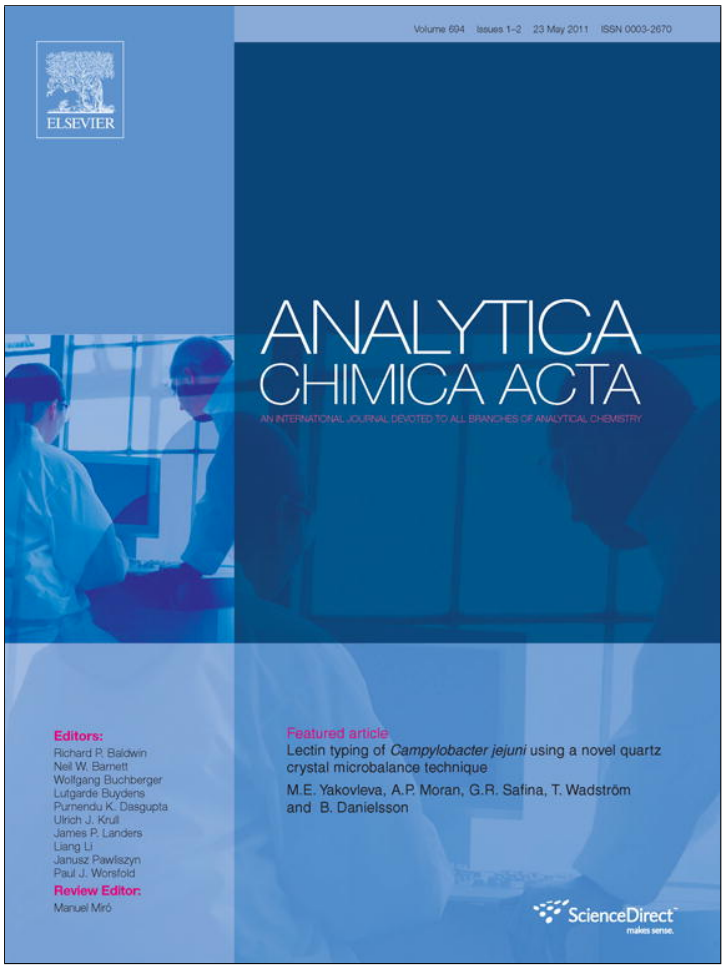

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Lectin typing of *Campylobacter jejuni* using a novel quartz crystal microbalance technique

Maria E. Yakovleva[a,*], Anthony P. Moran[b,1], Gulnara R. Safina[c], Torkel Wadström[a], Bengt Danielsson[d]

[a] Department of Infectious Diseases and Medical Microbiology, Lund University, 223 62 Lund, Sweden
[b] Department of Microbiology, School of Natural Sciences, National University of Ireland, Galway, Ireland
[c] Department of Analytical and Marine Chemistry, University of Gothenburg, 412 96 Gothenburg, Sweden
[d] Acromed Invest AB, Magistratsvägen 10, 226 43 Lund, Sweden

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ABSTRACT

Seven *Campylobacter jejuni* strains were characterised by a lectin typing assay. The typing system was based on a quartz crystal microbalance technique (QCM) with four commercially available lectins (wheat germ agglutinin, *Maackia amurensis* lectin, *Lens culinaris* agglutinin, and Concanavalin A), which were chosen for their differing carbohydrate specificities. Initially, the gold surfaces of the quartz crystals were modified with 11-mercaptoundecanoic acid followed by lectin immobilisation using a conventional amine-coupling technique. Bacterial cells were applied for lectin typing without preliminary treatment, and resonant frequency and dissipation responses were recorded. The adhesion of microorganisms on lectin surfaces was confirmed by atomic force microscopy. Scanning was performed in the tapping mode and the presence of bacteria on lectin-coated surfaces was successfully demonstrated. A significant difference in the dissipation response was observed for different *C. jejuni* strains which made it possible to use this parameter for discriminating between bacterial strains. In summary, the QCM technique proved a powerful tool for the recognition and discrimination of *C. jejuni* strains. The approach may also prove applicable to strain discrimination of other bacterial species, particularly pathogens.

