5S rRNA sequences of representatives of the genera *Chlorobium*, *Prosthecochloris*, *Thermomicrobium*, *Cytophaga*, *Flavobacterium*, *Flexibacter* and *Saprospira* and a discussion of the evolution of eubacteria in general

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5S rRNA sequences were determined for the green sulphur bacteria *Chlorobium limicola*, *Chlorobium phaeobacteroides* and *Prosthecochloris aestuarii*, for *Thermomicrobium roseum*, which is a relative of the green non-sulphur bacteria, and for *Cytophaga aquatilis*, *Cytophaga heparina*, *Cytophaga johnsonae*, *Flavobacterium breve*, *Flexibacter* sp. and *Saprospira grandis*, organisms allotted to the phylum 'Bacteroides–Cytophaga–Flavobacterium' and relatives as determined by 16S rRNA analyses. By using a clustering algorithm a dendrogram was constructed from these sequences and from all other known eubacterial 5S RNA sequences. The dendrogram showed differences, as well as similarities, with respect to results obtained by 16S RNA analyses. The 5S RNA sequences of green sulphur bacteria were closely related to one another, and to a cluster containing 5S RNA sequences from *Bacteroides* and its relatives, including *Cytophaga aquatilis*. 5S RNA sequences of all other representatives of the 'Bacteroides–Cytophaga–Flavobacterium' phylum as distinguished by 16S RNA analysis failed to group with *Bacteroides* and related clusters. On the basis of 5S RNA sequences, *Thermomicrobium roseum* clustered with *Chloroflexus aurantiacus*, as was expected from 16S RNA analysis.

Introduction

The order Chlorobiales (Gibbons & Murray, 1978) consists of the families Chlorobiaceae (green sulphur bacteria) and Chloroflexaceae (gliding, filamentous, non-sulphur green bacteria). The members of these families share a number of unique properties. For instance, the light-harvesting pigments are located in typical chlorosome structures (Cohen-Bazire *et al.*, 1964; Staehelin *et al.*, 1978), and members of both families contain monogalactosyl diglyceride, but not phosphatidylethanolamine in their lipids. Conversely, the families differ in numerous features, such as the structure of their photosynthetic reaction centres (Olson & Pierson, 1987) and their gliding ability. Furthermore, representatives of the Chlorobiaceae and Chloroflexaceae differ in their physiological and ecological properties. With the exception of *Chloroherpeton thalassium* (Gibson *et al.*, 1984) and *Chloroherpeton limnophilum* (Eichler & Pfennig, 1988), which demonstrate gliding motility, the Chlorobiaceae are non-motile, mesophilic, strictly anaerobic and obligately photo-autotrophic rods. In comparison,

Chloroflexus aurantiacus comprises gliding, thermophilic, facultatively chemo- or phototrophic filaments (Pierson & Castenholz, 1974). Members of both families inhabit different ecological niches (Gorlenko, 1988). Moreover, analyses of 16S rRNA oligonucleotide catalogues (Gibson *et al.*, 1985) and full sequences (Oyaizu *et al.*, 1987; Woese, 1987) have indicated a lack of phylogenetic relationship between the two families (Gibson *et al.*, 1985). Whereas the genera *Chlorobium* (Cb.), *Prosthecochloris* and *Chloroherpeton* form a tight phylogenetic unit, the thermophilic *Chloroflexus aurantiacus* is distantly related to two non-photosynthetic genera, the thermophilic *Thermomicrobium* and the mesophilic *Herpetosiphon* (Gibson *et al.*, 1985; Oyaizu *et al.*, 1987). Phenotypically, the latter arrangement is not immediately obvious (Oyaizu *et al.*, 1987). Indeed, the possession of unusual long-chain diols (Pond *et al.*, 1986) is the only phenotypic characteristic that links *Thermomicrobium roseum* to *Chloroflexus aurantiacus* (Stackebrandt *et al.*, 1988a), whereas *Herpetosiphon giganteus* and *Chloroflexus aurantiacus* possess similar carotenoids (Kleinig & Reichenbach, 1977). From 16S RNA analy-

