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Original article

Identification of *Propionibacteria* to the species level using Fourier transform infrared spectroscopy and artificial neural networks

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Abstract

Fourier transform infrared spectroscopy (FTIR) and artificial neural networks (ANN's) were used to identify species of *Propionibacteria* strains. The aim of the study was to improve the methodology to identify species of *Propionibacteria* strains, in which the differentiation index D, calculated based on Pearson's correlation and cluster analyses were used to describe the correlation between the Fourier transform infrared spectra and bacteria as molecular systems brought unsatisfactory results. More advanced statistical methods of identification of the FTIR spectra with application of artificial neural networks (ANN's) were used. In this experiment, the FTIR spectra of *Propionibacteria* strains stored in the library were used to develop artificial neural networks for their identification. Several multilayer perceptrons (MLP) and probabilistic neural networks (PNN) were tested. The practical value of selected artificial neural networks was assessed based on identification results of spectra of 9 reference strains and 28 isolates. To verify results of isolates identification, the PCR based method with the pairs of species-specific primers was used.

The use of artificial neural networks in FTIR spectral analyses as the most advanced chemometric method supported correct identification of 93% bacteria of the genus *Propionibacterium* to the species level.

Key words: *Propionibacteria*, FTIR spectra, PCR, artificial neural networks

Introduction

Microbial diversity results from variations in bacteria's morphological and biochemical characteristics. Bacterial Fourier transform infrared (FTIR) spectra are strain-specific, and they demonstrate the characteristic features of the strain's cellular components such as fatty acids, membrane proteins, intracellular proteins, polysaccharides and nucleic acids (Naumann et al. 1991b, Schmitt et al. 1998). The spectra of intact bacterial cells are a specific representation of the studied cell's phenotypic and genotypic properties. Naumann et al. (1991a) proposed to analyze bacterial FTIR spectra as dactyloscopic images. The differences between various microbial spectra are difficult to observe, which is why they have to be analyzed with the use of statistical methods. The most popular statistical techniques (Mariey et al. 2001,

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Table 1. List of specific primers for PCR identification of isolates.

Primer	Sequence 5' – 3'	Specifity	PCR Product [bp]	PCR reaction profile
PacI	CTGGAAGCTGGCCGTCG	*P. acidipropionici*	304	94°C, 2 min,
PacII	CTTGCAACACAACACATTAC			94°C, 30 s,
PfrI	AGGAGCCTTTTCGCCATC	*P. freudenreichii ssp.*	346	35x 60°C, 30 s
PfrII	TAGCTTGTCACACAAAACTC			72°C, 30
PjeI	CTAAGGAGCTGTGACTGTG	*P. jensenii*	331	72°C, 10 min
PjeII	AGCTTGCAATACACACAAAAC			
PthI	ATGGGCCCTGTGCTCAC	*P. thoenii*	267	
PthII	AGTAGCTTGCAATACACATAC			

Davis and Mauer 2010) involve hierarchical cluster analysis (HCA), discriminant analyses (DA), discriminant function analyses (DFA) and canonical variate analyses (CVA). In addition to traditional methods, microbial diversity is also determined with the involvement of artificial neural networks (Goodacre et al. 1998, Gupta et al. 2006, Mouwen et al. 2006, Rebuffo-Scheer et al. 2007, Correa and Goodacre 2011). A correctly trained network is characterized by high flexibility, it supports the recognition of even minor differences between spectra, and it enables researchers to ignore system errors such as noise or changes in baseline values. Such a network is capable of memorizing and generalizing knowledge based on a representative group of samples (Davis and Mauer 2010). ANNs produce more accurate results than traditional methods (Goodacre et al. 1998).

In our previous study (Dziuba and Nalepa 2012), FTIR and artificial neural networks supported the correct genus identification of 95% *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus* and *Propionibacteria* strains. The aim of the present study was to develop artificial neural networks for FTIR spectral analysis supporting the identification and differentiation of propionic acid bacteria to the species level. Our results were used to expand the library of FTIR spectra of lactic acid bacteria (Dziuba et al. 2007a). In the future, a computer application will be developed for identifying the analyzed microbial groups based on the database of FTIR spectra and trained artificial neural networks (BACTIFTIR database).

