Recommended standards for the description of new species of anoxygenic phototrophic bacteria

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Recommended standards for the description of new species of the anoxygenic phototrophic bacteria are proposed in accordance with Recommendation 30b of the International Code of Nomenclature of Bacteria. These standards include information on the natural habitat, ecology and phenotypic properties including morphology, physiology and pigments and on genetic information and nucleic acid data. The recommended standards were supported by the Subcommittee on the taxonomy of phototrophic bacteria of the International Committee on Systematics of Prokaryotes. They are considered as guidelines for authors to prepare descriptions of new species.

Introduction

Four major phenotypically and phylogenetically well-distinguished groups of bacteria are able to perform anoxygenic photosynthesis and to grow phototrophically under anoxic conditions. These are the Chlorobiales (green sulfur bacteria), the ‘Heliobacteriaceae’ (classified as Clostridiales), the ‘Chloroflexales’ (green filamentous bacteria) and the phototrophic purple bacteria belonging to the α-, β- and γ-Proteobacteria. The separation of these groups is well documented not only on the basis of 16S rDNA sequences, but also by a number of distinct phenotypic properties.

The most important common property of these bacteria is the possession of photosynthetic pigments, which is visible in their light-absorption spectra, and a photosynthetic apparatus, which enables the performance of light-dependent energy generation. With the exception of some of the phototrophic Proteobacteria, the so-called aerobic bacteriochlorophyll-containing bacteria (ABC bacteria; Shiba, 1992), they are anaerobic bacteria performing anoxygenic photosynthesis under anaerobic conditions in the light. Generally, pigment biosynthesis and formation of the photosynthetic apparatus are suppressed by oxygen and regulated by the light intensity (again in contrast to the ABC bacteria). However, in regard to pigment structure and composition, to structural features of the photosynthetic apparatus and to major biochemical pathways, the four groups show marked differences.

The current classification is based on nucleic acid analyses (G+C content, 16S rDNA sequences and, in some cases, also DNA–DNA relatedness) and on phenotypic properties such as physiology, growth characteristics, morphology and pigment composition. During the last few years, a fairly complete database of 16S rDNA sequences of available type strains (and additional other strains) of the species of Chlorobiales (Overmann & Tuschack, 1997; Alexander et al., 2002), purple sulfur bacteria (Imhoff & Süling, 1996; Guyoneaud et al., 1998; Imhoff et al., 1998), green filamentous bacteria (Keppen et al., 2000), ‘Heliobacteriaceae’ (Bryantseva et al., 1999, 2000; Madigan, 2001) and purple non-sulfur bacteria (Hiraishi & Ueda, 1994; Hiraishi et al., 1995; Imhoff et al., 1998; Kawasaki et al., 1993) has been built up. These data have revealed the phylogenetic relationships of these bacteria based on 16S rDNA sequences and are used today to determine the phylogenetic position of novel isolates. In addition, 16S rDNA-based sequence information has been used to reconsider the relative importance that is given to several phenotypic properties used in the taxonomic classification of phototrophic bacteria (see Imhoff et al., 1998; Imhoff, 1999).

General Comments

Recommended standards for the description of new species of the anoxygenic phototrophic bacteria are proposed in accordance with Recommendation 30b of the International Code of Nomenclature of Bacteria (the Bacteriological Code; Lapage et al., 1992). The purpose of these recommendations is to provide a framework for taxonomic studies of anoxygenic phototrophic bacteria and the description of new species. With the support of the Subcommittee on the taxonomy of phototrophic bacteria of the International Committee on Systematics of Prokaryotes, we recommend the inclusion of phenotypic, genotypic and ecological properties for the description of new species of these bacteria. It should be emphasized that most of the recommended data are essential for a species description to be accepted
for publication, while others are not obligatory and a few are optional, although they are valuable and important criteria for describing properties of phototrophic bacteria. The essential characters are marked in bold and the optional ones are indicated as such in Table 1.

The description of new species or subspecies of the anoxygenic phototrophic bacteria should be based on sufficient information to differentiate a new taxon from existing species of the genus. It should rely on a wide variety of phenotypic and molecular properties and their experimental

Table 1. Characteristics required for the description of new species of anoxygenic phototrophic bacteria

Properties shown in bold are essential for the description of a new species; others are highly recommended. Those marked as optional provide possibly important properties of the bacterium but are not regarded as essential for the species description.

