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Reactance and resistance: main properties to follow the cell differentiation process in *Bacillus thuringiensis* by dielectric spectroscopy in real time

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Abstract During growth, *Bacillus thuringiensis* presents three phases: exponential phase (EP), transition state (TS), and sporulation phase (SP). In order to form a dormant spore and to synthesize delta-endotoxins during SP, bacteria must undergo a cellular differentiation process initiated during the TS. Dielectric spectroscopy is a technique that can be utilized for continuous and in situ monitoring of the cellular state. In order to study on-line cell behavior in *B. thuringiensis* cultures, we conducted a number of batch cultures under different conditions, by scanning 200 frequencies from 42 Hz to 5 MHz and applying fixed current and voltage of 20 mA and 5 V DC, respectively. The resulting signals included *Impedance* (*Z*), *Angle phase* (*Deg*), *Voltage* (*V*), *Current* (*I*), *Conductance* (*G*), *Reactance* (*X*), and *Resistance* (*R*). Individual raw data relating to observed dielectric property profiles were correlated with the different growth phases established using data from cellular growth, *cry1Ac* gene expression, and free spores obtained with conventional techniques and fermentation parameters. Based on these correlations, frequencies of 0.1, 0.5, and 1.225 MHz were selected for the purpose of measuring dielectric properties in independent batch cultures, at a fixed frequency. *X* and *R* manifest more propitious behavior in relation to EP, TS, SP, and spore release, due to particular changes in their signals. Interestingly, these profiles underwent pronounced changes during EP and TS that were not noticed when using conventional methods, but were indicative of the beginning of the *B. thuringiensis* cell differentiation process.

Keywords Cell differentiation · Dielectric spectroscopy · *Bacillus thuringiensis* · On-line measuring · *Resistance* (*R*) · *Reactance* (*X*)

Introduction

A key question in biology relates to how it is that sister cells with identical genomes follow different patterns for gene expression (Iber et al. 2006). Bacteria utilize a variety of molecular mechanisms to achieve cell differentiation, and the resulting developmental transformations generate specialized cell types that increase their ability to survive in the environment (Moran 2002; Barák and Wilkinson 2005; Banse et al. 2008; Saujet et al. 2011). Cellular differentiation is the process during which relatively unspecialized cells acquire specialized structural and/or functional features that characterize the cells, tissues, or organs of the mature organism, or some other relatively stable phase during the life history of the organism. Differentiation includes the processes that induce a cell to a specific fate and its subsequent development to the mature state (Cammack et al. 2006), as in *Bacillus subtilis* where the formation of an asymmetric septum during sporulation is

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related to the control on the part of several genes that are involved in cellular division affected by sporulation factors (Errington 2003; Hilbert and Piggot 2004). Hence, this morphological process (sporulation process) is a powerful system for studying mechanisms that control cell division and development (Bejerano-Sagie et al. 2006).

The most successful insect pathogen used for insect control is *Bacillus thuringiensis*, currently comprising ~2 % of the total insecticide market (Bravo et al. 2011). During sporulation, *B. thuringiensis* produces parasporal inclusions (delta-endotoxins known as Cry proteins), with entomotoxic activity (Agaisse and Lereclus 1994; Aronson 2002; Soberón et al. 2007). Cry proteins are specifically toxic to different insect orders, such as *Lepidoptera*, *Coleoptera*, *Diptera*, *Hymenoptera*, and *Nematodes* (Soberón et al. 2007). *B. thuringiensis* undergo a life cycle that includes vegetative growth or exponential phase (EP), transition phase (TS), and sporulation phase (SP). The transition phase is characterized by a decrease in specific growth rate (μ) and metabolic changes (López-y-López and de la Torre 2005). The sporulation process in *B. thuringiensis* is very similar to that so well characterized in *B. subtilis* (Aronson 2002) and which has been extensively studied (Phillips and Strauch 2002). During transition from exponential to stationary growth phase, a complex regulatory network will determine which option (still growing or initiation of sporulation) is most suitable under the given conditions (Saujet et al. 2011). If the cell opts for sporulation (Lazazzera 2000; Phillips and Strauch 2002; Bassler and Losick 2006), one of the progeny will be a compact and durable spore, and the other the mother cell that aids in construction of the spore but is lysed at the completion of the cellular differentiation process (Jenal and Stephens 1996). Considerable work has been dedicated to depicting the mode of action of insecticidal Cry proteins (Vachon et al. 2012), as well as to the development of media culture for different strain production and research into insecticidal activity of Cry proteins (Frankenhuyzen 2009). Despite this information, there are no reports that address the cell differentiation process in *B. thuringiensis* cultures monitored on-line.

