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Ribosomal proteins as biomarkers for bacterial identification by mass spectrometry in the clinical microbiology laboratory

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ABSTRACT

Whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a rapid method for identification of microorganisms that is increasingly used in microbiology laboratories. This identification is based on the comparison of the tested isolate mass spectrum with reference databases. Using *Neisseria meningitidis* as a model organism, we showed that in one of the available databases, the Andromas database, 10 of the 13 species-specific biomarkers correspond to ribosomal proteins. Remarkably, one biomarker, ribosomal protein L32, was subject to inter-strain variability. The analysis of the ribosomal protein patterns of 100 isolates for which whole genome sequences were available, confirmed the presence of inter-strain variability in the molecular weight of 29 ribosomal proteins, thus establishing a correlation between the sequence type (ST) and/or clonal complex (CC) of each strain and its ribosomal protein pattern. Since the molecular weight of three of the variable ribosomal proteins (L30, L31 and L32) was included in the spectral window observed by MALDI-TOF MS in clinical microbiology, i.e., 3640–12000 m/z, we were able by analyzing the molecular weight of these three ribosomal proteins to classify each strain in one of six subgroups, each of these subgroups corresponding to specific STs and/or CCs. Their detection by MALDI-TOF allows therefore a quick typing of *N. meningitidis* isolates.
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1. Introduction

Whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) generates a spectrum based on proteins detected directly from intact microorganisms (Holland et al., 1996; Williams et al., 2003), allowing the rapid identification of bacterial isolates. This identification relies on comparison of the spectra of the sample with those of reference databases. The Andromas database was engineered using an algorithm that identifies a limited number of species-specific peaks for each entry (Carbonnelle et al., 2007; Degand et al., 2008). Briefly, to engineer the database, a set of reference isolates was chosen, and ten subcultures of each of these selected isolates, grown on different media, were analyzed. For each strain, only those peaks with a relative intensity above 0.07, and which that were constantly present in all 10 sets of data obtained for a given strain, were retained. With the Andromas database, accurate species identification is obtained if at least 68% of the species-specific peaks are present in the spectrum of the subject isolate. The failure to identify some specimens is explained by small protein variations among isolates of the same species, or by the fact that some peaks of the database cannot be observed because of the poor quality of spectra obtained from whole bacteria grown in primary culture.

In this work, we aimed at answering two questions regarding the implementation of mass spectrometry in clinical laboratories. (i) Although many studies have showed that peaks used for identification species of bacteria are ribosomal protein (Lay, 2001; Pineda et al., 2003; Ryzhov and Fenselau, 2001; Teramoto et al., 2007a, 2007b), we wanted to identify the exact nature of the biomarkers empirically used to build the Andromas database for bacterial species identification. (ii) We next aimed at identifying markers specific of strain and/or groups of strains and compare them to reliable epidemiological methods. Ribosomal proteins are good candidates for such an approach as they are universal amongst cellular life. Indeed, even though most ribosomal proteins are highly conserved within a bacterial species, some of these proteins are subject to slight variations at the strain level. As the variations of the ribosomal protein genes have been proposed for classification and typing purposes (Bennett et al., 2012; Jolley et al., 2012; Kozo, 1989; Matte-Tailliez et al., 2002; Roberts et al., 2008; Yutin et al., 2012), analysis of the ribosomal protein masses in a MALDI-TOF spectrum directly from intact bacterial cells could provide an interesting epidemiological tool for the classification of bacterial isolates

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to the sub-species or strain level. The use of ribosomal markers detected by MALDI-TOF would then dramatically speed up epidemiological studies in the clinical laboratory and in environmental microbiology.

For epidemiological studies, strains are routinely typed using multilocus sequence typing (MLST). They are subsequently compared by sequencing the internal fragment of seven housekeeping genes. Strains having similar sequence belong to the same sequence type (ST). STs are grouped into clonal complexes (CC) by their similarity to a central allelic profile (http://pubmlst.org/). In addition, using only ribosomal proteins, Jolley et al. (2012) showed that ribosomal multilocus sequence typing (rMLST) of *Streptococcus pneumoniae* had strain level resolution. Here, we examined isolates of the bacterial pathogen *Neisseria meningitidis* to determine the bacterial components corresponding to the species-specific peaks combining genomic and proteomic approaches (Demirev et al., 2005; Dworzanski et al., 2004; Ryzhov and Fenselau, 2001). Taking into account genomic sequence data of 100 isolates, we correlated the ribosomal protein profile of each isolates with its ST and CC. Use of these data allows classifying each isolate to a subgroup on the basis of spectra obtained in a routine clinical setting.

Table 1
Presence of the *Neisseria meningitidis* biomarkers among the 102 tested strains.

