Comparison of 16S rRNA Sequences from the Family Pasteurellaceae: Phylogenetic Relatedness by Cluster Analysis

By PAUL J. CHUBA, RENATE BOCK, GABRIELE GRAF,
THOMAS ADAM AND ULF GÖBEL*

Institut für Medizinische Mikrobiologie und Hygiene der Universität Freiburg, Herman Herder
Strasse 11, D-7800 Freiburg, FRG

(Received 25 January 1988; revised 28 March 1988)

The taxonomy of the family Pasteurellaceae has remained controversial despite investigations of biochemistry, serology, and nucleic acid relatedness. In an attempt to resolve some of this confusion, we have partially sequenced the 16S rRNAs of seven members of the family, representing all three genera. The sequences were aligned, similarity scores calculated, and single, average and complete linkage cluster analysis of the resulting distance matrix performed. In this way, an evolutionary branching pattern of these closely related species was reconstructed, and the approximate phylogenetic position of the family determined. *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans* clustered with *Haemophilus* instead of *Actinobacillus*, supporting transfer of this species to the genus *Haemophilus*. Thus cluster analysis of phylogenetic relatedness was found to be particularly useful for studying closely related organisms, and could be performed using a microcomputer.

INTRODUCTION

Pasteurellaceae is one of three families of Gram-negative facultative anaerobes, many of which are pathogenic for humans and animals. Phylogenetically these organisms belong to the gamma subgroup of the purple bacteria, clustering closely with the *Enterobacteriaceae* and the *Vibrionaceae* in 16S rRNA cataloguing studies (Woese *et al.*, 1985). They exhibit considerable interrelatedness as evidenced by biochemistry (Broom & Sneath, 1981; Brondz & Olsen, 1985; Sneath & Stevens, 1985), serology (Potts *et al.*, 1985; MacInnes & Rosendal, 1987), nucleic acid hybridization (Christiansen *et al.*, 1981; Potts & Berry, 1983; Coykendall *et al.*, 1983), and genetic transformation (Albritton *et al.*, 1986). Such data have indicated that the generic descriptions within this family (*Pasteurella*, *Haemophilus* and *Actinobacillus*) may need redefinition (Pohl *et al.*, 1983; Mutters *et al.*, 1984; Bisgaard *et al.*, 1986). In particular, it has been suggested that *Actinobacillus actinomycetemcomitans* be transferred to the genus *Haemophilus* (Potts *et al.*, 1985). As outlined below, it appears that direct sequencing of 16S rRNAs would be useful in better defining genetic relatedness within the Pasteurellaceae.

Mutations occur in macromolecules as a quasi clock-like process and the use of comparisons of primary sequences of homologous macromolecules to infer evolutionary relationships has attracted considerable attention (Zuckerlandl & Pauling, 1965; Wilson *et al.*, 1987). The 16S rRNA molecule is particularly well suited to such studies (for reviews see Olsen *et al.*, 1986; Woese, 1987), as relatively conserved regions of the 16S rRNA sequence can be used to study the relatedness of distantly related species, whereas variable regions can be used for the analysis of closely related species. To date, considerable progress has been made in establishing the major phylogenetic branches, based both on direct sequencing (Pace *et al.*, 1986) and on oligonucleotide cataloguing methods (Fox *et al.*, 1980). Recently, a method for the rapid

Abbreviations: I/L, identities per unit length; NAS, normalized alignment score.

(a)

GGGGGAACCCnGACGCAGCCUGCCGCGUGAAUGAAGAAGGCUUCGGGUU	50
.....n.....	2
.....n.....nA.....n.n.....	3
.....C.....A.....	4
.....U.....n.....	5
.....U.....A.....n.....	6
.....U.....A.....	7

AAGUUCUUUCGUAUGAGGAAGGGAUGUUGUAGAUAGCAUCAU	100=480
.....U.....UGA.GUG.UA.....CACAUCAA.....	2
.....G.C.....UGU.GUG.UA.....CGCAACAA.....	3
.....G.....UG.GUG.UA.....CAU..CAA.....	4
.....GC.....UGGUAUG.UA.....CAUUAUCA.....	5
.....GC.....U..CAAA..UA.....UUGG.A.....	6
.....GC.....U..CAAA..UA.....UUGG.A.....	7

