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Comparative proteomic map among *van*A-containing *Enterococcus* isolated from yellow-legged gulls

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ABSTRACT

The increase of vancomycin resistance enterococci (VRE) represents a threat for patient care and creates a reservoir of mobile resistance genes for other, more virulent pathogens. The existence of VRE in different ecologic niches complicates the understanding of its epidemiology. The aim of the present study was to analyse the proteome of 2 *van*A strains recovered from seagull faecal samples. The *van*A *E. durans* and *van*A *E. faecium* strains presented different genomic patterns: *tet*(M)-*tet*(L)-*erm* (B) and *tet*(M)-*tet*(L)-*erm*(B)-*hyl*, respectively. A total of 123 spots were excised from two-dimensional gel electrophoresis (2-DE) gel of *van*A *E. durans* SG 2 strain, and 16 were successfully identified by using MS, representing 42 different proteins. For the *van*A *E. faecium* SG 50 strain, 93 spots were excised from the 2-DE gel and 23 were identified, representing 47 different proteins. The vancomycin/teicoplanin A-type resistance protein VanA in *E. durans* SG 2 strain was present in two different spots. The identified proteins have shown diverse functional activities including glycolysis, conjugation, translation, protein biosynthesis, among others. This work reports the impact of proteomics on knowledge of *van*A-containing enterococci and will be helpful to further understand the expression of proteins in antibiotic-resistant bacteria.

Keywords: Antimicrobial resistance; Bioinformatics; Databases; Proteomics; *Van*A-containing *Enterococcus*; Virulence factors.

Abbreviations

2-DE: two-dimensional polyacrylamide gel electrophoresis; **PCR**: Polymerase Chain Reaction; **MALDI-TOF MS**: matrix-assisted laser desorption ionization time-of-flight mass spectrometry; **spp.**: subspecies; **MIC**: minimal inhibitory concentration

1. Introduction

Proteome analysis is a tool that can be used to visualize and, also, to compare complex mixtures of proteins, and to acquire important information about the individual proteins concerned in specific biological responses [1]. Proteomic is defined as the expressed complement of a genome. In comparison to the genome, the proteome is used to define a series of proteins expressed by a certain organism, under certain conditions, serving as unique and informative infor-

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mation of both its phenotypic state. This results in cell responses to physiological and environmental perturbations, and genomic information reflected in the amino acid sequences of expressed proteins. Therefore, the principal concern of proteomic resides, in the identification of proteins related to, in particular, cellular processes or presenting altered expression profiles as a consequence of different physiological conditions [2].

Various analytical tools such as two dimensional gel electrophoresis, mass spectrometry and searches in generalist and Expressed Sequence Tags (EST) databases, modified the protein identification process [3]. Although not usually used, proteomic approaches participate in determining antimicrobial resistance mechanism(s) and other cell metabolic alterations through the ability to study overall changes in bacteria [4]. The model of protein species linked to the antimicrobial resistance has been investigated in a diversity of microorganisms and with different antimicrobial agents [4-6]. The evaluation of changes in protein profiles in response to various mechanisms of stress, such as the susceptibility to antimicrobial agents or the modifications related to antibiotic resistance could represent an integrating method for the development of new therapeutic treatment and antimicrobial agents. Bacterial surface proteins are important for the host-pathogen interaction and they are commonly implicated in disease pathogenesis [4].

Enterococcus spp. live as commensals of the gastrointestinal tract of warm-blooded animals, being the most abundant Gram-positive cocci in humans and in animals [7]. Recently, the incidence of nosocomial enterococal infections has increased distinctly [8]. This genus is also recognized as important opportunistic pathogens, and reveals intrinsic resistance to a number of antimicrobial agents, in addition to the acquired multidrug resistance [9].

The increase of vancomycin-resistant enterococci (VRE) causes several challenges. Firstly, most of VRE are frequently also resistant to other available drugs e.g., aminoglycosides or ampicillin. Secondly, there is the possibility that the vancomycin resistance genes present in VRE could be transferred to other gram-positive microorganisms. In addition to the currently common detection of multiresistant bacteria in areas with high human density [10] their emergence in more remote areas like high mountain regions or natural reserve is even more alarming [11-13]. Monitoring the prevalence of resistance in indicator bacteria such as vancomycin-resistant enterococci in different populations, animals, patients and healthy humans, makes it feasible to compare the prevalence of resistance and to detect the transfer of resistant bacteria or resistance genes from animals to humans and vice versa [14]. Microbial resistance to antibiotics is a worldwide problem in human and veterinary medicine. Commonly, it is usual that the main risk factor for the increase of this situation is an extensive use of antibiotics that leads to the dissemination of resistant bacteria and resistance genes in animals and humans [15].