ses, both families have been provisionally assigned the rank of 'phylum' (Woese *et al.*, 1985). The lack of phylogenetic relationship between the two families is corroborated by lipopolysaccharide and peptidoglycan analyses (Jürgens *et al.*, 1987; Meissner *et al.*, 1987, 1988). The *Chloroflexus-Thermomicrobium-Herpetosiphon* phylum is one of the earliest branchings in eubacterial evolution (Oyaizu *et al.*, 1987). As a consequence *Chloroflexus*, rather than the cyanobacteria, has been suggested to be the photosynthetic component of the stromatolites (Oyaizu *et al.*, 1987). Another eubacterial phylum, termed '*Bacteroides-Cytophaga-Flavobacterium*', made up of genera such as *Bacteroides*, *Cytophaga* (C.), *Flexibacter*, *Saprospira*, *Sporocytophaga*, *Haliscomenobacter*, *Fusobacterium* and *Flavobacterium* (Flv.) (Paster *et al.*, 1985; Weisburg *et al.*, 1985) has been suggested to bear a somewhat closer relationship to the phylum of green sulphur bacteria than to any of the remaining eubacterial phyla (Woese, 1987).

In order to compare results obtained with 16S and 5S rRNA analyses, the 5S RNA sequences of *Cb. limicola*, *Cb. phaeobacteroides*, *Prosthecochloris aestuarii*, *Thermomicrobium roseum*, *Saprospira grandis*, *Flexibacter* sp., *Flv. breve*, *C. heparina*, *C. aquatilis* and *C. johnsonae* were determined. Using weighted average pairgroup clustering, a phylogenetic tree was constructed from these sequences, together with some 282 other eubacterial 5S RNA sequences, representative of eight of the ten major phyla distinguished by Woese (1987).

Methods

Bacterial strains. The following strains were used: *Chlorobium limicola* DSM 249, *Cb. phaeobacteroides* DSM 266^T (T, type strain), *Prosthecochloris aestuarii* strain SK 413, *Thermomicrobium roseum* ATCC 27502^T, *Cytophaga aquatilis* ATCC 29551^T, *C. heparina* ATCC 13125^T, *C. johnsonae* DSM 425, *Flavobacterium breve* ATCC 14234, *Flexibacter* sp. DSM 527 and *Saprospira grandis* ATCC 23119^T. These were obtained from the American Type Culture Collection (ATCC), Rockville, Md, USA, and from the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, FRG. Culture conditions were as follows. *Cb. limicola*, *Cb. phaeobacteroides* and *P. aestuarii* were grown using the method described by Pfennig & Trüper (1981). *T. roseum* was obtained from Dr Robert F. Ramaley, Department of Biochemistry, University of Nebraska, USA, and grown at 65 °C in a medium (pH 7.5) containing (l⁻¹) tryptone (Difco), 1 g, yeast extract (Difco), 1 g, and mineral salts as described by Zarilla & Perry (1984). *C. aquatilis*, *C. heparina* and *F. breve* were grown aerobically at 26 °C in 8 g nutrient broth (Merck) l⁻¹, pH 7.0. *Flexibacter* sp. was grown aerobically at 26 °C in medium (pH 7.2) containing (l⁻¹) Casitone (Difco), 3 g, and CaCl₂·2H₂O, 1.36 g. The same culture conditions applied to *C. johnsonae*, with the exception that yeast extract (Difco; 1 g l⁻¹) was added to the medium. *S. grandis* was grown according to Lewin (1972). Cultivated organisms were harvested by centrifugation and stored by freezing or freeze-drying until needed.

Preparation of 5S RNA. Cells were ruptured using either glass beads and a cell mill (Willekens *et al.*, 1986), or by grinding in a mortar with

alumina (Van den Eynde *et al.*, 1987). Sheared cells were then extracted with phenol (Willekens *et al.*, 1986) in order to obtain the rRNA fraction. 5S RNA was prepared by PAGE, as previously described (Fang *et al.*, 1982). 5S RNA was labelled at the 3'-terminus with cytidine 3',5'-[5'-³²P]bisphosphate (Amersham), purified on 8% (w/v) polyacrylamide gels, and subjected to partial chemical degradation (Peattie, 1979). Whenever necessary, partial enzymic degradation, as described previously (Dams *et al.*, 1983), was used to confirm the nucleotide pattern. In the case of length heterogeneity, components of different chain length were sequenced separately. With *Cb. limicola*, *Cb. phaeobacteroides*, *P. aestuarii*, *C. johnsonae*, *C. heparina*, *Flv. breve*, *S. grandis*, *Flexibacter* sp. and *T. roseum*, electrophoresis of the partial degradation products on 8–20% (w/v) polyacrylamide gels, kept at a constant temperature of 64 °C, proved sufficient to elucidate the entire sequence. In the case of *C. aquatilis*, the identity of the 5'-terminal nucleotide was confirmed by gel electrophoresis of enzymically degraded (Ap)₄A-extended, 5'-terminally labelled (Dams *et al.*, 1983) 5S RNA.

