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Use of *Bacillus subtilis* spores in printing and additive manufacturing as a robust, DNA-based anti-counterfeiting and identification feature: stresses, processing and evaluation

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Abstract

Part labeling is a crucial security feature as it can prevent product counterfeiting. Deoxyribonucleic acid (DNA), the information carrier of life, is started to be explored as an engineered information molecule with immense potential in respect to information density and encryption. Most research in this direction is concerned with how to encode binary data into DNA and read the stored information from this DNA. Little to no effort is made on how to apply DNA and the information stored within as an identification label for counterfeit protection. In this study, we explore DNA in various printing processes for its suitability as an anti-counterfeiting and identification tag. DNA is sensitive to environmental influences, which is why we compare the suitability of free DNA against using the spores of the bacterium *Bacillus subtilis* as a naturally evolved DNA protective shell. To integrate these two DNA species into products, we aim to use both conventional printing methods and additive manufacturing processes. Foremost we investigate the stresses on the DNA as well as spores, then derive suitable printing techniques and assess the practical application – processing, extraction and subsequent detection via polymerase chain reaction (PCR). The stresses are differentiated into four groups – solvents, UV irradiation, temperature and shear stress, to which both DNA species are exposed and characterized. In actual printing processes several kinds of stresses are combined and thus we test two exemplary and complementary methods. Namely gravure printing as a conventional 2D– and masked stereolithography as a 3D printing method. We were able to show that both free DNA as well as DNA encapsulated in spores can be readily integrated into printing processes and detected using PCR where there are some significant advantages for DNA protected in spores. Consequently, spores, which can be applied economically, fast and in large quantities using printing, offer great potential for counterfeit protection, for example on drug packaging.

Keywords: gravure printing, 3D printing, masked stereolithography, product piracy, counterfeit protection

1. Introduction

Product piracy is a global economic concern. According to OECD data of 2019, around 2.5 % of all products in international trade are counterfeit, which corresponds to a value of 414 billion EUR (OECD/EUIPO, 2021). Counterfeit products not only have a negative impact on sales revenue for companies, but also damage the reputation of the brand. In addition, low-quality products or harmful materials pose risks for the customer (Kennedy, 2020). This is especially true for counterfeit pharmaceuticals. It is estimated that about half of all medicines worldwide are counterfeits, resulting in several hundred thousand deaths per year due to substandard drugs (Glass, 2014; Shipalana, Matema and van der Westhuizen, 2020). Therefore, there is an industry-independent effort to provide products worth protecting with anti-counterfeiting features. Among the best-known and most widely used security features are holograms, seals and QR-codes, however, protection is limited as these can be imitated by product counterfeiters (Sung, et al., 2015; Fernandes, 2019).

We present a novel approach to anti-counterfeiting by applying deoxyribonucleic acid (DNA) protected in spores using 2D and 3D printing. We compare it with free, unprotected DNA to emphasize the advantages of our "DNA vault" concept. In addition to the potential for product counterfeit protection, it also offers the ability for product identification due to the possibility of storing information as a biological storage device.

The potential for commercial application of DNA as an anti-counterfeiting label had been described by Outwater and Tullis (2000). Preliminary work also exists on the application of DNA-tags in inks, e.g., by Hashiyada (2004). Even though the potential of DNA markers has been proven in many publications, DNA's lack of stability – and thus its suitability for long-term use under sometimes harsh environmental conditions – is still the greatest obstacle (Hashiyada, 2004; Altamimi, et al., 2019; Berk, et al., 2021; Sharief, Chahal and Alocilja, 2021). Embedding DNA in a protective shell (such as silica or nanomaterials) appears promising, but is accompanied by high manufacturing costs and an extensive extraction process (Sharief, Chahal and Alocilja, 2021). Nevertheless, a single commercialized DNA identifier appears to be resistant to high temperatures and UV radiation, but the technical implementation of this protection has not been described (Hayward and Meraglia, 2011).

Instead of the previously described approaches of providing DNA with an artificial protective layer, we take the approach of exploiting the ability of *Bacillus subtilis* to form so-called endospores and use these spores as a "DNA vault". The Gram-positive bacterium *B. subtilis* is one of the best studied microorganisms and widely used in industrial biotechnology (van Dijl and Hecker, 2013). It is able to differentiate between vegetative, i.e., normally reproducing cells, as well as a dormant state, the so-called spores. The process leading to this state is called sporulation and is the ultimate answer of the cell to harsh situations (Setlow, 2006). The cell copies its DNA and packs it into a protective shell equipped with many mechanisms to ensure the DNAs integrity (Nicholson, et al., 2000). Upon completion of this process, the spore destroys its mother-cell and is released. Spores can remain inactive due to their low water content for thousands of years (Sneath, 1962) while retaining their ability to germinate to vegetative cells within hours upon receiving the appropriate environmental signals (Setlow, 2014).

