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Sposensor: A Whole-Bacterial Biosensor That Uses Immobilized *Bacillus subtilis* Spores and a One-Step Incubation/Detection Process

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Key Words
Whole-cell biosensor · *Bacillus subtilis* · Spores

Abstract
A generic whole-cell bacterial sensor called sposensor was developed with immobilized spores from engineered *Bacillus subtilis*. Sposensor contains two different types of spores: reporting spores that contain a reporter gene fused to a promoter responding to a compound to be detected, and control spores use to monitor cell germination and viability. A one-step incubation/detection process was developed to meet the constraints of on-site analysis. Spores were directly incubated with culture medium containing the compound to be detected. β-Galactosidase was chosen as a reporter protein in both cases and its activity followed by a colorimetric assay. Results showed that sposensor was efficient in detecting two different compounds, a metal (Zn^{2+}) and a peptidic antibiotic (bacitracin). Owing to the stability and robustness of spores, sposensor is a very efficient and easy tool to manipulate for analyzing the presence of toxic compounds in natural settings.

Introduction

Biosensors are biological tools used to probe the physicochemical composition of the surroundings. Their potential use in detecting pollutants in natural settings has aroused much interest in recent years [for reviews, see Daunert et al., 2000; Ron, 2007; Yagi, 2007]. Indeed, biosensors have several features such as low cost and portability which endow them with a great potential in environmentally related issues, including on-site investigations. These features make biosensors more suitable for routine use than chemical or physical-based devices which need specific instruments that are often not easily transportable and usable on the site where the samples to be analyzed are harvested.

Numerous biological elements have served to build biosensors. Enzymes or antibodies, for instance, showed great efficiency owing to their specificity [for review, see Guilbault, 1982; for examples, see Vanderlaan et al., 1988; Gil et al., 1992]. However, the need to purify components first, associated with their fragility, has restricted their exploitation domain. With the advent of molecular technologies, the development of genetic tools and genome sequencing, whole-cell bacterial sensors have emerged as a most promising strategy [Daunert et al., 2000]. Basically, these are bacteria that express reporter genes fused to promoters chosen for their capacity to respond, hence to detect, a given compound. Nature provides us with a very large number of bacterial species, which have developed unmatchable abilities to adapt to any sort of environment, thanks to their sophisticated genetic regulatory circuits. Thus, a huge number of molecular mechanisms tailored to respond to environmental changes could conceivably be used as detectors for making whole-cell biosensors. Obvious additional advantages of whole micro-

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organisms relate to the fact that they do not need purification and can be rapidly grown in an inexpensive medium.

Reporter genes encoding enzymes, enzymatic complexes or fluorescent proteins have been used to construct whole-cell biosensors such as β-galactosidase, the well-known protein reporter for which numerous colorimetric substrates are available, and also the green fluorescent proteins, luciferase [Stocker et al., 2003], alkaline phosphatase [Kohler et al., 2000] or, as recently proposed, carotenoid [Maeda et al., 2006] or pyocyanin [Fletcher et al., 2007] synthesis systems. Crucial criteria for choosing or building a biosensor include sensitivity, time needed, portability, robustness and eventual reusability. All of these properties become even more important when the ultimate goal is to carry out the investigation in the field, e.g. in a spot distant from any laboratory settings. In this regard, colorimetric assay is highly recommended because no special tool is required to detect, at least qualitatively, the color of the product of the reaction. Immobilizing bacteria or the use of spores were proposed as issues regarding robustness problems [Date et al., 2007].

Our goal was to contribute to solving the problems of stability of biosensor and to design an assay that would be as simple as possible in order that anyone might use it for on-site analysis. For this we made use of spores of *Bacillus subtilis* containing dedicated gene fusion, which we immobilized on a filter paper and subsequently allowed to germinate in the presence of a toxic compound to be detected. To validate this approach, we used two compounds very different in chemical nature, a metal ion (Zn^{2+}) and an antibiotic peptide (bacitracin), and developed their respective biosensors which we referred to as sposensor. They proved to be efficient, sensitive, stable, portable and extremely easy to use. Thanks to the genetic amenability of *B. subtilis*, we believe this strategy is promising for the development of a large number of sposensor assays in the future.

