic ranks which may be applied to bacterial groupings are species, genus, family, order, class, phylum (= division), and kingdom (Sneath, 1984). The rank of domain has been

recently introduced above the rank of kingdom to apply to the separation of living organisms into three fundamental groupings, the *Bacteria*, the *Archaea* and the *Eukaryae*, although this proposal has not been universally accepted (Woese et al, 1990a; Cavalier-Smith, 1992).

Taxa at all ranks describe monophyletic groups of organisms. The degree of relationship between a monophyletic group of organisms, is reflected in the taxonomic rank chosen to describe that group. DNA-DNA hybridisation values in the order of 60 - 70% or greater have been proposed to define the degree of relationship applicable to the rank of species (Brenner, D. J. 1981; Johnson, 1984; Wayne et al, 1987). This level of DNA-DNA hybridisation was proposed on the basis of the phenotypic and genotypic characterisation of strains in many groups of bacteria, and so cannot be considered to be simply an arbitrary cut-off level (Johnson, 1984, Fox et al 1992). Exploration for a conceptual, rather than phenetic description for the bacterial species continues in the context of genetic exchange and the occupation of environmental niches (Young, 1989; Dykhuizen, 1991; Amann et al, 1992).

In comparison to species definition, the application of numerical homology values using 16S rRNA data has not been useful for the definition of genus or higher taxonomic ranks (Stackebrandt, 1988). The 16S rRNA sequence similarity among members of a genus varies from 78% to 95% for different genera (Stackebrandt, 1992). Stackebrandt (1988) suggested that this range of values reflects the difference in ages of different genera, and has broadly classified the ages of genera into three groups. These three groups reflect the emergence of genera during the periods, when the earth was anaerobic, when the transition from an anaerobic to an aerobic environment occurred, and since the earth has been largely aerobic. General features of the oldest genera are divergence at the molecular level and conservation at the phenotypic level. Conversely "young" genera have highly similar 16S rRNA sequences and yet exhibit phenotypic and chemotaxonomic variability.

Beyond the requirement for hierarchical monophyly, the ranks of genus and above remain undefined. This allows for considerable variance in the use of criteria for the delineation of taxa.

2.2 Taxonomy of Gram-negative, pigmented, aerobic bacteria with a mol %G+C in the range 30 - 53; members of the "flavobacteria-*Bacteroides*" phylum

2.2.1 An overview

The rather long heading for this section is an indication of the lack of meaningful definition in the taxonomy of this group of organisms at the sub-phylum level. The several genera which contain species of pigmented, aerobic bacteria, which have a low mol %G+C are listed in Table 2.1. Taxonomic difficulties with this group arise from inadequate description and differentiation between genera, a large amount of heterogeneity within genera, and regions of overlap between genera. Several members of this group of genera are sometimes collectively referred to as the *Flavobacterium-Cytophaga* complex (Reichenbach and Weeks, 1981). Related to this group of genera are the strictly fermentative species of the genus *Capnocytophaga*, and the strictly anaerobic species of the genus *Bacteroides*. As the genus name suggests, *Capnocytophaga* was considered to be related to the genus *Cytophaga*, because its member species were characterised by gliding motility, mol % G+C of their DNA in the range 33 - 41, and yellow-orange pigmentation (Leadbetter et al, 1979).

A relationship between the members of the *Flavobacterium-Cytophaga* complex and members of the genus *Bacteroides* was first recognised from a comparison of 16S rRNA oligonucleotide catalogues (Paster et al, 1985). The branch of the *Bacteria* which is comprised of members of the *Flavobacterium-Cytophaga* complex, the *Bacteroides* and related genera has often been referred to as a phylum, however as yet no taxonomic rank has been formally assigned to this branch. The 16S rRNA sequences of some members of all the genera listed in Table 2.1 have been determined, with the exceptions of the genera *Flexithrix* and *Chitinophaga*. These two genera contain a single species, represented by a single strain and five strains respectively. The 16S rRNA studies of members of the other genera have shown that they belong in the "flavobacteria-*Bacteroides*" phylum .

Table 2.1 Genera containing pigmented, aerobic, "low" mol % G+C bacteria[a]

Genus	mol % G+C
Cytophaga	30 - 45
Flavobacterium[b]	31 - 39
Flectobacillus	33 - 40
Cyclobacterium	34
Saprospira	35 - 48
Sporocytophaga	36
Flexithrix	37
Microscilla	37 - 44
Flexibacter	37 - 47
Sphingobacterium[b]	40 - 46
Chitinophaga	43 - 46
Haliscomenobacter	49
Runella	49 - 50
Spirosoma	51 - 53

a Data from Reichenbach, 1989; Holmes 1992; Mulder, 1989; McGuire, 1987; Raj and Maloy, 1990

b For the purposes of this table, the G+C values for *F. thalpophilum* and *F. yabuuchiae* were included in the genus *Sphingobacterium*

Several of the genera listed in Table 2.1, have been considered members of the order Cytophagales. In Bergey's Manual of Systematic Bacteriology (Reichenbach, 1989), the order Cytophagales, was comprised of the families *Cytophagaceae*, "*Flavobacteriaceae*" *Bacteroidaceae*, some related genera of the family *Cytophagaceae*, *Flexibacter*, *Microscilla* and *Chitinophaga*, and some genera of uncertain affiliation, *Saprospira*, *Taxeobacter*, *Haliscomenobacter*, and the organism Pl-12fs. In this arrangement the family *Cytophagaceae* was comprised of the genera, *Cytophaga*, *Capnocytophaga*, *Flexithrix* and *Sporocytophaga*. In The Prokaryotes 2nd ed (Reichenbach, 1992) the order Cytophagales was restricted to a single family, the *Cytophagaceae*, comprised of genera of unicellular gliding bacteria, *Cytophaga*, *Capnocytophaga*, *Flexithrix*, *Sporocytophaga*, *Flexibacter*, *Microscilla*, *Taxeobacter*, unnamed *Cytophaga*-like bacteria and possibly *Saprospira*. Reichenbach (1992) noted that this characterisation of the order should be considered preliminary.

The genera *Cytophaga*, *Flavobacterium*, *Flectobacillus*, *Cyclobacterium*, *Saprospira*, *Sporocytophaga*, *Flexithrix*, *Flexibacter*, *Chitinophaga*, *Haliscomenobacter*, *Runella* and *Spirosoma* are differentiated phenotypically, on the basis of gliding motility, cell morphology, presence of sheaths, formation of myxospores and habitat. The genus *Sphingobacterium* was created to accommodate species which contained sphingophospholipids, and originally classified as belonging to the genus *Flavobacterium* (Yabuuchi et al, 1981; Yabuuchi et al, 1982; Yabuuchi et al, 1983).

This review will focus on the genera *Cytophaga* and *Flavobacterium*. These two genera are selected as the Organic Lake, pigmented bacteria, which were studied in this work, most closely resembled members of these genera. In comparison with the other genera listed in Table 2.1, these two genera are comprised of a large number of species and reflect the taxonomic complexities of the whole group.

The genera *Cytophaga* and *Flavobacterium* are differentiated by a single phenotypic characteristic. Members of the genus *Cytophaga* exhibit gliding motility and members of the genus

Flavobacterium do not (Holmes et al, 1984a). Separation of these two genera, on the basis of a single characteristic has been questioned for over a decade, in light of many shared characteristics (Reichenbach and Weeks, 1981; Christensen, 1977). A simple combination of *Cytophaga* and *Flavobacterium* has been prevented, however, by a recognition of significant heterogeneity within each genus. Such heterogeneity has suggested rather, that these genera should be subdivided (Reichenbach, 1992; Holmes, 1992).

2.2.2 General characteristics of the genus *Flavobacterium*

The genus *Flavobacterium* was originally proposed as a colour genus in 1923, and consequently became a collection of heterogeneous, and often poorly described species (Holmes et al, 1984a). Progress towards a homogeneous taxon was made by successive removal of polarly-flagellate strains, Gram-positive organisms, gliding or spreading organisms and "high" G+C strains (Holmes et al, 1984a). The resultant genus description, in Bergey's Manual of Systematic Bacteriology (Holmes et al, 1984a), was of cells that were Gram-negative, aerobic, rods, which did not form endospores or granules of poly-ß-hydroxybutyrate, non-motile either by gliding or flagella, typically pigmented (yellow to orange), catalase-, oxidase- and phosphatase positive, non-agarolytic, chemoorganotrophic and mol% G+C of their DNA in the range 31 - 42. Seven species were listed in Bergey's Manual of Systematic Bacteriology and ordered into the following four groups:

1. *F. aquatile*

2. *F. breve*, *F. balustinum*, *F. meningosepticum*

3. *F. odoratum*

4. *F. multivorum*, *F. spiritovorum*

F. indoltheticum, a species represented by a single strain, was not included in this listing of species because it was phenotypically similar to *F. breve* and had a similar mol% G+C value (Holmes et al, 1984a). It was later listed as a separate species which could be differentiated from *F. breve* on the basis of esculin hydrolysis and a

slightly higher G+C value, 33.8 compared with 32.4 ± 0.6 (Holmes 1992).

F. aquatile was designated as the type species when the genus *Flavobacterium* was created. Holmes and Owen (1979) presented arguments to the Judicial Commission for the rejection of *F. aquatile* as the type species. These arguments elaborated problems associated with the history of isolation and early descriptions of the species, the atypical phenotype of the species in comparison with other *Flavobacterium spp.*, and the consequent difficulties that would be imposed on medical microbiology if it were to remain the type species.

None of the original isolates of *F. aquatile* are extant, and the current type strain (ATCC 11947), is an organism isolated from the same region as the original isolate 63 years after the time of the original isolation and description of the species (Holmes and Owen, 1979). The original description of *B. aquatilis* in 1889 (latter renamed *F. aquatile*), was, according to Holmes and Owen (1979), "meager and ambiguous", and referred to oscillatory movements and filaments suggesting that the original isolate may have been a cytophaga or flexibacter.

Disagreement existed as to whether the type strain, ATCC 11947, exhibited gliding motility or not (Holmes and Owen, 1979). This feature was critical as its presence would be in conflict with the description of the genus *Flavobacterium*, and would place the organism among the cytophagas. Holmes and Owen (1979) foreshadowed that the outcome of the determination of a close relationship between *F. aquatile* and the cytophagas would be the transfer of cytophagas into the genus *Flavobacterium* as this genus preceded the genus *Cytophaga* and the creation of a separate genus (or genera) to accommodate the other *Flavobacterium* species. This would result in confusion in the field of medical microbiology as all of the other species of *Flavobacterium*, except *F. balustinum*, were clinical isolates.

Holmes and Owen (1981) proposed the differentiation of the members of *Flavobacterium* into four genera. These genera A-D were comprised as follows:

A. *Flavobacterium breve, F. meningosepticum, F. indoltheticum, F. balustinum* and Group IIb

B. *F. odoratum*

C. Group IIk-2, "Group IIk-3"

D. Group IIf and Group IIj.

This scheme did not include *F. aquatile*. The proposal to reject *F. aquatile* as the type species and to substitute *F. breve* as the type species was not accepted by the Judicial Commission (Wayne, 1982). Consequently *F. aquatile* was included in the arrangement of the species in Bergey's Manual of Systematic Bacteriology (Holmes et al, 1984a), listed previously (page 20). The two groupings are equivalent in that group 2 = genus A, group 3 = genus B, group 4 = genus C. More recently, separation of species into the four genera A - D was the basis for the arrangement of species in The Prokaryotes, 2nd ed (Holmes, 1992). Holmes (1992) did not list *F. aquatile* among the members of *Flavobacterium* in this description of the genus, although he noted that if the genus was sub-divided, the genus name would remain with *F. aquatile*, and the other species of *Flavobacterium* would be transferred to new genera.

New taxa from clinical environments

During the last decade formal taxonomic proposals have been made for those groups of organisms referred to above as group IIb, group IIk-2, and groups IIf and IIj.

The species *F. indologenes* was proposed to accommodate organisms from Group IIb (Yabuuchi et al, 1983), and the species *F. gleum* was proposed to accommodate a subgroup of group IIb organisms (Holmes et al, 1984b). Yabuuchi et al (1990), differentiated the two species on the basis of phenotypic and DNA-DNA homology characterisation of 9 strains of *F. indologenes* and three strains of *F. gleum*. Ursing and Bruun (1991) similarly studied 31 Group IIb strains and identified 9 genotypic groups which could not easily be distinguished phenotypically. It was suggested that use of the species names *F. gleum* and *F. indologenes* be abandoned in favour of designation as "Flavobacterium group IIb", until the taxonomy of this

group of organisms could be further clarified (Ursing and Bruun, 1991; Holmes, 1992).

The genus *Sphingobacterium* was proposed to accommodate the group IIk-2 organisms which were characterised by the presence of sphingophospholipids (Yabuuchi et al, 1981; Yabuuchi et al, 1982; Yabuuchi et al, 1983). The species *S. multivorum*, *S. spiritovorum*, and *S. mizutae* have been described (Holmes et al, 1981; Holmes et al, 1982; Yabuuchi et al, 1983). The species *F. thalpophilum* and *F. yabuuchiae* also belong in this genus although a formal proposal to transfer these species to the genus *Sphingobacterium* has not been made (Holmes et al, 1983; Holmes et al, 1988). Holmes (1992) considered the proposal of the genus *Sphingobacterium* to be premature because the presence of sphingophospholipids cannot be determined easily. This group of species however is also characterised by the possession of menaquinone 7 as the major respiratory lipoquinone, in contrast to the other species of *Flavobacterium* which possess menaquinone 6 as the major respiratory quinone, and can be differentiated from other species of *Flavobacterium* on the basis of saccharolytic capacity, lack of indole production and lack of proteolytic activity (Dees et al, 1985; Bruun, 1988; Holmes, 1992). The members of this group are further characterised by their fatty acid profiles which contain significant amounts of 16:1 (11 - 56%), in addition to branched fatty acids (Dees et al, 1979; Dees et al, 1985, Dees et al, 1986; Shivaji et al, 1992).

The genus *Weeksella* was created to accommodate the group IIf and IIj organisms (Holmes et al, 1986a; Holmes et al, 1986b). The group IIf organisms have some features in common with members of the genus *Moraxella*, notably a small genome size (Owen and Holmes, 1981). Other similar features are lack of pigmentation, oxidase- and catalase positive, non-motile, asaccharolytic, sensitivity to penicillin and isolation from mucosal surfaces (Holmes and Owen, 1981; Doern, 1992). A notable difference between the members of the genera *Weeksella* and *Moraxella*, is the possession by the former of branched fatty acids including branched hydroxy fatty acids, and the possession by the latter of straight-chain fatty acids (Dees et al, 1981; Dees et al, 1986; Jantzen and Bryn, 1985). Another difference is the mol % G+C content of the DNA of members of the genus *Morexella*

(40 - 48) compared with members of the genus *Weeksella* (36 - 37) (Doern, 1992; Holmes et al, 1986a; Holmes et al, 1986b). Recent DNA:rRNA hybridisation studies indicated that the genus *Weeksella* belonged in the same rRNA homology group as *F. breve* (Segers et al, 1992).

The genus groups A - D, listed above, can be readily differentiated on the basis of phenotypic characteristics which include indole production, saccharolytic activity, proteolytic activity, urease activity, pigmentation and resistance to antimicrobials (Holmes, 1992; Bruun, 1988). Within these groupings, however, there are some species which are not easily differentiated. There are also genotypic groups which may represent new species, but for which distinguishing phenotypic characteristics have not been identified.

F. meningosepticum and the group IIb organisms are two species which are phenotypically similar. The main phenotypic difference between these two species is the intensity of their colony pigmentation (Owen and Holmes, 1981). The production of ß-galactosidase has also been identified as a discriminating feature, but this was not a completely consistent difference (Bruun, 1988; Holmes 1992). Bruun (1988) also noted the substantial phenotypic similarity between *S. multivorum* and *S. spiritovorum*, which were differentiated only on the basis of acid production from ethanol and mannitol.

Examples of genotypic groups for which no differentiating phenotypic features are known are found within *F. breve*, the group IIb organisms as described previously, *F. odoratum* and *F. meningosepticum* (Bruun, 1988). A study of 52 strains of *F. meningosepticum* found two DNA:DNA hybridisation groups which were related at a level of 40 - 55%. One of these groups was comprised of the type strain and three other strains, which were interrelated at a level of 90%. The other group was comprised of four subgroups. These subgroups were related to each other in the range 32 - 63%, and the level of intra-subgroup relatedness was in the range 72 - 100% (Ursing and Bruun, 1987). Extensive phenotypic characterisation, antimicrobial susceptibility testing, and crossed immunoelectrophoresis analysis did not identify any features which could discriminate the two hybridisation groups. However, it was found that 18 out of 20 strains isolated from cerebrospinal fluid

occurred in one subgroup, suggesting the genotypic delimitation of organisms occurring in a particular environmental niche, with in this case, pathogenic significance (Bruun and Ursing, 1987; Bruun, 1987; Bruun and Hoiby, 1987).

New taxa from non-clinical environments

The majority of taxonomic work performed on the genus *Flavobacterium* and related genera (genera A - D), since its limitation to low G+C species has involved organisms isolated from the clinical environment. In recent years two new species, *Flavobacterium branchiophila* and *Sphingobacterium antarcticus* have been described to accommodate organisms isolated from non-clinical environments.

Flavobacterium branchiophila was described to accommodate a group of organisms associated with gill disease in freshwater fish (Wakabayishi et al, 1989). The cell length of these organisms was 5 - 8 μm compared with the length of 1 - 3 μm in the description of the genus *Flavobacterium* (Holmes et al, 1984a). However these organisms were non-gliding and were consequently placed in *Flavobacterium*. Higher levels of DNA-DNA hybridisation have been observed between *F. branchiophila* and *Cytophaga* species, than *Flavobacterium* species, which has generated uncertainty about the placement of this organism (Holmes, 1992).

Sphingobacterium antarcticus was created to accommodate two strains isolated from Antarctic soil (Shivaji et al, 1992). These sphingophospholipid containing organisms most closely resembled *S. multivorum*, but showed negligible DNA:DNA hybridisation with *S. multivorum*. They could be differentiated from that species by their psychrotrophism, lack of acid production from a number of carbohydrates, and a higher proportion of 16:1 in their fatty acid profile.

Hayward and Sly (1984) characterised some dextranolytic soil organisms. The phenotype of these organisms, which were saccharolytic and indole negative, closely resembled the description of *S. multivorum*, although chemotaxonomic features were not determined.

Chemotaxonomy of the genus Flavobacterium (groups A - D)

Chemotaxonomic features play an important role in circumscribing the legitimate members of the *Flavobacterium-Cytophaga* complex. The chemotaxonomic features which primarily characterise these bacteria are the presence of substantial quantities of branched fatty acids, branched hxdroxy fatty acids and menaquinones.

Branched fatty acids are sparsely distributed among the genera of Gram-negative bacteria, and the presence of branched hydroxy fatty acids is rarer still, occurring only in members of the *Flavobacterium-Cytophaga* complex, *Bacteroides* and some myxobacteria (Kaneda, 1991). Similarly, among aerobic, Gram-negative bacteria, the occurrence of menaquinones as the major respiratory lipoquinone is limited to members of the *Flavobacterium-Cytophaga* complex and the myxobacteria (Mannheim, 1981; Reichenbach and Dworkin, 1992). Identification of these chemotaxonomic features supported the removal of high G+C species such as *F. devorans*, *F. capsulatum*, *F. flavescens*, *F fuscum*, and *F. lutescens* from the genus *Flavobacterium* (Fautz et al, 1981; Oyaizu and Komagata, 1981).

Flexirubin pigments are an apparently unique feature of some members of the *Flavobacterium-Cytophaga* complex. Flexirubin pigments generally occur in strains from soil, freshwater and clinical samples, but not in marine strains (Reichenbach et al, 1981; Weeks, 1981). Exceptions to this pattern have been reported. Reichardt et al (1983) in a study of chitinoclastic estuarine strains found a few strains which required NaCl for growth but contained flexirubin pigments and had a similar phenotype to the non-marine species *C. johnsonae*. Explanation of this phenotype was the possibility of plasmid-mediated regulation of salt tolerance. Reichardt et al (1983) also found many examples of flexirubin-producing strains which did not require NaCl for growth, but which would tolerate 3% NaCl. *F. breve* is similarly a flexirubin-producing organism which tolerates elevated concentrations of NaCl (Dobson, 1988).

Hamana and Matsuzaki (1991), examined *F. aquatile* and seven other legitimate species of *Flavobacterium* belonging to groups

A-C, and all contained homospermidine as the major polyamine. The occurrence of this polyamine is limited among bacteria, making it another useful chemotaxonomic marker for this group of organisms.

Chemotaxonomic features may also be employed to characterise the genus groups A - D. Group C, the genus *Sphingobacterium* is the best chemotaxonomically defined group of the four. It is identified by the presence of sphingophospholipids and menaquinone 7 as the major respiratory lipoquinone. Members of groups A and B contain menaquinone 6 as the major respiratory lipoquinone (as far as the author is aware the lipoquinone content of members of the genus *Weeksella*, group D, has not been reported). Fatty acid profiles of *F. aquatile* and members of groups A - D are listed in Table 2.2 The profiles for more than one strain, and profiles determined in separate studies are included to indicate the intra-species range of values which may be obtained. A summary of the major characteristics of the four groups, with the percentage quantities shown in brackets, is as follows:

- Group A i-15:0 (24-39); i-2-OH-15:0 (4-39); i-3-OH-17:0 (9-18); i-17:1 (4-26); i-3-OH-15 (4-12); 16:1 (0-21)
- Group B i-15:0 (48-59); i-17:1 (5-22); i-3-OH-17:0 (7-12); i-3-OH-15:0 (5-7)
- Group C i-15:0 (17-36); 16:1 (13-56); i-2-OH-15:0 (19-35)
- Group D i-15:0 (49-50); i-17:1 (13-18)

While there are similar profiles within each group, the distinctions between groups are not all clear. For example, both groups B and D are characterised by high ($\geq$ 48 %) levels of i-15:0. Group C organisms consistently contain significant levels of 16:1 (13-56), however, some strains of the group A species *F. breve* also contain significant levels of this fatty acid (5-21 %), although *F. breve* can be distinguished from group C organisms by a lower proportion of i-2-OH-15:0. The profile of *F. aquatile* is quite similar to that of *F. breve*, however, from the data of Fautz et al (1981) *F. aquatile* is differentiated from *F. breve* by the presence of small quantities of 17:1 and 3-OH-17:0, in its profile.

Table 2.2 Fatty acid composition of members of the genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*

	Straight-chain acids												Branched-chain acids								Hydroxy acids									
	12:0	13:0	14:0	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	i-13:0	i-14:0	i-15:0	a-15:0	i-15:1	i-16:0	i-17:0	i-17:1	2-OH 14:0	3-OH 14:0	2-OH 16:0	3-OH 16:0	3-OH 17:0	2-OH i-15:0	3-OH i-15:0	3-OH i-16:0	3-OH i-17:0	3-OH i-17:1
• *F. aquatile* [a]	1	-	1	12	-	4	1	-	-	4	8	-	-	1	22	2	-	-	-	-	-	-	-	-	-	-	8	1	10	-
• *F. aquatile* [h]	-	-	2	10	2	2	1	2	4	2	-	-	-	-	15	-	4	1	1	6	-	1	-	1	7	2	12	-	25	-
Group A:																														
• *F. breve* [a]	t	-	2	2	-	5	13	-	-	-	1	-	1	-	30	t	-	-	-	4	-	-	-	-	-	8	5	-	12	-
F. breve (3) [a]	t	-	t-1	2	-	1-4	5-7	-	-	-	t-2	-	t	0-t	28-36	t-3	-	-	-	4-7	-	-	-	-	-	5-16	6-7	-	11-14	-
F. breve (11) [f]	-	-	T	T	-	8	21	-	-	T	T	T	-	-	24	-	T	T	T	7	T	T	-	5	-	4	5	1	9	-
F. breve [h]	-	-	1	4	1	8	5	1	-	1	-	-	-	-	18	-	1	1	5	4	-	2	-	5	-	4	3	-	36	-
• *F. meningosepticum* [a]	t	-	-	-	-	1	4	-	-	-	-	-	-	-	30	3	-	-	-	6	-	-	-	-	-	22	4	2	18	-
F. meningosepticum (4) [b]	-	-	T	-	-	T	T	-	-	T	T	-	-	T	30-36	-	-	T	-	5-10	-	-	-	T	-	28-39	6-12	T-5	10-13	-
F. IIb (3) [b]	-	-	T	-	-	T	-	-	-	T	T	-	-	T	34-39	-	-	T	-	17-26	-	-	-	-	-	17-22	6-10	T	8-17	-
Group B:																														
• *F. odoratum* [a]	t	-	-	t	-	t	t	-	-	-	-	-	-	-	48	4	-	-	-	22	-	-	-	-	-	3	7	-	12	-
F. odoratum (2) [a]	0-1	-	1-2	2-3	-	2	2-3	-	-	-	-	-	t-8	t	56-59	t-8	-	-	-	5	-	-	-	-	-	1-2	5	-	7-8	-
F. odoratum (11) [f]	-	T	T	T	T	T	T	-	-	-	T	T	3	T	55	T	-	T	-	6	-	T	-	5	-	3	6	T	8	T
Group C:																														
• *S. multivorum* [c]	-	-	-	-	-	2	13	-	-	-	-	-	-	-	27	-	-	-	-	3	-	-	-	-	-	35	4	-	10	-
S. multivorum (4) [c]	-	-	4-6	-	-	8-11	27-29	-	-	-	-	-	-	-	17-23	-	-	-	-	-	1-3	-	-	2-3	-	19-21	6-7	-	1-3	-
S. spiritivorum (4) [c]	-	-	2-4	-	-	5-7	22-24	-	-	-	-	-	-	-	23-26	-	-	-	-	-	1	-	-	0-1	-	26-30	3-4	-	3-4	-
• *S. mizutae* [c]	-	-	-	-	-	3	17	-	-	-	-	-	-	-	26	-	-	-	-	2	-	-	-	1	-	30	3	-	11	-
S. mizutae (4) [c]	-	-	-	-	-	1-10	15-29	-	-	-	-	-	-	-	18-36	2-3	-	-	-	1-4	0-2	-	0-2	1-2	-	19-29	2-5	-	1-11	-
Group IIk-2 (6) [d]	-	-	3	T	-	10	27	T	-	T	T	-	T	-	37	-	T	-	-	T	-	-	-	2	-	28	9	-	12	-
S. antarcticus (2) [e]	-	-	-	-	-	-	56	-	-	-	-	-	-	-	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group D:																														
Group IIf (10) [f]	-	T	T	T	-	4	2	-	-	T	T	T	-	-	50	-	-	T	T	13	-	T	-	T	-	7	4	T	6	-
Group IIj (4) [g]	-	-	T	T	-	4	-	-	-	T	3	4	7	-	49	-	-	-	-	18	-	-	-	-	-	8	4	T	3	-

a Data from Oyaizu and Komagata (1981); • indicates type strain; (3) indicates number of strains; t acid present at < 0.5%; values have been rounded to the nearest integer.
b Data from Moss and Dees (1978); T acid present at < 2%.
c Data from Yabuuchi and Moss (1982).
d Data from Dees et al (1979); the average values are given.
e Data from Shivija et al (1992); note, partial data only.
f Data from Dees et al (1986); average values are given.
g Data from Dees et al (1981); average values are given.
h Data from Fautz et al (1981); values estimated from a diagrammatic representation of the fatty acid profile.

Rasoamananjara et al (1986) analysed volatile fatty acid (vfa) production from strains belonging to groups A-C to determine whether vfa profiles would differentiate between *Flavobacterium* species. An initial study separated 74 strains into species groups and subgroups, however the values chosen to separate groups were somewhat arbitrary to the extent that they were selected to accord with phenetic groups (Rasoamananjara et al, 1986). In a latter study, of a further 80 strains, the placement of 12 strains in the vfa groups did not accord with their phenetic characteristics (Rasoamananjara et al, 1988). Only one species of *Sphingobacterium*, *S. multivorum*, was examined and its vfa profile was quite distinct from the members of groups A and B. The results were inconclusive about the general application of vfas for the differentiation of species.

2.2.3 General characteristics of the genus *Cytophaga*

The genus *Cytophaga* is comprised of Gram-negative, rod shaped, gliding organisms which are usually pigmented yellow, orange or red, and which usually degrade one or more type of polysaccharide. The genus *Cytophaga* was created by Winogradsky in 1929 to accommodate a group of aerobic cellulolytic soil organisms (Reichenbach, 1989). The genus now accommodates both aerobic and facultatively anaerobic organisms which degrade one or more of a wide range of polysaccharides, including starch, cellulose, agar, chitin, pectin and heparin.

In contrast to members of the genus *Flavobacterium*, species belonging to the genus *Cytophaga* have been isolated only from non-clinical environments. A strain isolated from an industrial water spray air humidification system with characteristics similar to *C. aquatilis* has been implicated as the causative agent of a lung disease with hypersensitivity pneumonitis-like symptoms, however the disease is an allergenic reaction and the organism does not grow at 37 °C (Liebert et al, 1984).

Prior to the more recent taxonomic revisions of the genus *Flavobacterium*, the genus *Cytophaga* was considered a better defined and more homogeneous taxon. Consequently some workers placed non-gliding organisms in *Cytophaga* rather than in the genus

Flavobacterium which they considered heterogeneous (McDonald et al, 1963; Colwell et al, 1966; Inoue and Komagata, 1976). Some of these non-gliding species were later found to exhibit gliding motility (Perry, 1973; Reichenbach, 1989).

Gliding motility is an important defining characteristic of the genus *Cytophaga*. However the observation and interpretation of gliding motility and spreading colonies can difficult. For example, the absence of spreading growth does not necessarily imply the organism is non-gliding. Perry (1973) observed gliding motility of non-spreading organisms such as *F. aquatile* and *C. marinoflava*, by observing cells in hanging drops, and in a glass-liquid-agar sandwich, created by placing glass beads (0.10 - 0.11 mm) on an agar surface at the colony edge, and then covering with a coverslip. Some non-spreading mutants of *C. johnsonae* are motile when observed in wet mounts (Pate, 1988). The expression of gliding motility and spreading growth is also dependent on environmental conditions, and usually requires growth on low nutrient media under moist conditions (Henrichsen, 1972). Wolkin and Pate (1984) determined the concentrations of several sugars, Mg^{2+}, Ca^{2+} and SO_4^{2-}, which inhibited the spreading growth of *C. johnsonae*.

Additionally, a variety of different types of movement such as twitching, darting, sliding, and flexing as well as gliding motility, have been reported (Henrichsen, 1972; Pate 1988). It is not known whether more than one type of mechanism is responsible for all these translocational movements and therefore whether organisms which exhibit apparently different types of movement should be classified together, assuming that gliding motility is a phylogenetically significant character.

In 1989, Reichenbach included 20 species in the genus *Cytophaga*, listed a further 6 species of uncertain taxonomic placement as *species incertae sedis*, and identified 19 species from the literature which should be abandoned because cultures were not available. Reichenbach's (1989) list of 20 species was not substantially different from the list of extant cultures of *Cytophaga* prepared over a decade earlier by Christensen (1977) (Table 2.3).

Table 2.3 A comparison of species belonging to the genus *Cytophaga* in 1977[a] and 1989[b]

1977	1989
C. aurantiaca	*C. aurantiaca*
C. columnaris	*C. columnaris*
C. diffluens	*C. diffluens*
C. diffluens var. *aprica*	*C. aprica*
C. diffluens var. *carnea*	not available
C. fermentans	*C. fermentans*
C. flevensis	*C. flevensis*
C. hutchinsonii	*C. hutchinsonii*
C. johnsonae	*C. johnsonae*
C. latercula	*C. latercula*
C. lytica	*C. lytica*
C. marinoflava	*C. marinoflava*
C. psychrophila	*C. psychrophila*
C. salmonicolor	*C. salmonicolor*
C. salmonicolor var. *agarovorans*	*C. agarovorans*
C. succinicans	*C. succinicans*
	C. aquatilis
	C. marina
	C. pectinovora
	C. saccharophila
	C. uliginosa

a From Christensen (1977)
b From Reichenbach (1989)

Three new species have been described since 1977, *C. aquatilis* , *C. marina*, and *C. saccharophila*, the former two being associated with diseased fish (Reichenbach, 1989). The other additions to the list of *Cytophaga* species, *C. pectinovora* and *C. uliginosa* have resulted from the taxonomic revision of species formerly placed in the genus *Flavobacterium*. Two varieties, *C. diffluens* var. *aprica* and *C. salmonicolor* var. *agarovorans* have since been designated species in their own right (Reichenbach, 1989).

The 20 species of *Cytophaga* were separated by Reichenbach (1989) into 19 sub-generic groupings (groups A - T), which may form the basis of new genera. In this scheme only *C. hutchinsonii,* the type species, and *C. aurantiaca* are grouped together. These species are cellulose-degrading soil organisms and have always been considered the core organisms of the genus *Cytophaga*. Reichenbach (1989) has suggested the names *Agarophaga* and *Chthonophaga* for the groups containing *C. lytica* and *C. johnsonae* respectively. The separation of *Cytophaga* spp. into such a large number of groups reflects the heterogeneity of these organisms at the molecular level as revealed by DNA:DNA hybridisation studies (Reichenbach, 1989).

A variety of phenotypic features, habitat and mol% G+C values were employed to discriminate between species/sub-genus groups of the genus *Cytophaga* (Christensen, 1977; Reichenbach, 1989). Species were often created to accommodate organisms which degraded a particular macromolecule whereas it has been shown that organisms which degrade the same macromolecule may not necessarily be closely related (Hirsch and Reichenbach, 1981; Reichenbach, 1989). Hirsch and Reichenbach (1981) carried out a phenotypic examination of 185 strains of soil and freshwater *Cytophaga*-like bacteria with the aim of searching for key characters to group these organisms and to correlate groups with DNA:DNA hybridisation groupings. They found gliding motility, cell morphology, cellular shape-change and the presence of flexirubin-type pigments useful for the characterisation of major groupings. A number of enzymes (catalase, cytochrome oxidase, lecithinase, urease, phenylalanine deaminase, DNase, starch hydrolysis and gelatin liquefaction) were also useful. Their presence or absence was

consistent among strains in some of the major groups, or for the discrimination of subgroups. However, the delineation of subgroups using these characteristics was not always unambiguous, as was demonstrated by a comparison with groupings determined by DNA:DNA hybridisation. Reichenbach (1989) also considered that the differentiation of marine and non-marine (soil and freshwater) organisms, as often revealed by the presence of flexirubin pigments in the latter, was taxonomically significant.

Reichenbach (1989) described the concept of the genus *Cytophaga* as vague, and one which at best identified organisms which may belong together.

As noted previously phenotypic discrimination between members of the genera *Cytophaga* and *Flavobacterium* is limited to the capacity for gliding motility. Capacity for polysaccharide degradation is another differentiating feature between the two genera but is ambiguous. Members of the genus *Flavobacterium* in general do not degrade polysaccharides, although several strains of *Flavobacterium* group IIb degrade starch, and environmental strains of *S. multivorum* degrade starch and dextran (Holmes, 1992; Hayward and Sly, 1984). Conversely *C. marinoflava* degrades only starch and *C. marina* and *C. psychrophila* have not been shown to degrade any polysaccharides (Reichenbach, 1989).

Discrimination between the genera *Cytophaga* and *Flexibacter* (and *Microscilla*) is also somewhat uncertain. A feature which was previously considered important in the description of *Flexibacter* and *Microscilla* and in their differentiation from the genus *Cytophaga* was inability to degrade polysaccharides (Leadbetter, 1974). Reichenbach (1989) considered that this description of *Flexibacter* and *Microscilla* was unsatisfactory because it was ambiguous in terms of which polysaccharides were included in the definition, because some species of *Flexibacter* did degrade some polysaccharides, and because reliance on negative characteristics is inherently an unsatisfactory approach to classification.

The important characteristics which now define the genus *Flexibacter* and its marine counterpart *Microscilla* are cell morphology, mol% G+C and habitat. The genus *Flexibacter* is

differentiated from the genus *Cytophaga* on the basis of a higher mol% G+C content, however the range of species in both genera means that there is a considerable overlap of values (Table 2.1). Differentiation between the two genera is therefore primarily on the basis of cell morphology, with a small amount of overlap between the two genera in this regard. The cell dimensions of *Cytophaga* spp. are 0.3 - 0.8 x 1.5 - 15 μm and *Flexibacter* spp. 0.2 - 0.6 x 10 - 50 μm. *Cytophaga* cells may become elongate as cultures age, however, these longer cells are non-motile. One of the major groups observed by Hirsch and Reichenbach (1981) in their phenotypic study, was characterised as representing the genus *Flexibacter*. These strains had mol% G+C in the range 44 - 51% and were threadlike, actively gliding cells which underwent a morphological change to short, stout, non-motile cells as the cultures aged.

Chemotaxonomy of the genus Cytophaga

The general chemotaxonomic features of members of the genus *Cytophaga* with regard to fatty acid composition and major respiratory lipoquinones are similar to those of the members of the genus *Flavobacterium*. Reichenbach (1989) limited the genus *Cytophaga* to species containing menaquinone 6 as the major respiratory lipoquinone and excluded species such as *C. heparina* and *C. arvensicola*, which contain menaquinone 7 as the major respiratory lipoquinone. As fatty acid profiles are available for only a few *Cytophaga* spp., it is not possible to identify features which might define sub-groups within this genus within any certainty (Table 2.4). Within the limited data set available, *C. hutchinsonii* is characterised by the presence of 2-OH-i17:0 (Oyaizu and Komagata, 1981).

Aminosulfonates are a group of lipids, also known as capnines, which occur in *C. johnsonae*, a marine *Cytophaga* sp. and in some members of the genera, *Capnocytophaga*, *Flexibacter* and *Sporocytophaga* (Godchaux and Leadbetter, 1984). N-acylated versions of capnine predominated in all the species examined, except for *Capnocytophaga* spp., where capnine itself was predominant. Hydroxylated fatty acids in species containing N-acylated capnines, occurred predominantly as the N-acyl moiety of N-acylated capnines. In genera of Gram-negative bacteria not related to this group, hydroxylated fatty acids are largely associated with lipo-

Table 2.4 Fatty acid composition of members of the genus *Cytophaga*

	Straight-chain acids												Branched-chain acids										Hydroxy acids									Cyclopropane acid
	12:0	13:0	14:0	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	i-13:0	i-14:0	i-15:0	a-15:0	i-15:1	i-16:0	i-17:0	a-17:0	i-17:1	a-17:1	3-OH 14:0	2-OH 16:0	3-OH 16:0	3-OH 17:0	2-OH i-15:0	3-OH i-15:0	3-OH i-16:0	2-OH 17:0	3-OH i-17:0	cyc17:0
• *C aquatilis* [a]	-	-	1	15	-	13	12	-	-	-	1	-	T	T	29	3	-	-	-	-	3	-	-	-	-	-	1	7	-	-	5	-
C. aquatilis[c]	-	-	2	7	2	4	3	2	-	1	-	-	-	-	19	-	4	2	4	-	6	-	1	-	6	2	1	10	-	-	24	-
• *C. johnsonae* [b]	-	-	4	1	-	23	27	-	-	-	-	-	-	-	23	3	-	-	-	-	1	-	3	-	5	-	2	5	-	-	1	-
C. johnsonae (2) [b]	-	-	1-5	1-5	-	6-16	12-35	-	0-1	-	-	-	-	-	15-31	4-7	0-3	0-2	0-1	0-1	0-5	0-1		0-1	1-6	-	1-2	6-8	-	-	1-3	-
C. johnsonae(4)[a]	1-3	-	0-3	4-15	-	1-10	3-12	-	-	0-4	T-7	-	-	-	19-26	3-6	-	-	-	-	2-6	-	-	-	-	-	2-6	8-11	1-3	-	9-15	1-3
C. johnsonae[a] NCIB 10150	-	-	1	8	-	3	13	-	-	-	T	-	-	-	19	5	-	-	-	-	4	-	-	-	-	-	6	8	1	-	11	3
C. johnsonae[c] NCIB 10150	-	-	2	15	7	3	12	-	3	-	-	-	-	-	23	-	-	-	-	-	3	-	2	-	-	2	4	8	-	-	8	-
• *C. latercula*[c]	-	-	2	5	-	1	-	1	2	1	-	-	-	-	18	-	18	2	2	-	10	-	2	-	-	2	2	6	-	-	35	-
• *C. hutchinsonii*[d]	-	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-	20	-	6	-	-	-	-	-	-	-	-	5	-	19	6	-

a Data from Oyaizu and Komagata (1981); • indicates type strain; (3) indicates number of strains; T acid present at < 0.5%; values have been rounded to the nearest integer.
b Data from Yabuuchi and Moss (1982); (3) indicates number of strains.
c Data from Fautz et al (1981); values estimated from a diagrammatic representation of the fatty acid profiles.
d Data from Oyaizu and Komagata (1981); values estimated from a diagrammatic representation of the fatty acid profile.

polysaccharides. Apart from the bacterial genera mentioned and *Flavobacterium* spp., capnines have only been isolated from the diatom *Nitzchia alba* which exhibits a form of gliding motility (Godchaux and Leadbetter, 1984). Godchaux and Leadbetter (1984) noted that the presence of capnines in bacteria isolated from disparate environments, and suggested that they are a conserved feature which not only have taxonomic significance but which may be functionally associated with the capacity for gliding motility. Non-gliding species of *Flavobacterium*, *F. odoratum* and *F. breve*, however also contain substantial quantities of these lipids (Godchaux and Leadbetter, 1983).

2.2.4 Phylogenetic relationships among Gram-negative, pigmented, aerobic bacteria with a mol %G+C in the range 30 - 53, which are members of the "flavobacteria-*Bacteroides*" phylum

Bauwens and De Ley (1981) examined phylogenetic relationships among a group of low mol % G+C flavobacteria using the DNA:rRNA hybridisation technique, and found a heterogeneous group with a ΔT_m range of 10 °C, equivalent to the entire family of the *Enterobacteriaceae*. This group of organisms formed 5 hybridisation subgroups:

(i) *F. aquatile*, "*F. pectinovora*" (now *C. pectinovora*), "*F. tirrenicum*", and *C. johnsonae*

(ii) *C. marinoflava*, *C. lytica*, *C. salmonicolor*, "*F. uliginosum* " (now *C. uliginosa*), *Fx. aurantiacus* subsp. *excathedrus*

(iii) *F. odoratum*

(iv) *F. breve*

(v) *F. meningosepticum*

This arrangement demonstrated that there was no clear separation of the genera *Flavobacterium* and *Cytophaga*, particularly with respect to the occurrence of the *C. johnsonae* and the type species of *Flavobacterium*, *F. aquatile*, in the same hybridisation group.

An examination of 16S rRNA oligonucleotide catalogues established the phylogenetic relationship between the *Bacteroides* and members of the *Flavobacterium-Cytophaga* complex (Paster et al, 1985). An extensive signature occurred in the oligonucleotide catalogues of the *Bacteroides*. This group of organisms was not phylogenetically deep in comparison with other groups of anaerobic organisms, and appeared to be evolving at a faster rate relative to the flavobacterial branch. The flavobacterial branch, which did not have such an extensive signature, was comprised of the following two major subgroups:

(i) *C. johnsonae, Sporocytophaga myxococcoides, F. aquatile, C. lytica, "F. uliginosum* "(now *C. uliginosa*), *F. breve*

(ii) *F. heparinum, F. ferrugineum, F. elegans, Haliscomenobacter hydrossis, Saprospira grandis*

In this arrangement of a limited number of organisms, the species which are currently accommodated in the genera *Flavobacterium* and *Cytophaga* all occur in the first subgroup. In the internal structure of this subgroup *C. johnsonae, Sporocytophaga myxococcoides* and *F. aquatile* clustered together, *C. lytica* and *"F. uliginosum"* clustered together, and *F. breve* was on the periphery. This arrangement was consistent with DNA:rRNA hybridisation data (Bauwens and De Ley, 1981). A close relationship existed between of *C. johnsonsae* and *Sporocytophaga myxococcoides* which suggested that these organisms should belong in the same genus, and that it was not appropriate to define a separate genus, *Sporocytophaga* on the basis of formation of microcysts.

Weisburg et al (1985) cloned and sequenced a 16S rRNA gene from *F. heparinum* and one from *B. fragilis*. Their analysis of these sequences consolidated the conclusion of the oligonucleotide cataloguing study, that the *Bacteroides* and the flavobacteria are specifically related and comprise a distinct lineage. Weisburg et al (1985) further elaborated the sequence signatures for the *Bacteroides* and the flavobacteria, identifying the location of signature features, including distinctive secondary structure characteristics. There was subsequently some revision of this list of signature sequences, though 50% of the signature characteristics in the most recent listing were

identified in the earlier analyses (Woese et al, 1985a; Woese, 1987; Woese et al, 1990d). Of the signature characteristics associated with the "flavobacteria-*Bacteroides*" phylum, a deletion of 4-5 pairs in the stem of the 1426 - 1465 stem-loop structure, and an adenosine residue at 1532, were unique among the set of determined sequences. The presence of the latter signature has not often been determined as it is inaccessible by direct rRNA sequencing, or sequencing of PCR-generated material.

The sequences of the ß-subunit of the ATP-synthases from *Bacteroides fragilis* and *C. lytica* had a high degree of similarity and were characterised by insertions not found in other members of the *Bacteria* (Amann et al, 1988). The phylogenetic relationships derived from an analysis of the ATP-synthase ß-subunit sequences were the same as those derived from an analysis of the 16S rRNA sequences.

Woese et al (1990b) examined the phylogeny of the *Spirosomaceae* by 16S rRNA sequence comparison, and found that this family, comprised of the genera *Spirosoma*, *Flectobacillus* and *Runella*, formed a monophyletic branch within the "flavobacteria-*Bacteroides*" phylum. These genera are characterised by a ring-forming morphology, but also contain menaquinones and branched fatty acids. *Ancyclobacter*, another genus characterised by a ring-forming morphology, was not phylogenetically related to this group and contained ubiquinones and straight-chain fatty acids only (Urakami and Komagata, 1986). Raj and Maloy (1990) proposed that a new genus within the family *Spirosomaceae*, *Cyclobacterium*, be created to accommodate the species previously named *Flectobacillus marinus*, on the basis of morphological and phenotypic differences between *Fc. marinus* and the type species of *Flectobacillus*, *Fc. major*, and because the evolutionary distance between *Fc. marinus* and *Fc. major* was significant (18%).

