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Proteomic analysis of ampicillin-resistant oral *Fusobacterium nucleatum*

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Introduction: *Fusobacterium nucleatum* represents one of the predominant anaerobic species in the oral microbiota. Penicillin-resistant *F. nucleatum* have been isolated from intra- and extraoral infections. This study aimed to assess ampicillin resistance in *F. nucleatum* by investigating the synthesis of resistance-associated proteins.
Methods: Ampicillin-resistant and ampicillin-susceptible *F. nucleatum* isolates were obtained from 22 dental plaque samples. Two-dimensional gel electrophoresis and mass spectrometry were used to investigate bacterial protein synthesis. Proteins exhibiting statistically significant quantitative changes between sensitive and resistant isolates were identified using peptide mass mapping and matrix-assisted laser desorption/ionization – time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry.
Results: Twenty-three *F. nucleatum* isolates were recovered from plaque samples and their ampicillin minimum inhibitory concentrations ranged between 0.125 μg/ml and 256 μg/ml. Analysis of the bacterial cellular proteins by two-dimensional gel electrophoresis resolved 154–246 distinct protein spots (mean 212, $n = 9$). Between 32% and 83% of the protein spots were common for the *F. nucleatum* isolates. Comparisons of the protein profiles of sensitive and resistant isolates revealed the presence of a 29 kDa protein and significant increases in the synthesis of two proteins at 37 and 46 kDa in the ampicillin-resistant *F. nucleatum* isolates. These proteins were identified as a class D β-lactamase, ATP-binding cassette (ABC) transporter ATP-binding protein and enolase, respectively.
Conclusion: Synthesis of a class D β-lactamase by ampicillin-resistant *F. nucleatum* isolates could complicate antimicrobial treatment because these enzymes might confer resistance to many classes of β-lactam antibiotics. The differences observed in protein synthesis between ampicillin-resistant and ampicillin-susceptible *F. nucleatum* may contribute to the antibiotic resistance and virulence of these bacteria.

Key words: ampicillin; *Fusobacterium*; proteomics; resistance; Yemen

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Fusobacterium nucleatum is an anaerobic, non-sporing, non-motile, gram-negative rod bacterium with fused ends (2). It can survive aerobically for several hours and is therefore designated aerotolerant (6). The oral cavity is the main habitat of *F. nucleatum* and the bacterium represents one of the predominant anaerobic species in the oral microbiota. *F. nucleatum* is referred to as a putative periodontal pathogen belonging to the so-called orange complex of periodontal pathogens described by Socransky et al. (27). It acts as a bridge between early and late colonizers in dental plaque and co-aggregates with many species found in the oral cavity including the periodontal pathogens (13, 25, 28). The actual role of *F. nucleatum* in periodontal disease pathogenesis is probably masked by the bacterium being a common isolate in both healthy and diseased individuals (35). *F. nucleatum* has broader oral and medical involvements than just being a putative periodontal pathogen; it has been isolated from orofacial and skin infections, tonsillar abscesses, septic arthritis and bacterial endocarditis (2).

The genome of *F. nucleatum* subsp. *nucleatum* type strain American Type Culture Collection (ATCC) 25586 has been published (11). It contains 2.17 Mb

pairs encoding 2067 open reading frames. Shortly after publication of the genome sequence of *F. nucleatum* subsp. *nucleatum* type strain ATCC 25586, a draft of the *F. nucleatum* subsp. *vincentii* type strain ATCC 49256 genome became available (12). The heterogeneity of *F. nucleatum* is well known and five subspecies of *F. nucleatum* have been described (10). Recently, a genomic comparison conducted by Kapatral et al. showed that 441 open reading frames present in the *F. nucleatum* subsp. *vincentii* genome have no orthologs in the genome of *F. nucleatum* subsp. *nucleatum* (12).

Penicillin-resistant *F. nucleatum* have been isolated from both intraoral and extraoral infections, and can be encountered in their niches in the oral cavity of infants as young as 2 months; their proportions seem to correlate with previous antibiotic exposure and increase of the infants' age (15, 19). In a recent study, we found that 19% of *F. nucleatum* strains isolated from persons visiting dental offices in Yemen were resistant to 2 μg/ml ampicillin (1). Such a high prevalence of resistance could compromise the use of β-lactam-penicillins as the first-line option in treating infections caused by these anaerobes. β-lactamase enzymes were mostly blamed for *F. nucleatum* resistance to β-lactam antibiotics. Our data mining of *F. nucleatum* subsp. *nucleatum* ATCC 25586 annotated genes revealed the presence of a β-lactamase gene (FN1584) in addition to other β-lactamase-like proteins (FN0512, FN1848 and FN1162) that contain class A, B and C β-lactamase domains. However, the genetic basis of β-lactam resistance among clinical isolates of *F. nucleatum* is poorly characterized. In one study, using a panel of *bla* gene primers to characterize the genetic basis of β-lactamase production in one β-lactamase-positive clinical strain of *F. nucleatum*, there was no positive detection of any of the *bla* genes screened, suggesting that there might be uncharacterized genes behind β-lactamase production (8).