(b)

GUGCnAGCGUUnAUCGGAUnACUGGGCGUAAAGGGCACGCAGGCGGnCU	50
.....A.....n.....UUA	2
.....A.....n.....nn.....	3
.....n.....U.....C	4
.....A.....A.....UG	5
.....A.....A.....nn.UG	6
.....n.....nn.UG	7

UUUAAUGUGAGUGUAAAUCCCCGAGCUUAAUUGGGAACUGCAUUCAG	100
.....G.....G.....C.....n.U.....	2
.....G.....G.....n.C.....n.U.....	3
.....G.....G.....n.C.....n.U.....	4
A.....G.....G.....C.....n.U.....	5
A.....G.....G.....C.....U.U.....	6
A.....G.....G.....n.C.....n.U.....	7

ACUGGGAGUCUAGAGUCUGUAGAGGGGGGAAGAAUCCAGGUGUAGCGG	150
.....UAA.....ACUU..G.A..nU.....C.....	2
.....G.....ACUU..G.A..nnn.....C.....	3
.....G...G...ACUU..G.A..nU.....C.....	4
.....UCUA.....ACUU..G.A..U.....C.....	5
.....UCUA.....ACUU..G.A..nU.....C.....	6
.....UCUA.....ACU'U..G.A..nU.....C.....	7

UGAAAUGCGUAGAGAUUCUGGAGGAUACCGGUGGCGAAGGCGGCCCCCU	200
.....G.....AA.....n.....UUG	2
.....G.....AA.....A.....UUG	3
.....G.....AA.....n.....UUG	4
.....G.....AA.....A.....UUG	5
.....G.....AA.....n.....UUG	6
.....G.....AA.....A.....UUG	7

GGAAUGUACUGACGCUCUAGUGCUAAAGCGUGGGGAGCAAACAGGAUUG	250
.....C.....	2
.....G.....G.....n.....	3
.....G.....G.....n.....	4
.....G.....G.....n.....	5
.....G.....G.....n.....	6
.....n.....	7

AUACCCUGGUAGUCCACGCUGUAAACGCUGUCGAUUUGGGGAUUGGGCUA	300
.....U	2
.....U	3
.....G.....G.U	4
.....UCUA.....A..G	5
.....A..U	6
.....G	7

UAUGCUUGGUGCCnUAGCUAACGUGAUAAAUCGACCGCCUGGGG	345=888
..ACUC...CA..CG.....n.....	2
..GA.....G.....n.....	3
..GC.C.....C.A.....	4
..GA.UC.....CGA.....	5
..A.UC.....CGA.....	6
..GA.UC.....C.A.....	7

acquisition of 16S rRNA sequence data has become available (Lane *et al.*, 1985). The method is based on the bulk cellular RNA as template, oligonucleotides complementary to evolutionarily conserved regions as primers, and reverse transcriptase for chain elongation. We have applied this method to phylogenetic analysis of the Pasteurellaceae and report here partial sequence comparisons between *Pasteurella multocida*, *Haemophilus influenzae*, *H. aphrophilus*, *Actinobacillus (Haemophilus) actinomycetemcomitans*, '*A. hominis*', *A. equuli* and *A. lignieresii*.

METHODS

Bacterial strains and growth media. The following strains were obtained from the National Collection of Type Cultures (London, UK): *Actinobacillus (Haemophilus) actinomycetemcomitans* NCTC 9710 (type strain); *Actinobacillus equuli* NCTC 8529 (type strain); '*Actinobacillus hominis*' NCTC 11529 (type strain) (Friis-Møller, 1981); *Actinobacillus lignieresii* NCTC 4976; *Haemophilus influenzae* NCTC 8143 (type strain); *Haemophilus aphrophilus* NCTC 5906 (type strain); *Pasteurella multocida* NCTC 10322 (type strain). *Actinobacillus* and *Pasteurella* strains were cultured in yeast extract (0.5%) / Difco peptone (1%) / glucose (1%) (YPD) medium, which was supplemented with NAD (0.001%) and haemin (0.001%) to support the growth of *Haemophilus* species. For growth on solid medium Columbia blood agar (Difco), or chocolate agar (Difco) plates (*Haemophilus* species) were used. A battery of biochemical tests, including sugar fermentations, urease and H₂S production, and requirements for X and V factors, were routinely performed to ensure the purity of the cultures. All cultures were incubated at 37 °C with 5% carbon dioxide and without shaking.

