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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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Sposensor: a whole-bacterial biosensor that uses immobilized *B. subtilis* spores and a one step incubation/detection process.

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2 **Sposensor: a whole-bacterial biosensor that uses immobilized *B. subtilis* spores**
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4 **and a one step incubation/detection process.**
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33 **Summary**
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36 A generic whole-cell biosensor called Sposensor was developed with immobilized spores from
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38 engineered *Bacillus subtilis*. We developed a one step incubation/detection process. Spores
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40 germination takes place in the culture growth medium with the compound to be detected. Control
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42 spores allow a putative toxic effect of the medium analysed to be detected. Sposensor was
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44 developed as an as simple as possible tool for on site analysis and two different detection systems
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46 for a metal (Zn²⁺) or for a peptidic antibiotic (bacitracin) were built showing the versatility of the
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48 Sposensor methodology.
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Introduction

Biosensors are biological tools used to probe the physico-chemical composition of the surrounding. Their potential use in detecting pollutants in natural settings aroused much interest in the recent years (Yagi, 2007). Indeed, biosensors have several features, such as specificity, sensitivity, low cost, portability which endow them with a great potential in environmental related issues, including on-site investigations. Cost, portability and eventual on-site use make biosensors more suitable for routine use than chemical or physical-based devices. These later need large and expensive instruments which are not easily transportable and usable on the place where samples to analyze are harvested.

Numerous biological elements have served to build biosensors. Enzymes or antibodies for instance showed great efficiency, owing to their specificity (see as examples : Campanella *et al.*, 2007, Jeykumari & Narayanan, 2008, Jha *et al.*, 2008, Jiang *et al.*, 2008, Wang *et al.*, 2008, Ricci *et al.*, 2007). However, the need to purify components first, associated with their fragility have restricted the domain of their exploitation. With the advent of molecular technologies, development of genetic tools and genome sequencing, whole-cell bacterial sensors have emerged as a most promising strategy (Nakamura *et al.*, 2008, Reshetilov, 2007, Yagi, 2007). Basically, these are bacteria that expressed reporter genes fused to promoter chosen for their capacity to respond, hence to detect, a given compound. Nature is providing us with an infinite number of bacterial species, which developed unmatched 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 are out there waiting to be used as detectors for making whole-cell biosensors. Obvious additional advantages of whole microorganisms relate to the fact that they do not need purification and can be rapidly grown in non-expensive medium.

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One of the key question regarding whole cell biosensors is the choice of the reporter gene being used, thereby the assay needed to be developed for testing a given environment. β -galactosidase, a well known protein reporter for bacterial biosensors, has often been selected because numerous colorimetric substrates of this enzyme are available (Pocsi *et al.*, 1993). Green fluorescent protein, luciferase or recently a carotenoid synthesis gene were also proposed and used (Yagi, 2007). Others crucial criteria for choosing, or building, a biosensor include sensitivity, time needed, portability, robustness and reusability. All of these properties become even more important when the ultimate goal is to carry out the investigation on the field, say 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 colour of the product of the reaction. Immobilizing bacteria or use of spores were proposed as issues to the robustness and reusability problems (Date *et al.*, 2007).

Our goal was to contribute to solving the problems of stability of biosensor and to design a kit that would be as simple as possible for on site analysis. For this we made use of spores of *B. 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. The resulting biosensor which we referred to as Sposensor proved to be efficient, sensitive, stable, portable and extremely easy to use. Thanks to the genetic amenability of *B. subtilis*, we believe this strategy should be promising for the development of a large number of Sposensor kits in the future.

Results and Discussion

Developing a portable detection kit : Sposensor

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Our aim was to design a transportable kit based on *B. subtilis* spores that would be able to give a signal when germinating in the presence of a compound to be detected. The device once functioning should require as few manipulations as possible, should be cheap and exploitable by non specialists. The following prototype was designed. It is composed of 3 tubes (Fig. 1):

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- the first tube, referred to as the Starter/Controller, contains two Watman 3 paper discs on which *B. subtilis* spores have been immobilized. On the former, spores of the *B. subtilis* responsive strain, i.e. containing a regulatable reporter gene fusion, were spotted. On the latter, control spores from SG57 strain that contain a constitutively expressed reporter gene (Gorke *et al.*, 2004) were immobilized. Each disk is placed in a plastic ring. This configuration allows to test simultaneously 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.

