Fluorescence spectroscopy: a rapid tool for assessing tetracycline resistance in *Bifidobacterium longum*

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Abstract: The tetracycline uptake kinetics of 35 *Bifidobacterium longum* strains isolated from the human gastrointestinal tract were examined by fluorescence spectroscopy, and the suitability of the technique as a screening tool of tetracycline resistance or susceptibility was determined. The strains were first grouped into three classes based on their corresponding minimum inhibitory concentrations (MICs) of tetracycline, as established by the microdilution method: susceptible (MICs $\leq 1 \mu\text{g mL}^{-1}$), semi-resistant (MICs between 1 and $\leq 32 \mu\text{g mL}^{-1}$), and resistant strains (MICs $\geq 32 \mu\text{g mL}^{-1}$). The kinetics of tetracycline uptake for the strains in each resistance group were then analyzed over a 20 min period by fluorescence spectroscopy (absorbance wavelength 524 nm, excitation wavelength 400 nm) in a buffer system containing $100 \mu\text{g mL}^{-1}$ tetracycline. Principal component analysis and factorial discriminant analysis of the results showed excellent distinction among susceptible, semi-resistant, and resistant strains. The proposed method provides a powerful and convenient means of rapidly screening tetracycline resistance in *B. longum*.

Key words: fluorescence spectroscopy, *Bifidobacterium longum*, antibiotic resistance, tetracycline uptake, multidimensional data analysis.

Résumé : Les cinétiques d'absorption de la tétracycline chez 35 souches de *Bifidobacterium longum* isolées du tractus gastro-intestinal humain furent examinées par spectroscopie à fluorescence et la performance de cette technique comme outil de criblage de la résistance ou susceptibilité à la tétracycline fut déterminée. Les souches furent d'abord groupées en trois classes selon leurs concentrations inhibitrices minimales (CMI) à la tétracycline correspondantes telles que déterminées par la méthode de microdilution: souches susceptibles (CMI $\leq 1 \mu\text{g mL}^{-1}$), semi-résistante (CMI entre 1 et $\leq 32 \mu\text{g mL}^{-1}$) et résistantes (CMI $\geq 32 \mu\text{g mL}^{-1}$). Les cinétiques d'absorption de la tétracycline pour les souches dans chacun des groupes de résistance furent par la suite analysées sur une durée de 20 minutes par spectroscopie à fluorescence (longueur d'onde d'absorbance de 524 nm, longueur d'onde d'excitation de 400 nm) dans un système tamponné contenant $100 \mu\text{g mL}^{-1}$ de tétracycline. Une analyse des composantes principales et une analyse factorielle du discriminant des résultats ont démontré une excellente distinction entre les souches susceptibles, semi-résistantes et résistantes. La méthode proposée fournit un moyen performant et pratique pour cribler rapidement la résistance à la tétracycline chez *B. longum*.

Mots clés : spectroscopie à fluorescence, *Bifidobacterium longum*, résistance aux antibiotiques, absorption de la tétracycline, analyse multidimensionnelle des données.

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Introduction

Tetracyclines were discovered in the 1940s and shown to have activity against a wide range of microorganisms, including Gram-positive and -negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and even protozoan parasites (Chopra and Roberts 2001). These antibiotics have been extensively used in the prophylaxis and treatment of human and animal infections. They have also been used at the subtherapeutic

level as growth promoters in animal feeds (Wegener 2003). However, this intensive and extensive use has caused tetracycline resistance to spread to a large number of commensal, opportunistic, and pathogenic bacteria (Chopra and Roberts 2001; Roberts 2005). This resistance is mostly acquired by the horizontal transmission of genes coding for energy-dependent efflux systems or for proteins that protect the bacterial ribosomes from the blockage of protein synthesis (Chopra and Roberts 2001).