In order to successfully examine dynamic cellular processes in live cells, non-destructive real-time monitoring methods are required (Bagnaninchi and Drummond 2011; Park et al. 2011). Dielectric spectroscopy measurements have been applied to the analysis of biological materials such as cell suspension and tissues (Schwan and Takashima 1991; Morgan et al. 2007). These measurements are based on the application of an electric field to a cell suspension causing a shift in the ions present, which results in polarization of the cell membrane that behaves as a capacitor (November and Van Impe 2002). The cells are subsequently termed dielectric because they are located in the electric field between two electrodes for analysis (Justice et al. 2011), where ionic and electronic conduction is evident (Grimnes and Martinsen 2000; Luong et al. 2001; Bonmassar et al. 2010; Pänke et al. 2011). The electrical and morphological properties of the cell membrane and cytoplasm and extracellular media are assumed to represent sensitive parameters of the cellular state (Gheorghiu 1996). Currently, dielectric spectroscopy is used to determine biomass, living cell volume, cell length, etc. (Hunt et al. 2009), or for detection of bacteria in water (Timms et al. 1996), milk (Felice et al. 1999), or food (Gibson et al. 1992). Likewise, it is used for industrial microbial process control and sanitation microbiology (Swaminathan and Feng 1994; Silley and Forsythe 1996), as well as for microbial growth (Dézenclos et al. 1994) and to assess metabolic activity due to growth and physiological state among yeast (Owens et al. 1992; Deák and Beuchat 1993), bacteria, and fungi (Matanguihan et al. 1994). This technique has also been used as an on-line method for measuring the formation of biofilms by *Klebsiella rubiacearum* and for on-line control of these biofilms by the automated addition of chlorine in response to appropriate changes in electrical capacitance (Markx and Kell 1990). Similarly, Sarrafzadeh et al. (2005) mentioned that it is appropriate for monitoring growth and sporulation of *B. thuringiensis* in fed-batch culture. Biofilm formation and sporulation processes result from the adaptation of bacterial population to environmental conditions; these are regulated by quorum sensing. Other features regulated by quorum sensing in bacteria include the impulse to sporulate (Lazazzera 2000), as well as virulence, genetic competence, and bioluminescence, among other factors with potential application in biotechnology (Mangwani et al. 2012). Because bacteria use quorum sensing communication circuits that regulate a diverse array of physiological activities, these physiological changes modify the electrical capacity of the cell, so that dielectric spectroscopy can be used to follow these changes on-line. However, there is no available information regarding cell differentiation process in bacteria monitored by dielectric spectroscopy in real time and in situ. In order to address whether dielectric spectroscopy is appropriate for following a differentiation process in bacteria, we use *B. thuringiensis* as a study model and correlate the changes in dielectric properties when batch cultures are undergoing exponential growth phase, transition state, and sporulation phase, as well as analyzing data from cellular growth, *cry1Ac* gene expression and free spores obtained using conventional techniques, and fermentation parameters. We found good correlation between dielectric properties and cell differentiation process in *B. thuringiensis*. Interestingly, *Reactance* (*X*) and *Resistance* (*R*) manifested more propitious behavior in relation to exponential phase, transition state, sporulation phase, and spore release, due to particular changes in their signals. This suggests that *Reactance* and *Resistance* can be useful properties for identifying minimal changes in real time and in situ, in relation to the cell differentiation process, as well as for the initiation of this cellular event that is

not possible to evaluate using conventional techniques in real time.

Materials and methods

Microorganism

B. thuringiensis strain HD73 pHT1kAc derived from *B. thuringiensis* var. *kurstaki* HD73 (ATCC-35866) was used. The transformed strain has a 1000-bp fragment upstream of the *cry1Ac* promoter derived from *B. thuringiensis* HD73 fused to *lacZ* into plasmid pHT304-18Z (Agaisse and Lereclus 1994). Sierra-Martínez et al. (2004) constructed the transformed strain.

Media and culture conditions

Two culture media were utilized: Farrera medium (Farrera et al. 1998) and Clean medium. The composition of Farrera medium in ($g \cdot l^{-1}$) consisted of glucose, 13.42; soybean meal, 5.6; corn steep solids, 4.05; and yeast extract, 2.59. Clean medium in ($g \cdot l^{-1}$) consisted of glucose, 12.0; soybean peptone, 12.0; and yeast extract, 2.59. In addition, both media had the same composition of mineral salts in ($g \cdot l^{-1}$): $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.0058; $CuSO_4 \cdot 5H_2O$, 0.0075; KCl, 3.0; $FeSO_4 \cdot 7H_2O$, 0.00135; $CoCl_2 \cdot 6H_2O$, 0.03; and Mazu DF antifoaming agent, 1 ($ml \cdot l^{-1}$). For Farrera medium, the pH was adjusted to 2.0 with H_3PO_4 85 % (*w*/*v*), prior to sterilization. Before inoculation, the pH was adjusted to 7.2 with 5 M NaOH. Batch cultures were performed in a Sartorius Biostat A plus reactor, with 4-l of working volume. The pH was controlled automatically at 7.2 with 5 M NaOH and 0.67 M H_3PO_4. Foam was controlled by the addition of Mazu DF antifoaming agent. Operative conditions were 30 °C, 600 rpm, and 1 vvm. A log-phase inoculum was prepared from a spore disk with 10^7 spores with a one-stage culture in Farrera medium, incubated at 30 °C and 200 rpm for 14 h. The reactor was seeded with 6.25 % (*v*/*v*) for the one-stage culture. This culture condition was the same for all experiments. Biomass experimental data were adjusted to exponential growth model (Eq. 1):

$$X = X_0 e^{\mu t} \quad (1)$$

In Eq. (1), X are the cell counts at time t, X_0 are the initial cell counts, μ is the specific growth rate, and t is the time. It was assumed that the transition phase initiated when μ decreased and cell growth became linear (López-y-López and de la Torre 2005), whereas the onset of sporulation takes place 1 h prior to the detection of β-galactosidase activity from pHT1kAc, as transcription of *cry1A* from the BtI promoter has been reported to initiate 1.0 to 2.0 h after the onset of sporulation phase (Agaisse and Lereclus 1995; Schnepf et al. 1998; López-y-López and de la Torre 2005). Transition state is marked by a shaded area in growth profiles, determined by conventional analysis of each fermentation; this area delimits the exponential growth phase shown on the left side and the sporulation phase shown on the right side of each graph. For the same purpose, bacterial oxygen demand was maximal at the end of the log phase, remained high for a short time during the transition phase, and decreased during sporulation phase. To confirm the establishment of sporulation phase onset, the increase in the dissolved oxygen (DO) profile was also assessed. We use these guidelines to set the growth phases in the time course profile for all batch cultures in this work.