1. Introduction

The Gram-negative enteric pathogen, *Campylobacter jejuni*, is recognised as the most common cause of bacterial gastroenteritis in humans in developed and developing countries [1,2]. Infection with *C. jejuni* strains is characterised by profuse watery diarrhea accompanied by fever, severe abdominal pain and bloody stools [2]. Besides gastroenteritis, post-infectious sequelae of *C. jejuni* infection have been reported. These include reactive arthritis and Guillain–Barré syndrome that is defined clinically as a symmetrically progressive neuropathy of the peripheral nervous system and a related disorder consisting of ataxia, ophthalmoplegia, and areflexia called Miller Fisher syndrome [3,4]. Considering the worldwide occurrence and public health importance of *Campylobacter* infections a simple method for the discrimination of *C. jejuni* strains is required.

It has been shown previously that lectins can be a useful tool for the discrimination of strains of various bacterial species [5,6]. Lectins are proteins or glycoproteins of non-immune plant or animal origin which possess binding specificities for various carbohydrates and have more stable binding properties than antibodies [7]. Several publications have reported discrimination of bacterial strains, including *C. jejuni* isolates, by agglutination reactions with a panel of 6–12 lectins [8–10]. However, the required sample preparation involved in these techniques is a disadvantage for their widespread use for bacterial strain discrimination.

The present investigation was undertaken to develop a simple and robust method based on lectin typing for differentiation of untreated *C. jejuni* strains. In particular, the dissipation changes due to interactions between bacterial cells and different lectins were measured using a quartz crystal microbalance (QCM) technique. The QCM device is a mass sensor which is ideal for detecting high-molecular-mass analytes [11]. The main advantages of the application of QCM in biological measurements are its ability to measure changes of very small masses and to monitor mass deposition in real-time. Moreover, such measurements can be performed using native molecules without the requirement for labeling [12]. In addition to the QCM-based measurement, the presence of bacterial cells on the lectin-coated surfaces was assessed in atomic force microscopy (AFM) by scanning in the tapping mode.

* Corresponding author. Present address: Department of Biochemistry and Structural Biology, Lund University, 222 41 Lund, Sweden. Tel.: +46 76 105 4413; fax: +46 46 222 4116.
E-mail address: maria.yakovleva@gmail.com (M.E. Yakovleva).
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2. Materials and methods

2.1. Reagents

N-hydroxysuccinimide (NHS), 11-mercaptoundecanoic acid, ethanolamine hydrochloride, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), analytical grade, were purchased from Sigma (Stockholm, Sweden). Glycine, 25% ammonia, 30% hydrogen peroxide were supplied by Merck (Darmstadt, Germany). Ten millimolar phosphate-buffered saline, pH 7.4 (PBS) was prepared from analytical grade chemicals (Merck, Darmstadt, Germany) and contained 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.135 mM NaCl, 2.7 mM KCl, 1 mM Ca^{2+} and 1 mM Mn^{2+}. The pH values of 1 M ethanolamine hydrochloride (pH 8.5) and 10 mM glycine–HCl solution (pH 2.5) were adjusted with 0.1 M hydrochloric acid

Freeze-dried, native lectins (Table 1) from *Triticum vulgaris* (wheat germ agglutinin, WGA), *Maackia amurensis* (MAL) and lentils (*Lens culinaris* agglutinin, LCA) were purchased from Vector Laboratories (Burlingame, CA); and Concanavalin A (ConA) from Jack bean (*Canavalia ensiformis*) was obtained from Sigma. Lectins were dissolved in 10 mM PBS at concentrations 0.1 mg mL^{-1}.

X-Array gold-coated slides were obtained from Biomedical Technology AB (Lund, Sweden).

2.2. Preparation of bacterial strains

C. jejuni serostrains HS:2, HS:4, HS:6, HS:10 and HS:19 were obtained from the Culture Collection of the University of Gothenburg, Sweden (CCUG), isolate 81116 (NCTC 11828) from the National Culture Type Collection, UK and ATCC 43446 from the American Type Culture Collection. Strains from a frozen stock were cultured on blood agar (LAB M Limited, Lancashire, UK), containing 4% horse blood, for 14 h at 37 °C in a microaerobic atmosphere generated by a gas mixing system (Anoxomat®, MART BV, Lichtenvoorde, Holland). Cells were harvested in 10 mL of PBS and washed once in this solution. The biomass was stored at 4 °C prior to use, up to a maximum of 3 weeks.

