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A protein-based oxygen biosensor for high-throughput monitoring of cell growth and cell viability

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ABSTRACT

Fluorescently labeled hemocyanin has been previously proposed as an oxygen sensor. In this study, we explored the efficacy of this biosensor for monitoring the biological oxygen consumption of bacteria and its use in testing bacterial cell growth and viability of *Escherichia coli, Pseudomonas aeruginosa, Paracoccus denitrificans,* and *Staphylococcus simulans.* Using a microwell plate, the time courses for the complete deoxygenation of samples with different initial concentrations of cells were obtained and the doubling times were extracted. The applicability of our fluorescence-based cell growth assay as an antibacterial drug screening method was also explored. The results provide a proof-of-principle for a simple, quantitative, and sensitive method for high-throughput monitoring of prokaryotic cell growth and antibiotic susceptibility screening.

During the past decade, remarkable advances have been made in the design of methods for determining growth and viability of living cells. Measurements of the growth of microorganisms play a key role in a wide variety of applications such as development and testing of antibiotics, preservation or fermentation conditions of food [1,2], and optimization of the conditions for cell culture [3]. Cellular growth is typically measured by monitoring the optical density of a culture at 600 nm. However, this method does not account for the number of viable cells present in the culture; rather, it accounts for the total number of cells or cell detritus. To circumvent this limitation, cell-counting methods, such as microscopy and plate counting, are normally used. Although these methods can easily discriminate viable cells, they are not suitable for fast high-throughput screening [4]. In particular, in the commonly used plate-counting method, the time needed for the formation of visible colonies is relatively long and also lacks sensitivity [5]. A similar problem occurs with the widely used disk diffusion method for testing antibiotic sensitivity, which is also time-consuming and, in some cases, not very accurate [6]. Automated instruments, such as electric cell counters and flow cytometers, can partially alleviate these problems, but they require not only sophisticated and expensive equipment but also significant technical expertise [7]. These reasons motivate the development of faster and larger scale methods to measure cell growth and viability. Monitoring cellular metabolic activity has been recently proposed as a valid alternative to the assessment of cell viability and growth [5,8]. Oxygen is one of the key substrates in aerobic systems; therefore, the ability to consume oxygen is a good indicator of cellular metabolic activity and so can be correlated to cell viability and growth. Different methods for oxygen detection have been reported, and for a long time the Clark-type electrode has been considered as the method of choice for monitoring the respiration of cells [9].

During the past few years, new methods based on fluorescence-detected oxygen consumption have been developed for use in high-throughput systems. The BD BioSensor, commercialized by BD Bioscience, was the first reported example. This method uses 96- or 384-well plates containing a ruthenium-based fluorescent compound immobilized in an oxygen-permeable silicon matrix [8]. In the presence of oxygen, the fluorescence of the ruthenium compound is quenched; thus, an increase in the fluorescence signal indicates oxygen depletion. This system has been shown to be suitable for determining growth and viability of a variety of bacteria, mammalian cells, and fungi [8]. Phosphorescent porphyrin dyes [10,11] and a platinum complex [5] embedded in polymers have also been reported as oxygen-sensing materials. These studies have evolved

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during recent years to demonstrate the high sensitivity and versatility of optics-based cell assays [7,12].

Recently, we and others have shown that it is possible to detect oxygen using a type 3 copper protein labeled with a fluorescent dye [13,14]. Type 3 copper proteins possess a binuclear copper center that can specifically bind a molecule of oxygen [15]. On binding, the two reduced Cu(I) ions are converted into a [Cu(I)–O_2–Cu(I)] complex and two new absorption bands appear at 340 and 570 nm (Fig. 1A). This change in absorption can be translated into a change of fluorescence intensity of an attached label through a Förster resonance energy transfer (FRET)[2] mechanism [14]. In this way, when the protein is in its deoxygenated state, all the energy absorbed by the label is emitted as fluorescence. On oxygenation, some of this energy is transferred by radiationless decay to the metal center, resulting in a decrease in the fluorescence intensity (Fig. 1B). The high selectivity provided by the protein's oxygen binding in combination with the high sensitivity of fluorescence detection makes this method an excellent tool for the development of a new generation of oxygen biosensors. We have similarly shown that fluorescently labeled hemocyanin, immobilized within a silica matrix, can be used as a solid-state, reusable, and biocompatible oxygen-sensing device [16]. In the current work, we explore the efficacy of this biosensor for monitoring the biological oxygen consumption by bacteria and for testing cell growth and viability. We used hemocyanin from *Rapana thomasiana* fluorescently labeled with Cy3 dye. Three different gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa,* and *Paracoccus denitrificans*) and one gram-positive bacterium (*Staphylococcus simulans*) were tested for growth and antibiotic susceptibility. Three antibiotics (ampicillin, kanamycin, and chloramphenicol) and two lantibiotics (Pep5 and nisin) were used. These organisms and antibiotics were selected because of their ease of handling and frequent use in microbiological laboratories and because they represent major classes of microorganisms and antibiotics with different mechanisms of action, criteria that we considered as important for a proof-of-principle for the method. The simple preparation of our biosensor and its flexible application in standard cuvettes or microwell plates make it suitable for small- and large-scale measurements. Moreover, the protein-based selectivity to monitor oxygen as presented in this work can be converted into sensitivity for other compounds by using other suitably selected proteins.