Isolation of rRNA. Bulk cellular RNA was isolated using a differential LiCl extraction method essentially as previously described for eukaryotic cells (Auffray & Rougeon, 1980), followed by phenol and chloroform extractions. Cultures (500 ml) were grown to mid-exponential phase, washed once with phosphate-buffered saline (PBS), and the pellets were resuspended in 20 ml 4 M-LiCl/8 M-urea and incubated at 0 °C for 12–18 h. The mixture was centrifuged at 10000 r.p.m. for 20 min in a Sorvall SS34 rotor and the pellets resuspended in 10 ml TE buffer (20 mM-Tris/HCl, 1 mM-EDTA, pH 8.0), extracted two to four times with an equal volume of TE-saturated phenol (BRL), and finally extracted with chloroform/isoamyl alcohol (24:1, v/v). RNA of sufficient quality for use in sequencing was recovered by precipitation with two volumes of ice-cold ethanol and incubation for 30 min at –70 °C. The major contaminant in these preparations was bacterial lipopolysaccharide, as assessed by SDS gel electrophoresis and silver staining.

Partial sequencing of 16S rRNA. Partial sequences of the 16S rRNAs were obtained using avian myeloblastoma virus reverse transcriptase (Pharmacia), bulk cellular RNA as template (1–3 µg ml^{–1}), and oligonucleotides (0.1 µg ml^{–1}) complementary to conserved regions of the 16S rRNA molecules as primers in chain-terminating sequencing reactions as described by Lane *et al.* (1985), using [α -³²P]dCTP (800 Ci mmol^{–1}, 29.6 TBq mmol^{–1}; Amersham). The data shown in Fig. 1(a) were obtained using the oligonucleotide primer 5'GTAT-TACCGCGGCTGCTGGCAC 3', and the data shown in Fig. 1(b) were obtained using the oligonucleotides 5'TACCAGGGTATCTAATCCTGTT 3' and 5'TCGTTGCGGGACTTAACCCAACA 3'. Oligonucleotides were synthesized using phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer, and purified by electrophoresis on 20% (w/v) acrylamide gels followed by electroelution (Göbel *et al.*, 1987a).

Alignment of sequences and cluster analysis. A standard algorithm (Needleman & Wunsch, 1970) was used for sequence alignment using gap penalties (Feng *et al.*, 1985) assigned as described below. To calculate normalized alignment scores (NAS) for each pair of sequences, positions of identity and mismatch were assigned values of ten and zero, respectively, and gaps in the alignment were penalized with values from –10 to –90. The total was then divided by the length of the shorter sequence to give the NAS. The percentage of identities per unit length (*I/L*) was also calculated for all pairs of sequences. Positions which could not be unambiguously aligned, or where one of the bases was not determined, were not considered in the subsequent analysis. The resulting distance matrices were analysed using the Clustan 3/PC program suite (release 3.2; Wishart, 1987) using a Tandon microcomputer equipped with a math coprocessor. The Jaccard similarity coefficient was chosen for cluster analysis with single, average (unweighted pair group arithmetic average) and complete linkage conditions, as described previously for 5S rRNA sequences (Nearhos & Fuerst, 1987).

