The phylogenetic relationships of *Chlorobium tepidum* and *Chloroflexus aurantiacus* based upon their RecA sequences

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Abstract

Using RecA as the phylogenetic marker, the relationships of the green sulfur bacterium *Chlorobium tepidum* and the green non-sulfur bacterium *Chloroflexus aurantiacus* to other eubacteria were investigated. The *recA* genes of the two organisms were cloned, and the resulting protein sequences aligned with 86 other eubacterial RecA sequences. *Cb. tepidum* was placed as the nearest relative to the *Cytophaga/Flexibacter/Bacteroides* group, a relationship supported by results obtained with several phylogenetic markers. *Cf. aurantiacus* was placed near *Chlamydia trachomatis* and the high-GC Gram-positives; however, this branching pattern was not strongly supported statistically by bootstrap analyses. Possible reasons for this ambiguity are discussed. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

1. Introduction

The green sulfur bacteria (also called Chlorobiaciae) and the green non-sulfur bacteria comprise a relatively small number of identified genera which have not been exhaustively characterized phylogenetically. Using sulfide or sulfur as electron donors, the green sulfur bacteria are obligately anaerobic and photolithoautotrophic. Recently, the phylogeny of

several green sulfur bacteria was analyzed using SS-rRNA sequences [1,2]. *Chlorobium tepidum*, the only known thermophile of the genus *Chlorobium*, was found to be placed near the *Chlorobium limicola* cluster. The thermophilic nature of *Cb. tepidum* is believed to result from rapid, divergent evolution rather than from inherited growth characteristics derived from an ancestral thermophilic relative [1].

The green non-sulfur bacteria are composed of both phototrophic and non-phototrophic members. Pierson and Castenholtz [3] have recently suggested that the terms 'green non-sulfur bacteria' and 'gliding green bacteria' should no longer be used, and suggest the terms 'phototrophic flexibacteria' or 'filamentous anoxygenic phototrophs'. However, these terms do not properly describe the closely related

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organism, *Thermomicrobium roseum*, which is neither phototrophic nor filamentous. The phototrophic thermophile *Chloroflexus aurantiacus* is the best characterized member of this grouping. This organism is very interesting from an evolutionary perspective due to its combination of characteristics that are found in very different and diverse groups of phototrophic prokaryotes [3]. The phototrophic green non-sulfur bacteria have chlorosomes as their light-harvesting antenna system, like the green sulfur bacteria [4]. However, their reaction centers are similar to those of the phototrophic proteobacteria and to photosystem II of the cyanobacteria [5], and differ significantly from those of the green sulfur bacteria, the heliobacteria, and photosystem I of cyanobacteria. The overall cell morphology, carotenoid composition, and mat-forming behavior of these organisms resemble those of certain cyanobacteria [6]. *Cf. aurantiacus* also displays some features which are unique among autotrophs, such as its autotrophic CO_2 fixation mechanism by the 3-hydroxypropionate pathway [7].The RecA protein in *Escherichia coli* takes part in a number of cellular processes, among them homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis [8]. Although the RecA protein sequence and function is highly conserved within bacteria, it is not absolutely essential for cell survival in most organisms. Related proteins have also been found in archaea and eukaryotes [9]. Eisen [10] has shown that RecA comparisons are informative in studies of molecular systematics of bacteria. The molecule fulfils a number of criteria that make it a useful marker for phylogenetic analyses. Some of these are: the molecule is of reasonable size, thus allowing statistical analyses to be performed; some regions of RecA are conserved between species and other regions are highly variable, thus allowing comparisons between both close and distant relatives; and the gene is relatively easily cloned.

In this work the *recA* genes of *Cb. tepidum* and *Cf. aurantiacus* have been cloned and analyzed phylogenetically. It was our goal to use RecA as a marker to examine specifically the placement of these two organisms within the eubacterial kingdom. Furthermore, we updated the previous phylogenetic tree derived from RecA sequences [10] by including 26 additional eubacterial RecA sequences.