Data analysis. Dendrograms were constructed by using a weighted pairgroup clustering algorithm, as described previously (Huysmans & De Wachter, 1986; Dams *et al.*, 1987). In short, a dissimilarity matrix that takes into account multiple and back mutations serves as the input for the tree-construction algorithm. A correction, to allow for the effects of unequal evolutionary rates along different eubacterial lineages, is incorporated. The standard deviations on the substitution terms of the computed dissimilarities are indicated by error flags on all branching points of the resulting dendrogram.

Results and Discussion

Part of an alignment in which the newly determined sequences were aligned with approximately 600 5S RNA sequences of eubacterial, archaeobacterial, eukaryotic and organelle nature is included in Fig. 1. The complete alignment is available from the authors. Boxes superimposed on the alignment indicate putative double-stranded areas, according to a well-established secondary structure proposal (De Wachter *et al.*, 1982). Starting from an alignment containing the 303 eubacterial 5S RNA sequences and supplemented with 10 cytoplasmic 5S RNA sequences from red algae to permit correction of unequal evolutionary rates, a dendrogram was constructed (Fig. 2). The majority of the sequences included are found in the biannual compilation of Wolters & Erdmann (1988). The sequences are representative of eight of the ten major eubacterial phyla, as determined by 16S RNA analysis (Woese, 1987). When comparing the pattern deduced from 5S RNA sequence information with that based on 16S RNA analyses, a number of similarities and discrepancies become apparent. For example, no relationship was detected between plant mitochondrial 5S RNA sequences and the alpha subdivision of the recently installed (Stackebrandt *et al.*, 1988b; formerly the purple bacteria and their relatives) class Proteobacteria. This might be an aberration of the tree-construction algorithm, due to the extremely fast rate at which mitochondrial genomes appear to be evolving.

Fig. 1. Alignment of 5S rRNA sequences determined in the present study. Blank alignment positions are necessary to accommodate nucleotides of 5S RNAs from other taxonomic groups. Length heterogeneity is indicated by terminal residues printed in lower-case letters. Boxes labelled A and A', B and B', and so on, enclose the complementary strands of helices A, B, etc., in the secondary-structure model of De Wachter *et al.* (1982). Bulges within helices are enclosed in nested boxes, bases belonging to odd base pairs (pairs other than A-U, G-C and G-U), intercalated between Watson-Crick and/or G-U pairs, are in parentheses.

Fig. 2. Dendrogram constructed by weighted pairwise clustering of 5S rRNA sequences from eubacteria and plant organelles. The complete dendrogram was constructed starting from an alignment that contained 303 eubacterial and organelle sequences available to us at present, supplemented with 10 cytoplasmic 5S RNA sequences from red algae (not shown). The latter were used as an external reference group, allowing for a correction for unequal evolutionary rates in different eubacterial lineages. Each major cluster is represented by an isosceles triangle with its top at the dissimilarity value corresponding to the first divergence within the cluster, and with a base proportional to the number of sequences enclosed (indicated in parentheses after the cluster name). Standard deviations on the substitution terms of the computed dissimilarities are indicated by bars. Details on composition and topology of the cluster harbouring 5S RNA sequences from *Bacteroides* and relatives and green sulphur bacteria, and the cluster denoted 'miscellaneous', are given in Fig. 3.

Cyanobacteria and plastids (Van den Eynde *et al.*, 1988) are found as a monophyletic group, in accordance with the picture based on 16S RNA data. With 5S RNA, however, the cluster appears to branch off earlier in time than with 16S RNA. Furthermore, *Campylobacter* spp., on the basis of their 5S RNA sequence, exhibit no specific relationship to any of the eubacterial clusters (Fig. 2). Conversely, on the basis of 16S RNA sequences,

a slight relationship of *Campylobacter* to the class Proteobacteria has been proposed (Lau *et al.*, 1987).

The cluster that branches off next in Fig. 2 contains the majority of the 5S RNA sequences determined here. The cluster is defined by sequences from the '*Bacteroides*–*Cytophaga*–*Flavobacterium*' group of organisms (Paster *et al.*, 1985; Weisburg *et al.*, 1985), and by sequences from green sulphur organisms (Gibson *et al.*,

Fig. 3. (a) Detailed structure of the cluster containing the majority of the sequences determined in this study. Except for the sequences reported here, sequences were taken from Wolters & Erdmann (1988) or are unpublished (N. R. Pace, personal communication). (b) Detailed structure of the cluster termed 'miscellaneous' in Fig. 2. Except for the sequences reported here, sequences were taken from Wolters & Erdmann (1988), or are unpublished so far (H. Van den Eynde and others).

