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BchY-Based Degenerate Primers Target All Types of Anoxygenic Photosynthetic Bacteria in a Single PCR▽†‡

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To detect anoxygenic bacteria containing either type 1 or type 2 photosynthetic reaction centers in a single PCR, we designed a degenerate primer set based on the *bchY* gene. The new primers were validated in silico using the GenBank nucleotide database as well as by PCR on pure strains and environmental DNA.

Anoxygenic photosynthetic bacteria are diverse and important members of microbial communities (11, 13, 17, 20). There are five bacterial phyla containing anoxygenic phototrophs: *Proteobacteria* (purple bacteria), *Chlorobi* (green sulfur bacteria), *Chloroflexi* (green nonsulfur bacteria), *Acidobacteria* ("*Candidatus* Chloracidobacterium thermophilum" [7]), and *Firmicutes* (heliobacteria). While *Heliobacterium modesticaldum*, *Chlorobi*, and "*Ca.* Chloracidobacterium thermophilum" have a type 1 reaction center (RC1) similar to photosystem I in *Cyanobacteria* and higher plants, *Chloroflexi* and *Proteobacteria* possess a type 2 reaction center (RC2) similar to photosystem II of oxygenic phototrophs (7, 16).

Primers based on *pufM*, the gene encoding the M subunit of RC2, have been widely used to detect phototrophic purple bacteria (1, 4, 12, 19). However, phototrophic bacteria that do not possess RC2 are not retrieved when *pufM* is used as the target. Achenbach and coworkers (1) developed primers targeting rRNA genes of *Chlorobi*, *Chloroflexi*, and heliobacteria, while Alexander and coworkers (2) have developed primers to specifically detect green sulfur bacteria (*Chlorobi*) by using 16S rRNA and *fmoA* as gene targets and applied these primers in environmental studies (3). No currently available primer set can simultaneously target phototrophs containing either RC1 or RC2.

Since it is well established that both RC1- and RC2-containing anoxygenic phototrophs synthesize bacteriochlorophylls (BChls), we searched for a universal anoxygenic photosynthesis gene marker among all enzymes involved in BChl biosynthetic pathways. All known pathways for chlorophyll and BChl biosynthesis branch from the heme biosynthesis pathway at protoporphyrin IX and continue to chlorophyllide *a* (Chlide *a*) through the same intermediates (9). Chlide *a* is the branching point that separates chlorophyll and BChl biosynthetic pathways. Moreover, pathways for the synthesis of different BChls are also split at this stage: chlorophyllide oxidoreductase converts Chlide *a* to 3-vinyl-bacteriophyllide *a*, which is the precursor for BChls *a*, *b*, and *g*, while a yet unknown enzyme reduces Chlide *a* to 3-vinyl-bacteriophyllide *d*, a precursor for antenna BChls *c*, *d*, and *e* in *Chlorobium* spp. (9). Since 3-vinylbacteriophyllide *a* is the last common intermediate in the synthesis of BChl *a* and BChl *g*, and the latter is the only BChl in heliobacteria (14, 15), chlorophyllide oxidoreductase is the only enzyme that is (i) present in anoxygenic phototrophic bacteria and not in oxygenic phototrophs and (ii) common to all known anoxygenic phototrophic bacterial species (with the exception of "*Ca.* Chloracidobacterium thermophilum," where the pathway for BChl synthesis is not yet known). Analyzing multiple alignments of the subunits of chlorophyllide oxidoreductase, we found that only the Y subunit (encoded by the BchY gene) had two conserved regions distinguishing this protein from its closest homologs; therefore, the *bchY* gene was chosen as a universal marker for anoxygenic photosynthesis.