Results

Developing a Portable Detection Assay: Sposensor

Our aim was to design a transportable assay based on *B. subtilis* spores that would be able to give a signal when germinating in the presence of a compound to be detected. Once functioning it should require as few manipulations as possible, should be cheap and exploitable by non-specialists. The following procedure involving the manipulation of only 3 tubes was designed.

The first tube contains two Whatman 3 paper discs on which *B. subtilis* spores have been immobilized. On one of them, spores of the *B. subtilis* responsive strain, i.e. containing a regulatable reporter gene fusion, were spotted. On the other, control spores from SG57 strain that contain a constitutively expressed reporter gene [Görke et al., 2004] were immobilized. Each disk is placed in a plastic ring. This configuration allows simultaneous testing of the response to a given compound and to check that the compound-containing medium bears no toxic effect on spore germination or any biological processes required for functioning of the reporter, such as transcription or translation.

The second tube contains dehydrated growth medium. The principle of the detection system is to rehydrate the solid medium with a sample from the medium to be analyzed. Once the medium is rehydrated, the two paper discs assembled in the cap of the first tube are transferred on top of the second tube in order to close it. This closed tube is then incubated in a horizontal position which allows the paper disc to be kept at the surface of the medium, thereby preventing spore dissemination and instead allowing aerobic germination.

A third tube contains a bactericidal solution (mix of $CuCl_2$ and ascorbic acid [Shapiro et al., 2004]) that will be added to the assay tube after completion of the measurement.

Developing a Sposensor for Zinc, a Metal

To evaluate the efficiency of the assay described above we decided to use *czcD*, a gene previously described as being induced in the presence of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} [Moore et al., 2005]. Dr. J.D. Helmann kindly provided us with the HB5016 strain that contains a *czcD::lacZ* fusion integrated at the *amyE* locus on the chromosome of *B. subtilis* wild-type strain.

In a first step, HB5016 spores were grown on solid media containing Zn^{2+}, Ni^{2+}, Cd^{2+} or Cu^{2+}. β-Galactosidase activity was scored as described in Experimental Procedures. Surprisingly, the blue color, testifying to β-galactosidase activity, was only obtained on Zn^{2+}-containing medium. This indicated that, at least under our conditions, the HB5016 strain might be considered as a specific Zn^{2+} detection system.

In a second step, we followed the β-galactosidase activity obtained with vegetative HB5016 bacteria grown in liquid medium containing different concentrations of Zn^{2+}. The results shown in figure 1 indicated a linear relationship between *czcD* expression and Zn^{2+} concentration within a 10- to 300-μM range. These results demon-

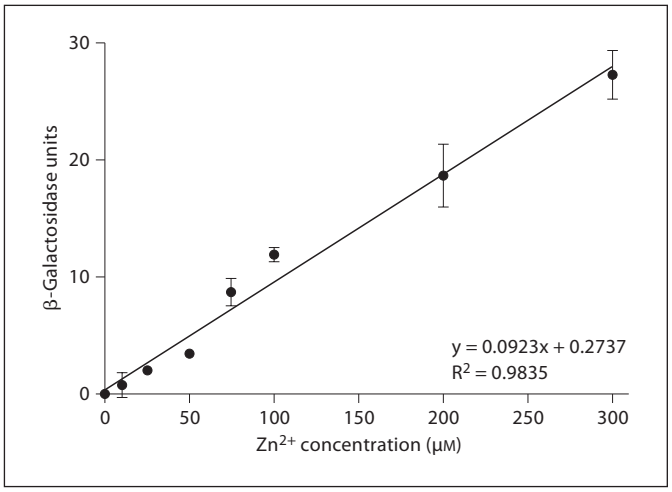

Fig. 1. Dose-response curves obtained in a liquid assay with HB5016 strain tested in presence of Zn^{2+}. Cells were incubated in LB medium containing various concentrations of zinc chloride for 30 min at 37°C. β-Galactosidase activities were then determined. Data points represent the average of specific activities from three replicates and linear regression is presented.