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- the second tube, referred to as the Developer, contains dehydrated growth medium. The principle of the detection system is to rehydrate the solid medium with a sample from the medium to be analysed. Once the medium gets rehydrated, the cap of the Starter/Controller that contains the two paper discs is transferred on top of and used for closing the Developer. The closed Developer is then incubated in an horizontal position. This positioning permits the paper disc to be kept at the surface of the medium, thereby preventing spore dissemination and instead allowing aerobic germination.

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- the third tube, referred to as the Killer, contains a bactericidal solution (mix of CuCl_2 and ascorbic acid, (Shapiro *et al.*, 2004)) that will be added to the Developer after completion of the measurement.

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Developping a Sposensor for Zinc, a metal

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

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11 In a first step, HB5016 spores were grown on solid media containing added Zn^{2+} , Ni^{2+} , Cd^{2+} or
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Surprisingly, blue colour, testifying of β -galactosidase activity, developed only on Zn^{2+} containing
medium. This indicated that at least in our conditions, the HB5016 strain can 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 the presence of different concentration of Zn^{2+} . The results shown in figure 2
indicated a linear relationship between *czcD* expression and Zn^{2+} concentration within a $10\mu M$
and $300\mu M$ range. These results demonstrated 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 on figure 3, differences in blue coloration intensity could clearly be
identified on filters. As a matter of fact, the authorized maximal Zn^{2+} concentration in water is
 $36,6\mu M$ which is 3 times higher than the minimal concentration detected by Sposensor ($10\mu M$ of
 Zn^{2+}). In each condition tested, the SG57 control grew perfectly indicating that the Zn^{2+}
concentration is not toxic for *B. subtilis* cells.

As micromolar amounts of free Zn^{2+} ion solution can kill many organisms (plants, invertebrate
and even vertebrate fish) Zn^{2+} detection in water is of great interest. 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 microbiologist people and it can be stored during a long period of time .

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 that purpose, we used the *bceRSAB* operon that allows *B. subtilis* to respond and to resist to the presence of bacitracin in the medium.

Previous studies 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 for an ABC transporter that confers high resistance to this antibiotic. To construct the bacitracin biosensor, the *B. subtilis* (BSGY005169 strain) was constructed placing a *bceA::lacZ* fusion at the *amy* locus (Ohki *et al.*, 2003). As shown on figure 4, when using this bacterial strain in a direct test, i.e. vegetative bacteria grown in the presence of the antibiotic, the threshold of detection was found to be inferior to 0,1µg/ml of bacitracin, showing that the biosensor is very sensitive. Increasing bacitracin concentration to reach 10µg/ml gave a linear dose-response curve. However, at higher bacitracin concentration a plateau was obtained reflecting a saturation of the system.

Spores of BSGY005168 strain were used to get a bacitracin Sposensor which was tested on samples each containing different bacitracin concentrations. As indicated in figure 5, no colour was observed in the absence of bacitracin whereas a blue colour is detectable with as little as a 0,5µg/ml bacitracin concentration. Although some differences in blue colour intensity could be observed when varying bacitracin concentrations, it seemed difficult to correlate the colour intensity to the bacitracin concentration. Nevertheless, Sposensor appears as a very sensitive bacitracin detection system with a 500 ng/ml threshold concentration limit.

To our knowledge the bacitracin Sposensor is the unique bacitracin detection kit available today. Bacitracin is a peptide antibiotic which is not use in human therapy due to its high nephrotocixicity (Drapeau *et al.*, 1992). However it is still used in several countries (but not anymore in Europe (Casewell *et al.*, 2003)) as food complement for growth stimulation in chicken and swine breedings (Corpet, 2000). As animals were shown previously to be reservoirs for antibiotic-resistant enterococci following the use of antimicrobial growth promotants and prophylactics (Bywater *et al.*, 2005), one can suspect that might be the case for bacitracin. In fact bacitracin resistant *E. faecalis* strains were already isolated from poultry in New-Zealand (Manson *et al.*, 2004). Thus, a Sposensor able to detect bacitracin would be of great interest to follow the behaviour of the antibiotic within the animal and in liquid discharge in breedings.