The wild birds seem to represent a significant reservoir, or at least source of *van*A enterococcal strains. Consequently, this may represent a significant hazard to human and animal health by transmitting these strains into waterways and other environmental sources via their faecal deposits. Although wild birds rarely come into contact with antimicrobial agents, arguing against the existence of direct selective pressure on birds, nevertheless they can be infected or colonized by resistant bacteria. Water contact and acquisition via food seem to be major aspects of transmission of the resistant bacteria of human or veterinary origin to wild animals [10,16]. Wild birds or wild animals in general could, therefore, serve as reservoirs of resistant bacteria and genetic determinants of the antimicrobial resistance [11].

In the present study, we examined the proteome of 2 *van*A strains recovered from seagull faecal samples. This evaluation was carried out in order to compare the proteins obtained from these strains with the results obtained in a previous published study using a different group of strains [13]. The combination of two high-resolution methods, isoelectric focusing and SDS polyacrylamide gel electrophoresis, permitted the separation of numerous proteins of *van*A-containing *Enterococcus* isolates and highlight the presence of vancomycin/teicoplanin A-type resistance protein *van*A.

2. Materials and Methods

2.1 *Samples and bacteria*

The phenotypic and genetic profiles of two *van*A-containing *Enterococcus* strains (*van*A *E. durans* SG2 and *van*A *E. faecium* SG50) as well as the SDS-PAGE of whole-cell extracts of them were studied in a previous report [13]. The complete proteomic analysis of these two *van*A-containing strains SG2 and SG50 has been the objective of the present study. These strains were previously obtained from faecal samples of yellow-legged seagulls, randomly recovered in the beaches of Berlengas Islands National Reserve of Portugal [13].

2.2 *Virulence factor genes*

The presence of genes encoding different virulence factors (*gelE*, *fsr*, *ace, cpd*, *agg and cyl*L_LL_SABM) was verified by PCR using primers and conditions previously described [17-19]. Positive and negative controls obtained from the collection of the University of Trás-os-Montes and Alto Douro (Portugal) were included in all assays. The presence of *hyl* or *esp* gene in these strains was previously reported [13].

2.3 *Assay of gelatinase activity*

Gelatinase production was detected by inoculating the enterococci onto freshly prepared tryptic soy agar plates (Difco; 236950, Le Pont de Claix, France) containing 1.5% of skim milk (Difco; 232100). Plates were incubated overnight at 37° C and then cooled to ambient temperature for 2 h. The ap-

pearance of a transparent halo around the colonies was considered to be a positive indication of gelatinase production [20].

2.4 *Assay of hemolytic activity*

The production of hemolysin was determined by streaking bacterial cultures, grown overnight at 37°C in brain heart infusion agar (Difco; 241830), on columbia agar plates supplemented with 5% of horse blood (BioMérieux; 43050, La Balme, Les Grottes, France). Plates were incubated at 37°C for 72h in aerobic conditions and after that the plates were examined for haemolysis. The haemolytic reaction was recorded by the observation of a clear zone of hydrolysis around the colonies (β-haemolysis), a partial hydrolysis (α-haemolysis) and a non reaction (γ-haemolysis). When observed, greenish zones around the colonies were interpreted as α-haemolysis and taken as negative for the assessment of β-haemolytic activity [19].

2.5 *PCR amplification of pbp5 gene*

Total DNA was extracted from *van*A *E. faecium* SG 50 isolate by the InstaGene Matrix (Bio-Rad; 732-6030, Hercules, United States), and 10µl of DNA was used for the PCR reaction, the $MgCl_2$ concentration being of 3.5 mM. The primers used for PCR amplification of *pbp*5 gene were the following ones: F 5´-AACAAAATGACAAACGGG-3´; R 5´-TATCCTTGGTTATCAGGG-3`. PCR conditions were as follows: 95°C initially for 15 min; 94°C for 30 s, 54°C for 30 s, 72°C for 2 min over 30 cycles followed by a final 7 min extension period at 72°C [21].