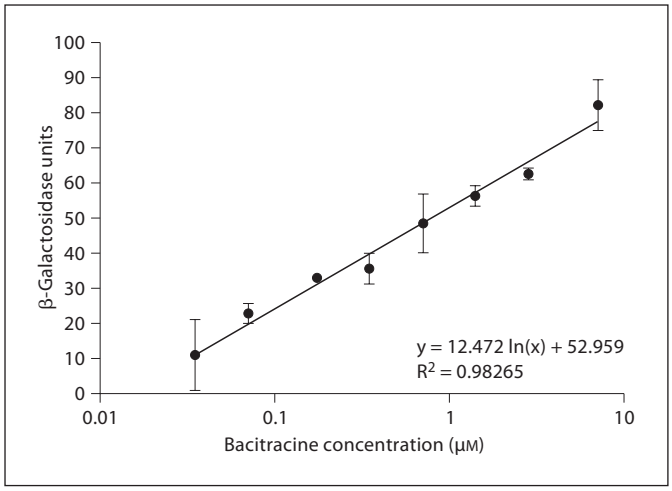

Fig. 3. Dose-response curves obtained in a liquid assay with the BSGY005168 strain in the presence of bacitracin. Cells were incubated with various concentrations of the antibiotic for 30 min at 37°C, then β-galactosidase activities were measured. Data points represent the average of specific activities from 3 replicates and linear regression is presented.

Zn^{2+} concentration	β-Galactosidase activity		
	37°C	30°C	24°C
0 μM			
20 μM			
50 μM			
100 μM			
200 μM			
Control (SG57)			

Fig. 2. Color development of the sposensor Zn^{2+} detection system (HB5016 spores). Sposensor was incubated at different temperatures using various zinc chloride concentrations over 16 h. A control using SG57 spores was used to follow cell viability at the highest zinc chloride concentration.

Bacitracin concentration	β-Galactosidase activity		
	37°C	30°C	24°C
0 μM			
0.035 μM			
0.35 μM			
3.5 μM			
35 μM			
70 μM			
Control (SG57)			

Fig. 4. Color development of the sposensor bacitracin detection system (BSGY005168 spores). Sposensor was incubated at different temperatures using various bacitracin concentrations over 16 h. A control using SG57 spores was used to follow cell viability at the highest bacitracin concentration.

strated that one could deduce the concentration of Zn^{2+} present in the medium from the amount of β-galactosidase activity measured.

In a third step, we used our Zn^{2+} sposensor detection system on samples containing different Zn^{2+} concentrations. As shown in figure 2, differences in blue coloration intensity could clearly be identified on filters. We obtained very similar results at 37 and 30°C with a blue coloration intensity increasing gradually with Zn^{2+} concentrations. We observed the same response at 24°C for Zn^{2+}

concentrations of <100 μM. Higher Zn^{2+} concentrations seem to be toxic as indicated by the absence of coloration with the control spores. The threshold of Zn^{2+} detection is the same at each tested temperature. As a matter of fact, the maximal Zn^{2+} concentration in drinking water is 5 mg/l [US Environmental Protection Agency, 2006] corresponding to 76.4 μM for Zn^{2+} which is almost 8 times higher than the minimal concentration detected by sposensor (10 μM Zn^{2+}).

Developing a Sposensor for Bacitracin, an Antibiotic

To assess the versatility of the sposensor approach, we developed a biosensor for the detection of a compound of a very different chemical nature than a metal. We choose bacitracin, a peptide antibiotic. For this purpose, we used the *bceRSAB* operon that allows *B. subtilis* to respond to and to resist the presence of bacitracin in the medium.

Previous studies including those obtained in our laboratory have demonstrated the specific induction of transcription of the *B. subtilis bceAB* genes in the presence of bacitracin [Bernard et al., 2003; Mascher et al., 2003; Ohki et al., 2003]. These genes encode an ABC transporter that confers high resistance to this antibiotic. To get the bacitracin biosensor, the *B. subtilis* (BSGY005169 strain) containing a *bceA::lacZ* fusion at the *amy* locus [Ohki et al., 2003] was used. As shown in figure 3, when using this bacterial strain in a direct test, i.e. vegetative bacteria grown in the presence of the antibiotic, the threshold concentration of detection was found to be lower than 70 nM of bacitracin, showing that the biosensor is relatively sensitive. Increasing the bacitracin concentration to reach 7 μM gave a linear dose-response curve when expressed in semi-logarithm plot. However, at a higher bacitracin concentration a plateau was obtained, reflecting saturation of the system.