Woese et al (1990d) using the reverse transcriptase methodology, obtained the 16S rRNA sequence of the type species of *Flexibacter*, *Fx. flexilis*, *F. aquatile*, *C. uliginosa*, *C. lytica* and the following *species incertae sedis*: *Fx. canadensis*, *Fx. sancti*, *Fx. aurantiacus*, and *F. ferrugineum*. In the phylogenetic tree derived from these sequences the flavobacteria clustered in the following groupings:

(i) *F. aquatile, Fx. aurantiacus, C. lytica, C. uliginosa*

(ii) *Fx. sancti, F. ferrugineum*

(iii) *Fx. canadensis, F. heparinum*

(iv) *Fx. flexilis, Fc. major, Rn. slithyformis, Fc. marinus, S. linguale*

Woese et al (1990d) identified stringently defined signatures to support these four groupings. The requirements of a stringently defined signature is that there is a constant composition at that position within the group, and a constant and different composition at that position in all the other members of the phylum. This study demonstrated that the members of the genus *Flexibacter* were not a monophyletic group. The type species of the genus *Flexibacter* clustered with the members of the family *Spirosomaceae*. Also apparent from this study and the study of Paster et al (1985) was that many of the species regarded as *species incertae sedis* from the genera *Flavobacterium*, *Cytophaga* and *Flexibacter* (often because their mol% G+C value would unreasonably expand the range of the genus) did in fact have a specific phylogenetic relationship to these genera if at a deeper level than an intra-generic relationship.

One apparent discrepancy between the 16S rRNA sequence study of Woese et al (1990d) and the DNA:rRNA hybridisation study of Bauwens and De Ley (1981) is the placement of *Fx. aurantiacus*. Woese et al (1990) determined the sequence of the type strain of this species and it clustered in the *F. aquatile* group. Bauwens and De Ley (1981) did not specify the strain they used in their hybridisation study but found it clustered with *Fx. roseolus* and *Fx. ruber* in a group far removed from the *Flavobacterium -Cytophaga* complex. This would suggest that Bauwens and De Ley (1981) did not use the type strain, and also that the phenotypic characteristics used to identify the strain as belonging to the species *Fx. aurantiacus* were not appropriate to define that species. Bauwens and De Ley (1981) did find that a subspecies *Fx. aurantiacus* subsp. *excathedrus* was closely related to *C. marina* and *C. marinoflava.*

Paster et al (1985) suggested that gliding motility was a poor criterion to define phylogenetic groups, because of the lack of relationship of the gliding bacteria belonging to the "flavobacteria-

Bacteroides" phylum and other gliding bacteria such as the *Beggiatoaceae*, *Lysobacteraceae*, *Leuchotrichaceae*, *Myxobacterales*, the chloroflexus-herpetosiphon group, the cyanobacteria and *Spirulina*, which belong in different phyla. Paster et al (1985) noted the close relationship of some gliding and non-gliding bacteria within the "flavobacteria-*Bacteroides*" phylum and suggested that pigmentation was a more phylogenetically valid character to define the flavobacterial branch of the phylum. Woese et al (1990d) however concluded from the observation that gliding organisms occur throughout the "flavobacteria-*Bacteroides*" phylum that gliding motility was an ancestral and defining feature of the phylum. Non-gliding species were organisms that had lost the capacity to glide, and therefore to define the genus *Flavobacterium* on the basis of non-gliding was not phylogenetically defensible.

Van den Eynde et al (1990) determined the 5S rRNA sequence of several members of the "flavobacteria-*Bacteroides*" phylum, including representatives of the genera *Flavobacterium*, *Cytophaga*, *Saprospira*, *Spirosoma*, *Flectobacillus* and *Bacteroides*. Surprisingly, their analysis using a weighted pairgroup clustering algorithm, produced a dendrogram in which these genera did not form a monophyletic unit. Unusual features of this dendrogram include *F. breve* clustering with high G+C Gram-positive organisms and *C. johnsonae* clustering with low G+C Gram-positive organisms. Van den Eynde et al (1990) suggested that a thorough investigation of the phylogenetic relationships among the members of the "flavobacteria-*Bacteroides*" phylum was called for due to the conflicting results from the 5S rRNA and 16S rRNA analyses. It seems more probable, however, that a phylogenetic analysis of this group is beyond the resolving capacity of 5S rRNA sequence comparisons, and that the results of the 5S rRNA analysis were not evidential that the phylogenetic relationships estimated from the 16S rRNA analysis might be invalid.

Woese et al (1990c) determined the 23S rRNA sequence of *F. odoratum* and *F. flexilis* with the common aim of Van den Eynde et al (1990) of investigating the phylogenetic position of the green sulphur bacteria (*Chlorobiaceae*), with respect to the "flavobacteria-*Bacteroides*" phylum. In contrast to the results of the 5S rRNA

sequence analysis, the results of the 23S rRNA sequence analysis were consistent with the 16S rRNA sequence analysis, and identified a specific relationship between the green sulphur bacteria and the "flavobacteria-*Bacteroides*" phylum.

Over the past two years a database of over 50 aligned 16S rRNA sequences of members of the "flavobacteria-*Bacteroides*" phylum has become available through the ribosomal RNA Database Project (RDP) (Olsen et al, 1991). An analysis of this extensive set of data has not been published to date. The determination and analysis of the 16S rRNA sequences of several species of *Cytophaga* has been recently reported (Kath and Reichenbach, 1992; Nakagawa and Yamasato, 1992). Kath and Reichenbach (1992) found the cellulose-degrading cytophagas to be closely related, and distantly related to *C. johnsonae* and *C. heparina*, confirming the hypothesis that the genus *Cytophaga* should be restricted to the cellulose-degrading organisms. A new genus name has been proposed to accommodate *C. heparina* (Steyn et al, 1992). Nakagawa and Yamasato (1992) observed seven lines of descent in a study of the 16S rRNA sequences of 20 *Cytophaga* spp. Two groups contained menaquinone 6. One of these groups was composed of terrestrial organisms and included *F. aquatile* and the other was comprised of marine strains. The other five groups were comprised of species containing menaquinone 7.

Segers et al (1992) have recently reported an extensive DNA:rRNA hybridisation study of several genera of flavobacteria and the genus *Bacteroides*. Their rRNA superfamily V corresponds to the "flavobacteria-*Bacteroides*" phylum. They identify a homology group that contains *F. aquatile*, most of the *Cytophaga* species they studied and some of the *Flexibacter* species. Another homology group contained *F. breve* and *Weeksella* spp.. *C. heparina*, *Sphingobacterium*, *Capnocytophaga*, *Moraxella anatipestifer*, *Saprospira* and *Bacteroides* were contained in separate homology groups with some *Flexibacter* spp. being closely related to members of the genus *Sphingobacterium*.

There is now a considerable body of data describing the phenotypic, chemotaxonomic, DNA:DNA hybridisation, rRNA:DNA hybridisation, and 16S rRNA sequence relationships between the Gram-negative, pigmented, aerobic bacteria with a mol %G+C in the

range 30 - 53, which are members of the "flavobacteria-*Bacteroides*" phylum. Analysis of this data has identified taxonomically coherent groupings. Proposals to formalise some of these groupings must be imminent, however, for many groupings there is not a sufficiently comprehensive set of data, in terms of the number of species which have been examined or the types of data available for each species to characterise and delineate properly new taxa (Holmes, 1992; Reichenbach, 1992).

2.2.5 The taxonomy of the Organic Lake pigmented bacteria

Organic Lake is located in the coastal Vestfold Hills region of the Australian Antarctic Territory. This lake is hypersaline and meromictic. The salts in the lake are derived from seawater and have been concentrated by evaporation. The anaerobic monimolimnion of the lake is approximately six times the concentration of seawater. The aerobic epilimnion is less concentrated, and supplemented with freshwater during the seasonal ice and snow melt. The density stratification in the lake maintains a stable meromictic state. Organic Lake is only 7.5 m deep, at its deepest point, however ice-cover for 9 months of the year protects it from mixing by substantial wind events during winter (Franzmann et al, 1987b). It is hypothesised that the origin of the meromictic state of this lake and other saline lakes in the region, is in the downward flow of concentrated brine, produced by the extrusion of salts during ice formation, particularly from the shallow regions of the lake. The occurrence of unusual temperature profiles in the water column during winter provides evidence of these density currents (Ferris et al, 1991).

The biodiversity in Organic Lake is restricted by a combination of high salinities and cold temperatures (Franzmann et al, 1987b). No invertebrate animals have been found in the lake. A species of the halophilic genus *Dunaliella*, is the dominant algal species. Despite the anoxic conditions of the bottom water no H_2S has been found in the lake. The absence of organisms capable of reducing sulphur compounds to H_2S is apparently due to the combination of cold temperatures and high salinity, as an H_2S-producing moderately halophilc organism has been isolated from a lake in a tropical region of even greater salinity than Organic Lake (Ollivier et al, 1991). Conversely other lakes in the Vestfold Hills region of a lower salinity

than Organic Lake, contain organisms which produce H_2S. Organic Lake is of interest because it contains the highest recorded level of dimethyl sulphide (DMS) in a natural water body (Franzmann et al, 1987b). As DMS production has not been observed from the *Dunaliella* sp. which colonises the lake, it is considered likely that bacteria are responsible for the DMS-production (Franzmann et al, 1987b).

Orange and yellow pigmented bacteria are readily cultured from Organic Lake during the summer. Fluorescent antibody studies have indicated that the population of these pigmented bacteria peaks during November near the oxycline region of the water column, and at that time they represent greater than 10% of the bacterial population (S. R. James, pers. comm.). A collection of strains of pigmented bacteria from Organic Lake, held in the Australian Collection of Antarctic Microorganisms have been the subject of taxonomic investigation (Dobson et al, 1991). These strains were psychrotrophic, rather than psychrophilic, suggesting that they were not entirely adapted to their cold environment. The theoretical minimum temperature for growth of one strain was determined to be -7 °C, and the optimum temperature for growth was 23 °C. The optimal salt concentration for growth of representative strains was 5%, in a medium in which NaCl, $MgCl_2.6H_2O$, $MgSO_4.7H_2O$ and KCl contributed to the total salt concentration. As organisms which have a requirement for salt for optimal growth, greater than seawater concentration, they are classified as moderate halophiles (Larsen, 1986). These bacteria also tolerated salt concentrations in the range 15 - 20%.

The likely origin of the Organic Lake bacteria is the Southern Ocean. It is considered that Organic Lake formed by the entrapment of seawater, in a depression in the landscape, during an isostatic readjustment in the region caused by the retreat of ice at the end of the last ice age (~ 8000 yr BP) (Adamson and Pickard, 1986). The proximity of Organic Lake to seawater would also allow for continuous inoculation by wind-blown aerosols. Gram-negative, orange-pigmented bacteria were found to comprise a surprisingly large (87%) component of the culturable heterotrophic population of the surface water of the Southern Ocean during a summer survey (Simudu, 1986). Halophilic bacteria, some of them pigmented, have

been cultured from intertidal pools and seawater (Forsyth et al, 1971; Ventosa et al, 1984). Ventosa et al (1984) have demonstrated the enrichment of moderately halophilic bacteria and *Dunaliella* from seawater by progressive salinity increases, and suggest that seawater is the means by which halophilic organisms are dispersed to hypersaline sites such as salterns.

The pigmented bacteria of Organic Lake were Gram-negative, non-motile, had an aerobic metabolism, and a mol % G+C of their DNA in the range 35 - 41, which suggested that they belonged to the *Flavobacterium-Cytophaga* complex. These bacteria clustered into two phenons in a numerical analysis of 134 phenotypic characteristics. A limited number of reference species belonging to the *Flavobacterium-Cytophaga* complex, which would tolerate the salinity of the basal medium, were included in the phenotypic study. The two phenons which contained the Organic Lake strains did not contain any of the reference species. *Cytophaga lytica* had the greatest phenotypic similarity to the Organic Lake strains, joining one of the phenons at a similarity level of 45% using the Jaccard coefficient and complete linkage clustering. However, *C. lytica* could be differentiated from the members of this phenon by several characteristics, including nitrate reduction, growth in 15% NaCl, agarolytic activity, sensitivity to antibiotics and growth stimulation by single carbon sources. The members of this phenon were therefore not considered to belong to the species *C. lytica* (Dobson et al, 1991).

Separation of pigmented Organic Lake strains into two phenons suggested that they may be new species, but a direct phenotypic comparison was limited to salt-requiring or salt-tolerant reference organisms. The expression of some phenotypic characteristics has been demonstrated to be variable according to the level of salt concentration in the growth media, suggesting that a strict phenotypic comparison can only be made among organisms with the same salt~growth profiles (Merkel, 1972; Hebert and Vreeland, 1987; Rosenburg, 1983). The same type of restrictions may apply to organisms with regard to temperature~growth profiles. Such conditions place limitations on the conclusions which may be drawn from phenotypic comparisons between these psychrotrophic,

moderately halophilic strains and strains from a diversity of other environments such as clinical, non-marine and temperate.

The assignment of the Organic Lake pigmented strains to the *Flavobacterium-Cytophaga* complex was confirmed by the characterisation of their phospholipid ester-linked fatty acid (PLFA) profiles and their respiratory lipoquinones. The PLFA profiles of representative strains of the two phenons contained a high proportion of branched C_{15} fatty acids, and their major respiratory lipoquinone was menaquinone 6 (Skerratt et al, 1991; P. D. Franzmann, pers. comm.). The PLFA profiles of the representative strains ACAM 44 and ACAM 48 contained mono-unsaturated branched C_{15} fatty acids, a15:1ω10c and i15:1ω10c respectively. Skerratt et al (1991) suggested that these fatty acids could serve as signatures for these organisms in ecological studies. The branched monounsaturated fatty acid, i15:1, had only previously been observed in trace amounts in the fatty acid profiles of *F. breve*, and group IIf, IIe and IIh strains. More recently i15:1ω10c has been found in *Flectobacillus glomeratus* (ACAM 171^T) (Nichols et al, 1993). The major PLFA in ACAM 44 was a15:0. No other member of the *Flavobacterium-Cytophaga* complex for which a fatty acid profile is available has a15:0 as the major fatty acid. A unique feature of the fatty acid profile of ACAM 48 among the library of available profiles, was the presence of i16:1ω7c. However, the fatty acid profiles of several species within the *Flavobacterium-Cytophaga* complex have not been determined, so the uniqueness of these features among the set of known species cannot be presumed. Branched 3-OH-fatty acids were also observed in the PLFA profiles of ACAM 44 and ACAM 48, including 3-OHa15:0 and 3-OHa17:0. These do not appear in the fatty acid profiles of other members of the *Flavobacterium-Cytophaga* complex (Fautz et al, 1981). The quantitative significance of the branched hydroxy fatty acids in the whole cells of the Organic Lake pigmented bacteria was not determined in the study of phospholipid ester-linked fatty acids as hydroxy fatty acids are predominantly found in the lipopolysaccharide or N-acyl-capnine fractions.

In summary, phenotypic, chemotaxonomic and genotypic (mol % G+C) data indicated that the pigmented bacteria from Organic Lake, belonged to the *Flavobacterium-Cytophaga* complex. However,

to assign these organisms to a genus in this complex on the basis of phenotypic data would be as doubtful as the genus descriptions themselves. The determination of the 16S rRNA sequences of the Organic Lake pigmented bacteria, would allow their phylogenetic position(s) within this group of organisms to be considered.

2.3 Taxonomy of the *Halomonadaceae*

2.3.1 Overview

The *Halomonadaceae* is comprised of the genera *Halomonas* and *Deleya*. In contrast to the genera *Flavobacterium* and *Cytophaga*, *Halomonas* and *Deleya* have short histories, being created in 1980 and 1983 respectively (Vreeland et al, 1980; Baumann et al, 1983). Nonetheless these two genera are a source of taxonomic confusion. Similarities between members of the two genera have been observed in phenotypic, chemotaxonomic, and genotypic studies, and clear discrimination between the two genera is not evident. The aim of this study was to clarify the phylogenetic relationships among members of the genera *Halomonas* and *Deleya* and determine whether generic distinctions within this group of organisms were warranted.

2.3.1 General characteristics of the genus *Halomonas*

The genus *Halomonas* was created to accommodate a group of moderately halophilic, Gram-negative, non-pigmented, rod-shaped strains which had been isolated from a solar saltern in the Netherlands Antilles (Vreeland et al, 1980). These strains were motile by either peritrichous or polar flagella, and long flexous cells were produced during the stationary phase of growth. These strains reduced nitrate to nitrite, and could grow anaerobically in the presence of nitrate, but not in its absence. They oxidised several sugars and fermented glucose, although this latter attribute was not observed in a subsequent phenotypic study (Franzmann et al, 1987a). Strains in this group tolerated NaCl concentrations in the range 20 - 32% at 30 °C, and the optimum NaCl concentration for growth was 8% (total salts 10%). The mol % G+C of their DNA was 61. Consideration was given to the possibility that these organisms may have been related to genera such as *Spirillum*, *Aquaspirillum*, *Oceanospirillum* and *Serpens* because of

their mol % G+C value, flexibility, and motility, however this was regarded as unlikely because of their fermentation of glucose, anaerobic growth on nitrate, urease production (6/9 strains) and lack of cytochrome oxidase. While these organisms were described as cytochrome oxidase negative based on the result of the test performed with the Patho-Tec Rapid ID System, they were positive by the Kovac's oxidase test. This suggests a difference in sensitivity between the two test methodologies, rather than a lack of the enzyme. Vreeland et al (1980) concluded that this group of strains should be classified as a novel genus of the family *Vibrionaceae*, and assigned them to a single species *Halomonas elongata.*

Rosenburg (1983) described *Pseudomonas halodurans* to accommodate a group of aerobic, polarly flagellate estuarine strains that were tolerant of concentrations of NaCl greater than 10 fold that which occurs in seawater. In a numerical analysis of phenotypic data these strains had a similarity to several *Pseudomonas* spp. in the range 70 - 75%, using the Jaccard coefficient, and the DNA:DNA hybridisation value with *Pseudomonas aeruginosa* was 58.5%. Hebert and Vreeland (1987) determined that the optimal NaCl concentration for growth of *P. halodurans* was 8% (10% total salts) and compared the phenotypes of *Ps. halodurans* and *Halomonas elongata* at that salt concentration. They found that the results of some phenotypic tests were opposite to those reported by Rosenburg (1983) in his examination of *Ps. halodurans* in media containing 1.7 % NaCl. Hebert and Vreeland (1987) found the phenotypes of *Ps. halodurans* and *H. elongata* to be 79 % similar using the Jaccard coefficient and on this basis proposed the transfer of *P. halodurans* to the genus *Halomonas*, as *H. halodurans*. Hebert and Vreeland (1987) did not refer to the DNA:DNA hybridisation data of Rosenburg (1983) which suggested a specific relationship between this group of estuarine strains and *P. aeruginosa.*

A comparison of 16S rRNA oligonucleotide catalogues demonstrated that the halotolerant species *Flavobacterium halmophilum* NCMB 1971^T (ATCC 19717^T, CCM 2833^T) an isolate from the Dead Sea, had a phylogenetic relationship to *H. elongata* (Woese et al, 1985a). The mol% G+C of this organism was corrected to 63, and it was transferred to the genus *Halomonas* as *H. halmophila*

(Franzmann et al, 1988b). In a numerical phenotypic study *H. halmophila* bore greatest similarity to *H.elongata* and *D. halophila* (James et al, 1990). *H. halmophila* NCMB 1971^T was non-pigmented, motile, produced acid from several sugars, utilised a wide range of carbon sources, and did not grow anaerobically with nitrate. *Flavobacterium halmophilum* had previously been described as yellow and non-motile (Weeks 1974, Ventosa, 1988). The fatty acid profile of *Flavobacterium halmophilum* CCM 2831 contains a large proportion of br-15:0 which is not typical of *Halomonas* spp. (Monteoliva-Sanchez and Ramos-Cormenzana, 1986; Franzmann and Tindall, 1990). The fatty acid profile indicates that strain CCM 2831 may be a member of the "flavobacteria-*Bacteroides*" phylum and should not be placed in *H. halmophila.*

Two species of *Halomonas* were described to accommodate groups of strains isolated from hypersaline Antarctic lakes (Franzmann et al, 1987a; James et al, 1990). *H. subglaciescola* was described from a phenotypic numerical taxonomic study of a group of non-pigmented strains isolated from Organic Lake (Franzmann et al, 1987a). The non-pigmented Organic Lake strains grew optimally in media containing 8% NaCl (10% total salts), tolerated 25% NaCl (27% total salts) and had a mol% G+C of its DNA in the range 61 - 63. The Organic Lake strains were assigned to the genus *Halomonas* on the basis of the mol% G+C content of their DNA, their largely oxidative metabolism and their halotolerance. *H. subglaciescola* had a much lower level of biochemical activity than *H. elongata* across the range of phenotypic characteristics determined. Strains belonging to *H. subglaciescola* produced negative results in the lysine decarboxylase, urease, esculin, ß-galactosidase, phosphatase, and gelatin hydrolysis tests, and utilised a lesser number of carbon sources in comparison to *H. elongata. H. subglaciescola* was comprised of two biovars. The strains in the biovar which contained the type strain were peritrichously flagellate, motile, and formed flexous filamentous cells in addition to shorter cells throughout the growth cycle. The strains in the other biovar did not have flagella, were non-motile and did not form filamentous cells.

The other Antarctic species, *Halomonas meridiana,* was characterised in a numerical phenotypic study of non-pigmented

strains isolated from Organic Lake, and four other hypersaline lakes of the Vestfold Hills region, "Horse" Lake, Burch Lake, "Lake Island" Lake, and Laternula Lake (James et al, 1990). Most of the strains from Organic Lake clustered with the *H. subglaciescola* reference strains. The strains isolated from the four other lakes, and one Organic Lake strain, did not cluster with the *H. subglaciescola* reference strains, and bore a greater phenotypic similarity to the non-Antarctic reference organisms, *H. elongata*, *D. aesta*, *H. halmophila*, and *D. halophila*, because of their utilisation of a wide range of carbon sources. These strains which were assigned to the species, *H. meridiana*, were slight halophiles, having optimal growth at salt concentrations similar to that of seawater, but were tolerant of NaCl concentrations in the range 20 - 25% (total salts 22 - 27%), and had a mol% G+C value of 59. James et al (1990) noted the lack of phenotypic and chemotaxonomic discrimination between the genera *Halomonas* and *Deleya*, and decided to place these organisms in the genus *Halomonas*, on the basis of the greater phenotypic similarity observed between them and *H. elongata* and *H. halmophila*, in comparison to *D. aesta* and *D. halophila*, and because the genus *Halomonas* had nomenclatural priority if the two genera were to be combined. The members of *H. meridiana* were characterised by the presence of 1 or 2 lateral flagella and were differentiated from other *Halomonas* spp. on the basis of biochemical characteristics, patterns of carbon source utilisation and antibiotic susceptibility, and growth at 0 °C. *H. meridiana* was comprised of two biovars which could be differentiated on the basis of Tween 20 and Tween 80 hydrolysis, and utilisation of some carbon sources.

2.3.2 General characteristics of the genus *Deleya*

In a numerical phenotypic analysis of non-fermentative marine strains isolated several miles off the coast of Oahu, Hawaii, 218 strains clustered into 22 groups, with a similarity of 68 - 76% using the simple similarity coefficient and complete linkage clustering (Baumann et al, 1972). Flagella type (polar, peritrichous), ability to accumulate poly-ß-hydroxybutyrate, and mechanism of aromatic ring cleavage (ortho, meta), were not included in the numerical analysis, but these characteristics conformed with the groupings. Mol% G+C values also supported most groupings. Baumann et al (1972)

suggested that these groups represented species. Groups with peritrichous flagella, and mol% G+C values in the range 53 - 68, were assigned to the genus *Alcaligenes* , and groups with polar flagella, and mol% G+C values in the range 56 - 63 were assigned to the genus *Pseudomonas* as new marine species of these genera.

The taxonomy of the marine *Alcaligenes* spp. was pursued by an examination of: metabolic processes, rRNA:DNA hybridisation relationships, protein electrophoregrams, and relatedness of enzymes using immunological methods. The aspartokinase from the marine *Alcaligenes* spp., *P. marina* and *P. nautica* had the same pattern of feedback inhibition/stimulation, with respect to L-threonine, L-lysine, L-methionine, and L-isoleucine, as the fluorescent pseudomonads and *Rhodospirillum rubrum* (Baumann and Baumann, 1974). The marine *Alcaligenes* and *P. marina* were distinctive from other *Pseudomonas* spp. and *Alteromonas* spp. in their fructose metabolism pathway which involved 1-phosphofructokinase and a pyrophosphate-dependent-6-phosphofructokinase (Sawyer et al, 1977). DNA:rRNA hybridisation studies demonstrated that the marine *Alcaligenes* spp. were a distinct lineage, within a branch that also contained the fluorescent pseudomonads. *Alcaligenes faecalis* belonged to a different RNA homology branch (De Ley, 1978). The protein electrophoregrams of the marine *Alcaligenes* spp. were distinguished from those of other *Alcaligenes* spp. by containing several bands closer to the anode (Kersters and De Ley, 1980). DeLong et al (1984) examined the immunological relationships of the superoxide dismutases (SOD) and glutamine synthetases from the marine *Alcaligenes* spp., and from marine species of *Alteromonas*, *Oceano-spirillium* and *Pseudomonas*. Four of the marine *Alcaligenes* spp. contained Mn-SOD. *Ac. pacifica*, and *P. marina* contained Fe-SOD. The reaction of the antisera to the Fe-SOD from *Ac. pacifica* showed that it was similar to the Fe-SOD from *P. marina*, and distinct from the enzymes from the representative species of the other genera. A close relationship between the marine *Alcaligenes* spp. and *P. marina* was indicated by the immunological reaction of their glutamine synthetases to antisera for the glutamine synthetase from *E. coli*.

The rRNA:DNA hybridisation studies determined that the marine *Alcaligenes* spp. and *P. marina* did not belong in the genera

Alcaligenes or *Pseudomonas*, and that they formed a distinct lineage. This conclusion was supported by the other taxonomic studies, and the genus *Deleya* was created to accommodate this group of species as, *D. aesta* (type species), *D. cupida*, *D. pacifica*, *D. venusta*, and *D. marina* (Baumann et al, 1983). These organisms were strictly aerobic, motile by flagella, accumulated poly-ß-hydroxybutyrate, had a requirement for Na^+, and a mol% G+C of their DNA in the range 52 - 68. The species belonging to this genus were differentiated on the basis of type of flagella, oxidase, growth at 4 °C, and carbon source utilisation. *Deleya* spp. were differentiated from marine *Pseudomonas* spp. by their utilisation of D-glucose, D-gluconate, and glycerol; and were differentiated from marine *Alteromonas* spp. by their accumulation of poly-ß-hydroxybutyrate.

Two new species, *D. halophila* and *D. salina* were later assigned to the genus. The description of the genus *Deleya* was expanded to include moderately halophilic organisms with the description of *D. halophila*, a species that accommodated a group of soil organisms which grew optimally in 7.5% marine salts, and were tolerant of 30% marine salts. The members of *D. halophila* had peritrichous flagella and a mol% G+C value of 67, and could be differentiated from other *Deleya* spp. on the basis of biochemical characteristics, carbon source utilisation and antibiotic susceptibility (Quesada et al, 1984). *D. salina*, also a moderate halophile, grew optimally at 5% marine salts, tolerated 20% marine salts, accumulated poly-ß-hydroxybutyrate, had a slightly yellow to cream pigmentation, was non-motile, and had a mol% G+C of its DNA in the range 60 - 64. *D. salina* had ≤ 28% DNA:DNA homology with other *Deleya* spp. and could be phenotypically differentiated from those species on the basis of biochemical characteristics, carbon source utilisation and antibiotic susceptibility (Valderrama et al, 1991).

Akagawa and Yamasato, (1989) found *Alcaligenes aquamarinus*, *Alcaligenes facaelis* subsp. *homari* and *Deleya aesta* to be synonymous from an examination of DNA:DNA homology values, fatty acid profiles and phenotypic characteristics. Nomenclatural precedence required that *D. aesta* (ATCC 27128^T), be replaced by *Deleya aquamarina* (ATCC 14400^T).

2.3.3 Phenotypic comparisons among members of the *Halomonadaceae*

All species belonging to the genera *Deleya* and *Halomonas* have been well characterised phenotypically. Most species of these genera were created to accommodate groups of strains, which were the subjects of numerical phenotypic analyses, in which they clustered into phenons separate from reference strains. Prior to the recognition of similarities between the two genera on the basis of phylogenetic data (Franzmann, 1988b), groups of unidentified strains were compared phenotypically with representative species of one genus or the other, but not both (Quesada et al, 1984; Franzmann et al, 1987a; Hebert and Vreeland, 1987; Akagawa and Yamasato, 1989). A phenotypic, numerical taxonomic study of representative species from both genera, which included *D. aesta*, *D. halophila*, *H. elongata*, *H. halmophila*, *H. subglaciescola* and *H. meridiana*, failed to find characteristics which would differentiate between the two genera (James et al, 1990; Dobson, 1988). *H. subglaciescola* was phenotypically distinct and consistently clustered at a low level of similarity with the cluster formed by the other species. The internal topology of the cluster formed by *D. aesta*, *D. halophila*, *H. elongata* and *H. halmophila* was unstable, varying according to the method of analysis used.

Baumann et al (1972) observed in their numerical phenotypic analysis of aerobic marine bacteria that groups which formed at lower similarity levels were not all coherent with regard to flagella type or mol % G+C values, and concluded that while this type of analysis was useful for clustering strains into species, it may not be useful for identifying genus groupings.

The genera *Deleya* and *Halomonas* were described on the basis of phenotypic characteristics and their mol % G+C contents (Baumann et al, 1983; Vreeland et al, 1980). Halotolerance, a defining characteristic of the genus *Halomonas*, does not differentiate it from the genus *Deleya*. This became evident from the inclusion of the moderately halophilic species, *D. halophila* and *D. salina*, in the genus, and also from the observation that the original type species, *D. aesta*, was tolerant of 20 % NaCl (James et al, 1990). Fermentation of glucose was a defining characteristic of the genus *Halomonas*, and

of the type species *H. elongata*, whereas the genus *Deleya* contained non-fermentative species. However, glucose fermentation has not been observed in any other species assigned to the genus *Halomonas*, and was not observed in a later phenotypic study of *H. elongata* (Franzmann et al, 1987a). Consequently glucose fermentation can neither be considered a defining characteristic of the genus *Halomonas*, nor a characteristic which differentiates this genus from the genus *Deleya*. Of the other characteristics which were included in the description of the genus *Halomonas*, they either do not occur uniformly among the other species assigned to the genus, or they are also present in members of the genus *Deleya*. Similarly the defining characteristics of the genus *Deleya* do not discriminate it from the genus *Halomonas*, with the possible exception of poly-ß-hydroxybutyrate accumulation, which occurs in all *Deleya* spp. tested but which has not been tested for in most *Halomonas* spp. and *D. halophila*. Both *Halomonas* spp. and *Deleya* spp. utilise ß-hydroxybutyrate (James et al, 1990). Furthermore, the ranges of mol % G+C values of members of the two genera overlap, and do not provide a means for discriminating between these genera.

2.3.4 Chemotaxonomic comparisons among members of the *Halomonadaceae*

The major fatty acids (9-35%) of *D. halophila*, cultured in media containing 7.5% salt at 32 °C, were 16:0, 18:1, 18:0, 16:1 and 17:0 cyclo (Monteoliva-Sanchez et al, 1988). Minor fatty acids (1-5%) were br-15:0, 14:0, cyclo19:0, br-14:0, 13:0, 12:0, and 20:0. Akagawa and Yamasato (1989) found all *Deleya* spp. to have similar fatty acid profiles with the major components being 16:0, 16:1 (+17:0cyc) and 18:1 (+19:0cyc), and 3-OH12:0 a minor component of all species. All *Deleya* spp. had ubiquinone 9 (Q9) as the major respiratory quinone (Akagawa and Yamasato, 1989). *H. elongata* and *H. halodurans* contained 12:0, 3-OH 12:0, 16:1, 16:0, 17:0, br-15:0, and 18:1 (Vreeland, 1992).

Fatty acid profiles are sensitive to culture conditions, as a comparison of profiles for *D. halophila* illustrates (Table 2.5). Monteoliva-Sanchez et al (1988) showed that for *D. halophila* the quantities of monounsaturated fatty acids decreased with a concomitant increase in the saturated fatty acids as temperature

Table 2.5 Fatty acid composition of *Deleya halophila*

% Salts	T (°C)	Saturated											Monounsaturated				Branched				Hydroxy
		10:0	11:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	19:0	20:0	16:1[a]	17:1	18:1[a]	20:1	14:0[g]	15:0[g]	17:0[g]	i17:1	3OH-12:0
10[b]	28	-	-	t[f]	-	-	-	23	-	t	-	-	11 (4)	-	65 (39)	-	-	-	-	-	-
12.6[c]	27	-	-	-	-	t	-	28	-	-	-	t	20 (nd)	-	52 (nd)	-	-	-	-	-	3
3[d]	32	t	t	2	3	2	1	35	-	4	t	2	14 (5)	-	30 (2)	-	t	4	1	-	-
7.5[d]	32	-	t	2	2	4	t	35	t	8	-	1	18 (9)	-	20 (3)	-	3	5	t	-	-
20[d]	32	2	t	2	3	4	t	34	-	10	1	t	17 (13)	-	18 (12)	-	1	6	t	-	-
12[e]	25	-	-	-	-	t	-	25	-	t	-	-	17 (0)	t	55 (3)	t	-	-	-	t	-

a Values include amounts of monunsaturated fatty acid and derived cyclopropane fatty acid. The amount of cyclopropane fatty acid is shown in brackets, nd=no data.
b Data from Franzmann and Tindall (1990).
c Data from Akagawa and Yamasato (1989).
d Data from Monteoliva-Sanchez et al (1988).
e Data from Skerratt et al (1991); phospholipid fatty acid composition only.
f t, less than 1%.
g Isomers not identified.

increased, and the quantities of cyclopropane fatty acids increased with a concomitant reduction in monounsaturated fatty acids, as salt concentration increased. The one-step conversion of monounsaturated fatty acids to cyclopropane fatty acids is also sensitive to oxygen tension, growth rate, and temperature (Wilkinson, 1988). The sum of the monounsaturated fatty acid and its derived cyclopropane fatty acid is a more stable characteristic than the quantification of the cyclopropane fatty acid alone (Jantzen and Bryn, 1985). Beyond the known variables which affect the proportions of the major fatty acids, the variation in the profiles from different laboratories with respect to the minor components suggest that the utilisation of these components for taxonomic purposes, without stringent standardisation of procedures, could not be recommended (Table 2.5).

Franzmann and Tindall (1990) compared the fatty acid profiles, respiratory lipoquinones and polar lipid profiles of all *Deleya* spp. and all *Halomonas* spp., under identical culture conditions. The major fatty acids in all species were 16:1 cis9 (2-28), 16:0 (16-32), 17:0 cyclo (2-27), 18:1 (20-69) and 19:0 cyclo11-12 (3-39). The relative proportions of these fatty acids varied within the ranges indicated in brackets, but there was no consistent variation between genera. All species from both genera contained Q9 as the major respiratory lipoquinone. There was some variation in the polar lipid patterns between species, but none that differentiated the two genera.

2.3.5 Phylogenetic relationships among members of the *Halomonadaceae*

A close phylogenetic relationship between *Flavobacterium halmophilum* and *"Alcaligenes" aquamarinus* was first identified in a DNA:rRNA hybridisation study undertaken prior to the creation of the genus *Deleya*, and the respective transfer of these species to the genera *Halomonas* and *Deleya* as *H. halmophila* and *D. aquamarinus* (Bauwens and De Ley, 1981). De Ley and his colleagues determined that the marine *Alcaligenes* were a distinct lineage within the rRNA superfamily II which also contained the fluorescent pseudomonads, *Azomonas*, *Azotobacter*, and *Xanthomonas* (De Ley, 1978; Kersters and De Ley, 1980).

Comparison of 16S rRNA oligonucleotide catalogues grouped *Halomonas elongata* and *F. halmophilum* as a distinct lineage within the gamma-subclass of the purple bacteria phylum, now known as the class *Proteobacteria* (Woese et al, 1985b; Stackebrandt et al, 1988). Six signature oligonucleotides, unique within the gamma-subclass of the *Proteobacteria* were identified.

Franzmann et al (1988b) produced the oligonucleotide catalogues for *H. subglaciescola* and *D. aesta* and compared them with those of *H. elongata* and *F. halmophilum*. The similarity coefficients (S_{AB} values) between these species were in the range 0.60 - 0.67. In the substructure of the tree derived from these similarity values, *H. elongata* and *F. halmophilum* formed a cluster, and *D. aesta* and *H. subglaciescola* formed a cluster. No conclusions were made about the relationships amongst this group, based on this clustering, as the differences in the similarity values between pairs of organisms was small. For example, the similarity values between *F. halmophilum* and *H. elongata* and between *F. halmophilum* and *H. subglaciescola* were 0.66 and 0.65 respectively. A new family *Halomonadaceae* was proposed to accommodate the genera *Halomonas* and *Deleya*.

The DNA:rRNA hybridisation values of *Deleya* spp. and *Halomonas* spp. with respect to *Deleya aquamarina* formed two groups (Kersters, 1992). One group which had $T_{m(e)} > 78$ °C, contained the synonyms of *D. aquamarina* (*D. aesta* and *Alcaligenes faecalis* subsp. *homari*) and *D. venusta*. The second group of species had $T_{m(e)}$ values in the range 75 - 77 °C, and contained *D. pacifica*, *D. cupida*, *D. marina*, *D. halophila*, *H. halmophila*, *H. subglaciescola*, and *H. elongata*. The membership of the second group indicated that species which had been assigned to the genus *Deleya* and the genus *Halomonas*, had the same level of rRNA relatedness to *D. aquamarina*. DNA:rRNA hybridisation values also indicated that the following species were members of the *Halomonadaceae* branch: "*Achromobacter viscosum*", "*Pseudomonas bathycetes*", "*Agarbacterium alginicum*", "*Achromobacter turbidis*", "*Achromobacter halophilus*", and "*Chromobacter marismortui*" (Kersters, 1992).

2.3.6 General characteristics of the genus *Halovibrio*

The genus *Halovibrio* was created to accommodate an obligately moderately halophilic organism with a vibroid morphology, isolated from the hypersaline (33% salinity) North Arm of Great Salt Lake, Utah, USA (Fendrich, 1988). This isolate was aerobic, had a single polar flagellum, was cytochrome oxidase positive, and did not produce acid from carbohydrates although its growth was stimulated by a limited number of carbon sources. Its mol% G+C value was 61. Assignment of this isolate to the genera *Aquaspirillum* or *Oceanospirillum* was precluded by its moderate halophilism, and mol% G+C value respectively. It was not considered appropriate to place this isolate in the genus *Halomonas* because it was obligately halophilic requiring 1.5 M salts for growth, did not grow anaerobically with nitrate, and its growth was stimulated by a limited range of carbohydrate sources. *Halovibrio variabilis* was included in a chemotaxonomic examination of members of the *Halomonadaceae* (Franzmann and Tindall, 1990). The fatty acid profile of *Halovibrio variabilis* was similar to the members of the *Halomonadaceae.* It had a significantly higher proportion of 19:0 cyclo, but the summed value of the 18:1 and 19:0 cyclo fatty acids was within the range observed for members of the *Halomonadaceae.* The major respiratory lipoquinone of *Halovibrio variabilis* was Q9, and its polar lipid pattern was identical to that of *H. halmophila.* It was suggested that *Halovibrio variabilis* should be transferred to a taxon within the *Halomonadaceae* (Franzmann and Tindall, 1990). The 16S rRNA of *Halovibrio variabilis* was determined in this study to confirm its placement within the *Halomonadaceae* and to determine its phylogenetic relationship to other members of the *Halomonadaceae.*

3 EXPERIMENTAL DESIGN

3.1 Strain selection

3.1.1 Organic Lake flavobacteria

The strains ACAM 44 and ACAM 48 were selected as representatives of two phenons derived in a numerical taxonomic study of the phenotype of a group of pigmented strains isolated from Organic Lake (Dobson et al., 1991). The mol% G+C of the DNA of ACAM 44 and ACAM 48 had been determined. They had been further characterised by their phospholipid fatty acid profiles, and the identification of their major respiratory lipoquinones (Skerratt et al, 1991, P. D. Franzmann, pers. comm.). Thus, their phylogenetic placement on the basis of 16S rRNA sequence comparison could be examined in relation to their phenotypic, gross genomic, and chemotaxonomic features. ACAM 44 and ACAM 48 were obtained from the Australian Collection of Antarctic Microorganisms, University of Tasmania, Australia.

3.1.2 Members of the *Halomonadaceae*

D. aquamarina, *D. halophila*, *D. marina*, *H. elongata*, *H. subglaciescola*, *H. meridiana*, and *Hv. variabilis* were the species selected for the determination of their 16S rRNA sequence. The culture collections from which these organisms were obtained are listed in Table 3.1. The 16S rRNA sequence of *Halomonas halmophila* had been previously determined and was obtained from the ribosomal RNA database project (RDP) (Olsen et al, 1991), for inclusion in the sequence comparisons.

The species selected for this study represented:
1) the type strains of the type species of the genera *Deleya* and *Halomonas*,
2) the diversity of environments from which members of the *Halomonadaceae* have been isolated, and which includes seawater, salterns, hypersaline Antarctic lakes, and hypersaline soils (Table 3.1),

Table 3.1 Source culture collection and source of isolation of strains of members of the genera *Deleya, Halomonas* and *Halovibrio* selected for inclusion in the 16S rRNA sequence comparisons.

Species	Source Culture Collection[a] Identification Number	Identification Number in Other Culture Collections[a]	Source of Isolation
D. aquamarina	DSM 30161^T	ATCC 14400^T, NCIMB 557^T	seawater[b]
D. halophila	DSM 4770^T	CCM 3662^T	hypersaline soil[c]
D. marina	DSM 4741^T	ATCC 25374^T, NCIMB1877^T	seawater[b]
H. elongata	DSM 2851^T	ATCC 33173^T, NCIMB 2198^T	saltern[d]
H. subglaciescola	ACAM 12^T	ATCC 43668^T, DSM 4683^T	hypersaline Antarctic lake[e]
H. meridiana	ACAM 246^T	ATCC 49692^T, DSM 5425^T	hypersaline Antarctic lake[f]
H. halmophila	-[i]	ATCC 19717^T, NCIMB 1917^T	Dead Sea[g]
Hv. variabilis	DSM 3051^T	-	saline lake[h]

a ACAM = Australian Collection of Antarctic Microorganisms, University of Tasmania, Australia
ATCC = American Type Culture Collection, Rockville, Maryland, USA
CCM = Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia
DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
NCIMB = National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK
b Baumann et al, 1983
c Quesada et al, 1984
d Vreeland et al, 1980
e Franzmann et al, 1987a
f James et al, 1990
g Franzmann et al, 1988b
h Fendrich 1988
i The 16S rRNA gene sequence of *H. halmophila* was not determined in this study. It was obtained from the rRNA Database Project (Olsen et al, 1991)

3) the diversity in the fatty acid profiles of members of the family *Halomonadaceae* (Table 3.2),
4) a large proportion of the breadth of mol% G+C values of the type strains of the species of the *Halomonadaceae* (Table 3.2).

Rules 15 and 17 of the Bacteriological Code state,

"The nomenclatural type, referred to in this Code as "type" is that element of the taxon with which the name is permanently associated. The nomenclature type is not necessarily the most typical or representative element of the taxon." and,

"The type determines the application of the name of a taxon if the taxon is subsequently divided or united with another taxon." (Lapage et al, 1975)

It was necessary therefore to include *Deleya aquamarina* and *Halomonas elongata*, the type species of the genera *Deleya* and *Halomonas*, among the species to be examined in this study. In the event of a proposal of formal taxonomic changes, the fate of the genus names is tied to these particular species.

Deleya marina was selected as being atypical among the members of *Deleya* in having polar flagella. The other members of *Deleya* have peritrichous flagella, with the exception of the recently described species *Deleya salina* which is non-motile (Valderrama, 1991). *Deleya halophila* was selected as a soil organism. Members of the *Halomonadaceae* have been predominantly isolated from water bodies. The Antarctic lake species, *Halomonas subglaciescola* and *Halomonas meridiana*, were included not only to represent the diversity within the members of the *Halomonadaceae*, but because their 16S rRNA sequence information would be useful for the development of species-specific oligonucleotide probes for application in ecological studies. The type strain of each species included in this study was used for the 16S rRNA sequence determination.

The species selected for the study represented the diversity in the fatty acid profiles of members of the family *Halomonadaceae*. The major fatty acids present in members of the *Halomonadaceae* occurred in the proportions 16:0 (15.5 - 30.3%), 16:1+17:cyc (7.7 -

Table 3.2. Mol% G+C values[a] and %proportions (wt/wt) of the major fatty acids[b] of the type strains of members of the *Halomonadaceae*

Species	Mol %G+C	Major fatty acids		
		16:0	16:1+17:0cyc	18:1+19:0cyc
• *D. aquamarina*[c]	57	17.4	8.4	70.7
D. cupida	61	22.6	17.5	56.1
• *D. halophila*	67	23.3	10.6	64.6
• *D. marina*	63	30.3	43.1	23.5
D. pacifica	68	26.2	15.6	55.7
D. salina	64	ND	ND	ND
D. venusta	54	15.9	10.7	68.6
• *H. elongata*	61	26.0	9.0	62.8
• *H. halmophila*	63	20.2	11.3	64.6
H. halodurans	63	27.3	41.7	26.7
• *H. meridiana*	59	15.5	7.7	73.4
• *H. subglaciescola*	62	25.4	19.7	52.3

a Data is from Baumann et al, 1972; Baumann et al, 1983; Vreeland et al, 1980; Franzmann et al, 1987a; Franzmann et al, 1988b; James et al, 1990, Rosenburg, 1983; Akagawa and Yamasato, 1989; Valderrama et al, 1991)

b Data is from Franzmann and Tindall (1990).

c • indicates species selected for 16S rRNA sequence comparisons; ND=not determined.

43.1%) and 18:1+19:0cyc (73.4 - 23.5%) (Franzmann and Tindall, 1990). The fatty acid profiles of *H. meridiana* and *D. marina* were at the opposite and extreme ends of these ranges of proportions (Table 3.2).

The species selected for the study represented a large proportion of the breadth of the range of mol% G+C values of the type strains of members of the *Halomonadaceae*. The type strains of the selected species had mol% G+C values in the range 57 - 67. The range of mol% G+C values among members of the family *Halomonadaceae* is 54 - 68 (Table 3.2). The type strains of *D. venusta* and *D. pacifica* have mol% G+C values of 54 and 68 respectively. These two species were the only members of the *Halomonadaceae* with type strains having mol% G+C values outside the range of values of the species included in the study.

Halovibrio variabilis was selected to determine its similarity to members of the *Halomonadaceae*. The genus *Halovibrio* is comprised of a single species.

3.2 16S rRNA sequence determination

3.2.1 Survey of methods

The complete sequence of a 16S rRNA gene of *E. coli* was obtained by the sequencing of cloned fragments of the gene (Brosius et al, 1978). The gene had been identified on a tranducing phage by hybridisation with rRNA (Lindahl et al, 1975). Locating a particular gene in the genome is often a long process and is not a practical approach for obtaining the sequence of the 16S rRNA gene from several different organisms for the purpose of taxonomic studies. The first 16S rRNA-based taxonomic studies (other than DNA:rRNA hybridisation studies), involved the determination of the sequences of catalogues of oligonucleotides which had been produced by digestion of the 16S rRNA with T1 ribonuclease (Fox et al, 1977). The catalogues were compared to obtain similarity values, and to identify signature characteristics (Woese, 1987).