Materials and methods

General

Hemocyanin from the mollusk *R. thomasiana* was prepared as described elsewhere [17]. Stock solutions of 10 mM Cy3 *N*-hydroxysuccinimide (NHS) ester (Amersham Biosciences, Freiburg, Germany) were prepared by dissolving the powder in water-free dimethyl sulfoxide (DMSO). PD-10 gel filtration columns (Amersham Pharmacia) were used for the purification steps after protein labeling. DMSO and mineral oil were obtained from Sigma–Aldrich (St. Louis, MO, USA). Kanamycin, ampicillin, and chloramphenicol were purchased from Duchefa (Haarlem, The Netherlands). Standard polystyrene 96-well U-bottom plates were purchased from Corning (Corning, NY, USA). Nisin was purified from batch fermentation as described previously [18]. Pep5 was purified as described by Sahl and Brandis [19].

Strains

E. coli (JM109, *Kan*^{r}), *P. aeruginosa* (CIT 135) [20], and *P. denitrificans* (PD 1235) [21] cells were grown on Luria–Bertani (LB) broth agar. Cultures of all the organisms were grown aerobically in LB medium in a shaker at 250 rpm and 37 °C (*E. coli*) or 30 °C (*P. aeruginosa* and *P. denitrificans*) until OD_{600} = 1. *S. simulans* [19] cells were grown in a similar way at 30 °C in tryptone–soy broth.

Protein labeling

According to literature procedures [22], hemocyanin was labeled with Cy3 in 100 mM potassium phosphate buffer at pH 6.8. Labeling ratios (dye molecule/protein) were kept in the range of 0.2 to 1, as quantified from the absorption spectra of the labeled samples by using the extinction coefficients of 280 nm protein absorption (ε_{280} = 75 mM^{-1} cm^{-1}) [23] and Cy3 (ε_{550} = 150 mM^{-1} cm^{-1}) (data provided by manufacturer). Bovine serum albumin (BSA) was labeled using the same conditions and served as a control.

FRET-based oxygen-sensitive assay

A typical experiment using a 96-well U-bottom plate was performed as follows. Serial dilutions of a freshly grown cell culture were made in LB to achieve 10^9 to 10^2 colony-forming units (cfu, confirmed by colony plate counting). Wells were prepared in triplicate by filling each well with 180 µl of the cell dilution plus 20 µl of Cy3-labeled hemocyanin solution (0.5–1 µM end concentration). To avoid evaporation and rapid oxygen diffusion, 50 µl of mineral oil was added on top of each well. For antibiotic resistance tests, the initial number of cells was 10^7 cfu/well; in the case of *S. simulans,* it was 10^6 cfu/well. Ampicillin, kanamycin, and chloramphenicol were added to a final concentration of 0.05 to 5000 µg/ml to the gram-negative bacteria, and Pep5 and nisin were added to a final concentration of 0.25 to 25,000 ng/ml for the gram-positive ones. Controls were performed by adding Cy3 or Cy3-labeled BSA instead of Cy3-labeled hemocyanin to the cell culture as well as by using unlabeled hemocyanin. The fluorescence of Cy3-labeled hemocyanin in LB without cells was also monitored as a control in the absence of oxygen consumption.

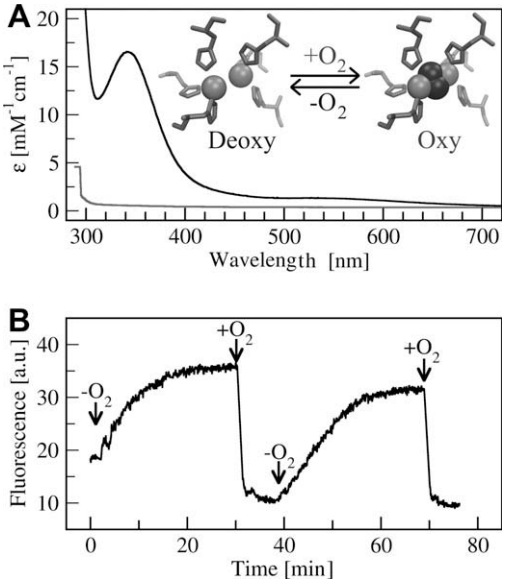

Fig. 1. (A) Absorption spectra of the deoxygenated (gray) and oxygenated (black) forms of hemocyanin. The structure of the metal center for each form is represented in the same panel showing the His, coppers (light gray), and oxygen (dark gray). (B) Example of a fluorescence time trace of Cy3-labeled hemocyanin in potassium phosphate buffer while bubbling argon (–O_2) or oxygen (+O_2).

