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Molecular characterization of an unauthorized genetically modified *Bacillus subtilis* production strain identified in a vitamin B_2 feed additive

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ABSTRACT

Many food and feed additives result from fermentation of genetically modified (GM) microorganisms. For vitamin B2 (riboflavin), GM *Bacillus subtilis* production strains have been developed and are often used. The presence of neither the GM strain nor its recombinant DNA is allowed for fermentation products placed on the EU market as food or feed additive. A vitamin B_2 product (80% feed grade) imported from China was analysed. Viable *B. subtilis* cells were identified and DNAs of two bacterial isolates (LHL and LGL) were subjected to three whole genome sequencing (WGS) runs with different devices (MiSeq, 454 or HiSeq system). WGS data revealed the integration of a chloramphenicol resistance gene, the deletion of the endogenous riboflavin (*rib*) operon and presence of four putative plasmids harbouring *rib* operons. Event- and construct-specific real-time PCR methods for detection of the GM strain and its putative plasmids in food and feed products have been developed.

1. Introduction

Riboflavin (7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxy pentyl]benzo[g]pteridine-2,4-dione, vitamin B_2) is a water-soluble vitamin naturally synthesized by many microorganisms and plants. Since not being produced by higher animals, it is an essential micronutrient in animal and human diets.

The product riboflavin is often used in food as an additive but also finds applications in small amounts as the colouring agent E101 or as a nutritional additive in animal feedstuffs (Abbas & Sibirny, 2011). In the past, riboflavin was mainly chemically synthesised for the production of very pure material. Biotechnological developments have resulted in microbiological processes that can compete with the chemical synthesis and nowadays commercial production of vitamin B_2 is mostly done by fermentation.

In most cases, microbial synthesis of riboflavin involves genetically engineered selected strains of *Escherichia (E.) coli*, *Bacillus (B.) subtilis*, *Ashbya (A.) gossypii*, and *Candida (C.) famata* (Abbas & Sibirny, 2011). Among this plethora of genetically modified micro-organisms (GMMs), GM strains of *E. coli* and *B. subtilis* are probably the best known (see (Burgess, Smid, & van Sinderen, 2009)for a review), as in these bacteria the riboflavin biosynthetic pathway has been studied extensively (reviewed in (Bacher et al., 2001)). *B. subtilis* is known as an aerobic endospore-forming bacterium commonly found in nature and generally not considered to have a pathogenic or toxigenic potential. There is a history of safe use in large-scale fermentation production of speciality chemicals of enzymes used in food production processes, and of several traditional ways of food preparation.

Usually, non-sporulating derivatives of the *B. subtilis* strain 168, which often carry natural mutations inducing riboflavin overproduction, were genetically modified (GM). Introduction of different plasmids harbouring (i) both a (recombinant) *B. subtilis* riboflavin biosynthetic operon (*rib* operon, also known as *ribDEAHT* operon, i.e. including the *ribD*, *ribE*, *ribA*, *ribH*, *ribT* genes) under the control

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of a strong promoter and (ii) antibiotic resistance genes as selection markers (e.g. *cat*, *tet*, *ermAM*), resulted in GM *B. subtilis* strains with multiple copies of the *rib* operon. These strains are able to amplify the riboflavin expression by a magnitude of 10- to 25-fold (Mander & Liu, 2010; Perkins et al., 1999; Smolke, 2009).

According to EFSA guidelines for additives produced with GMM, it is necessary to show that, in the final product, neither the production strain nor its recombinant DNA can be detected (EFSA, 2011). In September 2014, it was notified in the European Rapid Alert System for Food and Feed (RASFF) that a German official enforcement laboratory in Hesse detected viable GM *B. subtilis* spores in a consignment of vitamin B_2 feed additive (80% feed grade) imported from China (RASFF, 2014).

In April 2015, a report of a Belgian official control laboratory was published about the genome sequence of a GM *B. subtilis* strain (Barbau-Piednoir, De Keersmaecker, Wuyts, et al., 2015). This strain (isolate 2014-3557) was identified by a French competent authority in a lot of vitamin B_2 (riboflavin, 80% feed grade) imported to France from China. On basis of next generation sequencing (NGS) data of a Belgian institution a TaqMan® qPCR method (named VitB_2-UGM) for specific detection of this EU-unauthorized GM riboflavin-overproducing *B. subtilis* was developed (Barbau-Piednoir, De Keersmaecker, Delvoye, et al., 2015). However, the TaqMan® qPCR method targets a junction between the riboflavin biosynthesis genes and the vector backbone. It remains unsolved whether the targeted sequence is integrated into the bacterial genome or present on a plasmid. For the latter case, the detection might fail if the plasmid is lost and the corresponding target sequence is therefore missing. In addition, the authors have not reported the comprehensive molecular characterization of the GM strains' genome and plasmids.