Materials and Methods

Strains and growth conditions

A group of 9 reference *Propionibacteria* strains supplied by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and 28 food isolates deposited in the culture collection of Department of Industrial and Food Microbiology at the University of Warmia and Mazury in Olsztyn (Table 3) were used in the study. All isolates were previously typed by biochemical tests as *Propionibacterium*. Strains were cultured for 72 ± 2 hours on a growth medium containing 10 g of casein peptone, 5 g of yeast extract and 10 g of sodium lactate, at the temperature of 30°C in anaerobic conditions.

DNA extraction and PCR amplification

Loopfull amount of the culture was taken from the agar plate, suspended in 100 µL of Tris-HCl (10 mM, pH 8.5) buffer and incubated at 37°C for about 1 h in the presence of lysozyme added to a final concentration of 2 mg/mL. Further steps were done according to manufacturer instruction of Genomic Mini kit (A@A Biotechnology, Gdansk, Poland) for genomic DNA isolation.

PCR amplifications of specific DNA fragments were carried out in DNA thermal cycler (MJ Mini, BioRad). A reaction mixture (20 µl) containing 10 ng of each primer (Oligo, Poland), 200 µM of each nucleotide (Fermentas, Latvia), 1x PCR buffer with 2 mM MgCl2, 1 U of TAQ polymerase (Fermentas, Latvia) and 20-40 ng of bacterial DNA was prepared for the polymerase chain reaction. PCR methodology relies on primers and procedures developed by Tilsala-Timisjarvi and Alatossava (2001) presented in Table 1.

Preparation of samples for measurements of FTIR spectra

Using an inoculation loop, a small number of bacterial cells (150-300 µg d.w.) was collected from the surface of the agar plate and suspended in 200 µl of saline solution. The suspended cells were centrifuged

at 12000 rpm for 10 min and resuspended. This step was repeated two times. A sample of 200 µl was evenly distributed on the surface of a ZnSe window and dried at 50°C for 30 minutes (until transparent film was obtained). Three samples from two independent cultures (6 samples) were prepared for each bacterial strain. A total of 12 spectra were measured for every reference strain.

Spectroscopic measurements and spectrum processing

FTIR spectrophotometer Spectrum One (Perkin-Elmer) equipped with a beam spliter (KBr) and a DTGS (Deuterated TriGlycerine Sulfate) detector was used to measure in transmission mode spectra of the bacterial samples over the wavenumber range 4000 cm^{-1} to 500 cm^{-1}. Every sample was scanned 64 times at the resolution of 4 cm^{-1} and scanning speed of 0.2 cm/s. The background spectrum was measured with an empty ZnSe window.

To standardize data and minimize the influence of noise 5-points baseline correction at the wavelength of 400 cm^{-1}, 3700 cm^{-1}, 2700 cm^{-1}, 1800 cm^{-1} and 750 cm^{-1}, normalization to 1.0 value to the most intensive peak was applied. The first derivatives were calculated to increase the resolution of spectra (Savitzky-Golay algorithm, 9-point smoothing).

Five spectral regions were identified in each FTIR spectrum of the analyzed bacteria. Those regions were proposed by Naumann et al. (1991b) for spectral description and bacterial identification. The identified regions were: W1 – fatty acid region I between 3100 cm^{-1} and 2800 cm^{-1}, W2 – amide region between 1800 cm^{-1} and 1500 cm^{-1}, W3 – combination region between 1500 cm^{-1} and 1200 cm^{-1}, $W3_1$ – fatty acid region II between 1500 cm^{-1} and 1400 cm^{-1}, dominated by absorption bands corresponding to stretching vibrations identical to W1, W4 – polysaccharide region between 1200 cm^{-1} and 900 cm^{-1}, and W5 – dactyloscopic region between 900 cm^{-1} and 600 cm^{-1}.

Processing operations were performed using the Spectrum One 3.01.00 software (Perkin Elmer), and they were stored in ASCII format compatible with Excel (Microsoft) and Statistica (StatSoft Polska) applications.