Spores of the BSGY005168 strain were used to get a bacitracin sposensor which was tested on samples each containing different bacitracin concentrations. As indicated in figure 4, no color was observed in the absence of bacitracin. Although blue color intensity seems to deepen with increasing bacitracin concentrations for each incubation temperature tested, it was difficult to establish a firm correlation between the color intensity and the bacitracin concentration. The threshold of bacitracin detection decreases gradually when temperature increases, allowing the detection of as little as 0.035 μM bacitracin at 37°C. Note that the SG57 control is growing perfectly at all bacitracin concentrations tested. Thus, the bacitracin sposensor appears as a very sensitive detection system with a 0.035-μM threshold bacitracin concentration limit.

Discussion

In this work, we constructed a new tool, referred to as sposensor, that will enable anyone, including non-specialists, to check for the presence of a toxin in a natural setup. Sposensor is made of *B. subtilis* spores that are highly stable. The use of spores solves a key issue when developing bacterial biosensors, namely to keep bacteria in a state allowing long-term storage. These dormant highly resistant cells can easily differentiate into responding cells after a germination step. As a matter of fact, while this work was in progress, the ability of spore-based systems to retain their detection properties was investigated [Date et al., 2007]. The authors showed that spores were effectively able to retain their analytical performance (in terms of detection limit, dynamic range and reproducibility) after either a long storage period or several cycles of germination/sporulation. However, they did not propose a particular device to do the assay and used their spore in a classical way, i.e. liquid culture coupled with classical β-galactosidase activity measurement or fluorescence detection of EGFP. In the present article we propose a device called sposensor which contains immobilized spores and is directly used for analyte detection. A great advantage of the sposensor technology lies in its simplicity of use since (i) it requires few manipulations that all can be carried out by a non-expert manipulator, and (ii) it is associated with a one-step protocol allowing germination of the spores and detection of the target product at the same time. Together with its portability, these features make sposensor extremely handy and helpful for on-site investigations, one of the bottlenecks in biosensor technology. Moreover, sposensors were made up of *B. subtilis* spores which have been shown to be highly stable [Odlaug et al., 1981], and can be stored at room temperature for a very long period of time before use. During the process, *B. subtilis* spores have first to germinate to get vegetative cells which are the true responsive cells. To reduce the number of manipulations, this step is achieved directly in medium containing the compound to be detected. Nevertheless, the whole process requires at least 16 h of incubation at temperatures ranging from 24 to 37°C. This might constitute a major drawback when comparing incubation times needed with other whole-cell biosensors such as those made up of dried viable *Escherichia coli* cells or chemical and physical detection approaches. However, we believe that sposensor will constitute an interesting alternative when used in developing countries where (i) reading instruments are not always available even at long distances, and

Table 1. Strains used in this study

Strains	Relevant characteristics	References
HB5016	W168 *att*SPβ *trp*C2 *amyE::czcD*, *LacZ*	Moore et al. [2005]
BSGY005168	W168 *att*SPβ *trp*C2 *amyE::bceAp::lacZ*; Cmr	Ohki et al. [2003]
SG57	W168 *att*SPβ *trp*C2 Φ(*ptsH*, *lacZ*)(Hyb) *ery*(::pMutin4)	Görke et al. [2004]

(ii) climatic conditions make long-term storage of conventional whole-cell biosensors difficult.

Two *B. subtilis* sposensors each having a restricted specificity for a particular product were built. Zinc is an important element for living organisms and Zn deficiency or excess zinc intake might be deleterious for them [Fosmire, 1990]. Therefore, it is very important to know the Zn^{2+} concentration in water. Thus, the Zn^{2+} sposensor seems to be a good candidate because it is highly sensitive, its use does not present any difficulty even for non-microbiologists and it can be stored over a long period of time.

