Intracellular pH determination of pristinamycin-producing Streptomyces pristinaespiralis by image analysis


INTRODUCTION

Pristinamycins belong to the synergistin family of antibiotics and are composed of two distinct groups of molecules, pristinamycins I (P$_I$) and pristinamycins II (P$_II$) (Preud’homme et al., 1968), which act synergistically to exert their bactericidal action. For example in therapy against Staphylococcus aureus infection it has been found that the most efficient ratio is 60% P$_{II}$/40% P$_I$ (Videau, 1982). As for two-thirds of all antibiotics, pristinamycins are produced, in batch process, by a filamentous bacterium of the genus Streptomyces (Demain, 1999), namely Streptomyces pristinaespiralis (Rhône-Poulenc, 1961).

Because there has been an ever-increasing demand for antibiotics (new molecules and higher doses) over the last decade (Craig, 1996), the optimization of the existing processes and the modification (improvement) of bacterial strains by genetic engineering (Baltz, 1998) are of great interest. Fine optimization of the pristinamycin production process requires good knowledge of cell physiology during the fermentation. In the context of studies on the physiology of actinomycetes, research teams have focused on metabolic changes occurring when antibiotic biosynthesis takes place under the influence of numerous external factors (Paquet, 1990; Demain, 1972). Assuming the existence of a close link between morphology and secondary metabolite production, most reports on image analysis of filamentous micro-organisms, including Streptomyces, present their morphology (Paul & Thomas, 1998; Cox et al., 1997; Yang et al., 1996). Because Streptomyces filaments can have different morphologies (filaments, clumps and pellets), depending on culture conditions and the physiological state of cells, separate studies have been required to investigate the relationship between each morphological type and metabolite production by image analysis (Drouin et al., 1997; Durant et al., 1994a, b; Cox & Thomas, 1992). In addition, image analysis has been used to determine the location of respiration activity in Streptomyces ambofaciens filaments (Mauss et al., 1997) and the leakage of cellular components through the membrane of S. ambofaciens using carbon gentian violet staining (Pons et al., 1998).

**Abbreviations:** BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester; pH, intracellular pH; R$_{527/600}$ green/red fluorescence ratio (527/600 nm).
Among the parameters controlling metabolic activities, intracellular pH (pH$_i$) plays a major role in the regulation of enzyme activities and transport kinetics of nutrients and metabolites. Furthermore, the pH gradient across cell membranes (ΔpH) is related to cellular energetic mechanisms such as ATP generation (Levval et al., 1997; Imai & Ohno, 1995). pH$_i$ assessment in eukaryotic cells is now well documented in the literature; fluorescent probes coupled to spectrofluorometric methods and image analysis techniques have been successfully used to investigate pH$_i$ of several cell lines (Heiple & Taylor, 1980; Slavik, 1983; Paradiso et al., 1987; Dix & Verkman, 1990; Chelet et al., 1999). On the other hand, pH$_i$ determination in prokaryotic cells by means of flow cytometry (Levval et al., 1997) or microscopy coupled to image analysis tools (Siegumfeldt et al., 1999) remains sparse and pH$_i$ assessment in filamentous bacteria, such as S. pristinaespiralis, has not been reported so far. Due to a limited resolution time for distribution measurement of labelled weak acids or bases and NMR methods, other techniques must be considered for such studies. However, because most Streptomyces species tend to grow as pellet and filamentous forms in submerged culture (Whitaker, 1991), flow cytometry cannot be used. Therefore, epifluorescence microscopy combined with image analysis has emerged as an alternative method.

In this study a protocol was developed for pH$_i$ measurement in the pristinamycin-producing species S. pristinaespiralis by epifluorescence microscopy and image analysis using the fluorochrome BCECF-AM [2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxy-methyl ester]. A second consideration concerns the methodology applied to monitor the variations of pH$_i$ during pristinamycin-producing batch culture of S. pristinaespiralis.

**METHODS**

**Bacterial strain.** Streptomyces pristinaespiralis mutant strain Pr11 was used for all experiments and was obtained from the Rhône-Poulenc-Rorer research centre, Vitry sur Seine, France.