<table>
<thead>
<tr>
<th>Property</th>
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<td><strong>Morphology:</strong></td>
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| Cell shape
diameter, length                   |                                               |
| Motility and flagellation                   |                                               |
| Type of cell division                       |                                               |
| Cell differentiation and life cycle          |                                               |
| Formation of typical cellular aggregates    | e.g. Net or platelet formation, capsule and slime formation |
| Visible internal or external structures     | e.g. Spores, gas vesicles                     |
| Gram-stain                                   | Optional in the case of established *Proteobacteria* |
| Ultrastructure                               | e.g. Internal membranes, chlorosomes and others |
| **Chemical composition:**                   |                                               |
| Colour of cell suspensions                  |                                               |
| *In vivo* absorption spectra                |                                               |
| Type of bacteriochlorophyll                 |                                               |
| Carotenoids                                  | Optional                                      |
| Quinones                                     | Optional                                      |
| Fatty acids                                  | Optional                                      |
| Reserve materials                            | e.g. PHB, polyglucose, S0                     |
| DNA base ratio                               | i.e. G+C content                              |
| Phylogenetic analysis                        | 16S rDNA sequence, phylogenetic tree position |
| DNA–DNA hybridization                        | If applicable                                 |
| Cell wall analysis                           | Optional                                      |
| **Physiology:**                              |                                               |
| Growth medium                                |                                               |
| Temperature range and optimum                |                                               |
| pH range and optimum                         |                                               |
| NaCl range and optimum                       |                                               |
| Vitamin requirements                         |                                               |
| Carbon, nitrogen and sulfur sources          | Tolerance, use, oxidation products            |
| Relation to sulfide                          | Anaerobic, aerobic, microaerobic growth, tolerance |
| Relation to oxygen                           | Minimum requirement, light sensitivity: optional |
| Light responses                              |                                               |
| **Modes of energy generation:**              |                                               |
| Phototrophy                                  |                                               |
| Potential chemotrophy                        |                                               |
| Electron donors                              |                                               |
| Electron acceptors                           |                                               |
| **Ecology:**                                 |                                               |
| Natural habitat                              | Physico-chemical properties, pH, salinity, temperature |
| Source of isolates                           | Geographical location, GPS positioning         |
| Mineral salts composition of source          |                                               |
| Abundance at habitat                         |                                               |

*To be demonstrated by: a, a light micrograph; b, a light or electron micrograph; c, an electron micrograph.
comparison with the most similar described species. It is
proposed that the placement of a new species or genus
should preferentially be consistent with its phylogenetic
position according to 16S rDNA nucleotide sequences and,
if possible, to other gene sequences (optional). It is recom-
manded that several isolates, preferably from independent
sources, should be included in these studies, whenever
possible. The different sources and environments should
be indicated and major characteristics thereof should be
described. However, when a single strain has been retrieved
from a unique source or has important and unique pro-
erties, it may be described as a new species, if the descrip-
tion is of such detail to ensure significant differences from
all known members of phylogenetically related species.

The achievement of obtaining pure cultures, including
media and growth conditions, and the methods used to
prove purity should be described. A type strain has to be
designated for the new species and needs to be deposited
in at least two permanent culture collections (Rule 18 of
the Bacteriological Code). The type strains of related
species should be included in the study and differentiating
characteristics thereof compared to the new species.

The description of new species should be published in a
journal of wide distribution, preferably in the International
Journal of Systematic and Evolutionary Microbiology
(IJSEM). When published in another journal, a reprint
should be submitted to the IJSEM so that the name of the
new species may be validated. If the requirements of the
Bacteriological Code have been met, it will be included in
the Validation List of this journal as demanded by the

Putative new species of uncultured organisms for which
molecular sequence data such as 16S rDNA nucleotide
sequences are available may qualify for the status ‘Candi-
datus’ (Murray & Schleifer, 1994; Murray & Stackebrandt,
1995). It is required that at least three sequences from
different sources are provided that form a coherent cluster
in phylogenetic analyses. An important requirement of
the ‘Candidatus’ status is that the proposed organism is
identified with a specific probe by in situ hybridization
techniques. In addition to sequence data, morphological
properties and, if possible, other characteristic features
should be supplied.

