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Evolution, biodiversity, taxonomy

FTIR spectroscopy and taxonomic purpose: Contribution to the classification of lactic acid bacteria

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Abstract — The capacity of FTIR spectroscopy for identification and classification of lactic acid bacteria has been proved on brewery strains and more recently by ourselves on dairy strains. During our study, we underlined that IR spectra give very informative data which can be used for old or more recent taxonomical purposes. We tried to illustrate this aspect with two examples: the distinction between *Streptococcus thermophilus* and *Streptococcus salivarius*, the taxonomic range of *Lactobacillus casei, paracasei, zeae* and *rhamnosus*. The interest of this tool for taxonomic studies will be discussed.

taxonomy / FTIR spectroscopy / *Streptococcus* / *Lactobacillus*

Résumé — **Apport de la spectroscopie infrarouge à transformée de Fourier en taxonomie : contribution à la classification de bactéries lactiques.** La capacité de la spectroscopie IRTF pour l'identification et la classification de bactéries lactiques a été démontrée pour des souches de lactobacilles brassicoles et plus récemment par notre équipe en matière de souches laitières. Au cours de notre étude, il nous est apparu que les données spectrales obtenues, étaient riches en informations pouvant être utilisées pour étayer des discussions taxonomiques anciennes ou plus récentes. Nous illustrons notre propos par deux exemples : la distinction entre les souches types de *Streptococcus thermophilus* et *Streptococcus salivarius* et la position taxonomique des espèces *Lactobacillus casei, paracasei, zeae* et *rhamnosus.* L'intérêt et la place de cet outil en taxonomie bactérienne est présenté.

taxonomie / spectroscopie IRTF / *Streptococcus* / *Lactobacillus*

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1. INTRODUCTION

Bacteria FTIR spectra give a global picture of whole cellular components (fatty acids, intracellular and membrane proteins, polysaccharides, nucleic acids) [5, 16] and are considered to be a phenotypic method. Moreover, spectra give information relative to nucleic acids and in this way it may be proposed as a link between the both genomic and phenotypic approaches. In the last twenty years, genomic data has led to many taxonomic changes, and species usually confused can now be distinguished. This is the case for *S. salivarius* and *S. thermophilus*, and for *Lb. casei, Lb. zeae* and *Lb. rhamnosus* species involved in the dairy the industry that we have studied by FTIR spectroscopy [1, 2]. By using more and more efficient methods a lot of new species have been created each year, and systematic bacteriology is more and more complex. The interest of these new species is not always very clear for lack of easy methods to identify them routinely. The comparison of FTIR spectra using statistical analysis makes it possible to classify them. In a previous study, we recorded the spectra of 12 species of *Lactobacillus* involved in the dairy industry [1, 2]. In this paper, we point out the capacity of FTIR spectroscopy for taxonomic discussions about some species and we present the screening of wild strains belonging to these species.

2. MATERIALS AND METHODS

2.1. Bacteria growth and sample preparation

The strains come from international collections ATCC, CNRZ, LMG and CIP and were kept frozen (–80 °C) in Adria-Normandie. Wild strains were isolated from "Pont-L'Evêque" cheese (strains p64, p135) by Adria-Normandie and from "Camembert" cheese by LMA, University of Caen Basse-Normandie. All strains were grown for 24 h ± 2 h in optimal conditions: MRS, 37 °C for lactobacilli, M 17, 37 °C for streptococci. Cells are pelleted by centrifugation 4 000 rpm for 10 min and washed twice with saline solution as previously described [1]. Five microlitres of the concentrated bacteria were put on a ZnSe spectral window and dried for an hour at 50 °C.

2.2. Spectroscopic measurements

Bacterial spectra were recorded between 4 000 and 700 cm^{-1} using a FTIR spectrometer (Nicolet 250, Nicolet Instrument, Thermo-Optek, Montigny le Bretonneux, France) equipped with a KBr beamsplitter and a DTGS detector. Sixty-four scans were averaged per spectrum at a resolution of 4 cm^{-1}. All spectra were submitted to a "quality test" adapted from Helm et al. [10]. After this test, validated spectra were normalized to one absorbance unit using the amide I spectral band located at about 1640 cm^{-1}.

2.3. Statistical analysis

Three different methods were employed to compare spectra.

2.3.1. Discriminant analysis

Using Omnic TQ analyst software 1.2 (Nicolet instrument, Thermo-Optek, Montigny-le-Bretonneux, France), discriminant analysis is performed by the calculation of Mahalanobis distances between spectra [12].