Next to these highly intricate physiological features, *B. subtilis* spores are already produced in large scale industrial processes for agriculture (El-Bendary, 2006) and can also be used for various biotechnological applications (Setlow, 2006; Karava, Gockel and Kabisch, 2021). The safe use of spores is underlined by the fact that *B. subtilis* spores are prescribed as probiotics (Hong, Duc and Cutting, 2005). The use of spores has as well been demonstrated as 3D printable living materials (González, Mukhitov and Voigt, 2020).

Free genomic DNA can be applied to products using printing processes (see Figure 1a), but it may be damaged by the printing ink, processing or environmental influences. Our approach, shown in Figure 1b, is based on the fact that DNA as an ID is protected by *B. subtilis* in spores in such a way that it can be processed by industrial printing processes and is enduring on the products. Samples can then be taken from the protected product and the DNA ID of interest can be detected by a variety of methods. In this work, detection using polymerase chain reaction (PCR) was chosen as the method of choice, as it is well established in the lab. For future application in the field, a lower instrumented method such as loop-mediated isothermal amplification (LAMP) or even an instrumentation-free, on-site detection of the DNA could be established, using different lateral flow assays which have been developed to detect pathogenic bacteria (Lee, et al., 2016) and viruses (Piepenburg, et al., 2006).

Printing processes, such as gravure printing used in this work, seem suitable for the commercial application of spore-protected DNA identification features to products, as they are quick and cost-effective (Kipphan, 2001). However, in addition to the stresses to which the identification feature is subjected during storage, transport and use of the product, printing causes further stresses to be applied to the spores. Printing inks consist of various components, including

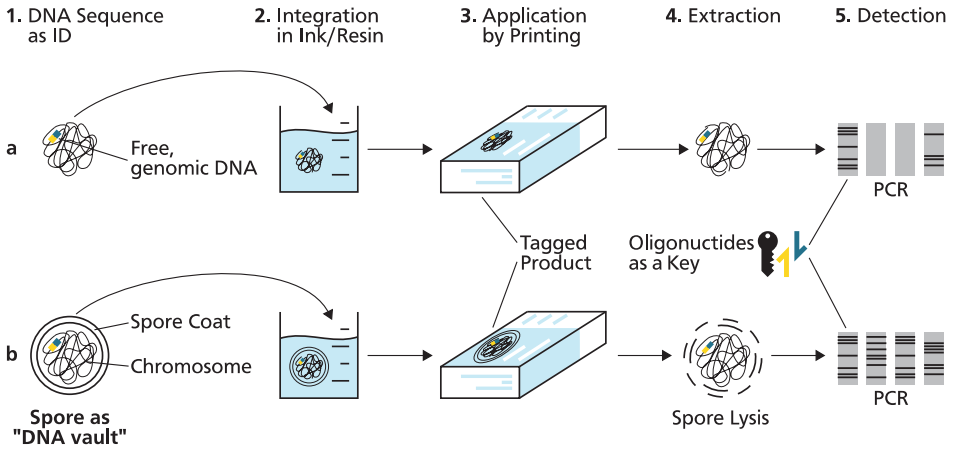

Figure 1: Process for the integration of DNA into an ink, subsequent processing by printing and later extraction and validation of the DNA for free genomic DNA (a), compared to the "DNA vault" concept, in which DNA is protected in spores (b); in this work, detection was performed by PCR; lateral flow assays represent a simpler solution in the field for future application

solvents, which are important for the inks' processability, the drying process or for dissolving additives (Kipphan, 2001). When spores are added to printing inks, they remain in contact with solvents for extended periods of time. The potentially damaging effect of the solvents on the investigated DNA must therefore be examined. Furthermore, in conventional printing, UV-curing inks may be used, in which polymerization is triggered by UV radiation (Kipphan, 2001). Likewise, UV-crosslinking resins are used in 3D printing by vat photopolymerization, including stereolithography (SLA) or digital light processing (DLP). Accordingly, to use spores as a counterfeit protection, they must withstand irradiation by UV light. Thermal stress poses a further challenge. It can occur both in conventional printing, such as for drying the inks, and in 3D printing, for example for melting the filament in fused filament fabrication (FFF). For instance, polylactic acid (PLA), a polymer frequently used as a 3D printing material, is processed at a temperature of 200 °C (Nienhaus, et al., 2019). Among the mechanical loads, shear is the dominant mechanical stress on inks in conventional printing processes. The range of values for typical shear rates varies heavily, depending on the printing process. In offset printing, shear rates of 1 000/s to 10 000/s can be assumed (Mewis and Dobbels, 1981; Pangalos, Dealy and Lyne, 1985), in flexographic printing, the range is between 1 000/s and 100 000/s (Lorenz, et al., 2013), and in gravure printing, shear rates as high as 1×10^6/s may be achieved (Jabrane, et al., 2008).