Due to likely codon variations coding identical amino acid sequences in different genomes (19), degenerate BchY primers were designed by reverse translation of two conserved regions of the BchY alignment (Fig. 1): bchY_fwd (5′-CCNCARACNATGTGYCCNGCNTTYGG-3′ [26 bases; 2,048 variants; corresponding amino acid sequence, PQTMCPAFG]) and bchY_rev (5′-GGRTCNRCNGGRAANATYTCNCC-3′ [23 bases; 4,096 variants; corresponding amino acid sequence, GE{I/M}FP{A/V}DP]). Each primer had no more than two bases deviating from known *bchY* sequences in the GenBank nr database (ex-

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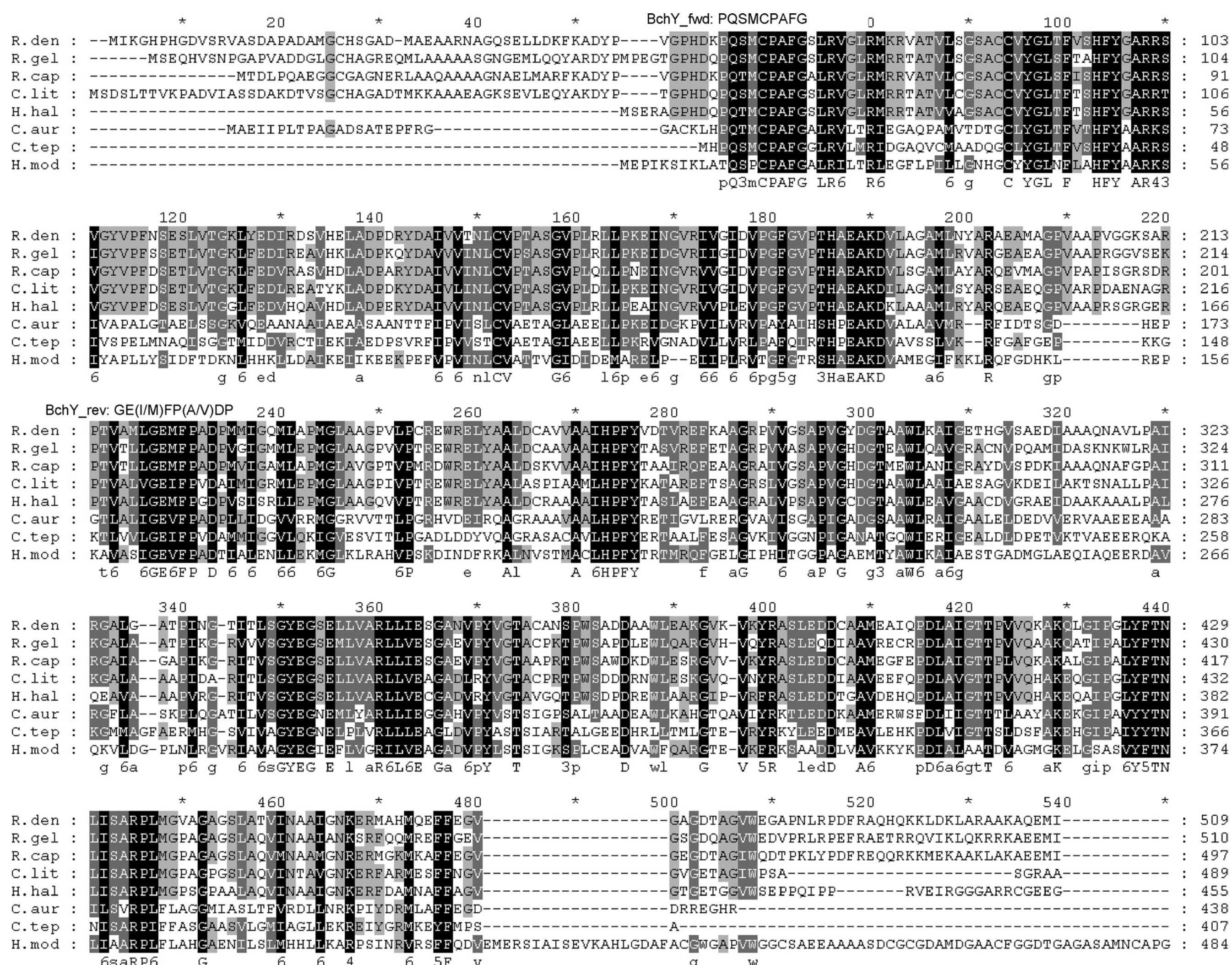