1985). A detailed picture of the cluster is presented in Fig. 3(a). In the cluster, 5S RNA sequences of *Chlorobium* and *Prosthecochloris* spp. are found closely related to one another, as was expected from 16S RNA analyses (Gibson *et al.*, 1985). The 5S RNA sequences of *Cb. limicola* DSM 249, *Cb. phaeobacteroides* and *Cb. vibrioforme* (N. R. Pace, unpublished results), and of *Cb. limicola* (Chumakov, 1987) are closely related.

The 5S RNA sequence of *Prosthecochloris aestuarii* was recovered next. As a whole, the green sulphur bacteria are associated with a cluster containing *Bacteroides fragilis*, *B. thetaiotaomicron*, *Porphyromonas gingivalis* and *B. veroralis*. This tendency towards a grouping of

Bacteroides and relatives with green sulphur bacteria has been observed previously, on the basis of both 16S RNA (Woese, 1987) and 5S RNA (Van den Eynde *et al.*, 1989) analyses. With 16S RNA, however, the genus *Fusobacterium* is included in the '*Bacteroides*'-like cluster, whereas with 5S RNA *Fusobacterium* is only peripherally related to the *Bacteroides*-green sulphur bacteria assemblage (Van den Eynde *et al.*, 1989). Furthermore, with 16S RNA sequences, *Flv. breve*, *Flexibacter* sp., *S. grandis*, *C. heparina* and *C. johnsonae* are recovered with the genus *Bacteroides* to form the so-called '*Bacteroides*-*Cytophaga*-*Flavobacterium*' phylum. That this is not the case with 5S RNA is documented below. The 5S RNAs

of *Streptococcus lactis* subsp. *cremoris* and of *Mycoplasma hyopneumoniae* were recovered, atypically, close to the *Fusobacterium* cluster, whereas they were expected to group with mycoplasmata and lactobacilli 5S RNA sequences.

The 5S RNA sequences from two out of three representative species of the phylum of green non-sulphur bacteria and relatives, namely *Chloroflexus aurantiacus* (D. A. Stahl, unpublished results) and *Thermomicrobium roseum*, were found (Fig. 2) to bear a specific relationship to one another, in accordance with the picture derived from 16S RNA analyses (Oyaizu *et al.*, 1987). The suggested ancient nature of the phylum of green non-sulphur bacteria and relatives (Oyaizu *et al.*, 1987) was not corroborated by 5S RNA analysis. In fact, both the cyanobacteria-plastid cluster and the cluster containing the green sulphur bacteria branched off earlier than the *Chloroflexus* cluster (Fig. 2). The results presented in this paper thus fail to affirm the proposal (Oyaizu *et al.*, 1987) of *Chloroflexus* being the photosynthetic component of the stromatolites. The 5S RNA of 'Herpetosiphon strain' Senghas Wie 2 (Van den Eynde *et al.*, 1987), originating from exactly the same strain (Senghas & Lingens, 1985) as was used for 16S RNA analysis, was not, contrary to 16S RNA results, recovered in close association with *Chloroflexus* and *Thermomicrobium* 5S RNA. Instead, the 'Herpetosiphon strain' Senghas Wie 2 sequence grouped with a cluster termed 'miscellaneous' in Fig. 2, and presented in detail in Fig. 3(b). Apart from the 'Herpetosiphon strain' Senghas Wie 2 sequence and a number of sequences from low G + C content Firmicuta, sequences from organisms belonging to the Proteobacteria delta group, from *C. heparina* and from *Flexibacter* sp. were scattered throughout this cluster. Thus, regarding the assignment of strain Senghas Wie 2 to the phylum of green sulphur bacteria and their relatives (Senghas & Lingens, 1985), and the connected suggestion for an assignment of the strain to the genus *Herpetosiphon* (Gibson *et al.*, 1985; Woese *et al.*, 1985), we urge caution. In any case, in the original paper of Senghas & Lingens (1985), the strain was set well apart from the genus *Herpetosiphon* on the basis of physiological differences and of differences in fatty acid composition and in DNA G + C content.