Resistance to ampicillin and other β-lactam antibiotics can be mediated by β-lactamase and/or other resistance mechanisms. However, the levels of β-lactamase production and resistance, expressed as the minimum inhibitory concentration (MIC), are not always in a linear relationship and other resistance determinants could exist simultaneously (18, 24). In general, proteins that are responsible for bacterial resistance to ampicillin are either β-lactamase enzymes, mainly in gram-negative bacteria, or altered penicillin-binding proteins (PBP), primarily in gram-positive bacteria, or mutant porins.

Some studies showed a relationship between the presence of resistance determinants and an altered expression of virulence factors or other proteins involved in different biological processes (3, 9, 17, 31, 36). In addition, the characterization of the proteome of an *Escherichia coli* strain, resistant to ampicillin, demonstrated the altered expression of eight outer membrane proteins of which TolC, OmpC and YhiU were previously known to be ampicillin-resistance determinants (36). The contribution of the other five ampicillin-resistance-related proteins to ampicillin resistance was not proven; should further investigations reveal their role in ampicillin resistance, then this would demonstrate the strength of 'proteomics' in identifying previously undocumented protein activities.

The aims of the current study were; first to assess the mechanism of ampicillin resistance in *F. nucleatum*, second to characterize the ampicillin-resistance determinants of highly resistant isolates, and finally to investigate the synthesis of resistance-associated proteins using high-resolution two-dimensional gel electrophoresis and mass spectrometry.

Material and methods

Isolation and identification of ampicillin-resistant *F. nucleatum*

The *F. nucleatum* isolates were obtained from 22 dental plaque samples. The selection criteria for the plaque donors, sampling procedure and transportation conditions are described elsewhere (1). In brief, three randomly selected posterior teeth in each patient were assigned for isolation and subgingival sampling. The subgingival samples were collected using sterile paper points size 50 (Dentsply, York, PA) that were inserted as far as possible subgingivally and removed after 20 s. The three samples thus obtained from each subject were pooled by placing them in a sterile vial containing 1.5 ml Viability Medium Gothenburg Anaerobe III transport medium.

The isolation of ampicillin-resistant *F. nucleatum* was performed by cultivating 10 μl of each plaque sample suspension on crystal violet erythromycin (CVE) plates supplemented with or without 2 μg/ml ampicillin (33). Subsequent identification of *F. nucleatum* was based on stereomicroscopic colony morphology, Gram-staining, anaerobiosis and a biochemical profile of 29 miniaturized enzymatic tests using a Rapid ID 32 A system (Biomerieux® Sa, Marcy l'Etoile, France).

MIC determination and assessment of β-lactamase production

MIC determinations of isolated strains were performed using the E-test (AB Biodisk, Solna, Sweden) on brucella agar plates (Sigma, St Louis, MO), 5% sheep blood and supplemented with 1 μg/ml vitamin K and 5 μg/ml haemin (4). The brucella plates were stored at 4°C and used within 1 week of preparation. Suspensions of the test strains were prepared with a turbidity equivalent to No.1 McFarland standard. A cotton swab was used to inoculate each strain on to the brucella agar plates in three different directions, and the plates were then allowed to dry for 10 min before the E-test strips were placed in the middle of each plate. All inoculated plates were incubated anaerobically (5% CO_2, 10% H_2 and 85% N_2) using the Anoxomat System™ (MART Microbiology BV, Lichtenvoorde, the Netherlands) for 48 h and then the MICs were read according to the manufacturer's instructions. MIC tests were performed as two independent duplicates and MICs were reported as a range if the readings differed. *Eggerthella lenta* ATCC 43055 and *Bacteroides fragilis* ATCC 25285 were used as quality control strains.

Ampicillin-resistant *F. nucleatum* isolates, defined as isolates with ampicillin MIC ≥2 μg/ml, were assessed for β-lactamase production using the chromogenic cephalosporin disk (Fluka, Steinheim, Germany) test according to the manufacturer's instructions. The chromogenic test results were read and interpreted within 5 min. *E. coli* strain ATCC 25922 was used as a negative control in this test and a β-lactamase-positive clinical strain of *F. nucleatum* was used as the positive control (19).

