Comparison of Flavobacterium and Sphingobacterium Species by Enzyme Profiles, with Use of Pattern Recognition of Two-Dimensional Fluorescence Data

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Enzyme profiles of eight Flavobacterium species and one Sphingobacterium species were compared after using a two-dimensional fluorescence technique. Enzyme contents and corresponding activities were rapidly determined for whole-cell preparations after incubation with a mixture of preselected fluorogenic substrates. A two-dimensional fluorescence spectrum of the resulting product mixture, measured with a video fluorometer, provided a characteristic "fingerprint" for each organism. Comparison of fluorescent spectra was facilitated by a Fourier-transform-based pattern-recognition algorithm and by a clustering technique involving the Pearson product-moment correlation coefficient. F. multivorum, F. thalpophilum, and S. mizutae formed one cluster; F. indologenes, F. spiritivorunz, F. odoratum, and F. balustinum formed a second. F. meningosepticum was intermediate between the first and second cluster, whereas F. breve was different from all other strains examined, based on their spectral dissimilarity indices and correlation coefficients.

The genus Flavobacterium generally refers to a group of Gram-negative, aerobic, non-spore-forming, yellow-pigmented, rod-shaped bacteria (1). They are clinically significant, in part, because of their resistance to a wide range of antimicrobial agents (2). Sphingobacterium, characterized by the presence of relatively high amounts of sphingophospholipids, has recently been described (3). Quantitative analysis of the phenotypic and chemical characteristics of Flavobacterium and Sphingobacterium suggested a close taxonomic relationship between these two genera (1-4).

Generally, bacteria are classified on the basis of overall morphological and biochemical characteristics, whereas definative taxonomic assignment is determined by nucleic acid hybridization (5). The latter technique, laborious and time-consuming, is not amenable to routine analysis. Alternatively, chemical data from whole-cell analysis, obtained by using rapid analytical methods that give results in good agreement with genetic analysis, have been adopted in some clinical laboratories for bacterial classification (6). Flavobacterium and Sphingobacterium species have recently been compared by determining the qualitative and quantitative differences in cellular fatty acid (4) and isoprenoid quinone content (1) by gas-liquid chromatography and "high-performance" liquid chromatography, respectively. Results of these analyses demonstrate the utility of chemical data and rapid analytical techniques for bacterial classification.

Recently, we reported a rapid enzymatic procedure for bacterial "fingerprinting," in which we used a multiparameter fluorescence technique coupled with a Fourier-transform-based pattern-recognition algorithm for two-dimensional fluorescence data (7). This method, based on the degree to which enzyme content and activity differ, is potentially useful for routine analyses.

In this study, we adopted this multiparameter fluorescence technique to compare species of Flavobacterium and Sphingobacterium. A procedure for clustering the two-dimensional fluorescence spectra, based on the Pearson product-moment correlation coefficient (8), is also described.

Materials and Methods

Instrumentation. The video fluorometer and computerized data-acquisition system have been described previously (7, 9).

Chemicals and reagents. All chemicals were analytical grade and were used without further purification. Deionized water purified with a "Milli Q" system (Millipore Corp., Bedford, MA 01730) was used throughout.

Fluorogenic enzyme substrates, N-benzoyl-DL-arginine-β-naphthylamide, L-alanine-7-amido-4-methylcoumarin, 3-indoxyl phosphate, L-leucine-4-methoxy-β-naphthylamide, and 4-methylumbellifer-β-D-galactoside were purchased from Sigma Chemical Co., St. Louis, MO 63178. N-Methylindoxyl butyrate and 5(+6)-carboxyfluorescein diacetate were obtained from ICN Biochemicals, Cleveland, OH 44128, and Molecular Probes, Inc., Junction City, OR 97448, respectively.

The following substrate stock solutions were prepared by dissolving stoichiometric amounts of each compound in 2-methoxyethanol: N-benzoyl-DL-arginine-β-naphthylamide (30 mmol/L), L-alanine-7-amido-4-methylcoumarin (1 mmol/L), 3-indoxyl phosphate (10 mmol/L), L-leucine-4-methoxy-β-naphthylamide (30 mmol/L), 4-methylumbellifer-β-D-galactoside (1 mmol/L), N-methylindoxyl butyrate (10 mmol/L), and 5(+6)-carboxyfluorescein diacetate (1 mmol/L). All stock solutions were stored at −20 °C and used within seven days.