Conclusion

In this work, we constructed a new tool, referred to as Sposensor, that will enable anyone, including non specialists, to check on the presence of toxic in a natural set up. Sposensor is made of *B. subtilis* spores, hence highly stable and reusable. The use of spores solves a key issue when developing bacterial biosensors, namely to keep bacteria in a responding state during 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, ability of spore-bases systems to retain their detection properties was reported, although the type of use proposed differed from ours presented here (Date *et al.*, 2007). A great advantage of Sposensors lies in their simplicity since they are associated to a one step protocol allowing to get germination of the spores and detection of the target product at the same time to facilitate their use. Evidently, the simplicity of its design makes Sposensor a perfect answer to the problem of portability and will make it extremely handy and helpfull for on-site investigations, one of the current bottleneck in

1 biosensor technology. Moreover, they can be stored at room temperature for a quasi infinite
2 period of time as responding cells are *B. subtilis* spores which were shown to be highly stable
3 (Odlaug *et al.*, 1981). As *B. subtilis* Sposensors are used at room temperature, they need to be
4 incubated longer than vegetative cells to produce detectable amount of the reporter enzyme.
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6 Nevertheless, incubation time does not exceed a few hours.
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13 Two *B. subtilis* sposensors each having a restricted specificity for a particular product were built.
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Chemical nature of the compounds to be detected, i.e. a metal and a dodecylpeptide, covers quite a range of pollutants demonstrating the versatility of this methodology. 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 exponentially

Experimental procedures

Bacterial strains and general growth conditions.

The strains used in this study are listed in table 2. 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-beta-D-galactopyranoside at 0,1mg/ml final concentration, allowing β -galactosidase blue coloration detection.

Sposensor detection kit

Spores to constitute the Starter/Controller tube were obtained by incubating HB5016, BSGY005168 and SG57 *B. subtilis* strains in Schaeffer's spore medium (SSM) (Schaeffer *et al.*, 1965) for 30 hours. Spores were then harvested by centrifugation at 3 000g for 15 minutes, resuspended in Tris-KCl buffer (0,1M pH=8) at an optimal density of 2 at 600nm (OD₆₀₀) and treated at 100 °C during 20 minutes. An equal volume of prewarmed (52°C) Tris-KCl buffer containing agar (4%) was added to spore suspension (volume/volume) and 20µl of this mix were spotted on autoclaved Whatman 3 paper disks.

The Developer tube contains powder medium composed with Bacto-Tryptone (100mg), Bacto-yeast extract (50mg), NaCl (10mg), glucose (20mg) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (1mg). The growth medium were reconstituted by adding 2 ml of various dilutions (in distilled water) of the solutions to be tested. Dose-response measurements were done with either ZnCl₂ or Bacitracin. All Developer tubes were then incubated for 18 to 20 hours in an horizontal position at 30°C.

The Killer tube contains 2 ml of bactericidal solution (108mM CuSO₄, 180mM ascorbic acid and 1M NaCl) as described by Shapiro and col. (2004). After performing the assay, this solution is added to the contain of the second tube in order to destroy spores and vegetative cells.

β-galactosidase bioreporter liquid assays

1µl of spore suspension obtained as described above was used for overnight LB cultures, then sub-cultured to adjust density of 0,05 at 600nm (OD₆₀₀) and grown at 37°C with rapid aeration until reaching an OD₆₀₀ of 0,4-0,6. Identical aliquots were harvested and exposed to various concentrations of ZnCl₂, of Bacitracin or left untreated. They were incubated during 30 minutes at 37°C, then rapidly chilled on ice. β-galactosidase activities were determined as described by