Two-dimensional gel electrophoresis and protein identification by peptide mass mapping and matrix-assisted laser desorption/ionization–time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry

Five highly ampicillin-resistant (MIC >8 μg/ml) and four susceptible *F. nucleatum* clinical isolates from the dental plaque samples were used for proteomic analysis. For soluble protein extraction, isolates were grown on fastidious anaerobic blood agar plates (Lab M, Bury, UK), which were incubated anaerobically (5% CO_2, 10% H_2

and 85% N_2) using the Anoxomat System™ for 48 h at 37°C. The colonies were removed from the agar surface using a disposable sterile plastic loop and suspended in phosphate-buffered saline-A (PBS) buffer for washing (three times for 5 min at 11,000 ***g***). Washed bacteria were suspended in a buffer composed of 7 M deionized urea, 2 M thiourea, 4% (weight/volume; W/V) 3-[3-(cholamidopropyl)-dimethylammonio]-1-propane sulphonate (CHAPS), 0.3% (W/V) dithiothreitol (DTT) and 0.2% carrier ampholyte (Bio-Lyte® 3/10 and/or 5/8 Ampholytes; all from Bio-Rad, Hercules, CA). Bacteria were disrupted using a hand-held homogenizer for 1 min and soluble proteins were clarified by centrifugation at 16,100 ***g*** for 15 min. The supernatants were collected and stored at –80°C for future analysis. Determination of the protein concentration in the supernatant was based on the Lowry methods using a Bio-Rad RC-DC™ protein assay. Immobilized pH gradient (IPG) gel strips (7 cm in length and pH ranges 3–10 and 5–8 linear gradient; Bio-Rad) were rehydrated in 125 µl 7 M deionized urea, 2 M thiourea, 4% (W/V) CHAPS, 0.3% (W/V) DTT, 2% carrier ampholyte (Bio-Lyte® 3/10 or 5/8 Ampholyte) and traces of bromophenol blue containing approximately 120 µg soluble bacterial protein. The IPG gel strips were overlaid with mineral oil and left for 15 h at room temperature. The proteins were then electrically focused using a PROTEAN IEF Cell (Bio-Rad) as follows: 200 V for 1 min, 3500 V for 1.5 h and a final focusing step of 3500 V for 3 h. The focusing mode was set in the rapid ramping mode. The IPG gel strips were then equilibrated in equilibration buffer [6 M urea, 2% sodium dodecyl sulphate (SDS), 0.05 M Tris–HCl pH 8.8 and 20% glycerol) containing 1% (W/V) DTT for 20 min with gentle agitation followed by a further 20-min equilibration in the same buffer but containing 2.5% (W/V) iodoacetamide. The equilibrated IPG gel strips were placed on the upper surface of the second dimension SDS–polyacrylamide slab gel (12% precast SDS–polyacrylamide gel electrophoresis gels; Bio-Rad). The second-dimension electrophoresis was carried out using the Mini Protean II gel system (Bio-Rad) as follows: 75 V for 1 h then 150 V for 2.5 h. A molecular weight marker (Precision Plus; Bio-Rad) was also run on each gel. Protein localization used the Bio-safe Coomassie blue stain (Bio-Rad) according to the manufacturer's instructions.

LabScan (GE Healthcare, Uppsala, Sweden) and IMAGEMASTER 2D PLATINUM software version 5 (GE Healthcare) were used for image acquisition and subsequent analysis of digitized images, respectively. For each *F. nucleatum* isolate that was analysed by two-dimensional gel electrophoresis, three biological replicate samples were prepared and run simultaneously. Differences in quantitative protein expression were determined by measuring the relative spot volume (% vol.) of normalized two-dimensional gel images. Statistical analysis of quantitative protein spot differences was conducted using Student's *t*-test; differences with $P < 0.05$ were considered significant.

Proteins exhibiting statistically significant changes in abundance between sensitive and resistant bacterial isolates were excised from gels and identified using peptide mass mapping and MALDI-TOF/TOF mass spectrometry. In brief, protein spots were excised from the gels using a sterile scalpel and the gel plugs were washed with 50% (V/V) acetonitrile and 25 mM ammonium bicarbonate. In-gel cysteine reduction and alkylation with 10 mM DTT and 55 mM iodoacetamide, respectively, were carried out before overnight trypsin digestion at 37°C (porcine trypsin; Promega, Madison, WI). Peptide extraction was initially performed with 1% trifluoroacetic acid (TFA) followed by 60% acetonitrile and 0.1% TFA. The recovered peptide extracts were then passed through a custom-made microcolumn of Poros 20 R2 (Applied Biosystems, Foster City, CA) in gel-loader tips. The adsorbed peptides were washed three to five times with 0.1% TFA and then eluted with 1 µl CHCA matrix (5–10 mg/ml α-cyano-4-hydroxycinnamic acid in 60% acetonitrile/15% methanol/0.1% TFA) onto the MALDI-TOF target. The mass spectra of the peptides were acquired on an Ultraflex MALDI-ToF/ToF mass spectrometer (BRUKER Daltonic GmbH, Leipzig, Germany). Peptide calibration standard (BRUKER Daltonic GmbH) dissolved in CHCA matrix was applied to the target near each sample and used for external calibration of the peptide spectra. The internal calibration of the spectra relied upon the trypsin auto-digestion products.

