Significance of absorption spectra for the chemotaxonomic characterization of pigmented bacteria

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Abstract: A method for characterizing pigmented bacteria based on the numerical analysis of their carotenoid absorption spectra was examined. To achieve this, some non-phototrophic pigmented bacteria that were isolated from various environments and closely related types of species were used. Bacterial carotenoid pigments were extracted with an acetone:methanol (7:2) mixture and the absorption spectra were determined. Data were normalized, and their first derivatives were analyzed using Ward clustering algorithms. Based on the spectral data, isolates were separated into 7 groups. Characterization based on derivative pigment spectral data showed good agreement statistically with biochemical characterization results. The Mantel test indicated that the dissimilarity matrices of phenotypic tests and the pigment spectral data were correlated (P < 0.001). Absorption spectra can simplify and accelerate the identification and characterization of pigmented bacteria, when used with other distinctive biochemical tests. This method might be useful for determination of the microbial community structure in a variety of ecological studies.

Key words: Bacterial carotenoids, chemotaxonomy, pigment absorption spectra

Pigmentli bakterilerin kemotaksonomik karakterizasyonu için absorbsiyon spektrumlarının önemi


Anahtar sözcükler: Bakteriyal karotenoidler, kemotaksonomi, pigment absorpsiyon spektrumları
Introduction

Since the beginning of the study of bacteriology, the production of pigments by bacteria has attracted attention, possibly because the presence of pigment is one of the most readily observed characteristics of microorganisms (1,2). Lipids are the most diverse molecules in prokaryotes, and pigments are the most conspicuous forms of lipids. Within this group, carotenoids are the most common naturally occurring terpenoid pigments and are present in a wide variety of prokaryotes, algae, fungi, and plants (3,4). Bacterial C_{40}-carotenoids, with 30, 45, or 50 carbon atoms, represent the majority of the more than 600 known structures. They occur as hydrocarbons and their oxygenated derivatives, which can be found in prokaryotes, fungi, algae, and higher plants (5).

Acetone–methanol extracts of the pigmented bacteria contain many visually distinct (chromophoric) fractions including carotenes, hydrocarbons, carotenoid epoxides, sterols, xanthophylls epoxides, mono- and dihydroxy carotenoids, and carotenoid-protein complexes (6). When petri dishes containing nutrient agar are exposed to air, or are inoculated with samples from a variety of origins, colored colonies of predominantly yellow, orange, pink, or red pigmentation often arise. Pigmented forms are common in such inocula, because the pigments are powerfully photo-protective against visible and near ultraviolet (UV) light. Thus, pigments confer a selective advantage over non-pigmented microorganisms.

In prokaryotic systematics, pigments serve as important chemotaxonomic markers for various taxonomic groups or genera that produce bacteriochlorophyll and carotenoids. These include the hydrogen (Knallgas) bacteria (7), Arthrobacter spp. (8), Chryseobacterium spp. (9), Sphingopyxis spp. (10), Sphingomonas (11), Micrococcus, Kocuria, and Planococcus spp. (12,13), Sphingobacterium (14), and Flavobacterium (15-17), among many others. Most Xanthomonas produce yellow, membrane-bound, brominated aryl-polyene pigments referred to as xanthomonadin (18,19). Xanthomonadins are unique to Xanthomonas bacteria and serve as useful chemotaxonomic (20,21) and diagnostic (22) markers. A correlation between the G+C content (<59 mol%) and the occurrence of the C_{40}, C_{30}, and C_{50} carotenoids has also been reported (23). Other pigments such as pyoverdins are also powerful taxonomic markers for a large majority of the fluorescent Pseudomonas species (24). Several species of the non-pathogenic Neisseria are pigmented, ranging from yellow to yellow-green. Thus, the use of this characteristic for species identification has been proposed (25).

Several obligately aerobic photosynthetic bacteria, particularly the purple sulfur and non-sulfur bacteria, produce pigments involving bacteriochlorophyll a and carotenoids that have played an important role in their taxonomy. The group contains the marine species of Erythrobacter (26,27) and Roseobacter (28) and the freshwater species Porphyrobacter (29), Erythromicrobiurn, Roseococcus (30-32), and Rhodobacter-Rhodovulum (33), which have a high degree of similarity at the 16S rDNA level. Erythrobacter strains especially are very similar with regard to their phenotypic and genetic characteristics (27). In eukaryotic microorganisms, the presence of carotenoid pigments is used as a taxonomic marker in the genus Rhodotorula from the genera Cryptococcus and Torulopsis (34).