Fig. 1 (on facing page). Alignments of 16S rRNA sequences determined for seven members of the Pasteurellaceae. The sequences are numbered as follows: 1, *P. multocida*; 2, *H. influenzae*; 3, *H. aphrophilus*; 4, *A. (H.) actinomycetemcomitans*; 5, '*A. hominis*'; 6, *A. equuli*; 7, *A. lignieresii*. Positions in the sequences which are identical to the *P. multocida* sequence are indicated with a period. The letter 'n' designates positions which were not determined. (a) Base positions in the sequences are numbered consecutively, where nucleotide 1 corresponds to nucleotide 380 in the standard *E. coli* 16S rRNA numbering (Brosius *et al.*, 1978). (b) Base positions in the sequences are numbered consecutively, where nucleotide number 1 corresponds to nucleotide 543 in the standard *E. coli* 16S rRNA numbering. The different oligonucleotide primers used to generate sequences for (a) and (b) are described in Methods.

RESULTS AND DISCUSSION

Alignments of partial 16S rRNA sequences from seven members of the family Pasteurellaceae are shown in Fig. 1. The sequences in Fig. 1(a) correspond to *Escherichia coli* 16S rRNA positions 380–480, and those in Fig. 1(b) correspond to *E. coli* 16S rRNA positions 543–888, so that about 60 bases between the two contiguous segments are missing. These two segments contain nearly all of the variable regions V3, V4 and V5 (Huysmans & De Wachter, 1986). It was not necessary to introduce gaps into these sequences in order to produce a good alignment; however, when a very low gap penalty (-10 , see Methods) was used for the pairwise alignments, gaps were introduced between *Pasteurella multocida* and all other sequences in the region around positions 450–480 (loop 17 after Huysmans & De Wachter, 1986). These gaps represent a compensatory insertion/deletion mutational event which retains the length of the loop (P. J. Chuba & U. Göbel, unpublished observations). From a total of 445 positions, 24 were uncertain (5%), and 67 of the remainder were different for the seven members of the family. Of these, 20 were different only for *P. multocida*, so that within the cluster haemophili/actinobacilli 47 of 421 positions in this region were variable. Many of these mutated positions are involved in base pairing, but we have not weighted base pairing positions differently in the construction of distance matrices as suggested by other authors (Olsen *et al.*, 1986), since an analysis of secondary structure does not indicate that a majority of these mutations have arisen in compensatory-type events.

Identities per unit length (I/L) and normalized alignment scores (NAS, gap penalty = 50) were calculated by pairwise comparison of the sequences determined here, plus seven additional sequences from the literature (compiled in Huysmans & De Wachter, 1986). These values are listed in Table 1. *E. coli* and *Proteus vulgaris* sequences served as representative of the Enterobacteriaceae (and the gamma subgroup of the purple bacteria). *Agrobacterium tumefaciens* and *Pseudomonas testosteroni* represented the alpha and beta subgroups of the purple bacteria, respectively. The Gram-positive bacterium *Bacillus subtilis*, the cyanobacterium *Anacystis nidulans*, and the archaeobacterium *Methanococcus vannielii* were also included. Alignment scores were also calculated using higher and lower gap penalties (data not shown).

Within the family Pasteurellaceae, *Pasteurella multocida* was most different from all the other organisms examined (I/L score ranging from 0.887 to 0.901), approximately defining the genetic variability in this region between bacteria at the family level (see below). With the exception of *A. (H.) actinomycetemcomitans*, the *Actinobacillus* sequences were remarkably similar, indicating close genotypic relatedness. The sequences of *A. equuli* and *A. lignieresii* differed at only four positions (99% similar), approaching the limits of accuracy of the method (Romaniuk *et al.*, 1987), and could therefore be considered as identical within experimental error. Nonetheless, rechecking the sequencing gels at the nonidentical positions indicated that the sequences were, in fact, different. *A. (H.) actinomycetemcomitans* was more closely related to *Haemophilus* spp. (I/L = 0.994 for *H. influenzae*, and 0.951 for *H. aphrophilus*) than to the other (true) actinobacilli (mean I/L = 0.917), but *H. influenzae* and *H. aphrophilus* were slightly more closely related to each other (I/L = 0.953) than to *A. (H.) actinomycetemcomitans*. Inspection of specific variable positions (Fig. 1) reinforced these observations. At certain positions (e.g. positions 51, 61 and 106 in Fig. 1b), *A. (H.) actinomycetemcomitans* had bases in common with the two *Haemophilus* sequences, which differed from the three other actinobacilli. At other positions, *A. (H.) actinomycetemcomitans* was the same as one or the other *Haemophilus* species (see positions 91 in Fig. 1a, and 107 in Fig. 1b), suggesting divergence at different times from a common ancestor.