β-galactosidase assay

cry1A expression was determined as described by López-y-López and de la Torre (2005), applying β-galactosidase assay; briefly, fermentation samples of 1.0 ml were centrifuged at 10, 000 rpm at 4 °C, and the cell pellet was washed twice and resuspended in 1.0 ml of phosphate buffer (0.1 M, pH 7.5). The reaction mixture (Sambrook et al. 1989) contained 30 μl, cell suspension; 201 μl, phosphate buffer; 3.0 μl, 100 × Mg solution; and 66 μl of *orto*-nitrophenyl-*β*-D-galactopyranoside (ONPG) and was incubated at 30 °C, for 30 min. The reaction was stopped with 500 μl of $NaCO_3$ 1 M, centrifuged under the same conditions mentioned above, and read at 420 nm. One unit of β-galactosidase (Miller unit, MU) was defined as the amount of enzyme hydrolyzing 1 μmol of ONPG min^{-1}. All assays were performed in duplicate.

Analytical methods

Quadruplicate bacilli and spore counts were performed by diluting broth samples in saline solution, followed by vigorous mixing to avoid cell or spore clumping and direct microscopic counting in a Neubauer chamber (Bravo et al. 1993; Flores et al. 1997; Farrera et al. 1998; Navarro et al. 2006; Boniolo et al. 2012). Sporulation efficiency was calculated by taking the sum of free spores obtained at the end of fermentation, dividing this by the bacilli count determined at the onset of sporulation phase and multiplying the quotient by 100 (López-y-López and de la Torre 2005). Residual glucose was analyzed in a biochemistry analyzer (YSI 2700 Select). The carbon dioxide (CO_2) in the exit gas was monitored in real time using an infrared CO_2 analyzer (Carbon dioxide analyzer Mod. 906, Quantek Instruments). Sartorius PC-Panel μDCU 6.34 was used for DO measurement and pH measurement-control.

Dielectric measurements

Dielectric parameters were measured by the Hioki 3532-50 LCR HiTester impedance meter. Figure 1 shows the reactor vessel and dielectric probes. The dielectric probes consisted of two stainless steel blades, 300 mm in length, 9.5 mm wide, and 2.38 mm thick. In order to avoid any contact with the metal of the reactor lid, the probes were fixed with an adapter constructed out of Teflon®. Each probe had a protrusion length of 35 mm from the lid, and was coupled to the impedance analyzer with a type A crocodile clip. Dielectric properties were measured by applying a fixed current of 20 mA and 5 V, scanning 200 frequencies at 4 ranges, initiating at 42 Hz and ending at 1 kHz, 5 kHz, 10 kHz, and 5 MHz. Each scan was performed for at least 19 measurements per fermentation process. Dielectric properties acquired from *B. thuringiensis* cultures included *Impedance* (*Z*), *Angle phase* (*Deg*), *Voltage* (*V*), and *Current* (*I*) as well as the parameters *Resistance* (*R*), *Reactance* (*X*), and *Conductance* (*G*) calculated by Eqs. (2), (3), and (4), respectively. Dielectric profiles were correlated with kinetic profiles obtained from conventional analysis such as bacilli and spore counts, *cry* expression, residual glucose, resulting CO_2, and DO. Specific frequencies were then selected based on the most pertinent correlations applying the correlation coefficient of Pearson for two data groups, according to the conditional Eq. (5).

$$R = Z{\cdot}\cos(Deg) \tag{2}$$

$$X = Z{\cdot}\sin(Deg) \tag{3}$$

$$G = \frac{I}{V} \tag{4}$$

$$\rho_{x,y} = \frac{\mathrm{Cov}(X,Y)}{\sigma_x{\cdot}\sigma_y} \geq |0.9| \tag{5}$$

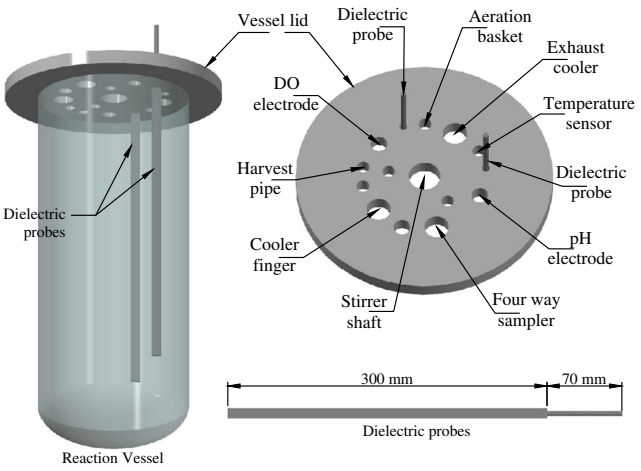

Fig. 1 Configuration of dielectric probes. The cooler finger and exhaust cooler are fixed to the lid, as are the four-way sampler, aeration basket, stirrer shaft, and harvest pipe for the pH, DO, temperature, and dielectric probes

In Eq. (5), ρ is the correlation coefficient, Cov is the covariance of *X* and *Y* values, *X* represents each fermentation parameter (bacilli, spores, *cry* expression, residual glucose, CO_2, and DO), and *Y* refers to each dielectric property (*Z*, *Deg*, *V*, *I*, *G*, *R*, and *X*) and σ is standard deviation of *X* and *Y*. Groups of data utilized are presented in Table 1. The Hioki LCR version, 4.03E was used to record the process data which sends individual raw data referring to dielectric properties to Excel® program.

