Expression of degQ gene and its effect on lipopeptide production as well as formation of secretory proteases in Bacillus subtilis strains

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Abstract
Bacillus subtilis is described as a promising production strain for lipopeptides. In the case of B. subtilis strains JABs24 and DSM10T, surfactin and plipastatin are produced. Lipopeptide formation is controlled, among others, by the DegU response regulator. The activating phospho-transfer by the DegS sensor kinase is stimulated by the pleiotropic regulator DegQ, resulting in enhanced DegU activation. In B. subtilis 168, a point mutation in the degQ promoter region leads to a reduction in gene expression. Corresponding reporter strains showed a 14-fold reduced expression. This effect on degQ expression and the associated impact on lipopeptide formation was examined for B. subtilis JABs24, a lipopeptide-producing derivative of strain 168, and B. subtilis wild-type strain DSM10T, which has a native degQ expression. Based on the stimulatory effects of the DegU regulator on secretory protease formation, the impact of degQ expression on extracellular protease activity was additionally investigated. To follow the impact of degQ, a deletion mutant was constructed for DSM10T, while a natively expressed degQ version was integrated into strain JABs24. This allowed strain-specific quantification of the stimulatory effect of degQ expression on plipastatin and the negative effect on surfactin production in strains JABs24 and DSM10T. While an unaffected degQ expression reduced surfactin production in JABs24 by about 25%, a sixfold increase in plipastatin was observed. In contrast, degQ deletion in DSM10T increased surfactin titer by threefold but decreased plipastatin production by fivefold. In addition, although significant differences in extracellular protease activity were detected, no decrease in plipastatin and surfactin produced during cultivation was observed.

Keywords
Bacillus subtilis, degQ, lipopeptide, plipastatin, secretory proteases, surfactin
1 | INTRODUCTION

*B. subtilis* is one of the best characterized gram-positive bacteria and serves as a model organism for fundamental and applied research. The knowledge about the physiology of *B. subtilis* made this strain an important microbial host in biotechnology (Stein, 2005). In this context, *B. subtilis* is used as a super-secreting cell factory due to benefits such as excellent fermentation properties, high product yields in gram per liter range, and the lack of toxic by-products (van Dijl & Hecker, 2013). In addition to the production of industrially relevant enzymes and vitamins (Cui et al., 2018), *B. subtilis* natively forms a variety of secondary metabolites. Among these compounds, three lipopeptide families, in particular, namely surfactin, iturin, and fengycin, are reported to have broad bioactivity based on a common amphipathic structure comprising a fatty acid linked to a peptide moiety (Geissler et al., 2019; Marvasi et al., 2010). Different amino acid sequences in the circular peptide and variable fatty acid chain lengths give each lipopeptide unique properties (Zhao et al., 2017). In the genome of *Bacillus* spp., bacteria encoding for fengycin biosynthesis also show the ability to produce surfactin (Kim et al., 2010; Yaseen et al., 2018). In this context, regulatory crosstalk of non-ribosomal peptide synthetases (NRPSs) is conceivable (Vahidinasab et al., 2020; Yaseen et al., 2018). Surfactin is described as one of the most powerful microbially produced biosurfactants and has great potential to be used in many industrial sectors such as cosmetics, pharmaceuticals, as well as food (Henkel et al., 2017; Hoffmann et al., 2021). The benefits of surfactin are not limited to emulsifying activity, as some studies reported antimicrobial and anticancer properties (Béven & Wroblewski, 1997; Kameda et al., 1974). Fengycins, including plipastatin as a member of this group, have been shown to have several antagonistic effects for soil-borne fungal phytopathogens and may act as elicitors for systemic plant resistance (Cawoy et al., 2015). Moreover, fengycin has been described to have antiviral, antibacterial, and anticancer properties (Huang et al., 2006; Ongena et al., 2007; Raaijmakers et al., 2010; Yin et al., 2013). Due to these characteristics, fengycin has great potential for future agricultural applications.