The processing of spores in printing inks and resins does not require any special machinery or processes. The size of *B. subtilis* spores, with a diameter of about 480 nm and a length of about 1070 nm (Carrera, et al., 2007), barely differs from ink pigments, which are usually in the range of 500 nm to 1500 nm (Szabo, 2002). In contrast to pigments, however, even very low concentrations of spores are sufficient, which we will discuss further below. The challenge rather is not to damage the DNA contained in the organism by processing it using printing methods, which is what we are addressing in this work. The fact that – as previously mentioned – *B. subtilis* is commercially available as a probiotic dietary supplement underlines its safe use. As long as no genetically modified organisms (GMO) are used, there are no special legal restrictions on their use, for example in commercial printshops. As a result of this, we use genetically non-modified wild type strains for producing the spores in order not to consider legislation for GMO in our work. The genetic diversity, i.e., different DNA sequences required to provide individual safety encoding can still be achieved this way by either using different locations to be detected on the strains genome or using strains with differing genomes.

Consequently, in this work we aimed to investigate the feasibility of different printing methods – including conventional printing and additive manufacturing using gravure printing and mask stereolithography (mSLA), respectively – for processing free DNA versus DNA protected by *B. subtilis* spores. Foremost we investigated the influence of the mentioned stresses on both DNA species.

2. Materials and methods

2.1 *Bacillus subtilis* spore and isolated genomic DNA preparation

Spores of *B. subtilis* were prepared as described in Karava, Gockel and Kabisch (2021) with the following modification. Strain S02003 was incubated for 48 h at

37 °C with 200 rpm shaking in Difco Sporulation Media (DSM; 8 g of Bacto nutrient broth, provided by Difco, Franklin Lakes, United States), 10 ml of 10 % KCl solution, 10 ml of 1.2 % $MgSO_4 \cdot 7H_2O$ solution and 0.5 ml of 1 M NaOH were dissolved in 1 l of deionized water and sterilized by autoclaving). For harvesting the spores, the culture broth was centrifuged for 15 minutes at 3 500 × g and the pellet resuspended in deionized water to yield an optical density measured at 600 nm (OD_{600}) of 0.1.

A custom washing solution (9 g/l dissolved by pipetting in deionized water, provided by NeoFroxx GmbH, Einhausen, Germany, available upon request), which safeguards integrity of the spores while eliminating free DNA, was used. This step was necessary to ensure that only spore DNA is detected in subsequent experiments. Therefore, the OD_{600} = 0.1 spore solution was mixed with 5 ml washing solution and incubated at 20 °C for 120 minutes with an agitation of 350 rpm. After this incubation the solution was washed three times with a double volume of deionized water by repeated centrifugation (5 minutes, 20 °C, 3 500 × g) and resuspension. The sample was finally concentrated by centrifugation as described above following a resuspension of the final pelleted spores in 1 ml of deionized water.

Free, genomic DNA (gDNA) from vegetative grown *B. subtilis* was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions with the following modifications. In order to ensure full lysis of the cell wall, the cells were incubated in the resuspension buffer with 10 µl of 10 mg/ml lysozyme (Sigma-Aldrich, St. Louis, USA) dissolved in deionized water for 60 minutes at 37 °C.

2.2 DNA detection

The DNA from spores was detected using colony PCR. Colony PCR involves cultivation on a growth medium, during which *B. subtilis* colonies are formed and subsequent PCR, during which a specific section of the DNA is amplified to such an extent that the detection limit is exceeded.

For this the spores in solution, e.g., organic solvents or from sample taking as described in the corresponding printing processes, were plated to LB-agar plates (7.5 g agar, 5 g tryptone, 5 g sodium chloride and 2.5 g yeast extract, autoclaved in 500 ml of deionized water) and germinated to colonies of vegetative cells in 16 hours at 37 °C. A pipette tip was used to transfer a minimal amount of cells to a 20 µl of *Taq* polymerase reaction mix (EURx, Gdańsk, Poland) and resuspended by pipetting. For gDNA, in contrast to spores, the cultivation step could be omitted.

Presence of sufficiently intact DNA was detected using PCR with the primers listed in Table 1 using *Taq* polymerase and thermocycling protocols as suggested by the manufacturer. The detected DNA sequence was a 424 bp stretch in the *amyE* gene of *B. subtilis*. PCR was evaluated by DNA gel electrophoresis.

Table 1: Oligonucleotides used in this study

Primer-ID	Used for	Sequence (5'→3')
14207	amplification of *amyE* reverse	CAGCGTGTAAATTCCGTCTGC
66032	amplification of *amyE* forward	GATGCGAATACAACAAAAGCCG

2.3 Stresses

Printing processes – regardless of whether they are conventional printing processes or 3D printing processes – have in common that stresses due to solvents, temperature, irradiation and shear occur when ink, resin or molten plastic filament is applied. These sometimes interacting stresses may have a negative effect on the free DNA as well as on the spores. Therefore, the different stresses are first analyzed individually in order to determine whether individual stresses already lead to damage to the DNA sequences to be detected. For all of the experiments described below regarding stresses, three independent experiments, each with spores and gDNA, were performed (n = 3). In addition, a negative control (samples containing neither spores nor gDNA) was included and analyzed.