Recommended Standards

Ecology
The natural habitat and its geographical location of the
proposed new species should be described in as much detail
as possible. It should include the pH, temperature range,
salinity and mineral salts composition. The GPS positioning
should also be included. If possible, information on the
bacterial abundance and distribution at the natural habitat
should be included as well as knowledge of the occurrence
in different habitats.

Phenotypic characterization
Guidelines for the phenotypic description of phototrophic
bacteria are found in various publications and it is recom-
manded to refer to the chapters of Bergey’s Manual of
Systematic Bacteriology for properties of relevance for the
differentiation from related species. It is proposed to use
the characters described below and summarized in Table 1
as features for the description of new species of anoxygenic
phototrophic bacteria. Different growth media are required
for the proper cultivation of the different physiological
types of phototrophic bacteria. Compilations of media
useful for their culture are given in chapters of The
Prokaryotes and Bergey’s Manual (Imhoff, 1992b; Imhoff
& Trüper, 1992; Madigan, 1992; Pfennig & Overmann,
2001; Pfennig & Trüper, 1992; Pierson & Castenholz, 1992;
Pierson, 2001) and by Imhoff (1992a). Media used for
isolation and growth need to be described precisely in their
composition and preparation, or references should be
given. It is necessary that basic conditions for optimum
growth (responses to pH, temperature, salinity, light and
oxygen) are reported. They should be established before
other phenotypic properties of the isolates are determined,
in order to ensure that these tests are performed at the
optima of these basic parameters.

Cell morphology
The properties of colony and cell morphology should be
determined under optimum pH, temperature, light and
salt concentration. The medium and culture conditions
under which these properties were determined have to be
described. Cell morphology should be studied by phase-
contrast microscopy of exponentially growing liquid
cultures. The shape and size, the staining behaviour in the
Gram-stain, the type of cell division, any special morpho-
logical properties (presence of flagella, formation of cell
aggregates, slime, capsules, cellular inclusions, spore forma-
tion) and motility of the cells have to be reported.

Gas vesicles are formed by a number of phototrophic
bacteria and can easily be recognized under the light micro-
scope. However, the property to form gas vesicles may
depend on the culture conditions and may be lost by some
strains. Therefore, not much weight should be given to the
presence or absence of gas vesicles in taxonomic decisions.
Specific culture conditions that initiate the formation of
gas vesicles should be given.

Negative stains under electron microscopic observation
can be used to supply information on the presence and
location of flagella. Care has to be taken, however, because
flagella may be lost by some bacteria quite easily during
long-term cultivation after several transfers in synthetic
media and this can lead to non-motile forms of basically
motile bacteria. It is therefore important to perform micro-
scopic examination of cultures in various growth stages and
under different culture conditions and to observe motility
under the microscope. Electron microscopic examination
of thin sections is required to demonstrate the presence
and form of internal membrane structures, of chlorosomes and other characteristic morphological features (gas vesicles, spores).

**Colour of cell suspensions and absorption spectra**

The colour of cell suspensions and *in vivo* light-absorption spectra from 370 to 1020 nm are indicative of the type and chemical structure of major photosynthetic pigments and have to be reported. An *in vivo* absorption spectrum needs to be published together with the species description. Absorption spectra of whole cells are measured with cell suspensions washed twice in medium or appropriate salt solutions and then suspended in 60% (w/v) sucrose solution (Biebl & Drews, 1969). Better results are often achieved by using isolated photosynthetic membranes suspended in buffer. For this purpose, it is sufficient to break the cells by ultrasonication or with a French press and to separate whole cells and large cell fragments from the internal membranes by centrifugation at 15 000 g. In some cases, light-absorption spectra of pigments after methanol or acetone/methanol extraction under anoxic conditions are necessary for identification of bacteriochlorophylls. It should be noted that the specific chemical identity of carotenoids cannot be delineated from absorption spectra and that more sophisticated chemical analyses are required to identify these pigments. Therefore, a qualitative and quantitative detailed pigment analysis is an optional property for the description of a new species.

**Physiological properties**

The exact composition of the culture medium used for growth experiments and the culture conditions in regard to pH, temperature, light and salinity for all nutritional tests have to be included in the species description. Factors affecting growth should be investigated under conditions as close as possible to the optimum of other growth factors and need to be described in detail. Growth experiments and determination of physiological properties should be performed at least in triplicate in liquid medium under anoxic conditions and in the light. They include ranges and optima of pH, NaCl concentration and temperature, light, vitamin requirements and utilization of carbon, nitrogen and sulfur sources, H₂ utilization, as well as the presence of nitrogenase and catalase.