[2] *Abbreviations used:* FRET, Förster resonance energy transfer; NHS, *N*-hydroxysuccinimide; DMSO, dimethyl sulfoxide; LB, Luria–Bertani; BSA, bovine serum albumin; cfu, colony-forming units; *Kan*^{r}, kanamycin resistance.

Control experiments

Controls were carried out by growing cells aerobically in a shaker at 250 rpm and 30 °C for *P. aeruginosa, P. denitrificans,* and *S. simulans* or 37 °C for *E. coli.* The growth of these cultures was followed by measuring the optical density at 600 nm (OD_{600}) (SmartSpec 3000, Bio-Rad, Hercules, CA, USA). If necessary, samples were diluted to maintain the measured OD_{600} below 0.5 to prevent deviations from linearity. To calculate the doubling time, 20 ml of fresh medium in 100-ml flasks were inoculated with 20 ml of a stationary culture. Samples (0.1 ml) were taken at given times, and the OD_{600} was measured. For each set of data, the doubling time was calculated from the exponential region of the growth curve. Antibiotic susceptibility assays were carried out in 2 ml of medium in 10-ml disposable plastic tubes and inoculated with 10^5 cfu. Antibiotics were added to the same concentration as used in the fluorescence experiments. Samples (0.1 ml) were taken at 0, 6, 12, and 24 h. In the case of *P. denitrificans,* an additional sample was collected at 48 h.

Absorbance and fluorescence measurements

Absorption spectra were recorded on a Cary 50 spectrophotometer (Varian, Walnut Creek, CA, USA). Fluorescence spectra and time traces were measured on a Cary Eclipse Fluorimeter (Varian) with a 96-well plate reader accessory. During the measurements, the temperature of the plate was kept constant using a thermostated water bath.

Results

Detection of bacterial oxygen consumption

As we have reported previously, a switching ratio of 55% for the construct of *R. thomasiana* hemocyanin labeled with Cy3 going from the O_2-bound form to the free form was found [16]. Although such a high switching ratio is advantageous, it is important to note that the only requirement for the method to work is the ability to detect a change in fluorescence due to a change in the oxygen concentration in the solution. This implies that the exact concentration of labeled protein or the amount of scattered light has no influence on the results. These properties make the current system ideal for detecting oxygen depletion in complex mixtures and turbid solutions such as bacterial cultures. When a bacterial culture grows aerobically, the oxygen in the medium is consumed. In our system, no shaking was applied to the 96-well plate used in the experiments, and a layer of mineral oil sealed each well, so the oxygen consumption was assumed to be faster than the diffusion of fresh oxygen from the air into the culture. This results in a drop of the oxygen concentration, eventually resulting in an anaerobic medium. When Cy3-labeled hemocyanin is present, this oxygen depletion causes an increase of the fluorescence intensity of the covalently attached label on the protein surface.

Fig. 2A shows the normalized fluorescence time traces of *E. coli* cultures for different initial numbers of cfu containing Cy3-labeled hemocyanin. The fluorescence intensity at 570 nm (excitation at 550 nm) was followed over approximately 10 h at 37 °C in a 96-well plate. The experiment was performed in triplicate for each initial cfu number. Fig. 2A shows that two distinct fluorescence intensity levels (low and high) can be distinguished. These were assigned to the oxygenated (low) and deoxygenated (high) form of Cy3-labeled hemocyanin, as described previously [14]. The increase in fluorescence intensity reflects the oxygen depletion from the medium. No significant changes in the fluorescence intensities were observed in the absence of cells or when Cy3, Cy3-labeled

