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Bacillus subtilis Biosensor Engineered To Assess Meat Spoilage

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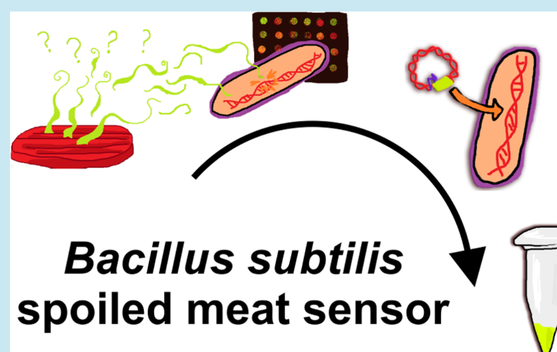
Supporting Information

ABSTRACT: Here, we developed a cell-based biosensor that can assess meat freshness using the Gram-positive model bacterium *Bacillus subtilis* as a chassis. Using transcriptome analysis, we identified promoters that are specifically activated by volatiles released from spoiled meat. The most strongly activated promoter was P_{sboA}, which drives expression of the genes required for the bacteriocin subtilosin. Next, we created a novel BioBrick compatible integration plasmid for *B. subtilis* and cloned P_{sboA} as a BioBrick in front of the gene encoding the chromoprotein amilGFP inside this vector. We show that the newly identified promoter could efficiently drive fluorescent protein production in *B. subtilis* in response to spoiled meat and thus can be used as a biosensor to detect meat spoilage.



Every year, one-third of global food production is unused and thrown away. The prime reason is that perfectly edible food is disposed of prematurely due to the "best before date" indicator system. Thus, fast and reliable systems are required to assess food spoilage to prevent health hazards and economic losses, as well as for ethical reasons. With food spoilage mainly being caused by degradative enzymes and toxins from food-associated bacteria and fungi, the classic "scientific" way to test whether food is spoiled is by counting colony forming units (CFU) in plating assays. While these tests give an estimate of the microbial load, they are very time-consuming, and furthermore cannot detect nonculturable microbes. It has long been appreciated that biological, cell-based biosensors could provide more sensitive and user-friendly devices compared to electronic ones, drawing on their evolutionary optimized systems to detect various analytes and the continuous self-renewal of the sensors within the living system.[1] Although cell-based biosensors have been successfully designed for a number of applications such as heavy metal or explosives detection and antibiotic quantification,[2] only few examples exist that can assess food spoilage such as a mammalian cell-system that reports on fruit quality.[3] So far, none have been commercially implemented in the food sector.[4]

Bacillus subtilis is a very promising organism for the development of novel biosensors since it has a GRAS status (generally regarded as safe), it is genetically tractable and is commonly used in industry. Several successful examples of *B. subtilis* as a biosensor exist. For instant, using firefly luciferase promoter fusions, it was shown that *B. subtilis* cells can be very effective biosensors toward several classes of antibiotics.[5] As a biosensor, *B. subtilis* offers the added benefit that it can produce dormant spores which allow for the long-term preservation, storage, and transport of the biosensor.[6] Together, this inspired us to explore the possibility whether we could engineer a new *B. subtilis* based biosensor that would be specifically responsive to spoiled meat.

Here, we describe the design, construction, and evaluation of a biosensor based on *Bacillus subtilis* cells that sense and respond to spoiled meat. Using DNA-microarray analysis we have identified several promoters that are upregulated when *B. subtilis* cells were exposed to volatiles coming from spoiled meat, but not from fresh meat. One of the most highly upregulated promoters, P_{sboA}, was selected and further characterized as a biosensor. To the best of our knowledge, this is the first example of using bacteria as a biosensor for food spoilage and the used strategy might be generally applicable to engineer other biosensors.

■ RESULTS AND DISCUSSION

Toward engineering *B. subtilis* for detecting and indicating spoiled meat (70%/30% pork/cow minced meat), we first developed an experimental approach with which we investigated *B. subtilis*' natural response to spoiled meat: the headspace gas of a container with either fresh (<10^3 CFU/gram) or spoiled (>10^6 CFU/gram) meat was flushed through

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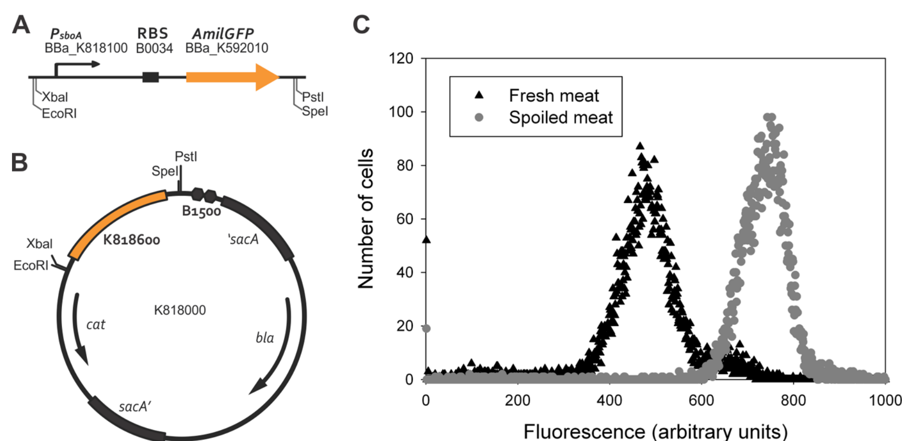