The monoisotopic mass spectra of the tryptic peptides were used to search in the NCBInr, MSDB and Swissprot protein databases using the MASCOT search engine (http://www.matrixscience.com/). The search parameters used were as follows: Taxonomy, other bacteria; Enzyme, trypsin; Fixed modifications, carbamidomethyl (C); Variable modifications, oxidation (M); Peptide tolerance, 50–150 p.p.m. No search restriction was applied to the isoelectric point parameter and a protein mass of up to 150 kDa was allowed.

Table 1. Ampicillin minimum inhibitory concentrations (MICs) of *Fusobacterium nucleatum* isolates determined by the E-test

Bacterial isolate number	MIC (µg/ml)	Bacterial isolate number	MIC (µg/ml)
2YM	0.125	35YM	8
5YM	0.25	36YM	32
13YM	1	37YM	4
14YM	2	41YM	4
17YM	0.5	42YM	1
22YM	2	43YM	1
28YM	>256	44YM	4
29YM	>256	47YM	0.25
30YM	4	57YM	1
32YM	4	70YM	2
33YM	4	77YM	0.25
34YM	8		

Results

Twenty-three *F. nucleatum* isolates were recovered from 11 of the 22 dental plaque samples processed as part of the current study. *F. nucleatum* isolates were able to grow on CVE plates, had characteristic stereomicroscopic colony appearance, were gram-negative bacilli with tapered ends, were growing anaerobically but not aerobically, and were indole-positive and lipase-negative. MIC values of the 23 *F. nucleatum* isolates are shown in Table 1. Resistant isolates turned the nitrocefin-impregnated discs red, indicating hydrolysis as the result of β-lactamase production. The five ampicillin-resistant *F. nucleatum* isolates used in the proteomic analysis were isolate numbers 28YM, 29YM, 34YM, 35YM and 36YM; isolates 35YM and 36YM were from one patient. The four susceptible isolates were numbered 2YM, 5YM, 47YM and 77YM.

Gel analysis showed 154–246 (mean 212, $n = 9$) distinct spots. Pairwise gel comparisons between different isolates showed between 32 and 83% matches, based on electrophoretic mobility. On the other hand, gel comparisons between two-dimensional images of resistant and susceptible isolates showed 32.3% matches between the two groups. Analysis of proteomic maps of resistant and susceptible *F. nucleatum* isolates revealed the presence of 29 kDa protein in ampicillin-resistant *F. nucleatum* isolates (Fig. 1; spot 8, and Fig. 2A). This protein was identified using MASCOT search of the obtained peptide mass fingerprint spectra as class D β-lactamase (Fig. 3). There were significant increases in the synthesis of two proteins that were 37 and 46 kDa (Fig. 1;

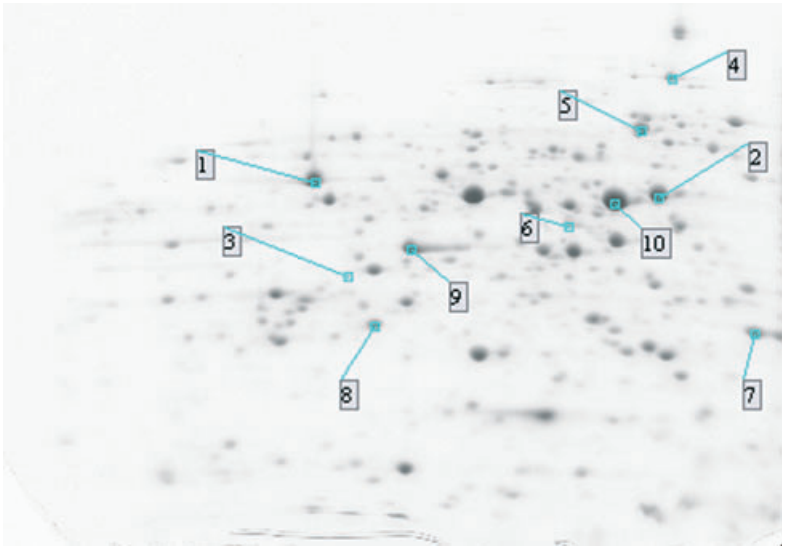

Fig. 1. Two dimensional gel electrophoresis map of ampicillin-resistant *Fusobacterium nucleatum* in the pH range 5–8. The proteins identified in the current study (Table 2) are indicated.