2.6 *DNA sequence analysis*

The *pbp5* PCR products were purified with the QiaQuick PCR purification kit (Qiagen Inc.) according to the instructions of the manufacturer. The purified products were sequenced in both strands on the ABI Prism 3700 DNA sequencer (Perkin-Elmer). The obtained sequences were compared through bioinformatics tools to that of *pbp*5 included in GenBank accession no. X84860.

2.7 *Protein extraction*

Frozen *van*A-containing *Enterococcus* cell stocks were streaked onto Luria-Bertani (LB) plates and grown at 37ºC. Single colonies of *van*A-containing *Enterococcus* strains were conducted in 250 mL of M9 minimal medium supplemented with 4 gL^{-1} glucose in covered 1 L Erlenmeyer flasks at 37 ºC. Cells were harvested from the exponential phase in all experiments. The cells were pelleted down at 10,000 rpm at 4ºC for 3 min. The pellet was supposed to be visible after spinning and resuspended in an equal volume of pre-warmed phosphate-buffered saline (PBS) pH 7.4 [22]. After new centrifugation pellet was suspended in 0.2 ml of SDS sample solubilization buffer. The sample was sonicated with an ultrasonic homogenizer (6 × 10 s, 4 °C at 100 W). The disrupted cells were centrifuged in an Eppendorf microfuge at maximum speed (14,000 rpm) for 30 minutes at 4°C. For SDS-PAGE experiment the supernatant was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue.

2.8 *Two-dimensional electrophoresis and proteomics*

The 2-DE was performed according to the principles of O'Farrell [23] but with IPG (Immobiline™ pH Gradient) technology [24]. Protein samples of *van*A *E. durans* isolate (SG 2) were used in parallel with those of *van*A *E. faecium* isolate (SG 50) proteins. For IEF, precast IPG strips with linear gradient of pH 4-7 were passively rehydrated overnight (12 to 16 hours) in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature and IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lyses buffer [9.5M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor] was added to the two *van*A-positive enterococci (1:1). Samples containing a total of 73.5 µg of protein were loaded into 13 cm IPG strips (pH 4–7 NL, Amersham Biosciences, UK) [25]. The sample solution was then applied to the previously rehydrated IPG strips pH 4-7 by cup loading and then proteins were focused sequentially at 500 V for 1 h, 1000 V for 2 h, 8000 V for 2 h, 1000 V for 4h and, finally, 1000 V for 55min incremented to 23208 V/h on an EttanTM IPGPhor IITM (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg [24] and the GE Healthcare protocol for IPG strips pH 4-7 of 13 cm, in order to obtain the optimized running conditions, resulting in a final around 10 hour run. Focused IPG strips were then stored at -80ºC in plastic bags. Before running the second dimension, strips were equilibrated twice for 15 minutes in an equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration, 1% DTT was added to the original equilibration buffer and 4 % iodoacetamide to the second one, and, also, bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied onto a 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli [26], that allowed its resolution to be increased with a proper insertion of the IPG strips in the stacking gel [26,27]. After SDS-PAGE, the 2-DE gels were fixated on 40% methanol / 10% acetic acid for one hour and, afterwards, stained overnight in Coomassie Brilliant Blue G-250 [22]. Coomassie-stained gels were scanned with a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image

Master 5.0 software (Amersham Biosciences; GE Healthcare).

2.9 *Protein identification by MALDI-TOF/TOF*

Spots of expression in all gels were manually excised from the gels and analyzed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). The gel pieces were washed three times with 25mM ammonium bicarbornate/50 % ACN, one time with ACN and dried in a SpeedVac (Thermo Savant). 25 mL of 10mg/mL sequence grade modified porcine trypsin (Promega) in 25mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37°C. The extraction of tryptic peptides was performed by adding 10% of formic acid (FA)/50% ACN three times and being lyophilised in a SpeedVac (Thermo Savant). Tryptic peptides were ressuspended in 10 mL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of a-α–cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/ 0.1% formic acid. Aliquots of samples (0.5µL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained through or from a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode.

Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm.