2. Materials and methods

2.1. Recombinant DNA procedures

Cb. tepidum was kindly provided by Dr. Michael Madigan (Southern Illinois University, Carbondale, IL), and *Cf. aurantiacus* J-10-fl kindly provided by Dr. Beverly Pierson (University of Puget Sound, Tacoma, WA). Total chromosomal DNA from *Cb. tepidum* and *Cf. aurantiacus* was isolated as described [11] with the inclusion of a CTAB (hexadecyl-trimethylammonium bromide) extraction. Clones containing the *recA* genes were isolated by using size-directed plasmid libraries as described [12]. DNA sequences were determined by the dideoxy chain termination method [13], with the Sequenase Version 2.0 DNA sequencing kit from U.S. Biochemical (Cleveland, OH) or were determined by an automated sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA). Oligonucleotides for sequencing were synthesized on a Model 392 Applied Biosystems (Foster City, CA) automated DNA/RNA synthesizer. Oligonucleotides for PCR were obtained from Genset Corporation. Sequence data were analyzed with MacVector Sequence Analysis Programs Version 6.0 (Eastman-Kodak, Rochester, NY).

2.2. Cloning of recA genes

Degenerate PCR primers were synthesized that span conserved regions of the RecA protein corresponding to amino acids 91–101 (primer sequence is 5′-GCITTYRTIGAYGCIGARCAYGCIYTIGAY-CC-3′) and amino acids 206–212 (primer sequence 5′-CCICCIGKIGTIGTRTCIGG-3′) of *E. coli* [10]. The resulting PCR products were used as hybridization probes to obtain genomic clones containing the complete *recA* genes. Southern blots with digests of *Cb. tepidum* and *Cf. aurantiacus* chromosomal DNAs were hybridized with the respective PCR products. Based on these hybridization experiments, a 3.2 kb *Eco*RI fragment of *Cb. tepidum* was cloned (see Fig. 1) to obtain the entire sequence of the *recA* gene. A portion of the *Cf. aurantiacus recA* gene was initially cloned on a 1.8 kb *Hin*cII fragment; subsequently, a 0.5 kb *Kpn*I-*Hin*cII fragment was cloned to obtain the remaining coding region of the gene (see Fig. 1). The DNA sequences for the
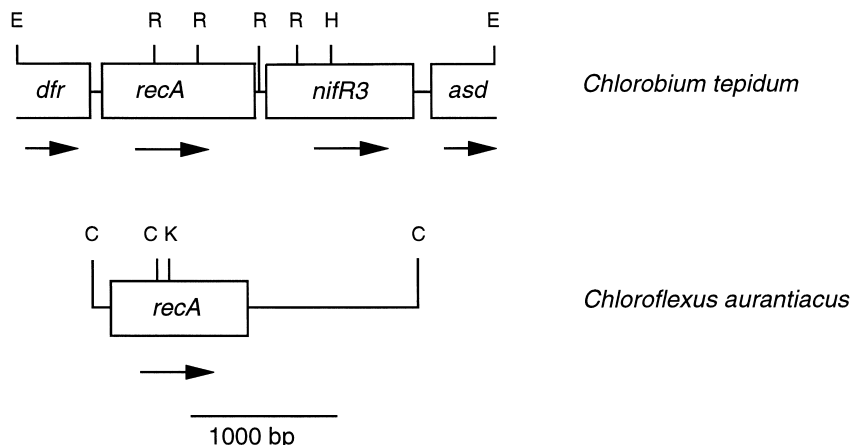
Fig. 1. Physical maps of the 3.2-kb *Eco*RI fragment encoding the *recA* gene of *Chlorobium tepidum* and of the 2.2-kb *Hin*cII fragment encoding the *recA* gene of *Chloroflexus aurantiacus*. The ORF found upstream of *recA* is homologous to dihydroflavonol-4-reductase (*dfr*) sequences. The ORFs downstream of *recA* are homologous to *nifR3* sequences and aspartate semialdehyde dehydrogenase (*asd*) sequences. Abbreviations for restriction enzymes are: C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; R, *Eco*RV.

Cb. tepidum recA gene and the *Cf. aurantiacus recA* gene have been deposited in GenBank under the accession numbers AF037259 and AF037258, respectively.