Statistical analysis of spectral data

Normalized FTIR spectra of the analyzed bacteria were transferred to Statistica spreadsheets (StatSoft) to perform the "quality test" proposed by Naumann et al. (1991b). The test accounted for reproducibility levels PP1 and PP2 calculated for the first spectral derivative in the range of 1500 cm^{-1} to 900 cm^{-1}. For data analyses involving artificial neural networks, only spectra with Pearson's correlation coefficient higher than 0.999 for PP1 and higher than 0.990 for PP2 were selected. A series of spreadsheets was developed with data (spectra and their first derivatives) from the entire spectral range (4000 cm^{-1} – 700 cm^{-1}) and from five spectral ranges and their combinations.

Development of artificial neural networks

Multilayer perceptrons (MLP) and probabilistic neural networks (PNN) were developed to identify bacteria based on their FTIR spectra using the STATISTICA Neural Network 4.01 application (StatSoft Poland). The structure of artificial neural networks used in this study consisted of three layers. The first layer (input) contained absorbance values of: entire spectra, selected spectral regions (W1 – 3100-2800 cm^{-1}, W2 – 1800-1500 cm^{-1}, W3 – 1500-1200 cm^{-1}, W4 – 1200-900 cm^{-1}, W5 – 900-600 cm^{-1}), combinations of spectral regions or wavenumbers selected by application of a genetic algorithm or stepwise regression. All neural networks had one hidden layer and four output nodes, each representing given species encoded in binary mode: *Propionibacterium acidipropionici* {*1000*}, *Propionibacterium freudenreichii* {*0100*}, *Propionibacterium jensenii* {*0010*} and *Propionibacterium thoenii* {*0001*}.

Input data were divided into three sets: training (72 spectra), validation (9 spectra) and test (9 spectra) containing spectra of every analyzed strain.

All neural networks were trained based on the FTIR spectra of reference strains until an error smaller than 0.05 was produced for training and validation sets. Wider description of artificial neural networks structure, learning and validation process can be found elsewhere (Goodacre et al. 1998, Dziuba et al. 2007b). The correctness of identification of isolates was verified with the use of molecular biology methods.

Results

The correctness of *Propionibacteria* identification to the species level was evaluated at two stages. At the first stage, artificial neural networks were tested based on the spectra of 9 reference bacterial strains. At the second stage, the evaluation was carried out with the involvement of 28 spectra of bacterial strains identified by PCR with species-specific primers. Exemplary diagram of electrophoretic separation of PCR reac-