The oligonucleotide cataloguing approach was replaced by direct sequencing of the 16S rRNA. This sequencing method involves the application of the Sanger dideoxynucleotide chain-terminating technique for sequencing DNA (Sanger et al, 1977) to the 16S rRNA molecule; through the use of reverse transcriptase and primers specific to conserved regions of the 16S rRNA (Lane et al, 1985). The majority of contiguous 16S rRNA sequence data has to date been obtained by this method.

The introduction of the polymerase chain reaction (PCR) using a thermostable polymerase (Saiki et al, 1988) has provided an alternative means of obtaining 16S rRNA sequence data. Multifold copies of a partial, or near complete segment of the 16S rRNA genes are produced with the use of pairs of primers specific to conserved regions within the 16S rRNA genes. The application of the PCR overcomes the rate-limiting step of locating the 16S rRNA genes within the genome.

The predominant advantages of sequencing PCR-amplified rRNA gene, rather than directly sequencing rRNA are:
1) The need to isolate large quantities of highly purified, undegraded RNA in an "RNase-free" environment is obviated. The PCR requires only small quantities of DNA, which need not be highly purified.
2) Problems of sequence ambiguities resulting from secondary structure or base modifications can be diminished. Two strands are available for sequencing, allowing regions of uncertainty to be sequenced in both directions and a greater flexibility of choice of primers to generate a contiguous sequence.
3) The PCR, with the appropriate choice of primers, can be used to amplify bacterial 16S rRNA genes from organisms embedded in eukaryotic tissue, allowing sequence data to be obtained for bacteria which may be difficult to culture (Chen et al, 1989). Similarly sequence information can be obtained for environmental organisms which are difficult to culture (Giovannoni, 1990).

Some initial attempts to amplify regions of the 16S rRNA gene were not for the purpose of obtaining sequence data for phylogenetic studies. Chen et al (1989) described oligonucleotides

(1175-1194, 1371-1390)[1] homologous to conserved regions of the 16S rRNA sequence specific to *Bacteria*. These oligonucleotides were used to amplify a 217 bp region of the 16S rRNA of bacteria from material containing an excess of eukaryotic DNA. Wilson et al (1989) identified two regions of the 16S rRNA sequence unique to rickettsiae, and amplified a 180 bp segment of the gene for the detection of these organisms.

Böttger (1989) amplified a 880 bp segment of the 16S rRNA gene from *E. coli*, *Legionella pneumophila*, and several species of the genus *Mycobacterium* using the "universal" primers A and C (Lane et al, 1985), and sequenced the amplified segment. The application of these primers based on regions of the 16S rRNA primary structure conserved in all living organisms, thus far examined, indicated the potential for obtaining sequence data for this gene from any organism, using this approach.

Edwards et al (1989) identified highly conserved regions close to both ends of the 16S rRNA gene of a diverse set of members of the domain *Bacteria*, and designed primers (8-27, 1541-1522)[2] to amplify a 1534 bp segment, which represented almost the entire gene. The near complete sequence of the 16S rRNA gene of *Mycobacterium kansasis* was determined. Wilson et al (1990) similarly designed primers for the 9-27, and 8-22[3] region with A, C degeneracy at 19, in addition to the 784-803, and 787-806 region with A, T, G degeneracy at 800, and the 1492-1507[4] region. Primers for these regions were used to amplify the 16S rRNA gene of members of a variety of genera as two segments of approximately 790 bp and 720 bp. Weisburg et al (1991) used the primer 8-27 designed by Edwards et al (1989) for the amplification of most of the *Bacteria*, and modified positions 19, 25, and 4 for use with the enterics and their relatives, members of the genus *Borrelia*, and members of the genus *Chlamydia* respectively. Weisburg et al (1991) suggested 1541-1525, a shorter version of the

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[1] The primary structure of the 16S rRNA of *E. coli* is shown in Fig 3.1. The numbering of the primers in the text is in accord with this diagram, and may differ from that of the original publication. Chen et al (1989) specified the positions of their primers at 1173-1192 and 1370-1389.

[2] See footnote 1. Edwards et al (1989) specified the positons of these primers at 8-28 and 1542-1522.

[3] See footnote 1. Wilson et al (1990) specified the position of this primer at 18-32.

[4] See footnote1. Wilson et al (1990) specified the position of this primer at 1487-1507.

Fig. 3.1 The primary structure of 16S rRNA from *E. coli* showing the numbering used in this study. The location of primers used for the determination of 16S rRNA sequences are indicated in bold type. The *E. coli* 16S rRNA sequence was obtained from the ribosomal RNA database project (Olsen et al, 1991).

```
        10          20          30          40          50          60
AAAUUGAAGA GUUUGAUCAU GGCUCAGAUU GAACGCUGGC GGCAGGCCUA ACACAUGCAA
       8.................27

        70          80          90         100         110         120
GUCGAACGGU AACAGGAAGA AGCUUGCUUC UUUGCUGACG AGUGGCGGAC GGGUGAGUAA
                                             103.................

       130         140         150         160         170         180
UGUCUGGGAA ACUGCCUGAU GGAGGGGGAU AACUACUGGA AACGGUAGCU AAUACCGCAU
122

       190         200         210         220         230         240
AACGUCGCAA GACCAAAGAG GGGGACCUUC GGGCCUCUUG CCAUCGGAUG UGCCCAGAUG

       250         260         270         280         290         300
GGAUUAGCUA GUAGGUGGGG UAACGGCUCA CCUAGGCGAC GAUCCCUAGC UGGUCUGAGA

       310         320         330         340         350         360
GGAUGACCAG CCACACUGGA ACUGAGACAC GGUCCAGACU CCUACGGGAG GCAGCAGUGG

       370         380         390         400         410         420
GGAAUAUUGC ACAAUGGGCG CAAGCCUGAU GCAGCCAUGC CGCGUGUAUG AAGAAGGCCU

       430         440         450         460         470         480
UCGGGUUGUA AAGUACUUUC AGCGGGGAGG AAGGGAGUAA AGUUAAUACC UUUGCUCAUU

       490         500         510         520         530         540
GACGUUACCC GCAGAAGAAG CACCGGCUAA CUCCGUGCCA GCAGCCGCGG UAAUACGGAG
                                        519.................536

       550         560         570         580         590         600
GGUGCAAGCG UUAAUCGGAA UUACUGGGCG UAAAGCGCAC GCAGGCGGUU UGUUAAGUCA

       610         620         630         640         650         660
GAUGUGAAAU CCCCGGGCUC AACCUGGGAA CUGCAUCUGA UACUGGCAAG CUUGAGUCUC

       670         680         690         700         710         720
GUAGAGGGGG GUAGAAUUCC AGGUGUAGCG GUGAAAUGCG UAGAGAUCUG GAGGAAUACC
                        685.................704

       730         740         750         760         770         780
GGUGGCGAAG GCGGCCCCCU GGACGAAGAC UGACGCUCAG GUGCGAAAGC GUGGGGAGCA

       790         800         810         820         830         840
AACAGGAUUA GAUACCCUGG UAGUCCACGC CGUAAACGAU GUCGACUUGG AGGUUGUGCC

       850         860         870         880         890         900
CUUGAGGCGU GGCUUCCGGA GCUAACGCGU UAAGUCGACC GCCUGGGGAG UACGGCCGCA

       910         920         930         940         950         960
AGGUUAAAAC UCAAAUGAAU UGACGGGGGC CCGCACAAGC GGUGGAGCAU GUGGUUUAAU
   907.................926
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Fig. 3.1 (cont'd)

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       970         980         990        1000        1010        1020
UCGAUGCAAC GCGAAGAACC UUACCUGGUC UUGACAUCCA CGGAAGUUUU CAGAGAUGAG

      1030        1040        1050        1060        1070        1080
AAUGUGCCUU CGGGAACCGU GAGACAGGUG CUGCAUGGCU GUCGUCAGCU CGUGUUGUGA

      1090        1100        1110        1120        1130        1140
AAUGUUGGGU UAAGUCCCGC AACGAGCGCA ACCCUUAUCC UUUGUUGCCA GCGGUCCGGC
                  1100.............1115

      1150        1160        1170        1180        1190        1200
CGGGAACUCA AAGGAGACUG CCAGUGAUAA ACUGGAGGAA GGUGGGGAUG ACGUCAAGUC

      1210        1220        1230        1240        1250        1260
AUCAUGGCCC UUACGACCAG GGCUACACAC GUGCUACAAU GGCGCAUACA AAGAGAAGCG
                        1226.................1245

      1270        1280        1290        1300        1310        1320
ACCUCGCGAG AGCAAGCGGA CCUCAUAAAG UGCGUCGUAG UCCGGAUUGG AGUCUGCAAC

      1330        1340        1350        1360        1370        1380
UCGACUCCAU GAAGUCGGAA UCGCUAGUAA UCGUGGAUCA GAAUGCCACG GUGAAUACGU

      1390        1400        1410        1420        1430        1440
UCCCGGGCCU UGUACACACC GCCCGUCACA CCAUGGGAGU GGGUUGCAAA AGAAGUAGGU
          1392...........1406

      1450        1460        1470        1480        1490        1500
AGCUUAACCU UCGGGAGgGC GCUUACCACU UUGUGAUUCA UGACUGGGGU GAAGUCGUAA

      1510        1520        1530        1540
CAAGGUAACC GUAGGGGAAC CUGCGGUUGG AUCACCUCCU UA
                    1522.................1541
```

primer used by Edwards et al (1989), was useful for many of the *Bacteria*, and that a primer at position 1492-1512, which overlaps that designed by Wilson et al (1990), was useful for enterics and most of the *Bacteria*, and that modification of position 1508 was useful for fusobacteria and most *Bacteria*.

There are three approaches for the determination of the sequence of PCR-amplified DNA:
1) To clone the DNA into a vector using either a sticky-end cloning technique, where restriction sites are incorporated into the PCR primers, or a blunt-end cloning technique (Giovannoni, 1991).
2) To produce single-stranded material to sequence directly. The technique of asymmetric-PCR has been used to generate a single-stranded product from the PCR. Alternatively a variety of methods have been used to generate single-stranded material from the double-stranded PCR product. Several of these methods have been reviewed by Gyllensten (1989).
3) To sequence the double-stranded PCR product directly.

The third approach is potentially the quickest route to obtaining sequence data. The additional steps involved in cloning material ready for sequencing, or of generating single-stranded material from the double-stranded PCR product are avoided. Also avoided is the increased potential for errors that occurs when sequencing individual clones generated by the PCR. Errors result from the misincorporation of nucleotides during the PCR. The possibility of errors requires that at least two clones be sequenced to confirm the accuracy of the sequence; and additional clones need to be sequenced if discrepancies are observed. Alternatively several clones can be mixed prior to sequencing (Sogin, 1990). When sequencing bulk double-stranded material directly, any misincorporation errors are effectively masked (Gyllensten, 1989). Asymmetric PCR has been reported to be unreliable for the generation of single-stranded material of length greater than approximately 1000 bp (McCabe, 1990).

While direct sequencing of the double-stranded PCR-amplified 16S rRNA gene appears to offer the quickest route to obtaining sequence data, very often the application of standard

sequencing protocols using α ^{35}S dATP and T7 DNA polymerase has not been successful. Generally, where double-stranded material has been sequenced a ^{32}P-labelled nucleotide has been employed, either by end-labelling of the sequencing primer or by incorporation during the polymerase reaction (Böttger, 1989; Edwards et al, 1989; Both et al, 1991; Embley, T. M., 1991). ^{35}S is the preferred radioisotope for sequencing because the bands in the sequence ladder derived by autoradiography are better resolved in comparison to ^{32}P (Bankier and Barrell, 1989). The lower energy of radioactive emissions from ^{35}S, also means that it is a less hazardous radioisotope to use, obviating the need to work with the material behind perspex screens, and reaction mixtures can be stored for longer periods in the event that they need to be utilised at a later date.

The difficulty in sequencing double-stranded PCR-generated material arises from the rapid reannealing of the short linear strands, resulting in only a small proportion of the material being involved in the sequencing reaction. The concentration of products from the reaction is small but detectable when the high-energy emitter ^{32}P is used. Weisburg et al (1991) report optimising the direct sequencing of PCR-amplified rRNA gene with α ^{35}S dATP by using 0.5 μg of template and annealing the template by heating the annealing mixture to 98 °C for 7 min and cooling to room temperature for 1 min, but recommended "cloning the fragments if a near-perfect sequence is desired". Bachmann et al (1990) demonstrated a significant improvement in the sequencing of a 115 bp double-stranded fragment generated by the PCR using α ^{35}S dATP, by the inclusion of the detergent non-idet P-40 (0.5%) in the sequencing reaction. The level of reannealing was further minimised by boiling the annealing mixture and snap-cooling on dry ice.

3.2.2 Methods development

The manner by which protocols were tested and modified is described in this section. The resultant protocols which were routinely used in this investigation are detailed in the Materials and Repetitive Methods section (s4).

3.2.2.1 DNA extraction

DNA was extracted by the rapid guanidium thiocyanate method of Pitcher et al (1989). Initial trials involved purification of this DNA by ultracentrifugation (45,000 rpm x 36 hrs, 20 °C; L8-70M Ultracentrifuge, Beckman Instruments Inc., Somerset, NJ, USA) in a CsCl-EtBr gradient (Sambrook et al, 1989). The PCR with the DNA extracted by the method of Pitcher et al (1989), without further purification by ultracentrifugation, was successful. Examination of the DNA extracted by the method of Pitcher et al (1989) after electrophoresis through an agarose gel, and staining with EtBr (s4.1.3), revealed an extra band which was presumptively identified as RNA. This band was absent after the extracted DNA had been treated with RNAase, confirming that it had been contaminated with RNA. The presence of RNA can be inhibitory to the PCR (I. Knight, pers. comm.). A comparison of the product from a PCR using *E. coli* DNA extracted in the manner described by Pitcher et al, (1989) without further purification, with that from a PCR using the same DNA but treated with RNAase, indicated a greater yield of the PCR product in the latter. The improved yield was significant as 0.5 - 1.0 μg of the PCR product was required for each sequencing reaction. An RNAase treatment added to the method of Pitcher et al (1989) was the standard procedure adopted for preparing DNA to be used as the template in the PCRs (s4.1.4). The purification of DNA by ultracentrifugation in a CsCl-EtBr gradient was unnecessary and not routinely applied.

3.2.2.2 Amplification of the 16S rRNA gene using the PCR

The PCR was first applied to DNA extracted from *D. halophila* and the two strains of Organic Lake flavobacteria. DNA extracted from *E. coli*, which had been successfully used in an amplification reaction by Edwards et al (1989) was included as a control. Primers 8-27 and 1541-1522 designed by Edwards et al (1989) were used to generate multifold copies of the 16S rRNA gene fragment via the PCR. Initially the protocol described by Edwards et al (1989) was followed. Amendments which became part of the standard protocol used in this investigation are described in section 4.1.6.

The PCRs were performed with materials from the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT, USA). The reaction mixture contained 10 µl 10 x Reaction Buffer (100mM Tris-HCl pH 8.3 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$ 0.01% (w/v) gelatin), 16 µl dNTPs (1.25 mM dATP, 1.25 mM dCTP, 1.25 mM dGTP, 1.25 mM dTTP), 100 pmol primer 8-27, 100 pmol primer 1541-1522, 1 µg template DNA, 2.5 units of AmpliTaq DNA polymerase and dd H_2O to 100 µl. The reaction mixtures were mixed in GeneAmp reaction tubes (Perkin-Elmer Cetus, Norwalk, CT, USA) and overlayed with 50 - 100 µl sterile mineral oil. The PCRs were run in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The thermal cycle was denaturation at 94 °C for 1 min, primer annealing at 37 °C for 2 min, and extension at 72 °C for 6 min. The cycle was repeated 36 times and then the mixtures were cooled to 4 °C.

The product was vortexed briefly with 100 µl of chloroform and the aqueous layer collected. This solution was extracted with one volume of TE -saturated phenol (APPENDIX A). The aqueous layer was collected and extracted with one volume of chloroform-isoamyl alcohol (24:1). The aqueous layer was collected and the DNA precipitated by the addition of 2 volumes of absolute ethanol (EtOH) and incubation at - 20 °C for at least 2 hours. The precipitate was collected by centrifugation at 12,000 rpm in a microfuge (Microcentaur, Fisons, England) at 4 °C for 30 min, washed with 75% EtOH, centrifuged for 15 min, washed with 100% EtOH and centrifuged. The pellet was dried and suspended in 50 µl of TE (APPENDIX A).

The product was examined by agarose gel electrophoresis using lambda Hind III-Eco RI markers. The major product band appeared between the 1584 and 1375 fragments of the lambda HindIII-EcoRI markers. The size of this fragment was consistent with the successful amplification of the target 16S rRNA gene of the test organisms. Secondary amplification products were also present. These were generally fragments of a smaller size than the desired target however a faint band was visible between the 2027 and 3530 fragments of the lambda Hind III-EcoRI markers.

The application of high annealing temperatures in the PCR minimises the production of non-specific products (Innis and Gelfand,

1990), however the application of too high a temperature will reduce the product yield. An annealing temperature of 5 °C below the T_m of amplification primers has been recommended (Innis and Gelfand, 1990). The melting temperatures (T_m) of primer 8-27 and primer 1541-1522 were estimated as 60 °C and 64 °C respectively (s4.1.5). Annealing temperatures of 50 °C and 55 °C using primers 8-27 and 1541-1522 were tested using *E. coli* DNA. No negative effect on the yield, as estimated by visualisation in an EtBr-stained agarose gel, was apparent, and the number of non-specific products from *E. coli* was reduced. An annealing temperature of 55 °C was adopted as standard protocol, however, even at this temperature non-specific products were generated from some organisms.

The presence of non-specific products was potentially a problem in sequencing the PCR-generated material, particularly as the PCR primers were to be used for sequencing. It was necessary therefore to isolate the desired fragment from agarose gel, following electrophoresis (s4.1.3). The band containing the desired product was excised from the gel. Three methods of extracting the DNA from this gel slice were compared. The methods were electroelution into dialysis bags (Sambrook et al, 1989), phenol-extraction from a low-melting point agarose gel, and GeneClean (Bio101). The yields from these three methods were similar. The GeneClean method was adopted as the standard protocol (s4.1.7), as it was a simple and quick technique in comparison with the other two methods.

Electrophoresis effectively isolated the 16S rRNA gene product which was subsequently purified adequately by the GeneClean method. It was therefore unnecessary to purify the PCR product prior to electrophoresis. Consequently as standard protocol the PCR product was collected from beneath the mineral oil layer and electrophoresed directly. Small amounts of mineral oil carried over, floated off in the electrophoresis buffer, and did not interfere with the electrophoresis.

The quantity of template DNA routinely used in PCRs was reduced to 10 ng and the quantity of the amplifying primers was reduced to 50 pmol without any negative effect on the yield of the product judged visually from an EtBr-stained agarose gel.

An attempt was made to generate ssDNA of almost the entire 16S rRNA gene using the primers of Edwards et al (1989) in the ratio 50:1 and 100:1; however no single-stranded product could be detected after staining with acridine orange (McMaster and Carmichael, 1977). Asymmetric PCR has been reported to be unreliable for the generation of ssDNA of length greater than approximately 1000 bp (McCabe, 1990). It would appear that two or more overlapping fragments would need to be generated to use asymmetric PCR for determination of the entire 16S rRNA sequence.

3.2.2.3 Direct sequencing of the PCR-amplified 16S rRNA gene using α ^{35}S dATP

Edwards et al (1989) used a standard dideoxy sequencing protocol with α ^{32}P dCTP and 0.05 - 0.2 pmol template DNA to sequence PCR-generated 16S rRNA gene fragment. In this investigation the first attempt to sequence the PCR generated material using α ^{35}S dATP similarly involved the application of a standard protocol (USB, 1989). The template DNA (0.1 pmol ~ 0.1 µg) was alkaline denatured and annealed with 2 pmol of the sequencing primer by heating to 65 °C for two minutes and slowly cooling to below 30 °C over a 30 minute period. Primer 8-27 of Edwards et al (1989) was used for all reactions during the development of the sequencing protocol. Standard labelling and termination reactions were performed. The reaction products were heated to 80 °C for 2 minutes prior to loading onto a pre-electrophoresed 0.2 mm 6% acrylamide gel, and electrophoresed for periods of 5 and 2 hours at 2100 V or 2700 V. The gel was dried and exposed to X-ray film for 40 hours. After development the autoradiograph showed only a few faint bands and some crossbands (bands that occurred in two or more lanes).

The following amendments were then tested:
1) Increasing the amount of the template to 0.2 µg.
2) Adding Mn^{2+} to the reaction mixture. It had been reported that the inclusion of Mn^{2+} to sequencing reaction mixtures improved the quality of the sequence close to the primer when there is a relatively low concentration of template DNA. The Mn^{2+} shortens the extensions that occur during the termination reactions (USB, 1989).

3) Diluting the labelling mix three fold. Dilution of the labelling mix also increases the concentration of shorter length products in the reaction products (USB, 1989).
After 2 weeks exposure of the electrophoresed products of these reactions, the result was still extremely poor. There was some improvement as a result of diluting the labelling mix, but generally the bands were faint and intermittent

The inclusion of non-idet P40 (NI) was then tested. Following the protocol of Bachmann et al (1990) the annealing process involved boiling the annealing mixture for three minutes and immediate snap-cooling in a dry-ice-ethanol bath. The concentration of NI was maintained at 0.5% during the annealing, labelling and termination reactions by additions of 5% NI. After 9 days exposure of the electrophoresed products, a much improved sequence ladder was obtained. Banding was clear only from the short electrophoresis (1.5 hours, 2900 V), representing sequence close to the primer. This may have been a result of the inclusion of Mn^{2+} in the reaction mixture.

A reaction with the inclusion of NI, the amount of the template DNA increased to 2 µg, the volume of $\alpha\ ^{35}S$ dATP (10 mCi/ml) tripled to 1.5µl and Mn^{2+} excluded, was then tested. A control reaction without NI was also performed. The exposed gel was readable after 2 days exposure, apparently a result of the increased concentration of $\alpha\ ^{35}S$ dATP. No readable sequence resulted from the reactions where NI was not included. These ladders consisted of crossbanding in two or more lanes. There were small regions of readable sequence in the ladders from the reactions were NI was included, although several areas were indistinct because of crossbanding or faint bands. Reactions with 1.0 µg of the 16S rRNA gene fragment from ACAM 44 and ACAM 48, with NI included, Mn^{2+} included, and 1.5 µl of $\alpha\ ^{35}S$ dATP were tested. After 24 hours exposure a good sequence ladder was observed from which 300 bases could be read. These reaction conditions became the basis of the standard protocol (s4.1.8) used for obtaining sequence data for the Organic Lake flavobacteria strains, and members of the genera *Deleya*, *Halomonas* and *Halovibrio*.

The inclusion of Mn^{2+} appeared to clarify the sequence ladder, however readable sequence was usually obtained without the

presence of Mn^{2+}. With the inclusion of Mn^{2+}, reaction products could not be stored longer than 2 - 3 days, and Mn^{2+} could produce weaker banding distant from the primer; therefore it was generally not included in the reaction mixtures.

Bachmann et al (1990) added the NI to the reaction mixture at the annealing, labelling and termination stages to maintain a concentration of 0.5% at each stage of the sequencing reaction. A single addition of 1 µl 20% NI to the annealing reaction was found to be as effective. The resultant concentration of NI in the reaction mixture was 2% at the annealing stage, 1.2% at the labelling stage and 0.5% at the termination stage.

As standard protocol, a second set of reactions where dITP replaced dGTP in the reaction mixtures, was performed. The presence of dITP effectively resolved band compressions in sequence ladders. The use of dITP can cause terminations of the polymerase reaction as a result of secondary structure (USB, 1989). This effect, which appears as crossbands in the sequence ladder, was observed. Also the banding from the dITP reactions was weaker. Therefore it was necessary to perform both dITP and dGTP reactions. Although the dITP sequence ladder contained imperfections, the banding in terms of spacing and resolution was often better than that of the dGTP sequence ladder and was helpful in reading the dGTP sequence.

The primers used to sequence ACAM 48 were 8-27 (Edwards et al, 1989), 519-536 (Lane et al, 1985), 536-519 , 685-704 (Embley et al, 1988b), 704-685, 907-926 (Lane et al, 1985), 1100-1115 (Embley et al, 1988b), and 1541-1522 (Edwards et al, 1989) (Fig. 3.1). The primer at 704-865 was modified at position 700 (C-› T) to match the sequence of the Organic Lake flavobacteria. A primer 122-103 (CGTTACGCACCCGTGCCCG) was designed to match the sequence of the Organic Lake flavobacteria, and used to read the sequence into the primer 8-27. This primer was based on the primer 107-126[1] designed by Stackebrandt and Charfreitag, 1990. The same set of primers were used to sequence ACAM 44, however a poor result was obtained from the priming reactions with the 907-926 and

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[1] See footnote 1. Stackebrandt and Charfreitag (1990) specified the position of this primer at 105-124.

1541-1522 primers with this template. Primers 1226-1245 (Stackebrandt and Charfreitag, 1990), 1245-1226 and 1392-1406 (Lane et al, 1985) were used to complete this sequence. The 1226-1245 (CACACGTGCTACAATGGTAG) and 1245-1226 primers were based on the primer 1223-1241[2] described by Stackebrandt and Charfreitag (1990), and designed to match the sequence of the Organic Lake flavobacteria.

Sequences for the members of the *Halomonadaceae* and *Halovibrio variabilis* were obtained using the primers 8-27, 536-519, 519-536, 685-704, 704-685, 907-926, 1100-1115, 1392-1406 and 1541-1522 (Fig. 3.1).

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[2] See footnote 1. Stackebrandt and Charfreitag (1990) specified the position of this primer at 1224-1242.

4 MATERIALS AND REPETITIVE METHODS

4.1 Determination of 16S rRNA sequences

Chemicals of analytical quality were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and BDH Australia, Pty Ltd (Kilsyth, Vic.), as indicated. Oligonucleotide primers were synthesised by C. -M Lin (Center of Marine Biotechnology, University of Maryland, MD, USA) and Bresatec Ltd (Thebarton, South Australia). α ^{35}S -dATP was purchased from Amersham Corp. (Arlington Heights, IL, USA) and Bresatec Ltd (Thebarton, South Australia).

4.1.1 Culture conditions

All strains were grown in Marine Broth (Difco Lab, Detroit, MI, USA), or on the same medium supplemented with agar (15g/l), at 25 °C.

4.1.2 Determination of the concentration of DNA solutions

The concentration of DNA solutions (DNA_{conc}) were determined by measurement of absorbance at 260 nm and use of the following equation:

$$DNA_{conc} (\text{ mg ml}^{-1} = \mu\text{g } \mu\text{l}^{-1}) = (A_{260} \text{ x } 50 \text{ } \mu\text{g ml}^{-1} \text{ x D})/1000 \text{ } \mu\text{g mg}^{-1}$$

where D = dilution factor

This equation is derived from the observation that a dsDNA solution of 50 μg ml^{-1} has an absorbance of 1 at 260 nm (Sambrook et al, 1989).

4.1.3 Agarose gel electrophoresis (Sambrook et al, 1989)

DNA preparations and the products of PCR reactions were routinely examined by electrophoresis through 1% (w/v) SeaKem agarose (FMC Bioproducts, Rockland, ME, USA) in a mini-gel apparatus (Horizon 58, Horizontal Gel Electrophoresis, BRL, Gaithersburg, MD, USA) in TAE buffer (APPENDIX A). Agarose gel

6x loading buffer (APPENDIX A) was mixed with the DNA solution in a ratio of 1:6 and loaded into the gel. The DNA was electrophoresed at 8 - 10 Vcm^{-1} for 30 - 45 minutes, using a Gene Power Supply GPS 200/240 (Pharmacia LKB, Uppsala, Sweden). The gel was stained with EtBr , either by soaking the gel in EtBr solution (0.5 μgml^{-1}) for 15 minutes following electrophoresis, or by including the EtBr at a concentration of 0.5 μgml^{-1}, in the molten gel, prior to electrophoresis (APPENDIX A). The nucleic acid material was visualised with UV light on a transilluminator (Model No. TF-20M, Vilber Lourmat, Cédex 2, France).

4.1.4 DNA extraction (Pitcher et al, 1989)

Several colonies were scraped from the agar surface and suspended in 100 µl TE pH 7.4 (APPENDIX A). The suspended cells were mixed with 0.5 ml GT lysis solution (APPENDIX A), and allowed to lyse (≈ 10 min) on ice. Following lysis of the cells, 0.25 ml 7.5 M CH_3COONH_4 (Sigma) and 0.75 ml chloroform:isoamyl alcohol (24:1) (BDH) were added. The solution was mixed well and centrifuged at 12,000 rpm for 10 minutes. The upper aqueous phase was collected and 2 volumes of cold (- 20 °C) EtOH (BDH) were added. The DNA precipitated on addition of the EtOH, and was collected by centrifugation at 12,000 rpm for 15 - 20 min (Microcentaur, Fisons, England). The resultant pellet of DNA was washed 3x with 70% EtOH, dried and dissolved in ddH_2O. RNAase A (APPENDIX A) was added to a concentration of 40 $\mu g\ ml^{-1}$, and incubated for 1 hr at room temperature. The DNA was reprecipitated, collected by centrifugation as before, and dissolved in ddH_2O.

4.1.5 Estimation of the T_m of the PCR primers (Sambrook et al, 1989)

The T_m of the primer-template hybrid was estimated using the following equation:

$$T_m = (N_{A+T} \times 2°C) + (N_{G+C} \times 4\ °C)$$

where N_{A+T} = number of A and T bases in the primer
N_{G+C} = number of G and C bases in the primer

4.1.6 PCR amplification of the 16S rRNA gene

10x PCR mixture

Reaction buffer*	100	µl
dNTPs**	160	µl
Primer 8-27 (10 pmol μl^{-1})	50	µl
Primer 1541-1522 (10 pmol μl^{-1})	50	µl
Template DNA (10 ng μl^{-1})	10	µl
AmpliTaq DNA polymerase	10	µl
ddH_2O	620	µl

The PCRs were performed with materials from the GeneAmp Reaction Kit (Perkin-Elmer Cetus, Norwalk, CT, USA). All the reactants, except the dNTPs, were mixed carefully, to minimise the formation of bubbles, and 10 x 84 µl aliquots pipetted into GeneAmp reaction tubes (Perkin-Elmer Cetus, Norwalk, CT, USA). These were placed in the DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) and heated to 80 °C. 16 µl of the dNTPs solution were then added to each tube. The reactants were mixed quickly and overlayed with 50 - 100 µl of mineral oil. The temperature was raised to 98 °C for 30 seconds, cycled through the denaturation (94 °C, 1 min), annealing (55 °C, 2 min), and extension (72 °C, 6 min) phases 36 times. At the completion of the 36 cycles the product was stored at 4 °C.

* Reaction buffer: 100 mM Tris-HCl pH 8.3 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin

** dNTPs: 1.25 mM dATP, 1.25 mM dCTP, 1.25 mM dGTP, 1.25 mM dTTP

4.1.7 Isolation of the PCR-amplified 16S rRNA gene fragment

The product from the PCRs was removed from beneath the mineral oil layer, pooled and then electrophoresed through either a large agarose gel or a few minigels. Materials in the GeneClean kit (BIO 101, La Jolla, CA, USA) were used to extract the DNA product from the agarose gel. The 16S rRNA gene fragment band in the agarose gel was excised and 3 volumes of 6 M NaI were added. The solution was heated to 60 °C until the gel dissolved. Glass milk

solution (≈ 35 µl) was added and the mixture incubated on ice for 20 min. The glass milk was pelleted by centrifugation and the supernatant discarded. The pelleted material was suspended in the wash solution, centrifuged and the supernatant discarded. This wash procedure was repeated 4 times. The pelleted material was then suspended in 2 volumes of ddH_2O, incubated at 50 °C for 3 min, pelleted by centrifugation and the supernatant collected. This extraction procedure was repeated and the collected supernatants were combined.

4.1.8 Sequencing reactions (Sanger et al, 1977; USB, 1989)

Sequencing reactions were performed with materials from the Sequenase 2.0 sequencing kit (USB, Cleveland, OH, USA). The contents of the reaction buffer, enzyme dilution buffer, labelling mixes, termination mixes, and stop solution are detailed in APPENDIX A.

Annealing Reaction:

Primer solution (10 pmol μl^{-1})	1	µl
Reaction buffer	2	µl
DNA template solution (0.17 pmol μl^{-1})	6	µl
20% Non-idet P40	1	µl

The reaction mix was heated to 100 °C in a boiling water bath for 3 minutes and immediately snap-frozen in a dry-ice-ethanol bath. The frozen mixture was centrifuged for a few seconds to collect any condensate and then stored on wet ice until ready to commence the labelling reaction.

Labelling Reaction:

Annealed template-primer solution	10	µl
0.1M Dithiothreitol	1	µl
Labelling mix (diluted 1:5)	2	µl
α ^{35}S -dATP (10 mCi/ml)	1.5	µl
T7 DNA polymerase (diluted 1:8)	2	µl

The T7 DNA polymerase was diluted 1:8 in the enzyme dilution buffer. The reactants were added in the order given above,

mixed carefully with a pipette and the labelling reaction allowed to proceed for 5 minutes at room temperature.

Termination reactions:

2.5 µl of the ddATP, ddCTP, ddGTP and ddTTP termination mixes were pipetted into separate tubes and warmed to 37 °C. Following the labelling reaction, 3.5 µl of the labelling reaction mix was added to each of the termination reaction mixes. The termination reactions were incubated for 5 minutes at 37 °C and then stopped by the addition of 4 µl of stop solution. The reaction mixtures were stored at - 20 °C, prior to electrophoresis.

Sequencing with dITP:

The annealing, labelling and termination reactions were performed according to the protocol described above except that the dITP labelling mix replaced the dGTP labelling mix and the dATP (dITP), dCTP (dITP), dGTP (dITP), dTTP (dITP) termination mixes replaced the dATP, dCTP, dGTP, dTTP termination mixes respectively.

4.1.9 Polyacrylamide gel electrophoresis (PAGE)

The sequencing reaction mixtures were electrophoresed through a 0.2 mm, 6% polyacrylamide gel (APPENDIX A) in TBE buffer (APPENDIX A). The Sequi-Gen Cell (Bio-Rad, Richmond, CA, USA) and LKB 2010 Macrophor (Pharmacia LKB, Uppsala, Sweden) sequencing systems were used for electrophoresis. The gel was pre-electrophoresed for 15 min at 1800 V. Prior to loading, the reaction mixtures were heated to 75 °C for 2 min. 1.5 µl of each of the reaction mixtures from the standard set of reactions, and the dITP set of reactions were loaded into adjacent wells. The reaction mixtures were electrophoresed for periods of 5 and 2 hrs at 1800 V. When the LKB 2010 Macrophor system was used, water heated to 55 °C was circulated through the thermostatic plate.

Following electrophoresis, when using the Sequi-Gen cell system, the gel was rinsed twice with 10% acetic acid (BDH), and removed to a backing of 3MM Whatman paper (W. & R. Balston Ltd., England). The gel was dried in a gel dryer (Model 583, Bio-Rad,

Richmond, CA, USA) at 80 °C for 30 min. When using the LKB 2010 Macrophor system the gel was bound to one plate with Bind-Silane (Pharmacia LKB, Uppsala, Sweden), according to the manufacturer's instructions. Following electrophoresis, the plate with the bound gel was soaked in 10% (v/v) acetic acid for 20 min, and left to dry in a fumehood overnight. The dried gels were exposed to X-OMAT AR film (Eastman Kodak Co, Rochester, NY, USA) for periods of between 24 hours and 2 weeks.

4.2 Analysis of sequence data

4.2.1 Sequence alignment

Sequences were read from the autoradiographs, checked, and recorded on computer disks. Nucleotide bases were recorded as capital letters, or as lowercase if there was a small amount of uncertainty as to their correct identity. Regions of overlapping sequence from different primers resolved several uncertain bases. Nucleotide bases which could not be identified were recorded as "N". Programs from the Wisconsin GCG set of programs (Devereaux et al, 1984) or the SeqSpeak 1.0 DNA Sequence Editor by Keith Conover (1991, Dalhousie University, Nova Scotia, Canada) were used to record sequences. Regions of overlap between sequences of different primers were determined using Bestfit from the Wisconsin GCG package or the Seqh program (Kanehisa et al, 1984). The Seqh program was accessed through the Australian National Genomic Information Service (ANGIS) (University of Sydney, NSW).

Sequences were aligned manually against pre-aligned sequences from the ribosomal RNA database project (RDP) (Olsen et al, 1991). The Homologous Sequence Editor (HOMED) program of Peter A. Stockwell (1986, University of Otago, New Zealand) was used for this purpose. The secondary structure of the sequences were drawn and taken into account in their alignment. The HOMED program was accessed through ANGIS.

The sequences of the members of the *D. aquamarina*, *D. halophila*, *D. marina*, *H. elongata*, *H. subglaciescola*, *H. meridiana* and *Halovibrio variabilis* were aligned against that of *Halomonas*

halmophila (ATCC 19717^T), and those of the following 38 organisms belonging to the gamma-subclass of the *Proteobacteria* (culture collection strain numbers, where identified in the database, are indicated): *Acinetobacter* sp. (ATCC 33604), *Aeromonas hydrophila* (ATCC 7966^T), *Alteromonas haloplanktis* (ATCC 14393), *Arhodomonas oleiferhydrans* (ATCC 49307), *Cardiobacterium hominis* (ATCC 15826^T), *Coxiella burnetii* str. Q177, *Chromatium tepidum* (ATCC 43061^T), *Chromatium vinosum* (ATCC 17899^T), *Citrobacter freundii* (ATCC 29935), *Ectothiorhodospira halochloris* (ATCC 35916^T), *Ectothiorhodospira halophila* str. SL 1, *Ectothiorhodospira shaposhnikovii, Erwinia carotovora* (ATCC 5713), *Erwinia herbicola, Escherichia coli, "Flavobacterium lutescens"* (ATCC 27951), *Haemophilus influenzae* (ATCC 33391^T), *Hafnia alvei* (13337^T), *Legionella pneumophila* subsp. *pneumophila* (ATCC 33152^T), *Marinobacter hydrocarbonoclasticus* (ATCC 49840^T), *Marinomonas vaga* (ATCC 27119^T), *Methylococcus capsulatus* str. BATH, *Methylomonas methanica* str. 81Z, *Oceanospirillum linum* (ATCC 11336^T), *Pasteurella multocida* (ATCC 43137^T), *Plesiomonas shigelloides* (ATCC 14029^T), *Proteus vulgaris* (IFAM 1731), *Pseudomonas aeruginosa* (ATCC 25330), *Pseudomonas mendocina* (ATCC 25411^T), *Ruminobacter amylophilus* (ATCC 29477), *Serratia marcescens* (ATCC 13880^T), str. symbiont P of *Acyrthosyphon pisum*, str. symbiont S of *Acyrthosyphon pisum, Vibrio parahaemolyticus* (ATCC 17802^T), *Wolbachia persica* (ATCC VR 331), *Xanthomonas maltophilia* (ATCC 13637^T), *Xylella fastidiosa* (ATCC 35880), and *Yersinia enterocolitica* (ATCC 9610^T). The sequences of all these species were obtained from the RDP, except for the sequences of *Alteromonas haloplanktis, Marinobacter hydrocarbonoclasticus* and *Marinomonas vaga*, which were obtained from Genbank and then aligned in the RDP format.

The sequences of the Organic Lake flavobacteria were aligned against those of 53 members of the "flavobacteria-*Bacteroides*" phylum available from the RDP which were the following (culture collection strain numbers, where identified in the database, are indicated): *Antarcticum vesiculatum* str. 23-P, *Bacteroides distasonis* (ATCC 8503^T), *Bacteroides fragilis* (ATCC 25285^T), *Bacteroides thetaiotaomicron* (ATCC 29148^T), *Bacteroides*

vulgatus (ATCC 8482^T), *Cyclobacterium marinus* (ATCC 43824), *Cytophaga aquatilis* (ATCC 29551^T), *Cytophaga diffluens* (ATCC 23140), *Cytophaga fermentans* (ATCC 19072^T), *Cytophaga flevensis* (ATCC 27944^T), *Cytophaga heparina* (ATCC 13125^T), *Cytophaga hutchinsonii* (ATCC 33406^T), *Cytophaga johnsonae* (ATCC 17061^T), *Cytophaga johnsonae* (DSM 425), *Cytophaga latercula* (ATCC 23177^T), *Cytophaga lytica* (ATCC 23178^T), *Cytophaga marinoflava* (ATCC 19326^T), *Cytophaga uliginosa* (ATCC 14397^T), *Flavobacterium aquatile* (ATCC 11947^T), *Flavobacterium balustinum* (ATCC 33487^T), *Flavobacterium breve* (ATCC 14234), *Flavobacterium ferrugineum* (ATCC 13524^T), *Flavobacterium gleum* (ATCC 35910^T), *Flavobacterium indologenes* (ATCC 29897^T), *Flavobacterium indoltheticum* (ATCC 27950^T), *Flavobacterium meningosepticum* (ATCC 13253^T), *Flavobacterium odoratum* (ATCC 4651^T), *Flavobacterium thalpophilum* (ATCC 43320^T), *Flectobacillus glomeratus* (ATCC 43844^T), *Flectobacillus major* (ATCC 29496^T), *Flexibacter aggregans* (ATCC 23162^T), *Flexibacter aurantiacus* (ATCC 23107^T), *Flexibacter canadensis* (ATCC 29591^T), *Flexibacter columnaris* (ATCC 43622), *Flexibacter elegans* (ATCC 23112^T), *Flexibacter filiformis* (ATCC 29495^T), *Flexibacter flexilis* (ATCC 23079^T), *Flexibacter litoralis* (ATCC 23117^T), *Flexibacter polymorphus* (ATCC 27820^T), *Flexibacter roseolus* (ATCC 23088^T), *Flexibacter ruber* (ATCC 23103^T), *Flexibacter sancti* (ATCC 23092^T), *Flexibacter tractuosus* (ATCC 23168^T), *Haliscomenobacter hydrossis* (ATCC 27775^T), "*Microscilla aggregans* subsp. *catalatica*" (ATCC 23190), "*Microscilla furvescens*" (ATCC 23129), *Microscilla marina* (ATCC 23134^T), "*Microscilla sericea*", *Runella slithyformis* (ATCC 29530), *Saprospira grandis* (ATCC 23119^T), *Sphingobacterium mizutae* (ATCC 33299^T), *Sphingobacterium spiritivorum* (ATCC 33861^T), and *Spirosoma linguale* (ATCC 23276). The sequences of the Organic Lake flavobacteria were also aligned against the sequence of *E. coli* in order to locate base positions in terms of *E. coli* numbering.

4.2.2 Phylogenetic analysis

The sequences of the Organic Lake flavobacteria were examined for the presence of signature nucleotides which had been

previously identified as characteristic of members of the "flavobacteria-*Bacteroides*" phylum.

The sequences of the members of the *Halomonadaceae* and *Halovibrio variabilis* were examined for the signature characteristics which had been previously identified as characteristic of members of the *Halomonadaceae* sub-lineage within the gamma-subclass of the *Proteobacteria* (Woese, 1985b). As all but one of these signatures were found not to be useful in characterising the members of the *Halomonadaceae*, the sequences were further examined to locate additional signatures for this group.

Distance analyses were performed with programs from PHYLIP v3.4 package (Felsenstein, 1989). Jukes-Cantor evolutionary distances were calculated by the DNADIST program. Phylogenetic trees were derived with the FITCH program using random order input of sequences and the global rearrangement option. Diagrams of the resultant trees were obtained using the DRAWTREE program. Up to 50 sequences were included in an analysis. The PHYLIP v3.4 programs were accessed through ANGIS.

Parsimony analyses were performed using PAUP v3.0 programs (Swofford, 1991). The most parsimonious tree(s) were found by branch and bound analyses. As branch and bound parsimony analysis guarantees finding the most parsimonious tree(s) amongst all possible tree topologies it involved a larger amount of processing than the distance analyses performed by the PHYLIP v3.4 programs. The PAUP v3.0 programs were run on a Macintosh IIsi (Apple Computer, Cupertino, CA, USA). Up to 12 sequences were included in the branch and bound analysis of the Organic Lake flavobacteria, and up to 13 sequences were included in the branch and bound analysis of the members of the *Halomonadaceae*. Outgroup species were identified in each analysis.

Some analyses were restricted to conserved positions. In these analyses variable length regions of uncertain alignment, and positions where < 50% of the taxa had the same nucleotide, were eliminated from the data set.

Bootstrap analyses (2000 replications) were performed using both the distance and parsimony methods. Limitations in processing time meant that heuristic rather than branch and bound searches were performed in the parsimony bootstrap analyses

4.2.3 Genus-specific and species-specific probes

The aligned sequences of the Organic Lake flavobacteria, and the members of the genera *Deleya*, *Halomonas* and *Halovibrio* were examined to identify regions which may be useful for species-specific probes, and a genus-specific probe for the genus *Halomonas*. The SSU (small-subunit) rRNA sequences of all the organisms within the RDP were examined to determine whether any matched the regions identified as potential probe sequences, and to determine which had the highest similarity to the potential probe sequence if they did not match exactly. This analysis of the SSU rRNA sequences within the RDP was done using the Lipman-Pearson algorithm (Lipman and Pearson, 1985), by the Match-Fast program, accessed through ANGIS. The Match-Fast program listed sequences with the greatest similarity to the potential probe sequences. Of these the probe with the greatest number of mismatches to the sequences with which it had the highest similarities, was chosen.

5 RESULTS

5.1 Organic Lake pigmented bacteria

An example of an autoradiograph of sequencing reactions after polyacrylamide electrophoresis is shown in Fig. 5.1. The simultaneous electrophoresis of sequencing reaction mixtures prepared using the standard set of nucleotides and those where dGTP was replaced with dITP was useful for resolving compressed bands. There was some variation in the quality of the sequence dependent on the template-primer combination, however at least 93.9% of the gene sequence within the region bounded by the PCR-amplification priming sites was determined, indicating the efficacy of the method of Bachmann et al (1990) for directly sequencing the PCR-amplified 16S rRNA gene.

The number of bases determined for the 16S rRNA gene of ACAM 44 and ACAM 48 were 1419 and 1466 respectively, which represented 96.1% and 99.2%, respectively, of the region bounded by the PCR-amplification priming sites. The sequences have been deposited in the GenBank database under accession numbers M92278 for ACAM 44 and M92279 for ACAM 48. The alignment of the 16S rRNA sequences of ACAM 44 and ACAM 48 against sequences for some members of the "flavobacteria-*Bacteroides*" phylum, and *E. coli* is shown in Fig. 5.2.