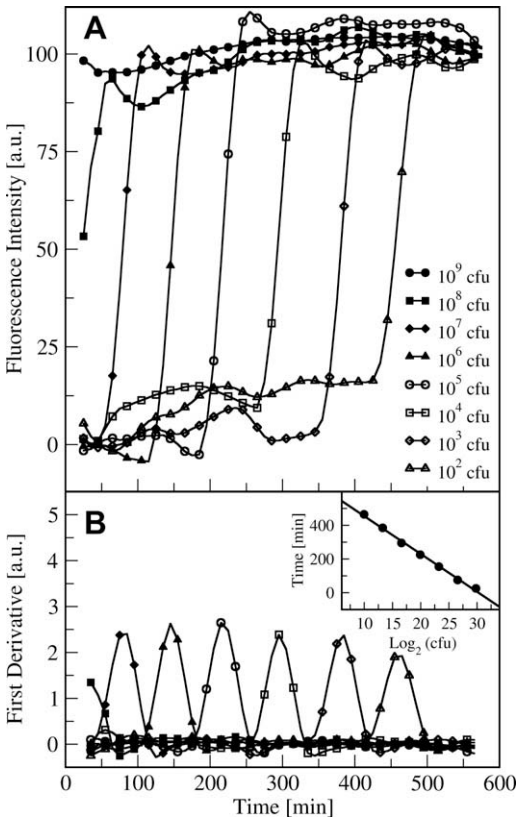

Fig. 2. (A) Fluorescence intensity time traces at 570 nm (excitation 550 nm) of cultures of *E. coli* containing Cy3-labeled *R. thomasiana* hemocyanin (~ 1 μM) as a function of the initial cfu (specified in the figure). (B) First derivative of the traces presented in panel A. Inset: estimation of the doubling time calculated from the absolute value of the slope of the linear regression (black line). The time to the midpoint transition is plotted versus the base 2 logarithm of the initial cfu.

BSA, or unlabeled hemocyanin was used as sensing material. The switching between these two levels took place at different times, depending on the initial number of cells present in the well. As can be seen in Fig. 2A, in the most concentrated culture the fluorescence is already maximal at the beginning of the experiment, whereas for the second highest cfu the switch occurred almost instantaneously. This indicates that the oxygen present in the well was consumed immediately. For more diluted cultures, the switching of the fluorescence took progressively longer, indicating that it took more time before the oxygen supply was exhausted. Even if the time traces were noisy, the well-defined fluorescence intensity levels made possible the determination of the midpoint transition from the first derivative of these traces (Fig. 2B). By plotting the time to the midpoint as a function of the base 2 logarithm of the initial cfu, it was possible to obtain the duplication time of the organism from the absolute value of the slope [12] (Fig. 2B, inset). The average duplication time for *E. coli* at 37 °C, determined from three independent experiments, was 22 ± 1 min, comparable to the 23 ± 1 min obtained in the control experiment where the optical density at 600 nm was followed in time (see Materials and Methods) and in agreement with published values [24]. This shows that our method is suitable for measuring *E. coli* growth quantitatively.

To test this system at different temperatures, the same experiment was carried out at 37, 32, 27, 24, and 18 °C (data not shown). For all temperatures tested, linear relationships resulted when plotting the time to reach the midpoint fluorescence versus the $\log_2$ of the initial cfu (Fig. 3). The obtained doubling times were in accordance with literature values [24]. It is known that the dou-

bling time of *E. coli* and other simple organisms decreases with temperature, following an activation type of equation similar to that proposed by Arrhenius for simple chemical reactions [25,26]. As shown in Fig. 3B, our data follow this equation, giving a straight line when the inverse of the natural logarithm of the doubling time was plotted versus the inverse of the absolute temperature. From the slope of the linear regression, an apparent activation energy for the duplication of *E. coli* of 58 ± 7 kJ K^{-1} mol^{-1} was obtained. The good agreement of this apparent activation energy with previously reported values [26] substantiates that no protein-related artifacts were interfering with the measurements.

Aiming at extending our protein-based growth assay to different gram-negative strains, *P. aeruginosa* and *P. denitrificans* cell cultures were also tested. The respiration profiles were measured at 30 °C for both strains, and the resulting plots resembled the ones obtained for *E. coli.* Doubling times of 30 ± 1 min for *P. aeruginosa* and 80 ± 1 min for *P. denitrificans* were obtained, with both values being in accordance with literature data [27,28]. From the control experiments in which the OD_{600} was followed in time, values of 29 ± 2 and 68 ± 5 min were obtained for *P. aeruginosa* and *P. denitrificans,* respectively.