Results

Scanning frequencies in batch cultures

Nine batch cultures were set up. A typical time course in Clean medium is shown in Fig. 2. The exponential growth phase lasted 4 h and was followed by the transition state with linear growth (4–6 h) (Fig. 2a). Glucose was depleted after 6 h, and at 14 h, free spores were detected (Fig. 2b). Oxygen demand was maximal at the end of the log phase, remained high during transition phase, and decreased during sporulation (Fig. 2b). Figure 2c shows 50 *Impedance* profiles obtained from individual raw data scans at 42 Hz to 5 MHz. These profiles showed a decreasing pattern with specific changes occurring in the periods from 0 to 1 and 1 to 4 h, which corresponded to the exponential growth phase, the transition phase (4 to 6 h), and during sporulation (6 to 14 h). The other dielectric properties profiles (*Deg*, *V*, and *I* as well as the calculated parameters *R*, *X*, and *G*) had similar profiles (data not shown). An example of fermentation with Farrera medium is shown in Fig. 3. In this case, the changes on *Impedance* profiles were not pronounced, as in the case of Clean medium. Although the decreasing pattern of impedance is noticeable in the range of 0 to 6Ω in Fig. 2c. This information suggests that the culture medium can affect the signal profiles of dielectric properties like *Impedance* in scanning frequencies. In Table S1, the value for the range of kinetic parameters from nine fermentations used for correlations are presented.

In order to reveal a correlation between the data acquired through dielectric spectroscopy and growth phases (Table 1), Eq. 5 was used, and 1,026,000 correlations were obtained for each fermentation; only 0.72 % of them had $\rho_{x,y} \geq 0.9$ (Fig. 4); most of them corresponded to frequencies in a range of 300 kHz to 1.8 MHz. For further experiments, clean medium and frequencies of 1.225 and 0.5 MHz were used, as well as 0.1 MHz, as in other studies (Gou et al. 2011; Schwarzenberger et al. 2011).

Table 1 Grouping of data used to calculate correlating parameters (Eq. 5) between dielectric properties obtained to *B. thuringiensis* fermentation parameters and cell differentiation process in time duration of fermentation and each growing phase established through conventional techniques for each of the frequencies tested. Both data groups (dielectric and fermentation) that were correlated are from the same fermentation age or fermentation segments and were obtained individually. The correlations obtained were from the combination of data groupings

Frequency range tested	Time of scanning (h)	Dielectric properties obtained	Fermentation parameter measured	Fermentation segment
42–1 kHz 42–5 kHz 42–10 kHz 42–5 MHz	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 22, 24	*Z*, *Deg*, *X*, *R*, *V*, *G*, *Capacitance* (*Cs*), *Dissipation factor* (*D*), *Current* (*I*)	Bacilli, spore, *cry* expression, dissolved oxygen, carbon dioxide, glucose	EP, TS, SP, Spores, Complete
200 frequencies	19 times	9 parameters	6 parameters	5 time segments

Batch cultures monitored at fixed frequencies of 1.225, 0.5, and 0.1 MHz

Figure 5 shows the time course for three batch cultures in Clean medium, monitoring at fixed frequencies of 1.225, 0.5, and 0.1 MHz in real time. Corresponding kinetic data are presented in Table 2, as well as those referring to the control fermentation. The period corresponding to the transition state as indicated by the growth rate and dissolved oxygen is shaded in the time courses. At 1.225 MHz, *Resistance* rapidly increased after 2.3 h and then decreased to a minimum of −5.94Ω at 3.71 h; subsequently, there was an increase and decrease in their signal during the period corresponding to the transition phase onset at 4 h, indicating reduction in μ (determined off-line) and oxygen consumption. Later (5.58 h), *Resistance* suddenly began to increase again and β-galactosidase from the reporter gene appeared, suggesting that sporulation onset had occurred. This increment was followed

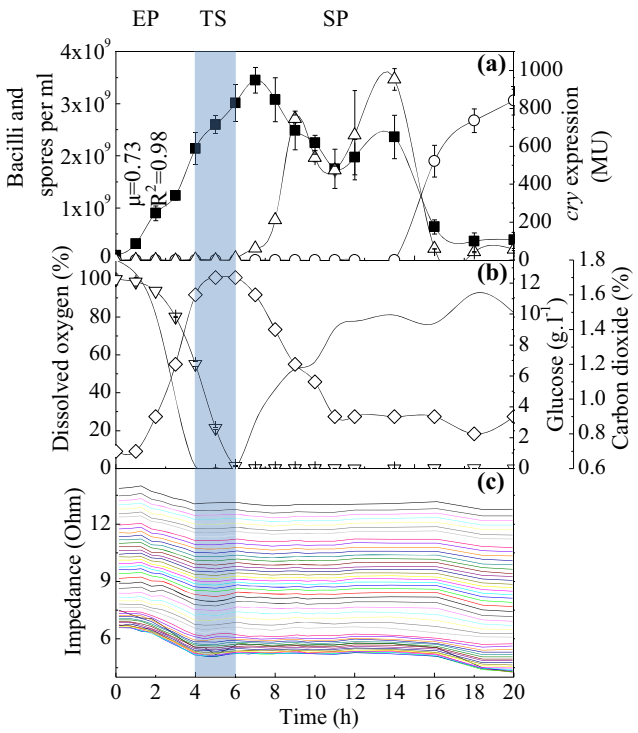

Fig. 2 Time course of *B. thuringiensis* HD73 pHT1kAc in clean medium. *Shaded area* represents the transition phase, determined by conventional analysis. Fifty profiles of *Impedance* measured in the range of 42 Hz to 5 MHz, registering 25 fermentation times. The symbols in the figure are **a** bacilli (*black square*), spores (*white circle*), and *cry* expression (*triangle*); **b** CO_2 production profile (*diamond*), residual glucose (*inverted triangle*), DO (*minus sign*), and **c** *Impedance* (*color lines*)