2.3. Phylogenetic analyses

In 1995 Eisen [10] aligned and analyzed phylogenetically 65 RecA sequences. Since then, 26 new sequences of RecA have been identified and deposited in the databases. These 26 sequences, as well as the sequences of *Cb. tepidum* and *Cf. aurantiacus*, have been added to most of the alignment obtained previously [10]. The alignment is available at http://www-leland.stanford.edu/~jeisen/RecA/RecA.Alignment.html. The phylogenetic tree was generated using algorithms available from the PHYLIP software package [14]. The pairwise distances between the RecA proteins were calculated with the *protdist* program in PHYLIP, using the PAM matrix-based distance correction [14]. The tree was generated by the neighbor-joining methods [15] as implemented in PHYLIP. Bootstrap replicates were carried out 100 times by the method of Felsenstein [16]. The RecA sequences were also analyzed phylogenetically using the parsimony inference method, as made available in PHYLIP [14]. The resulting tree was practically identical to the neighbor-joining tree shown in Fig. 2 (data not shown).

3. Results and discussion

As shown in Fig. 1, the *recA* gene of *Cb. tepidum* is flanked upstream by a gene with significant sequence similarity to dihydroflavonol-4-reductase (*dfr*) and downstream by genes with significant sequence similarity to the nitrogen regulatory gene *nifR3* and to aspartate semialdehyde dehydrogenase (*asd*). The *Cb. tepidum recA* gene predicts a protein of 346 amino acids with a predicted molecular mass of 37.1 kDa. No sequences with significant similarity to genes in the databases were identified downstream from the *recA* gene of *Cf. aurantiacus*; the *recA* gene of this bacterium predicts a protein of 351 amino acids with a predicted mass of 37.8 kDa. The deduced protein sequences were aligned with 86 other RecA sequences obtained from the databases. Fig. 2 shows the phylogenetic tree obtained for the RecA sequences, using the procedures described in Section 2.

3.1. Green sulfur bacteria

Using RecA as the phylogenetic marker (Fig. 2), *Cb. tepidum* is placed as the closest relative to the *Cytophaga-Flexibacter-Bacteroides* group. Based on the very close relationships of green sulfur bacteria among themselves [1,2], it can be assumed that the entire group will be closely related to the *Cytophaga-*