2.3. Quartz crystal preparation

Polished gold crystals of 5 MHz and 14 mm diameter were obtained (Q-Sense AB, Göteborg, Sweden). Prior to use, the surface of the crystals was cleaned for 10 min in a freshly prepared boiling mixture of 33% H_2O_2, 25% NH_3 and H_2O (in a ratio of 1:1:5) followed by washing with water and 99.5% ethanol. Subsequently, a thiolisation step was carried out, whereby chips were incubated with 1 mM 11-mercaptoundecanoic acid (prepared in ethanol) in the dark at room temperature overnight, to obtain self-assembled monolayers. Immediately prior to use, crystals were rinsed with ethanol and de-ionised water (Elga Maxima, 18 MΩ, *Elga* Processed Water *Ltd*., Marlow, UK) and finally dried in a gaseous stream of nitrogen. Crystals were then mounted into the QCM system and equilibrated with PBS.

Immobilisation was performed in the on-line mode at a flow speed equal to 50 μL min^{-1}. The surface was initially activated with 0.1 M NHS and 0.4 M EDC (in a ratio of 1:1) for 25 min. Then four lectins (WGA, Con A, MAL, LCA; all at 0.1 mg mL^{-1}) were passed over the chip surface for 1 h until no further frequency change was observed. Following complete blocking with 1 M ethanolamine hydrochloride (pH 8.5), the system was allowed to equilibrate with PBS until a stable baseline was observed. Bacterial cells were diluted (1:100) and passed through the QCM-D instrument at a flow rate of 50 μL min^{-1}. After 40 min, the surface of the quartz crystal was regenerated with 10 mM glycine–HCl solution (pH 2.5) for 10 min.

2.4. QCM measurement conditions

All QCM experiments were performed at 25 °C and at a flow speed of 50 μL min^{-1} using the Q-Sense E4 equipment (Q-Sense AB, Göteborg, Sweden). Four sensor channels immobilised with different lectins were used in parallel experiments with the same bacterial sample passed through each channel. Ten mM PBS (containing 1 mM Ca^{2+} and 1 mM Mg^{2+}) was used as running buffer and passed after each sample addition to eliminate the bulk effect of the sample. Adsorption/desorption on the crystal surface was recorded, using the QSoft programme (Q-Sense), as the resulting frequency (Δf, Hz) and dissipation shifts (ΔD, $E \times 10^{-6}$ Units), and was visualised and analysed by the QTools programme (Q-Sense). With the Q-Sense E4 system 14 incoming parameters are simultaneously registered (multiple overtones: 7 for frequency and 7 for dissipation values). Simultaneous measurement of multiple overtones provides information about the viscoelastic properties of the applied layers and, additionally, decreases noise to signal ratio when using higher overtones. Data is presented as a series of five curves for frequency and dissipation which correspond to five overtones: 3rd, 5th, 7th, 9th and 11th (1st and 13th overtones are not presented). All measurements were repeated at least twice.

2.5. AFM

All images were recorded at room temperature and normal pressure using an atomic force microscope (NTEGRA, NT-MDT Co., Zelenograd, Russia) mounted with a 50-μm scanner (Z-scanner) and a universal scanning probe microscopy head. Silicon cantilevers (NSG11, NT-MDT) with the following features were used: typical length, 100 and 130 μm; thickness, 2 and 2 μm; resonant frequency, 255 kHz and 150 kHz; and nominal force constants of 11.5 and 5.5 N m^{-1}, respectively.

For scanning, X-Array gold-coated slides were used whose surface had been cleaned, coated with the lectin layer (as described in Section 2.4), and to which bacterial cells were allowed to interact with the lectin-coated surface for 40 min in a humid atmosphere. Finally, slides were carefully washed with buffer solution in order to remove all loosely attached material from the lectin surface and allowed to dry in air before scanning. Freshly dried X-array slides were fixed to a special polycrystalline sapphire substrate (SU001, 24 mm × 19 mm × 0.5 mm, NT-MDT) and secured with the spring clips of the sample stage. Each AFM image was scanned in tapping

Table 1
Sugar specificities of lectins used in the present study.

