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Raman microspectroscopy as an identification tool within the phylogenetically homogeneous '*Bacillus subtilis*'-group

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Abstract

Vibrational methods have multiple advantages compared to more classic, chemotaxonomic and even molecular microbial tools for the identification of bacteria. Nevertheless, their definite breakthrough in diagnostic microbiology laboratories is determined by their identification potential. This paper reports on the profound evaluation of Raman spectroscopy to identify closely related species by means of 68 *Bacillus* strains that are assigned or closely related to the phylogenetically homogeneous '*Bacillus subtilis*'-group (sensu stricto). These strains were chosen to represent biological variation within the selected species and to create a realistic view on the possibilities of this technique

The evaluation resulted in 49/54 correct identifications at the species level for intern and 15/19 for extern testing. The correct identification of strains, which were not represented in the training set, supports the potential as an identification tool within the '*B. subtilis* group'. Considering the vague borderline between the species studied, Raman spectroscopy can be regarded here as a promising application for identifications at the species level.

Keywords: Raman spectroscopy; '*Bacillus subtilis*'-group; Identification; Species level; External evaluation; Blind study; Carotenoid structure

Introduction

Vibrational spectroscopic methods are currently studied and developed as powerful new techniques for identification of micro-organisms. The multiple advantages of these methods, compared to more classic, chemotaxonomic and even molecular tools, make them a great interest for modern diagnostic laboratories. A wide range of micro-organisms can be discriminated at the species level by Fourier Transform Infrared (FT-IR) spectroscopy [3,26,27] and Raman spectroscopy [14,19]. Both techniques provide chemical information regarding the complete molecular composition of the cells (carbohydrates, fatty acids, proteins, RNA/DNA) [23] and hence combine discriminatory abilities of various phenotypic characteristics (e.g. whole-cell fatty acid and protein composition, pigmentation, etc.).

Raman spectroscopy combines high information content, speed of analysis, minimal sample preparation and is, at the same time, less labor intensive than classic

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methods. Together, this has set the stage for the development of Raman spectroscopy into a powerful tool for rapid and inexpensive microbial analysis.

However, before this vibrational technique can be used as a more general routine screening tool, the borders of the taxonomic resolution of the method have to be evaluated for various well-chosen groups of Gram-positive and Gram-negative bacteria. Indeed, since it concerns a chemotaxonomic method, the reproducibility and the taxonomic resolution is affected by the molecular composition of the cell, which in turn depends on variations of growth conditions (medium composition, incubation time and temperature, etc.) [12].

To evaluate the feasibility of Raman spectroscopy as an identification tool, the genus *Bacillus* [4] was selected. This genus has been split at the generic level [2,8,11,24,36,41,44] while new relatives were discovered [17,25,29,35,37,38,40,43]. Among them, a group of eight species *Bacillus axarquiensis*, *B. malacitensis*, *B. mojavensis*, *B. vallismortis*, *B. amyloliquefaciens*, *B. atrophaeus*, *B. velezensis* and *B. subtilis* (with two subsp. *subtilis* and subsp. *spizizenii*) [21] exists that is homogenous at the phenotypic and phylogenetic level. These species represent the '*B. subtilis*'-group. Three additional species, *B. licheniformis*, *B. sonorensis* and *B. pumilus* are also closely related, yet they are phylogenetically clearly separated from the others. The majority of phenotypic surveys report that members of the '*B. subtilis*'-group are very difficult to discriminate. For example, Roberts et al. [31,32] demonstrated that, for the traits tested, fatty acid composition was the only phenotypic character that distinguished *B. mojavensis* and *B. vallismortis* from one another or from *B. subtilis*. Further, phenotypic discrimination between *B. atrophaeus* and *B. subtilis* was limited to pigmentation [22], fatty acid composition and a positive oxidase test [32]. In another extensive study, *B. amyloliquefaciens* could only be distinguished from *B. subtilis* by three of the 75 phenotypic traits tested [16]. Recently, Ruiz Garcia et al. [42] tested the type strains of all species closely related to *B. subtilis* on 112 characteristics. These authors found clear differences between the type strains of all species, e.g. four of the traits enabled the differentiation of *B. amyloliquefaciens* and *B. subtilis*. However, since this study included a single strain per species it did not consider the variation within the species and more extensive studies are needed to verify whether these differences are stable identifications for species differentiations. Palmisano et al. [28] described *B. sonorensis* as a novel species and could phenotypically only distinguish it from *B. licheniformis* by salt tolerance and pigmentation.

In modern bacterial classification, 16S rDNA sequence analysis is standardly used to allocate strains at various taxonomic levels. However, 16S rDNA sequences often show limited or no variation if closely related taxa are considered [6,9]. This is also the case for certain members of the '*B. subtilis*'-group. Indeed, the 16S rDNA sequences of the type strains of *B. subtilis* (subsp. *subtilis* and subsp. *spizizenii*), *B. amyloliquefaciens*, *B. atrophaeus*, *B. mojavensis*, *B. vallismortis* and the recently described *B. velezensis* [33] and *B. malacitensis* [34], show similarities ranging from 99.2% to 99.8% (pairwise similarities based on the UPGMA algorithm of 16S rDNA sequences obtained from the GenBank/EMBL/DDBJ database). In addition, the type strains of *B. licheniformis* and *B. sonorensis* show a 16S rDNA sequence similarity of 99.6%.

Based on the outlined taxonomic situation, the *'B. subtilis'*-group is considered as an excellent model in the evaluation of Raman spectroscopy as a technique to identify closely related species. The current classification of the strains that were used is well established due to polyphasic characterization. The study aims at testing the feasibility of identification by Raman spectroscopic analysis against a (limited) database. In order to evaluate the identification results in a realistic way, a blind approach is used.

Materials and methods

Strains and culture conditions

A total of 68 *Bacillus* strains assigned to the following eight species were used:

(1) *B. amyloliquefaciens* (LMG 12325, LMG 12329, LMG 12330, LMG 12331, LMG 12384, LMG 12385, LMG 17599, LMG 17600, LMG 17601 and LMG 9814^T);
(2) *B. atrophaeus* (LMG 16797^T, LMG 17795, LMG 17796 and LMG 8199);
(3) *B. licheniformis* (LMG 12247, LMG 12248, LMG 12360, LMG 12362, LMG 12363^T, LMG 17334, LMG 17337, LMG 17339, LMG 17340, LMG 17649, LMG 17652, LMG 17654, LMG 17656, LMG 17657, LMG 17658, LMG 17659, LMG 6934, LMG 7559, LMG 7560, LMG 7630 and LMG 7634);
(4) *B. mojavensis* (LMG 17797^T and LMG 17798);
(5) *B. pumilus* (LMG 10826, LMG 12258, LMG 12259, LMG 12372, LMG 12373, LMG 12374, LMG 12375, LMG 12376, LMG 18517, LMG 18928^T, LMG 3455 and LMG 8942);
(6) *B. sonorensis* (LMG 21636^T);
(7) *B. subtilis* (LMG 12260, LMG 12261, LMG 12262, LMG 12263, LMG 12264, LMG 12379, LMG 12380, LMG 12381, LMG 17722, LMG 17725, LMG 17726, LMG 17727, LMG 19154, LMG 19155, LMG 7135^T and LMG 8197);
(8) *B. vallismortis* (LMG 17800 and LMG 18725^T).