2.10 *Database search*

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence database was used for all searches under *Enterococcus*. Database search parameters are as follows: carbamidomethylation and propionamide of cysteine (+71Da) as a variable modification as well as oxidation of methionine (+16Da), and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and the fragment ion mass tolerance was 0.3 Da. Protein identifications were considered as reliable when the MASCOT score was > 70 (MASCOT score was calculated as - 10 × log P, where *P* is the probability that the observed match is a random event.). This is the lowest score indicated by the program as significant ($P < 0.05$) and indicated by the probability of incorrect protein identification. All spectra were also processed in a reversed decoy database created for Swiss-Prot (consisting of normal and reverse sequences) to allow estimation of the false discovery rate (false positive peptides/(false positive peptides + total peptides))*100) which is by routine below than 1%.

2.11 *Sequence alignments and construction of the phylogenetic tree*

The analysis was performed on the Phylogeny.fr platform and comprised the following steps. Sequences were aligned with MUSCLE (v3.7) configured for the highest accuracy (MUSCLE with default settings). After the alignment, the positions with gap were removed from the alignment. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for the rate heterogeneity across sites. The gamma shape parameter was fixed (alpha=12.4). The reliability for internal branch was assessed using the aLRT test (SH-Like). A graphical representation of the phylogenetic tree (phenogram) was performed with Drawgram from the PHYLIP package (v3.66) [26].

3. Results

3.1 *Characteristics of the two van*A *strains included in the study*

The phenotype and genotype of antibiotic resistance of the two *van*A *Enterococcus* strains included in this study were previously reported [13]. Both strains showed high level of vancomycin and teicoplanin resistance, as well as resistance to tetracycline and erythromycin. *E. faecium* strain SG 50 showed also resistance to ciprofloxacin and ampicillin. The *van*A *E. durans* and *van*A *E. faecium* strains presented different genomic patterns: *tet*(M)-*tet*(L)-*erm*(B) and *tet*(M)-*tet*(L)-*erm*(B)-*hyl*, respectively.

Genes encoding virulence factors were studied in the two *van*A-containing *Enterococcus* isolates (SG2 and SG50). The *cpd* gene was detected in both *van*A enterococci. The $cylL_L$ gene was identified in the *van*A *E. durans* SG 2 strain . The two *vanA* strains expressed gelatinase activity and carried the *gel*E gene. No beta-haemolytic activity was identified in both strains, although they showed alfa-hemolysis.

The sequence of the C-terminal region of *pbp5* was analyzed in our *van*A *E. faecium* SG 50 strain that showed ampicillin-resistance. This strain presented 11 amino acid substitutions in PBP5 protein (408Q → H, 427I → M, 470H → Q, 485M → A, 496N → K, 497F → I, 499A → T, 525E → D, 586V → L, 629E → V, 634N → Q), in relation with the reference one.

3. 2 *Two-dimensional Electrophoresis*

The SDS-PAGE of whole-cell extracts of the 2 *van*A-

containing enterococci strains are shown in a previous study [13]. In the present study, a comparative analysis among the strains has been carried out. The protein expressions of the two vancomycin-containing enterococci (*E. durans* SG 2 and *E. faecium* SG 50) strains were analysed. *E. faecium* SG 50 was presented on 2-DE gel (Figure 1). The use of pH 4-7 IPG strips resulted in a well spread protein spots map which contributed to accurate image identification and the safe excision of the spots. For each sample of SG 2 and SG 50 strains, a total of 123 and 93 relevant protein spots, respectively, were collected for analysis using MALDI-TOF mass spectrometry (Figure 2). These spots are consistently and systematically present in all replicates. The peptide mass peaks were compared with those in the NCBI database (http://www.ncbi.nlm.nih.gov/). The protein identification data including Genebank ID, MW, PI value, mascot score, number of matched peptides and sequence coverage ratio (%) are listed in Table 1 for and *E. durans* SG 2 and Table 2 for the *E. faecium* SG 50 proteins. The identified proteins were showing diverse functional activities, including glycolysis, conjugation, translation, protein biosynthesis, among others Replicate sequences, truncated sequences, and sequences with partial alignments were removed from the BLAST results (not shown). From the collected sequences were selected to represent the initial tree. These sequences were aligned and a phylogenetic tree was constructed by using the mini-