Lectin origin	Abbreviation	Molecular mass	Sugar specificities[a]
Triticum vulgaris	WGA	34 000	GlcNAcβ1-4GlcNAcβ1-4GlcNAc (*N*-acetylglucosamine-binding lectin)
Canavalia ensiformis	ConA	102 000	Branched α-mannosidic structures; high-mannose type, hybrid type and biantennary complex type *N*-glycans (mannose-binding lectin)
Maackia amurensis	MAL	140 000	Neu5Ac/Gcα2-3Galβ1-4GlcNAcβ1- (sialic acid-binding lectin)
Lens culinaris	LCA	48 000	Fucosylated core region of bi- and triantennary complex type *N*-glycans (mannose-binding lectin)

[a] Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac/Gc.

mode with a 0.55 Hz resolution. The error signal was minimised by optimising the feedback gain and using the protective hood. Height and lateral calibrations of the scanner were carried out using calibration gratings (TGQ and TQZ) of known period that had been provided with the instrument (NT-MDT). Overview images were acquired in order to select a smooth, flat area prior to high-resolution measurements.

3. Results

For the performance of QCM, as the gold surface is relatively chemically inert and unable to retain effectively adsorbed material, the surface of the quartz crystals initially was modified to make it possible to immobilise the recognition molecules (lectins). This was conveniently performed by a three-step adsorption procedure (Fig. 1). Firstly, the crystals were dipped into a solution of 11-mercaptoundecanoic acid in order to form a well-ordered and crystalline self-assembled monolayer that would incorporate different chemical groups. Although bonds between gold and sulphur form rapidly, typically within seconds to minutes, a few hours were required to introduce a significant amount of order into the assembly. Therefore, monolayer formation was performed in the off-line mode.

Subsequently, the crystals were mounted in the QCM system and the surface was modified in the on-line mode by passing a mixture of NHS and EDC through the instrument channels. Although a certain frequency shift was observed, the resulting baseline remained at the same value as before activation or was only slightly shifted upward, and thus it was concluded that some of the thiol-groups were being shed from the surface. Because of this, stable lectin layers were formed on the surfaces by a cross-linking reaction and non-specific sites were blocked with ethanolamine hydrochloride. For the present study, four lectins: WGA, Con A, MAL, and LCA and were chosen based on their specific glycan binding properties (Table 1). Using the QCM procedure, the maximum oscillation frequency shift was found to be different for each lectin (Fig. 2); 18 Hz, 34 Hz, 28 Hz and 11 Hz for WGA, ConA, MAL and LCA, respectively. Thus, the biggest frequency shift was achieved with the ConA lectin.

Previous studies on lectin typing of *Campylobacter* spp. [6,8–10] have required a 1–2 h pretreatment of the cell suspension with proteolytic enzymes and additional heating to 100 °C before lectin agglutination. In the present study, a simple procedure for bacterial sample preparation was utilised whereby samples were incubated for 30 min prior to the assay and then directly injected into the QCM instrument. The analytical response was measured in the on-line mode 40 min after injection, corresponding to the time when equilibrium had been achieved, after which a regeneration solution, 10 mM glycine–HCl solution (pH 2.5), was passed through the system in order to remove the bound bacteria from the lectin-coated surface. This regeneration solution was used since previously it had been shown optimal for bacterial removal without damaging the subsequent binding ability of the lectin-coated crystal surface, thereby allowing re-use in further experiments [13]. Interestingly, identical results to those shown in Fig. 3, were obtained with

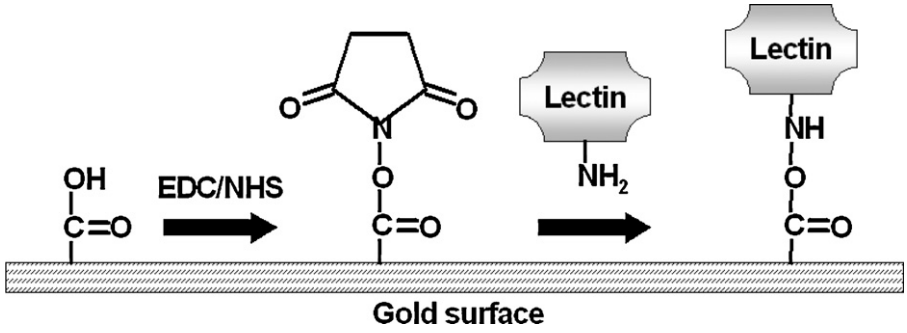

Fig. 1. Amine coupling procedure for immobilisation of lectins onto the thiolised golden surface.