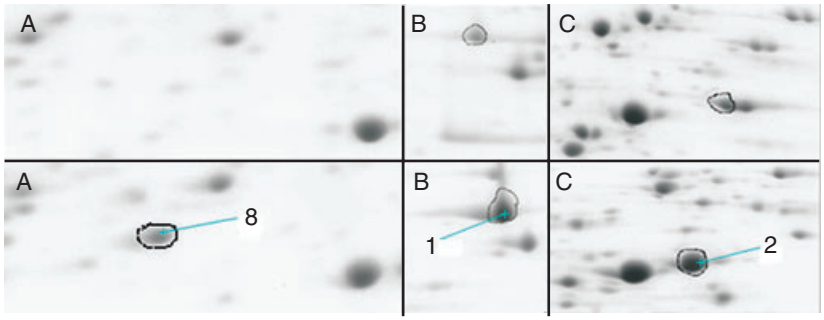

Fig. 2. Enlarged partial two-dimensional maps in the pH range 5–8 from susceptible, upper row, and resistant, lower row, *Fusobacterium nucleatum* showing the expression of class D β-lactamase (A), altered expressions of ABC transporter ATP-binding protein (B) and enolase (C).

spots 1 and 2, and Fig. 2B,C); these proteins were associated with resistant isolates of *F. nucleatum* ($P < 0.018$ and $P < 0.001$, respectively). The 37 and 46 kDa proteins were identified as ATP-binding cassette (ABC) transporter ATP-binding protein and enolase, respectively (Table 2). The mean normalized relative spot volume for enolase across the resistant isolates was 3.5 (range 2.51–4.53, $n = 5$) compared to 1.8 (range 1.24–3.36, $n = 4$) for the susceptible isolates. The corresponding values for the ABC transporter ATP-binding protein values were 1.9 (range 0.28–3.06, $n = 5$) and 0.6 (range 0.4–0.81, $n = 4$) for the resistant and sensitive isolates, respectively. Twenty-four spots were processed for peptide mass mapping and MALDI-TOF/TOF mass spectrometry. These spots were selected from both susceptible and resistant *F. nucleatum* isolates and represented landmarks, quantitative and qualitative differences as well as additional spots consistently expressed by *F. nucleatum*. Positive identifications were achieved for the 10 protein spots highlighted in Fig. 1 for an ampicillin-resistant *F. nucleatum* isolate (Table 2). Fig. 4 shows the NCBI amino acids sequence and MALDI-TOF/TOF peptide spectra of enolase.

Discussion

To the best of our knowledge, this study is the first to look at ampicillin-resistant *F. nucleatum* global gene expression at the level of the proteome, and to identify ampicillin-resistance-related proteins in *F. nucleatum*. Isolates of *F. nucleatum* have previously been shown to produce significant levels of β-lactamase enzymes (20). The molecular approaches to detect and characterize the genetic basis underlining β-lactamase activity are generally based on nucleic acid hybridization and amplification techniques. However, these methods depend on available gene sequence data to design labelled nucleic acid probes and primers for the targets of interest. Inspection of the complete genome sequence for the *F. nucleatum* subsp. *nucleatum* ATCC 25586 type strain demonstrated the presence of class A β-lactamase. However, it was not possible to detect the bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{AmpC} and bla_{CfxA} genes in a β-lactamase-positive strain of *F. nucleatum* (8).