2.3.1 Ambient conditions I: solvents

The solvents investigated in this work were selected because they are commonly found in commercially available inks and resins. Different groups of solvents were considered (alcohols, ketones, ethers, glycol ethers, aromatic hydrocarbons, esters, glycol esters, chlorinated hydrocarbons and water) resulting in the selection shown in Table 2.

Table 2: List of solvents used in this work to investigate the endurance of spores and gDNA

Solvent
Ethanol
Acetone
Tetrahydrofuran
2-Methoxyethanol
Toluene
Ethyl acetate
2-Methoxyethylacetate
Dichloromethane
Water

Two separate series of experiments were conducted, one with gDNA and one with *B. subtilis* spores. The spores were each provided in 100 µl of fully demineralized water with an optical density OD_{600} of approximately 0.1. According to Karava (2021), OD_{600} = 0.1 corresponds to about 10^7 – 10^8 spores per ml, resulting in 10^6 – 10^7 spores per sample. For gDNA, 10 µl of gDNA was provided in Tris-HCl buffer at a concentration of 30 µg/µl.

The samples were first dried under ambient conditions and then resuspended in the respective solvent (see Table 2). The exposure time to the corresponding solvent was 24 hours, including the time required for evaporation of the solvent under ambient conditions. This was achieved by initiating the evaporation of the solvents at different times to obtain samples that were finally dried after 24 hours. The spores respectively the gDNA, now again in dry condition, were then resuspended in fully demineralized water to their original concentrations and passed on to colony PCR as described in section 2.2. First, colonies are formed on LB-agar plates, and the effect of solvents on the ability of spores to germinate back into replicating cells can be evaluated at the same time.

To unambiguously assign the grown colonies to *B. Subtilis*, a PCR follows, which is evaluated by gel electrophoresis.

2.3.2 Ambient conditions II: irradiation

The energy input into inks and resins as a result of UV radiation varies with the process used. Therefore, two different scenarios were investigated.

First, UV irradiation in a conveyor drying system for printing inks (IST Metz GmbH, Nürtingen, Germany) with a power of 8 000 W (mercury-vapor lamps) distributed over a width of 40 cm at a wavelength spectrum of 180–450 nm and 5 m/min throughput speed was tested. This corresponds to a typical cross-linking process condition for an UV-curable ink.

On the other hand, a post-curing UV chamber for SLA components (Form Cure, Formlabs, Somerville, USA) was used. The device has a power of 9.1 W (from LEDs) at a wavelength of 405 nm. The selected exposure time to UV irradiation was 30 minutes at room temperature, which is a typical post-curing process for 3D printed parts.

Both spores (100 µl at an OD_{600} of 0.1 in demineralized water, corresponding to approximately 10^6 – 10^7 spores per sample) and gDNA (10 µl with a concentration of 30 µg/µl in Tris-HCl) were dried in aluminum trays at room temperature and then exposed to UV irradiation in the respective device. Samples were subsequently resuspended in 100 µl or 10 µl of demineralized water, respectively, and submitted for colony PCR.

2.3.3 Ambient conditions III: temperature

In printing processes, both for conventional and 3D printing, it can be assumed that the temperature exposure is only of short duration. Therefore, we consider that an exposure to heat of 60 seconds is appropriate. Again, 100 µl of spores with an OD_{600} of 0.1 in demineralized water were used corresponding to approximately 10^6 – 10^7 spores per sample. Similarly, 10 µl of gDNA with a concentration of 30 µg/µl in Tris-HCl buffer was examined. Both are first dried under ambient conditions in an aluminum tray. The aluminum tray was then placed on a hot plate and held at 200 °C for a period of 60 seconds. Finally, the dry sample was resuspended with 100 µl or10 µl of demineralized water, respectively, and transferred for colony PCR.

2.3.4 Mechanical loads: shear stress

A rotary rheometer (Malvern Kinexus lab+, Malvern Instruments, Malvern, United Kingdom) with cone-and-plate geometry (cone angle 1°) was used to apply defined shear rates to the liquid samples. In between the cone and plate geometry, 1 002 µl of spores in fully demineralized water with an OD_{600} of 0.1 were dispensed, corresponding to approximately 10^7 – 10^8 spores (Karava, 2021). The value of 1 002 µl results from the necessity to completely fill the gap between cone and plate. Similarly, the same volume of liquid containing gDNA in Tris-HCl buffer with a concentration of 3 µg/µl was assayed. After dispensing the liquid between the cone-plate geometry, the shear rate was incrementally increased over 400 s to the maximum possible value of 6 000/s and held at this rate for 20 seconds. At 6 000/s, the shear rate is within a typical range for offset and flexographic printing, as previously noted. The temperature of the liquid was kept constant at 25 °C. After the measurement the samples were submitted to colony PCR.