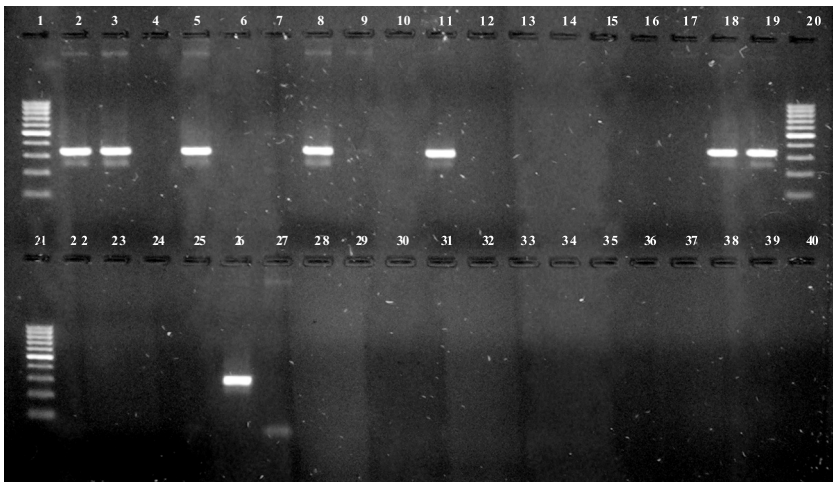

Fig. 1. Electrophoretic separation of PCR product obtained with primers PjeI and PjeI specific for *P. jensenii* (product 331 bp). Line: 1, 20, 21 – mass marker O'geneRuler 100bp DNA Lader; 2 – strain DSM 20274; 3 – strain DSM 20535; 4 – strain ID 34; 5 – strain ID 35; 6 – strain ID 36; 7 – strain ID 37; 8 – strain ID 38; 9 – strain ID 39; 10 – strain ID 40; 11 – strain ID 41; 12 – strain ID 42; 13 – strain ID 43, 14 – strain ID 44; 15 – strain ID 45; 16 – strain ID 46; 17 – strain ID 47; 18 – strain ID 48; 19 strain ID 49; 22 – strain ID 50;.23 – strain ID 51; 24 – strain ID 52; 25 – strain ID 53; 26 – strain ID 118; 27 – strain ID 55; 28 – strain ID 54.

Table 2. Set of artificial neural networks with best scores of *Propionibacterium* species identification.

Spectrum range	Network		RMSE[1]			Quality[2]				No. of epochs
	Type	Structure	1	2	3	1	2	3	4	
W5W4W3	MLP	679-38-4	0.006	0.050	0.041	1	1	1	0.93	$3.2x10^2$
W5W4W3W2	PNN	1049-72-4	0.000	0.000	0.001	1	1	1	0.71	–
$4000cm^{-1}$ -$500cm^{-1}$	MLP	895-92-4	0.000	0.040	0.110	1	1	1	0.79	$4.5x10^3$
W5W4W3	MLP	623-40-4	0.010	0.005	0.008	1	1	1	0.89	$7.0x10^2$
W5W4W2	PNN	741-72-4	0.008	0.008	0.005	1	1	1	0.86	–
AL.GEN	MLP	368-33-4	0.007	0.013	0.038	1	1	1	0.86	$3.0x10^2$

[1] Root mean square error: 1 – learning; 2 – validation; 3 – testing isolates

[2] Quality: 1 – learning; 2 – validation; 3 – test reference; 4 – test isolates

tion product specific for *Propionibacterium jenseni* is shown in Fig. 1. In the group of 28 *Propionibacteria* strains, 6 were classified as *P. jensenii*, 7 as *P. thoenii*, 7 as *P. acidipropionici*, 7 as *P. freudenreichii*, and one strain 119 (Table 3) did not produce a specific product with any primer in PCR amplifications. All tested neural networks were developed based on the first derivatives of bacterial FTIR spectra. The search for input variables, improvements in network structure and the training process led to the selection of the best neural networks shown in Table 2. The presented neural networks correctly identified all reference strains and 72% to 93% of isolates based on I derivatives of FTIR spectra. The best results were reported for the MLP 679-38-4 developed on the basis of the W5xW4xW3 combination of spectral ranges. The above network correctly identified all reference strains. In the group of 27 strains whose species identity was determined by PCR, the network correctly classified 24 strains (Table 3). Two strains, *P. jensenii* 48 and *P. freudenreichii* 46, were not unambiguously typed. In reference to *P. jensenii* 48, the network was unable to decide between *P. jensenii* and *P. acidipropionici*, and with regard to *P. freudenreichii* 46, the activation value on the output neuron was too low (below the threshold value of 0.7). With regard to the winning neuron, *P. jensenii* 48 was correctly classified, whereas the *P. freudenreichii* 46 strain was incorrectly identified. Strain 119 whose species was not determined by PCR was identified by the network as belonging to *P. acidipropionici*. The network correctly identified 93% of bacteria of the genus *Propionibacterium* to the species level.

Table 3. Identification of the *Propionibacterium* species as judged by MLP (679:38:4) analysis of I-st derivative of FTIR spectra.