Structurally, lipopeptides consist of a cyclic peptide and a fatty acid chain. In the case of surfactin and plipastatin, a hepta- or deca-peptide moiety, respectively, is linked to a β-hydroxy fatty acid chain of varying length (Cochrane & Vederas, 2016; Gao et al., 2018). The production of lipopeptides depends on NRPSs expressed by the *srfAA-AD*-operon for surfactin and by the *ppsA-E*-operon for plipastatin (Nakano et al., 1988; Tosato et al., 1997). Posttranslationally, NRPSs need to be activated by the 4-phosphopantetheinyl transferase Sfp (Nakano et al., 1992; Quadri et al., 1998). In addition, superordinated stimuli such as quorum sensing and nutrient availability influence lipopeptide biosynthesis. Specifically, several global regulators including SpoOA, AbdB, CodY, and DegU are involved in the control of NRPS expression (Nakano et al., 1992; Serrour & Sonenshein, 1996; Sun et al., 2021; Vahidinasab et al., 2020).

Different physiological adaptations are associated with the DegU regulon, including the formation of extracellular enzymes, genetic competence, and biofilm formation (Dahl et al., 1992; Kobayashi, 2007; Möder et al., 2002; Msadek et al., 1990; Shimotsu & Henner, 1986). Moreover, also surfactin and plipastatin production are affected by DegU regulation (Miras & Dubnau, 2016; Tsuge et al., 1999). As a response regulator, DegU is part of the two-component DegS-DegU system. After activating phospho-transfer from histidine kinase DegS to the response regulator DegU, the phosphorylated DegU version (DegU-P) can regulate the expression of various genes (Murray et al., 2009). In addition to this process, DegQ, a small protein of 46 amino acids, stimulates the autophosphorylation of DegS and is important for the complete activation of DegU by phosphorylation (Do et al., 2011; Yang et al., 1986). In the case of the domesticated laboratory model strain *B. subtilis* 168, a single base mutation in the −10 box silences *degQ* gene expression (Stanley & Lazazzera, 2005). As a result, phospho-transfer for DegU activation is reduced.

In this study, the lipopeptide-producing *B. subtilis* strain JABs24, an *sfp* derivative of *B. subtilis* 168, and the wild-type strain DSM107, which exhibits a native *degQ* expression, were used to analyze the effect of *degQ* expression on lipopeptide production and formation of secretory proteases.

2 | MATERIALS AND METHODS

2.1 | Chemicals, materials, and standard procedures

All chemicals were purchased from Carl Roth GmbH & Co. KG, if not otherwise mentioned. Standard molecular techniques were performed as described by Sambrook and Russell (2006). PCRs were carried out on a PCR thermal cycler (peqSTAR 96X VWR GmbH) using DNA polymerase (Phusion High-Fidelity #M0530S, New England BioLabs). PCR reactions were purified after agarose-based gel electrophoresis using QIAquick PCR & Gel Cleanup Kit (Qiagen). Plasmid DNA was extracted with innuPREP Plasmid Mini Kit (Analytik Jena AG), and chromosomal DNA was purified using the ready-to-use innuPREP Bacteria DNA Kit (Analytik Jena AG) according to the manufacturer's instruction.

2.2 | Strain construction, plasmids, and transformation method

All strains and plasmids used in this study are summarized in Table 1. The oligonucleotides used to construct the strains and plasmids are listed in Table 2. *Escherichia coli* JM109 was used for plasmid propagation and cloning. Transformation of naturally competent *B. subtilis* strains was performed according to the "Paris method" (Harwood & Cutting, 1990). Depending on the selection marker, transformants were selected on Lysogeny Broth agar supplemented with ampicillin (100 µg/ml), spectinomycin (100 µg/ml), or erythromycin (10 µg/ml for *E. coli* and 5 µg/ml for *B. subtilis*). All plates were incubated at 37°C.
For the construction of BCKN1, the degQ gene of *B. subtilis* DSM10^T^ including native promoter region (towards 510 bp) and terminator structure was integrated into the amyE locus of *B. subtilis* JABs24 using plasmid pMAV5 (Vahidinasab et al., 2020). BCKN2 was created by integrating the deletion of degQ gene in *B. subtilis* DSM10^T^ using chromosomal DNA of *Bacillus* knockout erythromycin (BKE) strain.