The 16S rRNA sequences of ACAM 44 and ACAM 48 contained the definitive flavobacterial signatures identified by C. Woese and his colleagues (Woese et al, 1990; Woese et al, 1985), that unequivocally place these organisms in the "flavobacteria-*Bacteroides*" phylum. These features include an A at position 38, a G-C base pair at 290-310, a loop size of 5 at 416-427, a loop of 4 and a 6 pair stem at 455-477, an U at 570 and an A at 866, AU at 995-6, an A at 1045, a deletion in the loop and a base added at position 1074 in the stem-loop helix at 1161-1175, and a deletion of 4 pairs at 1436-1465 (in comparison with the *E. coli* stem). A further signature at 1530-1533 was not determined as it occurs within the PCR primer site. The sequences of ACAM 44 and ACAM 48 contained a C at 1205 which

Fig. 5.1 Autoradiograph of sequencing reactions with the template ACAM 48 16S rRNA gene (PCR-amplified) and the primer 536-519. Reaction products from dGTP and dITP mixtures are in adjacent lanes. The full-arrows indicate the resolution of a compressed band (seen in the dGTP, G lane) in the dITP G lane. The open arrows indicate cross banding in the dITP lanes, which did not occur in the dGTP lanes. The reaction products in the left hand lanes (dGTP and dITP) were electrophoresed for 5 hours at 1800 V. The reaction products in the right hand lanes (dGTP and dITP) were electrophoresed for 2 hours at 1800 V.

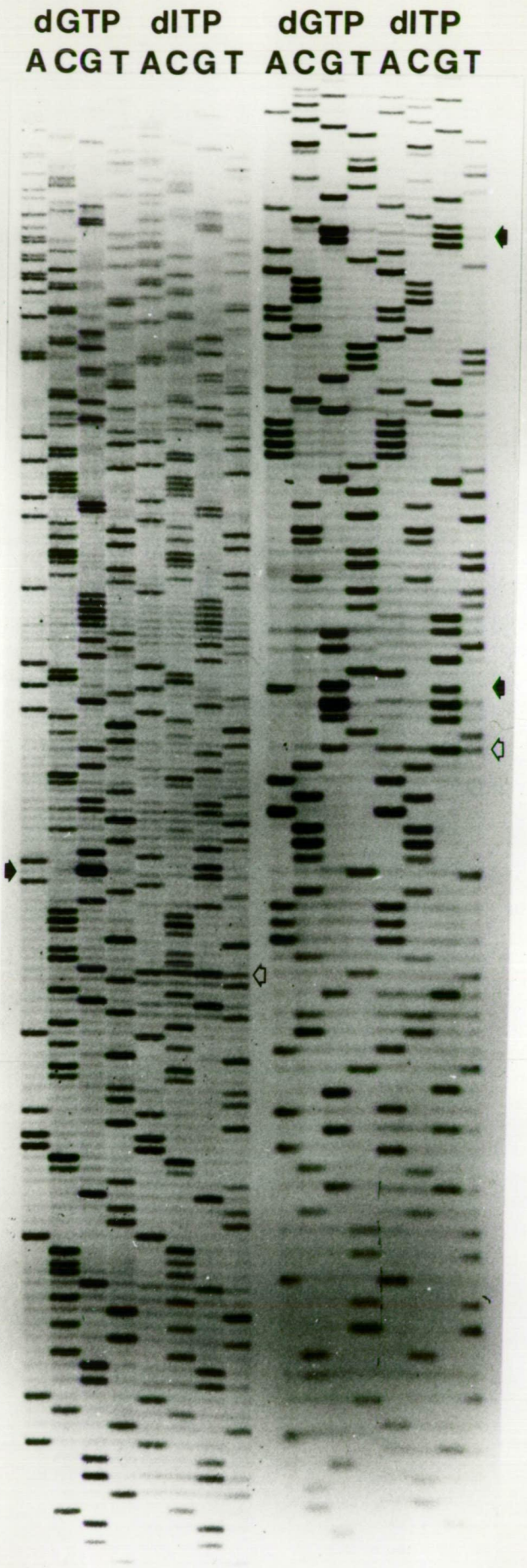
dGTP dITP dGTP dITP
A C G T A C G T A C G T A C G T

Fig. 5.2 Alignment of the 16S rRNA sequences of ACAM 44 and ACAM 48, with 16S rRNA sequences of some representatives of the "flavobacteria-*Bacteroides*" phylum and *E. coli* (RDP format). N= undetermined base; - = alignment gap.

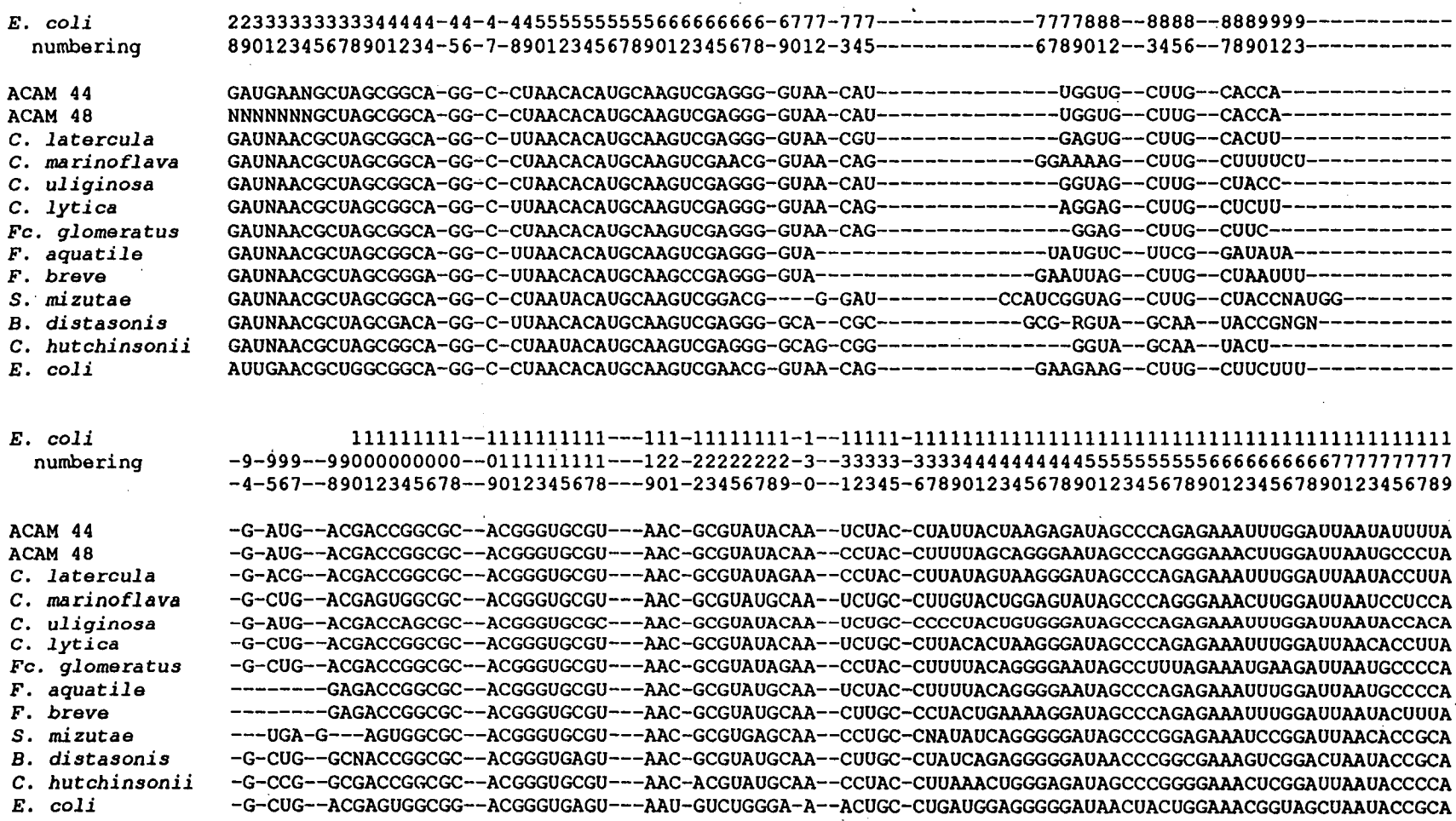

Fig. 5.2 (cont'd)

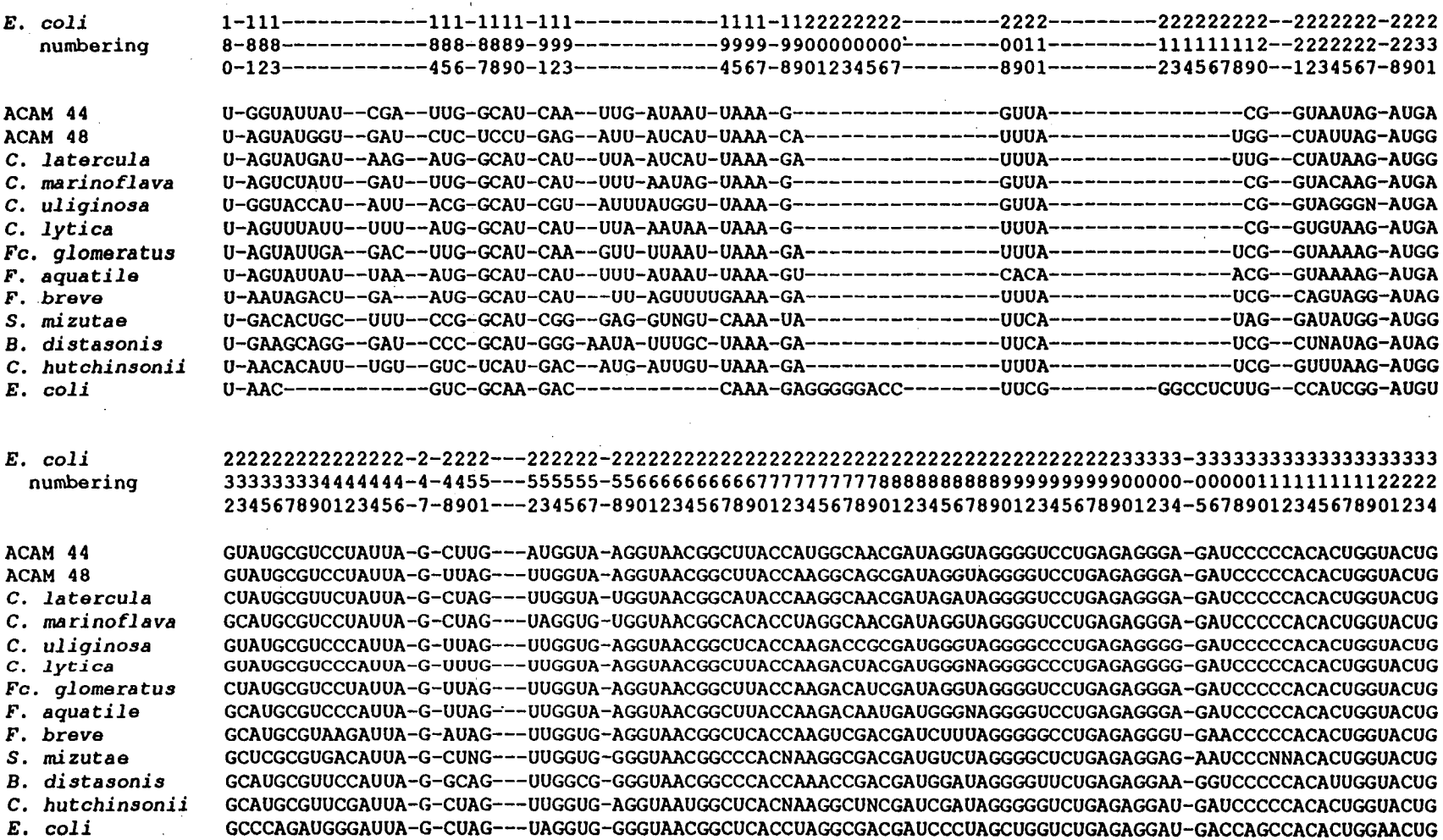
E. coli
numbering
1-111----------111-1111-111----------1111-1122222222--------2222---------222222222--2222222-2222
8-888----------888-8889-999----------9999-9900000000--------0011---------111111112--2222222-2233
0-123----------456-7890-123----------4567-8901234567--------8901---------234567890--1234567-8901
ACAM 44 U-GGUAUUAU--CGA--UUG-GCAU-CAA--UUG-AUAAU-UAAA-G----------GUUA----------CG--GUAAUAG-AUGA
ACAM 48 U-AGUAUGGU--GAU--CUC-UCCU-GAG--AUU-AUCAU-UAAA-CA----------UUUA----------UGG--CUAUUAG-AUGG
C. latercula U-AGUAUGAU--AAG--AUG-GCAU-CAU--UUA-AUCAU-UAAA-GA----------UUUA----------UUG--CUAUAAG-AUGG
C. marinoflava U-AGUCUAUU--GAU--UUG-GCAU-CAU--UUU-AAUAG-UAAA-G----------GUUA----------CG--GUACAAG-AUGA
C. uliginosa U-GGUACCAU--AUU--ACG-GCAU-CGU--AUUUAUGGU-UAAA-G----------GUUA----------CG--GUAGGGN-AUGA
C. lytica U-AGUUUAUU--UUU--AUG-GCAU-CAU--UUA-AAUAA-UAAA-G----------UUUA----------CG--GUGUAAG-AUGA
Fc. glomeratus U-AGUAUUGA--GAC--UUG-GCAU-CAA--GUU-UUAAU-UAAA-GA----------UUUA----------UCG--GUAAAAG-AUGG
F. aquatile U-AGUAUUAU--UAA--AUG-GCAU-CAU--UUU-AUAAU-UAAA-GU----------CACA----------ACG--GUAAAAG-AUGA
F. breve U-AAUAGACU--GA---AUG-GCAU-CAU---UU-AGUUUUGAAA-GA----------UUUA----------UCG--CAGUAGG-AUAG
S. mizutae U-GACACUGC--UUU--CCG-GCAU-CGG--GAG-GUNGU-CAAA-UA----------UUCA----------UAG--GAUAUGG-AUGG
B. distasonis U-GAAGCAGG--GAU--CCC-GCAU-GGG-AAUA-UUUGC-UAAA-GA----------UUCA----------UCG--CUNAUAG-AUAG
C. hutchinsonii U-AACACAUU--UGU--GUC-UCAU-GAC--AUG-AUUGU-UAAA-GA----------UUUA----------UCG--GUUUAAG-AUGG
E. coli U-AAC----------GUC-GCAA-GAC----------CAAA-GAGGGGGACC--------UUCG---------GGCCUCUUG--CCAUCGG-AUGU
E. coli
numbering
222222222222222-2-2222---222222-22222222222222222222222222222222222222233333-33333333333333333333
333333334444444-4-4455---555555-55666666666677777777778888888888999999999900000-00000111111111122222
234567890123456-7-8901---234567-89012345678901234567890123456789012345678901234-56789012345678901234
ACAM 44 GUAUGCGUCCUAUUA-G-CUUG---AUGGUA-AGGUAACGGCUUACCAUGGCAACGAUAGGUAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
ACAM 48 GUAUGCGUCCUAUUA-G-UUAG---UUGGUA-AGGUAACGGCUUACCAAGGCAGCGAUAGGUAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
C. latercula CUAUGCGUUCUAUUA-G-CUAG---UUGGUA-UGGUAACGGCAUACCAAGGCAACGAUAGAUAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
C. marinoflava GCAUGCGUCCUAUUA-G-CUAG---UAGGUG-UGGUAACGGCACACCUAGGCAACGAUAGGUAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
C. uliginosa GUAUGCGUCCCAUUA-G-UUAG---UUGGUG-AGGUAACGGCUCACCAAGACCGCGAUGGGUAGGGGCCCUGAGAGGGG-GAUCCCCCACACUGGUACUG
C. lytica GUAUGCGUCCCAUUA-G-UUUG---UUGGUA-AGGUAACGGCUUACCAAGACUACGAUGGGNAGGGGCCCUGAGAGGGG-GAUCCCCCACACUGGUACUG
Fc. glomeratus CUAUGCGUCCUAUUA-G-UUAG---UUGGUA-AGGUAACGGCUUACCAAGACAUCGAUAGGUAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
F. aquatile GCAUGCGUCCCAUUA-G-UUAG---UUGGUA-AGGUAACGGCUUACCAAGACAAUGAUGGGNAGGGGUCCUGAGAGGGA-GAUCCCCCACACUGGUACUG
F. breve GCAUGCGUAAGAUUA-G-AUAG---UUGGUG-AGGUAACGGCUCACCAAGUCGACGAUCUUUAGGGGGCCUGAGAGGGU-GAACCCCCACACUGGUACUG
S. mizutae GCUCGCGUGACAUUA-G-CUNG---UUGGUG-GGGUAACGGCCCACNAAGGCGACGAUGUCUAGGGGCUCUGAGAGGAG-AAUCCCNNACACUGGUACUG
B. distasonis GCAUGCGUUCCAUUA-G-GCAG---UUGGCG-GGGUAACGGCCCACCAAACCGACGAUGGAUAGGGGUUCUGAGAGGAA-GGUCCCCCACAUUGGUACUG
C. hutchinsonii GCAUGCGUUCGAUUA-G-CUAG---UUGGUG-AGGUAAUGGCUCACNAAGGCUNCGAUCGAUAGGGGGUCUGAGAGGAU-GAUCCCCCACACUGGUACUG
E. coli GCCCAGAUGGGAUUA-G-CUAG---UAGGUG-GGGUAACGGCUCACCUAGGCGACGAUCCCUAGCUGGUCUGAGAGGAU-GACCAGCCACACUGGAACUG

Fig. 5.2 (cont'd)

```
E. coli          333333333333333333-33333--3333333333-3-3333333333333333333-3333-3333-33333333333333344444-444-4444444
  numbering      222223333333333344-44444--4445555555-5-5566666666666777777-7777-8888-88888899999999900000-000-0011111
                 567890123456789012-23456--7890123456-7-8901234567890123456-6789-0123-45678901234567890123-567-8901234

ACAM 44          AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGA-GCAA-UCCUGAUCCAGCCAUGCCGNG-UGC-AGGAAGA
ACAM 48          AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGC-GAGA-GCCUGAUCCAGCCAUGCCGCG-UGC-AGGAAGA
C. latercula     AGACACGGACCAGNCUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGAG-GCAA-CUCUGAUCCAGCCAUGCCGCG-UGU-AGGAAGA
C. marinoflava   AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGC-GCAA-GCCNNAUCCAGCCAUGCCGCG-UGC-AGGAAGA
C. uliginosa     AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGC-GGGA-GCCUGAUCCAGCCAUGCCGCG-UGC-AGGAAGA
C. lytica        AGACACGGACCNGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGAG-GAGA-CUCUGAUCCAGCCAUGCCGCG-UGC-AGGAAGA
Fc. glomeratus   AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGGCAAU-GGAG-GAGA-CUCUGACCCAGCCAUGCCGCG-UGU-AGGAAGA
F. aquatile      AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGC-GCAA-GCCNGAUCCAGCCAUGCCGCG-UGC-AGGAAGA
F. breve         AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGACAAU-GGGU-GGAA-GCCUGAUCCAGCCAUCCCGCG-UGU-AGGAUGA
S. mizutae       AGACACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UAAGGAAUAUUGGUCAAU-GGGG-GCAA-CCCNGAACCAGCCAUGCCGCG-UGC-AGGACGA
B. distasonis    AGACACGGACCAAACUC-CUACG--GGAGGCAGCA-G-UGAGGAAUAUUGGUCAAU-GGCC-GAGA-GGCUGAACCAGCCAAGUCGCG-UGA-GGGAUGA
C. hutchinsonii  AGAUACGGACCAGACUC-CUACG--GGAGGCAGCA-G-UAGGGAAUAUUGGUCAAU-GGAG-GCAA-CUCUGAACCAGCCAUGCCGCG-UGU-AGGAAGA
E. coli          AGACACGGUCCAGACUC-CUACG--GGAGGCAGCA-G-UGGGGAAUAUUGCACAAU-GGGC-GCAA-GCCUGAUGCAGCCAUGCCGCG-UGU-AUGAAGA

E. coli          44444--4444-444444444-------------4444444444444444444--44-4444444444-4444444-444444-444-444444444444-
  numbering      11111--2222-222222333-------------3333333444444444445--55-5555555666-6666666-777777-777-788888888889-
                 56789--0123-456789012-------------3456789012345678901--12-3456789012-3456789-012345-678-901234567890-

ACAM 44          CUGCCC-UAU-GGGUUGUAAA-------------CUGCUUUUAUACGGGAAG--AAAAAGGUUCA-----CGUG----UGAA-CUG-UUGACGGUACCG-
ACAM 48          CUGCCC-UAU-GGGUUGUAAA-------------CUGCUUUUACAGAGGAAG--AACCACUCCCA-----CGUG----UGGG-AGU-UUGACGGUACUC-
C. latercula     CUGCCC-UAU-GGGUUGUAAA-------------CUACUUUUAUAGAGGAAG--AAACAGUUCCA-----CGUG----UGGA-ACU-CUGACGGUACUC-
C. marinoflava   CUGCCC-UAU-GGGUUGUAAA-------------CUNCUUUUAUACGGGAAG--AAUAAGGUCUA-----CGAG----UAGG-CUG-AUGACGGUACCG-
C. uliginosa     AUGCCC-UAU-GGGUAGUAAA-------------CUGCUUUUAUACGGGAAG--AAAAAGGGCUA-----CGUG----UAGC-CUA-CUGACGGUACCG-
C. lytica        CGGUCC-UAU-GGAUUGUAAA-------------CUGCUUUUAUACAGGAAG--AAUAAGGACUA-----CGUG----UAGU-CUG-GUGACGGUACUG-
Fc. glomeratus   AUGCCC-UAU-GGGUUGUAAA-------------CUACUUUUAUACAGGAAG--AAACACUGGUA-----UGUA----UACC-AGC-UUGACGGUACUG-
F. aquatile      CGGUCC-UAU-GGAUUGUAAA-------------CUGCUUUUAUACAGGAAG--AAACACUCCGA-----CGUG----UCGG-AGC-UUGACGGUACUG-
F. breve         CGGCCU-UAU-GGGUUGUAAA-------------CUACUUUUAUCUGGGGAU--AAACCUACUUA-----CGUG----UAAG-UAG-CUGAAGGUACCA-
S. mizutae       CUGCCC-UAU-GGGUUGUAAA-------------CUGCUUUUGUUAGGGAAU--AAACCCCGCUA-----CGUG----UAGC-GGG-CUGAAUGUACCU-
B. distasonis    AGGUUC-UAU-GGAUCGUAAA-------------CCUCUUUUAUAAGGGAAU--AAAGUGCGGGA-----CGUG----UCCN-GUU-UUGUAUGUACCU-
C. hutchinsonii  AGGCGU-UCU-GCGUUGUAAA-------------CUACUUUUGAUUGGGAAC--AAAU-GACUCU-----UGCG----AGAG-UAG-CUGAGUGUACCA-
E. coli          AGGCC--UUCG-GGUUGUAAA-------------GUACUUUCAGCGGGGAGG--AA-GGGAGUAAAG-UUAAUAC-CUUUGC-UCA-UUGACGUUACCC-
```

Fig. 5.2 (cont'd)

```
E. coli          4-444-4444455555-5555555555555555555555555555555555-5555555555555555555555555555555555555555555555
  numbering      9-999-9999900000-0000011111111112222222222333333333334-4444444445555555555666666666677777777778888888
                 1-234-5678901234-5678901234567890123456789012345678901-2345678901234567890123456789012345678901234567

ACAM 44          U-AAG-AAUAAGGACC-GGCUNNNNNNNNNNNNNNNNNNNNGGUAAUNNGGAG-GGUCCAAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGUAGGC
ACAM 48          U-GCG-AAUAAGGAUC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GAUCCAAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGUAGGC
C. latercula     U-ACG-AAUAAGGAUC-GGCUAACUCCGUGCCAGCAGCCGCGGUNAUACGGAG-NAUCCAAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGUAGGC
C. marinoflava   U-AAG-AAUAAGCACC-GGCUAACUCCGUGCCAGCAGCCGCGNNNAUACGGAG-NGUGCNAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGUAGGU
C. uliginosa     U-AAG-AAUAAGGACC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-NGUCCNAGCGUUAUCCGGAAUUAUUGGGUUUAAAGGGUCCGUAGGC
C. lytica.       U-AAG-AAUAAGGACC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-NGUCCNAGCGUUAUCCGGAAUUAUUGGGUUUAAAGGGUCCGUAGGC
Fc. glomeratus   U-AAG-AAUAAGGACC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GGUCCAAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGCAGGC
F. aquatile      U-AGG-AAUAAGGAUC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GAUCCAAGCGUUAUCCGGAAUCAUUGGGUUUAAAGGGUCCGUAGGC
F. breve         G-AAG-AAUAAGCACC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GGUGCNAGCGUUAUCCGGAUUUAUUGGGUUUAAAGGGUCCGUAGGC
S. mizutae       N-AAG-AAUAAGGAUC-GGCUAACUCCGUGCCAGCAGCCNCGGNNAUACGGAG-NAUCCNAGCGUUAUCCGGAUUUAUUGGGUUUAAAGGGUGCGUAGGC
B. distasonis    U-AUG-AAUAAGGAUC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GAUCCGAGCGUUAUCCGGAUUUAUUGGGUUUAAAGGGUGCGUAGGC
C. hutchinsonii  A-UAG-AAUAAGCCAC-GGCUAACUACGUGCCAGCAGCCNCGNNNAUACGUAG-GUGGCNAGCGUUGUCCGGAUUUAUUGGGUUUAAAGGGUGCGUAGGC
E. coli          G-CAG-AAGAAGCACC-GGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGAG-GGUGCAAGCGUUAAUCGGAATTACTGGGCGUAAAGCGCACGCAGGC

E. coli          55555-5555555566-6666666666666666666666666666666-6666666666666666-6666666666666666666-666666666666
  numbering      88899-9999999900-0000000011111111112222222222333-3333333344444444-4555555555566666666667-777777777788
                 78901-2345678901-2345678901234567890123456789012-3456789012345678-9012345678901234567890-123456789012

ACAM 44          GGGAC-AAUCAGUCAG-UGGUGAAAGUUUGCGGCUCAACCGUAAAAU-UGCCAUUGAUACUGUUG-UUCUUGAGUGCUUGUGAAGUG-GUUAGAAUGAGU
ACAM 48          GGGCA-GUUAAGUCAG-UGGUGAAAGUCUUCCGCUCAACGGGAGAAC-UGCCAUUGAUACUGAUU-GCCUUGAGUUAUUAUGAAGUG-GUUAGAAUGAGU
C. latercula     GGGUC-UGUAAGUCAG-UGGUGAAAGUUUUCGGCUCAACCGGAAAAU-UGCCAUUGAUACUGCAG-GUCUUGAAUCAUUAUGAAGUG-GUUAGAAUAAGU
C. marinoflava   GGACU-AAUAAGUCAG-UGGUGAAAGUCUGCAGCUUAACUGUAGAAU-UGCCAUUGAUACUGUUA-GUCUUGAAUUGUUAUGAAGUA-ACUAGAAUAUGU
C. uliginosa     GGGCC-GAUAAGUCAG-GGGUGAAAGUUUGCAGCUCAACUGUAAAAU-UGCCUUUGAUACUGUCG-GUCUUGAGUUAUAGUGAAGUU-GCCGGAAUAUGU
C. lytica        GGGCU-GUUAAGUCAG-GGGUGAAAGUUUGCAGCUCAACUGUAGAAU-UGCCUUUGAUACUGAUG-GUCUUGAAUUAUUGUGAAGUG-GUUAGAAUAUGU
Fc. glomeratus   GGUCA-AUUAAGUCAG-AGGUGAAAUCCCGUCGCUCAACGACGGAAC-UGCCUUUGAUACUGGUU-GACUUGAGUCAUAUGGAAGUA-GAUAGAAUGUGU
F. aquatile      GGUUU-AAUAAGUCAG-UGGUGAAAUCUGGUCGCUCAACGAUCAAAC-GGCCAUUGAUACUGUUA-GACUUGAAUUAUAAGGAAGUA-ACUAGAAUAUGU
F. breve         GGACU-GAUCNGUCAG-UGGUGAAAUCCUAUCGCUUAACGAUAGAAC-UGCCAUUGAUACUGUUA-GUCUUGAAUUAGUUUGAAGUG-GCUGGAAUGUGU
S. mizutae       GGCNC-UUUAAGUCAG-GGGUGAAAGACGGCAGCUCAACUGUCGCAG-UGCCCUUGAUACUGAAG-UGCUUGAAUGCGGCUGAAGAC-GGCGGAAUGAGA
B. distasonis    GGCCU-UUUAAGUCAG-CGGUGAAAGUCUGUGGCUCAACCAUAGAAU-UGCCGUUGAAACUGGGG-NGCUUGAGUAUGUUUGAGGCA-GGCGGAAUGCGU
C. hutchinsonii  GGCUU-AAUAAGUCAG-UGGUGAAAGCCGAUCGCUUAACGAUCGAAC-UGCCAUUGAUACUGCUA-GGCUUGAGUAUAGAUGAGGUA-GGCGGAAUUGAC
E. coli          GGUUU-GUUAAGUCAG-AUGUGAAAUCCCCGGGCUCAACCUGGGAAC-UGCAUCUGAUACUGGCA-AGCUUGAGUCUCGUAGAGGGG-GGUAGAATTCCA
```

Fig. 5.2 (cont'd)


E. coli
numbering
ACAM 44
ACAM 48
C. latercula
C. marinoflava
C. uliginosa
C. lytica
Fc. glomeratus
F. aquatile
F. breve
S. mizutae
B. distasonis
C. hutchinsonii
E. coli

Fig. 5.2 (cont'd)

```
E. coli          888888888888888888888888888888888-889999999999999-999-9999999-9999999999--99-9--999999-999999999999
  numbering      666666777777777788888888899999999-990000000000111-111-1111222-2222222333--33-3--333344-444444445555
                 456789012345678901234567890123456-789012345678901-345-6789012-3456789012--34-5--678901-234567890123

ACAM 44          AAAGUGAUAAGUAACCCACCUGGGGAGUACNUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACNGGNNCCC--GC-A--CAAGCN-GUNGAGCAUGUG
ACAM 48          AAAGUGAUAAGUAACCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGGGCCC--GC-A--CAAGCG-GUGGAGCAUGUG
C. latercula     AAAGUGAUAAGUAACCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGGGCCN--GC-A--CAAGCG-GUGGAGCAUGUG
C. marinoflava   AAAGUGAUAAGUAACCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGGGCCN--GC-A--CAAGCG-GUGGAGCAUGUG
C. uliginosa     AAAGUGAUAAGUAUCCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGNGCCN--GC-A--CAAGCG-GUGGAGCAUGUG
C. lytica        AAAGUGAUAAGUAUCCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGGGCCN--GC-A--CAAGCG-GUGGAGCAUGUG
Fc. glomeratus   AAAGUGAUAAGUAUCCCACCUGGGGAGUACGGUC-GCAAGACUGAAACUC-AAA-GGAAUUG-ACGGGGGCCC--GC-A--CAAGCG-GUGGAGCAUGUG
F. aquatile      AAAGUGAUAAGUAUCCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGNNGNCC--GC-A--CAAGCG-GUGGAGCAUGUG
F. breve         AAAGUUAUAAGUAUCCCACCUGGGGAGUACGUUC-GCAAGAAUGAAACUC-AAA-GGAAUUG-ACGGGGGCCN--GC-A--CAAGCG-GUGGAGCAUGUG
S. mizutae       AAAGCGUUAAGUCAUCCACCUGGGGAGUACGCCC-GCAAGGGUGAAACUC-AAA-GGAAUUG-ACNGGNGCCN--GC-A--CAAGCG-GAGGAGCAUGUG
B. distasonis    AAAGCGUUAAGUGAUCCACCUGGGGAGUACGCCG-GCAACGGUGAAACUC-AAA-GGAAUUG-ACGGGNGCCN--GC-A--CAAGCG-GAGGAACAUGUG
C. hutchinsonii  AAAGCGAUAAGUGAUCCACCUGGGGAGUACGCCC-GCAAGGGUGAAACUC-AAA-GGAAUUG-ACGGGNGUCN--GC-A--CAAGCG-GUGGAGCAUGUG
E. coli          AACGCGUUAAGUCGACCGCCUGGGGAGUACGGCC-GCAAGGUUAAAACUC-AAA-UGAAUUG-ACGGGGGCCC--GC-A--CAAGCG-GUGGAGCAUGUG

E. coli                                                                                1111---11111-1111-1111111-1-1111-11----11--1111--1
  numbering      99-999999999999999999999999999-999999999999999-9-0000---00000-0000-0000000-0-0000-00----00--0000--0
                 55-555566666666667777777778888-888888999999999-9-0000---00000-0111-1111111-2-2222-22----22--2333--3
                 45-678901234567890123456789012-3-456789012345678-9-0123---45678-9012-3456789-0-1234-56----78--9012--3

ACAM 44          GU-UUAAUUCGAUGAUACNCNAGGAACCUUA-CCANGGCUUAAAUNCAG-UUUG---ACNNN-NNUN-GAAACNN-C-UUUU-------UC--UUCG--G
ACAM 48          GU-UUAAUUCGAUNAUNCGCGAGGAACCUUA-CCAGGGCUUAAAUGUAG-UCUG---ACNGG-AGUG-GAAACAC-U-UUUU-------UC--UUCG--G
C. latercula     GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCAGGGCUUAAAUGUAG-UAUG---ACAGG-UUUA-GAGAUAG-A-CUUU-------UC--UUCG--G
C. marinoflava   GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCAGGGCUUAAAUGUAG-UCUG---ACAGG-AGUG-GAAACAU-U-CUUU-------UC--UUCG--G
C. uliginosa     GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCAGGGCUUAAAUGCAU-AUUG---ACAGG-GGUA-GAGAUAC-C-UUUU-------CC--UUCG--G
C. lytica        GU-UUAAUUCGAUGNUACGCGAGGAACCUUA-CCAGGGCUUAAAUGUAG-AUUG---ACAGG-UUUA-GAGAUAG-A-CUUU-------CC--UUCG--G
Fc. glomeratus   GU-UUAAUUCGAUNNUACGCGAGGAACCUUA-CCAGGGCUUAAAUGUAG-UCUG---ACAGC-UUUA-GAGAUAG-A-GUUU-------UC--UUCG--G
F. aquatile      GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCAAGGCUUAAAUGUAG-AUUG---ACAGG-ACUG-GAAACAG-U-UUUU-------UC--UUCG--G
F. breve         GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCAAGGCUUAAAUGCAU-AAUG---ACGUA-UUUG-GAAACAG-A-UAUU-------UC--UUCG--G
S. mizutae       GU-UUAAUUCGAUNNUACGCGAGNAACCUUA-CCCGGGCUUGAAAGU-U-ACUG---AAGGG-CGCA-GAGACGC-G-CCNN--U----CC--UUCG--G
B. distasonis    GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCCGGGUUUGAACGCAU-UCGG---ACCGA-GGUG-GAAACAC-C-UUUU-C-----UA--GCAA--U
C. hutchinsonii  GU-UUAAUUCGAUGAUACGCGAGGAACCUUA-CCUAGGCUAGAAUGU-G-AAGG---AAUGU-AUCA-GAAAUGG-U-GCAG--U----CA--GCAA--U
E. coli          GU-UUAAUUCGAUGCAACGCGAAGAACCUUA-CCUGGUCUUGACAUC-C-ACGG---AAGUU-UUCA-GAGAUGA-G-AAUG-UG----CC--UUCG--G
```

Fig. 5.2 (cont'd)

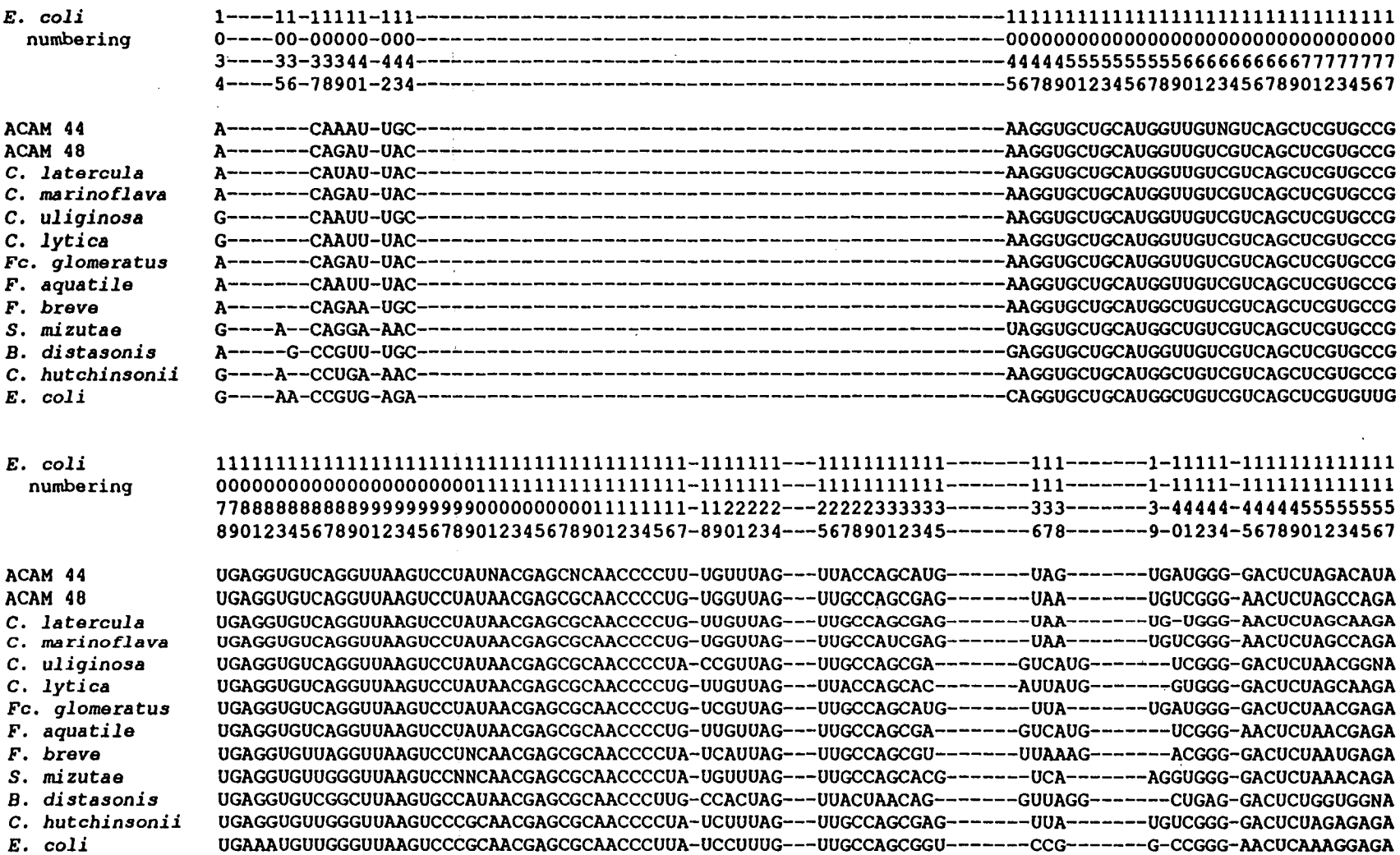
E. coli
numbering
ACAM 44
ACAM 48
C. latercula
C. marinoflava
C. uliginosa
C. lytica
Fc. glomeratus
F. aquatile
F. breve
S. mizutae
B. distasonis
C. hutchinsonii
E. coli

Fig. 5.2 (cont'd)

```
E. coli          1111111111-111-1111--1111-1-11111111-11111111--11111111111-111111111-11-11111111111111111111111111111
  numbering      1111111111-111-1111--1111-1-11111111-11111111--11112222222-222222222-22-22222222222222222222222222222
                 5566666666-667-7777--7777-7-88888888-88999999--99990000000-000111111-11-11222222222233333333333444444
                 8901234567-890-1234--5678-9-01234567-89012345--67890123456-789012345-67-89012345678901234567890123 45

ACAM 44          CUGCCAGUG--UAA-ACUGU-GAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCUACACACGUGCUACAAUGGUAG
ACAM 48          CUGCCGGUG--CAA-ACCGU-GAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCCACACACGUGCUACAAUGGUAG
C. latercula     CUGCCGGUG--CAA-ACCG-CGAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCUACACACGUGCUACAAUGGUAG
C. marinoflava   CUGCCAGUG--CAA-ACUG-CGAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCCACACACGUGCUACAAUGGUAG
C. uliginosa     CUGCCGGUG--CAA-ACCGU-GAGG-A-AGGUGGGG-ACGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCCACACACGUGCUACAAUGGCCG
C. lytica        CUGCCGGUG--CAA-ACCGU-GAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-UC-CUGGGCNACACACGUGCUACAAUGGUAG
Fc. glomeratus   CUGCCGGUG--CAA-ACCG-CGAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCNNUACG-UC-CUGGGCNACACACGUGCUACAAUGGUAU
F. aquatile      CUGCCAGUG--CAA-ACUGU-GAGG-A-AGGUGGGG-AUGACGUC--AAAUCAUCACG-GCCCUUACG-CC-UUGGGCUACACACGUGCUACAAUGGACG
F. breve         CUGCCAAUG--AAA-GUUGA-GAGG-A-AGGUGGGG-ACGACGUC--AAGUCAUCACG-GCCCUUACG-UC-UUGGGCUACACACGUGCUACAAUGGUAA
S. mizutae       CUGCCUGCG--CAA-GCAG-AGAGG-A-AGGCGGGG-ACGACGUN--NAGUCAUCAUG-GCCCNUACG-UC-CGGGGCUACACACGUGCUACAAUGGAUG
B. distasonis    CUGCCAGCG--UAA-GCUGC-GAGG-A-AGGCGGGG-AUGACGUC--AAAUCAGCACG-GCCCUUACA-UC-CGGGGCGACACACGUGUUACAAUGGCGU
C. hutchinsonii  CUGCCUGUG--UAA-ACAG-AGAGG-A-AGGAGGGG-ACGACGUN--AAGUCAUCAUG-GCCCUUACG-CC-UAGGGCUACACACGUGCUACAAUGGCGC
E. coli          CUGCCAGUGA-UAA-ACUG--GAGG-A-AGGUGGGG-AUGACGUC--AAGUCAUCAUG-GCCCUUACG-AC-CAGGGCUACACACGUGCUACAAUGGCGC

E. coli          111-1-1111111-111-1111111-1111111-----------111111111111-11111111111111111111111111111111111111111111
  numbering      222-2-2222222-222-2222222-2222222-----------222222222222-22222222222222333333333333333333333333333333
                 444-4-5555555-555-6666666-6667777-----------777777888888-88889999999999000000000011111111112222222222
                 678-9-0123456-789-0123456-7890123-----------456789012345-67890123456789012345678901234567890123456 78

ACAM 44          GGA-C-AGAGAGC-AGC-CACUGGG-UGACCAG-----------GAGCGAAUCUAC--AAACCCUAUCACAGUUCGGAUCGCAGUCUGCAACUCGACUGC
ACAM 48          GGA-C-AGAGAGC-AGC-CACUUCG-CGAGAAG-----------GAGCGAAUCUAC--AAACCCUAUCACAGUUCGGAUCGCAGUCUGCAACUCGACUGC
C. latercula     GUA-C-AGAGAGC-AGC-CACUUUG-CGAGAAG-----------GAGCGAAUCUAU--AAAACCUAUCACAGUUCGGAUCGGAGUCUGCAACUCGACUCC
C. marinoflava   GUA-C-AGAGAGC-AGC-CACUUUG-CAAAGAG-----------GAGCGAAUCUAU--AAAACCUAUCACAGUUCGGAUCGGGGUCUGCAACUCGACCCC
C. uliginosa     GUA-C-AGCGGGA-AGC-CAUGCGG-CAACGCA-----------GAGCGGAUCCAC--AAAACCGGUCACAGUUCGGAUCGGGGUCUGCAACUCGACCCC
C. lytica        GUA-C-AGAGAGC-AGC-CACUUAG-CGAUAAG-----------GAGCGAAUCUAU--AAAACCUAUCACAGUUCGGAUCGGAGUCUGCAACUCGACUCC
Fc. glomeratus   GGA-C-AAUGAGC-AGC-CAUCUGG-CAACAGA-----------GAGCAAAUCUAU--AAACCAUAUCACAGUUCGGAUCGGAGUCUGCAACUCGACUCC
F. aquatile      GUA-C-AGAGAGC-AGC-CACUGCG-CAAGCAG-----------GAGCGAAUCUAC--AAAACCGUUCUCAGUUCGGAUCGGAGUCUGCAACUCGACUCC
F. breve         GUA-C-AGAGGGC-AGC-UACUGGG-UGACCAG-----------AUGCGAAUCUCG--AAAACUUAUCUCAGUUCGGAUUGGAGUCUGCAACUCGACUCU
S. mizutae       GUA-C-AGCGGGC-AGC-UACACAG-CAAUGUG-----------GUGCCAAUCUCG-AAAAGCCAUUCACAGUUCGGAUCGGGGUCUGCAACUCGACCCN
B. distasonis    GGA-C-AAAGGGA-GGC-CACCUGG-CGACAGG-----------GAGCGAAUCCCC--AAACCACGUCUCAGUUCGGAUCGGAGUCUGCAACCCGACUCC
C. hutchinsonii  AUA-C-AGAGUGU-UGC-AAGCUAG-UGAUAGC-----------AAGCCAAUCACA-AAAAGUGCGUCUCAGUUCGGAUUGAAGUCUGCAACUCGACUUC
E. coli          AUA-C-AAAGAGA-AGC-GACCUCG-CGAGAGC-----------AAGCGGACCUCA-UAAAGUGCGUCGUAGUCCGGAUUGGAGUCUGCAACUCGACUCC
```

Fig. 5.2 (cont'd)

```
E. coli          11111111111111111111111111111111-11111111111111111111111111111111111111111111111111111111111111111111111
  numbering      33333333333333333333333333333333-33333333333333333333333333333333333333334444444444444444444444444444
                 23333333333444444444455555555566-66666666777777777788888888889999999990000000000111111111122222222
                 90123456789012345678901234567890l-23456789012345678901234567890123456789012345678901234567890123456789

ACAM 44          GUGAAGCUGGAAUCGCUAGUAAUCGCAUAUCAGCCAUNNNGCNGUGAAUACGUUCCCGGNCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCUGGGGGU
ACAM 48          GUGAAGCUGGAAUCGCUAGUAAUCGCAUAUCAGCCAUGAUGCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCUGGGGGU
C. latercula     GUGAAGCUGGAAUCGCUAGUAAUCGGAUAUCAGCCAUGAUCCGGUGAAUACGUUCCCNGGCNUUGUACACACCGCNCGUCAAGCCAUGGAAGCNNGGGGU
C. marinoflava   GUGAAGCUGGAAUCGCUAGUAAUCGCAUAUCAGCCAUGAUGCGGUGAAUACGUUCCCGGGCNUUGUACACACCGCCCGUCAAGNCAUGGAAGCNNGGGGU
C. uliginosa     GUGAAGCUGGAAUCGCUAGUAAUCGGAUAUCAGCCAUGAUCCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCCGGGAGU
C. lytica        GUGAAGCUGGAAUCGCUAGUAAUCGGAUAUCAGCCAUGAUCCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCUGGGGGU
Fc. glomeratus   GUGAAGCUGGAAUCGCUAGUAAUCGGAUAUCAGCCAUGAUCCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCUGGGGGU
F. aquatile      GUGAAGCUGGAAUCGCUAGUAAUCGGAUAUCAGCCAUGAUCCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGAAGCUNGGGGU
F. breve         AUGAAGCUGGAAUCGCUAGUAAUCGCAUAUCAGCCAUGAUGCGGUGAAUACGUUCCCGGGCNUUGUACACACCGCCCGUCAAGCCAUGGAAGCUGGGGGU
S. mizutae       GUGAAGUUGGAUUCGCUAGUAAUCGCGUAUCAGCAAUGACGCGGUGAAUACGUUCCCGGGCNUUGUACACACCGCNCGUCAAGNCAUGAAAGCUGGGGGU
B. distasonis    GUGAAGCUGGAUUCGCUAGUAAUCGCGCAUCAGCCAUGGCGCGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCAAGCCAUGGGAGCCGGGGGU
C. hutchinsonii  AUGAAGCUGGAAUCGCUAGUAAUCGCGUAUCAGCAAUGACGCGGUGAAUACGUUCCCNGACCUUGUACACACCGCNCGUCAAGCCAUGAAAGUCUGGUGG
E. coli          AUGAAGUCGGAAUCGCUAGUAAUCGUGGAUCAG-AAUGCCACGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCCGUCACACCAUGGGAGUGGGUUGC

E. coli          11111111-1111-111-11111-11----1111----111111111111-11111111111111111111111111111111111111111111111111
  numbering      44444444-4444-444-44444-44----4444----444444444444-44444444444444444444444444444444444455555555555555
                 22333333-3333-444-44444-44----5555----555555666666-66667777777777888888888899999999990000000000011111
                 89012345-6789-012-34567-89----0123----456789012345-67890123456789012345678901234567890123456789012345

ACAM 44          ACCUGAAG-UCGN-------UNN-CC----GCAA----GGAG----CCGC-CUAGGGUAAAACUAGUAACUGGGGCUAAGUCGUAACAAGGUAGCCGUAC
ACAM 48          ACCUGAAG-UCGG-------UCA-CC----GCAA----GGAG----CCGC-CUAGGGUAAAACUGGUAACUGGGGCUAAGUCGUAACAAGGUAGCCGUAC
C. latercula     ACCUGAAG-UCCG-------UCA-CC----GUAA----GGAG----CGGC-CUAGGGUAAAACUGGUAACUGGGGC
C. marinoflava   ACCUGAAG-UCCG-------UCA-CC----GUAA----GGAG----CGGC-CUAGGGUAAGACCGGUAACUGGGGC
C. uliginosa     GCCUGAAG-UCCG-------UCA-CC----GCAA----GGAG----CGGC-CUAGGGCAAGAUCGGUAACUAGGGC
C. lytica        ACCUGAAG-UUCG-------UCA-CC----GCAA----GGAG----CGAC-CUAGGGUAAAACUGGUAACUAGGGC
Fc. glomeratus   GCCUGAAG-UCGG-------UCA-CC----GCAA----GGAG----CCGC-CUAGGGUAAAACUGGUAACUAGGGC
F. aquatile      ACCUGAAG-UCGG-------UGA-CC----GCAA----GGAG----CUGC-CUAGGGUNNAACUGGUAACUNGGGC
F. breve         ACCUGAAG-UCGG-------UGA-CCG---UAAA---AGGAG----CUGC-CUAGGGUAAAACUGGUAACNAGGGC
S. mizutae       ACCUAAAG-CAUG-------UAA-CC----GCAA----GGAG----CGUG-UUAGGGUAAAACCGGUAAUUGGGGC
B. distasonis    ACCUGAAG-UCCG-------UAA-CC----GAAA----GGAU----CGGC-CUAGGGUAAAACUGGUGACUGGGGC
C. hutchinsonii  ACCUGAAG-GCCG-------UAA-CC----GCAA----GGAG----CGGC-UCAGGGUAAAACAGGUAAUUAGGGC
E. coli          AAAAGAAG-UAGG-UAG-CUUAA-CC----UUCG----GGAGGGCGCUUA-CCACUUUGUGAUUCAUGACUGGGGUGAAGUCGUAACAAGGUAACCGUAG
```

Fig. 5.2 (cont'd)

E. coli	1111111
numbering	5555555
	1111122
	5678901
ACAM 44	CGGAANG
ACAM 48	CGGAAGG
C. latercula	
C. marinoflava	
C. uliginosa	
C. lytica	
Fc. glomeratus	
F. aquatile	
F. breve	
S. mizutae	
B. distasonis	
C. hutchinsonii	
E. coli	GGGAACC

is a signature feature of the members of the genus *Bacteroides* and some groups of flavobacteria (Woese et al, 1985).