The analysis of gene expression at the level of the proteome is as technically straightforward as using nucleic acid amplification technologies. However, correlating protein spot differences in the two-dimensional protein profiles to the ampicillin-resistant phenotypes in our analysis was difficult mainly because of the heterogeneity that existed in the soluble protein extracts prepared from the ampicillin-resistant and ampicillin-susceptible *F. nucleatum* isolates. It was concluded that a significant proportion of this variability was unrelated to differences in ampicillin and was the result of background strain variation. Nevertheless, we identified a 29 kDa protein expressed in the ampicillin-resistant *F. nucleatum* isolates. This protein was identified by peptide mass mapping and MALDI-TOF/TOF mass spectormetry as a class D β-lactamase. This protein was recorded in the GenBank database in 2003. Although the genomes of the *F. nucleatum* subsp. *nucleatum* type strain ATCC 25586 and the *F. nucleatum* subsp. *vincentii* type strain ATCC 49256 are available, the β-lactamase protein identified in our study did not match any of the open reading frames available from the two genomes. This may be regarded as an additional sign of the heterogeneity among isolates of *F. nucleatum*. During the preparation of this manuscript, Voha et al. reported the genetic and biochemical properties of the same class D β-lactamase as found in our study, named FUS-1, that was purified from a clinical isolate of *F. nucleatum* from France (32). This enzyme showed narrow-spectrum hydrolysing activity, which is not consistent with the usual broad substrate specificity of many class D β-lactamases that, in some cases, can cover carbapenem antibiotics (34). In fact, FUS-1 is not the only enzyme found in *F. nucleatum*. Tunér et al. isolated a 26 kDa β-lactamase enzyme from a clinical isolate of *F. nucleatum* that has a strong hydrolysing activity on benzylpenicillin (30).

No additional qualitatively resistant proteins were identified in the current study other than the class D β-lactamase.

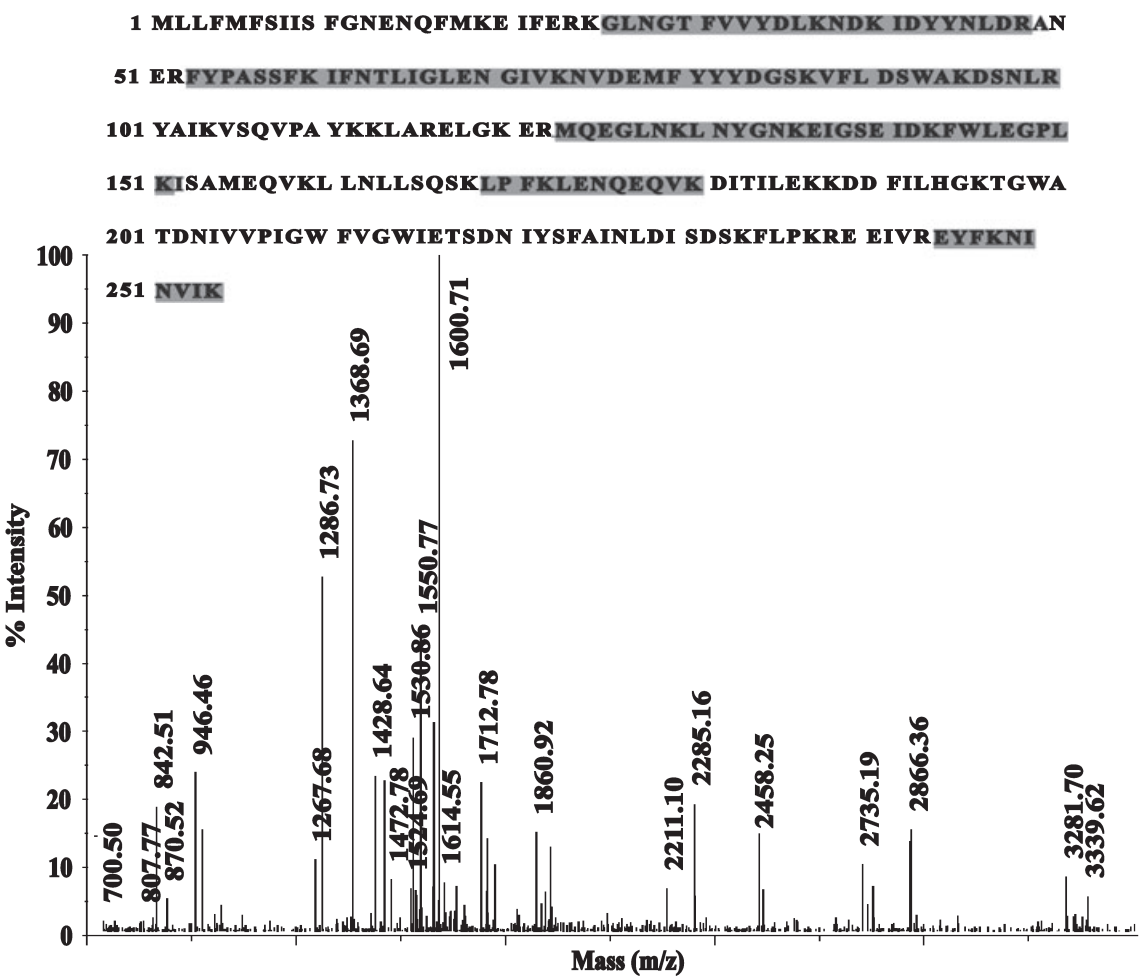

Fig. 3. NCBI amino acids sequence and MALDI-TOF/TOF peptide spectra of class D β-lactamase. Highlighted areas are the matching peptides predicted by MALDI-TOF/TOF peptide spectra.