Conditions of growth should also include the relations to oxygen. Qualitatively, these relations may be most easily determined in agar tubes set up with a suitable anoxic medium, inoculated and carefully mixed with a well-grown culture; the tubes are exposed to the air at the top to allow oxygen to diffuse into the agar and to establish an oxygen gradient. By incubation both in the light and in the dark, first hints on the response of the isolate to oxygen under both conditions can be visualized, i.e. possible microaerobic respiratory growth in the dark (chemotrophy) and tolerance of oxygen and possible microaerobic phototrophic growth in the light. Similar experiments can also be used to determine the effect of oxygen on the synthesis of photosynthetic pigments.

**Nutrition**

Because of the widely differing nutritional properties of different groups of anoxygenic phototrophic bacteria, the determination of their nutritional properties has to consider the different growth capabilities. Therefore, the major groups are treated separately below.

**Order Chlorobiales.** The Chlorobiales, with the *Chlorobioceae* as the only family, are strictly anaerobic and phototrophic bacteria. Photoautotrophic growth in the presence of sulfide, sulfur, thiosulfate, sulfite and hydrogen with CO₂ as sole carbon source should be verified in the presence of possible growth factors (vitamin B₁₂ and possibly others). Photoassimilation of acetate, propionate, formate, lactate, glycerol, pyruvate, succinate, fumarate, malate, glucose and other sugars should be analysed. The utilization of different nitrogen sources should be investigated (including ammonia, nitrate, glutamine and possibly other amino acids, optionally dinitrogen). The vitamin requirements (vitamin B₁₂ and others) should be established.

**Phototrophic purple bacteria.** Photoautotrophic growth in the presence of sulfide, sulfur, thiosulfate, sulfite and hydrogen with CO₂ as sole carbon source should be analysed in the presence of possible growth factors. Photoheterotrophic growth (in the absence of an inorganic electron donor) and the assimilation of organic substrates in the presence of an inorganic photosynthetic electron donor (sulfide, sulfur, thiosulfate, hydrogen) should be examined. Substrates to be tested are: sulfide (0.5–5 mM), sulfur (0.1%), thiosulfate (5 mM), sulfite (0.5 mM), formate (2 mM), acetate, propionate, butyrate, valerate, crotonate, citrate (all 5 mM), caprylate (2 mM), pelargonate (2 mM), palmitate (2 mM), lactate (5 mM), pyruvate (5 mM), succinate, malate, fumarate (all 5 mM), oxoglutarate, benzoate (2 mM), tartrate (5 mM), nicotinate (2 mM), glucose (5 mM), fructose (5 mM), sucrose (5 mM), trehalose (5 mM), glycolate (5 mM), glutamate, aspartate, gluconate (all 5 mM), methanol (5 mM), ethanol (5 mM), propanol (5 mM), butanol (5 mM), mannitol (5 mM), glycerol (5 mM), glycine betaine (5 mM), peptone (0.05%), cysteine (2 mM), methionine (2 mM), thiglycolate (2 mM), thioacetamide (2 mM), Casamino acids (0.05%) and yeast extract (0.05%). Recommended concentrations are indicated in parentheses. Growth with additional substrates may be analysed according to the physiological capacities of the bacteria under investigation.

In addition, the utilization of different nitrogen sources should be analysed (including ammonia, nitrate, glutamine and possibly other amino acids, optionally dinitrogen). The vitamin requirements should be examined, in particular of vitamin B₁₂ in purple sulfur bacteria and a number of vitamins in the purple non-sulfur bacteria.
Order ‘Chloroflexales’. Photoautotrophic growth with sulfide or hydrogen and CO$_2$ as sole carbon source should be verified in the presence of possible growth factors (yeast extract or mixtures of vitamins). Both photoheterotrophic (anoxic light conditions) and chemoheterotrophic (oxic dark conditions) growth of those representatives presently in culture occurs best in the presence of yeast extract (and Casamino acids). Experiments of carbon sources utilized should include glycerol, acetate, formate, glucose (and other sugars), glutamate, aspartate, pyruvate, lactate, ethanol, succinate, malate, fumarate, propionate and butyrate (in the presence of growth factors; 0-01 % yeast extract and/or vitamins). Vitamin requirements (biotin, vitamin B$_{12}$ and others) as well as the utilization of different nitrogen sources should be tested (including ammonia, nitrate, glutamine and possibly other amino acids, optionally dinitrogen).