As for the Proteobacteria, in agreement with the findings based on 16S RNA, the subdivisions alpha, beta and gamma were recovered intact, and in close connection with one another. However, with 5S RNA analysis, the alpha subdivision was severed from the beta and gamma clusters by a group containing radio-resistant organisms such as *Deinococcus* and their relatives. We can see no evidence for the existence of a fourth subdivision, delta, of the class Proteobacteria (Woese, 1987; Stackebrandt *et al.*, 1988b), since organisms of the

delta subgroup (H. Van den Eynde and others, unpublished) that were examined had 5S RNA sequences that grouped with, or, in the case of the *Sorangium cellulosum* 5S RNA, close to the miscellaneous cluster (Figs 2 and 3b).

Rather more in accordance with results based on 16S RNA sequencing is the occurrence in Fig. 2 of a cluster harbouring the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera*, which all lack peptidoglycan.

The phylum of Gram-positive organisms, or Firmicuta, a monophyletic grouping with four subdivisions according to 16S RNA analysis (Woese, 1987), showed a different arrangement on the basis of 5S RNA analysis, as can be seen from Fig. 2. The two most important subdivisions, namely Firmicuta with low, and Firmicuta with high DNA G + C contents, have a large number of representatives in the 5S RNA data bank. Results from 16S RNA analysis were corroborated to a certain extent by 5S RNA sequence data, although some of the 5S RNA sequences of Gram-positive organisms were recovered in unexpected places in the tree. Examples are the occurrence of some sequences of clostridia and bacilli, Firmicuta of low DNA G + C content, in the aforementioned 'miscellaneous' cluster (Fig. 3b), a cluster more closely related to the Firmicuta with high rather than to those with low DNA G + C content. Also unexpected, was the occurrence of the 5S RNA of *Flv. breve* and of *Saprospira grandis* among the Firmicuta with high DNA G + C content, and the occurrence of *Cytophaga johnsonae*, *Anaerorhabdus furcosus* (Van den Eynde *et al.*, 1989) and *Verrucomicrobium spinosum* (Bomar & Stackebrandt, 1987) 5S RNAs with those of Firmicuta with a low genomic G + C content. 16S RNA studies have previously shown the relatedness of the Gram-negative genera *Selenomonas*, *Sporomusa* and *Megasphaera*, to the Firmicuta and relatives phylum (Stackebrandt *et al.*, 1985). 16S RNA analysis showed these genera to be closely related, and to constitute one of the four subdivisions of the phylum (Woese, 1987). In contrast, several 5S RNA sequences from Gram-negative species, were scattered throughout the low and high G + C content subdivisions of the Gram-positive cluster, a clear discrepancy with results obtained by 16S RNA analysis. Moreover, since we chose exactly the same strains as were used for 16S RNA analysis (Paster *et al.*, 1985), in the case of *C. johnsonae*, *C. heparina*, *Flv. breve* and *S. grandis* 5S RNA, an association with the 'Bacteroides-Cytophaga-Flavobacterium' cluster, and not with the Gram-positive organisms, was most strongly expected. That this was not the case makes the observed discrepancy even more unusual. Moreover, 5S RNA analysis did not corroborate the ancient line of descent proposed for *Verrucomicrobium spinosum* by Albrecht *et al.* (1987).

We cannot offer a clearcut explanation for the differences revealed by analyses of 5S and 16S RNA sequences. The nature of the 5S RNA molecule, as opposed to the 16S RNA molecule, cannot, in our opinion be held responsible. Although 5S RNA is much smaller than 16S RNA (an average of 120 nucleotides for 5S RNA against an average of about 1550 nucleotides for eubacterial 16S RNA), 5S RNA is far more conserved than 16S RNA and has had to suffer less from extensive insertion- and/or deletion events. The alignment procedure with 5S RNA is thus much less prone to error than is the case with 16S RNA. Furthermore, it is not solely discrepancies that are encountered when results from 5S RNA sequence analysis are compared to those obtained using 16S RNA: many features of the 16S RNA evolutionary picture corroborated fairly well by analyses of 5S RNA sequences. The part played by unequal eubacterial evolutionary rates, is thought to be limited due to the application of a correction procedure for this phenomenon. In view of the conflicting results obtained with 5S RNA and 16S RNA, we believe that a renewed and thorough investigation of the phylogenetic interrelationships of the organisms assigned to the '*Bacteroides-Cytophaga-Flavobacterium*' is needed. Questions can also be raised regarding the extent to which the use of different tree-construction algorithms influences the inferred evolutionary picture. The differing results obtained using 16S RNA and 5S RNA as molecular tools for studying eubacterial evolution suggests, that the use of additional molecular clocks, such as 23S rRNA, may be required in order to arrive at a consensus picture of eubacterial evolution.

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