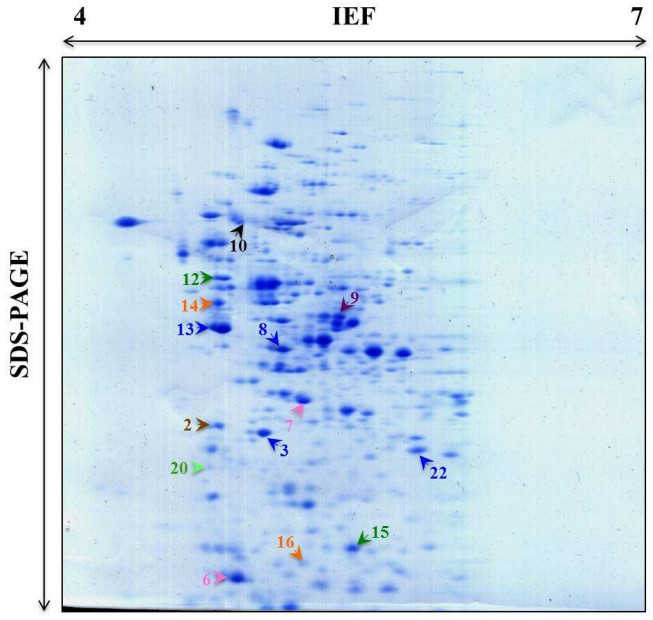

Figure 1. 2-DE gel image of SG 50 VRE with IPG strips pH4-7. Arrows indicates peptides that are part of one or more proteins whose have a role in such pathways: Blue: Glycolysis, Orange: Stress response, Pink: Protein folding/ Protein biosynthesis, Brown: Pyrimidine biosynthesis, Bright green: Transcription, Light green: ATP synthesis, Violet: Arginine metabolism, Black: Phosphotransferase system .