Peaks number	*m/z* (database Andromas)	% Detection of each peak among the 10 acquisitions of the 102 strains (i.e., 1020 acquisitions)	Numbers of strains which do not have the corresponding biomarker at least once, among the 10 acquisitions of each strain
1	4486	99	1
2	5049	100	0
3	5618	100	0
4	5936	100	0
5	6343	78	22
6	6434	97	2
7	7067	100	0
8	7230	99	0
9	8126	98	1
10	8230	100	0
11	8698	89	0
12	9360	100	0
13	9393	90	0

m/z: mass/charge.

2. Material and methods

2.1. Strains

One hundred clinical isolates of *N. meningitidis* included in the 107 strains collection used to establish multilocus sequence typing (Maiden et al, 1998) was obtained from D. Caugant, WHO Collaborating Center for Reference and Research on Meningococci, Norwegian Institute of Public Health, Oslo. In addition, two previously described isolates, *N. meningitidis* NEM 8013 and *N. meningitidis* Z2491, were used. All isolates were grown on GCB (Gonococcal Medium Base) agar plates (Difco) containing Kellog's supplements at 37 °C in 5% atmosphere for 18 h, harvested and inactivated in 70% ethanol. Pellets were conserved at −80 °C.

2.2. Proteolysis

Proteins of strains NEM 8013 and Z2491 were extracted from bacterial cells with 70% formic acid/acetonitrile (v/v) and the suspension centrifuged at 13000 g. The supernatant was then dried, and 5–100 μg of protein was dissolved in an appropriate volume of 10 mM Tris–HCl buffer, pH 7.0, containing 0.1% (w/v) SDS and 0.15% (w/v) dithioerythiol (DTT) to give a protein concentration of 1 μg/μl. The solutions were incubated at 90 °C for 60 s to reduce proteins and left to cool to room temperature. A volume of 5 μl of 0.5 M iodoacetamide (an excess) was added to the mixture, which was incubated at 37 °C for 30 min in the dark to carboaminomethylate the cysteine residues. Excess reagent and low molecular weight products were removed by ultrafiltration (Amicon, Millipore, Ireland), and the proteins was concentrated by centrifugal evaporation. Derivatised proteins were reconstituted in water to a final concentration of 1 μg/μl, and 5 μl was added to 190 μl of the appropriate reaction buffer: for endoproteinase GluC (protease V8), 50 mM sodium phosphate buffer, pH7.8, five microliters of the protease V8 (0.1μg/μl) (ThermoScientific, USA) were added to the reaction mixture. After incubation at 37 °C for 16 h, the reactions were stopped by heating at 90 °C for 30 s. The samples were concentrated and residual acetonitrile and TFA removed under vacuum before reconstitution in H20.

2.3. De-O-glycosylation and dialysis

Proteins of strains NEM 8013 and Z2491 were extracted from bacterial cells with 70% formic acid/acetonitrile (v/v). The suspension was centrifuged at 13000*g*. The resulting supernatant was dried out and the pellet resuspended in water. Twenty micrograms of extracted proteins were digested by endo-α-*N*-acetylgalactosaminidase (Biolabs, Great Britain). A slide-A-Lyser Dialysis Cassette 3.5K MCWO

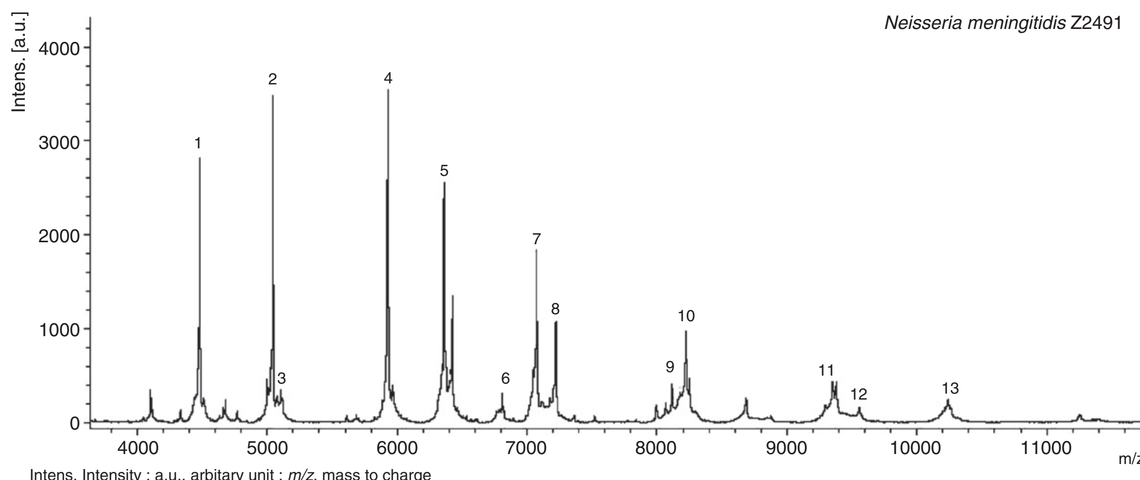

Fig. 1. Mass spectra of whole cell of *Neisseria meningitidis* Z2491.

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