The higher shear rates found in gravure printing and their effect on spores and gDNA were tested on a printability tester (IGT G1-5, IGT Testing Systems, Almere, Netherlands, see Figure 4). Tests were carried out using a gravure cylinder with a screen ruling of 40 lines/cm, a cell volume of 20 ml/m^2, a speed of 0.6 m/s and a printing force of 700 N, which is distributed over a width of 40 mm. A sheet of 125 µm thick polyethylene naphthalate film (PEN, Teonex Q65 HA, DuPont Teijin Films, Luxembourg) was used as a substrate for printing tests. The PEN sheet is provided with a surface treatment, not further specified by the manufacturer, which is intended to facilitate printing. The smooth

plastic film facilitates the extraction of spores or gDNA after printing. For the test, 100 µl of liquid (spores in demineralized water, OD_{600} = 0.1 or gDNA in Tris-HCl, 30 µg/µl) was applied between the gravure cylinder and the doctor blade and printed onto the substrate.

Extraction of the dried layer with spores or gDNA was performed by pipetting 100 µl of demineralized water onto the plastic film, then rubbing with the pipette tip and pipetting back again. The sample concentration is subject to fluctuations, however, in preliminary experiments we proved that the extracted sample amount was always sufficient for the subsequent colony PCR.

2.4 Gravure printing

Since gravure printing has the highest shear rates (Jabrane, et al., 2008) as well as the highest surface pressure (Kipphan, 2001) compared to other printing techniques such as offset or flexographic printing, we use it to investigate the processability of spores and gDNA.

For printing tests by rotogravure, the same IGT G1-5 printability tester as used in section 2.3.4 for the investigation of shear forces was chosen; the principle is shown in Figure 2.

For this purpose, the spores or gDNA were applied to the printing inks. Two different commercially available inks were investigated: a water-based coating (Senolith WB Gloss Coating FP DC, WEILBURGER Graphics, Gerhardshofen, Germany) and a solvent-based ink (Solvaplast P HR Cyan, SunChemical, Parsippany, US). For this purpose, 300 µl of spores in demineralized water (OD_{600} of 0.1, approx. $10^7 - 10^8$ spores) were dried and mixed with 300 µl of the respective coating or ink. Likewise, 100 µl of gDNA in Tris-HCl (30 µg/µl) were dried and mixed in 100 µl of coating or ink and printed on the PEN substrate (as specified in 2.3.4). In all cases, gravure printing was done as described previously (printing force of 700 N which is distributed over a width of 40 mm, substrate speed of 0.6 m/s, screen ruling of 40 lines/cm, cell volume of 20 ml/m²) on a 125 µm thick PEN substrate. Drying took place at room temperature. The changes in the rheological properties of the ink tagged with spores or gDNA were not further investigated due to the proof-of-concept scope of this work.

To assess whether the spores or gDNA embedded in the inks have withstood the loads, but also for later practical use to verify the authenticity of a spore-protected product, it is necessary to extract the spores or gDNA from the gravure printed substrate. Extraction was done as described in the previous section by pipetting 100 µl of demineralized water onto the dried film, dissolving the ink containing spores or gDNA and pipetting it back. For the solvent-based ink, the same procedure was performed using 100 µl of ethanol. In this case, the solvent subsequently needed to be evaporated at room temperature and then the extract to be resuspended with the same amount of demineralized water. Specimens were then transferred to subsequent colony PCR.

Again, three tests were performed for each ink with spores or gDNA, respectively. A thorough cleaning of all components in contact with spores or gDNA was performed between experiments to ensure independent tests, including the use of strong UV radiation for cleaning. Similarly, negative samples (ink/coating without gDNA or spores) were included and tested.

2.5 Additive manufacturing

Based on the results from the previous experiments with UV radiation in section 2.3.2, mSLA was chosen as a suitable 3D printing technique. The setup of an mSLA 3D printer can be seen in Figure 3.

A commercially available mSLA printer (Zortrax Inkspire, Zortrax, Olsztyn, Poland) and a common epoxy methacrylate based resin (Tough Transparent, Prusa Research, Prague, Czech Republic) were used. The

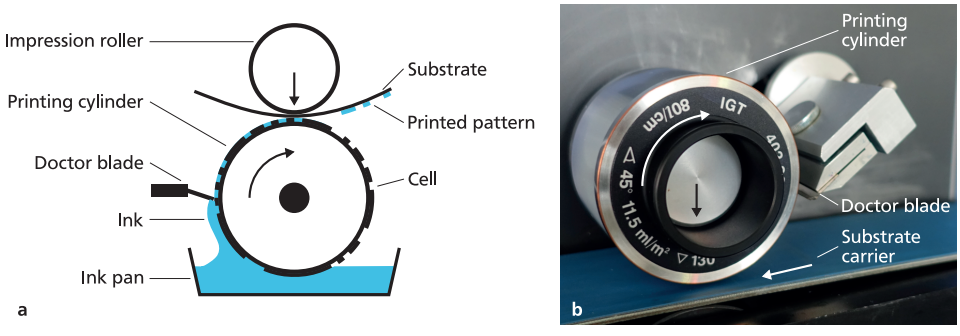

Figure 2: Principle of gravure printing as used in rotogravure printing machines (a); the printability tester used in this work (b), on the other hand, is slightly different in design, in particular the ink is applied to the doctor blade using a pipette