S. simulans was used as an example of the application of the sensor to gram-positive bacteria. Fluorescence time traces of *S. simulans* cultures were noisier than the ones obtained for the other organisms tested, and (differently) the fluorescence intensity went down after reaching a maximum (Fig. 4A). This is an indication that, after depletion from the culture, oxygen diffusion into the solution becomes perceptible and oxygen is no longer consumed. One of two different scenarios may explain this: (i) the cells die after reaching the stationary phase or (ii) an anaerobic metabolism sets in when the oxygen concentration drops below a certain threshold value. A close inspection of the fluorescence time traces shows in all cases that, at a later state, the fluorescence starts to increase again (Fig. 4A). This could indicate a return to aerobic metabolism, evidence for scenario (ii). Further investigation is required to better understand this behavior. Despite the complexity

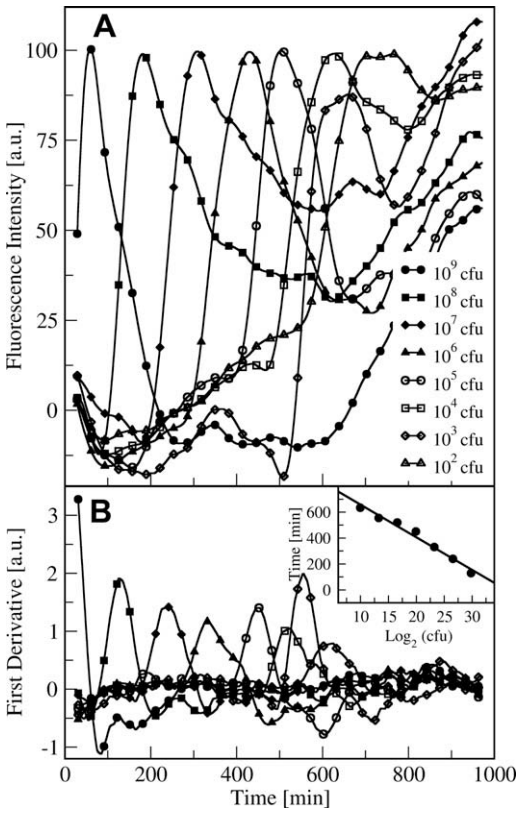

Fig. 4. (A) Fluorescence intensity time traces at 570 nm (excitation 550 nm) of cultures of *S. simulans* containing Cy3-labeled *R. thomasiana* hemocyanin as a function of the initial cfu (specified in the figure). (B) First derivative of the traces presented in panel A. Inset: estimation of the doubling time calculated from the absolute value of the slope of the linear regression (black line). The time to the midpoint transition is plotted versus the base 2 logarithm of the initial cfu.

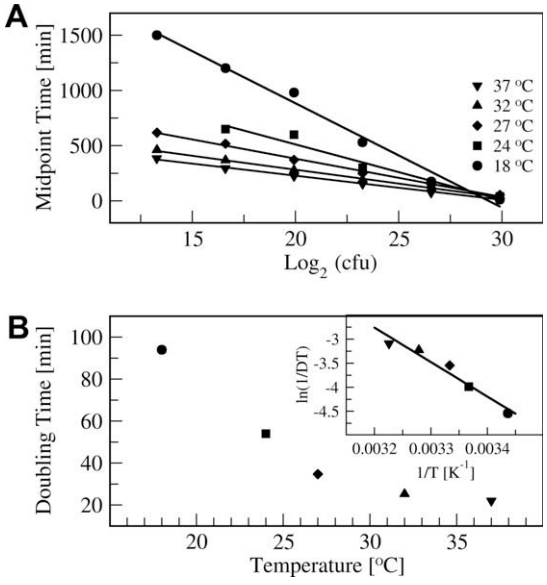

Fig. 3. (A) The time to the midpoint transition is plotted versus the base 2 logarithm of the initial cfu for the fluorescence time traces obtained at 18, 24, 27, 32, and 37 °C. For each series, the black line represents the linear regression from which the doubling time was determined. (B) The dependence of the calculated doubling times on the incubation temperatures is shown. Inset: Arrhenius plot of the doubling time versus the inverse absolute temperature. The black line represents the linear regression of the experimental data.

of the fluorescence time traces, the first derivative analysis still leads to well-defined maxima (Fig. 4B). A doubling time of 25 ± 2 min was calculated (Fig. 4B, inset), close to the 31 ± 2 min obtained in control experiments and in good agreement with reported values for similar organisms [29].

Antibiotic screening assay

Antibiotic susceptibility screening is an important application in both research and medicine. Thus, reliable, rapid, and high-volume processing systems are required. For this reason, we also tested our fluorescence-based cell growth assay as an antibacterial drug screening method. As a first approach, the commercially available antibiotics kanamycin, ampicillin, and chloramphenicol were used to test the antibiotic resistance of several gram-negative bacteria. For these experiments, an *E. coli* strain carrying a plasmid that confers kanamycin resistance (*Kan*^{r}) was selected. Fluorescence time traces of cultures with an initial concentration of 10^7 cfu, in the presence of different concentrations of the three antibiotics, were followed. The results are presented in Fig. 5 and Table 1 together with the optical density at 600 nm measured for the control aerobic cultures at 12 and 24 h. The oxygen consumption of *E. coli Kan*^{r}, and thus the growth, is not affected by doses of kanamycin below 50 μg/ml, commonly used for growing *E. coli Kan*^{r} cells. Above this concentration, an inhibitory effect was observed. At 500 μg/ml, a delay in time to reach the midpoint transition was observed, indicating that bacteria were able to grow but at a much slower rate. At 5000 μg/ml, no oxygen consumption occurred (Fig. 5A).