**TABLE 1 Bacterial strains and plasmids used in this study**

| Strain or Plasmid | Origin or Genotype | References |
|-------------------|--------------------|------------|
| **Strains**       |                    |            |
| *Escherichia coli*|                    |            |
| JM109             | mcrA recA1 supE44 endA1 hsdR17 (rK^-mK^+) gyA96 relA1 thi Δ(lac-proAB) F [traD36 proAB'* lacZ ΔlacZ ΔM15] | Yanisch-Perron et al. (1985) |
| *Bacillus subtilis*|                    |            |
| JABs24            | trp+, ΔmanPA; sfp+ | Geissler et al. (2019) |
| DSM10^T^          | wild-type strain   | German Collection of Microorganisms and Cell Cultures GmbH |
| BCKN1             | trp+: ΔmanPA; sfp+: ΔamyE::510 bp-degQ | This study |
| BCKN2             | DSM10^T^ ΔdegQ::erm | This study |
| BKE31720          | trpC2; ΔdegQ::erm | Bacillus Genetic Stock Center |
| BMV15             | DSM10^T^ wild-type; amyE::[P_{degQ}-lacZ, spcR] (degQ promoter region for lacZ fusion was derived from *B. subtilis* DSM10^T^) | This study |
| BMV16             | trp+: ΔmanPA; sfp+: amyE::[P_{degQ}-lacZ, spcR] (degQ promoter region for lacZ fusion was derived from *B. subtilis* DSM10^T^) | This study |
| BMV17             | DSM10^T^ wild-type; amyE::[P_{degQ}-lacZ, spcR] (degQ promoter region for lacZ fusion was derived from *B. subtilis* JABs24) | This study |
| BMV18             | trp+: ΔmanPA; sfp+: amyE::[P_{degQ}-lacZ, spcR] (degQ promoter region for lacZ fusion was derived from *B. subtilis* JABs24) | This study |
| **Plasmids**      |                    |            |
| pKAM446           | ori_{pUC18}, bla, rop, ermC, amyE::[P_{degQ}-lacZ, spcR] - amyE | Hoffmann et al. (2021) |
| pMAV5             | ori_{pBR322}, rop, ermC, bla, amyE::[P_{degQ}-lacZ, spcR] - amyE | Vahidinasab et al. (2020) |
| pMAV14            | ori_{pUC18}, bla, rop, ermC, amyE::[P_{degQ}-lacZ, spcR] - amyE (degQ promoter sequence derived from *B. subtilis* JABs24) | This study |
| pMAV15            | ori_{pUC18}, bla, rop, ermC, amyE::[P_{degQ}-lacZ, spcR] - amyE (degQ promoter sequence derived from *B. subtilis* DSM10^T^) | This study |
BKE31720 carrying the deletion of the degQ gene (ΔdegQ::erm) (Koo et al., 2017). The plasmids for the construction of the PdegQ reporter strains were cloned using Gibson Assembly (New England Biolabs). Therefore, the pKAM446 plasmid was digested with NheI and NdeI before integrating amplified degQ promoter regions from JABs24 and DSM10T, respectively. The correctness of all mutant strains was ensured by sequencing (Eurofins Genomics Germany GmbH).