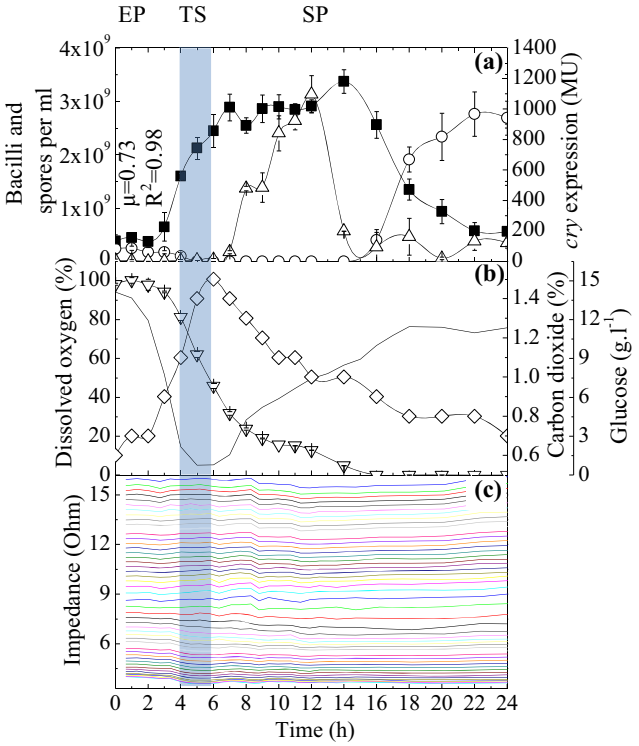

Fig. 3 Time course for *B. thuringiensis* HD73 pHT1kAc in Farrera medium. Shaded area is the transition phase determined by conventional analysis. Fifty profiles for *Impedance* measured within the range of 42 Hz to 5 MHz, registering 19 fermentation times. The symbols in the figure are **a** bacilli (*black square*), spores (*white circle*), and *cry* expression (*triangle*); **b** CO_2 production profile (*diamond*), residual glucose (*inverted triangle*), DO (*minus sign*), and **c** *Impedance* (color lines)

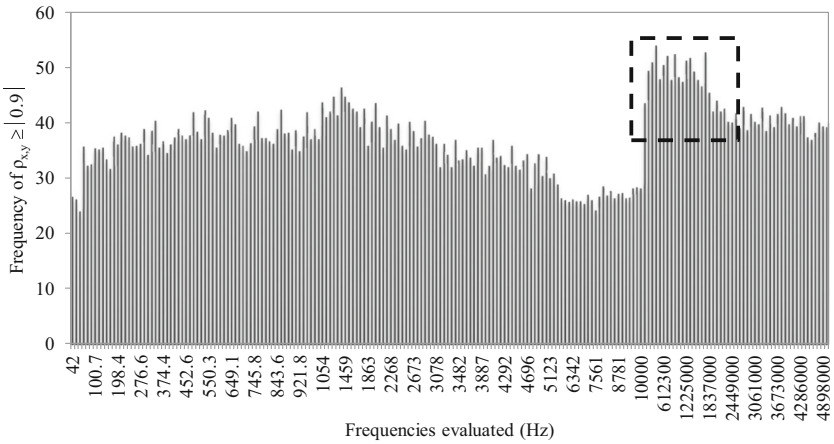

Fig. 4 Frequency distribution for absolute values of correlations, higher or equal to 0.9. The frequencies evaluated were 200, from 42 to 5,000,000 Hz. The frequency range that best fitted the condition was from 300 kHz to 1.837 MHz. From these results, three frequencies were selected: 0.1, 0.5, and 1.225 MHz

by a small decrement in *Resistance* and then a linear increment, followed by a linear decrement. During this interval, the reporter gene was expressed, i.e., sporulation took place and after β-galactosidase activity was no longer detected and refracting spores appeared. This period fits with reports regarding the time (about 6–8 h) necessary to complete the sporulation phase in *B. subtilis* (Losick et al. 1986; Phillips and Strauch 2002). As spores were detected, *Resistance* remained constant. Also, *Reactance* time course resembled the bacteria life cycle at 1.225 MHz (Fig. 5). It reached a maximum during the exponential growth phase and a minimum in the transition phase, while remaining fairly constant during sporulation and decreasing as spores became visible and the spore count increased.

The bacterial concentration at 0 h in the batch culture monitored at 0.5 MHz was 45.6 % of the concentration in the batch at 1.225 MHz; the transition and stationary phases were delayed for 1 h (Table 2), and this delay was observed in the *Resistance* profile (Fig. 5 at 0.5 MHz). An important difference between these batches is that β-galactosidase activity was detected and spore concentration increased; likewise, *Resistance* increased and remained higher during this period than in the batch at 1.225 MHz. These facts suggest that dielectric profiles at this frequency make it possible to detect small on-line differences in the time courses between batches. In contrast, although the *Reactance* profile during the exponential phase at 0.5 MHz showed a similar profile to the batch at 1.225 MHz, the signal response was less evident during the remainder of the bacilli life cycle. In contrast, correlation between *Resistance* and *Reactance* profiles at 0.1 MHz and the life cycle of the bacteria were not as clear as those at 1.225 and 0.5 MHz.

In spite of the frequency applied (0.1, 0.5, and 1.225 MHz), the profiles of the dielectric parameters *Impedance*, *Phase angle*, *Conductance*, and *Voltage* were associated with the exponential phase, transition state, and sporulation phase (Fig. 5). Furthermore, *Impedance* and *Voltage* had a tendency to decrease their signal value during exponential phase, with greater resemblance to a substrate consumption profile and were pronounced at 1.225 and 0.5 MHz. *Angle phase* and *Conductance* had a tendency to increase their signal with greater similarity to a characteristic growth curve and/or CO_2 production profile (Fig. 5).