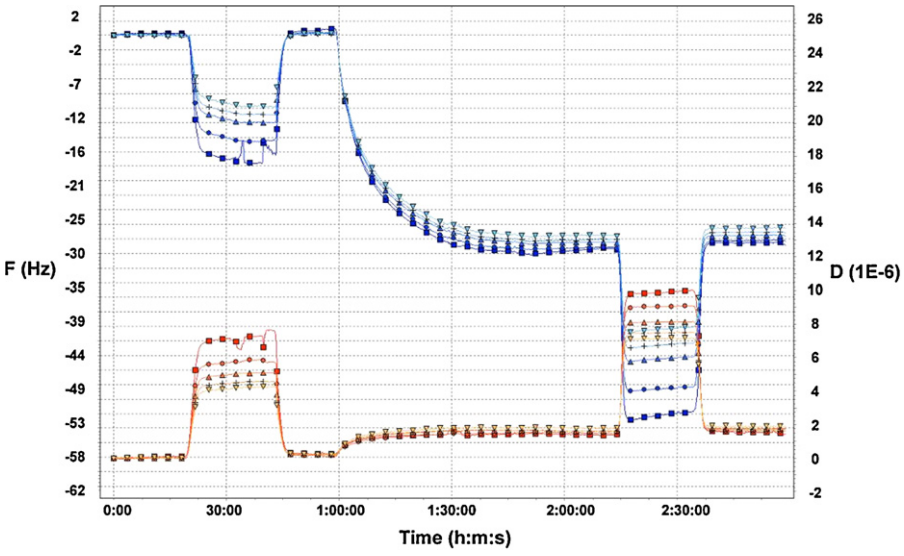

Fig. 2. Example of sensorgram representing on-line immobilisation of *Maackia amurensis* lectin (MAL) on a quartz crystal gold surface. Conditions: 0 min, buffer flow; 16 min, activation with 0.4 M EDC and 0.1 M NHS (1:1); 57 min, immobilisation of lectin; 2 h 10 min, blocking with 1 M ethanolamine-HCl (pH 8.5); 39 min, 1 h 47 min, 2 h 30 min, rinsing with buffer. Curves represent frequency (F, Hz) and dissipation (D, 1E-6) responses (upper and lower, respectively) for 3rd, 5th, 7th, 9th and 11th overtones.

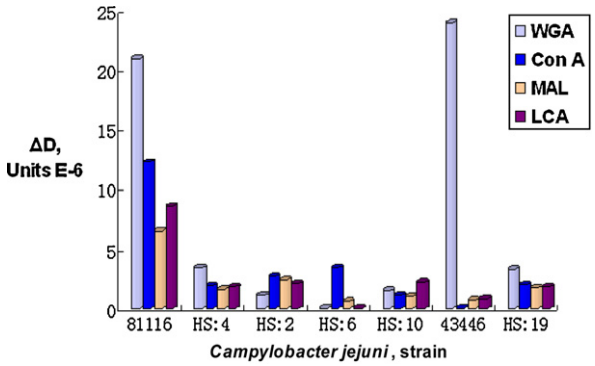

Fig. 3. Dissipation patterns from 5th overtone achieved from interaction between different *Campylobacter jejuni* strains and lectins. The analytical response was measured in the on-line mode 40 min after injection. Flow speed was set to 50 μL min^{-1}. Ten mM PBS (containing 1 mM Ca^{2+} and 1 mM Mg^{2+}) was used as a running buffer and 10 mM glycine–HCl (pH 2.5) was used as a regeneration solution.

rejuvenated replicate lectin-coated surfaces prepared on different days.

The presence of bacterial cells on the lectin-coated surfaces was verified in AFM by scanning in the tapping mode. This mode is advantageous for scanning soft biological samples as the cantilever touches the sample surface only at the very end of its downward movement and lateral forces during scanning are greatly reduced [14]. Thus, in this mode there is elimination of the lateral shear forces present in the contact-mode which, for many specimens, can damage the structure being imaged [15]. In the tapping mode, the imaging probe is vertically oscillated at or near the resonant frequency of the integrated cantilever, and an electronic feedback circuit maintains the oscillation at constant amplitude during scanning. Thus, the image is produced by mapping the vertical distance the scanner moves as it maintains constant oscillation amplitude at each lateral data point. Examples of the AFM images of bacterial cells bound to the lectin-coated layer are represented in Fig. 4A and B. Curved, rod-shaped cells which are 2–4 μm in length were observed over the surface. Of note, AFM scanning showed that the bacterial cells bound to the surfaces were not lysed.

