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Letter to the Editor

Antimicrobial resistance classification using MALDI-TOF-MS is not that easy: lessons from vancomycin-resistant *Enterococcus faecium*

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To the Editor,

Matrix-assisted Laser-Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) is a routinely applied technique to identify bacterial species. The acquired spectra show predominantly the molecular weights of ribosomal proteins. Once acquired, the spectra are compared with a database of either previously acquired spectra for which the organism is known or theoretical spectra based on predicted masses from sequence data, and identified based on identical peaks. Many efforts are undertaken to expand the information that can be extracted from these spectra to deduct further features regarding the antibiotic resistance phenotype or even the presence of virulence factors. To achieve this, large data sets of spectra of bacteria with different features are computationally analysed and their differences in masses are assumed to be distinctive for absence or presence of this feature.

Enterococci are commensal bacteria of animal intestines but may also cause nosocomial infections in immunocompromised patients [1]. *Enterococcus faecium* is naturally resistant against a number of antibiotics and easily acquires resistance to more antibiotics, e.g. vancomycin, by horizontal gene transfer. Even though alternative antibiotics for treatment of patients with vancomycin-resistant enterococcus (VRE) infections exist, VRE infections remain difficult to treat. *Enterococcus faecium* is a member of the ESKAPE group of pathogens that frequently display antibiotic resistance and are considered a major threat to modern medicine [2]. Griffin et al. analysed the MS spectra of *Enterococcus faecium* from a hospital outbreak and correlated these with the presence or absence of vanA- or vanB-type resistance against vancomycin. Using a supervised learning approach, the study found a strong correlation between the presence of a set of peaks and the antibiotic resistance [3]. Here we investigate the suitability of this approach further by examining a peak at 5092 m/z in a spectrum of vancomycin-resistant *Enterococcus faecium*.

Enterococcus faecium ST796 (*vanB*$^+$) and a vancomycin-sensitive *Enterococcus faecium* (Orla-Jensen, ATCC 19434) were grown in liquid medium or on plates and lysed in a 1:1 mixture of 70% formic acid in water and acetonitrile. MALDI-TOF spectra were acquired from 3 kDa to 20 kDa. Swiss *E. faecium* ST796 showed a peak at the same mass as published by Griffin et al. [3]. For intact, exact mass measurements, proteins of the lysate were precipitated using 20% trichloroacetic acid and resuspended in a 1:1 mixture of water and acetonitrile. A fraction of the sample was reduced by addition of Tris(2-carboxyethly)phosphine. One microlitre of the reduced and untreated samples was spotted on the target plate and overlaid with α-cyano-4-hydroxycinnamic acid, (Bruker Daltonik GmbH, Bremen, Germany) (see Supplementary material, Appendix S1).

The high signal intensity of the peak at 5092.12 m/z after protein precipitation allowed us to determine the protein sequence using MS/MS to a large extent and led to its identification as hiracin (Uniprot Q0Z8B6) or bacteriocin T8, a sec-dependent secretory protein [4] (Fig. 1). The pre-protein has a theoretical molecular weight of 8156.65 Da; however, without the signal peptide, its theoretical average mass ($MH^+_{avg/red/theo}$) is 5094.73 Da, which is in agreement with what is published by Griffin et al. (5094.7 m/z) (Fig. 1c) [3]. The high resolution of TOF mass spectrometers operating in reflectron-mode permitted us to measure the monoisotopic mass of the protein ($MH^+_{mono/ox}$) at 5089.64 Da (see Supplementary material, Fig. S1). This is below the theoretical monoisotopic mass ($MH^+_{mono/red/theo}$) of the reduced protein at

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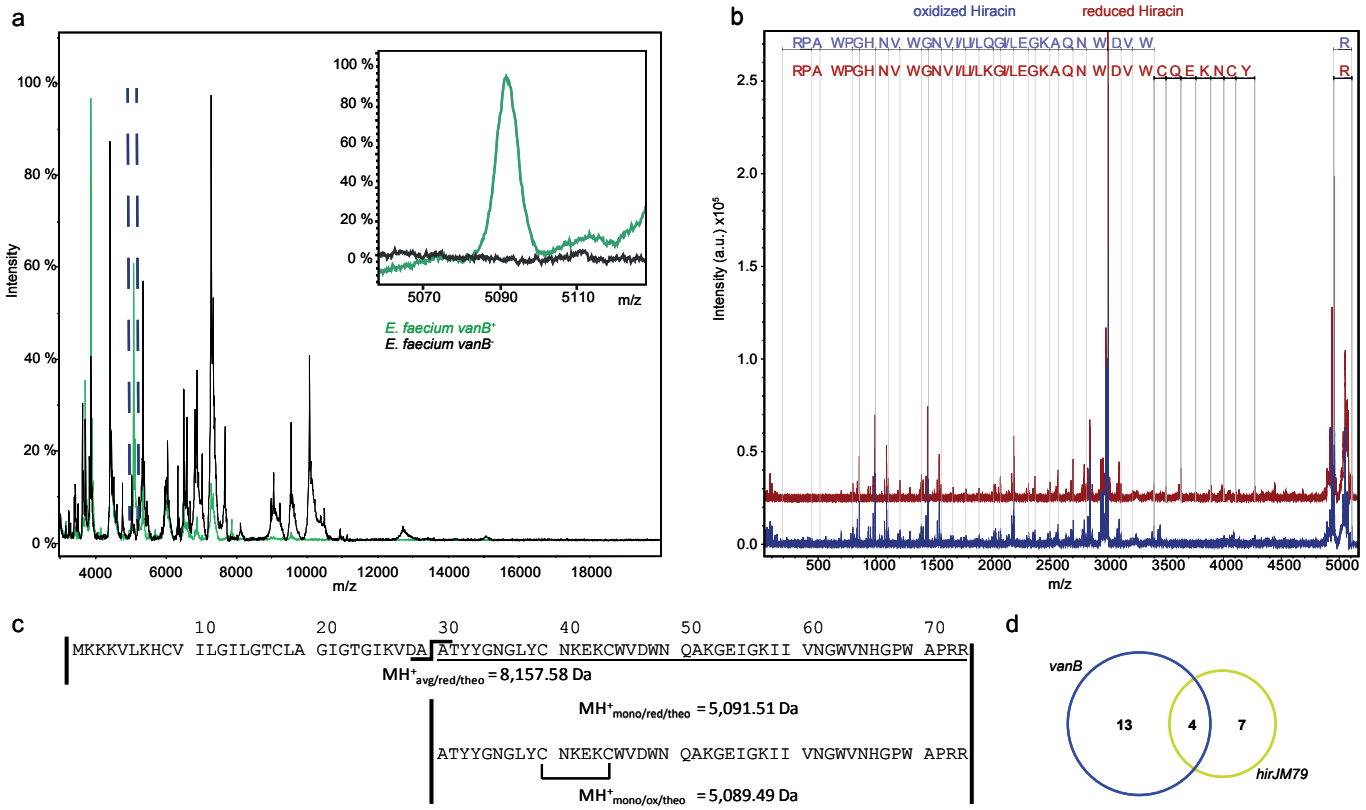