Pigment profiles have considerable potential value in the taxonomy and ecology of microbial communities in natural environments (35-37). The aim of the present article is to give an overview of carotenoid pigments in non-phototrophic prokaryotes with emphasis on their taxonomic potential.

Materials and methods

Isolation and cultivation of microorganisms

For isolation of bacteria, plant macerations, soil and water samples, leaf imprints, and air plates were used. Water and leaf samples were directly plated on R2A agar (Difco); soil samples were serially diluted in sterile distilled water. Plates were incubated at 25 °C for 4-6 days, and only non-phototrophic, pigmented bacterial colonies were isolated in pure form and maintained on tryptic soy agar (TSA) slants.

The pigmented bacterial isolates were grown on TSA containing 2% glycerol and adjusted to pH 7.2 ± 2. These were inoculated by streaking the surface of the medium with a 24 h tryptic soy broth culture. The isolates were streaked out using the 3-quadrant streak pattern and 3 independent parallel platings were prepared.
Phenotypic tests

Classical phenotypic tests, media preparation, and morphological and physiological characterizations were performed as described previously (17). The API 20NE micro test systems were used according to the manufacturer’s recommendations (bioMerieux). The isolates were placed in taxonomic groups and genera on the basis of their morphology and biochemical characteristics according to *The Prokaryotes* (17) and API identification manual. The Willcox probability ($P_W$) matrix used to assign and identify isolates indicated a positive identification at scores 0.8 and above (38). Some type strains were also used as a reference for comparisons.

16S rRNA gene sequencing

Some representative isolates were identified by their 16S rRNA sequencing. The genomic DNA were extracted using the Heliosis Genomic DNA Extraction Kit and PCR were conducted using the extracted DNA as a template to amplify the 16S rRNA gene with F (5'-AGTGGCGAAGGTTGAGTAA-3') and R (5'-AATCCTGTGGTCCACG-3') primers. DNA sequencing reactions were performed using the Thermo Sequenase Cy5.5 Dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, USA), using the OpenGene automated DNA sequencer.

Pigment extraction from bacterial cells

After incubation for 5 days at room temperature, the cells were scraped from the TSA plate surfaces and placed in test tubes. The pigments were extracted by the addition of 3 mL of acetone:methanol (7:2, v/v) to approximately 150 mL of aggregated cells. The test tubes were incubated at 50 °C for 5 min in a water bath to bring the solvents to the boiling point quickly. The pigments were extracted in a few minutes. The sample was centrifuged to remove the white cell pellet. The supernatant contained the total extractable pigments. The procedure was repeated at least 3 times for complete extraction of pigments from the bacterial cells. To avoid possible loss of other chromophores, no saponification or other procedure to remove lipid contaminants from the extracts was performed. All manipulations during preparation of the pigments were carried out rapidly, and the effects of light were avoided by carrying out the extractions in glassware covered with aluminum foil. The short exposure to an increased temperature was found to produce no injurious effects on the pigments (2,39,40). The detrimental effects of oxygen were avoided by flushing all solutions with nitrogen. Finally, samples were filtered through Teflon membranes and stored at -20 °C until analysis.

Spectral measurements

Spectra were recorded from 350 to 550 nm, with a 1 nm sampling interval using a Shimadzu 1601 UV−visible spectrophotometer fitted with a 10 mm path quartz cell and equipped with PC1201 personal spectroscopy software. Absorption spectra were recorded in either spectroscopic grade methanol or in an acetone/methanol mixture (7:2, v/v), which were also used as blanks when appropriate. Spectral characteristics of the extracts were compared with available type strains. The peak maxima were determined by their first derivatives.

Evaluation of spectral data

The ASCII spectral data were normalized such that the smallest recorded absorbance was set to 0 and the highest was set to 1 for each spectrum. This was done in order to remove variation due to differences in the biomass between the samples. In order to remove variations due to baseline shifts, the original spectra were converted to their first derivative using a Savitzky-Golay algorithm with 9 smoothing points (41). Ward’s algorithm and the Euclidean ($D_E$) coefficient were used to generate dendrograms of spectral distance. The variance of separation potential index (42) was chosen to find the optimal wavebands for spectral pigment analysis.