Succinate-Tris buffer (50 mmol/L, pH 6.3) was prepared by dissolving 5.9 g of succinic acid and 0.6 g of magnesium chloride hexahydrate in 1 L of water, then adjusting the pH to 6.3 with solid Tris. Magnesium chloride was used as an enzyme activator.

Bacterial culture and cell harvest. The type strains of eight Flavobacterium and one Sphingobacterium species were examined: F. thalpophilum National Collection of Type Cultures (NCTC) 11429, F. multivorum American Type Culture Collection (ATCC) 33613, F. spiritivorunz ATCC 33861, F. odoratum ATCC 4651, F. balustinum ATCC 33487, F. meningosepticum ATCC 13253, F. breve ATCC 14234, and F. indologenes ATCC 25897, and S. mizutae ATCC 33299. They were obtained from the culture collections of the Centers for Disease Control, Atlanta, GA.

Bacterial cells were grown on blood-agar plates (BBL, Cockeysville, MD 21030) for 48 h at 35 °C. At the end of this incubation, the cells were scraped from the agar plates, suspended in the succinate-Tris buffer, and diluted to an absorbance of 0.30, as measured with a Spectronic 20

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photometer at 580 nm. This suspension was used immediately.

**Bacterial enzyme analysis.** The procedure used for bacterial enzyme analysis has previously been described (7). Briefly, it is as follows. A 4-mL aliquot of the fresh bacterial cell suspension was pipetted into a test tube (13 × 100 mm) and placed in a water bath maintained at 37.0 °C. A working enzyme–substrate mixture was prepared by combining two parts of 3-indoxyl phosphate, N-methylindolyl butyrate, and 5(±)-carboxyfluorescein diacetate, three parts of 4-methylumbelliferyl-β-d-galactoside and L-alanine-7-amido-4-methylcoumarin, four parts of N-benzoyl-ω-arginine-β-naphthylamide, and six parts of L-leucine-4-methoxy-β-naphthylamide stock solutions. Then 110 μL of this substrate mixture was added to the bacterial suspension, and the mixture was incubated for 15 min at 37 °C. Bacterial cells were then removed by filtration through a "Dura-pore" hydrophilic disc filter (0.22-μm pore size) equipped with an AP15 prefiltcr (Millipore Corp.) and the filtrates were kept on ice until measured. Using a video fluorometer, we recorded a two-dimensional fluorescence spectrum (fluorescence intensity vs excitation and emission wavelength) of the filtrate (7, 9). A control blank was prepared by adding 110 μL of the enzyme–substrate mixture to 4 mL of succinate–Tris buffer and incubating for the same length of time. A spectrum of the control solution was acquired, and was later subtracted from each spectrum of the test organisms. Triplicate tests were performed for each organism and the average value of the three spectra was used for comparison.

Substrates were selected on the basis of their reactivities and the emission and excitation properties of their product. Preliminary studies showed that each substrate was hydrolyzed by at least one of the bacteria examined and that the products had different excitation–emission spectra, which allowed for their simultaneous measurements with a video fluorometer. Final substrate concentrations were chosen on the basis of the final fluorescence intensity so that reasonably high intensity was obtained in relatively short incubation interval.

**Spectral comparison by use of a Fourier-transform-based pattern-recognition algorithm.** We compared spectra, using a Fourier-transform-based pattern-recognition algorithm previously described (7, 10). A spectral dissimilarity index (P) between two spectra was calculated by using the following equation:

$$P = \frac{I}{R} (D)$$  \( (1) \)

where I is the sum of the imaginary coefficients of the frequency-domain correlation function, R is the sum of the absolute values of the real-negative coefficients of the frequency-domain correlation function, and D is the intervector distance between the Fourier transforms of the two spectra (10). This index provides for a quantitative measurement of the difference between two spectra. For two identical spectra, the dissimilarity index (P) would equal zero. On the other hand, a P value greater than zero indicates that two spectra are different (11).