4. Discussion

As was shown by Sauerbrey [16], changes in the resonant frequency (Δf, Hz) of the QCM are linearly related to the mass accumulated on the crystal surface:

$$\Delta f = -\frac{2f^2}{p_q v_q} m = -\frac{f}{p_q T_q} m = -Cm$$

where p_q and v_q are the specific density and the shear wave velocity in quartz, respectively, t_q is the thickness of the quartz plate, and m is the mass per unit are of the added film (with $p_q = 2648\ \mathrm{kg\,m^{-3}}$, $v_q = 3340\ \mathrm{m\,s^{-1}}$ and $f = 5$ MHz, we have $C = 57\ \mathrm{Hz\,\mu g^{-1}\,cm^2}$). However, the assumption of a linear relationship between attached mass and change in frequency is not generally applicable for biological systems, since the Sauerbrey equation is applicable only for rigid films coupled to the crystal surface [11]. Although bacterial cells are known for their non-rigid nature, inclusion of measurement of energy dissipation changes (ΔD, E $\times 10^{-6}$ Units) allows a more accurate estimate of mass changes and makes it possible to use these dissipation changes as an indicator of lectin–bacterium interaction and hence as a discriminative factor for bacterial strains. Dissipation measurements can be performed by periodically switching off the driving power to the QCM oscillator and recording the exponentially decaying signal [17]. In this study by using such an approach, the dissipation changes of interaction between bacterial cells and different lectins were measured.

The energy dissipation, which was measured simultaneously with the oscillation frequency on the QCM instrument, provided additional information about the viscoelastic properties of the surface layer. The sensorgrams for the lectin immobilisation did not show any significant increases of the dissipation response ($<5 \times 10^{-6}$ Units), and hence it was concluded that the lectins form a rigid layer on the gold surface. Both the frequency shift and dissipation shift displayed a behaviour corresponding to typical protein adsorption phenomena.

As expected, adsorption of *C. jejuni* strains on the lectin layer showed non-Sauerbrey behaviour and, as exemplified by the sensorgrams in Fig. 5, the addition of bacteria was characterised by a significant dissipation change. Moreover, the patterns of the dissipation shifts resulting from interactions between the individual test strains and the set of lectins were different, resulting from differences in bacterial strain glycosylation, reflective of inter-strain variations in lipooligosaccharide (LOS) structures, but also in capsular polysaccharide expression and *N*-glycosylation [4,18–20]. Thus, since lectins are characterised by different glycan affinity, it is apparent that interaction between bacterial glycomolecules and lectins will represent a different and unique dissipation pattern that can be used for strain discrimination. The measured data was