From the analysis of kinetic parameters (Table 2), we noted slight differences between cultures. Specific growth rates at 1.225, 0.5, and 0.1 MHz experiments were the same and higher than in fermentation with no dielectric sensing. These values are in accordance with the range reported by other researchers from 0.4 to 1.9 h^{-1} for *B. thuringiensis* (Avignone-Rossa and Mignone 1995; Rodriguez-Monroy and de la Torre 1996). Significative differences were found at the initial cellular counts between experiments ($p<0.5$, one-way ANOVA test), although they had the same magnitude order. Cellular counts were similar for different experiments at μ change and sporulation onset ($p<0.5$, one-way ANOVA test). Cellular counts evaluated at 1.225 and 0.5 MHz were comparable for *Resistance* change but not for *Reactance*. The time of transition phase onset was the same for those with no impedance sensing and 0.5 MHz experiments (at 5 h), 1 h earlier in experiment at 1.225 MHz and 1 h later at 0.1 MHz. The time of sporulation phase onset in the experiment evaluated at 1.225 MHz was established 1 h earlier than for the rest of the experiments. Residual glucose concentration was considered not to constitute a limit in terms of changes in *Resistance* and *Reactance* at 1.225 and 0.5, or concerning the change in μ (transition phase onset), in all experiments. Sporulation efficiency was considered acceptable for *B. thuringiensis* production. Notably, *cry1Ac* gene expression evaluated as β-galactosidase activity was higher in the fermentation evaluated at 1.225 MHz, followed by the *cry1Ac* expression between experiments at 0.5 and 0.1 MHz. Despite the fact of such differences between kinetic parameters obtained in described experiments, the dielectric properties used in this work, especially *Resistance* and *Reactance*, manifested more propitious behavior in relation to the exponential growth

Table 2 Kinetic parameters obtained in batch cultures of *B. thuringiensis* pHT1kAc in clean media without impedance measurement and monitored at 1.225, 0.5, and 0.1 MHz

	No impedance measurement	Frequency (MHz)		
		1.225	0.5	0.1
μ^a (h^{-1})	0.76 (R^2=0.93)	0.84 (R^2=0.98)	0.84 (R^2=0.99)	0.84 (R^2=0.98)
Bacilli counts (cell·ml^{-1})				
At time 0	6.00×10^7 ($\pm1.58\times10^7$)	8.50×10^7 ($\pm2.04\times10^7$)	3.88×10^7 ($\pm4.79\times10^6$)	1.86×10^7 ($\pm4.27\times10^6$)
At change in μ	2.16×10^9 ($\pm3.79\times10^8$)	2.04×10^9 ($\pm3.66\times10^8$)	2.69×10^9 ($\pm1.49\times10^8$)	2.08×10^9 ($\pm3.59\times10^8$)
At time of sporulation phase onset	3.58×10^9 ($\pm4.27\times10^8$)	3.48×10^9 ($\pm5.61\times10^8$)	3.34×10^9 ($\pm4.29\times10^8$)	3.88×10^9 ($\pm2.50\times10^8$)
Resistance change[c]	NM	5.13×10^8 ($\pm1.65\times10^8$)	6.00×10^8 ($\pm7.07\times10^7$)	ND
Reactance change[c]	NM	9.88×10^8 ($\pm4.79\times10^7$)	1.38×10^9 ($\pm1.85\times10^8$)	ND
Time of transition state (h)	5	4	5	6
Time of sporulation phase (h)	7	6	7	7
Glucose at μ change (g·l^{-1})	7.67	7.70	7.10	7.61
Glucose at *Resistance* change[c] (g·l^{-1})	NM	12.7	12.5	ND
Glucose at *Reactance* change[c] (g·l^{-1})	NM	10.4	10.9	ND
Maximum β-galactosidase activity (MU)	721 (±2.9)	1210 (±94)	996 (±64)	935 (±164)
Sporulation efficiency[b] (%)	80	82	86	100

ND not defined, *NM* not measured

[a] Values determined at the time of transition state onset

[b] Values calculated with the bacilli counts determined at the onset of sporulation phase and the final spore count