Bifidobacterium longum is among the dominant bifidobacterial species of the human gastrointestinal tract (GIT) (Ventura et al. 2004), where it is thought to have several health-promoting effects, including the prevention of diarrhea in antibiotic-treated patients, the reduction of cholesterol levels, the alleviation of lactose intolerance symptoms, and the stimulation of the immune system (Ouwehand et al. 2002). Given these possible properties, this species is frequently used as a probiotic in dairy products or is included

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in dietary supplements (Tuohy et al. 2003). The selection of suitable strains for this purpose is difficult, however, since the key characteristics necessary for survival and competition in the human GIT are poorly understood (O'Sullivan 2001). Nevertheless, agreement exists that the strains used in food systems should be free of potentially transferable determinants of antibiotic resistance (European Commission 2001). A few *B. longum* strains have already been characterized as resistant to certain antibiotics, including tetracycline (Delgado et al. 2005; Moubareck et al. 2005), and at least two tetracycline-resistant determinants in bifidobacterial species — *tet*(W) and *tet*(M) — have been characterized (Lacroix and Walker 1995; Scott et al. 2000). Thus, to avoid the spread of resistance via probiotics, systematic checking for antibiotic resistance (in particular to tetracycline) is essential.

The antibiotic resistance or susceptibility profiles of bifidobacteria have been assessed by many methods, e.g., disk diffusion (Matteuzzi et al. 1983; Charteris et al. 1998), agar dilution (Lim et al. 1993), microdilution (Delgado et al. 2005), and the E-test (Charteris et al. 2001). However, more robust methods would facilitate large-scale screening in the search for new industrial strains.

Tetracycline fluoresces after excitation with light of at least 400 nm. Therefore, fluorescence spectroscopy can be used to monitor its uptake in bacterial cells, i.e., the influx of the antibiotic from the external environment to the cell interior. The aim of the present work was to use fluorescence spectroscopy to follow tetracycline uptake in strains of *B. longum* with different mean inhibitory concentrations (MICs) for this antibiotic. The results show the suitability of this technique as a screening tool of tetracycline resistance or susceptibility.

Materials and methods

Strains and growth conditions

The bacteria used in this study were 35 *B. longum* isolates whose tetracycline MICs are known to be different (Delgado et al. 2005) (Table 1). The *B. longum* type strain LMG 13197^T was included as a control in all analyses.

Frozen bacteria were subcultured twice in De Man – Rogosa – Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France) containing 0.25% cysteine (Sigma Chemical, St. Louis, Missouri, USA) (MRS+C) prior to performing the assays. Isolated colonies were finally added to 10 mL of MRS+C broth and cultured at 37 °C for 48 h in an anaerobic chamber (MAC500; Down Whitley Scientific, West Yorkshire, UK) containing an atmosphere of 85% N₂, 10% H₂, and 5% CO₂.

MICs of tetracycline

The MICs of tetracycline for the different strains were determined with the Sensititre Anaero3 commercial system (Trek Diagnostic Systems, East Grinstead, UK), following the manufacturer's recommendations. Briefly, colonies of each strain grown on solid media were used to make a 0.5 McFarland suspension in Brucella Standard broth (Trek Diagnostic Systems, Cleveland, Ohio, USA), and 100 µL of this suspension was then transferred to 10 mL of the same medium, to which haemin and vitamin K₁ (Merck, VWR,

Table 1. Mean inhibitory concentration (MIC) of tetracycline, as determined by microdilution using the Sensititre Anaero3 system.

Isolate or strain	MIC of tetracycline (µg mL ⁻¹)
Susceptible strains (MIC ≤1)	
C51*	≤1
C54	≤1
C55	≤1
C56	≤1
C71*	≤1
C72	≤1
C73	≤1
C62 _{MC}	≤1
E44	≤1
L25	≤1
L43*	≤1
L45*	≤1
L46	≤1
M21*	≤1
M22	≤1
M23	≤1
M24	≤1
M25	≤1
M26	≤1
M27	≤1
LMG 13197 ^T	≤1
Semi-resistant strains (1 < MIC < 32)	
H91	4
H65	8
L41	8
B95	16
H68	16
H93	16
L42	16
L44	16
Resistant strains (MIC ≥32)	
E111	64
H66	128
H94	128
H67	≥256
H92	≥256
H95	≥256

*Test strains; all others were used to construct the model.