Family ‘Heliobacteriaceae’. Heliobacteria are strictly anaerobic, photoheterotrophic bacteria. Photoautotrophic growth (although not found so far in ‘Heliobacteriaceae’) and photoheterotrophic growth with a variety of carbon sources (including pyruvate, lactate, acetate, propionate, butyrate and ethanol) should be investigated in the absence and presence of CO$_2$. In addition, growth with yeast extract and casamino acids as carbon sources should be analysed. Also, the ability to grow under chemo-organotrophic conditions anaerobically as well as anaerobically (e.g. fermentation of pyruvate) should be established. Vitamin requirements (biotin and others) should be tested and growth should be established whenever possible in the absence of complex nutrients. Sulfide, thiosulfate, sulfate and cysteine or methionine should be established as possible sulfur sources. The utilization of different nitrogen sources should be analysed (including ammonia, nitrate, glutamine and other amino acids, optionally dinitrogen).

Lipid and fatty acid composition

The lipid and fatty acid composition of phototrophic bacteria is quite useful in the characterization of different species and groups of phototrophic bacteria (Imhoff, 1991; Imhoff & Bias-Imhoff, 1995; Thiemann & Imhoff, 1996). Fatty acid composition can be determined by gas chromatography using standard methods. Polar lipid patterns can be determined by one- or two-dimensional thin-layer chromatography (Imhoff et al., 1982; Imhoff, 1991). Information on fatty acids and polar lipids is considered as optional.

Nucleic acid characterization

The description of new species of anoxygenic phototrophic bacteria needs to contain information on the G+C content of their DNA and the nucleic acid sequence of the 16S rDNA (more than 1200 nt). The DNA base composition may be determined by any of the commonly used techniques, such as ultracentrifugation in a CsCl gradient, thermal denaturation or HPLC of nucleotides after hydrolysis. Reference DNA (e.g. Escherichia coli ATCC 11775$^T$: 51 mol% G+C) should be included in the analyses and the method used has to be given with the description.

The 16S rDNA nucleotide sequence is considered important to give information on the phylogenetic position of novel isolates. Sequences should be determined for representative strains included in the study. If a greater number of strains is available, alternative methods (e.g. genomic fingerprints) may be used to demonstrate the heterogeneity within the species and the differentiation from other species. Sequences need to be deposited with a recognized database. The database records and publications must include correct statements of the identity of the source strain. The accession numbers must be given together with the species description. The sequences of molecules other than 16S rDNA (such as puf gene sequences or others) may provide important additional phylogenetic information and should be included in considerations whenever possible. The sequences of fmo genes of the Chlorobiaceae, for example, have substantiated the phylogeny of this group as established by 16S rDNA sequences (Alexander et al., 2002).

The description of the methods used for alignment and the algorithms for tree construction should be included in the species description. Phylogenetic trees should be constructed using different methods, e.g. maximum-likelihood and distance-based methods. The reliability of the branching should be evaluated statistically by bootstrap analysis.

The discrimination of closely related species of the same genus should be demonstrated by DNA–DNA relatedness studies. DNA–DNA relatedness studies can be used to assess relationships only within narrow ranges of variation, because relatedness values fall to low levels (less than 30 %) for phenotypically moderately different species. By and large, values of more than 70 % DNA–DNA relatedness and differences in the denaturation temperatures of homoduplexes of less than 5 °C should be used as criteria for species recognition according to Wayne et al. (1987). Hybridization studies should include several strains of the newly proposed species, including the proposed type strain, as well as type strains of related species. DNA–DNA hybridization studies are required to ensure species identity when 16S rDNA sequences reveal similarities of more than 97 % (sequence similarity of more than 97 % does not necessarily mean identity at the species level). DNA–DNA hybridization has not been widely used in studies of anoxygenic phototrophic bacteria (e.g. de Bont et al., 1981; Ivanova et al., 1985; Ventura et al., 2000). If the hybridization level is above 70 %, a new species generally should not be proposed.

References

Alexander, B., Andersen, J. H., Cox, R. P. & Imhoff, J. F. (2002). Phylogeny of green sulfur bacteria on the basis of gene sequences of...