Flexibacter-Bacteroides group. The relationship between *Cb. tepidum* and the *Cytophaga-Flexibacter-Bacteroides* group is very highly supported in the RecA tree (bootstrap value of 100%), although the position of this entire clade within the tree lacks statistical significance (bootstrap value of 12%). The green sulfur bacteria have also been placed as the nearest relatives to the *Cytophaga-Flexibacter-Bacteroides* group based on SS-rRNA data [17,18]. This association has been confirmed by further studies, using the ATP-synthase β subunit and EF-Tu as markers, that included representative members of both phyla [19]. In analyses using sigma factors as the phylogenetic marker, the green sulfur bacteria are seen to be most closely related to the green non-sulfur bacteria (Gruber and Bryant, submitted); however, this study did not include any sequence from the *Cytophaga-Flexibacter-Bacteroides* group. Thus, there appears to be a consensus based upon several phylogenetic markers that the green sulfur bacteria and the *Cytophaga-Flexibacter-Bacteroides* group are close relatives.
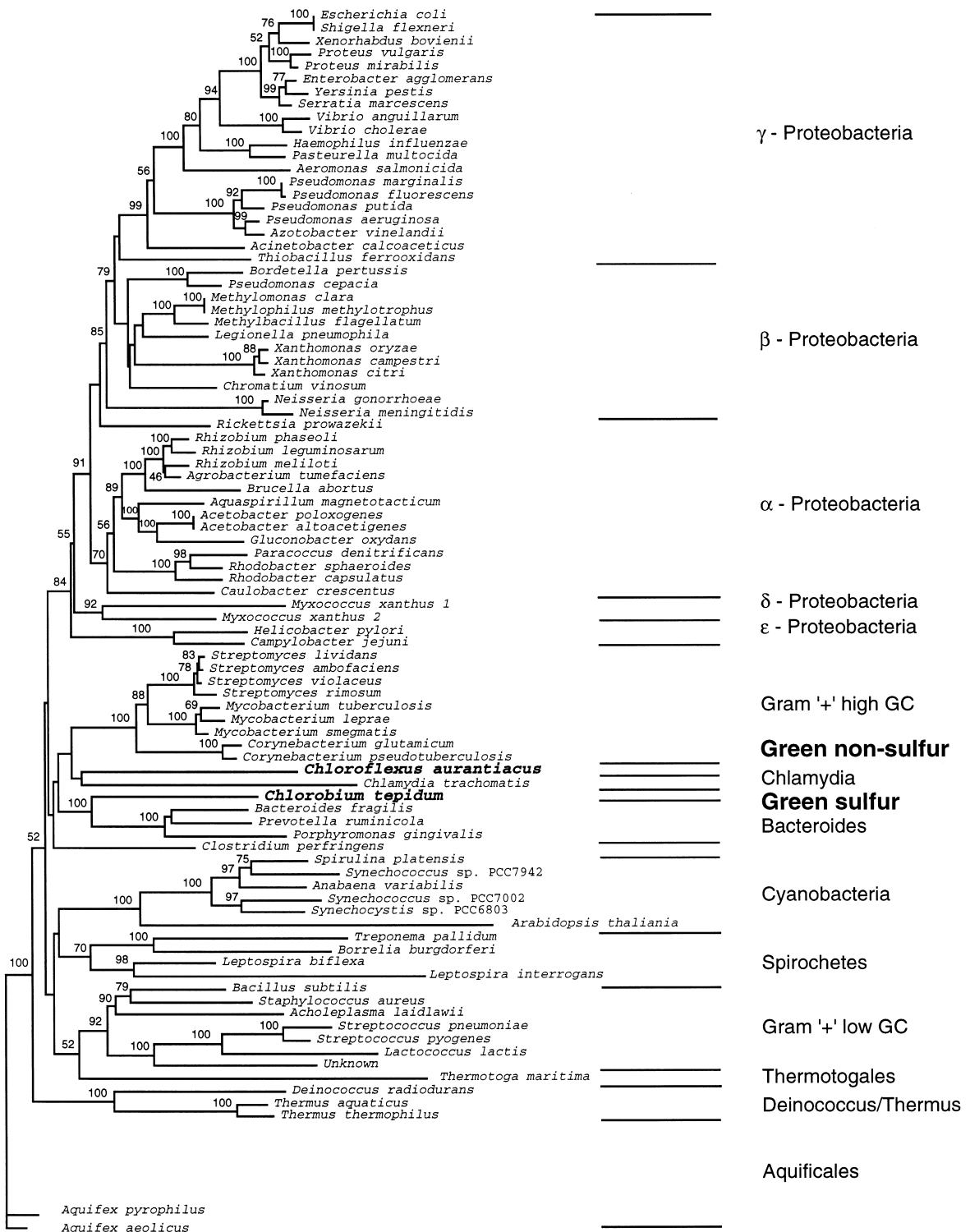
All green sulfur bacteria have similar physiological characteristics: all are strictly anaerobic, are obligately phototrophic, and can use carbon dioxide as the sole carbon source [20]. All species can use sulfide, which is oxidized to sulfate with the intermediate accumulation to elemental sulfur globules outside the cells, as the electron donor for growth. The *Cytophaga-Flexibacter-Bacteroides* group is composed of a mixture of physiological types [21]. The *Bacteroides* sp. are obligately anaerobic and primarily fermentative organisms, while the *Cytophaga* and *Flexibacter* sp. are heterotrophic gliding bacteria.

3.2. Green non-sulfur bacteria

The green non-sulfur bacteria are phylogenetically interesting organisms because different molecular markers place these organisms at different places in the eubacterial tree (see below). The best studied member of these organisms, *Cf. aurantiacus*, has even been termed a 'chimeric organism' due to its unique set of phenotypic properties [22]. In the present study using RecA as the marker, the position of *Cf. aurantiacus* is unfortunately not resolved. Although *Cf. aurantiacus* is placed nearest *Chlamydia trachomatis* and the high-GC Gram-positives, it may nevertheless still be fairly closely related to *Cb. tepidum* (Fig. 2). However, the bootstrap values supporting these relationships are low. In earlier studies using SS-rRNA as the marker, the green non-sulfur bacteria were placed as the closest relatives to the *Deinococcus/Thermus* group [18], whereas later studies showed the group to be positioned between the Thermotogales and the Planctomycetales [17]. In both analyses based upon SS-rRNA, the green non-sulfur bacteria were observed to diverge very early within the eubacterial line of descent. In an analysis using EF-Tu as the marker [19], the green gliding bacteria were placed between the *Deinococcus-Thermus* branch and the branch composed of the green sulfur bacteria and the *Cytophaga-Flexibacter-Bacteroides*. In a study using sigma factors, *Cf. aurantiacus* is the closest relative to *Cb. tepidum*, an association that is fairly well supported by bootstrap analyses (Gruber and Bryant, submitted). In a study using reaction center proteins as phylogenetic markers [22], it was unequivocally shown that the type II reaction center of *Cf. aurantiacus* is most closely related to the reaction centers of the phototrophic proteobacteria, in particular to *Rhodopseudomonas viridis* [22]. The reaction center of green non-sulfur bacteria also shows very distant similarity to Photosystem II of cyanobacteria, whereas green sulfur bacteria and heliobacteria have reaction centers which share a closer evolutionary relationship with Photosystem I of cyanobacteria [23]. There is also evidence that the membrane-bound bacteriochlorophyll *a*-containing antenna complexes and the membrane-bound cytochrome that donates electrons to the reaction center are similar in the phototrophic proteobacteria and *Cf. aurantiacus* [22]. Since none of the phylogenetic markers employed to date sug-