Fig. 2 shows dendrograms resulting from cluster analysis of the NAS matrix (gap penalty = 50) given in Table 1. Single linkage, average linkage and complete linkage results are given. Using all three methods, the branching pattern within the facultative anaerobes (*E. coli*, *Proteus vulgaris* and the Pasteurellaceae) was the same, and in all cases the archaeobacterium *Methanococcus vannielii* clustered apart from all other species tested, forming the 'root' of the tree. As expected, *A. (H.) actinomycetemcomitans* clustered with the members of the genus *Haemophilus*. The remaining three actinobacilli clustered together and separately from *Pasteurella multocida*. Importantly, the tree branching was consistent with previous studies of DNA/DNA hybridization between strains of Pasteurellaceae (Christiansen *et al.*, 1981;

Coykendall *et al.*, 1983; Potts & Berry, 1983; Escande *et al.*, 1984). The Enterobacteriaceae diverged from the Pasteurellaceae at an average NAS of 7.8, and it would be interesting to see if the Vibrionaceae also diverge at a similar level. Interestingly, the genera *Escherichia* and *Proteus* diverge from one another at a level similar to that of *Pasteurella* from *Haemophilus* and *Actinobacillus* (NAS of 8.9), but the genera *Actinobacillus* and *Haemophilus* clustered more closely at an NAS of about 9.3, perhaps justifying their inclusion in an intermediate group or 'tribe'.

In contrast to the branching pattern for the facultative anaerobes, the relative branching pattern for *Agrobacterium tumefaciens*, *Pseudomonas testosteroni*, *Bacillus subtilis* and *Anacystis nidulans* did not remain constant using the three linkage methods (Fig. 2). The situation did not improve when distance matrices were calculated using higher or lower gap penalties (data not shown). This may be explained by the fact that we used a relatively small number of positions to perform the analysis, and that these sequences were relatively variable, and therefore of more limited use in examining distantly related organisms.

We expect that the continued use of direct rRNA sequencing methods will simplify and improve the taxonomic analysis of a wide variety of micro-organisms. Although accurate phylogenetic analysis of distantly related species requires the analysis of a large number of relatively constant positions, the evolutionary branching patterns of closely related organisms can be quickly established as demonstrated here. The use of these methods is increasing rapidly. For example, similar sequencing studies have recently been reported for the protozoan

Table 1. Identities per unit length and normalized alignment scores for pairwise comparisons of 16S rRNA sequences of seven Pasteurellaceae and seven other prokaryotic species

Identities per unit length are given in the lower left portion of the table, and normalized alignment scores in the upper right portion. Bacterial species names have been abbreviated using the first four letters of the species designation. The sources of sequences not determined in this work are as follows: *Agrobacterium tumefaciens* and *Pseudomonas testosteroni*, Yang *et al.* (1985); *Anacystis nidulans*, Tomioka & Sugiura (1983); *Bacillus subtilis*, Green *et al.* (1985); *Methanococcus vannielii*, Jarsch & Böck (1985); *Escherichia coli*, Brosius *et al.* (1978); *Proteus vulgaris*, Carbon *et al.* (1981).