Evolutionary distances calculated by the Jukes-Cantor equation, and percentage sequence similarities between the Organic Lake flavobacteria and some members of the "flavobacteria-*Bacteroides*" phylum are shown in Table 5.1. The evolutionary distance between ACAM 44 and its nearest relative ACAM 48 was 0.091 (91.8% sequence similarity). The evolutionary distance between ACAM 48 and it nearest relative *C. latercula* was 0.095 (92.6% sequence similarity). A phylogenetic tree derived by distance analysis, which includes the Organic Lake flavobacteria, 47 representative species from the "flavobacteria-*Bacteroides*" phylum, and *E. coli* as the outgroup organism, is shown in Fig. 5.3. The Organic Lake flavobacteria clustered in a branch of this phylum which contained some marine *Cytophaga* spp., a subgroup of three marine species that included two species isolated from Antarctic environments, *Flectobacillus glomeratus* and "*Antarcticum vesiculatum*", and a subgroup of terrestrial species that included *F. aquatile*, the type species of the genus *Flavobacterium*. *F. odoratum*, a species isolated from the human urinary tract was a peripheral member of this branch.

The most parsimonious tree derived from a branch and bound analysis of a smaller number (12) of species is compared with the distance analysis tree for the same group of species in Fig. 5.4. The branching order in the trees derived by both approaches to phylogenetic analysis were similar with the exception of the positions of *C. marinoflava* and *C. lytica*.

Fig. 5.5 shows the result of parsimony analysis of the 12 species but derived from a subset of the sequence data which included only those positions identified as conserved. Three trees of equal and most parsimonious length resulted from this analysis. One of these trees (Fig. 5.5 c) had an identical branching order to the tree shown in Fig. 5.4 a, which indicated that restricting the analysis to conserved positions did not significantly affect the result. In all three trees ACAM 44 clustered with ACAM 48 and *C. latercula*. The branching order within the branch which contained the Organic Lake flavobacteria was more significantly affected by the elimination of

Table 5.1 16S rRNA evolutionary distances as calculated by the Jukes-Cantor equation (below the diagonal), and percentage similarities (above the diagonal), between the Organic Lake flavobacteria and some members of the "flavobacteria-*Bacteroides*" phylum.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	ACAM 44	-	91.8	91.5	90.6	90.0	88.2	88.2	88.8	88.5	89.0	88.3	88.3	88.5	88.4	88.2	86.9	86.3	83.2	82.4	80.3	80.2	80.8	81.0	78.9	80.3
2	ACAM 48	0.091	-	92.6	89.8	90.4	87.2	89.8	89.6	87.8	89.9	88.0	88.2	88.5	88.1	87.5	85.9	85.2	83.1	81.5	79.7	79.5	80.2	80.3	78.9	80.3
3	*C. latercula*	0.095	0.080	-	92.3	90.3	87.5	89.1	90.0	88.9	89.6	88.9	88.5	89.0	88.6	88.5	89.0	86.0	84.5	83.2	80.8	80.1	81.0	80.2	79.4	79.6
4	*C. lytica*	0.105	0.112	0.082	-	90.5	90.4	89.0	88.9	89.4	89.6	89.4	89.1	89.4	88.8	88.9	89.5	86.0	83.4	82.5	81.9	80.4	81.7	81.6	79.0	79.6
5	*C. marinoflava*	0.113	0.105	0.106	0.103	-	89.0	87.6	87.3	87.5	89.5	88.2	88.7	88.5	87.9	88.0	87.5	86.1	83.0	82.7	80.3	79.9	80.6	80.6	78.8	78.7
6	*C. uliginosa*	0.135	0.143	0.138	0.104	0.120	-	87.1	86.7	87.6	87.8	86.8	87.1	87.3	87.1	86.4	86.8	84.5	82.0	82.1	80.9	80.5	80.5	82.4	77.9	79.7
7	*Fc. glomeratus*	0.134	0.111	0.120	0.120	0.138	0.143	-	97.5	93.4	89.3	88.2	88.3	88.2	87.9	88.4	88.3	84.6	83.7	82.1	80.6	80.4	81.3	79.7	78.2	79.6
8	*"A. vesiculatum"*	0.127	0.114	0.109	0.121	0.141	0.148	0.026	-	93.1	89.5	88.7	88.9	88.5	87.8	88.5	88.1	85.4	84.0	82.6	80.5	80.3	81.7	80.0	78.1	79.9
9	*Fx. aggregans*	0.133	0.137	0.124	0.117	0.141	0.139	0.071	0.074	-	88.5	87.9	87.7	87.6	87.6	87.7	89.0	84.7	83.3	83.2	81.5	80.3	81.5	80.5	79.1	79.3
10	*F. aquatile*	0.125	0.111	0.113	0.114	0.115	0.135	0.117	0.115	0.129	-	95.3	95.2	95.5	94.5	94.1	90.0	86.4	85.0	84.0	80.3	79.3	80.2	79.7	79.3	78.8
11	*C. aquatilis*	0.135	0.134	0.122	0.117	0.131	0.147	0.131	0.124	0.136	0.050	-	97.6	98.3	96.9	96.0	89.7	86.4	83.9	83.5	80.5	79.7	81.6	79.9	79.1	79.2
12	*C. johnsonae*[a]	0.134	0.131	0.127	0.119	0.125	0.144	0.130	0.123	0.139	0.050	0.025	-	98.3	97.2	96.0	89.7	86.7	84.1	82.3	80.1	79.3	81.2	80.0	78.8	79.4
13	*C. columnaris*	0.131	0.127	0.121	0.116	0.126	0.141	0.131	0.127	0.139	0.047	0.017	0.017	-	97.2	96.0	89.7	86.3	83.4	83.2	80.0	79.2	81.2	79.8	79.6	79.2
14	*C. flevensis*	0.134	0.133	0.127	0.124	0.134	0.144	0.135	0.135	0.140	0.058	0.032	0.029	0.029	-	96.5	89.7	86.1	84.5	84.2	81.0	80.0	81.7	80.6	79.9	79.8
15	*Fx. aurantiacus*	0.135	0.140	0.127	0.122	0.133	0.153	0.129	0.127	0.138	0.062	0.042	0.042	0.042	0.037	-	89.2	86.3	83.8	84.2	80.8	80.8	81.7	80.0	80.2	80.0
16	*F. odoratum*	0.154	0.161	0.122	0.116	0.140	0.148	0.130	0.132	0.123	0.110	0.113	0.113	0.113	0.114	0.119	-	86.3	84.2	84.5	82.2	80.8	82.3	81.0	80.7	79.6
17	*F. breve*	0.160	0.168	0.157	0.157	0.156	0.176	0.175	0.165	0.176	0.152	0.153	0.149	0.154	0.157	0.155	0.155	-	86.9	86.9	81.9	81.4	81.5	80.8	80.5	78.8
18	*F. indoltheticum*	0.204	0.199	0.180	0.194	0.200	0.212	0.190	0.186	0.197	0.173	0.188	0.186	0.194	0.181	0.189	0.185	0.149	-	95.4	79.6	80.9	82.2	80.8	80.2	78.4
19	*F. gleum*	0.215	0.221	0.197	0.206	0.204	0.211	0.211	0.204	0.199	0.187	0.193	0.209	0.196	0.184	0.184	0.180	0.149	0.050	-	79.5	81.9	82.0	81.4	80.4	77.8
20	*C. heparina*	0.240	0.240	0.224	0.208	0.231	0.223	0.225	0.227	0.218	0.230	0.229	0.235	0.235	0.224	0.225	0.207	0.209	0.245	0.247	-	89.1	88.9	89.4	84.3	80.4
21	*S. spiritivorum*	0.244	0.245	0.236	0.231	0.238	0.229	0.231	0.232	0.236	0.248	0.242	0.248	0.247	0.238	0.226	0.226	0.218	0.228	0.214	0.120	-	88.1	92.5	82.3	78.8
22	*Fx. canadensis*	0.236	0.236	0.224	0.214	0.230	0.231	0.219	0.214	0.219	0.236	0.216	0.222	0.221	0.215	0.215	0.207	0.217	0.212	0.214	0.122	0.132	-	86.9	84.0	79.3
23	*S. mizutae*	0.236	0.238	0.237	0.218	0.233	0.206	0.244	0.241	0.236	0.245	0.243	0.241	0.244	0.233	0.241	0.228	0.229	0.233	0.223	0.118	0.082	0.149	-	81.1	80.8
24	*C. hutchinsonii*	0.262	0.253	0.245	0.251	0.254	0.265	0.261	0.263	0.253	0.247	0.250	0.254	0.241	0.239	0.234	0.228	0.229	0.239	0.234	0.178	0.205	0.184	0.225	-	80.0
25	*B. distasonis*	0.241	0.233	0.242	0.241	0.255	0.239	0.240	0.237	0.250	0.253	0.248	0.245	0.247	0.240	0.238	0.244	0.253	0.264	0.272	0.229	0.254	0.247	0.230	0.236	-

a *C. johnsonae* DSM 425

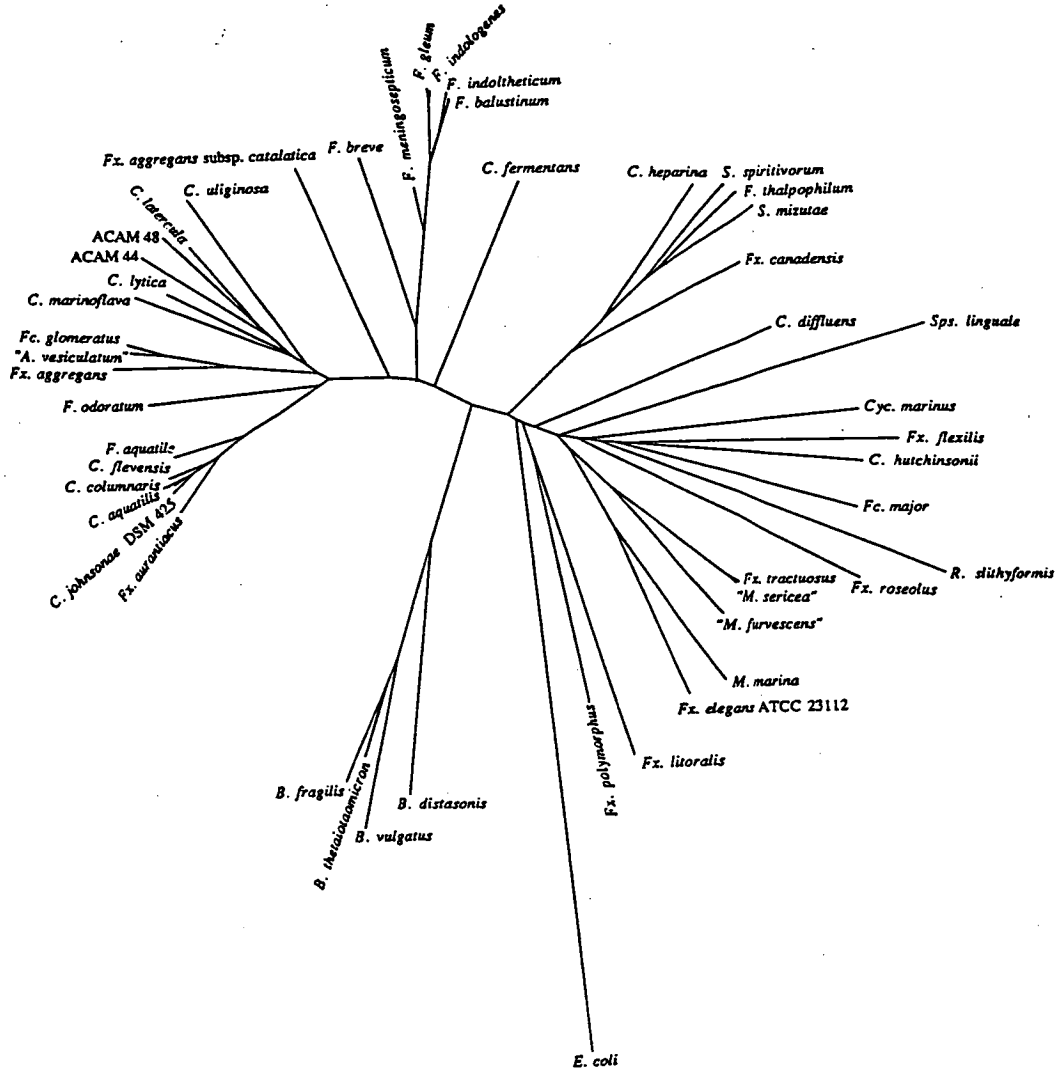

Fig. 5.3 Phylogenetic tree derived by distance analysis of 16S rRNA sequences, showing the relationship of ACAM 44 and ACAM 48 to members of the "flavobacteria-*Bacteroides*" phylum, with *E. coli* as the outgroup organism. Bar = 0.05 evolutionary distance units as calculated by the Jukes-Cantor equation.

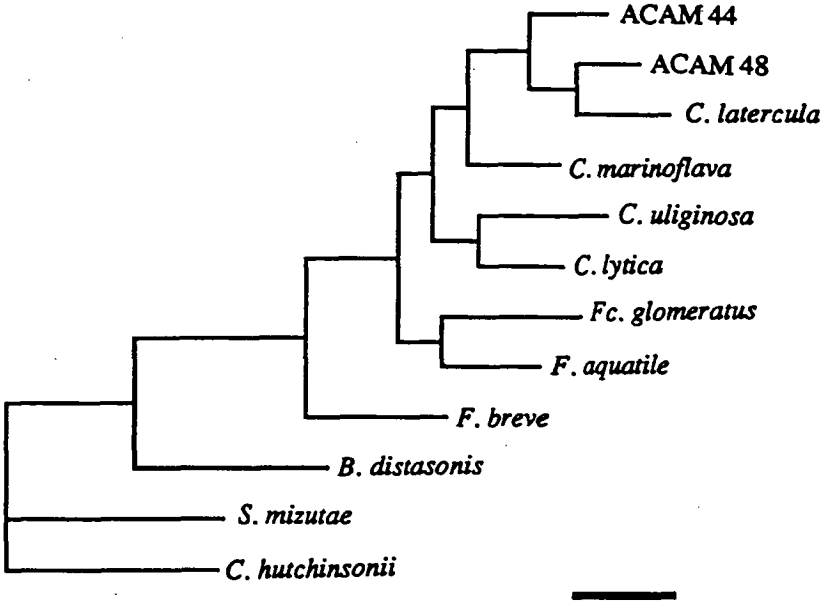

Fig. 5.4 a Most parsimonious tree derived from an analysis of the 16S rRNA sequences of ACAM 44, ACAM 48 and some members of the "flavobacteria-*Bacteroides*" phylum, with *C. hutchinsonii* as the outgroup organism. Bar (length of horizontal branches) = 70 nucleotide differences.

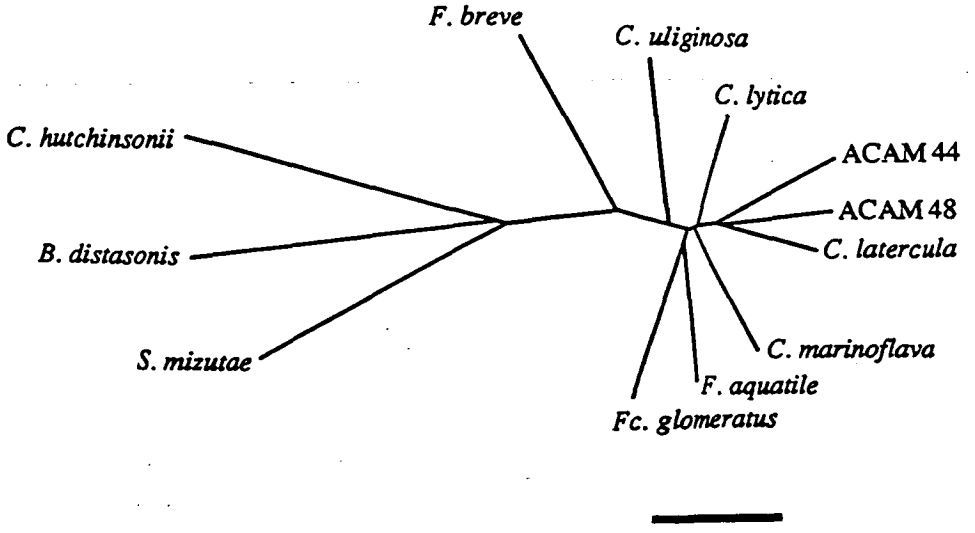

Fig. 5.4 b Phylogenetic tree derived by distance analysis of the same group of species included in Fig. 5.4 a. Bar = 0.05 evolutionary distance units as calculated by the Jukes-Cantor equation.

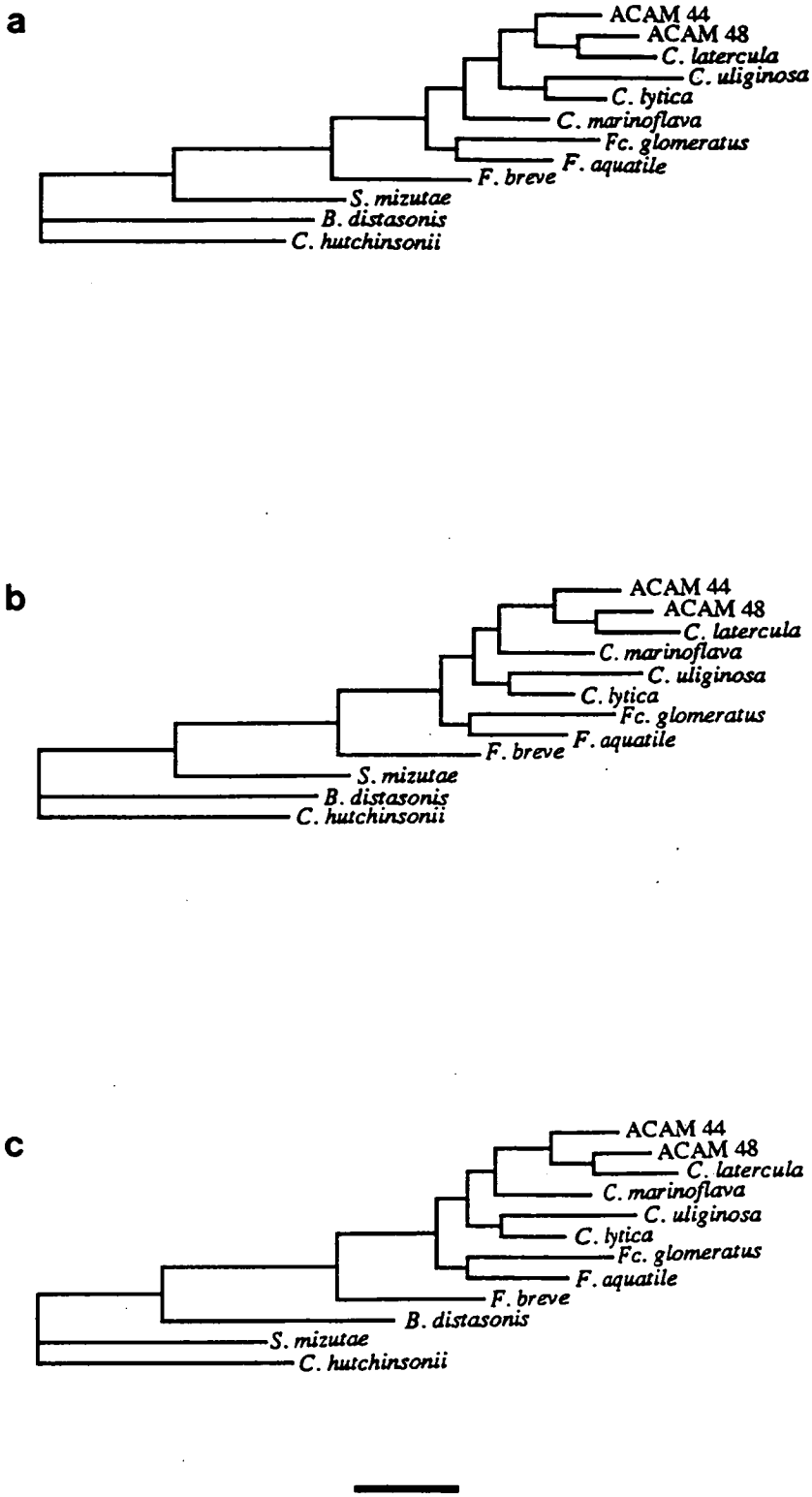

Fig. 5.5 Equally most parsimonious trees (a - c) derived from a character set limited to positions which were conserved (≥50%) amongst the 12 species included in the analysis. *C. hutchinsonii* is the outgroup organism. Bar (length of horizontal branches) = 50 nucleotide differences.

two species outside the branch, *S. mizutae* and *B. distasonis* (Fig. 5.6). In this tree ACAM 44 clustered with *C. lytica* and *C. uliginosa.* Similarly the topology of the tree derived from distance analysis (Fig. 5.4 b) was more significantly affected by the removal of *S. mizutae* and *B. distasonis* (Fig. 5.7 b), than by restricting the data to conserved positions (Fig. 5.7 a). The variation in the branching order of the trees containing 10 species and 12 species respectively, suggested that the branching order of some species within the branch containing the Organic Lake flavobacteria was unstable. Instability might also be expected by the small internodal distances between branching points. The instability within the branch containing the Organic Lake flavobacteria was evident from the result of bootstrap analyses using both the parsimony and distance methods (Figs. 5.8 and 5.9). The consensus trees derived from 2000 replicates showed that above the 90% confidence level the branching order of the Organic Lake flavobacteria with respect to the marine *Cytophaga* spp. was uncertain. The only clusterings of certainty were the *Fc. glomeratus*, "*A. vesiculatum*" and *Fx. aggregans* group and the terrestrial species group. The branching order of these groups with respect to the other marine species and the Organic Lake flavobacteria was also uncertain.

5.2 Phylogenetic relationships among members of the family *Halomonadaceae* and the genus *Halovibrio*

The number of determined bases, and the percentage of the sequence in the region bounded by PCR-amplification priming sites which this represented were as follows:

D.aquamarina, 1423 bases, 95.8%;
D. halophila, 1402 bases, 94.0%;
D. marina, 1402 bases, 93.9%;
H. elongata, 1455 bases, 97.7%;
H. meridiana, 1448 bases, 97.5%;
H. subglaciescola, 1458, 97.8%;
Hv. variabilis, 1438, 96.8%.

An average of 96.2% of the region bounded by the PCR-amplification priming sites was determined. These sequences have been deposited

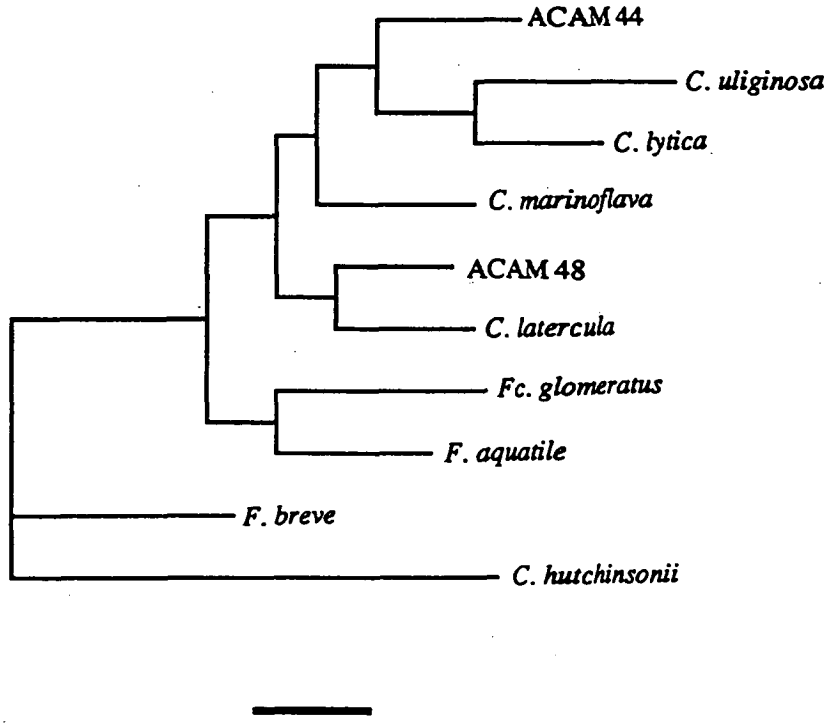

Fig. 5.6 Phylogenetic tree derived by parsimony analysis of a data set which excluded *S. mizutae* and *B. distasonis*. *C. hutchinsonii* is the outgroup organism. Bar (length of horizontal branches) = 70 nucleotide differences.

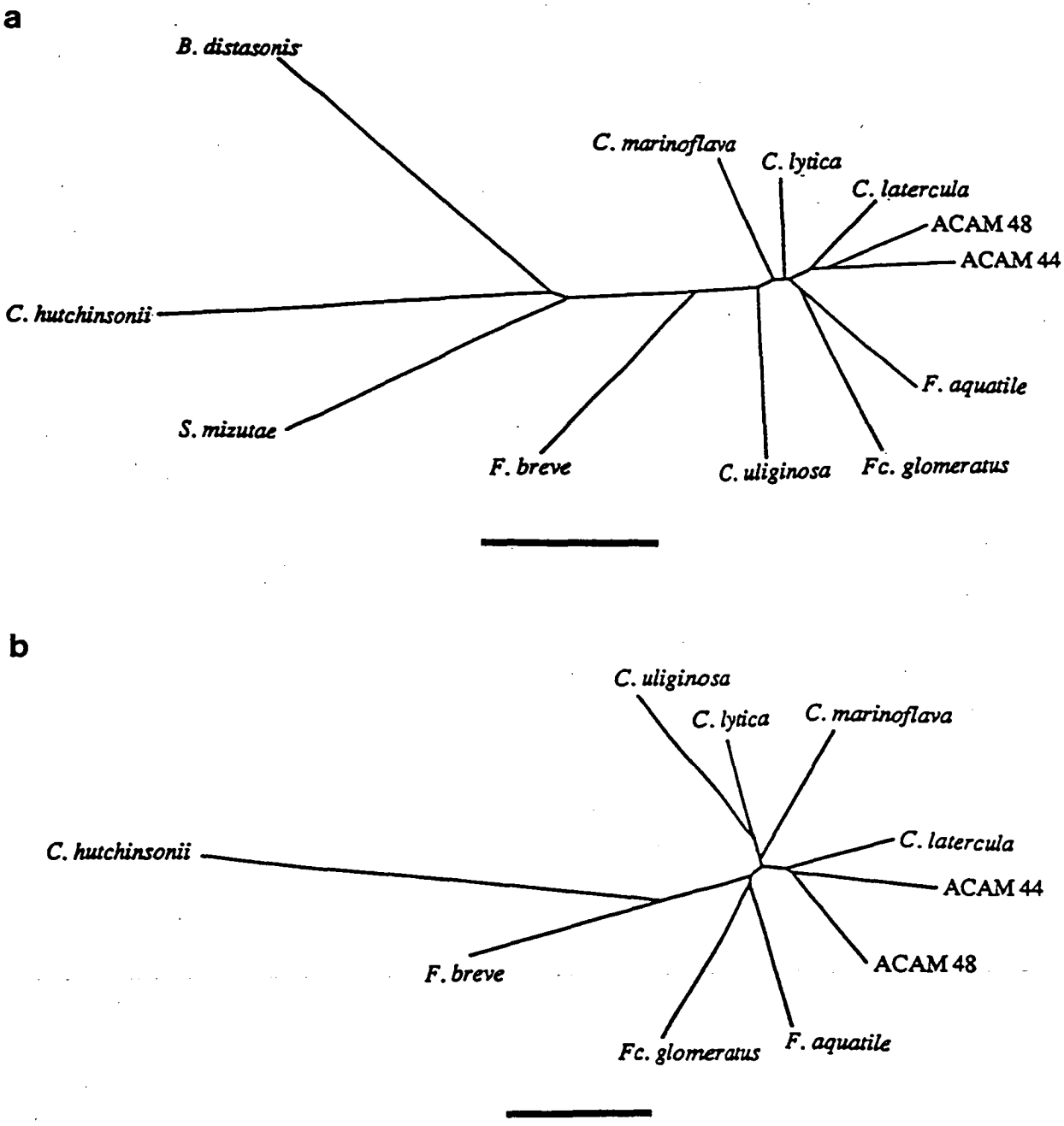

Fig. 5.7 Phylogenetic trees derived by distance analysis of (a) a character data set limited to positions which were conserved (≥50%) amongst the 12 species included in the analysis, (b) a data set which excluded *S. mizutae* and *B. distasonis*. *C. hutchinsonii* is the outgroup species. Bar = 0.05 evolutionary distance units as calculated by the Jukes-Cantor equation.

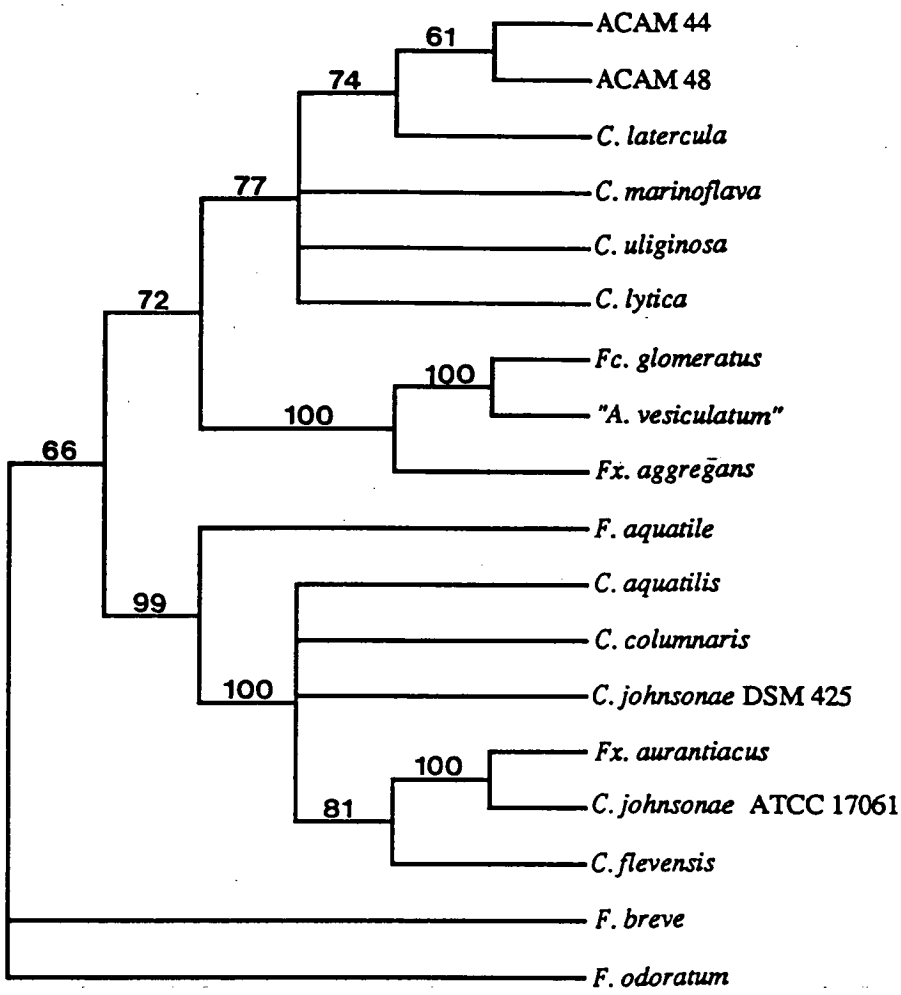

Fig. 5.8 Bootstrap consensus tree derived by parsimony analysis (2000 replications), showing % frequency of occurence of groups, where ≥ 50%.

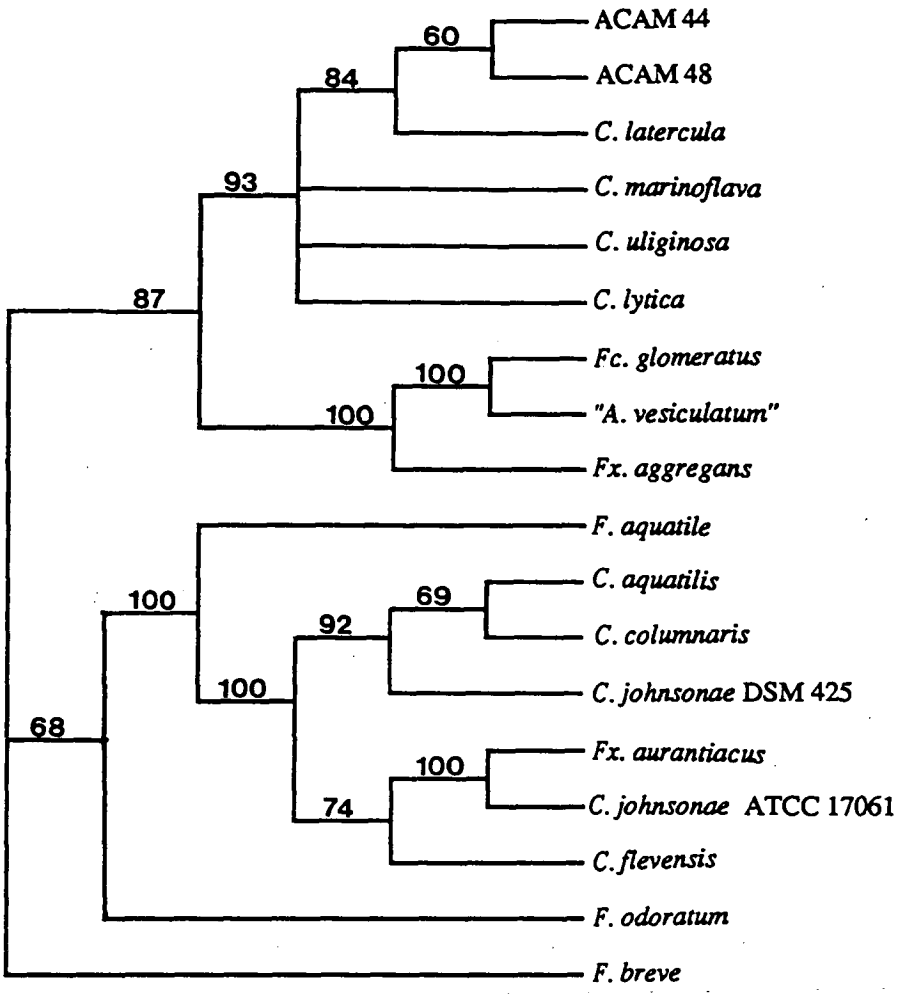

Fig. 5.9 Bootstrap consensus tree derived by distance analysis (2000 replications), showing % frequency of occurence of groups, where ≥ 50%.

in Genbank under accession numbers in the series M93352 - M93358. The alignment of these sequences against *H. halmophila*, a few other members of the gamma-subclass of the *Proteobacteria*, and *E. coli* is shown in Fig. 5.10. The 16S rRNA sequence of *H. elongata* has been determined by Gauthier et al (1992), and recently released into the Genbank database. Nine differences existed between the two sequences (Fig. 5.11). An examination of the autoradiographs resolved the differences in favour of the results of this study (Fig. 5.12).

The 16S rRNA sequences of all the members of the *Halomonadaceae* examined here had a cytosine (C) residue at 486, which represented a highly distinctive signature. An examination of 436 sequences in the ribosomal RNA database project (Olsen et al, 1991) did not locate any organism that possessed a C residue at this site. An exception is found in the recent publication of the partial 16S rRNA sequence of *Listonella anguillarum* which also contains a C at this position (Kita-Tsukamoto et al, 1993). The closest relatives of *L. anguillara*, *V. fischeri* and *V. logei* did not contain a cytosine residue at this position (Kita-Tsukamoto et al, 1993). All other members of the *Bacteria* possessed a uracil residue, with the exception of *Planctomyces staleyi* which had an adenine residue; a deletion occurred in the *Archaea*.

Comparison of the sequences of the members of the *Halomonadaceae* with 38 sequences of other members of the gamma-subclass of the *Proteobacteria* located several characteristics which form a distinctive signature for the *Halomonadaceae* within the gamma-subclass (Table 5.2). Of the seventeen identified characteristics, other members of the gamma-subclass contained a maximum of five. With one exception (*L. anguillarum*) within the gamma-subclass, the signature characteristic at 486, met the stringent condition of having a constant composition among members of the *Halomonadaceae*, and a different and constant composition in all other members of the subclass (Weisburg et al, 1985). Similarly, for the signature at position 776, there was only one other known member of the gamma-subclass which had the same compostion as the members of the *Halomonadaceae*; all the other members of the subclass had a different and constant composition (Fig. 5.10).

Fig. 5.10 Alignment of the 16S rRNA sequences of members of the *Halomonadaceae*, with 16S rRNA sequences of some representatives of the gamma-subclass of the *Proteobacteria* (RDP format). N= undetermined base; - = alignment gap.