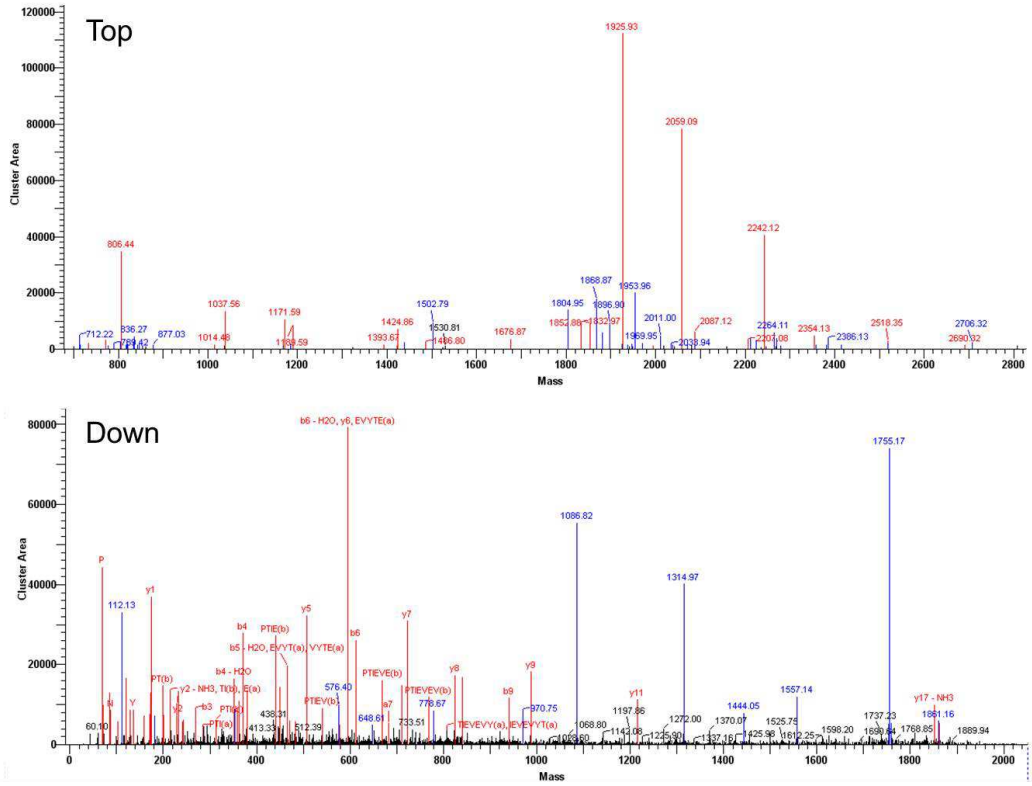

Figure 2. MALDI/MS spectra obtained for Enolase from *van*A *E. durans* SG 2 strain. Top - MS analysis of tryptic peptides. Peaks in red matching enolase. Down - MALDI-TOF-TOF MS tandem spectrum of tryptic peptide [M+H]+; m/z 1925.93 identified as enolase. The identified sequence is GNPTIEVEVYTESGAFGR. On spectrum, peaks in red correspond to y and b series .

mum-evolution method to root the tree. The clustering of the initial phylogenetic tree indicated that all of the proteins included in the data set diverged from a common ancestor (Figure 3).

4. Discussion

The worldwide appearance of antibiotic-resistant bacteria causes a severe threat to human health. Furthermore, it can add to the difficulties in controlling infectious diseases, the phenotype of resistance can generate metabolic changes that, in turn, can interfere with host-pathogen interactions. The commensal bacteria such as enterococci can carry virulence factors, and in that case, the pathogenic capacity can increase [17]. The two *van*A-containing *Enterococcus* strains included in the study were previously characterized for antibiotic resistance.

In our study, the *cpd* (a sex pheromone determinant) gene was detected in both *van*A strains (*E. durans* and *E. faecium*), which is similar to the results reported by others [28]. The isolates with sex-pheromone determinants have the potential to acquire the respective sex-pheromone plasmids and, hence, the associated virulence and resistance determinants. So, the sex pheromone production may promote the acquisition of vancomycin resistance and other linked traits from *E. faecium* strains and lead to an increased virulence [17].

Gelatinase, encoded by the *gel*E gene, is an extracellular zinc endopeptidase that hydrolyses collagen, gelatin, hemoglobin and other bioactive compounds, and it has been shown to exacerbate endocarditis in an animal model, although this activity is not required for pathogenesis [29]. Usually, the *gel*E gene has been detected with higher frequency suggesting that this virulence determinant is a common trait in the genus *Enterococcus*. In our study, the *gel*E was identified in both *van*A strains. Same results were observed in other reports [28,30]. The α-haemolysis could be a result of oxidation and a consequent lysis of the erythrocytes due to factors other than the production of enterococcal hemolysin [19]; therefore, the incubation under anaerobic conditions seems to be more reliable.

In this work, we performed a proteome analysis of two *van*A strains (*E. durans* SG 2 and *E. faecium* SG 50). A total of 123 spots were excised from 2-DE gel of SG 2 and 16 were successfully identified by MS, representing 42 different proteins. For the SG 50 strain, 93 spots were excised from the 2-DE gel and 23 were identified, representing 47 different proteins. It is important to highlight the presence of vancomycin/teicoplanin A-type resistance protein VanA in *E. durans* SG 2 strain, in two different spots (12 and 14). VanA is a protein capable of utilizing both hydroxyl acids and D-Ala as substrates with a concomitant switch from ester to peptide bond formation dependent on pH [28]. Vancomycin inhibits the extracellular steps of peptidoglycan synthesis by binding it to the C-terminal D-alanyl-D-alanine (DAla- D-Ala) residues of cell wall precursors in enterococci [32]. The D-Ala-D-Ala target residues are synthesized intracellularly as a dipeptide by a D-Ala:D-Ala ligase. Then, they are added to UDP-Nacetylmuramyl- L-Ala-g-D-Glu-L-Lys (UDP-MurNac-tripeptide) by using an adding enzyme [33]. The vancomycin/teicoplanin A-type resistance protein was also identified in *van*A-*E. durans* SG 3 isolate [13,34].

The presence of vancomycin/teicoplanin A-type resistance protein in vancomycin-resistant enterococci in natural environments could have impact on animal and human health. The emergence of vancomycin-resistant enterococci of human and veterinary origin [35], presumably leading to a great health concern [36].