Darmstadt, Germany) were added (approximate final bacterial concentration 1×10^6 cfu mL⁻¹). One hundred microlitres of this suspension was inoculated into each well of the Sensititre plates and incubated at 37 °C in anaerobic conditions for 48 h. The growth of the strains was recorded by visual inspection.

Fluorescence spectroscopy

Sample preparation

The strains were propagated in MRS+C agar for 24–48 h. One colony was placed in 10 mL of MRS+C broth and incu-

Fig. 1. Spectra of tetracycline uptake kinetics recorded following excitation at 400 nm in dilute suspensions of *Bifidobacterium longum* L46 (solid line) (MIC < 1 $\mu\text{g mL}^{-1}$), L42 (heavy grey line) (MIC = 16 $\mu\text{g mL}^{-1}$), and H67 (broken line) (MIC = 256 $\mu\text{g mL}^{-1}$).

bated overnight (16–18 h). Ten millilitres of fresh MRS+C was inoculated (2%) with this overnight culture on the following morning. The optical density (OD) of each culture was determined at 600 nm using a Kontron spectrophotometer (Tegimenta AG, Rotkreuz, Switzerland). When an OD_{600} of 0.5 ± 0.1 was reached, 8 mL of the culture was centrifuged at 7000 r/min ($1\text{ r} = 2\pi\text{ rad}$; 6800g) for 2 min. Cells were washed twice with 2 mL of 50 mmol L^{-1} potassium phosphate buffer containing 2 mmol L^{-1} MgSO_4 and 2 mmol L^{-1} glucose (pH 7), and the pellet was resuspended in 0.5 mL of the same buffer. The volume of this suspension necessary to obtain a final OD_{600} of 0.5 was added to 2 mL of the buffer solution prior to the measurement of fluorescence. Three independent cultures were assayed for each strain.

Fluorescence spectroscopy

The fluorescence spectra of the bacterial samples were obtained using a Cary Eclipse fluorescence spectrophotometer (Varian, Sydney, Australia) equipped with a thermostat-controlled, right-angled, single cuvette holder. Samples (2 mL) were placed in a Teflon[®] cuvette and the kinetics of tetracycline uptake measured over 20 min ($\lambda_{\text{Exc}} = 400\text{ nm}$, $\lambda_{\text{Emi}} = 524\text{ nm}$, slit width = 5 nm) at 37 °C. After a time period of 5 min of preincubation ($t_{5\text{min}}$), 100 $\mu\text{g mL}^{-1}$ of tetracycline

was added and incubation allowed to proceed for 10 min ($t_{5\text{min}}-t_{15\text{min}}$); 20 mmol L^{-1} of glucose was finally added to energize the cells, and fluorescence was monitored for a further 5 min ($t_{15\text{min}}-t_{20\text{min}}$).

Mathematical analysis of data

The spectral data were analyzed using XLStat pro 7.5 software (Addinsoft, Paris, France). Pearson principal component analysis (normed PCA) was performed to transform the large number of potentially correlated factors into a smaller number of uncorrelated factors (i.e., principal components) and thus reduce the size of the data set. This multivariate treatment allows score plots of the samples to be drawn representing the spectral patterns (Bertrand and Scotter 1992; Jolliffe 1986). Neighboring points on these score plots represent similar spectra.

The linearly independent principal components resulting from PCA were subjected to factorial discriminant analysis (FDA) by examining the spectral fluorescence data. The aim of this technique is to predict the likely belonging of an observation (spectrum data) to a previously defined qualitative group (Safar et al. 1994). Since the raw spectral data could not be used because of the strong correlations among variables (the measurement times), the uncorrelated principal component resulting from PCA was employed.

Fig. 2. Discriminant analysis similarity map determined with discriminant factors 1 and 2 for the spectral data of the different tetracycline resistance groups: $\square$, resistant strains ($\text{MIC} \geq 32 \mu\text{g mL}^{-1}$); $\triangle$, semi-resistant strains ($1 \mu\text{g mL}^{-1} \leq \text{MIC} < 32 \mu\text{g mL}^{-1}$); $\circ$, susceptible strains ($\text{MIC} < 1 \mu\text{g mL}^{-1}$).