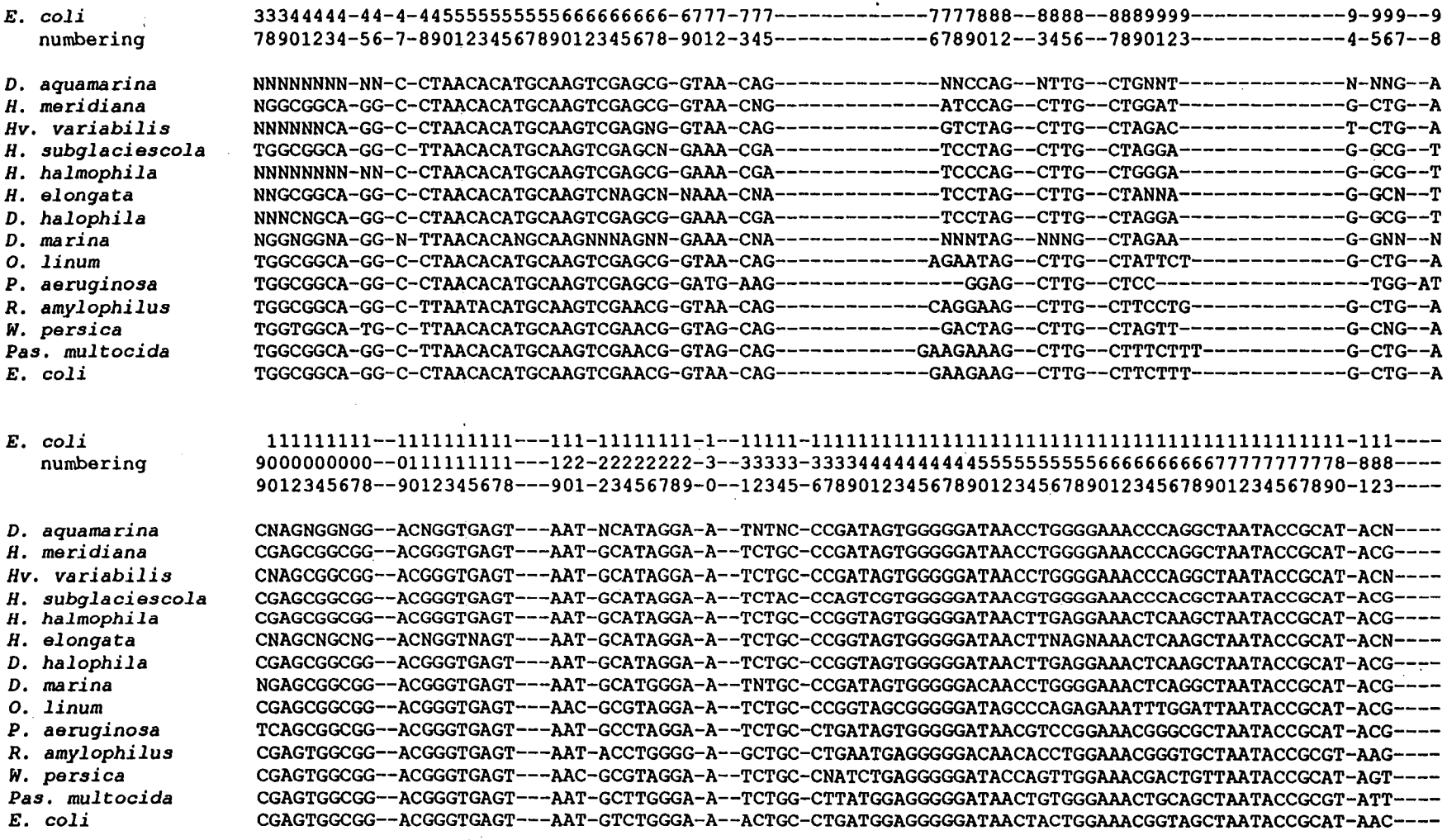

Fig. 5.10 (cont'd)

```
E. coli              --------111-1111-111------------1111-1122222222--------2222---------222222222--2222222-2222222222222
   numbering         --------888-8889-999------------9999-9900000000--------0011---------111111112--2222222-2233333333334
                     --------456-7890-123------------4567-8901234567--------8901---------234567890--1234567-8901234567890

D. aquamarina        --------NCC-TACG-GGA------------GANA-GGGGG----N--------TCCN---------N--CTCCCG--CTATNGG-ATGAGCCTATGTC
H. meridiana         --------TCC-TACG-GGA------------GANN-GGGGG----C--------TCCG---------G--CTCCCG--CTATGGG-ATGAGCCTATGTC
Hv. variabilis       --------TCC-TACG-GGA------------GAAA-GGGGG----C--------TTTG---------G--CTCCCG--CTATTGG-ATGAGCCTATGTC
H. subglaciescola    --------CCC-TACG-GGG------------GAAA-GAAGGGG-CC--------TTCN---------NNCCTTTCN--CNATNGG-ATNNNCCTATGTC
H. halmophila        --------CCC-TACG-GGG------------GAAA-GCAGGGGCTC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCTTATGTC
H. elongata          --------CCC-TACG-GGG------------GAAA-GCAGGGGCTC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCTTATGTC
D. halophila         --------CCC-CAAG-GGG------------GAAA-GCAGGGGATC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCCTATGTC
D. marina            --------TCC-TACG-GGA------------GAAA-GCAGGGGATC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCCCATGTC
O. linum             --------CCC-TACG-GGG------------GAAA-GCAGGGGATC--------TTCG---------GACCTTGCA--CTATCGG-ATGAGCCTGCGTG
P. aeruginosa        --------TCC-TGAG-GGA------------GAAA-GGGGGGGATC--------CTCG---------GACCTCACG--CTATCNG-ATGAGCCTAGGTC
R. amylophilus       --------CCT--GAG-GGG------------GAAA-GGCTGG-----------GCAA-----------CCAGTCG--CATTCAG-ATGCGCCCAGGTG
W. persica           --------ATC-TGTG-GAT------------TAAA-GGTAGC--T---------TTCG----------AGCTGTCG--CAGATGG-ATGAGCCTGCGTT
Pas. multocida       --------CTC-TGAG-GAG------------GAAA-GGGTGGGACC--------TTAG---------GGCCACCTG--CCATAAG-ATGAGCCCAAGTG
E. coli              --------GTC-GCAA-GAC------------CAAA-GAGGGGGACC--------TTCG---------GGCCTCTTG--CCATCGG-ATGTGCCCAGATG

E. coli              222222-2-2222---222222-22222222222222222222222222222222222222233333-33333333333333333333333333333
   numbering         444444-4-4455---555555-55666666666677777777778888888888999999999900000-00000111111111122222222223333
                     123456-7-8901---234567-89012345678901234567890123456789012345678901234-56789012345678901234567890123

D. aquamarina        GGATTA-G-CTAG---TTGGTG-AGGTAACGGCTCACCAAGGCNACGATCCGTAGCTGGTNTGAGAGGNT-GATCAGCCNNATCGGGACTGANACNCGGC
H. meridiana         GGATTA-G-CTAG---TTGGTG-AGGTAACGGCTCACCAAGGCCACGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
Hv. variabilis       GGATTA-G-CTAG---TTGGTG-AGGTAATGGCTCACCAAGGCAGCGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
H. subglaciescola    GNNTTA-G-CTGG---TTGGTG-AGGTAATGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
H. halmophila        GGATTA-G-CTAG---TTGGTG-AGGTAACGGCTTACCAAGGCGACGATCCGTAGCTNGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
H. elongata          GGATTA-G-CTGG---TTGGTG-AGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
D. halophila         GGATTA-G-CTAG---TTGGTG-AGGTAATGGCTCACCAAGGCCGCGaTCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGC
D. marina            GGATTA-G-CTTG---TTGGTG-AGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACACTGGGACTGAGACACGGC
O. linum             AGATTA-G-CTNG---TTGGTG-GGGTAAAGGCCTACCAAGGCGACGATCTCTAGCTGGTCTGAGAGGAT-GATCAGCCACACCGGAACTGAGACACGGT
P. aeruginosa        GGATTA-G-CTAG---TTGGTG-GGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGAC-GATCAGTCACACTGGAACTGAGACACGGT
R. amylophilus       GGATTA-G-CTAG---TTGGTG-GGGTAACGGCCTACCAAGGCGACGATCTCTAGCTGGTCTGAGAGGAT-GATCAGCCACACTGGAACTGAGACACGGT
W. persica           GGATTA-G-CTAG---TTGGTG-GGGTAANGGCCTACCAAGGCCACGATCCATAGCTGATTTGAGAGGAT-GATCAGCCACATTGGGACTGAGACACGGC
Pas. multocida       GGATTA-G-GTAG---TTGGTG-GGGTAAAGGCCTACCAAGCCTNCGATCTCTAGCTGGTCTGAGAGGAT-GACCAGCCACACTGGAACTGAGACACGGT
E. coli              GGATTA-G-CTAG---TAGGTG-GGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT-GACCAGCCACACTGGAACTGAGACACGGT
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Fig. 5.10 (cont'd)

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E. coli          33333333-33333--3333333333-3-333333333333333333-3333-3333-33333333333333344444-444-444444444444--44
   numbering     33333344-44444--4445555555-5-556666666666777777-7777-8888-88888899999999900000-000-001111111111--22
                 45678901-23456--7890123456-7-890123456789012345-6789-0123-45678901234567890123-567-890123456789--01

D. aquamarina    CCNNNCNC-CNNCG--GNAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GAAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CT
H. meridiana     CCGNACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GNAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CT
Hv. variabilis   CCGAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GCAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CT
H. subglaciescola CCGAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGC-GCAA-GCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CT
H. halmophila    CNGAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGC-GAAA-GCCTGATCCAGCCATGCCGNG-TGT-GTGAAGAAGGCC--CT
H. elongata      CCGAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GCAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CT
D. halophila     NNNAACTC-NTACG--GGAGGNANCA-G-TGGGGAATATTGGACAAT-GGGC-GAAA-GCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--TT
D. marina        CCAGACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGC-GAAA-GCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--TT
O. linum         CCGGACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGCACAAT-GGGC-GCAA-GCCTNATGCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--TT
P. aeruginosa    CCAGACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGC-GAAA-GCCNGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGTC--TT
R. amylophilus   CCAGACTC-CTACG--GGAGGCAGCA-G-TAGGGAATATTGCACAAT-GGGG-GAAA-CCCTGATGCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--TT
W. persica       CCAAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GAAA-CCCTGATCCAGCAATGCCATG-TGT-GTGAAGAAGGCC--TT
Pas. multocida   CCAGACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGCGCAAT-GGGG-GGAA-CCCTGACGCAGCCATGCCGCG-TGA-ATGAAGAAGGCC--TT
E. coli          CCAGACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGCACAAT-GGGC-GCAA-GCCTGATGCAGCCATGCCGCG-TGT-ATGAAGAAGGCC--TT

E. coli          44-444444444-------------4444444444444444--44-4444444444-4444444-444444-444-444444444444-4-444-444
   numbering     22-222222333-------------3333333444444444445--55-5555555666-6666666-777777-777-788888888889-9-999-999
                 23-456789012-------------3456789012345678901--12-3456789012-3456789-012345-678-901234567890-1-234-567

D. aquamarina    CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-CGCCTAGCGG-TTAATAC-CCGCTA-GGA-AAGACATCACTC-G-CAG-AAG
H. meridiana     CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-CGCCTNNNGG-TTAATAC-CCGCTA-GGA-AAGACATCACTC-G-CAG-AAG
Hv. variabilis   CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-CGCCTGTCGG-TTAATAC-CCGGCA-GGA-AAGACATCACTC-G-CAG-AAG
H. subglaciescola CG-GGTTGTAAA-------------GCACTTTCAGTGAGGAAG--AA-CGCCGTCGGG-TTAATAC-CCCGGC-GGA-AAGACATCACTC-A-CAG-AAG
H. halmophila    CG-GGTTGTAAA-------------GCACTTTCAGTGAGGAAG--AA-GGCTTGTCGG-TTAATAC-CCGGCA-GGA-AGGACATCACTC-A-CAG-AAG
H. elongata      CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-TGCTTGTCGG-TTAATAC-CCGGCA-AGG-GAGACATCACTC-G-CAG-AAG
D. halophila     CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-CGCTTGTGGG-TTAATAC-CCTGCA-AGA-AGGACATCACTC-G-CAG-AAG
D. marina        CG-GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-CGCTTCGGGA-TTAATAC-TCCCGA-GGA-AAGACATCACTC-G-CAG-AAG
O. linum         AG-GGTTGTAAA-------------GCACTTTCAGCGAGGAGG--AA-AGGTTATTGA-TTAATAC-TCAATA-GCT-GTGACGTTACTC-G-CAG-AAG
P. aeruginosa    CG-GATTGTAAA-------------GCACTTTAAGTTGGGAGG--AA-GGGCAGTAAG-TTAATAC-CTTGCT-GTT-TTGACGTTACCA-A-CAG-AAT
R. amylophilus   TG-GGTTGTAAA-------------GCACTTTCAGTATGGAGG--AA-GTGTAGTATG-TTAACAG-CATGCT-GCA-TTGACGTTACAT-A-CAG-AAG
W. persica       AG-GGTTGTAAA-------------GCACTTTAGTNGGGGAGG--AA-AGCCTTGAGG-TTAATNG-CCTTTA-GGA-ATGACGTTACCC-N-AAG-AAT
Pas. multocida   CG-GGTTGTAAA-------------GTTCTTTCGGTAATGAGG--AA-GGGATGTTGT-TAAATAG-ATAGCA-TCA-TTGACGTTAATT-A-CAG-AAG
E. coli          CG-GGTTGTAAA-------------GTACTTTCAGCGGGGAGG--AA-GGGAGTAAAG-TTAATAC-CTTTGC-TCA-TTGACGTTACCC-G-CAG-AAG
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Fig. 5.10 (cont'd)

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E. coli          4455555-555555555555555555555555555555555555-555555555555555555555555555555555555555555555555555555555-555
   numbering     9900000-000001111111111222222222233333333334-444444445555555555666666666677777777778888888888999-999
                 8901234-567890123456789012345678901234567890-123456789012345678901234567890123456789012345678901-234

D. aquamarina    AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTT-GAT
H. meridiana     AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTT-GAT
Hv. variabilis   AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTT-GAT
H. subglaciescola AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCNGTAATACGGAG-GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTT-GAT
H. halmophila    AAGCCCC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGGGCNAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGCTT-GGT
H. elongata      AAGCACC-GGCTAACTCCGTGCCAGCAGCCNCNGTAATACGGAG-GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGCTT-GAT
D. halophila     AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTT-GAT
D. marina        AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTA-AGT
O. linum         AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTT-GTT
P. aeruginosa    AAGCACC-GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG-GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTC-AGC
R. amylophilus   AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGAGCTCGTAGGCGGTTT-GTC
W. persica       AAGCACC-GGCTANCTCCGTGCCAGCAGCCGCGGTAATACGGGG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGGGTCTGTAGGTGGTTT-GTT
Pas. multocida   AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCACGCAGGCGGACT-TTT
E. coli          AAGCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTT-GTT


E. coli          5555566-666666666666666666666666666666-66666666666666666-6666666666666666666-666666666666-----6666
   numbering     9999900-000000001111111111222222222233-33333333444444444-455555555556666666666-777777777788-----8888
                 5678901-234567890123456789012345678901-23456789012345678-901234567890123456789-012345678901-----2345

D. aquamarina    AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTCA-AGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
H. meridiana     AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTCA-AGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
Hv. variabilis   AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTCA-GGCTAGAGTACAGGAGAGGAA-GGTAGAATTCCC-----GGTG
H. subglaciescola CAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-AGCATCCGGAACTGTCA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
H. halmophila    AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAT-GGCATCCGGAACTGTCA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
H. elongata      AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTTA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
D. halophila     AAGCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCNTCCGGAACTGTCA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
D. marina        CAGCCAG-GTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCTGGAACTGCTT-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTG
O. linum         AAGCCAG-ATGTGAAAGCCCCGGGCTTAACCTGGGNAC-TGCATTTGGAACTGGCA-GGCTAGAGTACAGAAGAGGGT-GGTAGAATTTCC-----TGTG
P. aeruginosa    AAGTTGG-ATGTGAAATCCCCGGGCTCAACCTGGGAAC-TGCATCCNAAACTACTG-AGCTAGAGTACGGTAGAGGGT-GGTGGAATTTCC-----TGTG
R. amylophilus   AAGTCAG-ATGTGAAAGCCCCGGGCTTAACCTGGGAAC-CGCATTTGAAACTGACA-GACTAGAGTACTGTAGAGGGA-GGTAGAATTCCA-----GGTG
W. persica       AAGTCAG-ATGTGAAAGCCCAGGGCTCNACCTTGGAAC-TGCATTTGATACTGGCA-AACTAGAGTACGGTAGAGGAA-TGGGGAATTTCT-----GGTG
Pas. multocida   AAGTGAG-ATGTGAAATCCCCGAGCTTAACTTGGGAAC-TGCATTTCAGACTGGGA-GTCTAGAGTACTTTAGGGAGG-GNTAGAATTCCA-----CGTG
E. coli          AAGTCAG-ATGTGAAATCCCCGGGCTCAACCTGGGAAC-TGCATCTGATACTGGCA-AGCTTGAGTCTCGTAGAGGGG-GGTAGAATTCCA-----GGTG
```

Fig. 5.10 (cont'd)

```
E. coli          6666666666666-67777777777777777777777--77777777777777777777777777777777777777777777777777777-777777
   numbering     8888999999999-90000000000111111111222--22222223333333333444444444455555555556666666666777777-777788
                 6789012345678-90123456789012345678901 2--34567890123456789012345678901234567890123456789012345-678901

D. aquamarina    TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGG-TAGCAA
H. meridiana     TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCNGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGG-TAGCAA
Hv. variabilis   TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAANGCGGCCTTCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG-TAGCAA
H. subglaciescola TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGG-TAGCAA
H. halmophila    TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTACGAAAGCGTGGG-TAGCAA
H. elongata      TAGCGGTNAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCNAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTACNAAAGCGTGGG-TAGCAA
D. halophila     TAGCGGTNAAATG-CGTAGAGATNNNGNNNAATACCAG--TNGCNAANGCNGCCTTCTNGACTGACACTGACNCTGAGGTGCGAAAGCGTGGG-TAGCAA
D. marina        TAGCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGG-TAGCAA
O. linum         TAGCGGTGAAATG-CGTAGATATAGGAAGGAATACCAG--TGGCGAAGGCGGCCACCTGGTCTGATACTGACGCTGAGGTGCGAAAGCGTGGG-GAGCAA
P. aeruginosa    TAGCGGTGAAATG-CGTAGATATAGGAAGGAACACCAG--TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG-GAGCAA
R. amylophilus   TAGCGGTGAAATG-CGTAGATATCTGGAGGAATACCGG--TGGCGAAGGCGGCCTCCTGGACAGAGACTGACGCTGAGGAGCGAAAGCGTGGG-GAGCAA
W. persica       TAGCGGTGAAATG-CGTAGAGATCAGAAGGAACACCAA--TGGCGAAGGCAACATTCTGGACCGATACTGACNCTNAGGGACGAAAGCGTGGG-GATCAA
Pas. multocida   TAGCGGTGAAATG-CGTAGAGATGTGNAGGAATACCGA--AGGCGAAGGCAGCCCCTTGGGAATGTACTGACGCTCATGTGCNAAAGCGTGGG-GAGCAA
E. coli          TAGCGGTGAAATG-CGTAGAGATCTGGAGGAATACCGG--TGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGG-GAGCAA


E. coli          7777777777777777778888888888888888888888888-888888888888888---88888-----888888888888888888888888888
   numbering     8888888899999999990000000000011111111111222222-222233333333334---44444-----444455555555556666666666777
                 2345678901234567890123456789012345678901234 5-678901234567890---12345-----678901234567890123456789012

D. aquamarina    ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CCAGCCGTTGGGTGC---CTAGC-----GCACTTTGTGGCGAAGTTAACGCGATA
H. meridiana     ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CCAGCCGTTGGGTGC---CTAGC-----GCACTTTGTGGCGAAGTTAACGCGATA
Hv. variabilis   ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CCAGCCGTTGGGTGC---CTAGA-----GCACTTTGTGGCGAAGTTAACGCGATA
H. subglaciescola ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CCAGCCGTTGGGATC---CTTGA-----GATCCTTGTGGCGAAGTTAACGCGATA
H. halmophila    ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTAGCCGTTGGGGTC---CTTGA-----GACCTTTGTGGCGCAGTTAACGCACTA
H. elongata      ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTAGCCGTTGGGGTC---CTCGA-----GACCTTTGTGGCGCAGTTAACGCACTA
D. halophila     ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA-CTAGCCGTTGGGTCC---CTTGA-----GGACTTAGTGGCGCAGCTAACGCAATA
D. marina        ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA-CTAGCCGTTGGGTCC---CTTGA-----GGACTTAGTGGCGCAGCTAACGCAATA
O. linum         ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTA-CTAGCCGTTGGGAAC---CTTGA-----GTTCTTAGTGGCGCAGCTAACGCGATA
P. aeruginosa    ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTAGCCGTTGGGATC---CTTGA-----GATCTTAGTGGCGCAGCTAACGCGATA
R. amylophilus   ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAA-TTAGAAGCATGTTGC---CATGA-----GTAGTGTGTTTCTAAGCTAACGCGATA
W. persica       ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTA-CTAGCTGTTGGAGTCG--GTGTNA---AGGCTCTAGTGGCGCAGCTAACGTGATA
Pas. multocida   ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGCTGTCGA-TTTGGGGATTGGGC----TATAT------GCTTGGTGCCCGAAGCTAACGTGATA
E. coli          ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTTGGAGGTTGTGCC---CTTGA-----GGCGTGGCTTCCGGAGCTAACGCGTTA
```

Fig. 5.10 (cont'd)

```
E. coli          88888888888888888888888-889999999999999-999-9999999-9999999999--99-9--999999-99999999999999-999999
   numbering     77777778888888888999999999-990000000000111-111-1111222-2222222333--33-3--333344-44444444555555-555566
                 34567890123456789012345678-901234567890123-345-6789012-3456789012--34-5--678901-23456789012345-678901

D. aquamarina    AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
H. meridiana     AGTCGACCGCCTGGGGAGTACNGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
Hv. variabilis   AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
H. subglaciescola AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
H. halmophila    AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGNNN--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
H. elongata      AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
D. halophila     AGTTGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
D. marina        AGTTGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
O. linum         AGTAGACNGCCTGGGGAGTACGGTC-GCAAGATTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GNGGAGCATGTGGT-TTAATT
P. aeruginosa    AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCNN--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
R. amylophilus   AATTGACCGCCTGGGGAGTACGGCCGGCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
W. persica       AGTACTCCGCCTGGGGACTACGGCC-GCAAGGCTAAAACTC-AAA-GGAATTG-ACGGGGACCN--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
Pas. multocida   AATCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-NCGGGGGCCN--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT
E. coli          AGTCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATT

E. coli                                                    1111---11111-1111-1111111-1-1111-11----11--1111--11----11-1
   numbering     99999999999999999999-999999999999999-9-0000---00000-0000-0000000-0-0000-00----00--0000--00----00-0
                 66666666777777777888-888888999999999-9-0000---00000-0111-1111111-2-2222-22----22--2333--33----33-3
                 23456789012345678901 23-456789012345678-9-0123---45678-9012-3456789-0-1234-56----78--9012--34----56-7

D. aquamarina    CGATGCAACGCGAAGAACCTTA-CCTACTCTTGACATC-C-TGCG---AATTT-GGTA-GAGATAC-C-TTAG-TG----CC--TTCG--GG----AA-C
H. meridiana     CGATGCAACGCGAAGAACCTTA-CCTACTCTTGACATN-C-TGCG---AATTT-GGNA-GAGATAC-C-TTAG-NG----CC--TTNG--GG----AA-C
Hv. variabilis   CGATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-T-ACAG---AAGCC-GGAA-GAGATTC-T-GGTG-TG----CN--TTCG--GG----AA-C
H. subglaciescola CGATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-C-TGCG---AACTT-GGTA-GAGATNC-C-NTGG-TG----CN--TTCG--GG----AA-C
H. halmophila    CGATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-G-TGCG---AACTT-GGTA-GAGATAC-C-TTGG-TG----CC--TTCG--GG----AG-C
H. elongata      CGATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-G-TGCG---AACTT-TCCA-GAGATGG-A-TTGG-TG----CC--TTCG--GG----AG-C
D. halophila     CGATNCAACGCNAAGAACCTTA-CCTACTCTTGACATC-G-NGNG---AACCN-TCCA-GAGATGG-A-TGGG-TN----CC--TTCN--NG----AA-C
D. marina        CGATNCAACGCNAAGAACCTTA-CCTACNCTTGACATC-C-AGAG---GACTT-TCCA-GAGATNG-A-TTGG-TN----CC--TTCG--GG----AA-C
O. linum         CGAAGCAACGCGAAGAACCTTA-CCTACTCTTGACATC-C-AGAG---AACTT-GGTA-GAGATAC-C-TTGG-TG----CC--TTCG--GG----AA-C
P. aeruginosa    CGAAGCAACGCGAAGAACCTTA-CCTGGCCTTGACATG-C-TGAG---AACTT-TCCA-GAGATGG-A-TTGG-TG----CC--TTCG--GG----AA-C
R. amylophilus   CGATGCAACGCGAAGAACCTTA-CCTGGACTTGACATA-T-TGAG---AAGTA-TTTA-GAGATAG-A-TACG-TG----CC--GCAA--GG----AG-C
W. persica       CGATGCAACGCGAAGAACCTTA-CCTGGTCTTGACATC-C-TGCG---AACTT-TCTA-GAGATAG-A-TTGG-TG----CC--TTCG--GG----AA-C
Pas. multocida   CGATGCAACGCGAAGAACCTTA-CCTACTCTTGACATC-C-TAAG---AAGAG-CTCA-GAGATGA-G-CTTG-TG----CC--TTCG--GG----AA-C
E. coli          CGATGCAACGCGAAGAACCTTA-CCTGGTCTTGACATC-C-ACGG---AAGTT-TTCA-GAGATGA-G-AATG-TG----CC--TTCG--GG----AA-C
```

Fig. 5.10 (cont'd)

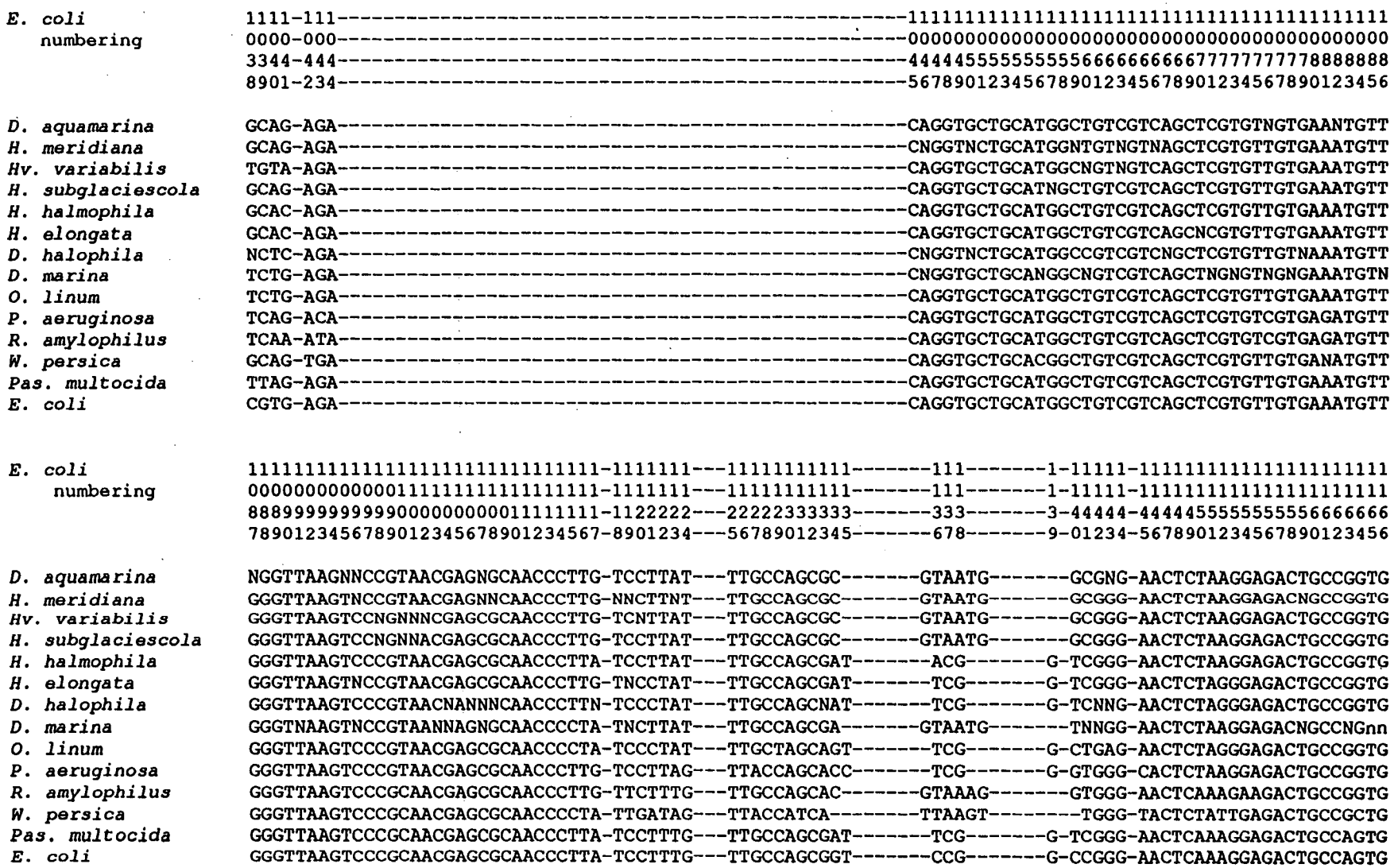

Fig. 5.10 (cont'd)

```
E. coli          1-111-1111--1111-1-11111111-11111111--11111111111-111111111-11-1111111111111111111111111111111-1-111
   numbering     1-111-1111--1111-1-11111111-11111111--11112222222-222222222-22-22222222222222222222222222222-2-222
                 6-667-7777--7777-7-88888888-88999999--99990000000-000111111-11-11222222222233333333334444444444-4-555
                 7-890-1234--5678-9-01234567-89012345--67890123456-789012345-67-8901234567890123456789012345678-9-012

D. aquamarina    A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCATG-GCCCTTACG-AG-TAGGGCTACACACGTGCTACAATGGTCGGTA-C-AAA
H. meridiana     A-CAA-ACCG--GAGG-A-AGGTGGGG-ACNACGTC--AAGTCATCATG-GCCCTTACG-AG-TAGGGCTACACACGTGCTACAATGGTCGGTA-C-AAA
Hv. variabilis   A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGANGTC--ANGTNNNNNNN-CNNNNNNNN-GG-TAGGNCTACACACGTGCTACAATGGCCGGTA-C-AAA
H. subglaciescola A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCANCATG-GCCCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCCGGCA-C-AAA
H. halmophila    A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCATG-GCCCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCCGATA-C-AAT
H. elongata      A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCANG-GCCCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCCGGTA-C-AAT
D. halophila     A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AGGTCATCATG-GCCCTTACG-AG-TAGGGCTACACACGTGCTACAATGGCCGGTA-C-AAT
D. marina        N-NNN-NNNN--NNNN-N-NNNNNNNN-NNNNNNNN--NNNNNNTCATG-GCCCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCAAGTA-C-AAA
O. linum         A-CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCATG-GCCCTTACG-AG-TAGGGCTACACACGTGCTACAATGGCCGGTA-C-AGA
P. aeruginosa    A-CAA-ACCG--GAGG-A-AGGTGGGG-ATGACGTC--AAGTCATCATG-GCCCTTACG-GC-NAGGGCTACACACGTGCTACAATGGTCGGTA-C-AAA
R. amylophilus   A-CAA-ACCG--GAGG-A-AGGCAGGG-ATGACGTC--AAGTCATCATG-GCCCTTACG-TC-CAGGGCTACACACGTGCTACAATGGGTTGTA-C-AGA
W. persica       A-CAA-GGCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCATG-GCCCTTACG-AC-CAGGGCTACACACGTGCTACAATGGGTATTA-N-AGA
Pas. multocida   A-CAA-ACTG--GAGG-A-AGGNGGGG-ATGACGTC--AAGTCATCATG-GCCCTTACG-AG-TAGGGCTACACACGTGCTACAATGGTGCATA-C-AGA
E. coli          A-TAA-ACTG--GAGG-A-AGGTGGGG-ATGACGTC--AAGTCATCATG-GCCCTTACG-AC-CAGGGCTACACACGTGCTACAATGGCGCATA-C-AAA

E. coli          1111-111-1111111-1111111----------111111111111-11111111111111111111111111111111111111111111111111111
   numbering     2222-222-2222222-2222222----------222222222222-22222222222222333333333333333333333333333333333333333
                 5555-555-6666666-6667777----------777777888888-88889999999999000000000011111111112222222222333333333
                 3456-789-0123456-7890123----------456789012345-67890123456789012345678901234567890123456789012345678

D. aquamarina    GGGT-TGC-CAACTCG-CGAGAGT----------GAGCCAATCCCG-AAAAGCCGATCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
H. meridiana     GGGT-TGC-CAACTCG-CGAGAGT----------GAGCCAATCCCG-AAAAGCCGATCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
Hv. variabilis   GGGC-TGC-GAGCTCG-CGAGAGT----------CAGCGAATCCCT-TAAAGCCGGTCTCAGTCCGNATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
H. subglaciescola GGGC-TGC-NAACTCG-CGAGAGT----------AAGCGAATCCCA-TAAAGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
H. halmophila    GGGT-CGC-AAAGCGG-CGACGTG----------GCGCTAATCCCA-TAAAGTCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
H. elongata      GGGT-TGC-AAAGCGG-CGACGTG----------GAGCTAATCTCA-TAAAGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCG
D. halophila     GGGC-TGC-CATCCCG-CNAGGGG----------GAGCTAATCTCA-TAANGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTNAAGTCG
D. marina        GGGT-TGC-AATACGG-CGACGTG----------GAGCCAATCCCA-TAAAGCTTGCCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
O. linum         GGGC-TGC-AATATCG-CGAGATG----------GAGCTAATCCCA-CAAAACCGGNCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
P. aeruginosa    GGGT-TGC-GAAGCCG-CGAGGTG----------GAGCTAATCCCA-TAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG
R. amylophilus   GGGA-AGC-GAAGTCG-CGAGGTA----------GAGCGGAACCCA-GAAAGCAACTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
W. persica       GGGC-TGC-GAAGGAG-CGATCTG----------GAGCGAAACTCA-AAAAGGTACTCTTAGTCCGGATTGCAGTCTGCAACTCGACTGCATGAAGTCG
Pas. multocida   GGGC-AGC-GAGAGTG-CGAGCTT----------GAGCGAATCTCA-GAAAGTGCATCTAAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
E. coli          GAGA-AGC-GACCTCG-CGAGAGC----------AAGCGGACCTCA-TAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
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Fig. 5.10 (cont'd)

```
E. coli          111111111111111111111111-1111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111-
   numbering     333333333333333333333333-3333333333333333333333333333333333333344444444444444444444444444444444444444-
                 334444444445555555555566-6666666677777777778888888888999999999900000000001111111111222222222233333333-
                 890123456789012345678901-2345678901234567890123456789012345678901234567890123456789012345678901234567-

D. aquamarina    GAATCGCTAGTAATCGTGGATCAG-AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
H. meridiana     GAATCGCTAGTAATCGTGGATCAG-AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
Hv. variabilis   GAATCGCTAGTAATCGTGAATCAG-AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
H. subglaciescola GAATCGCTAGTAATCGTGAATCAG-AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTGGACTGCACCAGAAG-
H. halmophila    GAATCGCTAGTAATCGTGCATCAG-AATGGCACGGTGAATACGTTCCCGGGCNTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
H. elongata      GAATCGCTAGTAATCGTGCATCAG-AATGGCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
D. halophila     NAATCGCTAGTAATCGTGCATCAg-AATGGCACNGtNAATACGTTCCCgNGccNNNNNNNNNNNNNNNNNNNNNNNNNNGGGAGTGGACTGCACCAGAAG-
D. marina        GAATCGCTAGTAATCGTGGATCAG-AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-
O. linum         GAATCGCTAGTAATCGTGAATCAG-AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAG-
P. aeruginosa    GAATCGCTAGTAATCGTGAATCAG-AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAG-
R. amylophilus   GAATCGCTAGTAATCGCGAATCAG-AATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGAATTGCACCAGAAG-
W. persica       GAATCGCTAGTAATCGCAGGTCAG-AATACTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCNTGGGAGTGGGTTGCTCCAGAAG-
Pas. multocida   GAATCGCTAGTAATCGCAAATCAG-AATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGTACCAGAAG-
E. coli          GAATCGCTAGTAATCGTGGATCAG-AATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAG-

E. coli          1111-111-11111-11----1111----111111111111-1111111111111111111111111111111111111111111111111111111111111111111111
   numbering     4444-444-44444-44----4444----444444444444-4444444444444444444444444444444444445555555555555555555555
                 3333-444-44444-44----5555----555555666666-6666777777777788888888889999999999000000000011111111112 2
                 6789-012-34567-89----0123----456789012345-6789012345678901234567890123456789012345678901234567890 1

D. aquamarina    TGGT-TAG-CCTAA-C-----GCAA-----GAGGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
H. meridiana     TGGT-TAG-CCTAA-C-----GCAA-----GAGGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
Hv. variabilis   TGGT-TAG-CCTAA-C-----GCAA-----GAGGGCGATNA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
H. subglaciescola TGNT-TAG-CCTAA-C-----TTCG-----GAAGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
H. halmophila    TGGT-TAG-CCTAA-C-----TTCG-----GAGGGCGATCA-CCACGGTGTGGTTCATGGTAACAAGGAACCTGGATCACCTCCTTA.....
H. elongata      TGGT-TAG-CCTAA-C-----TTCG-----GAGGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
D. halophila     TGGT-TAG-CCTAA-C-----TTNG-----GAGGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
D. marina        TGGT-TAG-CCTAA-CC----TTCG----GGAGGGCGATCA-CCACGGTGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGGAACC
O. linum         TGGC-TAG-TCTAA-CC----TTCG----GGAGGACGGTCA-CCACGGAGTGATTCATGACTGGGGGTAACAAGGAACCTGGATCACCTCCTTA....
P. aeruginosa    TAGC-TAG-TCTAA-CC----GCAA----GGGGGACGGTTA-CCACGGAGTGATTCATGGTAACAAGGAACCTG.....................
R. amylophilus   TAGT-TAG-CTTAA-CCC---GCAA---GGGAGGGCGATTA-CCACGGTGTGGTTTATGACTGGGGTGAAGTCGTAACAAGGTAACCATAGGGGAACC
W. persica       TAGA-TAG-CTTAA-------CGAA------TGGGCGTTTA-CCACGGAGTGATTCA.....................................
Pas. multocida   TAGA-TAG-CTTAA-CC----TTCG----GGGGGGCGTTTA-CCACGGTATGATTCATGACNNGGGGTAACAAGGATCACCTCCTTA.....
E. coli          TAGG-TAG-CTTAA-CC----TTCG----GGAGGGCGCTTA-CCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACC
```

Fig. 5.11 A comparison of the 16S rRNA gene sequence of *H. elongata* determined in this project (1) and the sequence determined by Gauthier et al (1992) (2). Positions where the sequences differed are indicated with an asterisk (*).

```
H. elongata (1)  GCGGCA-GG-C-CTAACACATGCAAGTCNAGCN-NAAA-CNA--------------TCCTAG--CTTG--CTANNA--------------G-GCN--TCN
H. elongata (2)  GCGGCA-GG-C-CTAACACATGCAAGTCGAGCG-GAAA-CGA--------------TCCTAG--CTTG--CTAGGA--------------G-GCG--TCG

H. elongata (1)  AGCNGCNG--ACNGGTNAGT---AAT-GCATAGGA-A--TCTGC-CCGGTAGTGGGGGATAACTTNAGNAAACTCAAGCTAATACCGCAT-ACN------
H. elongata (2)  AGCGGCGG--ACGGGTGAGT---AAT-GCATAGGA-A--TCTGC-CCGGTAGTGGGGGATAACTTGAGGAAACTCAAGCTAATACCGCAT-ACG------

H. elongata (1)  ------CCC-TACG-GGG------------GAAA-GCAGGGGCTC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCTTATGTCGG
H. elongata (2)  -------CC-TACG-GGG------------GAAA-GCAGGGGCTC--------TTCG---------GACCTTGCG--CTATCGG-ATGAGCTTATGTCGG
                       *
                     184

H. elongata (1)  ATTA-G-CTGG---TTGGTG-AGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGCCC
H. elongata (2)  ATTA-G-CTGG---TTGGTG-AGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGAT-GATCAGCCACATCGGGACTGAGACACGGCCC

H. elongata (1)  GAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GCAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CTCG
H. elongata (2)  GAACTC-CTACG--GGAGGCAGCA-G-TGGGGAATATTGGACAAT-GGGG-GCAA-CCCTGATCCAGCCATGCCGCG-TGT-GTGAAGAAGGCC--CTCG

H. elongata (1)  -GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-TGCTTGTCGG-TTAATAC-CCGGCA-AGG-GAGACATCACTC-G-CAG-AAGAA
H. elongata (2)  -GGTTGTAAA-------------GCACTTTCAGCGAGGAAG--AA-TGCTTGTCGG-TTAATAC-CCGGCA-AGG-GAGACATCACTC-G-CAG-AAGAA

H. elongata (1)  GCACC-GGCTAACTCCGTGCCAGCAGCCNCNGTAATACGGAG-GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGCTT-GATAA
H. elongata (2)  GCACC-GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG-GGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGTAGGCGGCTT-GATAA

H. elongata (1)  GCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTTA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTGTA
H. elongata (2)  GCCGG-TTGTGAAAGCCCCGGGCTCAACCTGGGAAC-GGCATCCGGAACTGTTA-GGCTAGAGTGCAGGAGAGGAA-GGTAGAATTCCC-----GGTGTA

H. elongata (1)  GCGGTNAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCNAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTACNAAAGCGTGGG-TAGCAAAC
H. elongata (2)  GCGGTGAAATG-CGTAGAGATCGGGAGGAATACCAG--TGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTACGAAAGCGTGGG-TAGCAAAC

H. elongata (1)  AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTAGCCGTTGGGGTC---CTCGA-----GACCTTTGTGGCGCAGTTAACGCACTAAG
H. elongata (2)  AGGATTAGNTACCCTGGTAGTCCACGCCGTAAACGATGTCGA-CTAGCCGTTGGGGTC---CTCGA-----GACCTTTGTGGCGCAGTTAACGCACTAAG
```

Fig. 5.11 (cont'd)

```
H. elongata (1)  TCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TGAATTG-ACGGGGGCCC--GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATTCG
H. elongata (2)  TCGACCGCCTGGGGAGTACGGCC-GCAAGGTTAAAACTC-AAA-TNAATTG-ACGGGGGCC---GC-A--CAAGCG-GTGGAGCATGTGGT-TTAATTCG
                                                                                *
                                                                               932

H. elongata (1)  ATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-G-TGCG---AACTT-TCCA-GAGATGG-A-TTGG-TG----CC--TTCG--GG----AG-CGC
H. elongata (2)  ATGCAACGCGAAGAACCTTA-CCTACCCTTGACATC-G-TGCG--GAACTT-CCCA-GAGATGG-A-TTGG-TG----CC--TTCG--GG----AG-CGC
                                                        *      *
                                                      1004   1009

H. elongata (1)  AC-AGA-----------------------------------------------------CAGGTGCTGCATGGCTGTCGTCAGCNCGTGTTGTGAAATGTTGG
H. elongata (2)  AC-AGA-----------------------------------------------------CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGG

H. elongata (1)  GTTAAGTNCCGTAACGAGCGCAACCCTTG-TNCCTAT---TTGCCAGCGAT-------TCG-------G-TCGGG-AACTCTAGGGAGACTGCCGGTGA-
H. elongata (2)  GTTAAGTCCCGTAACGAGCGCAACCCTTG-TCCCTAT---TTGCCAGCGAT-------TCG-------G-TCGGG-AACTCTAGGGAGACTGCCGGTGA-

H. elongata (1)  CAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCANG-GCCCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCCGGTA-C-AATGG
H. elongata (2)  TAA-ACCG--GAGG-A-AGGTGGGG-ACGACGTC--AAGTCATCATG-G-CCTTACG-GG-TAGGGCTACACACGTGCTACAATGGCCGGTA-C-AATGG
                 *                                              *
              1168                                           1208

H. elongata (1)  GT-TGC-AAAGCGG-CGACGTG-----------GAGCTAATCTCA-TAAAGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGA
H. elongata (2)  GT-TGC-AAAGCGG-CGACGTG-----------GAGCTAATCTCA-TAAAGCCGGTCTCAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGA

H. elongata (1)  ATCGCTAGTAATCGTGCATCAG-AATGGCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG-TG
H. elongata (2)  ATCGCTAGTAATCGTGCATCNG-AATGGCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCACGTCACACCATGGGAGTGGACTGCACCAGAAG-TG
                                                                                *
                                                                              1403