**Spectral comparison by using correlation coefficients and cluster analysis.** A two-dimensional fluorescence spectrum was acquired and stored in the form of an excitation–emission matrix (EEM) of 64 rows (excitation channels) and 64 columns (emission channels) (7, 10). We calculated the Pearson product-moment correlation coefficient (r) between two EEMs by using the equation:

$$r = \frac{\sum \sum \sum \sum X_{ij} Y_{ij} - \frac{1}{N} \sum \sum \sum X_{ij} \sum \sum \sum Y_{ij}}{\sqrt{\left(\sum \sum \sum \sum X_{ij} - \frac{1}{N} \sum \sum \sum X_{ij}^2\right) \left(\sum \sum \sum \sum Y_{ij} - \frac{1}{N} \sum \sum \sum Y_{ij}^2\right)}}$$  \( (2) \)

where $X_{ij}$ and $Y_{ij}$ denote the fluorescence intensities of the two EEMs at the corresponding excitation and emission channels, i and j, respectively. The parameter $n$ is equal to 64, and is the maximum number of channels, and $N = n^2$ (i.e., $64 \times 64$) is the number of data pairs. Spectral comparison can be obtained by clustering the correlation coefficients among spectra of all bacterial strains with the unweighted pair-group method using arithmetic averages (UPGMA) (8, 12). This clustering method has been applied to one-dimensional protein electrophoretograms or profiles after sodium dodecyl sulfate/polyacrylamide gel electrophoresis (13).

**Results and Discussion.**

Figure 1 depicts typical two-dimensional fluorescence spectra of the resulting product mixtures from enzymatic hydrolysis of the fluorogenic substrates from *S. mizutae*, *F. multivorum*, *F. thalophilum*, and *F. spiritivorum*. Figure 2 shows those from *F. indologenes*, *F. odoratum*, *F. balustinum*, *F. meningosepticum*, and *F. breve*. It is apparent from both figures that no two spectra are identical. All four spectra in Figure 1 have three major emission peaks above 500 nm with excitation maxima at approximately 430 nm, 460 nm, and 490 nm, respectively, and one minor emission peak below 500 nm. Emission peaks with excitation wavelengths at 430, 460, and 490 nm indicate enzymatic hydrolysis of 3-indoxyl phosphate, N-methylindolyl butyrate, and

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**Notes:**

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**Fig. 1.** Typical isometric projections of two-dimensional fluorescence spectra of: (a) Sphingobacterium mizutae (ATCC 33299), (b) Flavobacterium thalophilum (NCTC 11429), (c) Flavobacterium multivorum (ATCC 33613), and (d) Flavobacterium spiritivorum (ATCC 33861)

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**Fig. 2.** Typical isometric projections of two-dimensional fluorescence spectra of: (a) Flavobacterium indologenes (ATCC 29887), (b) Flavobacterium odoratum (ATCC 4851), (c) Flavobacterium balustinum (ATCC 33487), (d) Flavobacterium meningosepticum (ATCC 13253), and (e) Flavobacterium breve (ATCC 14234)
5(6)-carboxyfluorescein diacetate, respectively. Hydrolysis of either of the other four compounds in the substrate mixture would result in fluorescence peaks with emission maxima below 500 nm and excitation maxima below 430 nm. None of the spectra in Figure 2 have an emission peak with excitation wavelength of 460 nm. Spectra d and e in Figure 2 do not have an emission peak at 490 nm either.

Spectral dissimilarity indices (P) between F. thalophilum and the other organisms examined are summarized in Table 1. F. multivorum has the lowest P value \(4 \times 10^{-11}\), followed by S. mizutae, F. spiritivorum, F. indologenes, F. odoratum, F. balustinum, F. meningosepticum, and F. breve. Table 1 also gives the Pearson product-moment correlation coefficients (r), calculated by using equation 2. It is apparent from these data that F. multivorum has the highest \(r\) (0.9906) and lowest P value, indicating that its spectrum is highly correlated with that of F. thalophilum. The decrease of \(r\) from F. multivorum to F. breve is in good agreement with the increase in the P values. These results indicate that P and r are consistent with each other in spite of the fact that the former is derived by using the Fourier transforms of the spectra and the latter is obtained by using the real data. There is no simple linear relationship between these two sets of values. However, it seems that the P values are more sensitive to small spectral changes when two spectra are highly correlated. It can be seen from Table 1 that P increases from \(4 \times 10^{-11}\) for F. multivorum to \(13 \times 10^{-11}\) for S. mizutae (threefold increase), whereas \(r\) decreases only from 0.9906 to 0.9676. On the other hand, a larger percentage change is observed for the \(r\) value than the P value when two spectra are more different, as in the case of F. breve and F. meningosepticum.