Species	TUME	TEST	SUBT	NIDU	VANN	COLI	VULG	MULT	INFL	APHR	ACTI	HOMI	EQUU	LIGN
<i>Agrobacterium tumefaciens</i>	7.51	7.48	6.96	5.76	7.31	7.48	7.23	7.13	7.03	7.16	7.03	7.03	6.98	
<i>Pseudomonas testosteroni</i>	0.763		6.98	6.84	6.07	7.19	7.12	7.18	7.16	7.14	7.07	7.09	7.14	7.09
<i>Bacillus subtilis</i>	0.786	0.721		7.26	6.02	6.75	6.74	6.71	6.71	6.64	6.71	6.69	6.74	6.76
<i>Anacystis nidulans</i>	0.721	0.709	0.751		5.99	6.57	6.54	6.44	6.49	6.35	6.23	6.49	6.47	6.47
<i>Methanococcus vannielii</i>	0.602	0.633	0.641	0.625		5.56	5.58	5.58	5.45	5.56	5.68	5.63	5.58	5.56
<i>Escherichia coli</i>	0.793	0.789	0.745	0.719	0.620		8.97	7.98	7.68	7.72	7.65	7.75	7.82	7.79
<i>Proteus vulgaris</i>	0.798	0.771	0.773	0.704	0.610	0.932		7.89	8.00	7.91	7.89	8.15	8.12	8.07
<i>Pasteurella multocida</i>	0.761	0.754	0.718	0.682	0.597	0.857	0.836		8.87	9.01	8.92	8.76	8.92	8.92
<i>Haemophilus influenzae</i>	0.750	0.751	0.718	0.686	0.584	0.826	0.847	0.887		9.53	9.44	9.39	9.32	9.30
<i>Haemophilus aphrophilus</i>	0.740	0.761	0.688	0.659	0.594	0.831	0.838	0.901	0.953		9.51	9.34	9.20	9.23
<i>Actinobacillus actinomycetemcomitans</i>	0.753	0.742	0.695	0.667	0.607	0.824	0.836	0.892	0.944	0.951		9.23	9.16	9.13
<i>Actinobacillus hominis</i>	0.741	0.756	0.716	0.686	0.602	0.833	0.861	0.876	0.939	0.939	0.923		9.72	9.74
<i>Actinobacillus equuli</i>	0.741	0.749	0.721	0.684	0.584	0.840	0.859	0.842	0.932	0.920	0.916	0.972		9.93
<i>Actinobacillus lignieresii</i>	0.736	0.744	0.723	0.684	0.581	0.838	0.854	0.842	0.930	0.923	0.913	0.974	0.993

Fig. 2. Dendrograms illustrating the results of cluster analysis of the normalized alignment score matrix from Table 1. The lengths of the branches are equivalent to the normalized alignment scores given on the scale at the bottom.

Toxoplasma gondii (Johnson *et al.*, 1987) and the prokaryotic genus *Campylobacter* (Lau *et al.*, 1987; Romaniuk *et al.*, 1987). The PC version of the Clustan program allows cluster analysis of sequence data on a microcomputer and this will make the rRNA sequencing approach less expensive and more widely accessible to taxonomists. Aside from taxonomic and evolutionary applications, the rapid accumulation of rRNA sequence data is also important for the

production of specific oligonucleotide probes complementary to variable regions of rRNA (Göbel *et al.*, 1987b), which may prove to have wide applications in diagnostic medicine.

Our thanks go to Professor W. Mannheim and to Bodo Rak for their assistance and encouragement. Financial support for this project was provided by the Bundesministerium für Forschung und Technologie.