2.3 | Cultivation and growth conditions

The composition of the mineral salt medium used in this study was based on the fermentation medium containing 8 g/L glucose of Willenbacher et al., (2014) with slight modifications: 4.0 × 10⁻⁶ M Na₂EDTA × 2 H₂O, 7.0 × 10⁻⁶ M CaCl₂, 4.0 × 10⁻⁶ M FeSO₄ × 7 H₂O, 1.0 × 10⁻⁶ M MnSO₄ × H₂O, 50 mM (NH₄)₂SO₄, 0.03 M KH₂PO₄, 0.04 M Na₂HPO₄ × 2 H₂O and 8.0 × 10⁻⁶ M MgSO₄ × 7 H₂O. An overnight culture in Lysogeny Broth medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) was used for the first preculture. The second preculture using a mineral salt medium was inoculated with exponentially growing cells from the first preculture. When the cell culture reached the exponential phase, the main culture was inoculated with exponentially growing cells from the first preculture. The main culture was adapted from Charney and Tomarell (1947) and applied by Baur et al., (2015). In detail, 100 ml of the supernatant was transferred to a microtiter plate and mixed with 50 μl NaOH (1 M). The absorbance was measured in a microtiter plate spectrophotometer (MULTISKAN GO, Thermo Scientific) at 450 nm. A blank for the measurement was performed with the cell-free supernatant after the addition of trichloroacetic acid. A calculation of the protease activity is summarized in Equations (2) and (3).

2.4 | Data analysis

For the conversion of OD₆₀₀ into cell dry weight (CDW), the correlation factor of 3.762 was determined in a preliminary experiment described by Willenbacher et al., (2014). The product yield of biomass $Y_{p/x}$ (g g⁻¹) was calculated using Equation 1. For the calculation, the mean values of the total mass of the product (mSurfactin, mPlipastatin) and CDW (mCDW) from the beginning of cultivation to the time point at the end of the exponential phase were used.

$$Y_{p/x} = \frac{\Delta m_{\text{Surfactin, Plipastatin}}}{m_{\text{CDW}}} \text{[g g}^{-1}]$$

2.5 | Assay for extracellular protease activity

The total activity of the degrading proteins in the cultivation medium was analyzed by azocasein assay. The measurement method was adapted from Charney and Tomarell (1947) and applied by Baur et al., (2015). In detail, 100 μl of cell-free supernatant was mixed with an equal volume of a pre-warmed (40°C for 5 min) azocasein solution (5 g/L, pH 7, dissolved in H₂O) and subsequently incubated for 1 h at 37°C and 1.07 g. The reaction was stopped by adding 20 μl trichloroacetic acid (2 M). Precipitated azocasein was removed by centrifugation at 1715 g for 10 min at 4°C. Subsequently, 150 μl of the supernatant was transferred to a microtiter plate and mixed with 50 μl NaOH (1 M). The absorbance was measured in a microtiter plate spectrophotometer (MULTISKAN GO, Thermo Scientific) at 450 nm. A blank for the measurement was performed with the cell-free supernatant after the addition of trichloroacetic acid. A calculation of the probe activity is summarized in Equations (2) and (3).
The calculation of the absorption difference (ΔA) is described in Equation (2). The following volumetric peptidase activity is defined in Equation (3) and is determined by the absorption difference (ΔA), a dilution factor (F), the total measurement volume (V) [µl], the incubation time (t) [h], and the volume of the cell-free supernatant used for the assay (v) [µl].

3 | RESULTS

3.1 | Expression of degQ gene in B. subtilis strains JABs24 and DSM10T

As a derivative of B. subtilis 168, the surfactin-forming strain JABs24 exhibits a point mutation within the –10 box of the degQ gene compared to B. subtilis wild-type strains such as B. subtilis DSM10T (Figure 1). This base substitution (T::C) in the promoter region of degQ was previously described by Stanley and Lazazzera (2005) and leads to significantly reduced gene expression of degQ in strain 168. This point mutation is also prominent when comparing the genome sequences of B. subtilis strains 168 and DSM10T (Kunst et al., 1997; Lilge et al., 2021).