H. elongata (1)  GT-TAG-CCTAA-C-----TTCG-----GAGGGCGA-TCACCACGGTGT
H. elongata (2)  GT-TAG-CCTAA-C-----TTCG-----GAGGGCGAGTCA-CACGGAGT
                                             *    *
                                          1462 1466
```

Fig. 5.12 Identity of nucleotides at positions where the results of this study differed from the results of Gauthier et al (1992). The positions indicated are as follows (*E. coli* numbering - see Fig. 5.11):

a) 184

b) 932

c) 1004, 1009

d) 1168

e) 1208

f) 1403; the reverse-complement of the sequence is,
ACACCGCCCGTCA
*

g) 1462, 1466; the reverse-complement of the sequence is,
GGAGGGCGATCACCAC
* *

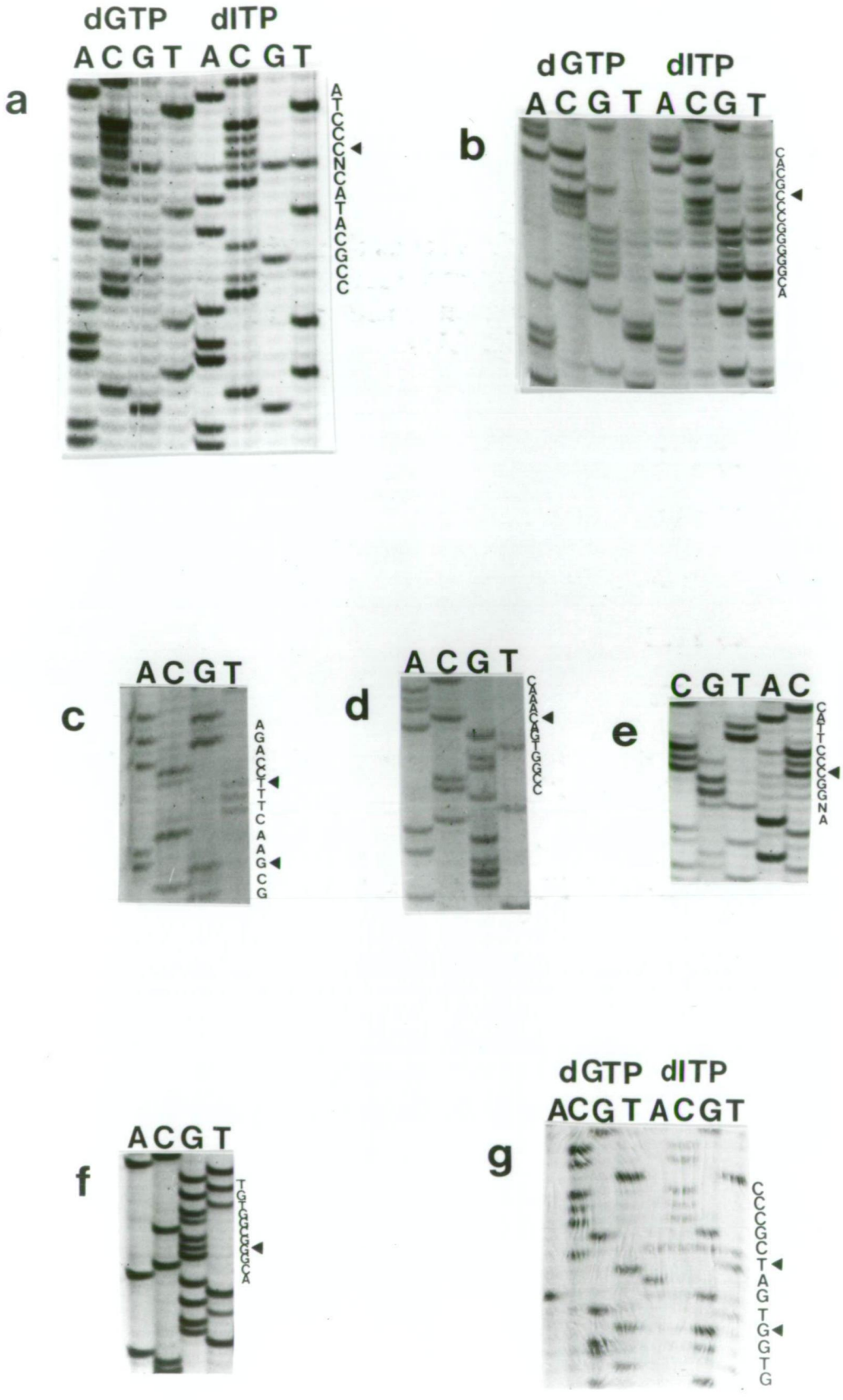
a
dGTP dITP
A C G T A C G T
ATCCCNCATACGCC
b
dGTP dITP
A C G T A C G T
c
A C G T
AGACCTTTCAAGCG
d
A C G T
e
C G T A C
f
A C G T
g
dGTP dITP
A C G T A C G T

Table 5.2 Signature characteristics of the 16S rRNA gene for members of the *Halomonadaceae*, and frequency of occurrence among other members of the gamma-subclass of the *Proteobacteria*.

Signature site[a]	*Halomonadaceae*	aci[b]	aha	ard	car	cox	ecc	ecp	ecs	erh	fll	hin	leg	mba	mlc	mlm	mva	osp	pas	psa	psm	rum	syp	vpa	wlb	xan	xyl	Total[c]
76 - 93	6 pair stem									+				+			+								+			4
484	A															+												1
486	C																											0
640	G													+				+										3
660[d]	A		+															+										1
668~738	A~U	+			+				+							+									+			5
669~737	A~U			+	+	+																+	+		+			6
745[d]	U	+							+		+		+					+		+	+							7
776	U							+																				1
1124	U																+	+						+				3
1297	U											+			+	+			+						+	+	+	7
1298	C			+												+										+	+	4
1423	A					+								+			+					+						4
1424	C			+			+	+	+															+				5
1439	U													+								+						2
1462	A													+								+						2
1464	C					+												+										2

a *E. coli* numbering.

b Sequences examined were from the RNA database project (Olsen et al, 1991), aci=*Acinetobacter calcoaceticus*, ard=*Arhodomonas oleiferhydrans*, car=*Cardiobacterium hominis*, cox=*Coxiella burnetii* str.Q177, ecc=*Ectothiorhodospira halochloris* str. A, ecp=*Ectothiorhodospira halophila* str. SL 1, ecs=*Ectothiorhodospira shaposhnikovii*, erh=*Erwinia herbicola*, fll="*Flavobacterium*" *lutescens*, hin=*Haemophilus influenzae*, leg=*Legionella pneumophila*, mlc=*Methylococcus capsulatus* str. BATH, mlm=*Methylomonas methanica* str 81Z, osp=*Oceanospirillum linum*, pas=*Pasturella multocida*, psa=*Pseudomonas aeruginosa*, psm=*Pseudomonas mendocina*, rum=*Ruminobacter amylophilus*, syp=str. symbiont P of *Acyrthosyphon pisum*, vpa=*Vibrio parahaemolyticus*, wlb=*Wolbachia persica*, xan=*Xanthomonas maltophilia*, xyl=*Xylella fastidiosa* str. PWT-100; and from Genbank, aha=*Alteromonas haloplanktis*, mba=*Marinobacter hydrocarbonoclasticus*, mva=*Marinomonas vaga*.

c The number of organisms belonging to the gamma subclass of the *Proteobacteria* out of a sample of 38 available sequences (excluding members of the *Halomonadaceae*) which contained the same 16S rRNA signature characteristic as the members of the *Halomonadaceae*.

d 660~745 are paired.

Evolutionary distances calculated by the Jukes-Cantor equation, and percentage sequence similarities between members of the genera *Deleya*, *Halomonas* and *Halovibrio*, and some representatives from the gamma-subclass of the *Proteobacteria* are shown in Table 5.3. *D. aquamarina* and *H. meridiana* were the most highly related organisms, having identical sequences over the determined region. The distances between the other members of the three genera were in the range 0.026 - 0.086, and the percentage sequence similarities were in the range 92.5 - 97.6. The closest relative of the *Halomonadaceae* was *Oceanospirillum linum*. The evolutionary distances between *O. linum* and members of the *Halomonadaceae* were in the range 0.11 - 0.125, and sequence similarities were in the range 88.8 - 90.4%.

A phylogenetic tree derived by distance analysis is shown in Fig. 5.13. Two trees of equal minimal length were found by parsimony analysis (Fig. 5.14). One of these (Fig. 5.14 b) had the same topology as the tree derived from distance analysis. This topology also resulted from distance and parsimony analysis of the conserved data set (Fig. 5.15). The members of the genera *Halomonas* and *Deleya* did not form separate monophyletic subgroups in these trees and confirmed the lack of any phylogenetic support for their retention as separate genera.

Halovibrio variabilis clustered among the members of the *Halomonadaceae* (Fig. 5.13) and was most closely related to *D. aquamarina* and *H. meridiana*, with 16S rRNA sequence similarity of 96.7%. *Halovibrio variabilis* was clearly a member of the *Halomonadaceae*, and is treated as such in the discussion that follows.

The members of the *Halomonadaceae* separated into the following three clusters:

1) *D. aquamarina*, *H. meridiana*, *Hv.variabilis*, *H. subglaciescola*;

2) *H. elongata*, *H. halmophila*, *D. halophila*;

3) *D. marina*.

Bootstrap analyses using both the parsimony and distance methods however indicated that the internal topology of the *Halomonadaceae*

Table 5.3 16S rRNA evolutionary distances as calculated by the Jukes-Cantor equation (below the diagonal), and 16S rRNA % similarities (above the diagonal), between members of the genera *Deleya*, *Halomonas* and *Halovibrio*, and some other members of the gamma-subclass of the *Proteobacteria*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*D. aquamarina*	-	100	96.7	95.3	94.0	94.0	94.0	93.6	89.5	88.4	88.2	87.5	85.3	84.9	82.8
2	*H. meridiana*	0.000	-	96.7	95.0	93.7	93.8	93.9	93.6	89.1	88.2	88.0	87.2	85.0	84.6	82.7
3	*Hv. variabilis*	0.036	0.035	-	94.5	92.5	93.1	94.0	92.6	88.8	88.1	87.5	86.9	84.4	83.1	82.6
4	*H. subglaciescola*	0.051	0.053	0.059	-	94.8	94.8	94.5	93.2	89.2	88.1	87.4	88.1	83.8	83.2	82.0
5	*H. halmophila*	0.065	0.068	0.082	0.055	-	97.6	95.7	93.5	89.0	86.8	87.3	87.3	83.3	83.0	81.9
6	*H. elongata*	0.066	0.068	0.075	0.056	0.025	-	96.7	94.1	89.4	87.6	87.7	87.7	83.5	83.5	82.1
7	*D. halophila*	0.068	0.069	0.068	0.061	0.047	0.036	-	95.2	90.2	88.7	87.8	88.5	84.0	83.7	82.7
8	*D. marina*	0.073	0.073	0.085	0.077	0.073	0.067	0.056	-	90.4	89.0	87.9	88.2	84.7	84.1	82.8
9	*O. linum*	0.118	0.121	0.125	0.120	0.120	0.118	0.111	0.110	-	89.7	88.4	89.4	84.2	84.2	83.8
10	*Mb. hydrocarbonoclasticus*	0.132	0.133	0.134	0.134	0.148	0.140	0.132	0.128	0.113	-	88.1	88.3	84.1	83.6	82.2
11	*P. aeruginosa*	0.133	0.134	0.141	0.142	0.140	0.137	0.142	0.140	0.127	0.133	-	88.2	86.0	84.3	82.6
12	*Mm. vaga*	0.142	0.145	0.149	0.132	0.142	0.138	0.133	0.137	0.115	0.130	0.130	-	84.3	85.3	83.8
13	*R. amylophilus*	0.170	0.171	0.180	0.186	0.191	0.190	0.192	0.183	0.178	0.182	0.156	0.178	-	82.8	85.3
14	*W. persica*	0.176	0.179	0.199	0.196	0.197	0.192	0.198	0.193	0.180	0.191	0.179	0.167	0.198	-	81.7
15	*Pt. multocida*	0.203	0.202	0.204	0.211	0.210	0.210	0.211	0.210	0.185	0.208	0.201	0.185	0.164	0.214	-

D.= Deleya, H.= Halomonas, Hv.= Halovibrio, O.= Oceanospirillum, Mb.= Marinobacter, P.= Pseudomonas, Mm.= Marinomonas, R.= Ruminobacter, W.= Wolbachia, Pt.= Pasteurella.

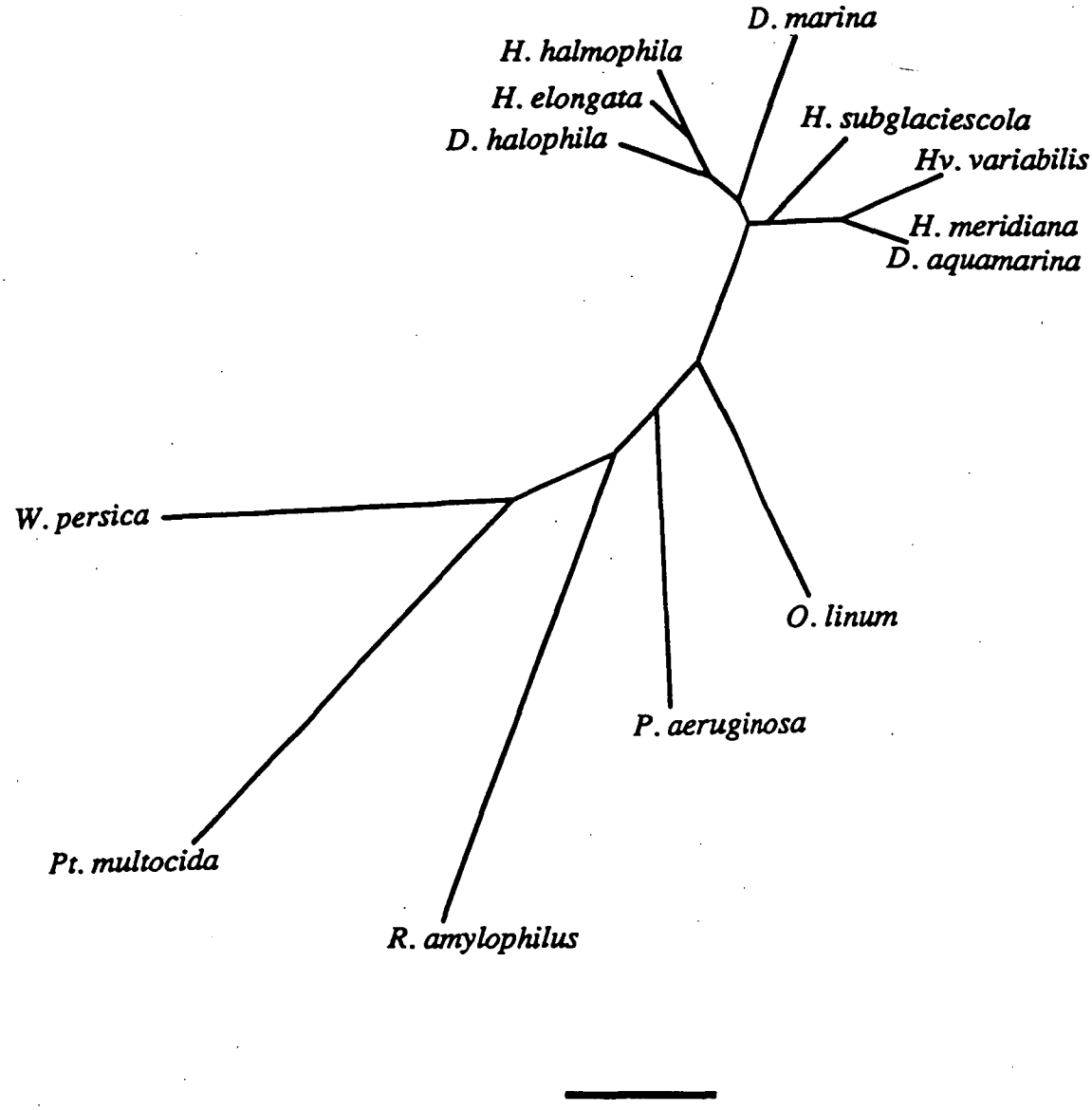

Fig. 5.13 Phylogenetic tree derived by distance analysis of 16S rRNA sequences, showing the relationships between members of the genera *Deleya*, *Halomonas* and *Halovibrio*, and some other members of the gamma-subclass of the *Proteobacteria*. *Pasteurella multocida* is the outgroup organism. Bar = 0.03 evolutionary distance units as calculated by the Jukes-Cantor equation.

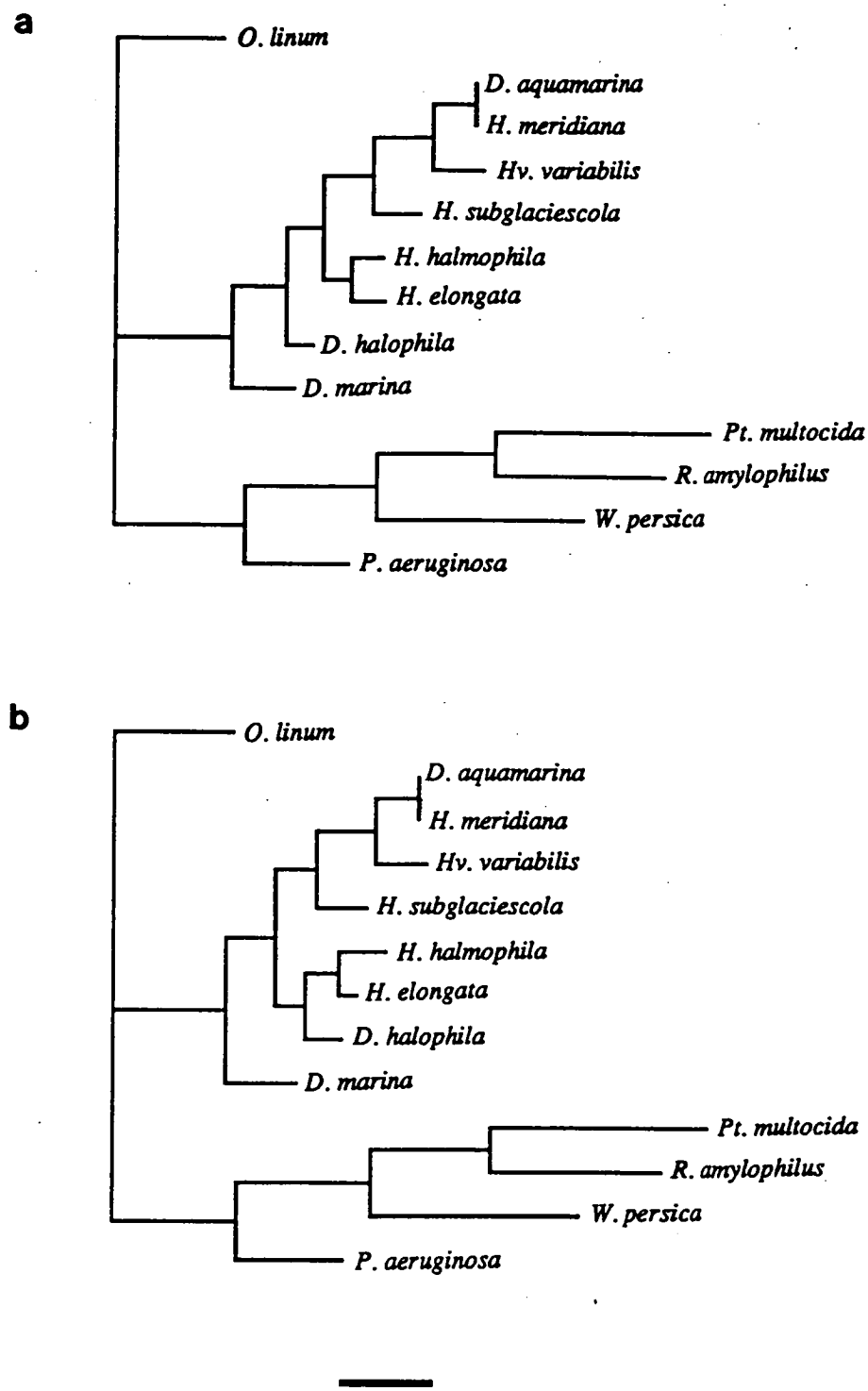

Fig. 5.14 Equally most parsimonious trees (a, b) derived from an analysis of the 16S rRNA sequences of members of the genera *Deleya*, *Halomonas* and *Halovibrio*, and some other members of the gamma-subclass of the *Proteobacteria*. *Pasteurella multocida* is the outgroup organism. Bar (length of horizontal branches) = 50 nucleotide differences.

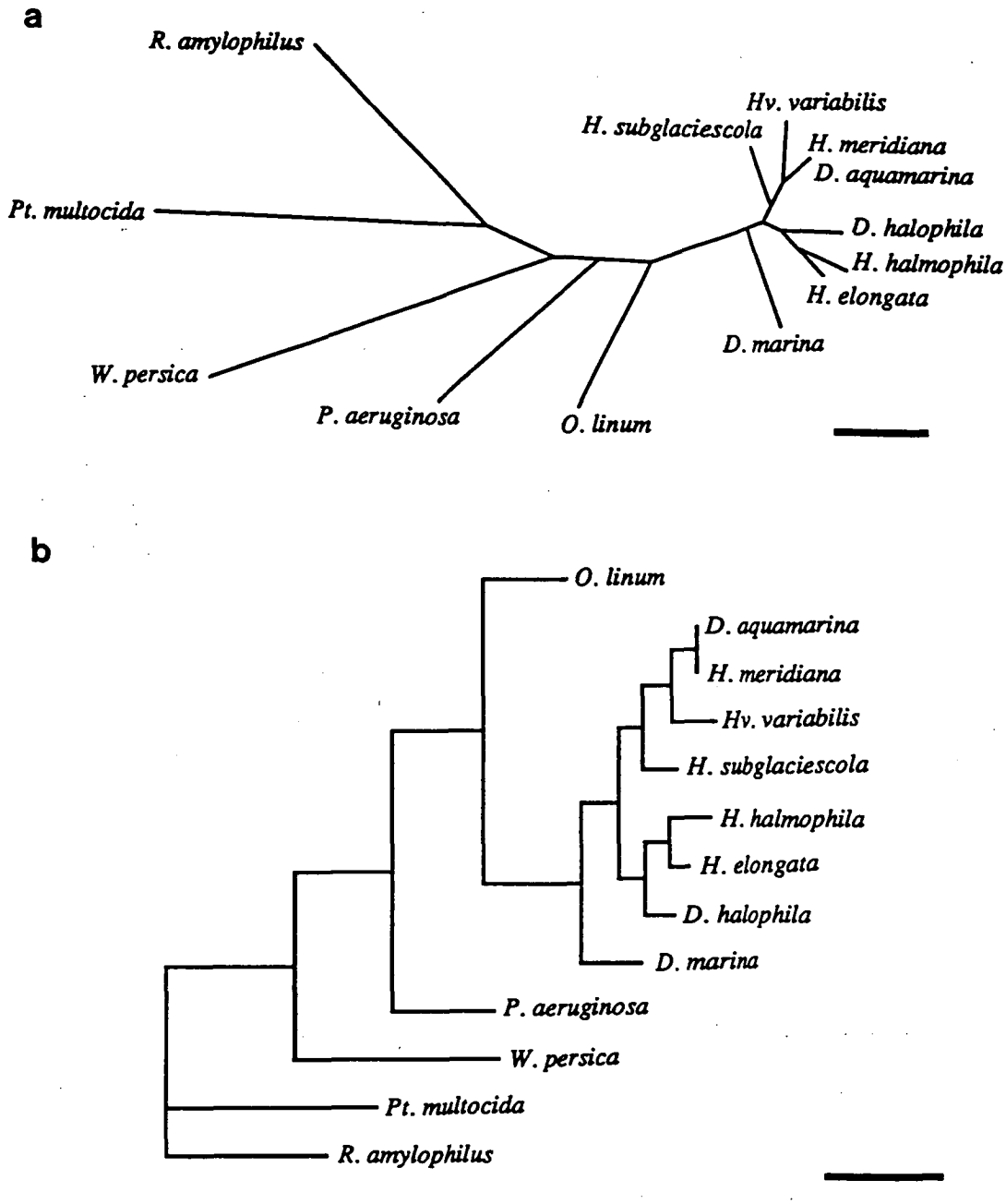

Fig. 5.15 Phylogenetic trees derived by (a) distance analysis, and (b) parsimony analysis, from a character set limited to positions which were conserved (≥ 50%) amongst members of the genera *Deleya*, *Halomonas* and *Halovibrio* and 35 other members of the gamma-subclass of the *Proteobacteria*. (a) Bar = 0.02 evolutionary distance units as calculated by the Jukes-Cantor equation. (b) Bar = 50 nucleotide differences.

group was not stable (Fig. 5.16). Strong support existed only for the *D. aquamarina*, *H. meridiana* and *Hv. variabilis* sub-cluster within cluster 1 described above.

An examination of the sequence similarity values also suggested that the clusters did not represent unreservedly distinct subgroups within the *Halomonadaceae* (Table 5.3). The differences in the distances of *H. subglaciescola* and *D. halophila* to members of different subgroups were marginal. *H. subglaciescola* was related to the other members of the *D. aquamarina* subgroup in the range 94.5 - 95.3%, and to the *H. elongata* and *H. halmophila* at 94.8%. *D. halophila* was related to the other members of the *H. elongata* subgroup in the range 95.7 - 96.7% and to *D. marina* at 95.2%. Therefore *H. subglaciescola* and *D. halophila* were not clearly aligned to one subgroup, but were intermediary between subgroups.

The inclusion of *H. subglaciescola* in cluster 1 was also not recommended from an examination of the character data (Fig. 5.10). There were a greater number of positions where *H. subglaciescola* was monophyletic with regard to *H. elongata*, *H. halmophila* and *D. halophila*, than the number where it was monophyletic with regard to the other members of cluster 1. Similarly the uncertainty about the placement of *D. halophila* was evident from the character data. The number of sequence positions where the identity of the base in *D. halophila* was the same as *H. halmophila* and *H. elongata*, and different to all other members of the *Halomonadaceae* was 5, compared with the 7 positions where the identity of the base in *D. halophila* was the same as *D. marina*, and different to all other members of the *Halomonadaceae*.

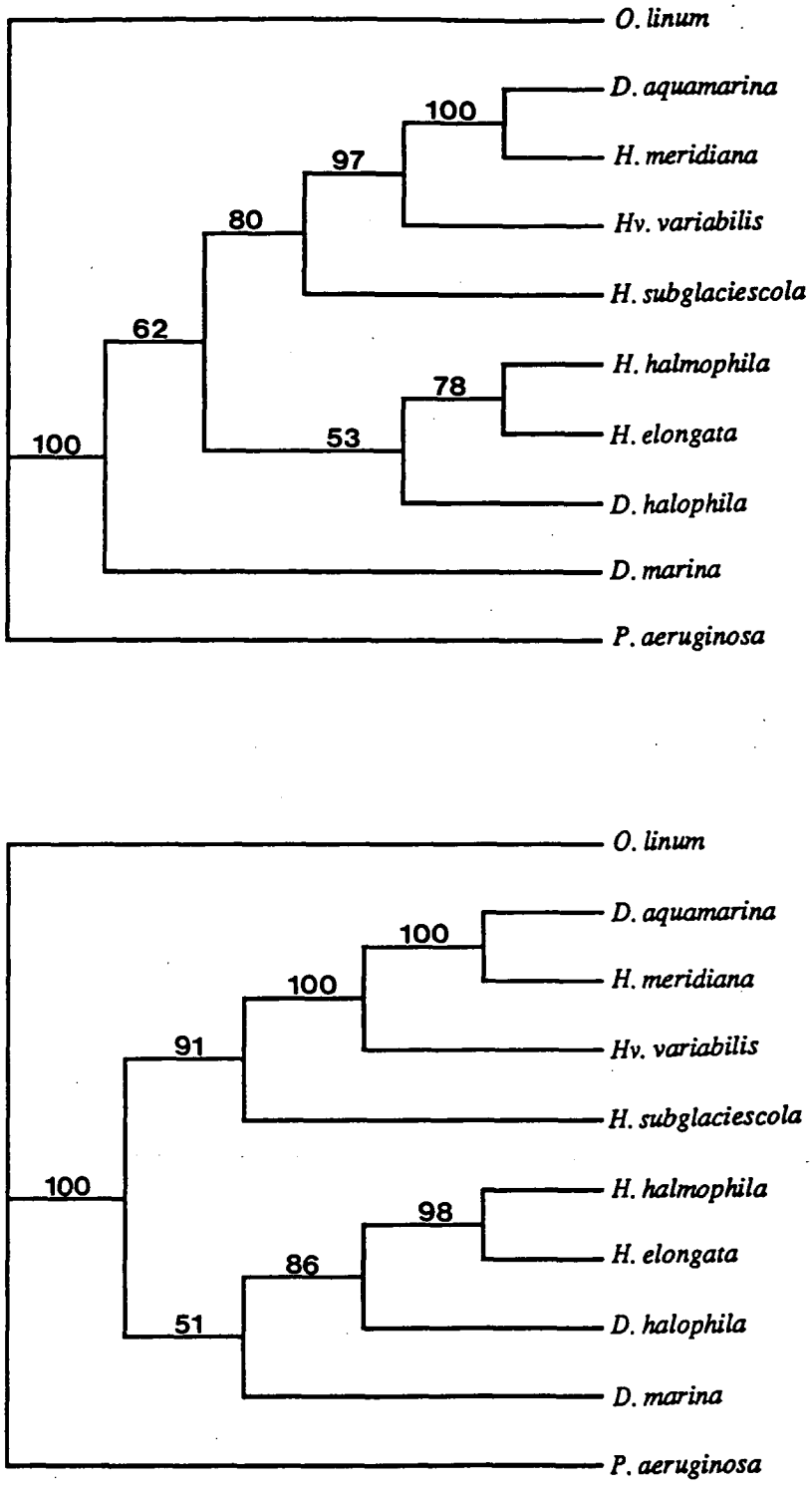

Fig. 5.16 Bootstrap consensus trees derived from (a) parsimony analysis and (b) distance analysis (2000 replications), showing the % frequency of occurrence of groups, where ≥ 50%.

6 DISCUSSION

6.1 Organic Lake flavobacteria

6.1.1 Generic assignment of the Organic Lake flavobacteria

The tree derived from all the available 16S rRNA sequences of members of the "flavobacteria-*Bacteroides*" phylum (Fig. 5.3) indicates that the phylogenetic relationships between some members of the aerobic, pigmented bacteria belonging to the phylum, is of the same depth or deeper, than the relationship between some aerobic pigmented species and the anaerobic *Bacteroides* spp. In comparison with the phylogenetically and phenotypically coherent grouping of the *Bacteroides* spp., the spread of species belonging to such genera as *Cytophaga*, *Flavobacterium*, *Flectobacillus* and *Flexibacter* throughout the tree underlines the inadequacies of the phenotypic descriptions which delimit these taxa.

Given the heterogeneity that exists within the genera of aerobic, pigmented bacteria belonging to the "flavobacteria-*Bacteroides*" phylum, it would only be appropriate to assign the Organic Lake flavobacteria to an existing genus if it had a close relationship to the type species of an existing genus. The marine *Cytophaga* spp., to which the Organic Lake flavobacteria were most closely related, were only very distantly related to the type species of *Cytophaga*, *C. hutchinsonii*. Thus these marine *Cytophaga* spp. do not belong in the genus *Cytophaga*, and the Organic Lake flavobacteria could not be assigned to this genus. Similarly, the Organic Lake flavobacteria were only distantly related to the type species of the genera, *Flexibacter*, *Sphingobacterium*, *Flectobacillus*, *Runella*, *Cyclobacterium*, *Spirosoma*, and *Microscilla*.

The Organic Lake flavobacteria were more closely related to the type species of the genus *Flavobacterium*, *F. aquatile*. ACAM 44 and ACAM 48 were located in a branch of the "flavobacteria-*Bacteroides*" phylum that contained *F. aquatile* and several species isolated from marine and terrestrial environments. Woese et al (1990c) found that this branch of the phylum was well supported by

ten signature features which met the stringent condition of having a constant composition at the signature positions among the members of the branch, and a different and constant composition among the other members of the phylum examined. ACAM 44 and ACAM 48 had respectively 89.0% and 89.9% 16S rRNA sequence similarity to *F. aquatile*. The question to be considered was whether these similarity levels were sufficient to assign the Organic Lake flavobacteria to the genus *Flavobacterium*.

The levels of 16S rRNA sequence similarity between member-species of a genus vary dramatically, in the range 78 - 95%, for many well-defined genera. This range is indicative of the variation in age of different genera (Stackebrandt, 1992). While the level of relationship between the Organic Lake flavobacteria and *Flavobacterium aquatile* is in the upper part of this range, it is not necessarily evident that these species should automatically be placed in this genus. An appropriate level of 16S rRNA sequence similarity for genera within this phylum might be indicated by another branch of the "flavobacteria-*Bacteroides*" phylum, such as the branch which contains a group of medically important *Flavobacterium* spp., *F. breve*, *F. meningosepticum*, *F. indoltheticum*, *F. balustinum*, *F. gleum* and *F. indologenes*, identified as group A by Holmes and Owen (1981). The similarity levels between the members of this branch are ≥ 86.9%. On this basis it would be appropriate to assign the Organic Lake flavobacteria to the genus *Flavobacterium*, on the basis of its 16S rRNA sequence similarity to *F. aquatile*.

It is evident from the close relationship of the type species of *Flavobacterium*, *F. aquatile* and gliding species such as *C. aquatilis*, that in the future, the genus description of *Flavobacterium* will be amended to include gliding organisms. Gliding motility has been observed for many of the species most closely related to the Organic Lake flavobacteria, but not their closest relative, *C. latercula* (Reichenbach, 1989). Gliding motility or spreading growth has not been observed for the Organic Lake flavobacteria, although their cell morphology and cell arrangement are similar to their gliding relatives (Dobson, S. J., 1988).

The terrestrial (freshwater and soil) species belonging to the branch which contains the Organic Lake flavobacteria, form a

phylogenetically coherent cluster within the branch, as has been noted previously (Nakagawa and Yamasato, 1992). In the future, consideration may be given to selection of a higher 16S rRNA similarity level between member-species of the genus *Flavobacterium*, and limiting the membership of this genus to terrestrial species. Currently, habitat is the only phenotypic characteristic which would differentiate the group of terrestrial species from the other members of the branch. Possession of flexirubin pigments is often associated with terrestrial species, in contrast to marine species which lack these pigments, however *C. flevensis* is an exception. *C. flevensis* does not contain flexirubin pigments, is agarolytic and grows in full-strength seawater media. In light of these characteristics and its isolation from a freshwater lake which was originally a marine environment, it has been suggested that this species may be a marine organism (Reichenbach, 1989). The marine-origin hypothesis for this species is however doubtful given its position among the terrestrial species in the 16S rRNA phylogenetic tree (Fig. 5.3). The phenotypic characteristics of *C. flevensis*, which are unusual for a freshwater species indicate that unequivocal phenotypic discrimination between marine and terrestrial groups may be difficult.

Reichenbach (1989) suggested that *C. lytica*, *C. marinoflava*, *C. latercula*, and *C. uliginosa* were subgroups of the genus *Cytophaga*, and may form the basis of new genera. If a high level of 16S rRNA sequence similarity were chosen to define generic groups, then this would be appropriate. The bootstrap analyses indicated that there was no support for the clustering of any pair or larger group of these marine *Cytophaga* spp (Figs. 5.8 and 5.9). The 16S rRNA sequence distances between some of these marine species was greater than the distance between some of the terrestrial species and some of the marine species. *F. odoratum* may also form a separate genus as has been proposed (Holmes and Owen, 1981; Holmes, 1992). It was equidistant from *F. aquatile* and *C. lytica* (Table 5.1). The other marine cluster in this branch, comprised of *Fc. glomeratus*, *"A. vesiculatum"*, and *Fx. aggregans* may also define a new genus. *Fc. glomeratus*, *"A. vesiculatum"* and *Fx. aggregans* have been characterised respectively as being curved or ring-shaped cells , containing gas vesicles, and being long threadlike cells (20 to >

100 μm), indicating that morphological characteristics will not define this group.

ACAM 44 and ACAM 48, like the marine *Cytophaga* species to which they are most closely related, may represent new genera, if a high 16S rRNA similarity was considered appropriate to define genera within this branch. The Organic Lake flavobacteria are the only moderately halophilic flavobacteria thus far described; however the significance of this characteristic in defining taxa at the generic level has not been established. As descriptions of flavobacterial genera are replete with inappropriately chosen phenotypic traits, the choice of moderate halophilism/halotolerance to identify these organisms as representative of new genera could not be justified, without any evidence that this characteristic is associated with a monophyletic group at the genus level. The existence of a higher 16S rRNA similarity level between *C. latercula*, a species with a NaCl concentration tolerance limit of 3.5 % (Reichenbach, 1989), and ACAM 48, than that between the two moderately halophilic organisms ACAM 44 and ACAM 48, suggests that moderate halophilism/ halotolerance is not a phenotypic character useful in defining a phylogenetic group at the genus level or higher taxonomic levels, within the "flavobacteria-*Bacteroides*" phylum.

Some features of the fatty acid profiles of ACAM 44 and ACAM 48 are unique within the library of profiles available for aerobic, pigmented members of the "flavobacteria-*Bacteroides*" phylum, and these features may define phylogenetic groups for these organisms at the genus level. ACAM 44 uniquely contains a15:0 as the major fatty acid, and also contains a15:1ω10c. ACAM 48 uniquely contains i16:1ω7c. However, the occurrence of these fatty acid signatures in other aerobic, pigmented members of the "flavobacteria-*Bacteroides*" phylum cannot be ruled out as *F. aquatile*, *C. aquatilis*, *C. johnsonae*, *C. latercula*, and *Fc. glomeratus* (Tables 2.2 and 2.4; Nichols et al, 1993) are the only close relatives of the Organic Lake flavobacteria for which profiles are available. The variation between the phospholipid ester-linked fatty acid profiles of ACAM 44 and ACAM 48 is notable however and may indicate that fatty acid characteristics could be useful in describing new genera amongst this group (Table 6.1). The fatty acid

Table 6.1 Percentage phospholipid ester-linked non-hydroxy[a] fatty acid composition of representative strains of the Organic Lake flavobacteria[b].

	Straight-chain acids						Branched-chain acids												
	14:0	15:0	15:1	16:0	16:1	17:0	17:1	i-14:0	i14:1	i-15:0	a-15:0	i-15:1	a15:1	i-16:0	i16:1	i-17:0	a-17:0	i-17:1	a-17:1
ACAM 44	1	1	T	-	-	1	-	11	4	3	53	T	15	12	-	-	-	-	T
ACAM 48	T	5	1	4	10	-	1	-	-	25	12	4	-	8	2	3	2	14	8

a The hydroxy fatty acids, 3-OHa15:0, 3-OHi16:0, 3-OH16:0, 3-OHi17:0 and 3-OHa17:0 were present in the PLFA profiles of both strains.
b Data from Skerratt et al, 1991; T < 0.5.

profile of ACAM 48 is distinguished from that of its nearest relative, *C. latercula*, by the presence of a15:0, i16:1, 16:1, a17:1, and a17:0 (Table 2.4).

The fatty acid composition of bacteria is sensitive to the parameters of culture, including composition of the culture media, pH, temperature, oxygen tension and the phase of growth when harvested. Additionally, some fatty acid types in early analyses were not identified. An extreme example is the profile of *C. hutchinsonii* obtained by Oyaizu and Komagata (1981) which included an unidentified component that represented 20% of the total fatty acids. Unidentified components are usually minor species, however the identity of minor components will be significant in the comparison of groups of bacteria which have similar profiles with respect to their major components. A definitive analysis of the fatty acid composition of the members of the branch which contains the Organic Lake flavobacteria therefore requires a comparison of all species grown under standardised conditions.

ACAM 44 and ACAM 48 had a 16S rRNA similarity of 91.8%. ACAM 48 and *C. latercula* had a 16S rRNA similarity of 92.6%. If these organisms were considered representatives of different genera, it would suggest that a 16S rRNA % similarity ≥ 93, would exist between members-species of a genus in the branch of the "flavobacteria-*Bacteroides*" phylum to which the Organic Lake bacteria belonged. At this similarity level the members of the branch would separate into eight genera. A full set of chemotaxonomic and phenotypic data, obtained under standardised conditions, is required before the subdivision of this branch into several genera could be properly considered; as well as agreement among practitioners as to the nature and extent of chemotaxonomic and phenotypic variability which discriminates between genera for this group. Consideration also needs to be given to the possibility that as sequences of additional organisms are determined, that the distinctions evident in this data set may fade.

In light of the current taxonomy it is proposed that the Organic Lake flavobacteria be assigned to the genus *Flavobacterium*, on the basis of their location in a branch of the "flavobacteria-

Bacteroides" phylum which contains the type species *Flavobacterium aquatile*, and their possession of phenotypic and chemotaxonomic characteristics consistent with the description of the genus *Flavobacterium*. These characteristics are Gram-negative cell wall, a low G+C content of their DNA, aerobic metabolism, yellow/orange pigmentation, menaquinone 6 as the major respiratory lipoquinone, and fatty acid profiles dominated by branched-chain fatty acids.

6.1.2 New species descriptions for the Organic Lake flavobacteria

The 16S rRNA similarity levels between the Organic Lake flavobacteria and their nearest relatives indicated that they could not be placed within any existing species represented in the 16S rRNA database. The 16S rRNA sequences of some flavobacterial species have not been determined, and their similarity to the Organic Lake flavobacteria need to be considered on the basis of other taxonomic data. One of these species *Sphingobacterium multivorum* contains sphingophospholipids. Species which contain this lipid, *S. spiritovorum*, *S. mizutae*, and *F. thalpophilum*, form a phylogenetically coherent branch within the "flavobacteria-*Bacteroides*" phylum, distinct from the branch which contains the Organic Lake flavobacteria (Fig. 5.3). Therefore the Organic Lake flavobacteria could not be placed in this species. Chemotaxonomic data is not available for the other flavobacterial species not represented in the 16S rRNA database. These species are compared with the Organic Lake flavobacteria on the basis of phenotypic characteristics in Table 6.2. Given the separation of marine and terrestrial species in the branch of the "flavobacteria-*Bacteroides*" phylum to which the Organic Lake flavobacteria belong, it is highly improbable that the Organic Lake flavobacteria belong in any of the terrestrial species listed in Table 6.2, so consideration will be confined to the marine species.

The expression of some phenotypic characteristics such as antibiotic susceptibility (Merkel, 1972), motility (Rosenberg, 1983), urease activity, reduction of nitrate and production of acid from carbohydrates (Hebert and Vreeland, 1987) is sensitive to the salt concentration of the growth media. Phenotypic comparisons between organisms observed in studies conducted at different salt concentrations should therefore be treated cautiously. The phenotypic

Table 6.2 Some characteristics which differentiate between the Antarctic flavobacterial species, *Flavobacterium gondwanense* and *Flavobacterium salegens*, and other flavobacterial species, for which chemotaxonomic and 16S rRNA sequence data is not available.

	F. gon.[a]	F.sal.	C. aur.[d]	C. sal.	C. aga.	C. sac.	C. apr.	C. pec.	C.psy.	C. mar.	C. suc.	Fx. fil.
Catalase	+ (9/11)[b]	+	-	+	+	-	-	+	-	+	+	-
Oxidase	+	+	+	N	N	-	+	+	+	+	+	+
H_2S production	-	-	N[e]	N	N	+	+	-	-	-	N	-
Glucose sole carbon and energy source	-	- (4/5)	+	+	+	+	-	+	-	-	+	N
Degradation of												
Casein	- (10/11)	-	N	N	N	-	+	+	+	+	+	
Agar	-	-	-	-	+	+	+	-	-	-	-	N
Chitin	-	-	N	-	-	-	-	+	-	-	-	+
Starch	+	+	-	+	+	+	+	+	-	-	+	-
Strict aerobe (a)/ facultaive anaerobe (f)	a	a (4/5)	a	f	f	a	a	a	a	a	f	a
Flexirubin reaction	-	-	-	N	N	+	-	+	+	N	-	+
Highest NaCl (%) concentration tolerated	15 (9/11)	20 (3/5)	N	3	3	2	6	1	0.8	N	N	0.3
Mol% G+C of type strain	39	41	42	37	41	32	35	34	32	31	38	47
Mol% G+C range	35-39[c]	39-41[c]	N	N	N	32-36	35-37	N	32	31-32	38	46-47

a F. gon. = *F. gondwanense*, F. sal.= *F. salegens*, C. aur.=*C. aurantiaca*, C. sal.=*C.salmonicolor*,C. aga.=*C. agarovorans*, C. sac.=*C. saccharophila*,C. apr.=*C.aprica*, C. pec.=*C. pectinovora*, C. psy.=*C.psychrophila*, C. mar.=*C. marina*, C. suc. =*C. succinicans*, C. col.=*C. columnaris*, Fx. fil.=*Flexibacter filiformis*.

b The result given is for the type strain.The fraction in brackets indicates the number of strains which had this reaction.

c The mol %G+C range for this species is based on three strains (Dobson et al, 1991).

d The data for the non-Antarctic species is from Reichenbach, 1989.

e N=no result.

characteristics of the Organic Lake flavobacteria were determined in 3% Artificial Organic Lake Peptone media (3% AOLPM) which contained 3% NaCl and 2% other salts (Dobson, 1988). The phenotypes of the marine species in Table 6.2 were determined in media containing seawater salt concentrations, 3.5% (Lewin and Lounsbery, 1969; Veldkamp, 1961; Hikadi et al, 1979). This difference in salt concentrations is not substantial. The reported salt-determined phenotypic variations have been associated with more significant differences in salt concentrations. The vibriostatic agent, 2,4-diamino-6,7-diisopropyl pteridine, is effective in freshwater, but not seawater (Merkel, 1972). The phenotypic variations noted by Hebert and Vreeland (1987) were related to growth in media containing 2.6% total salts, compared with growth in media containing 10% total salts. A further indication that the salt concentration difference between seawater and 3% AOLPM, does not produce a significant impact on phenotypic expression, is given by a comparison of the phenotype of *C. lytica* grown on seawater-based media and 3% AOLPM. The phenotype of *C. lytica* grown at these two salt concentrations was the same across the range of examined characteristics, with the exception of glucose utilisation as a sole carbon source (Lewin and Lounsbery, 1969; Dobson, 1988). A phenotypic comparison between the marine species in Table 6.2 and the Organic Lake flavobacteria appears valid. This phenotypic comparison shows that the Organic Lake flavobacteria are phenotypically distinct from *C. salmonicolor*, *C. agarovorans*, *C. aprica* and *C. marina* on the basis of catalase, casein degradation, starch degradation, agar degradation, facultatively anaerobic metabolism, and halotolerance. *C. marina* exhibits the closest phenotypic similarity to the Organic Lake bacteria across the range of characteristics examined; however, *C. marina* has a significantly lower G+C content.

ACAM 44 and ACAM 48 were representative strains from two clusters observed in a numerical phenotypic study (Dobson, 1988). The phenotypic characteristics which differentiated between the members of these two clusters are listed in Table 6.3. On the basis of these phenotypic differences, differences in their fatty acid profiles and the level of the 16S rRNA sequence similarity of the

Table 6.3 Phenotypic characteristics which differentiate between the members of the two Organic Lake flavobacterial phenons represented by ACAM 44 and ACAM 48 respectively[a].

	ACAM 44 Phenon (11 strains)	ACAM 48 Phenon (5 strains)
Colony pigmentation:		
Orange	+	-
Yellow	-	+
Nitrate reduction to nitrite	-	+
Growth in 20% NaCl	-	+ (3/5)[b]
Growth stimulated by:		
D-gluconic acid	-	+
Maltose	-	+
Acid production from:		
L-arabinose	- (6/7)[b]	+
Mannose	- (6/7)[b]	+

a Data from Dobson, 1988.
b No. of strains/no. ofstrains for which a result was obtained.

representative strains it is proposed that two new species, *Flavobacterium gondwanense* and *Flavobacterium salegens* be created to accommodate the Organic Lake flavobacteria. The descriptions of these species are as follows:

***Flavobacterium gondwanense* sp. nov.** *Flavobacterium gondwanense* (Gondwanaland or Gondwana n. one of the two ancient supercontinents comprising what are now Africa, South America, Australia, Antarctica and the Indian subcontinent (Hanks, 1986)) cells are rod-shaped and occur as single cells, pairs and short chains. Cell widths range from 0.4 μm - 0.8 μm and cell lengths range from 1.7 μm -11.7 μm. Cells are Gram-negative, non-motile and have a strictly aerobic metabolism. Colonies on solid media are orange, circular and entire. Members of this species are moderately halophilic, and grow optimally in media containing 5% total salts. Pigment production is inhibited on media containing Tween 20. Members of this species produce cytochrome oxidase, phosphatase, DNAase and hydrolyse esculin and starch. They do not reduce nitrate to nitrite, produce ß-galactosidase, ornithine or lysine decarboxylase, utilise malonate, grow in 20% NaCl, grow at 37 °C or hydrolyse chitin or agar. They do not produce spreading growth on low-peptone agar. No bathochromic shift is observed on the addition of 20% KOH to colonies. All strains grow in 10% NaCl . The growth of all members of the species is not stimulated by a wide range of single carbon sources, including proline, sucrose, l-rhamnose, salicin, d-glucose, d-galactose, glycine, hydroxy-l-proline, d-mannose, d-gluconic acid, maltose, serine, d-trehalose, tryptophan, alanine, cysteine, histidine, l-arabinose, tyrosine, glycerol, inositol, isoleucine, maltose, leucine and valine. Acid is not produced from galactose by any strain. All members of the species are sensitive to novobiocin (30 μg), chloramphenicol (30 μg) and erythromycin (15 μg); and resistant to 0/129 (10 μg), polymyxin B (300 U), and gentamicin (10 μg). The mol% G+C of this species, determined for three strains, ranges from 35 to 39. The type strain is ACAM 44 which has a mol% G+C of 39. Distinctive features of the fatty acid profile of ACAM 44 are the occurrence of a15:0 as the major fatty acid and the presence of a15:1ω10c, 3-OHa15:0 and 3-OHa17:0. The major respiratory

lipoquinone is menaquinone 6. The species is represented by 11 strains (ACAM 1, 40, 41, 43, 44, 45, 46, 49, 51, 56, 62).

***Flavobacterium salegens* sp. nov.** *Flavobacterium salegens* (L. n. *sal*, salt; L. a. *egens*, needy) cells are rod-shaped and occur as single cells, pairs and sometimes chains. Cell widths range from 0.5 μm to 0.8 μm and cell lengths range from 1.2 μm to 11.5 μm. Cells are Gram-negative and non-motile. Members of the species are aerobic. Colonies on solid media are yellow, circular, entire and convex. Members of this species are moderately halophilic, and grow optimally in media containing 5% total salts. Growth on Tween 20 agar inhibits pigment production. All members of this species produce catalase, cytochrome oxidase, phosphatase, DNAase, ß-galactosidase, reduce nitrate to nitrite, grow in 15% NaCl, hydrolyse Tween 20, starch and esculin, and liquefy gelatin. No strains produce ornithine or lysine decarboxylase, utilise malonate, hydrolyse chitin, agar or casein or produce spreading growth on low peptone agar. No bathochromic shift is observed on the addition of 20% KOH to colonies. The growth of all members of the species is stimulated by arginine, d-gluconic acid, pyruvate, maltose, ornithine and lactose. The growth of all strains is not stimulated by adonitol, l-rhamnose, salicin, glycine, hydroxy-l-proline, d-mannose, d-xylose, serine, d-trehalose, l-asparagine, l-lysine, histidine, l-arabinose, cysteine, threonine, tyrosine, glycerol, inositol, isoleucine, maltose, leucine and valine. All strains produce acid from l-arabinose, maltose and mannitol. All strains are sensitive to novobiocin (30 μg), chloramphenicol (30 μg), ampicillin (10 μg), erythromycin (15 μg) and cephalothin (30 μg) and are resistant to 0/129 (10 μg) and neomycin (30 μg). The mol% G+C of this species, determined for three strains, ranges from 39 to 41. The type strain is ACAM 48 which has a mol% G+C of 41. Distinctive features of the fatty acid profile of ACAM 48 are the presence of 16:1ω7c, 3-OHa15:0 and 3-OHa17:0. The major respiratory lipoquinone is menaquinone 6. The species is represented by 5 strains (ACAM 2, 48, 52, 53, 54).

6.2 The *Halomonadaceae*

6.2.1 Phylogenetic relationships among members of the genera *Deleya*, *Halomonas* and *Halovibrio*

Six oligonucleotides from the catalogues of *H. elongata*, and *H. halmophila* were identified previously as unique to these organisms within the gamma-subclass of the *Proteobacteria* (Woese, 1985b), however within the wider set of organisms examined in this study, only one of these signatures, occurring at 1297-8 (Table 5.2), had value as a signature for the *Halomonadaceae*. An additional 16 characteristics present in the 16S rRNA sequences of all examined members of the genera *Deleya*, *Halomonas* and *Halovibrio*, and sparsely distributed among the other members of the gamma-subclass of the *Proteobacteria* provide a defining signature for this family (Table 5.2). One of these signature characteristics, a cytosine residue at 486, was extremely rare among the prokaryotes, and has been reported for only one other species, *Listonella anguillara.*

The identical 16S rRNA sequences of *D. aquamarina* and *H. meridiana* suggested that these two species may be synonymous. The 16S rRNA data was consistent with similar fatty acid profiles for the two organisms (Table 6.4). *H. meridiana* and *D. aesta*, a synonym of *D. aquamarina*, also have identical polar lipid patterns (Franzmann and Tindall, 1990). The current, widely accepted definition of a bacterial species is, organisms exhibiting approximately ≥70% DNA-DNA hybridisation and $\Delta T_{m(e)} \leq 5$ °C (Wayne et al,1987).

Amman et al (1992) found 50% DNA-DNA hybridisation corresponded to approximately 99% 16S rRNA sequence similarity among strains of *Fibrobacter*, and suggested that genomic sequence conservation varies among groups of strains having similar evolutionary depth as estimated from 16S rRNA sequence similarity. Fox et al (1992) reported a 99.8% 16S rRNA sequence similarity between two *Bacillus* sp. which exhibited 23 - 50% DNA-DNA hybridisation and concluded that 16S rRNA sequence identity within the limits of experimental error is not sufficient to guarantee species identity, where species are defined by ≥ 70% DNA-DNA hybridisation. A numerical taxonomic analysis (James et al, 1990)

Table 6.4 Percentages (wt/wt) of the major fatty acids of members of the genera *Deleya, Halomonas* and *Halovibrio* [a].

Species[b]	16:0	16:1+17:0cyc	18:1+19:0cyc
H. meridiana	15.5	7.7	73.4
D. aesta	17.0	9.1	71.2
D. aquamarina	17.4	8.4	70.7
D. venusta	15.9	10.7	68.6
Hv. variabilis	17.5	13.8	66.0
H. halmophila	20.2	11.3	64.6
D. halophila	23.3	10.6	64.6
H. elongata	26.0	9.0	62.8
D. cupida	22.6	17.5	56.1
D. pacifica	26.2	15.6	55.7
H. subglaciescola	25.4	19.7	52.3
H. elongata DSM 3043	29.9	16.5	49.8
H. subglaciescola ACAM 21	32.0	28.9	38.2
H. halodurans	27.3	41.7	26.7
D. marina	30.3	43.1	23.5

a Data is from Franzmann and Tindall (1990).
b Type strain unless otherwise indicated.

showed *Halomonas meridiana* and *Deleya aesta* to be phenotypically distinct. Therefore DNA-DNA hybridisation data is required to clarify whether *D. aquamarina* and *H. meridiana* are synonymous. Fox et al (1992) state " In the absence of definitive DNA-DNA data, strains with effectively identical 16S rRNA sequences are best regarded as belonging to the same "rRNA species complex" or "rRNA superspecies". Such strains can be expected to exhibit considerable levels of DNA-DNA homology (typically at least 30%), which need not exceed 70%." According to this definition *D. aquamarina* and *H. meridiana* belong to the same rRNA species complex.

A comparison of DNA:rRNA hybridisation values (Kersters, 1992) with 16S rRNA percentage similarity values showed that of the species common to both studies *H. subglaciescola* was the least distant to *D. aquamarina* (Table 6.5). There were some differences in the arrangement of the other species common to both studies with respect to their similarity to *D. aquamarina*. *H. halmophila*, *H. elongata*, and *D. halophila* had the same level of 16S rRNA sequence similarity with respect to *D. aquamarina*, whereas there was a difference of 1°C in the $T_{m(e)}$ values of the hybrids. The $T_{m(e)}$ values for *H. subglaciescola* and *H. hamophila* were identical whereas there was a 1.3% difference in the 16S rRNA sequence similarities between these species with respect to *D. aquamarina*. Additionally *D. marina* had a higher $T_{m(e)}$ value than that of *D. halophila* by 0.4 °C, but had a lower 16S rRNA sequence similarity by 0.4%. These differences were marginal, and possibly reflect the use of 23S rRNA for the hybridisation studies, and the small sensitivities of hybridisation to the distribution and identity of mismatched bases. The possibility that these differences might have arisen from the presence of undetermined bases in the 16S rRNA sequences was considered. However, the arrangement of 16S rRNA sequence similarity values was identical when the values were calculated from a data set where all positions where there was an undetermined base in any of the sequences were eliminated (data not shown).

DNA:rRNA hybridisation data for members of the *Halomonadaceae* with respect to *D. aquamarina* showed the presence of two subgroups (Kersters, 1992). Species closely related to *D. aquamarina* (3 °C $\Delta T_{m(e)}$) were its synonyms *D. aesta* and

Table 6.5 A comparison of % 16S rRNA sequence similarity, DNA:rRNA $T_{m(e)}$ values[a], and DNA:DNA % homolgy values[b] between *D.aquamarina* and some members of the genera *Deleya, Halomonas* and *Halovibrio* [c].

	% 16S rRNA sequence similarity	DNA:rRNA $T_{m(e)}$ °C	DNA:DNA %homolgy
H. meridiana	100.0	-[d]	-
Hv. variabilis	96.7	-	-
D. venusta	-	79.0	41
D. cupida	-	76.4	16
D. pacifica	-	76.6	12
H. subglaciescola	95.3	76.2	-
H. halmophila	94.0	76.2	-
H. elongata	94.0	75.6	-
D. halophila	94.0	75.2	-
D. marina	93.6	75.6	12

a From Kersters (1992).
b From Akagawa and Yamasato (1989).
c All values indicate relatedness of type strains. Values are not available for *H. halodurans* and *D. salina*.
d - = not available.

Alcaligenes faecalis subsp. *homari*, *D. venusta* and the misnamed taxa "*Achromobacter viscosum*", "*Pseudomonas bathycetes*", "*Agarbacterium alginicum*" and "*Achrombacter turbidus*". Related to *D. aquamarina* in the second subgroup (5 - 7 °C) were *D. pacifica*, *D. cupida*, *H. halmophila*, *H. subglaciescola*, *H. elongata*, *D. marina*, *D. halophila*, and the misnamed taxa "*Achromobacter halophilus*" and "*Chromobacterium marismortui*". The 16S rRNA sequence comparisons determined in this study provided information on the relatedness between all combinations of pairs of organisms, and not just with respect to a single reference organism. While it appeared from the phylogenetic trees that the members of the *Halomonadaceae* clustered into three subgroups, bootstrap analysis and an examination of distance and character data showed that these clusters were not well supported. On the basis of this phylogenetic analysis, the division of the members of the *Halomonadaceae* into three genera in accord with the composition of the three clusters could not be justified.