REFERENCES

- ALBRITTON, W. L., SETLOW, J. K., THOMAS, M. L. & SOTTNEK, F. O. (1986). Relatedness within the family Pasteurellaceae as determined by genetic transformation. *International Journal of Systematic Bacteriology* **36**, 103–106.
- AUFFRAY, C. & ROUGEON, F. (1980). Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *European Journal of Biochemistry* **107**, 303–314.
- BISGAARD, M., PHILLIPS, J. E. & MANNHEIM, W. (1986). Characterization and identification of bovine and ovine Pasteurellaceae isolated from the oral cavity and rumen of apparently normal sheep. *Acta pathologica et microbiologia scandinavica* **B94**, 9–17.
- BRONDZ, I. & OLSEN, I. (1985). Differentiation between major species of *Actinobacillus*–*Haemophilus*–*Pasteurella* group by gas chromatography of trifluoroacetic acid anhydride derivatives from whole-cell methanolysates. *Journal of Chromatography* **342**, 13–23.
- BROOM, A. K. & SNEATH, P. H. A. (1981). Numerical taxonomy of *Haemophilus*. *Journal of General Microbiology* **126**, 123–149.
- BROSIOUS, J., PALMER, J. L., KENNEDY, J. P. & NOLLER, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 4801–4805.
- CARBON, P., EBEL, J.-P. & EHRESMANN, C. (1981). The sequence of the ribosomal 16S RNA from *Proteus vulgaris*: sequence comparison with *E. coli* 16S RNA and its use in secondary structure model building. *Nucleic Acids Research* **9**, 2325–2333.
- CHRISTIANSEN, C., HANSEN, E. & FRIIS-MOLLER, A. (1981). Homology between DNA from selected strains of the genera *Pasteurella*, *Actinobacillus*, and *Haemophilus*. In *Haemophilus, Pasteurella, and Actinobacillus*, pp. 158–160. Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.
- COYKENDALL, A. L., SETTERFIELD, J. & SLOTS, J. (1983). Deoxyribonucleic acid relatedness among *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and other *Actinobacillus* species. *International Journal of Systematic Bacteriology* **33**, 422–424.
- ESCANDE, F., GRIMONT, F., GRIMONT, P. A. D. & BERCOVIER, H. (1984). Deoxyribonucleic acid relatedness among strains of *Actinobacillus* spp. and *Pasteurella ureae*. *International Journal of Systematic Bacteriology* **34**, 309–315.
- FENG, D.-F., JOHNSON, M. S. & DOOLITTLE, R. F. (1985). Aligning amino acid sequences: comparison of commonly used methods. *Journal of Molecular Evolution* **21**, 112–125.
- FOX, G. E., STACKEBRANDT, E., HESPELL, R. B., GIBSON, J., MANILOFF, J., DYER, T. A., WOLFE, R. S., BALCH, W. E., RANNER, R. S., MAGRUM, L. J., ZABLEN, L. B., BLAKEMORE, R., GUPTA, R., BONEN, L., LEWIS, B. J., STAHL, D. A., LUEHRSEN, K. R., CHEN, K. N. & WOESE, C. R. (1980). The phylogeny of prokaryotes. *Science* **209**, 457–463.
- FRIIS-MOLLER, A. (1981). A new *Actinobacillus* species from the human respiratory tract: *Actinobacillus hominis* nov. sp. In *Haemophilus, Pasteurella, and Actinobacillus*, pp. 151–157. Edited by M. Kilian, W. Frederiksen & E. L. Biberstein. London: Academic Press.
- GÖBEL, U., MAAS, R. & CLAD, A. (1987a). Quantitative electroelution of oligonucleotides and large DNA fragments from gels and purification by electro dialysis. *Journal of Biochemical and Biophysical Methods* **14**, 245–260.
- GÖBEL, U., GAISER, A. & STANBRIDGE, E. J. (1987b). Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. *Journal of General Microbiology* **133**, 1969–1974.
- GREEN, C. J., STEWART, G. C., HOLLIS, M. A., VOLD, B. S. & BOTT, K. F. (1985). Nucleotide sequence of the *Bacillus subtilis* ribosomal RNA operon *rrnB*. *Gene* **37**, 261–266.
- HUYSMANS, E. & DE WACHTER, R. (1986). Compilation of small ribosomal subunit sequences. *Nucleic Acids Research* **14** (suppl.), r73–r118.
- JARSCH, M. & BÖCK, A. (1985). Sequence of the 16S ribosomal RNA gene from *Methanococcus vannielii*. *Systematic and Applied Microbiology* **6**, 54–59.
- JOHNSON, A. M., MURRAY, P. J., ILLANA, S. & BAVERSTOCK, P. J. (1987). Rapid nucleotide sequence analysis of the small subunit ribosomal RNA of *Toxoplasma gondii*: evolutionary implications for the Apicomplexa. *Molecular and Biochemical Parasitology* **25**, 239–246.
- LANE, D. J., PACE, B., OLSEN, G. J., STAHL, D. A., SOGIN, M. L. & PACE, N. R. (1985). Rapid determination of 16S rRNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6955–6959.
- LAU, P. P., DEBRUNNER-VOSSBRINK, B., DUNN, B., MIOTTO, K., MACDONNEL, M. T., ROLLINS, D. M., PILLIDGE, C. J., HESPELL, R. B., COLWELL, R. R., SOGIN, M. L. & FOX, G. E. (1987). Phylogenetic diversity and position of the genus *Campylobacter*. *Systematic and Applied Microbiology* **9**, 231–238.
- MACINNES, J. I. & ROSENDAL, S. (1987). Analysis of major antigens of *Haemophilus (Actinobacillus) pleuropneumoniae* and related organisms. *Infection and Immunity* **55**, 1626–1634.
- MUTTERS, R., PIECHULLA, K. & MANNHEIM, W. (1984). Phenotypic differentiation of *Pasteurella sensu strictu* and the *Actinobacillus* group. *European Journal of Clinical Microbiology* **3**, 225–229.