[c] Values determined at change during exponential phase

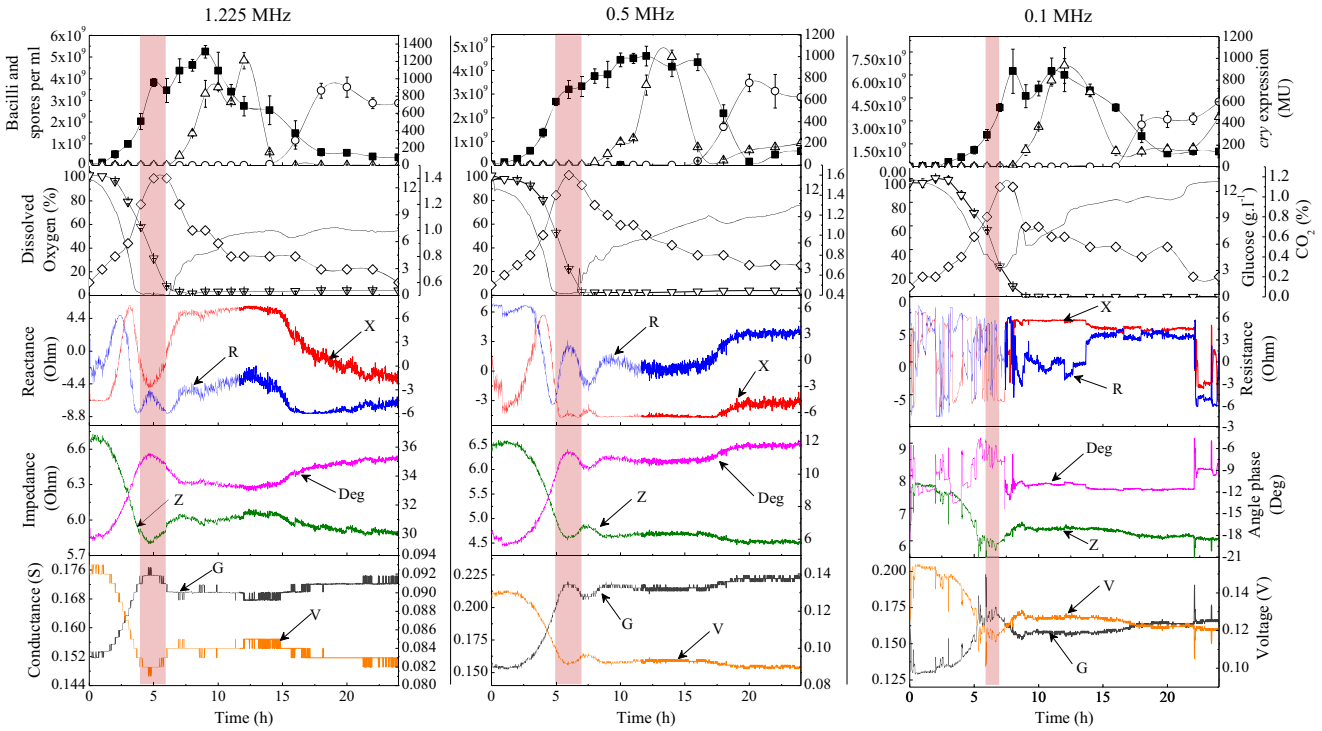

Fig. 5 Time course for *B. thuringiensis* HD73 pHT1kAc batch cultures in clean medium monitored at 1.225, 0.5, and 0.1 MHz. *Shaded area* represents the transition phase, determined by conventional analysis. Data for dielectric properties were acquired every 60 s during the entire fermentation process. The symbols in the figure are bacilli (*black square*), spores (*white circle*), and *cry* expression (*triangle*); CO_2 production profile (*diamond*), residual glucose (inverted triangle), DO (minus sign), and dielectric properties: *Reactance* (*red line*), *Resistance* (*blue line*), *Impedance* (*green line*), *Angle phase* (*pink line*), *Conductance* (*gray line*), and *Voltage* (*orange line*)

phase, transition state, sporulation phase, and spore release, due to particular changes in their signals.

Discussion

Bacilli have the ability to undergo a cellular differentiation process leading to the formation of a spore that will germinate when growth-favoring conditions return. In order to cope with nutrient limitations, bacterial cells have to change their metabolism, therefore when the transition phase is settled up, sets of genes are turned off and others are turned on sequentially, and finally part of the bacterial population sporulates (Saujet et al. 2011). Additionally, as a population of quorum sensing bacteria grows, a proportional increase in the extracellular concentration of the signaling molecule occurs. When a threshold concentration is reached, the group detects the signaling molecule and responds to it with a population-wide alteration in gene expression (Bassler and Losick 2006). During vegetative growth, *B. thuringiensis* simultaneously consume glucose and amino acids (Farrera et al. 1998), Krebs cycle activity is almost null, and cells produce pyruvate, acetate, and lactate (Anderson 1990; Rowe 1990). During the transition phase, glycolysis flux is diminished, metabolites produced during vegetative growth are consumed, Krebs and glyoxylate cycles are active (Aronson et al. 1975), and citrate and butyrate can be produced (López-y-López and de la Torre 2005). In sporulation phase, Krebs, glyoxylate, and γ-aminobutyric cycles are still active. All these changes may be detected by impedance spectroscopy (Michaelis et al. 2013).

In spite of the studies and applications of dielectric spectroscopy used for monitoring microorganisms, as mentioned in the "Introduction" section, information regarding cell differentiation in bacteria throughout dielectric spectroscopy is non-existent. In this study, we found that frequencies with a range of 0.3 to 1.8 MHz correlate with the changes associated with the cell differentiation process of *B. thuringiensis* (Table 1, Fig 4). Therefore, to compare dielectric parameters, we selected the fixed frequencies of 1.225, 0.5, and 0.1 MHz, the latter two have been reported in other dielectric studies (Benoit et al. 1998; Guan et al. 1998; Gou et al. 2011; Schwarzenberger et al. 2011). At these frequencies, there is no apparent damage to the cell (Sanchis 2009).

Frequency utilized at fixed value is an important fact for obtaining a better relationship between dielectric properties and growth phases (Fig. 5). Usually, capacitance change (ΔC) or relative permittivity (ε) have been used as indicators of biomass growth, at frequencies ranging from 0.5 to 3.0 MHz (Kiviharju et al. 2008). Interestingly, Sarrafzadeh

et al. (2005) reported in fed-batch cultures of *B. thuringiensis* that the transition state can be detected in real time by means of dielectric permittivity. The authors mentioned that in such transition state, vegetative cells stayed almost constant and sporulated cells begun to appear. In the present work, we do not detect spores in the transition state. It is worth mentioning that the transition state per se in the *Bacillus* genera is the moment when the cell reorganizes their metabolic and genetic efforts to prepare to cells for sporulation, and it has been suggested by several works in the topic (Sonenshein 2000; Phillips and Strauch 2002; Banse et al. 2008) that sporulated bacilli neither spores exist during transition state in batch cultures.