Several proteins were found in multiple spots on the two gels. From the 42 different proteins identified in *van*A *E. durans* SG 2 isolate, five of them were involved in stress response. Usually, stress response requires heat molecular chaperones or shock proteins that preserve protein function or repair damage after cell injury. As such, the integrity of chaperone systems can seriously modify the progression of diseases associated with ageing, DNA damage and chronic injury [37]. While the molecular chaperone proteins are among the most evolutionarily preserved proteins and have a ubiquitous function in all repair processes, there is a high degree of tissue specificity in chaperone induction [38,39] showing that some cells have developed unique stress responses due to unique micro-environmental pressures. DnaK are abundant heat shock proteins that function as chaperones inside the bacterial cytoplasm [40]. The bacteria that overproduce the DnaK protein at all temperatures undergo a considerably reduced heat-shock response at high temperature. The DnaK protein is identified as an inhibitor of the heat-shock response [41] a very important reaction for the survival of bacteria such as enterococci, and that contributes to the antibiotic resistance. The protein DnaK was detected in spot 3 as linked to the *Enterococcus faecalis* strain (accession number Q835R7), the *Streptococcus agalactiae* serotype III strain (accession number P0A3J2), the *Streptococcus thermophilus* strain (Q5M6D1), the *Streptococcus pyogenes* serotype M12 strain (Q1JKD6) and the *Streptococcus mutans* (O06942).

It is important to underline the presence of both DnaK and DnaJ proteins (spot 14 and spot 16) in *E. faecium* SG 50 strain as related to the *Yersinia pseudotuberculosis* serotype O:1b and to the *Enterococcus faecalis* (*Streptococcus faecalis*), respectively. DnaJ proteins participate actively in the response to hyperosmotic and heat shock by preventing the aggregation of the stress-denatured proteins and by disaggregating proteins, also in an autonomous, DnaK-independent fashion. DnaJ proteins are identified as co-chaperones because they help another family of chaperones (DnaKs) with protein folding. The DnaJ and DnaK proteins must act together to facilitate protein folding [42].

In vitro the GroEL proteins raise the yield of functional protein during refolding by suppressing aggregation as a side-reaction and, probably, by shifting the substrate protein from off-pathway reactions back to the productive folding