- NEARHOS, S. P. & FUERST, J. A. (1987). Reanalysis of 5S rRNA sequence data for the *Vibrionaceae* with the clustan program suite. *Current Microbiology* **15**, 329–335.
- NEEDLEMAN, S. B. & WUNSCH, C. D. (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of Molecular Biology* **48**, 443–453.
- OLSEN, G. J., LANE, E. J., GIOVANNONI, S. J., PACE, N. R. & STAHL, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annual Review of Microbiology* **40**, 337–355.
- PACE, N. R., OLSEN, G. J. & WOESE, C. R. (1986). Ribosomal RNA phylogeny and the primary lines of evolutionary descent. *Cell* **45**, 325–326.
- POHL, S., BERTSCHINGER, H. U., FREDERIKSEN, W. & MANNHEIM, W. (1983). Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica* like organisms causing porcine necrotic pleuropneumonia to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. *International Journal of Systematic Bacteriology* **33**, 510–514.
- POTTS, T. V. & BERRY, E. M. (1983). Deoxyribonucleic acid–deoxyribonucleic acid hybridization analysis of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *International Journal of Systematic Bacteriology* **33**, 765–771.
- POTTS, T. V., ZAMBON, J. J. & GENCO, R. J. (1985). Reassignment of *Actinobacillus actinomycetemcomitans* to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov. *International Journal of Systematic Bacteriology* **35**, 337–341.
- ROMANIUK, P. J., ZOLTOWSKA, B., TRUST, T. J., LANE, D., OLSEN, G. J., PACE, N. R. & STAHL, D. A. (1987). *Campylobacter pylori*, the spiral bacterium associated with human gastritis, is not a true *Campylobacter* sp. *Journal of Bacteriology* **169**, 2137–2141.
- SNEATH, P. H. A. & STEVENS, M. (1985). A numerical taxonomic study of *Actinobacillus*, *Pasteurella* and *Yersinia*. *Journal of General Microbiology* **131**, 2711–2738.
- TOMIOKA, N. & SUGIURA, M. (1983). The complete nucleotide sequence of a 16S ribosomal RNA gene from a blue-green alga, *Anacystis nidulans*. *Molecular and General Genetics* **191**, 46–50.
- WILSON, A. C., OCHMAN, H. & PRAGER, E. M. (1987). Molecular time scale for evolution. *Trends in Genetics* **3**, 241–247.
- WISHART, D. (1987). *Clustan User Manual* (Release 3.2). Edinburgh: Program Library Unit, Edinburgh University.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221–271.
- WOESE, C. R., WEISBURG, W. G., HAHN, C. M., PASTER, B. J., ZABLEN, L. B., LEWIS, B. J., MACKE, T. J., LUDWIG, W. & STACKEBRANDT, E. (1985). The phylogeny of the purple subgroup bacteria: the gamma subdivision. *Systematic and Applied Microbiology* **6**, 25–33.
- YANG, F., OYAIZU, Y., OYAIZU, H., OLSEN, G. J. & WOESE, C. R. (1985). Mitochondrial origins. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4443–4447.
- ZUCKERKANDL, E. & PAULING, L. (1965). Molecules as documents of evolutionary history. *Journal of Theoretical Biology* **8**, 357–366.