Results and discussion

MICs of tetracycline

Table 1 shows the MICs of tetracycline for the different strains. The results obtained agree well with those reported by Delgado et al. (2005). Differences with respect to the results of the latter authors were always less than two Log_2 dilutions, which is normal for MICs obtained by the micro-dilution method (Delgado et al. 2005; Flórez and Mayo 2005).

A tetracycline resistance gene (*tet(W)*) was detected in all the semi-resistant and resistant isolates (Flórez and Mayo 2005). In fact, it has been reported in several *B. longum* strains (Moubareck et al. 2005; Scott et al. 2000) and seems to be the most common tetracycline resistance gene in other intestinal bacterial genera (Scott et al. 2000; Roberts 2005).

Fluorescence spectroscopy

Tetracycline uptake kinetics

Figure 1 shows the spectra recorded for three representative isolates of the different susceptibility groups. In general, no correlation was found among fluorescence intensity and tetracycline resistance. However, the resistant strains reached

fluorescence equilibrium soon after the addition of the antibiotic, preceded by a peak of antibiotic uptake. Resistant strains were also characterized by a small increase in uptake after the cells received glucose. In contrast, susceptible strains reached the equilibrium more slowly after the addition of tetracycline and were characterized by a greater increase in uptake after the addition of glucose.

Previous studies have shown that the movement of tetracycline across the plasma membrane is an energy-dependent process driven by the ΔpH component of proton motive force (Nikaido and Thanassi 1993). The influx of the antibiotic is fast enough for fluorescence equilibrium to be reached in just a few seconds. In contrast, when ribosome-binding proteins are present (which form stable complexes with ribosomes; Spahn et al. (2001)), fluorescence equilibrium is reached much later because the binding of the tetracycline to the 30S subunit is inhibited (Nonaka et al. 2005). Based on the present observations and given that all resistant strains harbor the *tet(W)* gene (encoding a ribosomal protection protein), the fluorescence equilibrium may be affected by the efficiency of the binding of the antibiotic to the ribosome. Since ribosomal binding sites are protected in resistant strains, tetracycline cannot bind to them, and the equilibrium between the internalized tetracycline and the out-

Table 2. Classification, membership probability, supplementary observation scores, and squared distances to group centroids.

Test strain*	Group	Probability			Factor 1	Factor 2	D^2		
		R	S	SR			<i>i</i> , Centroid (R)	<i>i</i> , Centroid (S)	<i>i</i> , Centroid (SR)
M21-1	S	0	1	0	−11.26	−4.32	2328.39	515.51	2198.59
M21-2	S	0	1	0	−15.89	−5.82	2842.91	422.13	2671.67
M21-3	SR	0	0	1	6.92	−12.95	1321.26	1645.61	922.49
C51-1	S	0	1	0	−16.81	3.70	2640.45	266.26	2752.63
C51-2	S	0	1	0	−48.05	1.94	6811.88	481.32	6893.21
C51-3	S	0	1	0	−22.66	0.99	3336.66	180.56	3372.60
C71-1	S	0	1	0	−14.66	5.73	2487.67	418.82	2658.87
C71-2	S	0	1	0	−49.45	−3.70	7137.06	533.65	7051.74
C71-3	SR	0	0	1	90.86	−4.54	3602.09	14621.20	3395.78
L43-1	S	0	1	0	−14.29	−4.29	2531.58	337.38	2404.76
L43-2	S	0	1	0	−34.62	−1.05	4864.23	170.38	4847.60
L43-3	S	0	1	0	−20.68	7.46	3092.34	295.53	3319.28
L45-1	S	0	1	0	−2.79	1.39	1502.56	850.65	1536.68
L45-2	S	0	1	0	−11.56	−8.17	2464.29	547.60	2220.07
L45-3	S	0	1	0	−11.44	−0.96	2206.56	428.56	2176.83

Note: S, susceptible; SR, semi-resistant; R, resistant.
*The numbers 1, 2, and 3 refer to distinct replicate observations for every test strain.