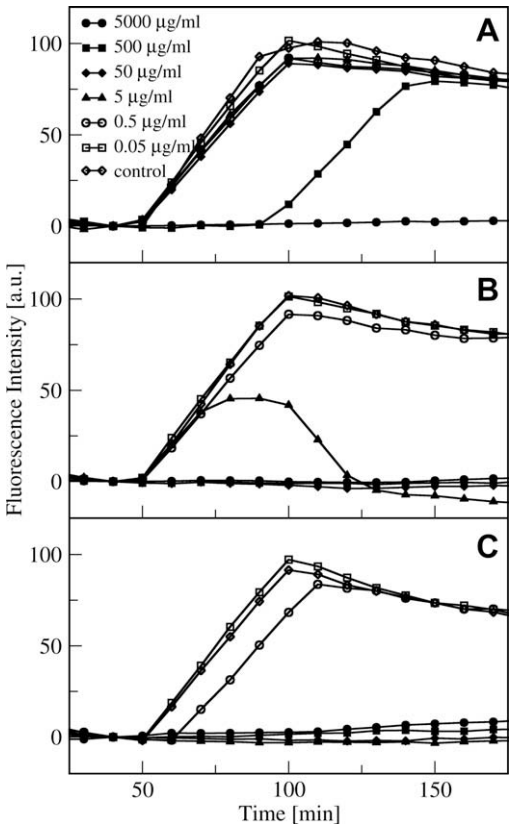

Fig. 5. Fluorescence intensity time traces at 570 nm (excitation 550 nm) of cultures of *E. coli* containing Cy3-labeled *R. thomasiana* hemocyanin as a function of antibiotic concentration. Antibiotic concentrations are stated for kanamycin (A) and maintained for ampicillin (B) and chloramphenicol (C).

In the case of ampicillin, a bactericidal effect was observed. Concentrations within the range of 5000 to 50 µg/ml killed all microorganisms, and wells that were proliferating in the presence of 5 µg/ml returned to baseline, indicating cell death (Fig. 5B). This trend was similar to the *S. simulans* growth profiles (Fig. 4), although in the case of *E. coli* no secondary increase in the fluorescence intensity was observed. In the case of chloramphenicol, just 0.5 µg/ml slowed down the oxygen consumption (Fig. 5C). Above this chloramphenicol concentration, no oxygen consumption was observed at all. A close inspection of the traces reveals that our method is suitable for distinguishing small changes in the concentration of drugs added. In fact, for some concentrations (e.g., 500 µg/ml kanamycin, 5 µg/ml ampicillin, 0.5 µg/ml chloramphenicol), a delay in the deoxygenation time response was observed. Control experiments measuring OD_{600} confirmed these results (Table 1). Even the cases where a slower oxygen depletion rate or an initial oxygen consumption followed by the reoxygenation of the culture was observed, this effect was mimicked by a smaller OD_{600} when compared with the OD_{600} reached in the absence of antibiotics (Table 1). This indicates that our method is sensitive enough to detect variations in the oxygen consumption rate.

Analogous experiments were carried out with *P. aeruginosa* and *P. denitrificans* (data not shown), and data are summarized in Table 1. *P. aeruginosa* showed resistance up to 50 µg/ml kanamycin, 500 µg/ml ampicillin, and 5 µg/ml chloramphenicol. This finding is in accordance with the well-known inherent resistance of this strain to many antibiotics [28]. *P. denitrificans* showed resistance up to 5 µg/ml kanamycin, 0.5 µg/ml ampicillin, and 0.5 µg/ml chloramphenicol, showing higher sensitivity than *P. aeruginosa* toward these antibiotics. For *P. aeruginosa* at 500 mg ampicillin and *P. denitrificans* at 5 mg kanamycin, a discrepancy between the oxygen depletion data and the optical density measurements was observed (Table 1). This difference could be attributed to an inoculum effect given that in the fluorescence measurements a higher number of initial cells was used (10^7 vs. 10^5). An alternative explanation is that even if the cells are not able to replicate at a fast rate, they will still consume some oxygen and this consumption is also detected by the fluorescence method.