Strain	PCR typing	ANN Typing	Answer	RMSE	Activation on outlet neuron			
					P.aci.	*P.fre.*	*P.jen.*	*P.tho.*
44	*P. freudenreichii*	*P.fre.*	Correct	0.021	0.00	0.96	0.00	0.00
118	*P. jensenii*	*P.tho.*	False	0.707	0.00	0.00	0.00	1.00
35	*P. jensenii*	*P.jen.*	Correct	0.017	0.01	0.00	0.97	0.01
50	*P. freudenreichii*	*P.fre.*	Correct	0.026	0.03	0.98	0.00	0.00
55	*P. freudenreichii*	*P.fre.*	Correct	0.050	0.00	0.96	0.04	0.08
47	*P. acidipropionici*	*P.aci.*	Correct	0.005	0.99	0.00	0.00	0.00
48	*P. jensenii*	*P.jen.*	?	0.169	0.41	0.00	0.77	0.00
38	*P. jensenii*	*P.jen.*	Correct	0.010	0.00	0.00	0.98	0.00
46	*P. freudenreichii*	*P.fre.*	?	0.526	0.00	0.02	0.00	0.38
34	*P. thoenii*	*P.tho.*	Correct	0.010	0.00	0.01	0.00	0.98
36	*P. thoenii*	*P.tho.*	Correct	0.055	0.00	0.08	0.00	0.93
119	*Propionibacterium*	–	?	0.706	1.00	0.00	0.00	0.00
52	*P. thoenii*	*P.tho.*	Correct	0.001	0.00	0.00	0.00	1.00
42	*P. acidipropionici*	*P.aci.*	Correct	0.000	1.00	0.00	0.00	0.00
45	*P. acidipropionici*	*P.aci.*	Correct	0.037	0.93	0.00	0.01	0.00
40	*P. acidipropionici*	*P.aci.*	Correct	0.004	1.00	0.01	0.01	0.00
54	*P. acidipropionici*	*P.aci.*	Correct	0.010	1.00	0.00	0.00	0.02
49	*P. jensenii*	*P.jen.*	Correct	0.005	0.01	0.00	1.00	0.00
41	*P. jensenii*	*P.jen.*	Correct	0.095	0.00	0.00	0.81	0.01
338	*P. thoenii*	*P.tho.*	Correct	0.000	0.00	0.00	0.00	1.00
53	*P. freudenreichii*	*P.fre.*	Correct	0.005	0.00	1.00	0.01	0.00
51	*P. freudenreichii*	*P.fre.*	Correct	0.002	0.00	1.00	0.00	0.00
341	*P. thoenii*	*P.tho.*	Correct	0.001	0.00	0.00	0.00	1.00
342	*P. thoenii*	*P.tho.*	Correct	0.011	0.00	0.00	0.02	1.00
37	*P. freudenreichii*	*P.fre.*	Correct	0.024	0.00	1.00	0.05	0.00
337	*P. acidipropionici*	*P.aci.*	Correct	0.011	1.00	0.00	0.02	0.00
339	*P. acidipropionici*	*P.aci.*	Correct	0.029	0.94	0.00	0.01	0.00
340	*P. thoenii*	*P.tho.*	Correct	0.016	0.00	0.00	0.02	0.98
DSM20270	*P. freudenreichii*	*P.fre.*	Correct	0.001	0.00	1.00	0.00	0.00
DSM20271	*P. freudenreichii*	*P.fre.*	Correct	0.002	0.00	1.00	0.00	0.00
DSM20274	*P. jensenii*	*P.jen.*	Correct	0.002	0.00	0.00	1.00	0.00
DSM20275	*P. thoenii*	*P.tho.*	Correct	0.000	0.00	0.00	0.00	1.00
DSM20276	*P. thoenii*	*P.tho.*	Correct	0.002	0.00	0.00	0.00	1.00
DSM20535	*P. jensenii*	*P.jen.*	Correct	0.014	0.02	0.01	0.98	0.00
DSM4900	*P. acidipropionici*	*P.aci.*	Correct	0.005	1.00	0.00	0.01	0.00
DSM4902	*P. freudenreichii*	*P.fre.*	Correct	0.020	0.04	1.00	0.00	0.00
DSM20272	*P. acidipropionici*	*P.aci.*	Correct	0.012	0.99	0.00	0.23	0.00