FIG. 1. Multiple-amino-acid alignment of BchY proteins. Sequence abbreviations: R.den, *Roseobacter denitrificans* (gi|110677524); R.gel, *Rubrivivax gelatinosus* (gi|29893484); R.cap, *Rhodobacter capsulatus* (gi|114868); C.lit, *Congregibacter litoralis* KT 71 (gi|88706663); H.hal, *Halorhodospira halophila* (gi|121998388); C.aur, *Chloroflexus aurantiacus* (gi|163849328); C.tep, *Chlorobium tepidum* (gi|66576270); and H.mod, *Heliobacterium modesticaldum* (gi|167629410).

cept for *H. modesticaldum*) as well as to environmental BchY variants in the GenBank env_nr database. None of these deviations were located in the 3′ ends of the primers (see Tables S2 and S3 in the supplemental material). These primers, therefore, were predicted to amplify a wide diversity of *bchY* genes under nonstringent PCR conditions (50 to 52°C annealing temperature). The lengths of the expected PCR products were either 480 bp (for green sulfur, green nonsulfur bacteria, and heliobacteria) or 510 bp (for purple bacteria).

In order to check primer specificity in silico, a screening procedure was developed. Putative primer sites (tags) for both the bchY_fwd and the bchY_rev primers were gathered from the GenBank nucleotide collection (nt) by BLAST with relaxed search conditions; the tags having mismatches at the 3′ end or more than five overall mismatches from their primer were filtered out, and the remaining tags were mapped to their sequences mimicking PCR primer annealing. Fragments ranging from 300 to 700 bp (virtual "PCR products") were retrieved from GenBank and annotated (see Table S4 in the supplemental material). All *bchY* genes present in the GenBank nt database were virtually "amplified," pointing to the robustness of the primers and our in silico PCR analysis. On the other hand, all nonspecific "amplicons" have major deviations from the primer sequences and would likely not be amplified by a real PCR. The same screening procedure was performed against the GenBank environmental nucleotide collection (env_nt) (see Table S5 in the supplemental material), and as in the case with the nt database, only *bchY* fragments were virtually "amplified."

The BchY primer set was validated using five key control organisms, including the RC2-containing the purple sulfur bacterium *Allochromatium vinosum* and the purple nonsulfur bacterium *Rhodobacter capsulatus* as well as the RC1-containing green sulfur bacterium *Chlorobium limicola*, green nonsulfur bacterium *Chloroflexus aurantiacus*, and the heliobacterium *H. modesticaldum*. Amplifications yielded the predicted products