2.3.2. Search standard method

Using the same software, we can obtain the percentage of similitude between an unknown spectrum and spectra of a database. Results are noted from 1 to 100 (100 is the best correlation between spectra) [1, 2].

2.3.3. Hierarchical cluster analysis

Using Omnic software (Nicolet instrument, Thermo-Optek, Montigny-le-Bretonneux, France), spectra were first smoothed and the first derivative was calculated. Then, using SPSS 8.0 for Windows software, each spectra was considered as an observation, and each point of the spectra as a variable. Euclidian spectral distances were calculated and Ward algorithm was performed to obtain the dendrogram.

3. RESULTS AND DISCUSSION

3.1. Discrimination between *S. salivarius* and *S. thermophilus*

Normally only one species of the genus *Streptococcus* (*S. thermophilus)* is found in the dairy industry [7, 13] but the species *S. salivarius,* very close to *S. thermophilus*, is an opportunistic bacteria in man and that is why it is so important to separate them. The taxonomic border between the two species has been discussed for years: in 1984, on the basis of DNA hybridization and fatty acid results, Farrow and Collins [9] proposed to classify them in the same species. Later, Schleifer et al. [17] underlining a DNA homology of only 60% in optimal conditions and 30% in stringent conditions between the two species, refiled them as distinctive species. This last taxonomic position was validated in 1980 by Skerman [18]. However, in practice, strains belonging to these two species are difficult to separate by phenotypic tests.

Using FTIR spectroscopy, both type strains (NCDO 573^T and ATCC 7073^T) show spectral differences visible to the naked eye, particularly between 3 100–2 800 cm^{-1}, a region corresponding to fatty acids vibrations, and around 1 200–900 cm^{-1} (polysaccharides vibrations) (Fig. 1). Calculation of Mahalanobis distances between the spectra of the two strains (over 100 versus 0.67 for distances between spectra of each strain) in the region 1 500–700 cm^{-1}, translates a good discrimination of the two strains (Fig. 2, Tab. I). Likewise, the percentage of homology between the two strains is less than 90%, even though in our experience the limit for the same species is 95% [1, 2]. These three arguments, obtained from the IR spectrum alone, are sufficient to confirm the argument of Schleifer et al. rather than the one of Farrow et al. and to propose FTIR spectroscopy as a complementary tool for the discrimination of the two species.

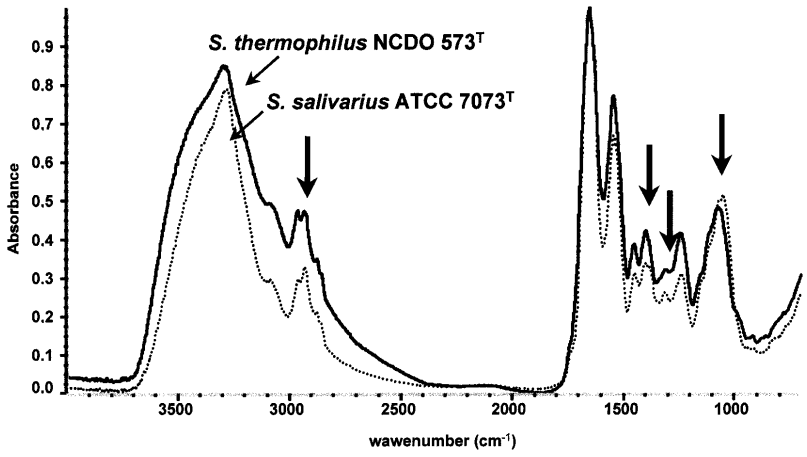

Figure 1. FTIR spectra of *S. thermophilus* (——) and *S. salivarius* (·····) type strains.

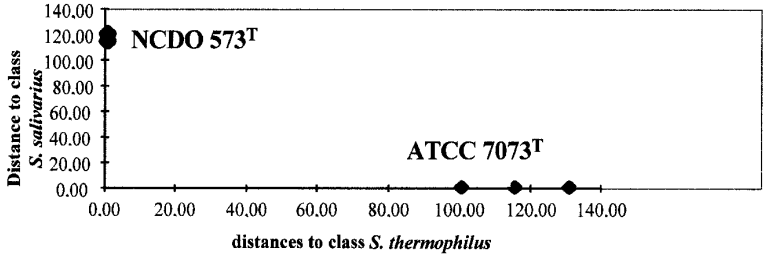

Figure 2. Discriminant analysis of spectra from *S. thermophilus* and *S. salivarius* type strains.