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Fig. 2. Neighbor-joining tree for RecA. The distances were calculated using the *protdist* program of PHYLIP with a PAM matrix-based distance correction. Bootstrap values were obtained after 100 replications and are indicated when over 40. (References for the sequences used can be obtained in [9], in Genbank, in appropriate webpages of completed genome sequences, and at http://www-leland.stanford.edu/∼jeisen/RecA/RecA.html)

Phylogenetic tree showing bacterial relationships. Major groups labeled on the right:

γ - Proteobacteria
β - Proteobacteria
α - Proteobacteria
δ - Proteobacteria
ε - Proteobacteria
Gram '+' high GC
Green non-sulfur
Chlamydia
Green sulfur
Bacteroides
Cyanobacteria
Spirochetes
Gram '+' low GC
Thermotogales
Deinococcus/Thermus
Aquificales

Taxa shown in the tree include:
Escherichia coli, *Shigella flexneri*, *Xenorhabdus bovienii*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter agglomerans*, *Yersinia pestis*, *Serratia marcescens*, *Vibrio anguillarum*, *Vibrio cholerae*, *Haemophilus influenzae*, *Pasteurella multocida*, *Aeromonas salmonicida*, *Pseudomonas marginalis*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Azotobacter vinelandii*, *Acinetobacter calcoaceticus*, *Thiobacillus ferrooxidans*, *Bordetella pertussis*, *Pseudomonas cepacia*, *Methylomonas clara*, *Methylophilus methylotrophus*, *Methylbacillus flagellatum*, *Legionella pneumophila*, *Xanthomonas oryzae*, *Xanthomonas campestri*, *Xanthomonas citri*, *Chromatium vinosum*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Rickettsia prowazekii*, *Rhizobium phaseoli*, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Agrobacterium tumefaciens*, *Brucella abortus*, *Aquaspirillum magnetotacticum*, *Acetobacter poloxogenes*, *Acetobacter altoacetigenes*, *Gluconobacter oxydans*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Caulobacter crescentus*, *Myxococcus xanthus 1*, *Myxococcus xanthus 2*, *Helicobacter pylori*, *Campylobacter jejuni*, *Streptomyces lividans*, *Streptomyces ambofaciens*, *Streptomyces violaceus*, *Streptomyces rimosum*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium smegmatis*, *Corynebacterium glutamicum*, *Corynebacterium pseudotuberculosis*, **Chloroflexus aurantiacus**, *Chlamydia trachomatis*, **Chlorobium tepidum**, *Bacteroides fragilis*, *Prevotella ruminicola*, *Porphyromonas gingivalis*, *Clostridium perfringens*, *Spirulina platensis*, *Synechococcus sp. PCC7942*, *Anabaena variabilis*, *Synechococcus sp. PCC7002*, *Synechocystis sp. PCC6803*, *Arabidopsis thaliana*, *Treponema pallidum*, *Borrelia burgdorferi*, *Leptospira biflexa*, *Leptospira interrogans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Acholeplasma laidlawii*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Lactococcus lactis*, *Unknown*, *Thermotoga maritima*, *Deinococcus radiodurans*, *Thermus aquaticus*, *Thermus thermophilus*, *Aquifex pyrophilus*, *Aquifex aeolicus*