Statistical analysis

Biochemical test results and pigment spectral data were converted into numeric data matrices in order to allow for detailed statistical analyses with cluster analysis and Mantel test statistics. The Mantel test is a regression procedure in which the variables are themselves distance or dissimilarity matrices summarizing pairwise similarities among objects. In order to learn whether the distances according to the phenotypic test results between the studied bacteria were related to the distances according to the pigment spectral data, the distance matrices were constructed based on the Euclidean coefficient. The Mantel test
statistics and the correlation between the lower diagonal elements of the 2 similarity matrices were calculated, and the significance of association was determined with 10,000 randomizations. A one-tailed test was used to test for a positive correlation between the 2 matrices. A detailed approach of the principle of the Mantel test has been previously described (43,44).

Results and discussion

Some properties of the bacteria used in this study are shown in the Table. Optimal wavebands that showed a high degree of spectral diversity for spectral pigment analysis were determined to lie within 350-550 nm. The pigment spectra indicated that the yellow and pink colors of the isolates are chiefly due to carotenoids (Figure 1). Closely related strains had similar pigment spectra.

Cluster analysis of pigment spectral data (Figure 2) revealed very high similarity to the dendrograms obtained with phenotypic tests. The Mantel test correlation coefficients of $r = 0.32$ ($P < 0.001$) between the pigment spectra and phenotypic tests confirmed the consistency of these 2 fingerprinting methods and the power of the Mantel test for objective analysis of dendrogram similarities. This suggests that the distances between the studied bacterial isolates from the 2 test systems were in good agreement. While cluster analyses revealed similar results for phenotypic tests and pigment spectral data, pigment spectral data yielded a different dendrogram topology, possibly due to different biological characteristics being detected with each method. There was also some variability in the biochemical characteristics of the isolates grouped in the same clusters according to their pigment spectral data.

Figure 1. Absorption spectra of some pink (a) and yellow (b, c) pigmented isolates, as well as related reference strains and a representative of the first derivative (d) of absorbance spectra.
Isolates NS6, NS7, NS8, NS9, DSM 1337\textsuperscript{T}, and PS1 contained pink water-insoluble pigments, while TA17\textsuperscript{T}, N3, NS13, YOx\textsuperscript{T}, SA1549, OD1\textsuperscript{T}, SA15413, NS14, A2B, NEU2137\textsuperscript{T}, NEU2113\textsuperscript{T}, and SA14 contained yellow or orange water-insoluble pigments. Absorption spectra of the pigment extracts in acetone:methanol mixture (7:2, v/v) from pink-pigmented isolates had absorbance maxima in the region 494-497 nm (Table). Absorption spectra of the main yellow and pink pigments from some isolates and related reference strains illustrate the characteristic differences in the absorption properties of the carotenoids (Figure 1).

The absorption spectra of the pigment extracts of yellow-pigmented isolates had heterogeneous absorption maxima. They are grouped in 4 major and 1 single member cluster groups. Clusters A and B (\(D_E<4\)) consisted of 4 (N3, NS13, YOx\textsuperscript{T}, and OD1) slow- and 2 (SA15413, SA1549) moderately slow-growing isolates that were closely related to the type strain of Oxalibacterium flavum TA17\textsuperscript{T} (sharing more than 99% 16S rRNA gene sequence similarity values). Clusters C (NS14, A2B) and D (NEU2137\textsuperscript{T}, NEU2113\textsuperscript{T}) were heterogeneous, and contained 2 yellow-pigmented isolates and 2 reference type strains from the genus Xanthobacter at a \(D_E\) value of <6, respectively. Among them, NS14, isolated from soil, showed completely different cell and colony morphology and substrate utilization patterns and a different pigment spectrum. The yellow pigment was soluble in acetone:methanol (7:2, v/v), where it showed distinct peaks at 437 and 466 nm and a shoulder at 415 nm (Figure 1c). The pigment type might be identical to that of Xanthobacter spp., a mixture of zeaxanthin dirhamnoside and its monorhamnoside and free carotenoid derivatives (45-47).

Isolate SA14 (single member Cluster F) could not be included in any cluster at a distance level of \(D_E<6\). This gram-positive, endospore-free rod-shaped isolate showed a characteristic rod-coccus growth cycle during the mid-log phase of growth. On the basis of these special characteristics, SA14 was identified as Arthrobacter sp.