In the current study, microbiological and molecular analyses of the GM *B. subtilis* strain found in Germany in 2014 are presented. Whole genome sequencing (WGS) was performed with DNA extracted from two independent isolates to characterize in detail the genome of these riboflavin-overproducing GM *B. subtilis* strains, and to reconstruct the putative plasmids present. Subsequently, construct- and event-specific PCR-based methods for its detection in food and feed were developed and applied.

2. Materials and methods

B. subtilis living cells were isolated independently by the Hessian State laboratory (LHL) and the Bavarian Health and Food Safety Authority (LGL) in Germany from a product lot of vitamin B_2 feed additive (80%) powder imported from China and analysed in the framework of the RASFF notification reference number 2014.1249 (RASFF, 2014). Microbiological and molecular methods for the cultivation and identification of the microorganism and procedures for DNA extraction are described as Supplementary Material.

2.1. PCR analyses

Real-time PCR methods were applied to screen for the presence of DNA sequences from recombinant pUC plasmids (Table 1).

Primers for screening and detection of an erythromycin resistance gene (*ermAM*) and of the chloramphenicol acetyl transferase gene (*cat*) were designed using the Primer Express 3.0 software (Life Technologies Inc.) on the basis of the *Streptococcus faecalis* plasmid pAM-beta1 adenine methylase gene (GenBank:Y00116) and the sequence information for plasmid pC194 of *Staphylococcus (S.) aureus* (GenBank:K01998.1), respectively (Table 1).

WGS data were used to develop a GMM event-specific real-time PCR assay targeting the integration site of the chloramphenicol resistance gene (*cat*) in the genome of the *B. subtilis* isolate. Further real-time PCR assays were designed to detect the putative recombinant extra-chromosomal plasmids (see Supplementary Material).

Conventional PCR was done in 25 μL using a 10× PCR buffer (Qiagen Inc.) with 15 mM $MgCl_2$, 0.5 μM of each primer, 0.625 U Taq polymerase (HotStar, Qiagen Inc.) and 5 μL of template DNA corresponding up to 500 ng DNA. For thermal cycling, an initial denaturation step of 15 min at 95 °C was followed by 45 cycles of 30 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C with a final elongation step of 7 min at 72 °C.

Real-time PCR was performed in an ABI PRISM 7500 (Applied Biosystems) and 25 μL PCR buffer (QuantiTect Multiplex PCR Mix, Qiagen Inc.) with 0.4 μM of each primer, 0.1 μM probe and 5 μL of template DNA corresponding up to 500 ng DNA. Thermal cycling conditions were a denaturation step of 15 min at 95 °C followed by 45 cycles of 1 min at 94 °C and 1 min at 60 °C. Template DNAs tested by the different PCR methods were either extracted from the different isolates of LHL and LGL or from isolate 2014-3557 of the French competent authority (kind gift of the Scientific Institute of Public Health, Brussels – WIV-ISP).

2.2. Whole genome sequencing (WGS)

Three WGS experiments using NGS were performed starting from *B. subtilis* DNA isolated by the two German laboratories (LHL and LGL) and by the Joint Research Centre (JRC) of the European Commission (Italy).

The analysis of the DNA sample isolated by the LHL were performed by a NGS service provider (StarSeq Inc., Germany) using a MiSeq apparatus (Illumina Inc.). For library preparation, 1 ng of extracted DNA was used for application in the Nextera XT DNA library preparation kit (Illumina Inc.). The generated genomic library was sequenced using the MiSeq Reagent Nano Kit v2 300 cycles (Illumina Inc.) and the pair-end option of 2 × 150 bp of the MiSeq sequencing system. Sequencing was monitored using the 'Sequencing Analysis Viewer' program (Illumina Inc.).

The NGS analysis of the DNA sample isolated by LGL was also carried out using Illumina Nextera XT library preparation of 1 ng purified DNA. After quality control, the library was sequenced using an Illumina HiSeq 1500 device using the pair-end flowcell v4 and the HiSeq SBS kit v4, 2 × 50 bp chemistry.

The third NGS analysis was performed by the JRC using another DNA sample from LHL. Here, NGS was done using a GS Junior System (GS Junior System, 454 Life Sciences, Roche Applied Sciences). Rapid libraries (medium length 400–600 bp) were prepared using Rapid library preparation kit (Roche) and all the steps were conducted in accordance with the manufacturers' instructions.

2.3. Bioinformatics

2.3.1. Quality of NGS reads

After adapter trimming, the quality of the NGS Illumina reads was analysed with the Fast QC program (version 1.2.10, default setting) (Andrews, 2010).

For the NGS Roche 454 reads, the quality was inspected by using Roche software (gsRunBrowser, version 2.9).

2.3.2. Assembly and mapping of NGS reads

The following assemblers and mapper tools were used to analyse the produced NGS reads:

- Burrows-Wheeler Aligners program (MiSeq Reporter adapted version, default setting) for mapping of Illumina reads. The genome of *B. subtilis* subsp. *subtilis*, strain AG1839 (NCBI No. CP008698) was used as a reference;