Fig. 3. Discriminant analysis similarity map of principal (constructed model) and supplementary (test strains) data determined with discriminant factors 1 and 2 for the spectral data of the different tetracycline resistance groups: □, resistant strains, principal observations ($MIC \geq 32 \mu g mL^{-1}$); △, semi-resistant strains, principal observations ($1 \mu g mL^{-1} \leq MIC < 32 \mu g mL^{-1}$); ▲, semi-resistant strains, supplementary observations; ○, susceptible strains, principal observations ($MIC < 1 \mu g mL^{-1}$) ●, susceptible strains, supplementary observations.

side pool is quickly reached. In contrast, in the susceptible strains, tetracycline binds to the ribosome and fluorescence equilibrium is reached later (Fig. 1: see $t_{5\min}-t_{15\min}$).

Tetracycline uptake was also found to be positively correlated to the amount of MgSO_4 in the buffer. In fact, fluorescence in the presence of 2 mmol L^{-1} of MgSO_4 was approximately 5-fold greater than in the presence of 0.4 mmol L^{-1} MgSO_4 . This agrees with previous observations suggesting that the complexation of tetracycline with divalent metal ions, such as Mg^{2+} or Ca^{2+} , greatly increases fluorescence (Schneider et al. 2003).

Model building

To ensure robustness, the model was constructed using the results for 30 strains and validated with five test strains. The spectral kinetics data obtained for the 30 *B. longum* strains were pooled in one matrix and the data examined by PCA. The linear independent factors resulting from the PCA were then used as new variables to perform FDA based on the defined group constituting the dependent variable.

In a first step, the dependent variable was composed of 30 groups representing the number of considered strains. The aim was to ensure that the results of the three replicate assays for each strain were attributed to the group formed by the corresponding strain, thus confirming the reproducibility of the method. The confusion matrix resulting from the FDA provided 100% correct classification of the results to the corresponding strain. The first two discriminant factors were able to explain more than 86.7% of the total variance (results not shown), thus confirming reproducibility.

In a second step, the dependent variable was composed of three groups according to the resistance level: susceptible ($\text{MICs} \leq 1 \mu\text{g mL}^{-1}$), semi-resistant (MICs between 1 and $32 \mu\text{g mL}^{-1}$), and resistant ($\text{MICs} \geq 32 \mu\text{g mL}^{-1}$). The confusion matrix of the FDA results indicated 100% correct classification of the strains in these groups (results not shown). Figure 2 shows the map defined by discriminant factors 1 and 2; these two factors explained 100% of the total variance. The model was therefore considered well constructed; validation experiments were therefore performed.

Model validation

The spectral kinetics data for the five test strains were collected and pooled in a matrix. PCA was then applied to a model and validating data matrices. The model matrix provided the principal observations and the validating matrix provided the supplementary observations; thus, the supplementary observations did not contribute to the inertia of the model. The linearly independent factors (principal and supplementary data) were used as new variables (principal and supplementary data) in FDA analysis. Although, 2 of the 15 observations corresponding to these supplementary observations were misclassified (Table 2), all five test strains were assigned a position close to other strains of their phenotypic group (Table 1). Both misclassified observations corresponded to the semi-resistant group (Fig. 3), but they did not invalidate the overall results. This misclassification might be explained by the small number of strains used in the construction of the model. However, since more data can be added to the model, the analysis of new *B. longum* strains

showing differences in tetracycline resistance should certainly increase its robustness.

Conclusions

The results show that fluorescence spectroscopy is a rapid and accurate method for screening *B. longum* strains for resistance to tetracycline. Models similar to that constructed for *B. longum* could easily be developed for other bacterial species. The method is amenable to semi-complete or complete automation; this should be useful in large-scale tetracycline resistance surveys, complementing or perhaps replacing the more expensive and time-consuming phenotypic assays currently used.

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