In other studies, the calculated normalized *Impedance* has been used to follow the cell differentiation process of mesenchymal stem cells (Hildebrandt et al. 2010; Park et al. 2011) and embryonic cancer stem cells (Öz et al. 2013). Other authors used the normalized values of *Resistance* to follow the cell differentiation of mesenchymal stem cells (Cho et al. 2009) and for monitoring cell culture of insects (Luong et al. 2001). In both normalized properties, the signal response tends to increase as a function of time. Normalized values refer to the *Impedance* or *Resistance* values of culture medium prior to inoculation, a measurement at a determined frequency or a measurement of a particular condition of the system. Our results showed that the *Impedance* profiles (raw data) tend to decrease (Figs. 2c and 5), indicating that in initial conditions, the *Impedance* of medium culture is higher and attributable to the nutrients only. As the process continues, cellular growth, nutrient consumption (glucose, oxygen, etc.), as well as production of metabolites such as organic acids (López-y-López and de la Torre 2005) and CO_2 facilitate electron transport by means of dielectric effect, so that the *Impedance* value decreases. *Impedance* changes have been related to growth and alterations to the physiological state of the cells during cultivation (Ehret et al. 1997; Felice et al. 1999; Valero et al. 2010; Pänke et al. 2011; Whulanza et al. 2011; Gou et al. 2011; Justice et al. 2011; Michaelis et al. 2013).

In a way inversely proportional to *Impedance*, *Conductance* indicates the ease with which a material conducts an electrical current that modify the media composition changing the ionic content and provoking a change in the media conductivity (Ramírez et al. 2009). This contribution is principally due to metabolic activity and secretion of metabolite, as well as the release of intracellular material during cell lysis (Matanguihan et al. 1994), and the movement of ions through the membrane (Justice et al. 2011). In this work, the increase in *Conductance* is evident during the exponential phase and transition state, but during the sporulation phase, it remains almost constant, showing a slight increment during the release of spores (Fig. 5). Therefore, we suggest that increase in *Conductance* correlates with the metabolic changes during each growth phase (mentioned above) and cell lysis of *B. thuringiensis*. *Angle phase* is one of the clinically established dielectric parameters that indicate cellular health, cell membrane integrity, and cellular function in tissues and patients with different diseases, at a frequency of 50 kHz (Abad et al. 2011; Tanabe et al. 2012; Norman et al. 2012). There is no information regarding this dielectric property in the case of cell culture per se. However, *Angle phase* showed almost the same behavior as *Conductance*, and as frequency increases the signal value increases.

Interestingly, *Resistance* and *Reactance* at 1.225 and 0.5 MHz were the more suitable properties for monitoring changes related to the cell differentiation process, including events during exponential phase and transition state that were not elucidated using conventional techniques (Fig. 5). Electrical *Resistance* is the impediment to the flow of an electric current through a component consisting of a circuit, a medium, or a substance (Cammack et al. 2006), whereas *Reactance* is the non-resistive component of *Impedance* in an AC circuit, arising from the effect of inductance or capacitance or both, and causing the current to be out of phase with the electromotive force that causes it (Cammack et al. 2006). The changes in *Resistance* and *Reactance* profiles during exponential growth may suggest the triggering of cellular events that cannot be elucidated through conventional analysis techniques but must be taking place because of the onset of *B. thuringiensis* growth in batch cultures, as these events depend on the increased number of biochemical reactions and genetic responses that may include synthesis of genetic, signaling, metabolic, and structural molecules of cells, some of them independent of growth per se, but related to the programmed cell cycle of *B. thuringiensis*, indicating an easier electron transport in both culture medium and within the cells. Biochemical reactions within the cells can provoke high ionic activity throughout the cellular membrane, causing a reduction in *Resistance* and *Reactance* profiles due to easier transport of electrons (Luong et al. 2001; November and Van Impe 2002; Bonmassar et al. 2010; Pänke et al. 2011). Besides metabolic changes, one example of the possible explanations for changes in *Reactance* and *Resistance* during the exponential growth from a genetic regulation point of view is the effect of the transition state regulator, AbrB. Some reports indicate that maximal expression of AbrB occurred during early exponential growth phase in *B. subtilis* (Fürbaβ et al. 1991; O'Reilly and Devine 1997) and in *B. thuringiensis* (Lozano et al. 2014). The AbrB protein is involved in preventing inappropriate gene expression in actively growing cells and during the transition phase reorganizes the expression of more than 100 post-exponential genes with different biological functions, including biofilm formation, transport functions, antibiotic production, motility, development of competence for DNA uptake, synthesis of extracellular and degradative enzymes, and sporulation (Strauch and Hoch 1993; Lazazzera

2000; Chumsakul et al. 2011). Therefore, AbrB has an impact on the cell differentiation process of *B. thuringiensis*.

The negative values registered for *Resistance* and *Reactance* in experiments at fixed frequencies mean that cells are in an active network, and this does not imply biological system instability (Conciauro and Puglisi 1981). Hence, an analysis of the dielectric data obtained for the negative slope region makes it possible to establish the value of the external resistance of the measure device, at which oscillations are being generated and to estimate frequencies for these oscillations. Survila and Mockus (1999) mentioned that the negative values are related to the potential-dependent adsorption of the process involved in the system. Therefore, we suggest that the oscillations showed in Fig. 5 refer to high ion transfer through the membrane of bacilli for reduction in *Resistance* and *Reactance*, whereas increase values indicate a minus ion transfer due to metabolic reactions and genetic efforts depending of the exponential phase and transition state, which become constant during the sporulation phase. This indicates that the dielectric profile of *Resistance* and *Reactance* makes it possible to relate the different growth stages of the cell.

Identifying the moment when the cell differentiation process initiates is crucial and decisive to obtaining optimum results quickly in different studies at the cellular level for different organisms, as well as for many applications where it has practical consequences and which are important for many human interests. In our case, our principal interest was to observe the profile changes related to the cellular differentiation process, but further studies are required to further investigate the dielectric values obtained, therefore a robust online monitoring application of this technique must be developed. This study demonstrates that dielectric spectroscopy can be used for time-continuous, label-free monitoring of cellular events in the cell cycle, including the cell differentiation process in *B. thuringiensis*. *Resistance* and *Reactance* may represent useful properties for identifying minimal changes in real time and in situ, in relation to the cell differentiation process, as well as concerning the initiation of a cellular event that is not possible to evaluate using conventional techniques.

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