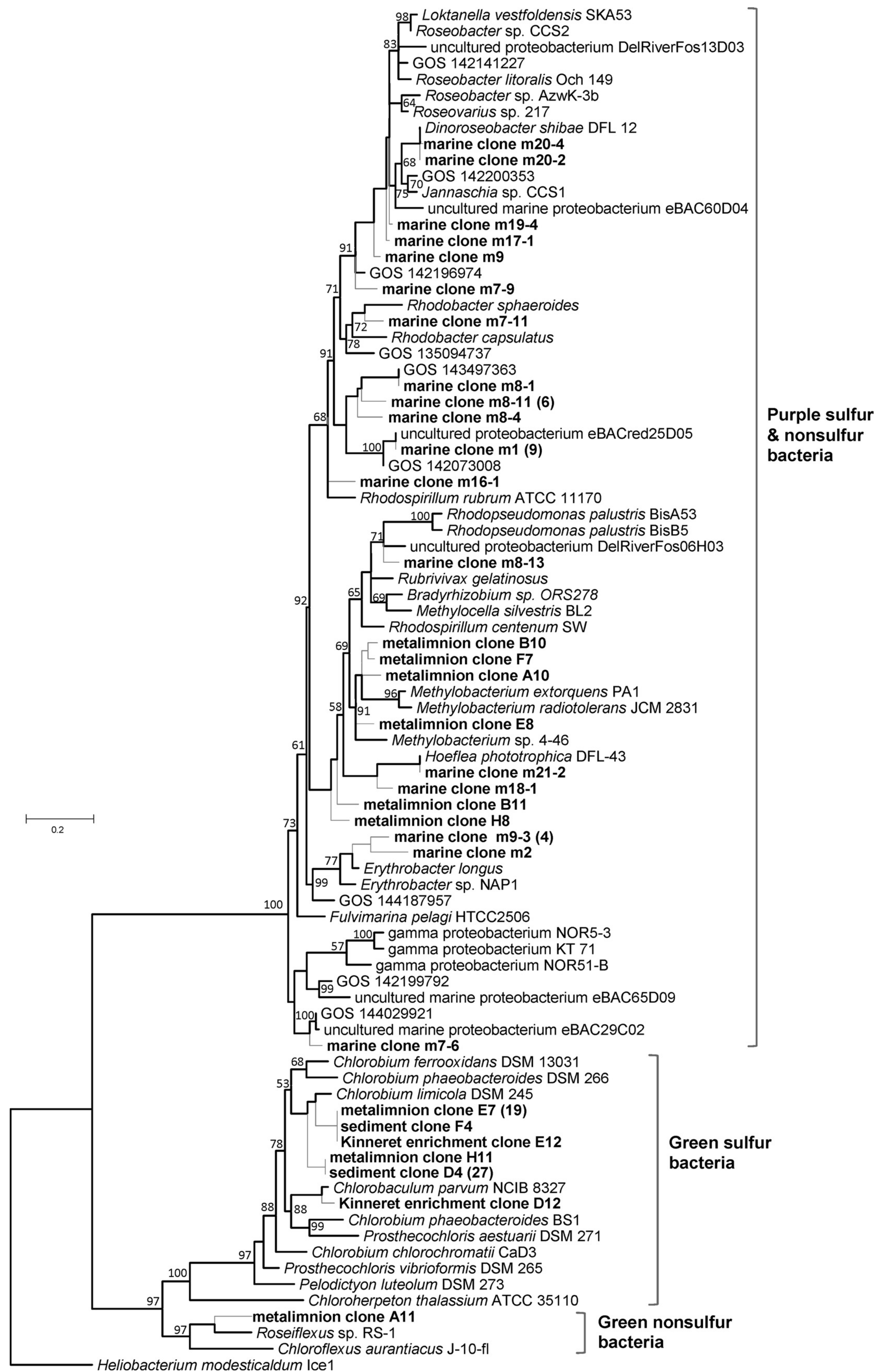

FIG. 2. BchY phylogenetic tree based on a maximum likelihood tree to which short sequences were added by ARB parsimony. The branches that appeared on the original maximum likelihood tree are shown with thicker lines. Bootstrap values greater than 50% are indicated next to the branches. Sequences obtained in this study are shown in bold. For reasons of clarity, not all BchY sequences retrieved are shown in the tree. For cases in which a BchY fragment was found in more than three clones, the numbers of clones are given in parentheses. Clones m21_2 and m21_3 are identical to the *bchY* gene of *Hoeflea phototrophica* strain DFL-43 (6); the m20_2 clone was identical to the *bchY* gene of *Dinoroseobacter shibae* (5).

of 510 bp from the purple bacteria and 480 bp from the green sulfur and nonsulfur bacteria and *H. modesticaldum*. Negative-control *Escherichia coli* and *Synechocystis* sp. strain PCC 6803 did not yield amplification products when the *bchY* primers were used.

The designed BchY primer set successfully amplified *bchY* genes from DNA obtained from both marine (East Mediterranean Sea) and freshwater (Lake Kinneret) environments (see Table S6 in the supplemental material for best BLASTX hits for selected sequenced fragments). These habitats were chosen for testing due to the previously reported wide diversity of their anoxygenic phototrophs (8, 10, 18, 19). A phylogenetic tree of *bchY* gene fragments amplified from both freshwater and marine DNA samples is shown in Fig. 2.

Our study underlines the utility of the *bchY* gene as a molecular marker for revealing genetic heterogeneity in phototrophic microbial populations. Using both wide-scale bioinformatic analysis and PCR on control strains and naturally occurring microbial community DNA, we have confirmed the specificity and coverage of the proposed degenerate BchY primers.

Nucleotide sequence accession numbers. The *bchY* sequences were deposited in GenBank under accession numbers EU854432 (*Allochromatium vinosum*), EU888421 and EU888422 (*Chlorobium* enrichments), EU888377 to EU888420, EU888424 to EU888440 (Lake Kinneret), and GQ861394 to GQ861424 (Mediterranean Sea).

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