Cluster E (\(D_E \leq 2\)) included pink-pigmented isolates (NS6, NS7, NS8, NS9) from air plates and soil samples. The results of the 16S rRNA gene sequence analysis showed that these isolates shared more than 99.9% sequence similarity values and showed the highest similarity values (99.9%-100%) to M. extorquens, M. thiocyanatum, M. populi, and M. organophilum. Results of spectral carotenoids analysis
#### Table. Some properties and spectral characteristics of the bacteria used in this study.

<table>
<thead>
<tr>
<th>Abbreviation used in study</th>
<th>Species name</th>
<th>Origin or culture collection numbers(^\text{a})</th>
<th>Color profiles and clusters</th>
<th>(\lambda_{\text{max}})</th>
<th>(\lambda_{\text{III}}/\lambda_{\text{II}}) ratio</th>
<th>Main absorption bands (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM1337(^\text{a})</td>
<td><em>Methylobacterium extorquens</em></td>
<td>DSM 1337(^\text{a})</td>
<td>Pink E</td>
<td>496</td>
<td>82</td>
<td>466, 496, 527</td>
</tr>
<tr>
<td>NS6</td>
<td><em>Methylobacterium organophilum</em></td>
<td>NEU 1213, NBRC 103119</td>
<td>Pink E</td>
<td>494</td>
<td>84</td>
<td>463, 494, 525</td>
</tr>
<tr>
<td>NS7</td>
<td><em>Methylobacterium organophilum</em></td>
<td>NEU 1214, NBRC 103120</td>
<td>Pink E</td>
<td>494</td>
<td>83</td>
<td>464, 494, 524</td>
</tr>
<tr>
<td>NS8</td>
<td><em>Methylobacterium organophilum</em></td>
<td>NEU 1215, NBRC 103121</td>
<td>Pink E</td>
<td>494</td>
<td>83</td>
<td>465, 494, 523</td>
</tr>
<tr>
<td>NS9</td>
<td><em>Methylobacterium thiocyanatum</em></td>
<td>NEU 1216, NBRC 103122</td>
<td>Pink E</td>
<td>495</td>
<td>83</td>
<td>461, 495, 524</td>
</tr>
<tr>
<td>PSI</td>
<td><em>Kocuria rosea</em></td>
<td>0.92 Leaf imprint</td>
<td>Pink G</td>
<td>497</td>
<td>75</td>
<td>470, 497, 529</td>
</tr>
<tr>
<td>TA17(^\text{a})</td>
<td><em>Oxalibacterium flavum</em></td>
<td>DSM 15506(^\text{a})</td>
<td>Yellow A</td>
<td>447</td>
<td>95</td>
<td>400, 438, 447</td>
</tr>
<tr>
<td>N3</td>
<td><em>Pseudomonas</em> sp.</td>
<td>0.85 Waste water</td>
<td>Yellow A</td>
<td>452</td>
<td>74</td>
<td>403, 424, 452</td>
</tr>
<tr>
<td>NS13</td>
<td><em>Oxalibacterium flavum</em></td>
<td>DSM 15507</td>
<td>Yellow A</td>
<td>448</td>
<td>93</td>
<td>405, 432, 448</td>
</tr>
<tr>
<td>YOX(^\text{a})</td>
<td><em>Oxalibacterium faecigallinarum</em></td>
<td>CCM 2767(^\text{a})</td>
<td>Yellow A</td>
<td>452</td>
<td>97</td>
<td>434, 452</td>
</tr>
<tr>
<td>SA1549</td>
<td><em>Sphingomonas</em> sp.</td>
<td>0.84 Air plate</td>
<td>Yellow B</td>
<td>453</td>
<td>89</td>
<td>436, 453</td>
</tr>
<tr>
<td>OD1(^\text{a})</td>
<td><em>Oxalibacterium horti</em></td>
<td>NBRC 13594(^\text{a})</td>
<td>Yellow A</td>
<td>446</td>
<td>99</td>
<td>432, 446, 473</td>
</tr>
<tr>
<td>SA15413</td>
<td><em>Sphingobacterium multivorum</em></td>
<td>0.98 Freshwater</td>
<td>Yellow B</td>
<td>451</td>
<td>88</td>
<td>427, 451, 479</td>
</tr>
<tr>
<td>NS14</td>
<td><em>Microbacterium</em> sp.</td>
<td>NEU 1221</td>
<td>Yellow C</td>
<td>437</td>
<td>91</td>
<td>415, 437, 466</td>
</tr>
<tr>
<td>A2B</td>
<td><em>Pseudomonas</em> sp.</td>
<td>0.91 Freshwater</td>
<td>Yellow C</td>
<td>470</td>
<td>81</td>
<td>444, 470, 503</td>
</tr>
<tr>
<td>NEU2137(^\text{a})</td>
<td><em>Xanthobacter autotrophicus</em></td>
<td>DSM 432(^\text{a})</td>
<td>Yellow D</td>
<td>451</td>
<td>86</td>
<td>452, 477</td>
</tr>
<tr>
<td>NEU2113(^\text{a})</td>
<td><em>Xanthobacter flavus</em></td>
<td>DSM 338(^\text{a})</td>
<td>Yellow D</td>
<td>453</td>
<td>83</td>
<td>453, 479</td>
</tr>
<tr>
<td>SA14</td>
<td><em>Arthrabaacter</em> sp.</td>
<td>0.91 Soil</td>
<td>Yellow F</td>
<td>436</td>
<td>91</td>
<td>400, 436, 464</td>
</tr>
</tbody>
</table>