gest a close relationship between the proteobacteria and *Cf. aurantiacus*, the simplest explanation for these observed differences is that a lateral gene transfer event may have been responsible for the transfer of these photosynthesis genes into or out of an ancestor of *Cf. aurantiacus*. In fact, in the phototrophic proteobacteria these genes are clustered as a 46 kb region [24], and a similar clustering of photosynthesis genes has also recently been demonstrated in *Heliobacillus mobilis*, a member of the low-G+C Gram-positives (Dr. C. Bauer, personal communication). Such clustering of photosynthesis genes has not been observed in *Cf. aurantiacus* [25], *Cb. tepidum* [25], or cyanobacteria [26,27]; however, transfer followed by subsequent dispersal can not be excluded. Sequence comparisons of some of the proteins found in the chlorosomes of green sulfur bacteria and green non-sulfur bacteria indicate an evolutionary relatedness, albeit limited, between some proteins of the antenna complexes of the two groups [28].

In the present study, *Cf. aurantiacus* is the only member of the green non-sulfur bacteria included. Before any consensus regarding the placement of the green non-sulfur bacteria in phylogenetic trees can be established, it will be necessary to include more examples of the green non-sulfur bacteria, both phototrophic and non-phototrophic, in the phylogenetic analyses using various markers. Since the photosynthetic apparatus appears to be chimeric (or alternatively gave rise to different lineages through divergence), it seems as if the components of the photosynthetic apparatus are not appropriate markers to represent the organism as a whole in comparative phylogenetic studies. Different components would give very different results; and although this is very intriguing, it would not be particularly informative. It would be very interesting to include non-phototrophic green non-sulfur bacteria, such as *T. roseum*, in studies using the sigma factor marker. *Cf. aurantiacus* is now the sole representative [12] in such analyses, and it should be possible to determine if the group would still be most closely related to the green sulfur bacteria and the cyanobacteria. Due to the apparently chimeric nature of *Cf. aurantiacus*, it would be very useful to obtain the sequence of the entire genome to examine completely the origins of this sequence diversity. Such an analysis could help to define specifically the phylogeny of green non-sul-

fur bacteria, but might also provide more clues to the origins and evolution of this puzzling genome.

3.3. Other phyla

A detailed description and discussion of the use of RecA as a phylogenetic marker can be found in Eisen [10]. More than half of the species displayed in the tree (Fig. 2) belong to proteobacteria phylum. Of the 26 newly added species to the alignment, 14 represent proteobacterial species. The general relationships in the proteobacteria have not changed significantly due to these additions, and the five distinctive subgroups (α, β, γ, δ, and ε) are maintained. Four Gram-positive organisms have been added, and it is notable that the Gram-positives still do not form a monophyletic clade, as was observed in the previous study using RecA [10]. Although the high-GC Gram-positives cluster together, the low-GC Gram-positives do not cluster with the high-GC Gram-positives and are not themselves monophyletic, consistent with SS-rRNA phylogenies [29]. *Clostridium perfringens* is not placed within the Gram-positive bacteria in the RecA tree, whereas this organism is placed within the low-GC Gram-positive organisms based on SS-rRNA data [18]. The cyanobacteria form a coherent group, with the nuclear-encoded chloroplast RecA from *Arabidopsis thaliana* falling within this group. The RecAs of *Deinococcus radiodurans* and two *Thermus* species form a well supported group, which is placed near the Aquificales (as seen previously [10]). A number of phyla are presently represented by only one or two sequences (e.g. the Thermotogales, Chlamydia, green-gliding bacteria, and green sulfur bacteria) which contributes to the inability to produce a meaningful and precise placement of some of these organisms within the tree. This is reflected by relatively low bootstrap values for most of these species. The placement of these groups should increase in statistical significance once additional RecA sequences have been analyzed.

3.4. Conclusions

The position of *Cb. tepidum*, the representative of the green sulfur bacteria used in these studies, as the closest relatives to the *Cytophaga-Flexibacter-Bacteroides* group, is very well supported in the phyloge-

netic tree based on RecA sequences. This result is consistent with a number of other analyses using several phylogenetic markers. On the other hand, the position of the green non-sulfur bacterium *Cf. aurantiacus* remains highly ambiguous, and at the present time no definite conclusions regarding the relationship of this organism to other eubacteria can be drawn from the analyses using RecA or other phylogenetic markers.

Acknowledgments

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