Table I. Mahalanobis distances between type strains of *S. thermophilus* and *S. salivarius*.

Strain		Distance to class	
		S. thermophilus	*S. salivarius*
S. salivarius	spectra 1	101.74	**0.67**
ATCC 7073^T	spectra 2	115.61	**0.67**
	spectra 3	131.03	**0.67**
S. thermophilus	spectra 1	**0.67**	112.73
NCDO 573^T	spectra 2	**0.67**	120.56
	spectra 3	**0.67**	115.09

3.2. Contribution to the discussion about the taxonomic position of *Lb. casei* and related taxa

Taxonomic discussion about *Lb. casei* species and related taxa is old and always uncertain: five subspecies admitted to the approved list of Bacterial Names in 1980 [18], four in the last edition of the Bergey's Manual of Systematic Bacteriology [11], and, more recently, three separate species (*Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*), on the basis of molecular arguments proposed by Collins et al. [4]. At the same time, since 1971, the proximity of the type strains ATCC 393^T (*Lb. casei*) and ATCC 15820^T (*Lactobacterium zeae*, reclassified as *Lb. rhamnosus*) has been underlined by different research teams [3, 6, 8, 14, 15]. Most of the authors proposed the combination of ATCC 393^T and ATCC 15820 in the species *Lb. zeae* (type strain ATCC 15820). Moreover, Dellaglio's team proposes the rejection of the species *Lb. paracasei* and the recognition of ATCC 334 as the new type strain of *Lb. casei*. However, these changes have been rejected by the Juridicial Commission of the International Committee on Systematic Bacteriology [19] for fear of confusion in the existing taxonomy.

Comparison of FTIR spectra of the type strains considered and some other collection strains does not show many differences between them except for *Lb. rhamnosus* CNRZ 212^T (Fig. 3). Similarly, the hierarchical cluster obtained from spectral data, using the Ward method, confirms the

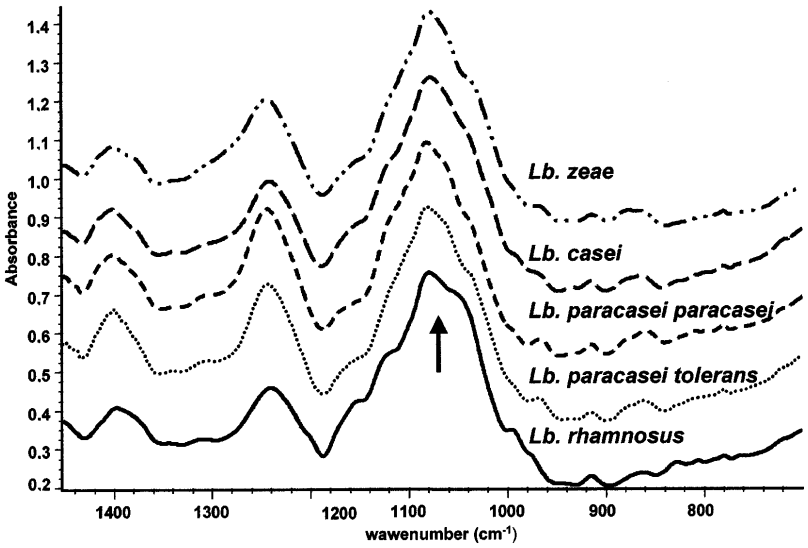

Figure 3. FTIR spectra of *Lb. zeae* (CIP 103253: –···–), *Lb. casei* (CNRZ 313^T ––––), *Lb. paracasei* subsp *paracasei* (CNRZ 62^T ----), *Lb. paracasei* subsp *tolerans* (CIP 102309^T ……) and *Lb. rhamnosus* (CNRZ 212^T ——).

proximity between CNRZ 313^T and CIP 103253, the singularity of ATCC 334, and the real distance of CNRZ 212^T from other strains (Fig. 4). Yet, contrary to the results of Dicks et al. [8], strains CNRZ 62^T and CIP 103024^T are closer to the casei-zeae cluster than to strain ATCC 334. Since our results are partly in accordance with those of Dellaglio's team we decided to consider as *Lb. zeae* all strains closely related to ATCC 103253 and/or CNRZ 313^T; to consider as *Lb. paracasei* all strains related to CNRZ 62^T,