The only group of species that was well supported in the phylogenetic trees was comprised of *D. aquamarina*, *H. meridiana* and *Hv. variabilis*, which were related at a 16S rRNA sequence similarity of 96.7% (Jukes-Cantor values 0.035 - 0.036) (Fig. 5.16). If this similarity level were considered appropriate for the formation of a genus, this would imply that *H. subglaciescola* and *D. marina* be assigned to two separate genera, *H. halmophila* and *H. elongata* would be assigned to another separate genus, also not well supported from the parsimony bootstrap analysis (Fig. 5.16 a), and the position of *D. halophila* would remain ambiguous. *D. halophila* had a sequence similarity to the *H. halmophila -H.elongata* group in the range 95.7 - 96.7%, and to *D. marina* of 95.2% and the clustering of *D. halophila* with either the *H. halmophila- H. elongata* group or *D. marina* was not supported by the bootstrap analyses. Notwithstanding these uncertainties which would arise from the choice of a similarity level of 96% to define genera within the *Halomonadaceae*, the *D. aquamarina*, *H. meridiana* and *Hv. variabilis* group might be considered a separate genus if its members were phenotypically or chemotaxonomically distinct from the other members of the *Halomonadaceae*.

In a numerical phenotypic comparison which included *H. meridiana*, *Deleya aesta* a synonym of *D. aquamarina*, *H. elongata*, *H halmophila* and *D. halophila*, *H. meridiana* was more distant from *D. aesta* than it was from the other species of the *Halomonadaceae* (James et al, 1990). This indicates a lack of phenotypic characteristics which would distinguish the phylogenetically highly related *H. meridiana* and *D. aquamarina* group from the other species belonging to the *Halomonadaceae*, among those phenotypic characteristics traditionally employed to describe bacterial taxa. A consideration of the phenotypic description of the genus *Halovibrio* is further evidence of the difficulty of finding traditional phenotypic characteristics to define the *D. aquamarina*, *H. meridiana*, *Hv. variabilis* cluster. The characteristic which distinguished the genus *Halovibrio* from most members of the genus *Deleya* was flagella arrangement (polar vs. peritrichous), and the characteristics which differentiated it from the genus *Halomonas* were obligate halophilism, inability to reduce nitrate, and inability to grow on several carbohydrates (Fendrich, 1988). Given the close phylogenetic relationship of *Hv. variabilis* to *D. aquamarina* and *H. meridiana*, it is evident that these phenotypic characteristics are not appropriate for the delineation of genera within the *Halomonadaceae*.

All the members of the *Halomonadaceae* contained the same major fatty acids (Franzmann and Tindall, 1990). Thus, subgroups within the *Halomonadaceae* could not be characterised by signature fatty acids. The major fatty acids, 16:0, 16:1+17:0cyc and 18:0+19:0cyc, represented 95.2 - 99.1% of the total fatty acids. The relative proportions of the fatty acids within the profiles of the members of the *Halomonadaceae* did vary, and they are presented in Table 6.4, in decreasing order of the proportion 18:1+19:0cyc. In this arrangement the *D. aquamarina*, *H. meridiana*, and *Hv. variabilis* group have higher proportions of 18:1+19:0cyc, and lower proportions of 16:0 than the other members of the *Halomonadaceae*, however the differences are marginal. The differences in the proportions of 16:0, 16:1+17:0cyc and 18:1+19:0cyc in *Hv. variabilis* and *H. halmophila* are in the range 1 - 3%. Whether this level of difference is significant would require an estimation of error from repeat determinations of these profiles. If evidence of this difference was repeatable, larger than experimental error, and larger than intra-

species variation, the identification of an unknown strain would still require a concomitant determination of the fatty acid profiles of several reference species of the *Halomonadaceae*, to account for the effect of small differences in culture conditions with each experiment. The creation of a separate genus on the basis of such a small difference in fatty acid profiles is not justified in the opinion of the author. The majority of minor fatty acid components were present at levels < 1% (Table 6.6). Utilisation of these components to define new genera would similarly be unreliable.

The Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics recommended that,

"a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until they can be differentiated be some phenotypic property" (Wayne et al, 1987)

The Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria* expressed a similar opinion with respect to higher taxonomic levels, concluding that,

"the integrated use of phylogenetic and phenotypic characteristics, or polyphasic taxonomy, is necessary for the delineation of taxa at all levels from kingdom to genus", and that,

"The first step in the identification of bacteria is the assignment of organisms to genera. Therefore the greatest clarity in circumscription and utility in the choice of characteristics must be accorded to the level of genera. It is completely impracticable to define genera solely on the basis of phylogenetic data." (Murray et al, 1990)

Although the *D. aquamarina*, *H. meridiana*, *Hv. variabilis* cluster was well supported phylogenetically, phenotypic characteristics could not be identified which would define this group, polar lipid patterns and the identity of respiratory lipoquinones did not discriminate the group from the other members of the *Halomonadaceae*, and the differences in the proportion of fatty acids were marginal and could not practicably employed to define the group. In accord with the opinion of the Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria*, it is concluded

Table 6.6 Minor fatty acid components of the members of the genera *Deleya*, *Halomonas*, and *Halovibrio* [a].

Species [b]	10:0	12:0	14:0	15:0	15:0 anti	17:0 iso	17:0	17:0 anti	18:0	16:1 b	16:1 a	17:1 b	17:1 a	18:1 b	18:1 cis9	F9
H. meridiana	-	-	1.0	+	-	-	+	+	+	-	-	-	-	-	+	-
D. aesta	-	-	1.0	+	-	-	+	+	+	-	-	-	-	-	-	-
D. aquamarina	-	-	1.4	-	-	-	+	-	+	-	-	-	-	-	+	-
D. venusta	-	-	+	+	-	+	+	-	+	-	+	+	+	+	+	+
Hv. variabilis	-	-	+	-	-	-	+	-	+	-	-	-	-	-	+	+
H. halmophila	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+
D. halophila	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+
H. elongata	-	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-
H. elongata DSM 3043	-	+	+	-	-	-	-	-	1.6	-	-	-	-	-	+	+
D. cupida	-	1.1	+	+	-	-	+	-	1.1	-	-	+	-	-	+	-
D. pacifica	-	-	+	-	-	-	+	-	+	-	-	-	-	+	-	+
H. subglaciescola	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	+
H. subglaciescola ACAM21	-	-	+	-	-	-	-	-	+	-	-	-	-	-	1.5	-
H. halodurans	-	+	-	-	-	+	+	-	+	-	-	-	-	-	+	+
D. marina	-	1.0	-	-	-	-	+	-	+	-	-	-	-	-	+	-

a Data from Franzmann and Tindall (1990); "a" or "b" represent unidentified isomers; "anti" = anteiso, "+" = present at less than 1%; "-" = not detected; F9 = Feature 9, unidentified peak.

b Type strain unless otherwise indicated.

that *D. aquamarina*, *H. meridiana*, and *Hv. variabilis* should not be described as a separate genus.

DNA:rRNA hybridisation studies and 16S rRNA oligonucleotide cataloguing studies have previously demonstrated that the genera *Deleya* and *Halomonas* comprise a distinct lineage, the family *Halomonadaceae*, within the gamma-subclass of the *Proteobacteria* (Woese et al, 1985b; Franzmann et al, 1988b). This study further clarifies the phylogenetic relationship between species within this family and demonstrates that the genus *Halovibrio* also belongs within it, as had been suggested from an examination of its chemotaxonomic features (Franzmann and Tindall, 1990).

The greatest distance between two species belonging to the *Halomonadaceae* was 0.086, between *D. marina* and *Hv. variabilis*, which had a 92.6% 16S rRNA sequence similarity. The level of 16S rRNA similarity that has been observed between member-species of well described genera ranges from 78 - 95% (Stackebrandt, 1992). At 95% sequence similarity the relationships among the members of the *Halomonadaceae* were not well resolved. At this level it was unclear whether *H. subglaciescola* belonged to the *D. aquamarina-H. meridiana-Hv. variabilis* subgroup or to the *H. halmophila-H. elongata* subgroup, and it was unclear whether *D. halophila* clustered with *D. marina* or belonged with the *H. halmophila-H. elongata* cluster. This ambiguity was reflected in low levels of support for clusters within the phylogenetic tree, and was evident from an examination of the character data.

Resolution of the placement of *H. subglaciescola* and *D. halophila* would be achieved by selecting a similarity level of 97% to define subgroups within the *Halomonadaceae*. However other problems would remain if this similarity level were used to separate the eight member-species of the *Halomonadaceae* examined. At 97% 16S rRNA sequence similarity *H. subglaciescola*, *D. halophila*, and *D. marina* would separate into individual groups; *H. elongata* and *H. halmophila* would form a cluster (sequence similarity 97.6%), that was not well supported by bootstrap analysis using the parsimony method; and the *D. aquamarina-H. meridiana-Hv. variabilis* group, which was well supported phylogenetically, would break down as

Hv. variabilis had a 16S rRNA sequence similarity to the other two species of 96.7%.

An examination of the fatty acid profiles of member-species of the *Halomonadaceae* not included in the 16S rRNA sequence analysis suggests that the addition of these species to the 16S rRNA phylogenetic tree would result in even less resolution of groups within the tree. The species are arranged in Table 6.4 in order of decreasing amount of 18:1+19:0cyc. The concomitant trends with respect to the other major fatty acids are increasing amounts of 16:0, and increasing amounts of 16:1+17:0cyc, although these are not entirely consistent. The proportions of 16:1+17:0cyc and 18:1+19:0cyc in ACAM 21 are intermediary in the range of those present in *D. marina* and a group of species which included *D. halophila*, while the proportion of 16:0 is similar to that in *D. marina*. Given the similarities that exist between the arrangement of species in Table 6.4 and in the phylogenetic trees, *H. subglaciescola* (ACAM 21) may be phylogenetically intermediary between *D. halophila* and *D. marina*. The fatty acid profiles of *D. cupida* and *D. pacifica* were most similar to *H. subglaciescola* (ACAM 12^T), and the proportions of their 16:1+17:0cyc and 18:1+19:0cyc components were intermediary in the range of those fatty acids in *H. subglaciescola* and *H. elongata*. The exact phylogenetic position of *D. cupida* and *D. pacifica* is not predictable. Their profiles fall within a group that contains *H. halmophila*, *H. elongata*, *D. halophila*, and *H. subglaciescola* (ACAM 12^T). The differences in the proportions of fatty acid types are not large amongst this group and not consistent between trends for each of the three major fatty acid types.

The members of the genera *Halomonas*, *Deleya*, and *Halovibrio*, form a monophyletic phylogenetic group, and share common chemotaxonomic and phenotypic characteristics. Sub-groups among the members of the genera *Halomonas*, *Deleya* and *Halovibrio* cannot be resolved on the basis of phylogenetic data, chemotaxonomic data or phenotypic data. It is therefore proposed that the members of these three genera be united in a single genus.

Rule 44 of the Bacteriological Code (Lapage 1975) states,

"If two or more species of different genera are brought together to form a genus and if these species include the type species of one or more genera, the name of the genus is that associated with the type species having the earliest legitimate generic name."

Halomonas elongata is the type species having the earliest legitimate name, among the three genera (Vreeland, 1980), consequently the generic name *Halomonas* is retained, and the description of the genus emended as follows:

Emended description of the genus *Halomonas* Vreeland 1980

Gram-negative, straight or curved rod-shaped cells. Motile by peritrichous or polar flagella, or non-motile. Slight or moderate halophiles, showing optimal growth in the range 0.3 - 2.0 M NaCl and halotolerant in the range 3.4 - 5.5 M NaCl. Aerobic respiration, but some species can grow anaerobically in the presence of nitrate.

The major respiratory lipoquinone is ubiquinone 9 and the major fatty acids are 16:1ω7c, 16:0, and 18:1ω7c. Most species form 17:0cyc, and 19:0cyc at increased salt concentrations with a concomitant reduction in the proportion of the mono-unsaturated moieties. The G+C content of the DNA ranges from 52 - 68 mol%.

The genus is a member of the family *Halomonadaceae* of the gamma-subclass of the *Proteobacteria*. The 16S rRNA sequences of members of the genus have a cytosine (C) residue at 486 (*E. coli* numbering), and a signature of a further sixteen other features, which characterise this genus within the gamma-subclass of the *Proteobacteria* (Table 5.2).

Emendation of the family *Halomonadaceae* Franzmann, Wehmeyer and Stackebrandt 1988.

The family *Halomonadaceae* is now comprised of a single genus, *Halomonas*. The description of the family *Halomonadaceae* is therefore emended to be the same as the description of the genus *Halomonas* given above.

The species for which 16S rRNA sequences were determined, represented the diversity of environments from which the members of the *Halomonadaceae* have been isolated, which includes seawater, salterns, hypersaline lakes and hypersaline soils; and a large proportion of the breadth of mol% G+C values of the type strains of the species of the *Halomonadaceae*. Type strains selected had mol% G+C values in the range 57 - 67. The range among the type strains of the species of the *Halomonadaceae* is 54 - 68. The species of the *Halomonadaceae* for which 16S rRNA sequences were not determined are *H. halodurans* , *D. cupida*, *D. pacifica*, *D. venusta* and *D. salina.*

A DNA-DNA hybridisation value between *H. halodurans* and *Pseudomonas aeruginosa* of 58.5 ± 2 % has been reported (Rosenburg, 1983). On this basis the placement of *H. halodurans* in the genus *Pseudomonas*, as was originally proposed seems justified. The %16S rRNA sequence similarity values between *P. aeruginosa* and the members of the *Halomonadaceae* are in the range 87.3 - 88.2 (Table 5.3). If *H. halodurans* is a member of the *Halomonadaceae* it would therefore be expected to exhibit 0% DNA-DNA hybridisation with *P. aeruginosa*. Conflicting with the DNA-DNA hybridisation data is the fatty acid profile of *H. halodurans*, which contains the same isomers of the same fatty acids found in all the other members of the *Halomonadaceae* (Franzmann and Tindall, 1990). In particular, the fatty acid profiles of *H. halodurans* and *D. marina* are highly similar which suggests that these two species are very closely related (Tables 6.4 and 6.6). Redetermination of the DNA-DNA hybridisation value between *H. halodurans* and *P. aeruginosa* or determination of the 16S rRNA sequence of *H. halodurans* would resolve this apparent anomaly. Until this anomaly is resolved *H. halodurans* will remain in the genus *Halomonas*.

DNA:rRNA hybridisation data (Kersters, 1992) have indicated that *D. cupida*, *D. pacifica* and *D. venusta* are more closely related to *D. aquamarina*, than are *H. subglaciescola* and 4 other species included in the 16S rRNA sequence comparisons (Table 6.5). That *D. cupida*, *D. pacifica* and *D. venusta* are within the range of variability observed from the 16S rRNA comparisons of the other species was also evident from their fatty acid profiles (Table 6.4). A

fatty acid profile and DNA:rRNA hybridisation data are not available for the recently described *D. salina* (Valderrama et al, 1991). This species had a phenotype consistent with the description of the genus, *Deleya*, and a mol % G+C in the range 61 - 64. As a legitimate species of *Deleya* it is considered appropriate to include it in the proposed taxonomic revisions.

The following new combinations are therefore proposed, *Halomonas aquamarina* (Akagawa and Yamasato, 1989) comb. nov., *Halomonas cupida* (Baumann et al, 1983) comb. nov., *Halomonas halophila* (Quesada et al, 1984) comb. nov., *Halomonas marina* (Baumann et al, 1983) comb. nov., *Halomonas pacifica* (Baumann et al, 1983) comb. nov., *Halomonas venusta* (Baumann et al, 1983) comb. nov., *Halomonas salina* (Valderrama et al , 1991) comb. nov. and *Halomonas variabilis* (Fendrich, 1988) comb. nov. The species descriptions are unchanged.

The difference in the proportions of the major fatty acids (Table 6.4) between the type strain of *H. subglaciescola* (ACAM 12^T), and *H. subglaciescola* ACAM 21, which is a representative strain of a biovar of the species suggests that this biovar may represent a separate species. The only characteristics that were present in all the members of the biovar represented by the type strain ACAM 12, and absent in all the members of the biovar represented by ACAM 21, were motility and peritrichous flagella (Franzmann et al, 1987a). DNA-DNA hybridisation data or 16S rRNA sequence data would be required to confirm that the biovar represented by ACAM 21 represents a new species of the genus *Halomonas*.

6.2.2 Differentiation of the genus *Halomonas* (emended as proposed) from other genera containing aerobic marine bacteria, within the gamma-subclass of the *Proteobacteria*.

Several genera of aerobic marine bacteria exist within the gamma-subclass of the *Proteobacteria*. Differentiation of some of these genera on the basis of phenotypic characteristics and mol% G+C has often been problematic. Many uncertain affiliations have been resolved by examination of these species using DNA:rRNA hybridisation, 16S rRNA oligonucleotide cataloguing, and 16S rRNA sequencing. Utilisation of the 16S rRNA moiety to examine

relationships among members of these genera has revealed several phylogenetically distinct lines of descent (De Ley, 1978; Kersters and De Ley, 1980; Van Landschoot et al, 1983). It has become apparent from these phylogenetic groupings that phenotypic characteristics such as presence and arrangement of flagella which have been commonly used to define these genera, are inappropriate for that purpose. Consequently the descriptions of some genera are not adequate for their differentiation and a search for suitable characteristics is required.

The nearest relatives to the genus *Halomonas* (emended as proposed) in the RDP were *Oceanospirillum linum* (88.8 - 90.4% similarity) and the terrestrial organism *Pseudomonas aeruginosa* (87.3 - 88.4% similarity). The genus *Oceanospirillum* is differentiated from the genus *Halomonas* on the basis of the morphology of its members, being rigid helical rods with bipolar tufts of flagella, and their lower G+C content of 45 - 50%.

Kita-Tsukamoto et al (1993) determined the partial sequence (600 bases) of *Pseudomonas nautica*. This marine species was distinct from *Halomonas marina* comb. nov. (87.4% sequence similarity), however it was also distinct from the terrestrial species *P. aeruginosa* and *P. fluorescens*, having 86.5% and 88.5% similarity to those species respectively.[1] Therefore Kita-Tsukamoto et al (1993) suggest that marine *Pseudomonas* spp. should be placed in a separate genus.

Morphology, arrangement of flagella, respiratory lipoquinones, and mol% G+C do not differentiate the genera *Halomonas* and *Pseudomonas*. The genus *Halomonas* is differentiated from terrestrial *Pseudomonas* spp. on the basis of the slight-moderate halophilism and halotolerance of its members. Differentiation of the genus *Halomonas* from marine *Pseudomonas* spp. is more difficult. DNA:rRNA homology studies have demonstrated that "*P. bathycetes*", *P. beijerinckii*, and "*P. halosaccharolytica*" belong to the *Halomonadaceae* branch (Kersters, 1992), indicating that use of the presence of polar flagella and mol% G+C content to assign aerobic marine bacteria to the genus *Pseudomonas* has the potential of

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[1] While these values will change when full sequences are determined, the difference is unlikely to be large. For example the similarity of the partial sequences of *H. marina* comb. nov. and *P. aeruginosa* was 86.2%, and the similarity based on the nearly-complete sequences was 87.9%.

resulting in the misclassification of organisms. Members of the genus *Halomonas* are characterised by their halotolerance. It is therefore important to determine whether a high degree of halotolerance would discriminate between this genus and the true marine *Pseudomonas* spp., that is, those species related to *P. nautica*. The phylogenetic position of the halotolerant species *P. halophila* has not been determined. If it were demonstrated that *P. halophila* was related to *P. nautica*, and did not belong within the genus *Halomonas*, then halotolerance would not be a useful characteristic to differentiate the genera. The fatty acid profile of *P. halophila* has suggested that it does not belong within the *Halomonadaceae* (Franzmann and Tindall, 1990). Although *P. halophila* contained the same major fatty acids as the members of the *Halomonadaceae* (16:1, 16:0, 18:1), the isomers of those fatty acids were different from all the members of the *Halomonadaceae* which uniformly contained the same isomers. In addition *P. halophila* contained 18:0-10Me which was not present in the fatty acid profiles of any of the members of the *Halomonadaceae*. These features may be useful for differentiating marine *Pseudomonas* spp. from the genus *Halomonas*. Detailed fatty acid profiles of all marine *Pseudomonas* spp. should be determined, and examined with respect to 16S rRNA sequences or DNA:rRNA homology characteristics to determine whether a correlation exists between these features.

Gauthier et al (1992) have determined the 16S rRNA sequence for *Marinomonas vaga* and the 16S rRNA sequence for *Marinobacter hydrocarbonoclasticus*, a new genus and species created to accommodate a halotolerant, hydrocarbon-degrading marine bacterium. The 16S rRNA sequence similarities between *Marinomonas vaga* and members of the genus *Halomonas* (emended as proposed) were in the range 86.9 - 89.4% (Table 5.3). The genus *Marinomonas* is distinguished from the genus *Halomonas* on the basis that its members contain ubiquinone 8 (Q8) as the major respiratory lipoquinone, in comparison with the members of the genus *Halomonas* which contain Q9 as the major respiratory quinone, and on the basis of a lower G+C content of 44 - 48% (Akagawa-Matsushita et al, 1992; Franzmann and Tindall, 1990). The genus *Alteromonas* is distinguished from the genus *Halomonas* on the basis of these same characteristics, however discrimination of the genera

Alteromonas and *Marinomonas*, which represent two distinct groups on the basis of rRNA:DNA homology remains problematic (Akagawa-Matsushita et al, 1992; Gauthier and Breittmayer, 1992).

The 16S rRNA sequence similarities between *Marinobacter hydrocarbonoclasticus* and members of the genus *Halomonas* were in the range 87.5 - 90.4% (Table 5.3). *Marinobacter hydrocarbonoclasticus* like *O. linum* contained only 5 of the 17 signature characteristics which defined the genus *Halomonas* (Table 5.2). *Marinobacter hydrocarbonoclasticus* was distinguished from the genus *Halomonas* on the basis of a lower G+C content of 52.7, and also noted were its higher temperature tolerance (30 °C, compared with 32 °C), absence of anaerobic growth on glucose, and inability to utilise carbohydrates and amino acids as sole carbon sources in comparison with *H. elongata*. Absence of anaerobic growth, and inability to use many carbohydrates and amino acids would not however distinguish *Marinobacter hydrocarbonoclasticus* from *H. subglaciescola* (Franzmann et al, 1987; James et al, 1990). Given the proposed amalgamation of the genera *Deleya* and *Halomonas*, the difference between *Marinobacter* and *Halomonas* is poorly resolved on the basis of mol% G+C. The mol% G+C range of *Halomonas* is 54 - 68, based on the type strains of its member-species. Among the type strains of the member-species of *Halomonas*, the type strain of *Halomonas venusta* comb. nov. has the lowest mol% G+C content, 54.1, and other strains assigned to this species have a mol % G+C content as low as 52.6 (Baumann et al, 1972). It would therefore be desirable to determine other characteristics which would differentiate between these two genera. These characteristics might be found among the chemotaxonomic features of *Marinobacter hydrocarbonoclasticus* which have not yet been determined.

6.3 Phylogenetic probes

Oligonucleotide probes based on ribosomal RNA sequences have been termed phylogenetic probes or stains (Stahl et al, 1988; Giovannoni et al, 1988; DeLong et al, 1989). Several genus-specific and species-specific phylogenetic probes have been developed (Hahn et al, 1990; Haun and Göbel, 1987; Hensik et al, 1992; Pütz et al,

1990; Spring et al, 1992; Wang et al, 1991; Williams and Collins 1992). Forsman et al (1990) were able to detect selectively *Francisella tularensis* var. *palaeartica* and *Francisella tularensis* var. *tularensis* with two oligonucleotide probes having a single nucleotide difference.

Potential species-specific probes for *F. gondwanense* sp.nov. and *F. salegens* sp. nov. were identified (Fig 6.1). The most similar sequences to the *F. gondwanense* and *F. salegens* probes, among organisms in the RDP, contained 4 mismatches, and 8 mismatches respectively.

A genus-specific probe for *Halomonas* (emended as proposed) was identified, for which organisms outside this genus contained a minimum of 2 mismatches (Fig. 6.2). Potential species-specific probes were identified for those member-species of the genus *Halomonas* for which a 16S rRNA sequence has been determined (Fig. 6.2).

Determination of the efficiency of the species-specific probes will require testing against closely related species for which 16S rRNA sequences are not available. These probes will be useful in determining the quantitative significance of these organisms in Antarctic lake environments.

Fig. 6.1 Phylogenetic (16S rRNA) probes for *F. gondwanense* sp. nov. and *F. salegens* sp. nov. Positions which are mismatched with the probe are indicated in bold-type. Positions of probes within the 16S rRNA gene are indicated by *E. coli* numbering. Organisms within the ribosomal RNA Database Project which have less than 4 mismatches are listed; where the minimum number of mismatches found were 4 or greater, the organism with the minimum number of mismatches is listed.

F. gondwanense, species-specific probe (138-158)

	ATTACTAAGAGATAGCCCAGA
F. salegens	**TTTAGCAGGGAA**TAGCCCAGG
C. lytica	**TAC**ACTAAGGGATAGCCCAGA

F. salegens, species-specific probe (184-198)

	CTCTCCTGAGATTATCATTAAAC
F. gondwanense	**TTGGCATCAATTGATAA**TTAAAG
Fusobacterium simiae	**AATA**CCTGATATTATGATTA**CAA**

Fig. 6.2 Phylogenetic (16S rRNA) probes for members of the genus *Halomonas* (emended as proposed). Positions which are mismatched with the probe are indicated in bold-type. Positions of probes within the 16S rRNA gene are indicated by *E. coli* numbering. Organisms within the ribosomal RNA Database Project which have less than 4 mismatches are listed; where the minimum number of mismatches found were 4 or greater, the organism with the minimum number of mismatches is listed.

Halomonas, genus-specific probe (1462-1481)

	ATCACCACGGTGTGGTTCAT
H. aquamarina	ATCACCACGGTGTGGTTCAT
H. variabilis	ATCACCACGGTGTGGTTCAT
H. subglaciescola	ATCACCACGGTGTGGTTCAT
H. halmophila	ATCACCACGGTGTGGTTCAT
H. elongata	ATCACCACGGTGTGGTTCAT
H. halophila	ATCACCACGGTGTGGTTCAT
H. marina	ATCACCACGGTGTGGTTCAT
Legionella pnuemophila	**TT**TACCACGGTGTGGTTCAT
Ruminobacter amylophilus	AT**T**ACCACGGTGTGGTT**T**AT
Spirillum volutans	AT**T**ACCACGGTGNG**A**TTCAT
Oceanospirillum linum	**G**TCACCACGG**A**GTG**A**TTCAT
Aeromonas hydrophila	**TTT**ACCACGGTGTG**A**TTCAN

H. aquamarina* - *H. meridiana,
"rRNA species complex"-specific probe (1273-1294)

	TGAGCCAATCCCGAAAAGCCGA
H. variabilis	T**C**AGC**G**AATCCC**TT**AAAGCCG**G**
H. subglaciescola	T**A**AGC**G**AATCCC**A**TAAAGCCG**G**
H. halmophila	**GGC**GC**T**AATCCC**A**TAAAG**T**CG**G**
H. elongata	**GGA**GCTAATC**TCA**TAAAGCCG**G**
H. halophila	**G**GAGC**T**AATC**TCA**TAANGCCG**G**
H. marina	**G**GAGCCAATCCC**A**TAAAGC**TT**G
Azospirillum lipoferum	**G**GAGCCAATCCCC**C**AAAAGCCG**T**
Desulfosarcina variabilis	T**A**AGCCAATCCC**A**AAAAGCCG**T**
Simonsiella muelleri	**G**GAGCCAATCCC**A**AAAAA**A**CCGA

Fig. 6.2 (cont'd)

H. variabilis, species-specific probe (999-1020)

	TACAGAAGCCGGAAGAGATTCT
H. aquamarina	**CTGC**GAA**TTT**GGT**A**GAGAT**ACC**
H. subglaciescola	**CTGC**GAA**CTT**GGT**A**GAGAT**N**CC
H. halmophila	**GTGC**GAA**CTT**GGT**A**GAGAT**ACC**
H. elongata	**GTGC**GAA**CTTTCC**AGAGAT**GGA**
H. halophila	**GNGN**GAA**CCNTCC**AGAGAT**GGA**
H. marina	**CAGAGGACTTTCC**AGAGATN**GA**
Alcaligenes xylosoxidans	**TCTGGAATCCTGAAGAGATTTA**

H. subglaciescola, species-specific probe (124-143)

	ATAGGAATCTACCCAGTCGT
H. aquamarina	ATAGGAATNTNCCC**GATA**GT
H. variabilis	ATAGGAATCT**G**CCC**GATA**GT
H. halmophila	ATAGGAATCT**G**CCC**GGTA**GT
H. elongata	ATAGGAATCT**G**CCC**GGTA**GT
H. halophila	ATAGGAATCT**G**CCC**GGTA**GT
H. marina	AT**GGGAATNTG**CCC**GATA**GT
Rhodopseudomonas marina subsp. *agilis* str. G	**GTGGGAATCTACCCAGTGGT**

H. halmophila, species-specific probe (1275-1292)

	CGCTAATCCCATAAAGTCGG
H. aquamarina	**AGCCAATCCC**GA**AAAGCCGA**
H. variabilis	**AGC**GAA**TCCCTTAAAGCCGG**
H. subglaciescola	**AGC**GAA**TCCCATAAAGCCGG**
H. elongata	**AGCTAATCTCATAAAGCCGG**
H. halophila	**AGCTAATCTCATAANGCCGG**
H. marina	**AGCCAATCCCATAAAGCTTG**
Mycoplasma sp. str. M1	**AGCTAATCTCATAAAGTCGG**
Acholeplasma entomophilum	**AGCTAATCTCAAAAAGTCGG**
Arthrobacter globiformis	**AGCTAATCCCAAAAAGCCGG**
Pseudomonas testosteroni	**AGCTAATCCCATAAAGCCAG**
Micrococcus luteus	**AGCTAATCCCAAAAAGCCGG**
Mycoplasma sp. str. 831-C4	**AGCAAATCTCATAAAGTCGG**

Fig. 6.2 (cont'd)

***H. elongata*, species-specific probe (461-480)**

	GGTTAATACCCGGCAAGGGA
H. aquamarina	GGTTAATACCCG**CTAGGAAA**
H. variabilis	GGTTAATACCCGGCA**GGAAA**
H. subglaciescola	GGTTAATACCC**CGGCGGAAA**
H. halmophila	GGTTAATACCCGGCA**GGAAG**
H. halophila	GGTTAATACCC**T**GCAAG**AAG**
H. marina	G**A**TTAATAC**TCCCGA**GGAAA
Chromobacterium violaceum	GGTTAATANCCGGC**GGGGAT**

***H. halophila*, species-specific probe (456-478)**

	TTGTGGGTTAATACCCTGCAAGA
H. aquamarina	**CTAGC**GGTTAATACCC**GCTAG**GA
H. variabilis	**C**TGTC**G**GTTAATACCC**G**GCA**G**GA
H. subglaciescola	**CGTC**GGGTTAATACCCC**GGCG**GA
H. halmophila	TTGT**C**GGTTAATACCC**G**GCA**G**GA
H. elongata	TTGT**C**GGTTAATACCC**G**GCAAG**G**
H. marina	TT**CGGGA**TTAATACTC**CCGA**GGA
Alcaligenes xylosoxidans	T**C**GTGGGTTAATACCCCGC**GGC**A

***H. marina*, species-specific probe (629-648)**

	AACGGCATCTGGAACTGCTT
H. aquamarina	AACGGCATC**C**GGAACTG**TCA**
H. variabilis	AACGGCATC**C**GGAACTG**TCA**
H. subglaciescola	AAC**A**GCATC**C**GGAACTG**TCA**
H. halmophila	AA**T**GGCATC**C**GGAACTG**TCA**
H. elongata	AACGGCATC**C**GGAACTG**TTA**
H. halophila	AACGGCNTC**C**GGAACTG**TCA**
Lactobacillus acidophilus	AAC**T**GCATC**GGA**AACTG**T**TT

SUMMARY OF CONCLUSIONS

1. The protocols of Pitcher et al (1989), Edwards et al (1989), and Bachmann et al (1990), for the extraction of DNA, PCR-amplification of the 16S rRNA gene, and sequencing of PCR-generated dsDNA, efficiently produced near-complete sequences of 16S rRNA genes.

2. The 16S rRNA sequences of the Organic Lake strains, ACAM 44, and ACAM 48 contained the signature characteristics of members of the "flavobacteria-*Bacteroides*" phylum. The Organic Lake flavobacteria clustered in a branch of the "flavobacteria-*Bacteroides*" phylum which contained the type species of the genus *Flavobacterium*, *F. aquatile*. The Organic Lake flavobacteria were assigned to the genus *Flavobacterium*, because of their phylogenetic relationship with the type species of that genus, and chemotaxonomic and phenotypic characteristics consistent with the description of the genus. ACAM 44 and ACAM 48, were phylogenetically, chemotaxonomically, and phenotypically distinct from other flavobacterial species. Two new species *Flavobacterium gondwanense* and *Flavobacterium salegens* are proposed to accommodate the Organic Lake flavobacteria.

3. The branch of the "flavobacteria-*Bacteroides*" phylum in which the Organic Lake flavobacteria clustered may, in the future, be subdivided into several genera. The phospholipid fatty acid profiles of ACAM 44 and ACAM 48 contained some features unique to fatty acid profiles of flavobacterial species. *F. gondwanense* and *F. salegens* may form the basis of new genera, if these fatty acid features remain unique to the Organic Lake flavobacteria after a comprehensive determination of the fatty acid profiles of all other flavobacterial species.

4. *Halovibrio variabilis* clustered among members of the genera *Deleya* and *Halomonas* in 16S rRNA based-phylogenetic trees, confirming that this organism belonged within the *Halomonadaceae*.

5. *D. aquamarina* and *H. meridiana* had identical 16S rRNA sequences. DNA:DNA hybridisation data is required to determine if these two species are synonymous.

6. The members of the genera *Deleya* and *Halomonas* did not form separate monophyletic groups in phylogenetic trees derived by distance and parsimony analysis of 16S rRNA sequence data. This was consistent with the results of phenotypic, chemotaxonomic and rRNA:DNA hybridisation studies. Seventeen signature features were identified which were present in the examined members of the genera *Deleya*, *Halomonas*, and *Halovibrio*, which characterised this group of species within the gamma-subclass of the *Proteobacteria*.

7. Eight members of the genera *Deleya*, *Halomonas* and *Halovibrio* had 16S rRNA sequence similarities in the range 92.6 - 100% (Jukes-Cantor distances were in the range 0.086 - 0.000). The only sub-group within this group of species which was well-supported in the phylogenetic trees, was comprised of *D. aquamarina*, *H. meridiana*, and *Hv. variabilis*. The members of this sub-group could not be differentiated from the other members of the *Halomonadaceae* on the basis of phenotypic or chemotaxonomic data. The unification of the genera *Deleya*, *Halomonas* and *Halovibrio* is proposed on the basis of an examination of phylogenetic, chemotaxonomic and phenotypic characteristics. The descriptions of the genus *Halomonas*, and the family *Halomonadaceae* are emended. The following new combinations are proposed, *Halomonas aquamarina* (Akagawa and Yamasato, 1989) comb. nov., *Halomonas cupida* (Baumann et al, 1983) comb. nov., *Halomonas halophila* (Quesada et al, 1984) comb. nov., *Halomonas marina* (Baumann et al, 1983) comb. nov., *Halomonas pacifica* (Baumann et al, 1983) comb. nov., *Halomonas venusta* (Baumann et al, 1983) comb. nov., *Halomonas salina* (Valderrama et al , 1991) comb. nov. and *Halomonas variabilis* (Fendrich, 1988) comb. nov.

8. Mol% G+C content of DNA, and phenotypic characteristics such as arrangement of flagella and utilisation of carbohydrates do not clearly differentiate the genus *Halomonas* (emended as proposed) from marine *Pseudomonas* spp. and the genus *Marinobacter*.

Fatty acid data may be useful for the description and differentiation of these phylogenetically distinct genera.

9. Potential phylogenetic probes, for application in ecological studies, were identified from an examination of the 16S rRNA sequences of *F. salegens*, *F. gondwanense* and eight member-species of the genus *Halomonas* (emended as proposed).

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APPENDIX A: Buffers and Solutions

Chemicals of analytical quality were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and BDH Australia, Pty Ltd (Kilsyth, Vic.), as indicated. Deionised, glass-distilled water is indicated by ddH_2O.

1. Tris-acetate buffer (TAE): 0.04 M Tris-acetate, 0.001 M EDTA (Sambrook et al, 1989)

   To prepare a 50x stock solution:

Tris base (Sigma)	242	g
Glacial acetic acid (BDH)	57.1	ml
0.5M EDTA (pH 8.0)	100	ml
ddH_2O to a final volume	1000	ml

2. Tris-borate buffer (TBE): 0.045M Tris-borate, 0.001M EDTA (Sambrook et al, 1989)

   To prepare a 5x stock solution:

Tris base (Sigma)	54	g
Boric acid (Sigma)	27.5	g
0.5M EDTA (pH 8.0)	10	ml
ddH_2O to a final volume	1000	ml

3. 0.5M EDTA

   Dissolve 181.1 g $EDTA.2H_2O$ (Sigma) in 800 ml ddH_2O. Adjust pH to 8.0 by the addition of NaOH pellets (BDH) ($\approx$ 20 g). Add ddH_2O to a final volume of 1000 ml.

4. Agarose 1% (w/v), 1xTAE, 0.5 μg ml^{-1} EtBr (Sambrook et al, 1989)

Agarose	1.0	g
50x TAE	2.0	ml
ddH_2O to a final volume	100	ml

Boil the solution to completely dissolve the agarose. Cool to < 60 °C. Add 5 μl of EtBr (10 mg ml^{-1})(Sigma). Mix and pour into gel mold. Position well-forming comb.

5. Agarose gel 6x loading buffer (Sambrook et al, 1989)

Bromophenol blue (Sigma)	0.125	g
Xylene cyanol FF (Sigma)	0.125	g
Sucrose (Sigma)	20	g
ddH_2O to a final volume	50	ml

6. Tris-EDTA buffer (TE pH 7.4): 0.01 M TrisCl (pH 7.4), 0.001 M EDTA (pH 8.0) (Sambrook et al, 1989)

To prepare a 50x stock solution:

Tris base (Sigma)	60.6	g
0.5M EDTA	100	ml

Dissolve the Tris base in 800 ml ddH_2O. Adjust the pH to 7.4 by the addition of concentrated HCl (BDH). Add ddH_2O to 1000 ml.

7. GT lysis solution: 5M guanidine thiocyanate, 100 mM EDTA, 0.5% sarkosyl (Pitcher et al, 1989)

Dissolve 60.0 g guanidine thiocyanate (Fluka Chemie AG, Buchs, Switzerland) in 20 ml of 0.5 M EDTA and 20 ml ddH_2O at 60 °C. Cool solution and add 5.0 ml 10% (v/v) sarkosyl. Add ddH_2O to a final volume of 100 ml. Sterilise by passage through a 0.45 μm filter. Store at room temperature.

8. TE-saturated phenol (Sambrook, 1989)

   Dissolve crystalline phenol (BDH) by stirring in an excess of TE (pH 8.0). Leave overnight to equilibrate. Remove the supernatant. Add fresh TE (pH 8.0) and stir overnight. Remove the supernatant. Add 0.1 volume of fresh TE (pH 8.0). Store at 4 °C in a darkened bottle for up to 4 weeks.

9. RNAase A (10 $mgml^{-1}$) (Sambrook, 1989)

   Dissolve 10$mgml^{-1}$ RNAase A (Sigma) in 0.01M Tris pH 7.5, 0.015M NaCl. Heat to 100 °C for 15 min to denature any contaminating DNAase.

10. Sequencing reagents from the Sequenase 2.0 Sequencing Kit (USB, Cleveland, OH, USA) (cont'd)

    Sequenase 2.0 reaction buffer: 200 mM tris.HCl pH7.5, 100 mM $MgCl_2$, 250 mM NaCl
    Sequenase 2.0 enzyme dilution buffer: 10 mM tris.HCl pH 7.5, 5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin
    dGTP labelling mix: 1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP
    ddATP termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddATP, 50 mM NaCl
    ddCTP termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddCTP, 50 mM NaCl
    ddGTP termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddGTP, 50 mM NaCl
    ddTTP termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddTTP, 50 mM NaCl
    dITP labelling mix: 3 µM dITP, 1.5 µM dCTP, 1.5 µM dTTP
    ddATP(dITP) termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dITP, 80 µM dTTP, 8 µM ddATP, 50 mM NaCl
    ddCTP(dITP) termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dITP, 80 µM dTTP, 8 µM ddCTP, 50 mM NaCl
    ddGTP(dITP) termination mix: 80 µM dATP, 80 µM dCTP, 160 µM dITP, 80 µM dTTP, 1.6 µM ddGTP, 50 mM NaCl
    ddTTP(dITP) termination mix: 80 µM dATP, 80 µM dCTP, 80 µM dITP, 80 µM dTTP, 8 µM ddTTP, 50 mM NaCl

10. Sequencing reagents from the Sequenase 2.0 Sequencing Kit (USB, Cleveland, OH, USA) (cont'd)

    Stop solution: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

11. 6% (w/v) Polyacrylamide sequencing gel

    Dissolve 28.5g of acrylamide (Sigma) and 1.5g bis-acrylamide (Sigma) in 70 ml ddH_2O. Stir for 30 min. Dissolve with gentle heating (60 °C), 210 g urea (Sigma) in 100 ml 5X TBE and a minimum quantity of ddH_2O. Mix the acrylamide and urea solutions and add ddH_2O to a final volume of 500 ml. Pass the solution through a 0.45 µm filter and degas for 20 min. Store at 4 °C in a darkened bottle for up to 4 weeks. Immediately prior to pouring a gel add 50 µl of 50% (w/v) ammonium persulphate (Sigma) and 30 µl TEMED (N,N,N',N' tetramethylethylene-diamine) (Sigma) to 50 ml of the acrylamide gel solution.