Table 2. Proteins identified in the current study by peptide mass mapping and MALDI-TOF/TOF mass spectrometry

Spot numbers[1]	Protein name	Mascot score	Sequence coverage (%)
1	ABC transporter ATP-binding protein	68	38
2	Enolase	171	48
3	Pyridoxal biosynthesis lyase pdxS	142	60
4	Protein translation elongation factor G (EF-G)	112	27
5	Glutaconyl-coenzyme A decarboxylase A subunit	87	24
6	Zinc-dependent alcohol dehydrogenases and related dehydrogenases	66	33
7	Electron transfer flavoprotein β-subunit	143	67
8	Class D β-lactamase	114	48
9	Fructose-bisphosphate aldolase	148	52
10	Acyl-coenzyme A dehydrogenase, short-chain specific	270	70

[1]See Fig. 1.
MALDI TOF/TOF, matrix-assisted laser desorption/ionization–time of flight/time of flight.

However, significantly increased synthesis of other proteins was demonstrated in ampicillin-resistant *F. nucleatum*. One of these proteins was identified as enolase, which is an essential glycolytic enzyme that catalyses the interconversion of 2-phosphoglycerate and phosphoenolpyruvate. Enolase has also been reported to be present at the cell surfaces of bacteria, particularly on gram-positive mucosal pathogen group A streptococci and pneumococci (21). The surface-expressed enolase is believed to play a role in bacteria–host tissue interactions, in terms of invasion and generation of the inflammatory response. The plasminogen/plasmin system *in vivo* is involved in this interaction. This presumably aids penetration and dissemination of these pathogens into host tissues during infection (21, 22). Interestingly, the possession of this bacterial-plasmin activity was also reported for *F. nucleatum* and lysine residue protein was speculated to be the protein involved in this interaction (5). It is, however, worth noting that the amino acid sequence of the enolase identified from our data has a double lysine residue at its C-terminal (Fig. 4). Thus, it is probable that the enolase identified in our study is the same protein proposed to be involved in the interaction between the cell surface of *F. nucleatum* and the plasminogen/plasmin system *in vivo*. The increased expression of enolase in ampicillin-resistant *F. nucleatum* isolates, either for energy or other unknown function, might increase the ampicillin-resistant *F. nucleatum* dissemination in host tissues during infection.