\(^{a}\) DSM (DSMZ), Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CCM, Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic; NBRC, NITE Biological Resource Center, Chiba, Japan; NEU, Neuchatel University Collection of Microorganisms, Switzerland. \(^{\text{a}}\) indicates type strains.
performed with *M. extorquens* suggest that the total acetone:methanol extract represents a mixture of pigmented substances. However, absorption spectra obtained with this total extract reflect a similarity of pattern among strains within a species. Curves of the *Methylobacterium extorquens* DSMZ 1337, was included in this cluster. Pigment spectra of *Methylobacterium* species were very similar; only differences in the degree of absorption were detected. If the Euclidean distance of 6.0 units ($D_E \leq 6$) was used as a cut-off value (48), the gram-positive coccus strain PS1 (single member Cluster G) could be separated from the Cluster E isolates. However, the pigment was soluble in acetone:methanol (7:2, v/v), where it showed distinct peaks at 497 nm and shoulders at 470 and 529 nm. According to morphological and biochemical test results, the strain was identical to *Kocuria rosea* (formerly *Micrococcus roseus*), which contains bacterioruberin as a major pigment (49,50).

**Conclusion**

During the course of this study, we found that each *Methylobacterium* strain produced the same pigments repeatedly, and yellow-pigmented isolates were characterized by a specific spectral pattern, suggesting the production of a different carotenoid for each of the species. Several of the species of bacteria contained identical pigments. Consistency of pigment production is important in this respect. Though data are too sparse to be conclusive, some previous work has shown that specific growth conditions and the composition of the medium have no effect on the kinds of pigment formed by a prokaryote (1,51). Light absorption spectra of the phenazine (*Pseudomonas aeruginosa*) and pyrrolic (*Serratia marcescens*) type pigments and other non-carotenoid polyenes are markedly pH-dependent in neutral and alkaline solutions and will change during the pigment isolation from cells, disallowing comparison of the spectral data by numerical taxonomy and reducing the utility of these pigments for our purpose.

Although some of the pigments in yellow- and red-pigmented isolates bear resemblance to substances present in other pigmented bacteria, the spectral qualities of the total extracts are very different (Table 1). The most obvious characteristic of all carotenoids is their intense coloration resulting from the chain of conjugated double bonds that acts as the chromophore. The color differences of carotenoids arise from differences in their numbers of conjugated bonds. The shape of the absorption spectrum ($\% A_{III}/A_{II}$) is a function of the structure of the carotenoid (52). In this convention, the height ($A_{III}$) of the longest wavelength absorption band is expressed as a percentage of that ($A_{II}$) of the middle (usually $\lambda_{max}$) absorption band. It is therefore useful to compare carotenoid spectra not only with respect to the positions of the absorption maxima but also in terms of their shape. For example, lutein and zeaxanthin differ very slightly in color ($\lambda_{max} = 445$ and $450$ nm, respectively), but the shapes of their absorption spectra are 60 and 38, respectively (53). The $\% A_{III}/A_{II}$ values may be more helpful in taxonomic comparisons of closely related species.

In this study, the diversity of the individual carotenoid pigments within each genus made it possible to use them as chemotaxonomic markers. Although the same pigments may occur in distantly related genera, the types of carotenoids and their quantitative composition may be valuable criteria for classification.

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