Bacitracin is a peptide antibiotic which is not used in human therapy due to its high nephrotoxicity [Drapeau et al., 1992]. However, it is still used in several countries (but no longer in Europe [Casewell et al., 2003]) as a food complement for growth stimulation in chickens and swine [Corpet, 2000]. As animals were shown previously to be reservoirs of antibiotic-resistant enterococci following the use of antimicrobial growth promotants and prophylactics [Bywater et al., 2005], one can suspect that this might be the case for bacitracin. In fact, bacitracin-resistant *Enterococcus faecalis* strains have already been isolated from poultry in New Zealand [Manson et al., 2004]. To construct our biosensor, we choose the *B. subtilis bceAB* promoter rather than the *B. subtilis liaIH* promoter [Mascher et al., 2004], as it does not respond exclusively to bacitracin but it is also induced by nisin, vancomycin and ramoplanin. The biosensor described here is to our knowledge the first device constructed towards monitoring the bacitracin concentration. It should be of great interest to follow the behavior of the antibiotic within the animal and in the liquid discharge of breeds.

The chemical nature of the compounds tested here, i.e. a metal and a dodecyl peptide, covers quite a range of pollutants demonstrating both the feasibility and versatility of the sposensor-based methodology. We are currently developing several sposensor systems to detect Co^{2+}, estradiol or atrazin, the two latter being of great importance in human health. The presence of an internal control allows us to detect a possible toxicity of the compound. It is our belief that any regulatory system of *B. subtilis* which exhibits the required properties in terms of detection, e.g. specificity and sensitivity, could be turned into a responsive element towards building a new sposensor. Moreover, *B. subtilis* being a model bacteria for which performant genetic tools are available, it could be used as a host for introducing heterologous regulatory responding systems. Thus, the repertoire of sposensor that might be realized in the near future might grow very quickly.

Experimental Procedures

Bacterial Strains and General Growth Conditions

The strains used in this study are listed in table 1. Luria-Bertani (LB) broth was routinely used for bacterial growth. When needed, the media were supplemented with chloramphenicol at 5 mg/ml. For solid medium, LB broth was supplemented with agar and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at 0.1 mg/ml final concentration, allowing β-galactosidase blue coloration detection after overnight incubation at 30°C.

Sposensor Detection Kit

Spores were obtained by incubating HB5016, BSGY005168 and SG57 *B. subtilis* strains in Schaeffer's spore medium (SSM) [Schaeffer et al., 1965] for 48 h. Spores were then harvested by centrifugation at 5,000 *g* for 15 min, resuspended in Tris-KCl buffer (0.1 M, pH 8) at an optical density of 2 at 600 nm (OD_{600}) and treated at 80°C during 10 min. An equal volume of pre-warmed (52°C) Tris-KCl buffer containing agar (4%) was added to the spore suspension (volume/volume) and 20 μl of this mix were spotted on autoclaved Whatman 3 paper disks.

The dried powder medium is composed of Bacto-Tryptone (100 mg), Bacto-yeast extract (50 mg), NaCl (10 mg), glucose (20 mg) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (1 mg). The growth medium was reconstituted by adding 2 ml of various dilutions (in distilled water) of the solutions to be tested. Dose-response measurements were done with either $ZnCl_2$ or bacitracin. Incubation of the responding and control spores, in the presence of medium containing the substance to be detected, was done at three different temperatures (24, 30 and 37°C) for 16 h in an horizontal position.

After performing the assay, in order to destroy vegetative cells as well as spores, 2 ml of bactericidal solution was added (108 mM $CuSO_4$, 180 mM ascorbic acid and 1 M NaCl) as described by Shapiro et al. [2004].

β-Galactosidase Bioreporter Liquid Assays

1 μl of spore suspension, obtained as described above, was used for overnight LB cultures, then sub-cultured to adjust to a density of 0.05 at 600 nm (OD_{600}) and grown at 37°C with rapid aeration until reaching an OD_{600} of 0.4–0.6. Identical aliquots were harvested and exposed to various concentrations of $ZnCl_2$ or bacitracin or left untreated. They were incubated for 30 min at 37°C, then rapidly chilled on ice. β-Galactosidase activities were determined as described by Miller [1972] using o-nitrophenyl-β-galactopyranoside as a substrate. Data from 3 independent experiments are presented.

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