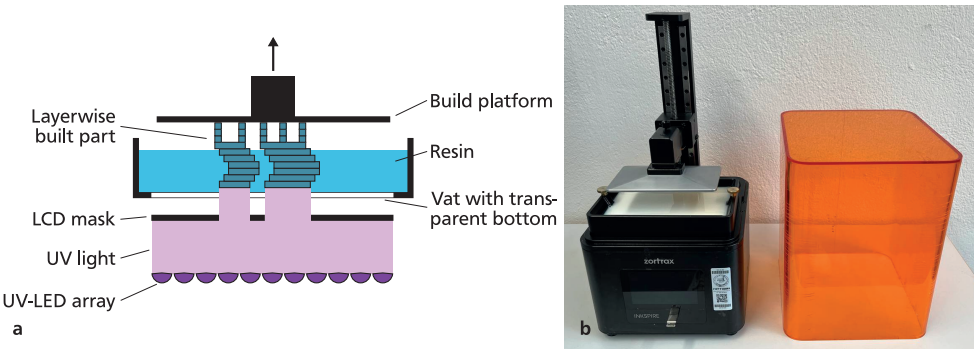

Figure 3: Principle of operation of an mSLA 3D printer (a), which is characterized in particular by the use of an LCD mask that allows the UV light to pass selectively so the resin polymerizes in the desired locations; Zortrax Inkspire mSLA-printer (b), as used by the authors

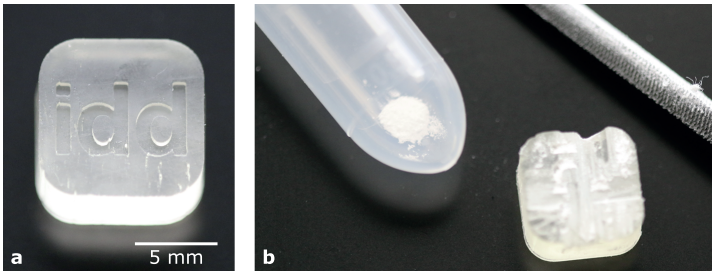

Figure 4: Specimen produced using mSLA (dimensions 10 mm × 10 mm × 5 mm) incorporating DNA protected in spores over its entire volume (a), specimen after sample extraction using a file, the resulting powder is passed to colony PCR in a microcentifuge tube (b)

printer manufacturer's software (Z-Suite) was used together with typical settings (layer thickness of 50 µm, layer exposure time of 10 s, five bottom layers with 120 s exposure time). These constraints were deliberately chosen to demonstrate the practical application of the spores as an anti-counterfeiting device without modifications to the equipment and the processing parameters. To fill the resin vat of the printer sufficiently, 10 ml of spores with $OD_{600} = 1$ were dried at room temperature, mixed with 100 ml of resin and poured into the vat. Three identical specimen geometries (10 mm × 10 mm × 5 mm, see Figure 4) were manufactured. Because of the good resistance to solvents of the cured resin, a different procedure was chosen than for the samples produced by gravure printing and the sample was extracted mechanically using a file. The resulting powder was passed to colony PCR in 100 µl of demineralized water. Due to the high amount of material required, no experiments were performed with gDNA in this case.

3. Results and discussion

The complete results of the PCR and corresponding gel electrophoresis can be found in supplemental data, Table S1 <https://doi.org/10.48328/tudatalib-977>.

First, the stress due to contact with solvents was evaluated for spores and gDNA. Nine different representatives of solvents frequently used in printing inks including water were tested. After 24 hours in contact with the respective solvents, *B. subtilis* spores were able to germinate thereafter, regardless of the solvent, and the DNA sequence was detected by PCR. An example of the formation of colonies can be seen in Figure 5. Only very few outliers not showing a corresponding signal after gel-electrophoresis occurred (i.e., see Figure 6 "spores in Ethanol"). This is likely due to a pipetting error while setting up the PCR reactions, but is mitigated by using appropriate numbers (i.e., $n = 3$) of technical replicates. There is an uncertainty about the absolute amount of DNA tested in PCR. The amounts of free DNA used vary significantly, as there is 30 µg/µl of free gDNA versus ~10–15 ng of DNA from spores per sample. However, germinating the spores results in replicating cells, which in turn results in additional DNA material. Therefore, amounts of gDNA as well as spores utilized were chosen based on their ease of application in a technical process. The sensitivity of PCR allows detection of as little as 35 molecules of DNA (Purcell, et al., 2016), which are well surpassed in any sample taken by several orders of magnitude, thus allowing us to make qualitative statements. When testing for resistance to solvents, spores were shown to be