**Media.** The following media were used in this study. Complex medium (g l$^{-1}$): sucrose, 15; corn steep (Roquette), 5; (NH$_4$)$_2$SO$_4$, 10; K$_2$HPO$_4$, 1; NaCl, 3; MgSO$_4$7H$_2$O, 0.2; CaCO$_3$, 1.25; pH 6.8; sterilized for 20 min at 120°C. Synthetic medium (g l$^{-1}$): glucose, 30; malonic acid, 7.5; l-arginine, 3; l-glutamate, 1.5; K$_2$HPO$_4$, 0.75; MgSO$_4$7H$_2$O, 0.3; FeSO$_4$, 7H$_2$O, 0.15; ZnSO$_4$.7H$_2$O, 0.55; CaCl$_2$.2H$_2$O, 0.4; 3-morpholinopropanesulfonic acid, 20; pH 6.8; sterilized for 20 min at 110°C. K$_2$HPO$_4$ and CaCl$_2$ in concentrated solutions were sterilized by filtration on 0.22 µm filters (Millipore) and added separately to avoid precipitation. All chemicals were analytical grade.

**Culture conditions.** Inocula were prepared by pouring 0.8 ml of a thawed spore solution calibrated at 3 × 10$^7$ c.f.u. l$^{-1}$ into 500 ml baffled shake flasks containing 80 ml complex medium. Flasks were shaken at 250 r.p.m. and 28°C for 44 h using an orbital shaker (Ika).

Bacteria used in the experiments dedicated to method development were grown in 300 ml Erlenmeyer flasks, whereas monitoring of pH$_i$ of pristinamycin-producing S. pristinaespiralis was performed on cultures grown in a 2-l bioreactor (CMF 100; Alpha-Laval-Chemap). Inocula (2 ml) were poured into 250 ml Erlenmeyer flasks containing 30 ml synthetic medium. Cells were grown with agitation at 250 r.p.m. and 28°C. For bioreactor batch cultures, 80 ml preculture was inoculated into 2 l synthetic medium at 28°C. The oxygen level was maintained at a minimum of 30% of air saturation and controlled through stirring conditions. Agitation with Rushton impellers ranged between 300 and 1500 r.p.m.

**Cell staining.** All experiments were carried out at 4°C in a dark room. Stock solutions (50 µl samples) of 1 mM BCECF-AM (Molecular Probes) in DMSO were stored in the dark at −20°C. Culture samples were directly diluted in PBS to obtain a final OD$_{590}$ of 1. Cells were incubated at 28°C on a rotary shaker with different concentrations of BCECF-AM (2.5–50 µM) for incubation times ranging from 0 to 40 min.

**In vivo calibration.** The [H$^+$/K$^+$] carboxylic ionophore nigericin and neutral ionophore valinomycin (Sigma) were used for pH$_i$ calibration as described elsewhere (Musgrove et al., 1986; Fressman, 1976). Nigericin was dissolved in absolute ethanol and valinomycin in DMSO at final concentrations of 10 and 20 µM, respectively. Both solutions were stored at −20°C. The cells used for the establishment of calibration curves were stained as described above. After a 30 s centrifugation at 6000 g and the removal of supernatant, cells were resuspended in high [K$^+$] buffers at different pH values (6.5–8.5). High [K$^+$] buffers were obtained by mixing appropriate quantities of 135 mM KH$_2$PO$_4$/20 mM NaOH and 110 mM K$_2$HPO$_4$/20 mM NaOH. Buffer solutions were filtered before use through 0.22 µm filters and stored at 4°C. When stated, osmolality of each buffered solution (measured with a Roebling osmometer) was adjusted to that of the fermentation broth by adding xylose. Nigericin was added at a final concentration ranging from 5 to 40 µM for 10 min. Valinomycin was added simultaneously to 20 µM nigericin at concentrations varying from 0 to 10 µM for different incubation times (5–30 min). Cells were then centrifuged again as described above, resuspended in the same high [K$^+$] buffer and kept on ice.