Antibiotic susceptibility of *S. simulans* was tested using Pep5 and nisin. The experiment was performed in the same manner as for the other organisms. In this case, the initial cfu was 10^6 and the antibiotic concentrations were varied from 0.25 to 25,000 ng/ml (Fig. 6 and Table 1). The fluorescence time traces were not as well defined as for the other organisms, and a wide distribution of switching times was observed, especially in the case of Pep5 (Fig. 6A). Pep5 prevented oxygen consumption at the two highest concentrations (Fig. 6A). Although the error in the measurement is relatively large, 250 ng/ml Pep 5 appeared to decrease the rate of oxygen consumption to some extent (Fig. 6A), whereas below this concentration no effect was observed. In the case of nisin (Fig. 6B), the switching times were less scattered than for Pep5 and concentrations below 2500 ng/ml clearly did not affect the oxygen consumption by *S. simulans*. These findings were confirmed by control experiments in which the OD_{600} was measured (Table 1). Our results are in good agreement with previous reports [30,31], although there is some discrepancy in the literature about the exact minimal inhibitory concentration and the mode of action of these two antibiotics. An important difference between the Pep5 and nisin results is what happens after the switching of the fluorescence. In the case of Pep5 the fluorescence intensity stays high, whereas for nisin it drops rapidly. As discussed above, the exact nature of the drop of the fluorescence intensity after reaching the maximum will require further studies, but the experimental findings suggest a different mechanism of action for these two antibiotics.

Conclusions

A simple, quantitative, and sensitive method suitable for high-throughput screening of prokaryotic cell growth has been developed. The application of this method for antibiotic susceptibility

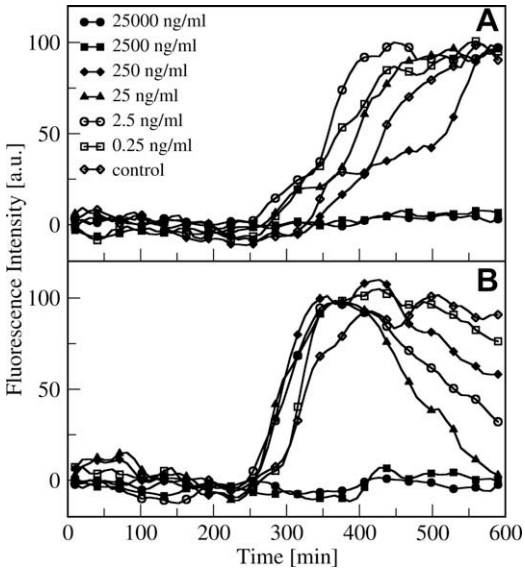

Fig. 6. Fluorescence intensity time traces at 570 nm (excitation 550 nm) of cultures of *S. simulans* containing Cy3-labeled *R. thomasiana* hemocyanin as a function of antibiotic concentration. Antibiotic concentrations are stated for Pep5 (A) and maintained for nisin (B).

Table 1
Antibiotic drug screening results.

Concentration of antibiotic added (μg/ml)								
		5000	500	50	5	0.5	0.05	0
E. coli Kan^r								
Kan	Oxygen depletion	No	Yes[a]	Yes	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.01	0.50/1.37	2.85/4.31	2.65/4.74	2.52/4.97	2.74/4.99	2.80/5.40
Amp	Oxygen depletion	No	No	No	No[b]	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.00	0.00/0.00	0.01/0.03	0.46/3.62	2.92/4.41	2.85/5.01	2.80/5.40
Chl	Oxygen depletion	No	No	No	No	Yes[a]	Yes	Yes
	OD_{600} (12 h/24 h)	0.01/0.00	0.00/0.00	0.00/0.00	0.00/0.01	2.21/4.12	2.69/4.80	2.80/5.40
P. aeruginosa								
Kan	Oxygen depletion	No	No	Yes	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h).	0.00/0.00	0.02/0.04	0.30/5.04	0.55/4.51	0.51/5.03	0.49/4.99	0.46/5.02
Amp	Oxygen depletion	No	Yes	Yes	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.00	0.02/0.02	0.55/5.23	0.50/4.54	0.41/4.53	0.43/4.87	0.46/5.02
Chl	Oxygen depletion	No	No	No	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.00	0.01/0.00	0.02/0.04	0.18/5.89	0.49/4.60	0.48/4.50	0.46/5.02
P. denitrificans								
Kan	Oxygen depletion	No	No	No	Yes[a]	Yes	Yes	Yes
	OD_{600} (24 h/48 h)	0.00/0.00	0.01/0.00	0.01/0.01	0.05/0.04	0.65/3.83	0.74/3.00	0.72/3.45
Amp	Oxygen depletion	No	No	No	No[b]	Yes	Yes	Yes
	OD_{600} (24 h/48 h)	0.00/0.00	0.01/0.01	0.01/0.01	0.01/0.01	0.11/3.01	0.13/3.30	0.72/3.45
Chl	Oxygen depletion	No	No	No	No	Yes	Yes	Yes
	OD_{600} (24 h/48 h)	0.00/0.00	0.01/0.00	0.01/0.01	0.01/0.00	0.14/3.65	0.63/3.74	0.72/3.45
Concentration of antibiotic added (ng/ml)								
		25,000	2500	250	25	2.5	0.25	0
S. simulans								
Pep5	Oxygen depletion	No	No	Yes[a]	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.00	0.00/0.01	0.05/0.22	0.29/4.62	0.35/4.44	0.33/4.66	0.47/4.92
Nisin	Oxygen depletion	No	No	Yes	Yes	Yes	Yes	Yes
	OD_{600} (12 h/24 h)	0.00/0.01	0.00/0.03	0.031/4.73	0.41/5.27	0.39/5.07	0.42/5.11	0.47/4.92