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2 Miller (1972) using ONPG (o-Nitrophenyl-beta-galactopyranoside) as a substrate. Data from 3
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4 independent experiments are presented.
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9
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Metallic ions	Blue coloration
No ion	-
Zn ²⁺	+
Ni ²⁺	-
Cd ²⁺	-
Cu ²⁺	-
Ni ²⁺ , Cu ²⁺	-
Ni ²⁺ , Cd ²⁺	-
Zn ²⁺ , Ni ²⁺	+
Zn ²⁺ , Cd ²⁺	+
Cd ²⁺ , Cu ²⁺	-
Zn ²⁺ , Cu ²⁺	+
Zn ²⁺ , Ni ²⁺ , Cd ²⁺	+
Zn ²⁺ , Cd ²⁺ , Cu ²⁺	+
Zn ²⁺ , Ni ²⁺ , Cu ²⁺	+
Zn ²⁺ , Ni ²⁺ , Cd ²⁺ , Cu ²⁺	+

Table 1: Colour obtained after overnight incubation of HB5016 strain containing the *czcD::lacZ* fusion on LB, Agar solid medium containing various metal concentrations : Ni²⁺ (500μM), Zn²⁺ (300μM), Cd²⁺ and Cu²⁺ (10μM). – white, + blue.

Strains	Relevant characteristics	References
HB5016	W168 <i>attSPβ trpC2 amyE::czcD, lacZ</i>	(Moore <i>et al.</i> , 2005)
BSGY005168	W168 <i>attSPβ trpC2 amyE::bceAp::lacZ; Cmr</i>	(Ohki <i>et al.</i> , 2003)
SG57	W168 <i>attSPβ trpC2 Φ(<i>ptsH, lacZ</i>)(Hyb) <i>ery</i> (::pMutin4)</i>	(Gorke <i>et al.</i> , 2004)

Table 2 : Strains used in this study

For Review Only

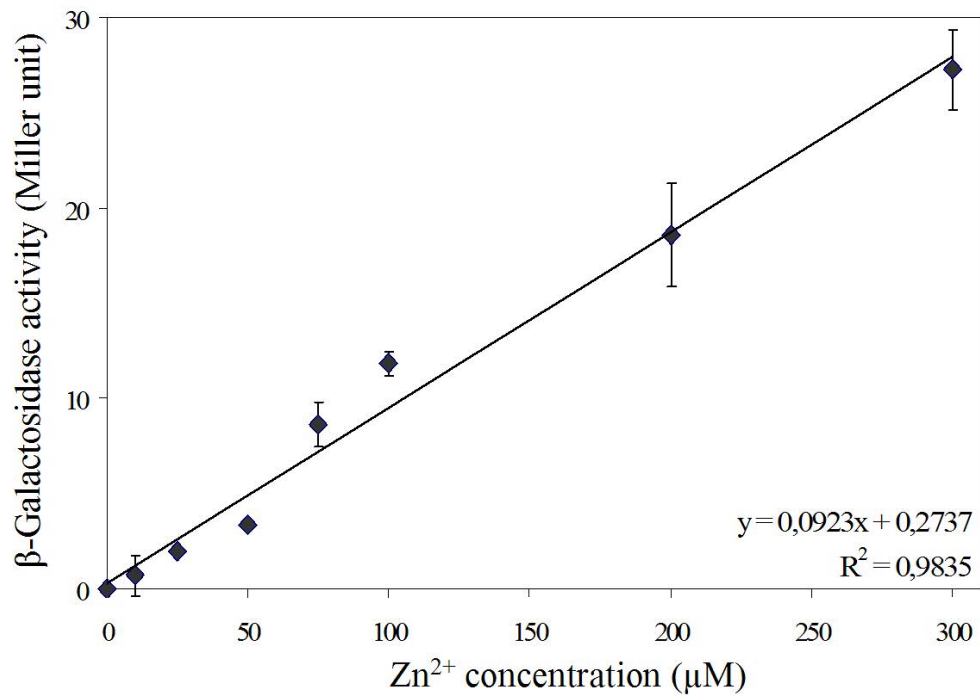


Figure 1 : Dose, response curves obtained in a liquid assay with HB5016 strain tested in presence of Zn²⁺. Cells were incubated in LB medium containing various concentrations of zinc chloride for 30 minutes at 37°C. β-galactosidase activities were then determined. Data points represent the average of three replicates and linear regression is presented.