Within analytical runs, the variability of the fluorescence spectra was low, the overall correlation coefficient and dissimilarity index being 0.997 ± 0.003 and \((4 \pm 6) \times 10^{-12}\), respectively, for the nine species of bacteria examined. Thus, the spectra were highly reproducible, and minor differences between two spectra could be easily distinguished.

F. indologenes, F. odoratum, and F. balustinum have very similar P values as well as \(r\) values, suggesting that their spectra are similar. However, we must note that all P and \(r\) values in Table 1 are comparisons between only F. thalophilum and the other organisms listed. It is necessary to compare all spectra directly and compute all similarity and dissimilarity indices. With a large number of organisms, a large data matrix of P and \(r\) values would result and it would be impossible to mentally distinguish and remember differences between all the organisms. Therefore, a quantitative approach is needed to delineate clusters based on these values.

Figure 3 is a dendrogram displaying the similarity relationships between the two-dimensional fluorescence spectra of the organisms, prepared by using the unweighted pair-group method with arithmetic average (UPGMA). F. thalophilum, F. multivorum, and S. mizutae form one cluster and F. spiritivorum, F. indologenes, F. odoratum, and F. balustinum form a second. F. meningosepticum is intermediate between the first and the second cluster, whereas F. breve is different from all other strains examined. The spectra of S. mizutae, F. thalophilum, and F. multivorum are similar, suggesting that they are closely related. These findings are in agreement with the results of cellular fatty-acid analysis (1, 4) and fluorescent pigment-profile measurements (14). In spite of the fact that S. mizutae and F. spiritivorum have been observed to resemble each other in their phenotypic and other biochemical characteristics (1–4), we found that these two species did not cluster in the same group. This finding is in agreement with the results of fluorescent pigment-profile analysis (14).

In conclusion, we have developed a rapid procedure to compare bacterial-enzyme profiles of eight species of Flavobacterium and one species of Sphingobacterium, using a multiparameter fluorescence technique and pattern recognition of the fluorescence data. The results are in agreement with previous studies. Comparison was based on quantitative measurements of spectral differences (P) and similarity value (\(r\)) rather than by subjective interpretation of biochemical and morphological characteristics. P and \(r\) values may both be useful in bacterial classification by a spectral comparison procedure described previously (7), once a large standard library of spectra has been established. The use of the UPGMA to delineate clusters based on the correlation coefficient may be useful as adjuncts to conventional bacterial classification procedures. Finally, once computerized data-acquisition systems and algorithms for data reduction have been established, this technique would be amenable to automation in clinical laboratories.

![Dendrogram displaying the relationships among spectra of organisms based on correlation coefficients calculated using equation 2 in the text](image)

Cluster analysis was done with the unweighted pair-group method, with use of arithmetic averages (upas).

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**Table 1. Spectral Dissimilarity Indices (P) and Pearson Product-Moment Correlation Coefficients (r) Comparing F. thalophilum and Other Organisms Examined**

<table>
<thead>
<tr>
<th>Organism</th>
<th>P × 10⁻¹¹</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavobacterium multivorum</td>
<td>4</td>
<td>0.9906</td>
</tr>
<tr>
<td>Sphingobacterium mizutae</td>
<td>13</td>
<td>0.9876</td>
</tr>
<tr>
<td>F. spiritivorum</td>
<td>17000</td>
<td>0.8801</td>
</tr>
<tr>
<td>F. indologenes</td>
<td>110 000</td>
<td>0.8328</td>
</tr>
<tr>
<td>F. odoratum</td>
<td>130 000</td>
<td>0.8226</td>
</tr>
<tr>
<td>F. balustinum</td>
<td>150 000</td>
<td>0.8130</td>
</tr>
<tr>
<td>F. meningosepticum</td>
<td>480 000</td>
<td>0.6123</td>
</tr>
<tr>
<td>F. breve</td>
<td>500 000</td>
<td>0.4266</td>
</tr>
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References