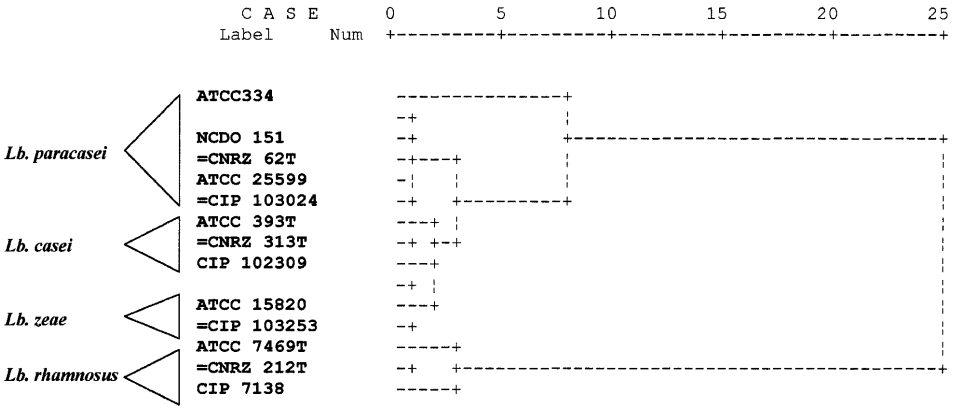

Figure 4. Hierarchical cluster analysis using Ward algorithm on Euclidian spectral distances.

and CIP 103402^T; and to keep the strains close to CNRZ 212^T as *Lb. rhamnosus*. The remaining strains, including ATCC 334, are kept as *Lb. casei*.

In this way, when we try to identify wild strains by comparing their spectra with those of type strains, we can easily distinguish the *Lb. rhamnosus* strains from the *zeae* and the *casei* strains. Table II gives the results of FTIR identification in comparison with the results of phenotypic tests (Api 50 CHL) and for some strains with the RAPD method. The correlation between RAPD and Api methods was good for five strains among the six tested. The correlation between the RAPD and FTIR methods was good for the six strains. The differences observed between the FTIR and Api methods can be explained insofar as phenotypic tests do not take into account the species *Lb. zeae* and consider as *Lb. rhamnosus* the strains which ferment rhamnose. In fact, ATCC 15820 and related strains ferment rhamnose too.

With this precision, FTIR spectroscopy allows a more precise classification of the casei-group than phenotypic tests. This classification is as efficient as those obtained with genomic methods such as RAPD. In both cases, FTIR spectroscopy is more rapid.

4. CONCLUSION

Concerning the taxonomic subject, our results confirm only in part those of Dellaglio et al. but don't allow the grouping together of *Lb. casei* and *Lb. paracasei* strains around the type strain ATCC 334,

Table II. Comparison of identifications with different methods.

Strain	Identification by		
	Api50CHL	RAPD	IRTF
435	*paracasei*	*paracasei*	*paracasei*
468	*paracasei*	*paracasei*	*paracasei*
733	*paracasei*	*paracasei*	*paracasei*
736	*paracasei*	*paracasei*	*paracasei*
833	*paracasei*	*paracasei*	*paracasei*
936	*paracasei*	ND	"*casei* 334"
p64	ND	ND	"*casei* 334"
102	*paracasei*	ND	"*casei* 334"
103	*paracasei*	ND	"*casei* 334"
196	*paracasei*	ND	"*casei* 334"
82	*paracasei*	ND	"*casei* 334"
83	*paracasei*	ND	"*casei* 334"
84	*paracasei*	ND	"*casei* 334"
62	*rhamnosus*	ND	*rhamnosus*
80	douteux (rhamnose+)	ND	*rhamnosus*
81	*rhamnosus*	ND	*rhamnosus*
272	ND	ND	*rhamnosus*
85	*rhamnosus*	ND	*casei/zeae*
79	*rhamnosus*	*casei*	*casei/zeae*
p135	*paracasei*	ND	*casei/zeae*
837	*paracasei*	ND	*casei/zeae*

since two distinct clusters are present. Nevertheless, the method allows one to set apart easily *Lb. zeae* strains from *Lb. rhamnosus* strains, contrary to Api 50CHL which cannot distinguish them. Moreover in the case of *streptococci*, FTIR spectroscopy allows one to set apart type strains of *S. thermophilus* and *S. salivarius* so we can propose to study these two species more precisely with this method.

The information given by FTIR spectra is complementary to genomic information and can be introduced in a polyphasic taxonomic approach.

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