Note. No, no oxygen consumption was observed; Yes, oxygen consumption was observed.
[a] Slower than the normal rate.
[b] After initial oxygen consumption, the culture became oxygenated again. See text for details.

screening was shown to work for both gram-negative and gram-positive bacteria and for antibiotics with different mechanisms of action. The results provide a solid proof-of-principle for the presented methodology and suggest that there are no impediments to applying it to other kinds of cells. To validate the method for a broader range of applications, studies with clinically relevant strains and antibiotics, as well as a direct comparison with existing methodology, will be undertaken at a later stage in collaboration with the University of Leiden Medical Center. This work also shows that Cy3-labeled hemocyanin can be used as an oxygen sensor even in conditions that are far from ideal, during long periods of time, and at relatively high temperatures. In comparison with other available methods that require special microwell plate equipment with an integrated oxygen sensor [5,8,10–12], the soluble nature of our system offers versatility for use in any kind of standard fluorescence device. Moreover, the concentration of labeled protein can be adjusted according to the specific needs of a given experiment. The low concentrations of sensor needed and the simplicity and rapidity in setting up the system provide a realistic alternative to conventional cell proliferation and viability assays. As shown in the antibiotic susceptibility tests, the proper choice of the initial cell number allows relatively fast results within a couple of hours. In fact, a close inspection of Fig. 5 shows that it is possible to perform a susceptibility test in less than 100 min. This is just four doubling cycles for *E. coli* at 37 °C. Further adaptation using, for instance, engineered hemocyanin with a higher K_d for oxygen, analysis of the initial oxygen consumption rate rather that waiting for oxygen depletion, and optimization of the initial number of cells could further reduce the time required to perform the test. One possible problem in performing the test as quickly as possible is the high initial number of cells needed that could induce some error when compared with standard methodology in which a much smaller inoculum is used. This was indeed the case for *P. aeruginosa* at 500 mg ampicillin and for *P. denitrificans* at 5 mg kanamycin. However, at this stage, we did not attempt to validate our method as a standard for IC_{50} determination; rather, we did so as a fast way to perform antibiotic screening for drug discovery and possible clinical applications.

It is important to realize that the actual measured parameter is the oxygen concentration, which also opens the door to other kinds of metabolic studies. The observation of oxygen depletion followed by reoxygenation of the medium in some of the fluorescence time traces suggests that this method could be used to distinguish between different metabolic strategies. This observation also indicates that by fine-tuning the experimental conditions, it might be possible to distinguish between bactericidal and bacteriostatic effects, although further studies would be required. On the other hand, the fact that our method relies on the measurement of the oxygen concentration can also be a limitation. In particular, our method is not suitable for anaerobic organisms and should be used with care when working with facultative aerobes. In addition, a lack of oxygen consumption is not necessarily related to cell death; additional information about the antibiotics being tested might be required for a correct interpretation of the results. Despite these limitations, the broad applicability of our FRET-based method is a notable advantage. The benefits of measuring fluorescence over absorption are highlighted when the volume of the sample is small.

Other than absorption, fluorescence can be measured down to the single-molecule limit, allowing the miniaturization of the wells (i.e., the increase in the number of samples in a plate) to sizes where absorption measurements are just not possible. Although the system presented here measures oxygen depletion, the principle can easily be adapted to other kinds of metabolites, inhibitors, or any other compounds to be studied by substituting the hemocyanin for a protein that interacts with the substrate of interest. This opens the door to a new generation of biosensors by combining protein selectivity with fluorescence sensitivity.

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