49x34mm (600 x 600 DPI)

Only



Figure 2 : Sposensor detection kit. Tube one is the Starter/Controller that contains control and sensing spores (disk A and B respectively), each immobilized on a Watman 3 paper discs placed in plastic rings. Tube two is the Developer, it contains dehydrated growth medium which is rehydrated with 2 ml of the water solution to analyse. In such condition germination and detection are performed in one step. Tube three is the Killer which contains 2ml of bactericidal solution composed of CuSO_4 (108mM), ascorbic acid (180mM) and NaCl (1M).

49x56mm (600 x 600 DPI)






Zn ²⁺ concentration	β-galactosidade activity
0μM	
20μM	
50μM	
100μM	
200μM	

Figure 3 : Colour development after over night incubation by Sposensor Zinc detection kit using various zinc chlorure concentrations.

64x30mm (600 x 600 DPI)

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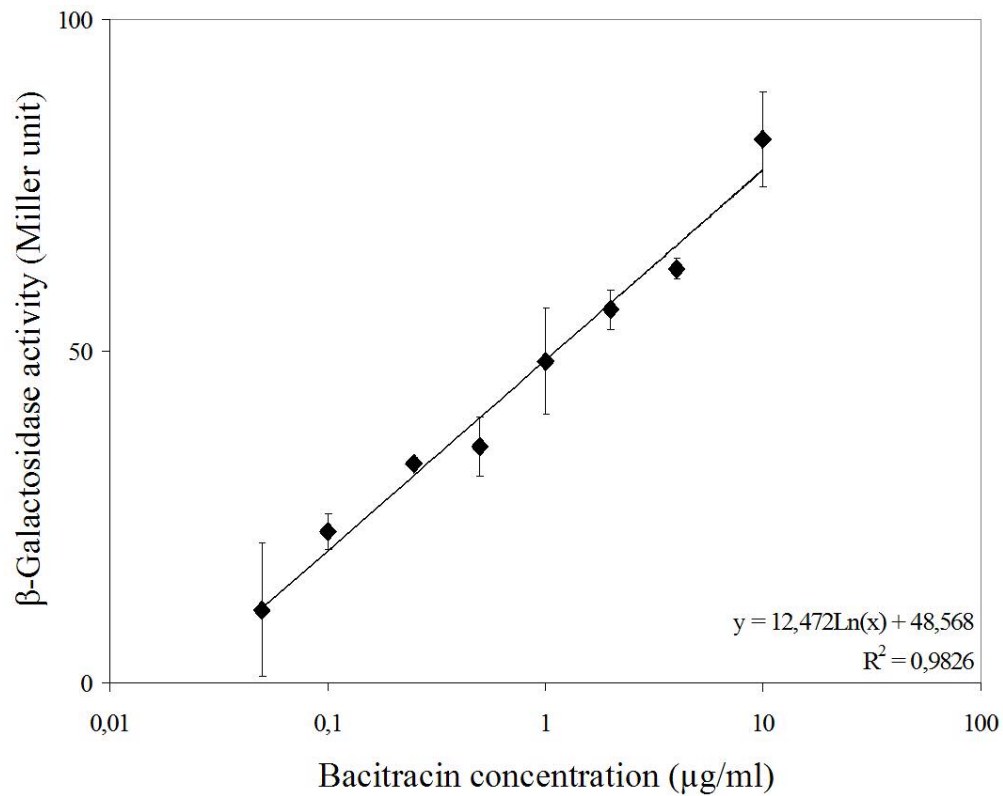


Figure 4 : Dose, response curves obtained in a liquid assay with BSGY005168 strain in presence of Bacitracin. Cells were incubated with various concentrations of the antibiotic for 30 minutes at 37°C then β -galactosidase activities were measured. Data points represent the average of three replicates and linear regression is presented.

45x35mm (600 x 600 DPI)






Bacitracin concentration	β -galactosidase activity
0 μ g/ml	
0,05 μ g/ml	
0,5 μ g/ml	
5 μ g/ml	
50 μ g/ml	

Figure 5 : Colour development after over night incubation by Sposensor Bacitracin detection kit using various bacitracin concentrations.

63x29mm (600 x 600 DPI)

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