Fig. 1. MS peak at 5092.12 m/z of *Enterococcus faecium* vanB^{+} is a bacteriocin and secreted in an oxidized form. (a) MS spectra of vancomycin-resistant (green) and vancomycin-sensitive (black) *Enterococcus faecium* from 3 to 20 kDa. The insert shows the region of the peak of interest. (b) MS/MS-spectra and sequence assignment of the hiracin in oxidized (blue) and reduced (red) forms. Three ion series are partially assigned but only one is shown. Peaks around 5050 Da are peaks from neutral losses. For visualization purposes, the spectrum of the reduced hiracin has a *y*-offset of 25 000 au. (c) Sequence of the immature hiracin (with signal sequence, the cleavage site of the signal peptide is marked with a bar) and its mature form with their respective theoretical monoisotopic masses. The part between the two cysteines is not completely sequenced in the oxidized form but can be sequenced in the reduced form. (d) Venn-diagram of presence of *vanB* or *hirJM79* genes in several complete genomes of *Enterococcus faecium* using tBLASTN on the REFSEQ complete genomes database with the sequence of the mature hiracin and complete VanB.

5091.51 Da, but fairly close to the monoisotopic mass of the oxidized protein at 5089.49 Da ($MH^{+}_{mono/ox/theo}$) (Fig. 1c and see Supplementary material, Fig. S1).

Hiracin harbours two cysteines, which are expected to be oxidized when the protein is secreted. Reduction of the protein sample before analysis resulted in an increase of molecular weight by two hydrogen atoms ($MH^{+}_{mono/red}$ 5091.65 Da) (see Supplementary material, Fig. S1). Additionally, after reduction, the sequence coverage by MS/MS was increased mainly in the region between the two cysteines (Fig. 1b).

To further analyse the usability of this protein as a marker for the identification of vanB-type vancomycin-resistant *E. faecium*, we computationally analysed all available *vanB*- or *hirJM79*-encoding genomes on REFSEQ (tBLASTN on REFSEQ, complete genomes, *Enterococcus faecium*, Uniprot accession numbers Q0Z8B6 (HirJM79) and P97205 (VanB), >90% query cover, >90% identity) and checked if these two genes co-occur by comparison of the strains' BIOSAMPLE accession numbers (Fig. 1d). We found 17 *vanB*-harbouring genomes and 11 that contained the *hirJM79* gene. Interestingly, they co-occurred in four strains, yielding a predictive power of 36% (4/11 *vanB*/*hirJM79* to *hirJM79*, Fig. 1d) [5].

We have shown here the identity of a presumed indicative protein peak for vancomycin resistance using state-of-the-art MALDI-TOF-MS/MS. The corresponding hiracin gene is not directly linked to the presence of vanB-type vancomycin resistance, so cannot be routinely used to classify resistance phenotypes. However, we expect that it can be used to investigate and diagnose VRE in an outbreak setting, where the strain is sequenced and presence or absence of the *hirJM79* gene has been established, for both the VRE-outbreak-causing strain and a reference background. We strongly advocate to investigate the functional relationship between MALDI-MS peaks and resistance phenotype other than by pure correlation, so that these results can potentially be transferred to other diagnostic centres. Our study is limited by the measurement of only two strains and MS/MS sequencing of one of their proteins and so lacks statistical implications. We also did not use the strain of the original publication [3]. However, this study underscores the need for characterization of MALDI protein peaks to deduce meaningful information about the strain's phenotype. Another caveat of our bioinformatic analysis is a regional bias, as many of the strains with complete sequence stem from the Netherlands (49 of 126) [5]. Analysis of more MS spectra of several clinical microbiology laboratories together with either genome sequencing or antimicrobial susceptibility testing will further enhance our understanding of the distribution of these genes as well as their suitability as markers for resistance.

Transparency declaration

The authors declare no conflict of interest. No external funding was received for this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2019.10.027.

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