**Double emission ratio technique.** An epifluorescence microscope (DMRB; Leica Leitz) with ×10 magnification and a 0.3–0.4 numerical aperture PL Fluorot objective was used. The final magnification was ×100, because of the camera and the ×10 magnification of the objective. The light source was a 50 W Hg lamp (Osram). Excitation and emission band pass filters were assembled. Whereas the excitation filter was the same for both modules (480/40 nm), two different emission filters were used: 527/30 nm (green) and 600/40 nm (red). Stained cells (20 µl) were carefully spread onto a glass slide. After the 527 nm image (green image) was taken and saved, another image of the same field (same pellet) was acquired at 600 nm (red image). Photobleaching of the stained samples was considered as negligible because only a 5% decrease in fluorescence intensity was registered after five ratio measurements on the same field (10 successive excitations). Auto fluorescence of the cells was shown to have no influence on the calculated ratio, since the mean fluorescence ratio at 527/600 nm (R$_{527/600}$) was 1.00 (sd < 0.01) and pixel level intensity was inferior to image processing threshold values. No correlation could be found between ratio values and pellet size, suggesting that the methodology can be used for pellets of different sizes. For each experiment, 20–30 images were recorded at each emission wavelength. Error bars on the graphs represent the SD of mean R$_{527/600}$ values.

**Image processing.** An integrated controlled CCD monochrome camera (Cohu) was fitted onto the epifluorescence...
microscope. It was controlled via a PC through an acquisition card (Secad Vision) which allowed capture of 768 × 576 pixel TIFF images coded on 256 grey levels. The integration time was set to 25 ms for each image. Image treatment was carried out on a PC using professional image analysis software (Visilog; Noesis).

The aim was to obtain images with pixel values directly connected to the pH value (pH image) by using the double ratio technique described. The first part of the treatment deals with the segmentation of these images. This operation was carried out by an automatic thresholding technique based on image entropy. Concerning the image, entropy is maximum for an equally distributed grey level histogram and equal to zero for an image with only one grey level. This technique is used to segment images into two regions by maximizing the total entropy (Coster & Chermant, 1989). It has been shown that such a segmentation best fits images containing pellets (Pons & Vivier, 1999). Consequently, two binary images were obtained, one for each initial grey level image. The binary image only contains the relevant information (object), with pixels set to 1 and background pixels set to 0. Then, a logical operation (intersection) between these images gives a single binary image representing the common part of the initial images. After morphological operations (dilation and reconstruction) to eliminate artefacts due to the thresholding, a final binary image is obtained and used as a mask for further operations. Masking is a point operation between a grey level and a binary image that produces a grey level image. In such an image, only pixel parts of the object (i.e. the pellet) are visible in grey level, the background pixels being set to black (0). Then, both grey level images (527 and 600 nm) were masked and the mean grey levels of the pellets and the SD values were computed. These values are good indicators of the quality of calibration images and they were used as criteria for the final pH calculation. Finally, after transformation in floating point images to allow arithmetic operations, a pixel to pixel division between images was performed to get the pH image. Mean grey level and SD values were also calculated on this final image to give values related to pH.

Additional analytical methods. During batch fermentations samples were collected to determine glucose, pristinamycin and dry weight biomass levels.

OD₅₈₆ was measured according to the method of Lubbe et al. (1985) by means of a Beckman spectrophotometer, DU 7500. Dry weight biomass was determined gravimetrically by filtering 10 ml fermentation broth on pre-weighed 0.45 µm cellulose acetate membranes (Sartorius). Membranes were washed twice with 0.85% NaCl and placed in an oven at 100 °C for 12 h before being weighed again. An OD₅₈₆ vs dry weight correlation curve allowed dry weight biomass determination.

Pristinamycins were extracted and analysed by HPLC as described by Thibaut et al. (1995) with a Spectra-Physics HPLC system.

Glucose concentration was determined by HPLC according to the protocol described by Rondags et al. (1998).

RESULTS

Method development

Microscopic and morphological parameters. Due to the characteristics of the genus Streptomyces, S. pristinaespiralis tends to grow as a pellet of several hundred micrometres in submerged culture. Moreover, during the pristinamycin production process, most of the cells are aggregated and form small pellets. Consequently, pHᵢ was determined only on pellets, the predominant morphological form in this study.

Because magnifications of ×1000 and ×400 led to a dramatic photobleaching of the fluorescent probe, a final magnification of ×100 was chosen. Using this magnification, a 25 ms exposure time was used to capture images.