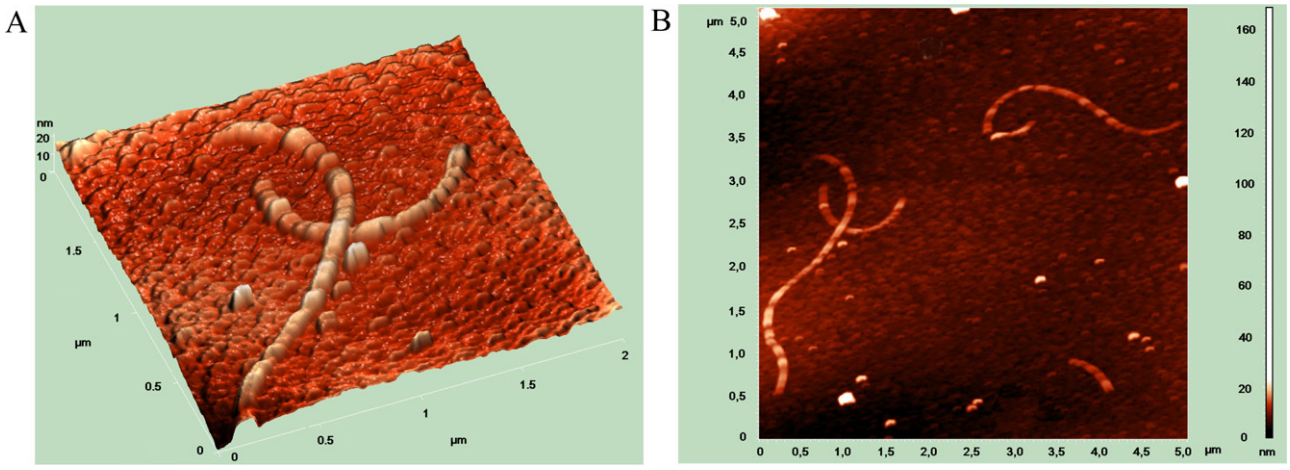

Fig. 4. High-resolution AFM images of *C. jejuni* HS:2 strain bound on a lectin surface. (A) AFM scan acquired by tapping mode in air (2 μm × 2 μm area). (B) AFM image of same sample location (5 μm × 5 μm) with a 0.55 Hz resolution.

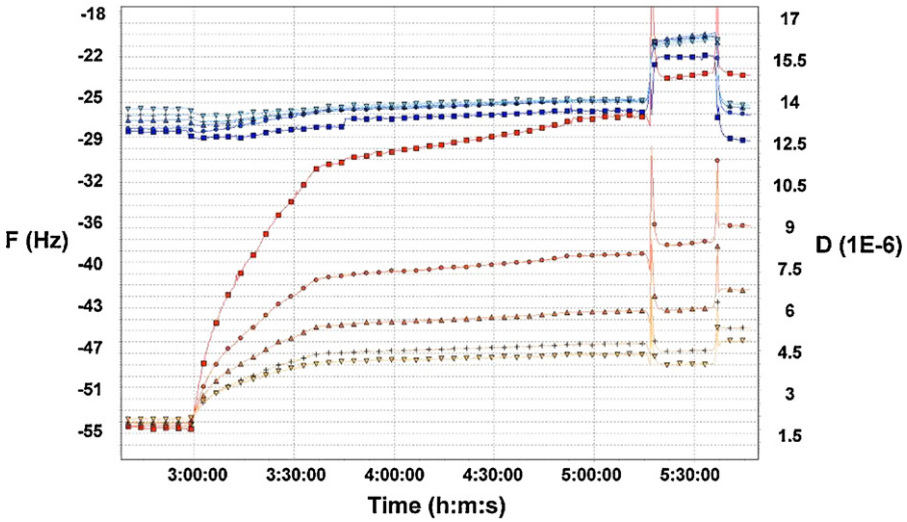

Fig. 5. Example sensorgram representing the changes in energy dissipation during interaction between *Campylobacter jejuni* 81116 and *Maackia amurensis* lectin (MAL). Conditions: 2 h 30 min, buffer flow; 3 h, bacteria addition (1:100 in buffer); 5 h 10 min, regeneration with 10 mM glycine–HCl (pH 2.5); 5 h 40 min, rinsing with buffer. Curves represent frequency (F, Hz) and dissipation (D, 1E-6) responses (upper and lower, respectively) for 3rd, 5th, 7th, 9th and 11th overtones.

analysed in the QTools programme (Q-Sense, Sweden) to determine the exact values of the dissipation shift (ΔD). Changes in dissipation due to interaction between the seven *C. jejuni* strains and the four tested lectins are shown in Fig. 3. Identical results were obtained with replicate lectin-coated surfaces prepared on different days and with different batches of the same bacterial strain (data not shown). Of the lectins used, the strongest lectin interaction with almost all strains was observed for WGA (Fig. 3), whose binding specificity is *N*-acetylglucosamine. This is consistent with results from previous lectin agglutination studies [9], as well as known surface glycosylation in *C. jejuni*, particularly that of capsular polysaccharides and protein *N*-glycosylation [18,20,21].

5. Conclusions

In summary, in this study a new methodology for the discrimination of *C. jejuni* strains based on lectin-bacterial interaction was achieved since the dissipation patterns measured by the QCM sensor were different for each strain. To our knowledge, this is the first report on the use of any QCM sensor for bacterial discrimination based on measurements of dissipation shift (ΔD). This approach has the advantages of robust and reproducible analysis and avoidance of the necessity for a bacterial sample preparation procedure, in contrast to lectin agglutination. The approach may also prove applicable to strain discrimination of other bacterial species, particularly pathogens, to which lectin agglutination has previously been applied [e.g., see Refs. [10,22]] and which is worthy of further investigation.

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