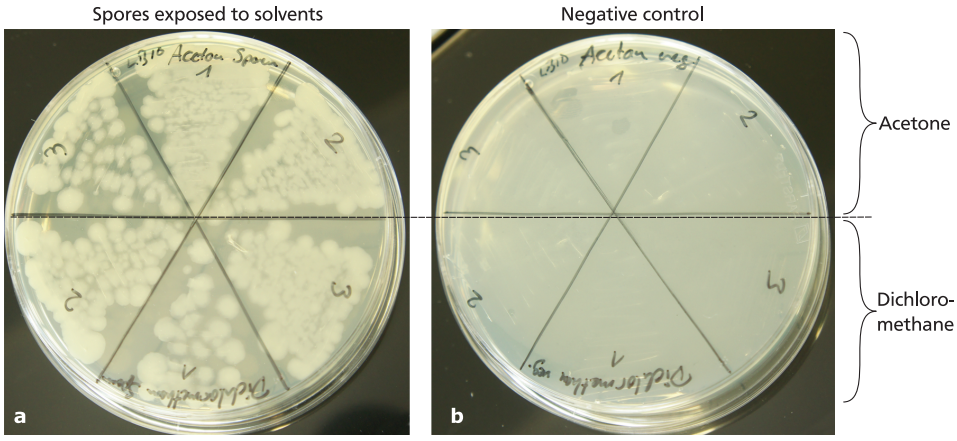

Figure 5: The formation of colonies on a growth medium (LB-agar, see section 2.2) shows in (a) that the spores were able to germinate and grow after contact with solvents (top: acetone, bottom: dichloromethane), while the negative samples (no spores) can be seen on the right side (b); each sample was in triplicate indicated by the intersecting lines between them

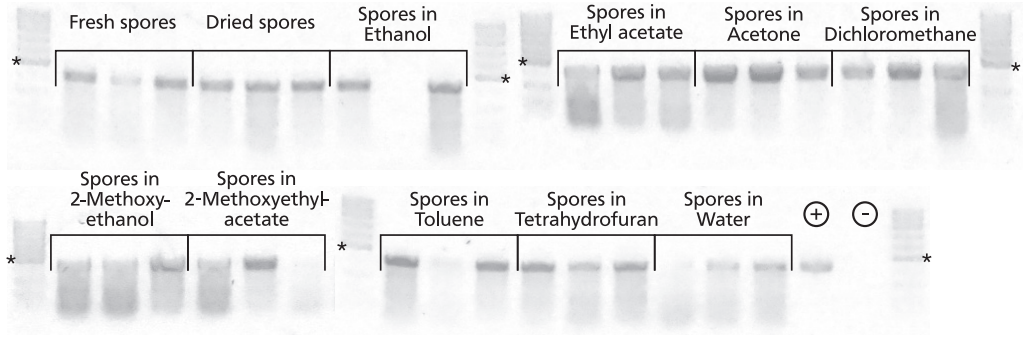

*Figure 6: Gel electrophoresis image of the detection of specific spore DNA with PCR after the labeled treatment; *: reference DNA band at 500 bp; ⊕: positive control of untreated DNA; ⊖: negative control not containing any added DNA (n = 3)*

unaffected by the substances studied for the selected period of time. For gDNA, resistance could also be shown, with the exception of 2-Methoxyethanol. It can therefore be concluded that spores are suitable for use in printing inks. This also applies to gDNA, however, with the exception mentioned.

The resistance against UV irradiation was investigated with two different intensities. In the post-curing chamber ("UV low" in Table 3), the DNA sequence from both spores and gDNA could be detected after irradiation. For the higher UV exposure of the continuous dryer ("UV high" in Table 3), no DNA sequence could be detected for either spores or gDNA. The different results indicate that for UV exposure of spores and gDNA, respectively, there seem to be threshold values up to which a DNA sequence can be detected by PCR. Thresholds were not investigated within the scope of this work, but it has been shown that the performance of commercially available drying units for UV-crosslinking coatings appears to be too high for spores and gDNA in a dry, non-embedded condition. Therefore, processing of spores or gDNA in UV-curing coatings does not seem to be appropriate, whereas processing by 3D printing using mSLA appears to be feasible.

Short-term exposure to elevated temperatures (200 °C, 60 s, "Temperature" in Table 3) also resulted in no DNA sequence being detected for either spores or gDNA. This suggests that damage or destruction of the DNA segments occurred during the heat treatment. Therefore, the use of spores or gDNA in processes where the investigated temperatures may occur (for example, in 3D printing using FFF) should not be considered.

Mechanical stress, on the other hand, was well endured by both spores and gDNA, as in both cases the detection of the DNA sequence was successful – for low as well as for high shear rates ("Shear low" and "Shear high" in Table 3). Hence, it can be concluded that conventional printing processes, which involve high shear rates, are suitable for processing both spores and gDNA.

The combination of different loads was investigated in actual printing tests. In conventional gravure printing with water- and solvent-based inks, samples of spores were extracted from the printed film and DNA sequences were detected by PCR. Spores could also be taken from components produced by additive manufacturing (mSLA) and DNA detection could be performed successfully (see "Gravure" and "mSLA" in Table 3). However, using gDNA, only printing tests with water-based coatings were carried out, but already at this stage no DNA sequence could be detected.