Optimization of the BCECF-AM staining technique. The protocol for pHᵢ measurement was first optimized by testing the effect of various dye concentrations and staining times on the measured fluorescence of S. pristinaespiralis. Cell samples were submitted to increasing concentrations of BCECF-AM, from 1 to 60 µM, for 30 min at 28 °C. The measured mean R₅₂₇/₆₀₀ values of the samples are reported in Fig. 1.

For fluorochrome titres below 20 µM, the fluorescence ratio varies with the dye concentration, whereas at higher concentrations it remains rather constant. Because titres over 20 µM gave an increasing SD and an...
important stain release, 20 µM BCECF-AM was retained as a compromise concentration for further experiments. The optimized staining time with the fluorescent dye was determined using the same criteria (Fig. 2): 30 min staining was chosen for further experiments.

**In vivo calibration.** The effect of nigericin concentration was first assessed on stained samples suspended in high [K⁺] buffer (pH 7.0) (Fig. 3). The optimal nigericin concentration was found to be 20–30 µM, since concentrations above or below reduced the \( R_{527/600} \) values. Concentrations higher than 40 µM led to cell lysis. However, the \( R_{527/600} \) SD values found for different nigericin titres remained rather high. In addition, cell suspensions in high [K⁺] buffers at different pH values (ranging from 6.5 to 8.5) using only nigericin did not show a linear relationship between \( R_{527/600} \) and external pH.

Thus, the effect of simultaneous addition of 20 µM nigericin and valinomycin concentrations ranging from 0 to 10 µM on the \( R_{527/600} \) values of the cells was assessed. It appeared that valinomycin at low concentrations had no significant influence on ratio values, but the heterogeneity of pellet \( R_{527/600} \) values was diminished considerably, i.e. at 1 µM valinomycin the SD of the mean ratio was reduced fivefold \((1.41 ± 0.1)\) compared to the assay without valinomycin \((1.43 ± 0.5)\). When higher valinomycin concentrations were used, dye was released, which considerably decreased the precision of the measurements. An investigation into the sequence of ionophore addition revealed that the best combination was a simultaneous addition of nigericin and valinomycin (data not shown). Thus, simultaneous addition of 20 µM nigericin and 1 µM valinomycin was chosen for further experiments.

To optimize the ionophore incubation time, suspensions of *S. pristinae spiralis* were stained with BCECF-AM and incubated with 20 µM nigericin/1 µM valinomycin at 28 °C in high [K⁺] buffer (pH 7.0) for times varying from 10 to 30 min. \( R_{527/600} \) was constant \((1.46 ± 0.05)\) whatever incubation time was used. On this basis, a 10 min incubation time was chosen for further experiments.

For the calibration of the technique, cells were resuspended in high [K⁺] buffers at different pH values ranging from 6.5 to 8.5. The osmolality of the buffers was adjusted to that of the culture medium by means of xylose to avoid osmotic shock. Nigericin and valinomycin were added to the samples to equilibrate the pH of the stained cells to the pH of the surrounding buffer.

Calibration curves – green/red fluorescence ratios vs pH – were established for three samples of growing cells sampled after 24 h culture (Fig. 4). After being captured by the monochrome camera, image analysis showed that the equation of the calibration curve was slightly different for pellets with grey level images between 20 and 25, and between 25 and 30, at 600 nm. Thus, two calibration curves were used. One curve was drawn for pellets having mean grey levels between 20 and 25 at 600 nm and another was determined for mean grey levels between 25 and 30 (Fig. 5). For pellets with grey
levels at 25 at 600 nm, pH\textsubscript{i} values were calculated using both equations.

Interestingly, calibration curves established for bacteria sampled at different culture times showed the same relationship between \( R_{527/600} \) and pH\textsubscript{i}, indicating that the age of the \textit{S. pristinae spiralis} culture has no influence on the \textit{in vivo} calibration.

To investigate a possible effect of the three-dimensional size and shape of the pellets on axial resolution (depth of field), topographic sections across pH pellet images were taken (Fig. 6a). If there was an effect, a central pixel displaying a pH\textsubscript{i} similar to another at the periphery of the pellet would have a higher \( R_{527/600} \). In fact, as shown in Fig. 6(b), the mean \( R_{527/600} \) values of the peripheral pixels (at the termini of the straight line) were identical to the values of the pixels from the centre of the pellet (middle of the line), indicating that the surface fluorescence is not affected by the depth of the pellet. Pellets presented a homogeneous pH\textsubscript{i} over a large part of their surface and the sd of mean \( R_{527/600} \) values for pixels of a given pH\textsubscript{i} remained very low over the whole surface of the pellets.