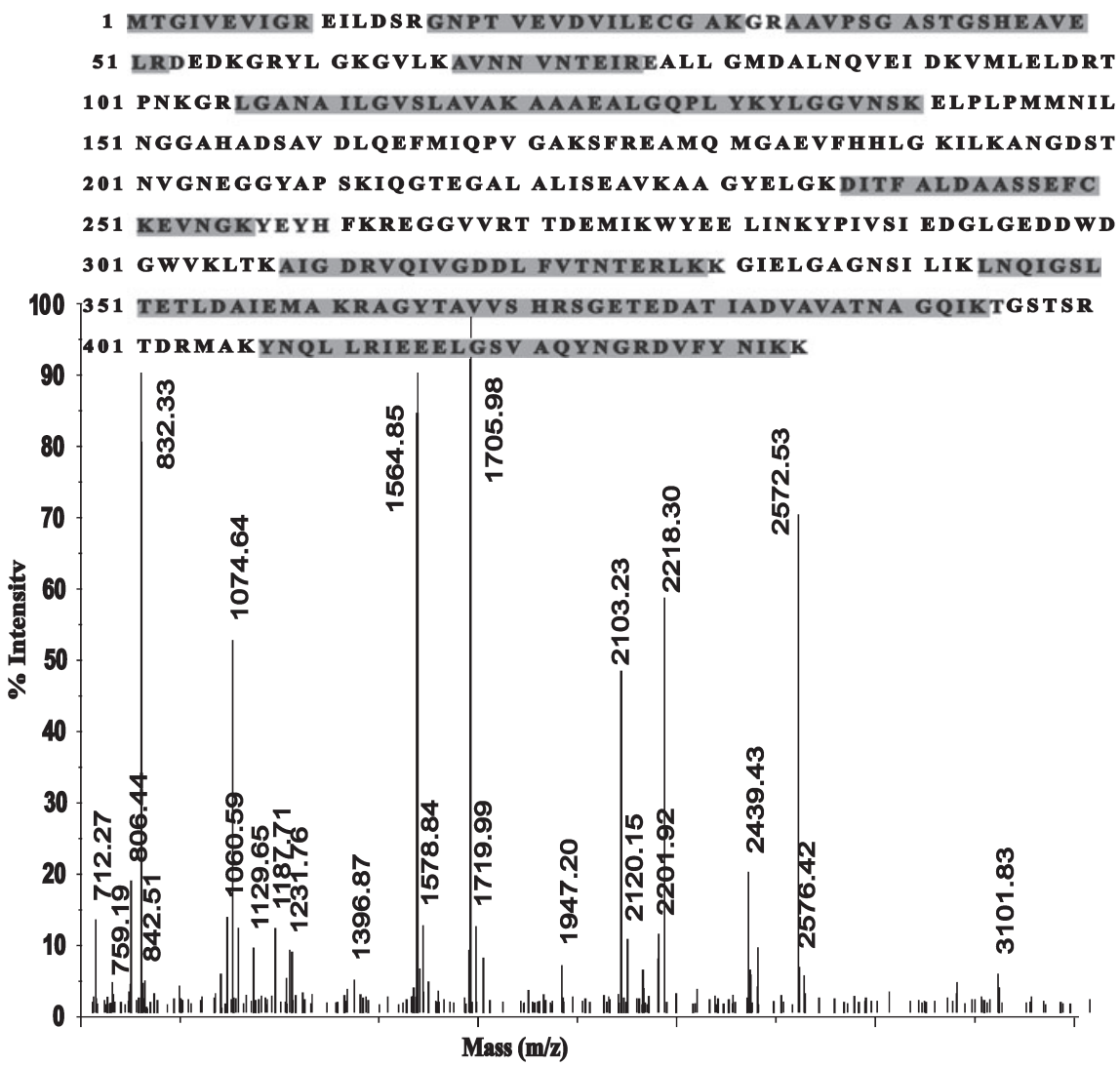

Fig. 4. NCBI amino acids sequence and MALDI-TOF/TOF peptide spectra of the enolase reported in the current study. Highlighted areas are the matching peptides predicted by MALDI-TOF/TOF peptide spectra.

The ABC transporter ATP-binding protein was found to be quantitatively increased in ampicillin-resistant *F. nucleatum*. This protein is a membrane protein that belongs to the ABC transporter protein family. ABC transporters are multidomain membrane proteins, responsible for many biological processes as diverse as cell division, control of cell volume and the controlled efflux and influx of substances across cellular membranes (37). These proteins have been implicated as playing a role in antimicrobial resistance in bacteria, fungi and parasites (26). The increased expression of efflux pump proteins is usually constitutive in otherwise susceptible isolates (23). It is important to note that the role of an ABC transporter efflux pump in β-lactam antibiotic resistance is not as well documented as its contribution to resistance in other classes of antibiotics (26). However, analysis of the outer membrane proteome of ampicillin-resistant *E. coli* showed an increased expression of the TolC protein, which is considered part of the ABC superfamily efflux pump in gram-negative bacteria (23, 36). The antibiotic substrate profile of ABC transporters differs in gram-positive bacteria compared to gram-negative bacteria. In fact, only macrolides have been reported as being affected by ABC transporter efflux pumps in gram-negative bacteria, whereas numerous classes of antibiotics, including β-lactams, are effluxed by the ABC transporter pumps in gram-positive microbes (14, 23). Although *F. nucleatum* is a gram-negative bacterium, interesting phylogenetic inferences, based on conserved indels, place *Fusobacteria* at an intermediate position between gram-positive and gram-negative taxa (7). In addition, based on 16S ribosomal RNA sequence analysis, *Fusobacterium* appears as a separate cluster only distantly associated to the low G + C Gram-positive bacteria (10, 16, 29). Thus, if further studies support the finding that the increased expression of the ABC transporter demonstrated from our work contributes to ampicillin resistance in *F. nucleatum*, an additional unusual feature of this gram-negative bacterium will be highlighted.

The association between enolase and ABC transporter ATP-binding protein and ampicillin-resistant *F. nucleatum* in our study might or might not *per se* contribute to ampicillin resistance in *F. nucleatum*

and our findings deserve further investigation to disclose the extent of such an association. On the other hand, our study underscores the presence of a newly reported class D β-lactamase in a number of ampicillin-resistant *F. nucleatum* isolates from Yemen. In conclusion, differences in protein synthesis were found between ampicillin-resistant and ampicillin-susceptible *F. nucleatum* clinical isolates. The synthesis of a class D β-lactamase by ampicillin-resistant *F. nucleatum* isolates could complicate antimicrobial treatment because these enzymes might confer resistance to many classes of β-lactam antibiotics.

Acknowledgment

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