The complete list of all tests on single and combined loads on spores as well as on gDNA can be found in Table 3. An exemplary figure of the PCR results analyzed by gel electrophoresis which form the basis for Table 3 is depicted in Figure 6.

Table 3: Detection of DNA after stresses on free genomic DNA (gDNA) and spore-protected DNA (spores) after PCR; due to the qualitative nature of the measurement, successful detection (●) or partially successful detection with a single outlier (●[1]) is distinguished from unsuccessful detection (○) resp. not tested (–); corresponding gel electrophoresis images can be found in supplementary data (n = 3)

			Spores	gDNA
Single stress	**Solvents**	Ethanol	●[1]	●
		Acetone	●	●
		Tetrahydrofuran	●	●
		2-Methoxyethanol	●	○
		Toluene	●	●
		Ethyl acetate	●	●
		2-Methoxyethylacetate	●[1]	●
		Dichloromethane	●	●
		Water	●[1]	●
	UV	UV low	●	●
		UV high	○	○
		Temperature	○	○
	Mech. stress	Shear low	●	●
		Shear high	●	●
Comb. stresses	**Printing**	Gravure (water)	●	○
		Gravure (solvent)	●	–
		mSLA	●	–

So far, based on the previous experiments, no particular benefit of spores as a "DNA vault" has emerged in comparison to gDNA, assuming that only individual stresses are investigated. Only for 2-Methoxyethanol an advantage of DNA protected in spores is found for individual stresses. This assumption could be refuted in the printing experiments, where combined loads are present. In the case of gravure printing, it can be seen that gDNA was no longer detectable after processing in water-based coating, while DNA extracted from spores could be detected. This also suggests that a simple superposition of the results from the previous experiments is not possible.

In addition, the potential of DNA protected in spores as an anti-counterfeiting agent has also been demonstrated in additively manufactured components. The DNA extracted from spores could be detected despite the combined exposure to resin ingredients and UV radiation. However, the spores in the mSLA-manufactured specimens are distributed over the entire volume of the part, making it seem inappropriate in terms of cost-effectiveness and the number of spores required. A local application of resin incorporating spores, for example by using two-component mSLA, might be appropriate.

4. Conclusion

We accomplished the processing of DNA protected in spores using gravure and 3D printing, and successfully detected the DNA after extraction from the sample. To demonstrate the benefit of spores and the potential of spores as an anti-counterfeiting feature applied by printing, all experiments were also performed and compared with free, genomic DNA.

Single stresses that can occur in conventional and 3D-printing (solvents, UV irradiation, temperature and mechanical stress as shear) were applied to spores and gDNA. For a single solvent (2-Methoxyethanol), an advantage in terms of resistance of the DNA protected in spores compared to gDNA could be shown. For other individual stresses, whether solvents, UV radiation, temperature or shear stress, no advantage has been found for DNA protected in spores compared to free DNA. The knowledge about the resistance of the spores to environmental conditions obtained in this work allows, in reverse, recommendations for cleaning, which is also relevant for an application in print shops. Spore-protected DNA can be successfully removed by high UV irradiation and elevated temperatures. However, in the case of combined loads, such as those found in actual printing processes, the advantage of DNA protected in spores is more evident. In gravure printing of a water-based coating, no DNA segments from unprotected gDNA could be detected by PCR. Though, when spores were used, detection by colony PCR was successful, thus demonstrating that not only the DNA but the complete spore survived. This allows using germination to "amplify" the DNA signal by natural DNA replication of growing cells. In addition, the usability of spores in a solvent based ink applied via gravure printing was shown. Likewise, the suitability of spores for processing by mSLA has been proven. In particular, it could be demonstrated that spores – unlike free genomic DNA – offer a significant advantage under

realistic processing conditions due to their robustness. In future studies, other printing techniques with a wider range of inks should be investigated.

Spores as a "DNA vault" thus offer great potential for use as counterfeit protection when applied using inexpensive and productive industrial 2D- and 3D-printing processes. Especially in conventional printing techniques, such as gravure printing shown in this work, spores may be applied locally and thus economically. This does not require any modifications to the machinery, which facilitates the future implementation of this technology. Because of the non-pathogenic nature of *Bacillus subtilis*, it may be used without concern in print shops and additive manufacturing facilities. In fact, they are already used in e.g. agriculture and are commercially available as a probiotic dietary supplement. Genetically modified organisms are restricted in use, so a special designed DNA sequence is not possible under current legislation. Right now, this is not needed, as the natural sequence length in *B. subtilis* already provides a high number of possible variants by using different locations to be detected on the strains genome or using strains with differing genomes. The detection and validation of the DNA right now needs equipment and knowledge (PCR, etc.), but should in future approaches be realized by e.g. lateral flow assays, which can be used by untrained users.

Supplemental data

Supplemental data (Table S1 including gel electrophoresis images) can be accessed through DOI <https://doi.org/10.48328/tudatalib-977>.

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