Using these experimental conditions, pH\textsubscript{i} was determined with satisfactory precision, since the error did not exceed 0·3 pH units for pH\textsubscript{i} values between 6·5 and 8·5.

**Bioreactor batch culture of pristinamycin-producing \textit{S. pristinae spiralis}**

The protocol presented for pH\textsubscript{i} determination was applied to the pristinamycin-producing \textit{S. pristinae spiralis} grown under stable pH conditions (external pH 6·8). To evaluate growth, glucose consumption, pristinamycin production and pH\textsubscript{i} of \textit{S. pristinae spiralis}, a bioreactor was operated in batch mode over a 40 h period (Fig. 7a, b). The pH\textsubscript{i} of the pellets of each culture sample was calculated with the calibration curve corresponding to the mean grey level at 600 nm. The dry weight biomass level, initially 0·4 g l\textsuperscript{−1}, increased to a maximum of 8·0 g l\textsuperscript{−1} within 40 h (Fig. 7a). Growth stopped independently of carbon limitation as the glucose concentration was still around 20 g l\textsuperscript{−1} at that time.

Contrary to the external pH, pH\textsubscript{i} showed important variations during the course of fermentation (Fig. 7b). Starting from an initial value of 7·2, pH\textsubscript{i} quickly increased to values around 8·2 during the first 10 h. Then it dramatically decreased, to reach 6·2 after 17 h culture. This value remained stable for a 10 h period and finally came back to near its initial value during the last period of the process. Further pH\textsubscript{i} determinations during the stationary phase were not possible due to significant probe leakage. Because the extracellular pH was quite constant around 6·8, the pH\textsubscript{i} and ΔpH profiles were similar during the batch process. As seen, the cells were capable of maintaining a positive ΔpH throughout the culture period, except during the 10 h period preceding the excretion phase.

This culture was performed in triplicate (data not shown) and identical pH\textsubscript{i} profiles were observed, though minor differences regarding the pH\textsubscript{i} values were obtained during the three processes (± 0·4 pH units).
DISCUSSION

The first aim of this study was to develop a new method for the measurement of \( \text{pH}_i \) in filamentous streptomycetes. Because flow cytometry cannot be used for filamentous bacteria, and NMR or radiolabelled weak acid/base distribution analyses are even more difficult and tedious techniques requiring high cell densities and specific materials, an image-analysis-based fluorometric method was developed.

The fluorochrome BCECF-AM, with a \( pK_a \) of 7.0, was found to be efficient for \( \text{pH}_i \) determination of neutrophilic bacteria, such as \( S. \) pristinaespiralis, grown in a bioreactor: the fluorescence was proportional to the \( \text{pH}_i \) of the cells between \( \text{pH} \) values of 6.5 and 8.5, and the fluorescence of the stained cells was stable for at least 30 min.

During this study only pellets of \( S. \) pristinaespiralis were taken into account because the bacteria mainly grow in this form during the pristinamycin-producing process. Another reason for this choice is that the origin of dispersed filaments present in the culture medium is not always clear. They can be present as a consequence of the alteration of fluffy pellets caused by shearing forces produced by the increased agitation necessary for the regulation of dissolved oxygen. Moreover, simultaneous analysis of all morphologies present in the culture has been reported to be impossible since a \( \times 100 \) oil immersion objective \( (\times 1000 \text{ final magnification}) \) generally requires the use of coverslips for filament analysis which can disturb the physiology of the cells in the pellets (Nielsen et al., 1995; Cox et al., 1998).

The \( \text{pH}_i \) of \( S. \) pristinaespiralis cells only from the surface of the pellets (projected area) was considered. In fact, surface cells within the pellets can be considered as the most active cells because pellets have been described to have hollows in their centre (Thomas, 1992). These hollows form due to cell necrosis as a result of limitations in oxygen and nutrients.