Figure 1. (A) Schematic representation of the meat-biosensor BioBrick BBa_K816000. (B) Schematic representation of the *sacA B. subtilis* integration vector BBa_K818000. The *sacA* homology regions are indicated. The *cat* gene provides chloramphenicol resistance in *B. subtilis* and the *bla* gene confers ampicillin resistance in *E. coli*. (C) *B. subtilis* cells harboring the biosensor-construct were grown to midexponential growth phase in LB-medium, split in two cultures and the headspace of either fresh meat (kept on ice) or of spoiled meat (kept at room temperature) was flushed continuously through the shaking cultures. Fluorescence (arbitrary units) of 10 000 cells was determined by flow cytometry at timely intervals and a typical outcome of data from cells incubated for 5 h is shown.

an exponentially growing *B. subtilis* culture (Supporting Information Movie S1). To identify whether *B. subtilis* contains an endogenous response to volatiles present in the spoiled meat headspace, we performed a transcriptome analysis. After 2 h of incubation, *B. subtilis* cells were harvested and total RNA was isolated and compared to that of *B. subtilis* cells flushed with the headspace of fresh meat using DNA-microarrays (GEO accession number: GSE50538). 297 genes were more than 2-fold up- or down-regulated. Using Genome2d,[7] we identified 19 operons of which all genes where more than 2-fold upregulated. We ranked the operons by fold change and ruled out the operons related to general stress response. One of the strongest up-regulated promoters (12.5-fold, $p < 0.0001$) remaining was P_{sboA} which, interestingly, drives expression of the genes required for the bacteriocin subtilosin.[8] Subtilosin is a peptide with antimicrobial activity against a wide range of bacteria and subtilosin expression is under complex gene-regulatory control.[8]

BioBrick prefix and suffix were added to P_{sboA} by PCR resulting in the part BBa_K818100. Next, we generated a BioBrick compatible *B. subtilis* integration vector BBa_K818000 by addition of the BioBrick prefix and suffix and the transcriptional terminator (part BBa_B0015) to plasmid pSac-*Cm* (Genbank accession number: AY464562).[9] BBa_K818000 integrates via double crossover at the non-essential *sacA* locus. As a visual readout of the promoter activity, we employed the chromoprotein amilGFP, which is fluorescent and, when expressed in bacteria, pigments cells yellow visible to humans with the naked eye.[10] Using BioBrick assembly, we combined amilGFP (part BBa_K592010), the ribosome binding site (part BBa_K592010) and $P_{sboA,}$ resulting in part BBa_K818600 (P_{sboA}-*amilGFP,* Figure 1A), which was stably integrated in the *B. subtilis* chromosome using plasmid BBa_K818000 resulting in the meat-spoilage biosensor (Figure 1B).

To test the functionality of the biosensor, cells were incubated as described above (Supporting Information Movie S1) and fluorescence from amilGFP was examined using flow cytometry. As shown in Figure 1C, after 5 h of incubation, biosensor cells showed strong fluorescence in the presence of spoiled meat but not in the presence of fresh meat.

This is a proof of principle project that shows that *B. subtilis* can be used as a biosensor to assess meat spoilage. Our strategy to identify the genetic program of *B. subtilis* in response to volatiles coming from spoiled meat is potentially universal and could be used to identify other specific transcriptional responses of *B. subtilis* (e.g., to spoiled fish or heavy metals) or of other organisms.

METHODS

DNA Techniques, Media and Growth Conditions. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, transformation, and growth of *E. coli* and *B. subtilis* were carried out as described before.[11] The biosensor construct (BBa_K818600) was sequence verified and is shown in the Supporting Information.

DNA Microarrays. *B. subtilis* cultures were grown shaking in 40 mL of LB medium 40 mL in 100 mL Schott-Duran bottles (Schott, U.S.A.) at 37 °C. At midexponential growth (OD600 nm ∼0.1) either the headspace of a container with fresh (<10^3 CFU/g) meat (70/30 pork/cow minced meat) or spoiled meat was flushed through the culture using a peristaltic pump system (see Supporting Information Movie S1). After 2 h of incubation when cells were approximately OD 0.8, cells were collected by centrifugation at 7500 rcf for 5 min and frozen for RNA isolation with liquid nitrogen. RNA isolation, cDNA preparation, labeling, hybridization, scanning, and data analysis was performed as described on GEO (accession number GSE50538). Two biological replicates and 2 technical replicates were performed.

Total Aerobic Microbial Count Assays (TAMC). Meat was considered spoiled according to EU regulation ISO 16140:2003(E) (containing >10^6 CFU/g in a TAMC assay). TAMC's were performed by resuspending 1 g of meat in 100 mL of Trypton Soy Broth and serial dilutions were plated in triplicate in Trypton Soy Agar and incubated at 37 °C. After 3 days of incubation, CFU's were counted.