Discussion

The results of this study demonstrate that FTIR spectra are highly species-specific. The FTIR spectra of bacteria comprise hundreds or even thousands of overlapping absorption bands that are impossible to separate. Their analysis requires image recognition methods which examine spectra as fingerprints (dactyloscopic images). At present there is a wide range of FTIR applications in microbial studies (Davis and Mauer 2010). Some of the examples are: detection and quantification of bacteria from culture and food; discrimination of viable, injured, and dead bacteria; analysis of structural components of bacteria; taxonomic classification of bacteria. In respect to the last example also our previous works (Dziuba 2007, Dziuba et al. 2007a,b) demonstrated that Fourier transform infrared spectroscopy is a useful technique for identifying lactic acid bacteria. The above study was performed at two stages. The method of measuring the spectra of reference strains and the strategies for FTIR spectra analysis were developed at the first stage. The resulting spectral measurement methods and strategies were used to create a library of the examined bacteria's FTIR spectra. The results were analyzed and used to build artificial neural networks, which were than validated against the unknown spectra of isolates at the second stage. At present study we applied FTIR spectroscopy and artificial neural networks for identification of *Propionibacteria* strains at the species level. The method of measuring the spectra of reference strains and the strategies for FTIR spectra analysis were similar to that presented earlier (Dziuba et al. 2007a). The most problematic in

preparation of the sample for measurement of *Propionibacteria* was that some of them produce substantial amounts of exopolysaccharides during growth (Dobruchowska et al. 2008) which presence influence the quality of obtained spectra used for identification. Washing the cells in distilled water and centrifugation allowed overcoming this problem. Twelve spectra were measured for every reference strain and subjected to "quality test" proposed by Naumann et al. (1991b) calculated based on Pearson's correlation coefficients. This test allows to evaluate measurement reproducibility and to estimate the discriminatory power of measurements in the long-term perspective. The aim of the above procedures was to account for microbial variability, to separate natural responses from experimental artifacts and equipment defects. The resulting spectral measurement methods and strategies were used to create a library of the examined bacteria's FTIR spectra. The best ten spectra out of twelve were than used to develop artificial neural networks. For the purpose of generalizing the knowledge acquired in the training process, one of the main requirements set for artificial neural networks is an appropriate ratio of the number of cases (bacterial FTIR spectra) to the number of variables (absorbance values at given wavelength). An excessively high number of variables prevent the generalization of knowledge. Before training the dimensionality of the data was reduced by application of a genetic algorithm or stepwise regression. The training processes relied on an error back propagation algorithm or a conjugate gradient algorithm for MLP. Probabilistic neural networks use kernel-based approximation to form an estimate of the probability density function of classes in a classification problem. Smoothing factor for learning this type of ANN was set between 0.3 and 1.0. After the learning process was finished the spectral data in the test set were introduced to the network and analyzed.

All tested neural networks were designed based on the first derivatives of FTIR spectra of propionic acid bacteria. In analyses of bacteria of the genus *Propionibacterium*, the best results were reported for the MLP network and the W5xW4xW3 combination of spectral ranges. Our observations are largely consistent with the results reported by other authors for other groups of microorganisms. The most generalized conclusions were presented by Mariey et al. (2001) in a review paper. The above authors argued that the most satisfactory results were generated in respect to the first spectrum derivatives and for the W5xW4xW3 combination of spectral ranges.

The MLP network correctly identified all reference strains. In the group of 28 strains whose species identity was determined by PCR, the network correctly classified 24 strains. With the exception of strain P114 (ID 119) which was not identified to the species level by PCR, the network correctly identified 93% of bacteria of the genus *Propionibacterium*. Presented accuracy is very high and within the range of that reported by other researchers (Davis and Mauer 2010), however difficult to compare as its first report concerning identification of *Propionibacteria* species based on their FTIR spectra.

In conclusion, the discussed method may be recommended in analytical laboratories for identifying propionic acid bacteria, monitoring the purity of cultures in strain collections and fast screening selected bacterial groups. The proposed technique is also suitable for monitoring the quality of products and raw materials in the food processing industry. FTIR delivers a variety of advantages, including simple technology, low cost, high specificity and a wide range of industrial applications.

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