In vivo calibration is essential to avoid response variability due to differences in microscope focusing between BCECF solutions \( \text{(in vitro calibration)} \) and BCECF-AM-stained pellets.

Satisfactory correlation coefficients and linearity were found with the proposed nigericin-valinomycin protocol over the \( \text{pH} \) 6.5–8.5 range. Contrary to the results of Boyarsky et al. (1996a, b) using eukaryotes, enough \( K^+ \) was able enter the pellets to equilibrate extracellular and \( \text{pH}_i \) even at basic \( \text{pH} \) values. Addition of xylose, resulting in increased osmolality of high \([K^+]_o \) buffers, could be used to avoid \( K^+ \) efflux in response to hypotonic shock. Such inward or outward cationic fluxes have been described in response to medium osmolality changes (Guillouet, 1996). One explanation for the efficient combination of valinomycin and nigericin at these concentrations could be their overwhelming action on mechanisms of cell regulation, allowing equal distribution of \( K^+ \) at intracellular and extracellular levels, without any residual \( K^+ \) gradient, contrary to the results of Boyarsky et al. (1996a, b).

Slight differences in 600 nm pixel intensity for images of pellets resuspended in buffer with the same \( \text{pH} \) could generate slightly different \( R_{527/600} \) ratios, as seen during this study. Differences in staining have been reported for cells having different esterase activities in the culture (Franch et al., 1996). However, the difference in 527 nm fluorescence should have been of the same order. One explanation could be a different sensitivity of the camera, which is described as more efficient around 527 nm, at the two wavelengths. Another reason is that 600/40 nm fluorescence is not really an isosbestic point (Franch et al., 1996). Actually, this artefact could be corrected using two different calibration curves, according to the level of fluorescence at 600 nm.

The advantage of the image analysis algorithm developed here is that it does not modify any pixel level. This results in the collection of unmodified data and it gives a more accurate estimation of actual \( \text{pH}_i \). Since fluorescence was pellet-area-independent, contrary to other studies dealing with morphology by image analysis (Sieracki et al., 1989; Durant et al., 1994a, b), no delimitation problems were encountered by using an automated entropy threshold. The simplicity and rapidity (less than 1 min for calculation) of this procedure are favourable to on-line routine analysis during fermentation processes.

This paper presents for the first time a method for the determination of \( \text{pH}_i \) of filamentous bacteria by means of epifluorescence microscopy and image analysis using a \( \text{pH} \)-sensitive fluorescent probe. Although a positive control was not employed during this study \( \text{(use of a bacterium with a known \( \text{pH}_i \))}, \) the different tools and techniques utilized have already been reported to be efficient for the determination of \( \text{pH}_i \) in bacteria. First, BCECF is a valuable fluorochrome to determine \( \text{pH}_i \) since it has been shown to perfectly corroborate \( ^{31} \text{P-NMR} \) spectroscopy data obtained for Propionibacterium acnes (Futsaether et al., 1993). Moreover, Slavik (1997) has shown that the BCECF emission ratio technique can be employed for confocal microscopy to determine \( \text{pH}_i \). In addition, \( \text{pH} \) determination of \( Listeria innocua \) using epifluorescence microscopy and image analysis (Siegumfeldt et al., 1999) confirmed the results obtained by spectrofluorimetry (Breeuwer et al., 1996).

During this study, the \( \text{pH}_i \) of \( S. \) pristinaespiralis grown in batch culture was measured by an image analysis-based method using the fluorochrome BCECF-AM at a final concentration of 20 \( \mu M \) at 28 °C and for 30 min. As shown, \( \text{pH}_i \) could be correlated to pristinamycin excretion and glucose consumption in batch process. In fact, \( \text{pH}_i \) was found to be a good marker of the physiological state of the cells cultivated under pristinamycin-producing conditions. Furthermore, \( \text{pH}_i \) assessment can contribute to the understanding of variations in carbon fluxes through the enzymes involved in glucose metabolism, and \( \Delta \text{pH} \) calculation may help in the
establishment of kinetic models for excretion and consumption of metabolites. Finally, the new method presented in this paper for pH$_i$ assessment of *S. pristinaespiralis* could be extended to filamentous bacteria of fungal cells cultivated *in vitro*, provided that the staining protocol was adapted to each cell line.

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