Construction of Plasmids and Strains. To generate a BioBrick compatible integration vector for *B. subtilis*, we first introduced a RFC(10) standard compatible multiple cloning site (*Eco*RI, XbaI, SpeI, *Pst*I) together with a double transcriptional terminator into plasmid pSac-*Cm*,[9] resulting in

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Table 1. Parts and BioBricks Used in This Study

part/plasmid	BioBrick number	description	source
P_{sboA}	BBa_K818100	promoter induced by rotten meat volatiles	this study
P_{sboA}-*amilGFP*	BBa_K818600	production of yellow pigment amilGFP induced by rotten meat volatiles	this study
K818000	BBa_K818000	plasmid replicates in *E. coli* and integrates into the *Bacillus subtilis* genome via double crossover; contains BioBrick MCS and double terminator B0015	this study
amilGFP	BBa_K592010	fluorescent and bright colored protein	iGEM Uppsala 2011
K818000-*amilGFP*		intermediate plasmid for creation of P_{sboA}-*amilGFP* in K818000	this study
B0015	BBa_B0015	double terminator	parts registry
RBS	BBa_B0034	ribosome binding site used widely in the iGEM competition	parts registry

vector BBa_K818000. The double terminator and prefix and suffix were amplified by PCR using primers AP_BB-B0015_fwd (5′-GCATAGAATTCACAGGTCTAGAGTGCAATAACT-AGTATCATCTGCAGCCAGGCATCAAATAAAAC-GAAAGG-3′) and AP_BB-B0015_rev (5′-ATCGAAAGCTT-AATATAAACGCAGAAAGGCCCACC-3′) and BioBrick B0015 as a template. The amplified fragment was subsequently cleaved with *Eco*RI and *Hin*dIII and ligated into the corresponding sites of pSac-*Cm*, resulting in plasmid BBa_K818000. This vector replicates in *E. coli* (selection with ampicillin) but not in *B. subtilis* where it will integrate at the *sacA* locus via double homologous recombination (upon selection with chloramphenicol) provided by the two *sacA* flanking regions present on BBa_K818000 (see Figure 1B). The BioBrick compatible MCS is present between the *sacA* integration regions.

To construct BioBrick BBa_K818100 (P_{sboA}) which encodes the *B. subtilis sboA* promoter with the BioBrick prefix and suffix according to the RFC(10) standard, a PCR with the primers AO_20120816_P_sboA_fw (5′- CTATCGGAATTCTCTA GACTGCTTCTATCTTACCATCATTGC-3′) and AO_20120821_P_sboA_rev (5′- CATGCCTGCAGAC-TAGTGACAGCTTTTTTCATAATTG-3′) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. To amplify *amilGFP*, a PCR using primers AP_amilGFP_fwd (5′-GCGGTGAATTCTCTAGAAAAGAGGAGAAAATGT CTTATTCAAAGCATGGCATCG-3′) and AP_amilGFP_rev (5′-GCTGCACTAGTC TGCAGTTATTATTTAACCTT-CAAAGGG-3′) was performed using BioBrick BBa_K592010 as a template. *amilGFP* and plasmid BBa_K818000 were digested with XbaI and *Pst*I. The two fragments where ligated and used to transform *E. coli*. The resulting plasmid K818000-*amilGFP* was isolated and digested with *Eco*RI and XbaI and P_{sboA} was digested with *Eco*RI and SpeI. The two fragments were ligated and used to transform *E. coli*, resulting in plasmid BBa_K818600 (P_{sboA}-*amilGFP*).

The *B. subtilis* spoiled meat biosensor strain (*sacA*::P_{sboA}-*amilGFP*, *cat*) was obtained by a double crossover recombination event between the *sacA* regions located on plasmid BBa_K818600 (Figure 1B) and the chromosomal *sacA* gene of strain 168. Transformants were selected on LB agar plates containing chloramphenicol after overnight incubation at 37 °C. Correct integration into the *sacA* gene was tested and confirmed by PCR using primers prIDJ215 (5′-GTGTCAGCGTTCATTGCAGC-3′) and prIDJ216 (5′-GAATAGCACAGATGGCTCAG-3′). All constructed parts are listed in Table 1.

Flow Cytometry. Cells were diluted 100 fold in 0.2 μM filtered minimal medium and scatter and emission were directly measured on a BD FACSCanto Flow Cytometer (BD Bioscience, NL) equipped with a 488 nm solid state, 20 mW laser. Fluorescence intensity of 10^5 cells was measured with at medium flow and forward scatter, side scatter and fluorescence (FL-1) was recorded. Data was analyzed using matlab (Mathworks, U.S.A.) and plotted using Sigmaplot (Systat Software Inc., U.S.A.).

■ ASSOCIATED CONTENT

Supporting Information

Movie S1, a plasmid map and the sequence of the biosensor construct. Movie S1 shows the experimental setup: the headspace of trays containing either fresh meat (on ice) or spoiled meat (>10^6 CFU/g meat) (room temperature) are pumped through shaking cultures of the *B. subtilis* biosensor. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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