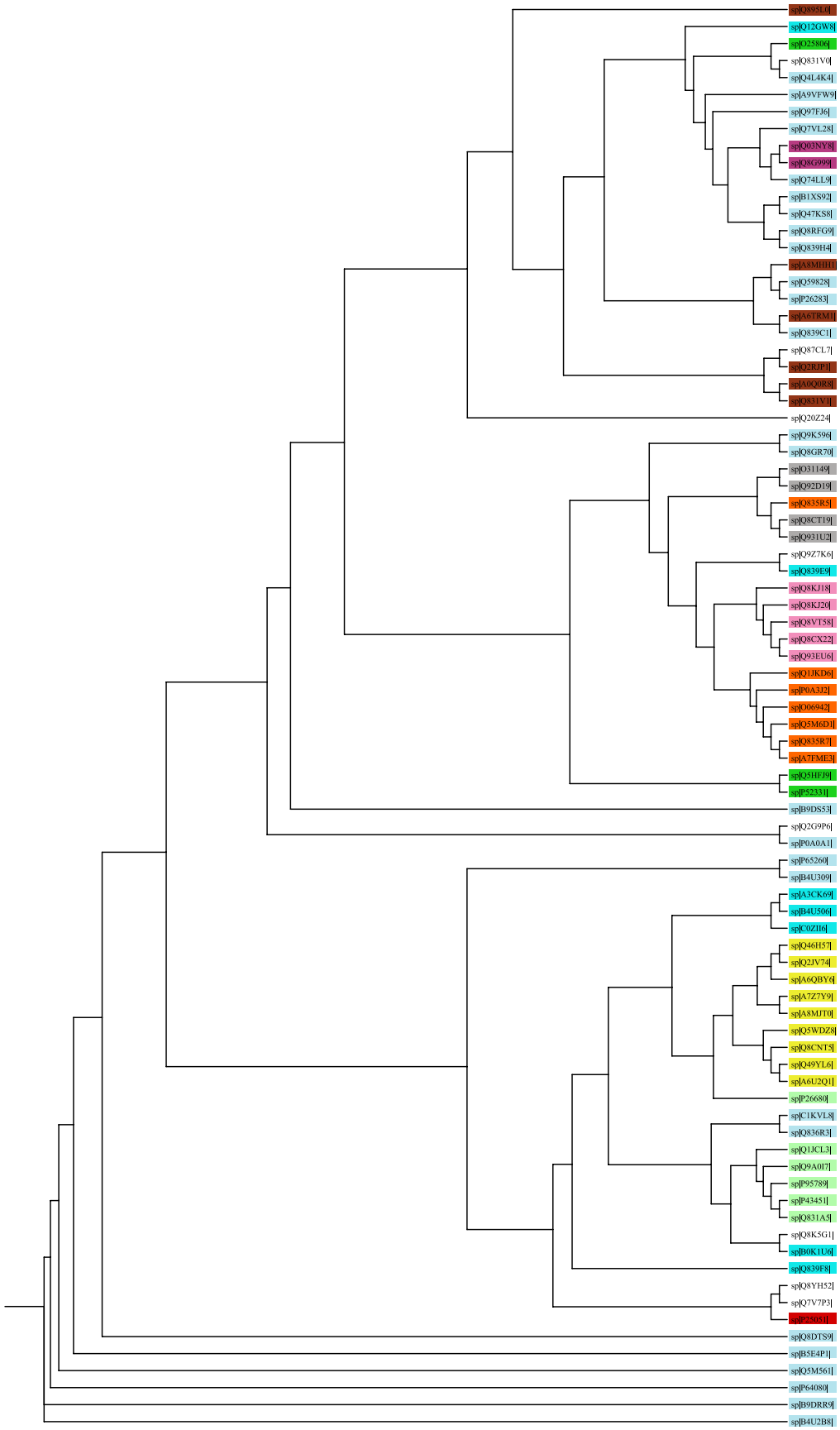

Figure 3. Phylogenetic tree of FASTA protein sequences of all proteins identified. The full alignment of these sequences were done with MUSCLE (v3.7) configured for highest accuracy.

pathway [43]. The GroEL protein is the major heat shock protein of a large number of bacteria and belongs to the chaperonin family. This protein avoids the misfolding of proteins and promotes the refolding and the proper assembly of unfolded polypeptiedes caused under stress condition [42]. In the *van*A *E. faecium* SG 2 isolate, the spot 4 showed the presence of the GroL protein (Q93EU6, Q8KJ20, Q8KJ18, Q8VT58 and Q8CX22) associated to the *Enterococcus faecalis*, the *Streptococcus anginosus*, the *Streptococcus constellatus*, the *Streptococcus gordonii*, the S*treptococcus agalactiae* serotype III, respectively. The dnaK and groL proteins were also detected in the enterococci strains from seagulls [13] and in the *Salmonella* strains from wild boars [44].

In the analysis of the obtained proteomes, the most abundant proteins identified in our *van*A SG 2 and *van*A SG 50 strains were those involved in glycolysis. In our study, the high number of proteins linked to the ATP synthesis, transferase, translation and protein folding is emphasized.

It is highly important to point out that proteins from *van*A enterococcal strains were identified in the obtained proteomes, some of which are involved in the antibiotic resistance. These proteins were controlled by vancomycin which, also, triggered innate signal regulators, adhesion factors, and metabolic gene expression in *E. faecalis*. Therefore, these responses may enable *Enterococcus* spp. to adapt, survive and remain pathogenic even under the pressure of the vancomycin treatment. Our results are in accordance with those observed in another report [45].

5. Concluding remarks

Our report showed that it became possible through a detailed proteomic approach and a 2-DE combined with mass spectrometry (MALDI/TOF-TOF) to obtain important information for further understanding of the antibiotic-resistant mechanism(s), but also for the evaluation of protein profiles in response to various stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance. All of these could represent a valid and integrating approach to the development of new therapeutic strategies [46]. In fact, the elaboration of a 2-D electrophoresis gel of the 2 *van*A enterococcal strains with phenotypic and genotypic profiles, indicating antimicrobial resistance, permitted us to identify and characterize the present proteins.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/86/0

Table 1. Protein spots identification of 2-DE gels and MALDI-TOF sequencing results from *van*A *E. durans* SG 2 isolate .

Table 2. Protein spots identification of 2-DE gels and MALDI-TOF sequencing results from *van*A *E. faecium* SG 50 isolate .

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Competing interests

The authors declare that they have no competing interests.

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