To analyze the effect of promoter point mutation on degQ gene expression, reporter strains with chromosomally integrated P_{degQ}-lacZ fusions were constructed. Accordingly, time-resolved expression patterns were measured for both degQ promoter versions in JABs24 (BMV16 and BMV18) and DSM10T (BMV15 and BMV17). The corresponding β-galactosidase activity showed that the P_{degQ} promoter region of DSM10T exhibited a significantly higher expression level compared with that of JABs24 (Figure 2). In the transition between the exponential and stationary phase, approx. 14-fold higher Miller Units were detected for the lacZ fusion with degQ promoter from DSM10T.

3.2 | Effect of degQ expression on the formation of lipopeptides and secretory proteases

It is already known that DegQ acts as a stimulator for autophosphorylation of DegS signal kinase leading to enhanced activation of DegU response regulator (Do et al., 2011). In the active state, DegU-P controls a variety of genes encoding secretory proteases, flagellin proteins, and non-ribosomal peptide synthetases for the biosynthesis of plipastatin and surfactin (Hsueh et al., 2011; Mäder et al., 2002; Miras & Dubnau, 2016; Tsuge et al., 1999; Wang et al., 2015). To get an overview of the influence of different degQ gene expressions on biotechnologically relevant production of plipastatin and surfactin as well as secretory proteases, production strains with different degQ expression capabilities were analyzed. For this purpose, the wild-type strains JABs24, DSM10T, and their degQ mutant strains BCKN1 and BCKN2 were examined. While BCKN1 represents strain JABs24 with native degQ gene expression, BCKN2 is the DSM10T strain with degQ deletion.

Using 8 g/L glucose, cell dry weights reduced drastically after complete glucose depletion during the cultivation, resulting in detectable cell lysis without any stationary phase (Willenbacher et al., 2015). However, as previously shown by Vahidinasab et al., (2020), the concentrations of surfactin and plipastatin are not negatively affected by the reduction of cell dry weight (CDW) during glucose
During the late exponential phase (Figure 3a). In contrast, strain DSM10 demonstrated a dramatically increased plipastatin biosynthesis (Geissler et al., 2017). This observation indicates a significant reduction in the expression of the DegQ regulatory domain, which might contribute to the observed increase in lipopeptide production.

In the case of BCKN1, the strain DSM10 showed a remarkable increase in extracellular protease activity sevenfold compared to JABs24. This suggests that secretion of proteases has a significant impact on lipopeptide production.

In comparison, integration of a natively expressed degQ version from the DSM10 strain into JABs24 increased secretory protease activity 2 times (17.6 ΔA/(h·mL) after 24 h) compared to JABs24 (Figure 3c). A comparably great effect was observed for BCKN2, resulting in a continuous basal level of up to 6.2 ΔA/(h·mL) at the end of cultivation (Figure 3d). In this way, deletion of the degQ gene in DSM10 reduced extracellular protease activity sevenfold.

Table 3 gives an overall summary of the effect of degQ gene expression on the lipopeptide productivity and secretory protease formation of the wild-type strains JABs24 and DSM10 and their degQ mutant strains.

4 | DISCUSSION

Due to the point mutation within the degQ promoter region, B. subtilis JABs24, the lipopeptide-forming derivative of B. subtilis 168, shows a drastically reduced degQ gene expression. This circumstance was already described by Stanley and Lazazzera (2005) and confirmed by using lacZ reporter strains for a time-resolved comparison of the expression of the two degQ promoter versions until the transient growth phase (Figure 2). In this process, the wild-type strain DSM10 showed much higher PdegQ promoter activity compared to JABs24. Since DegQ is directly involved in the activation of the DegU response regulator, it is reasonable to assume that DSM10 also displays a more stimulated DegU regulation. The positive feedback regulation of DegU-P on degQ gene expression amplifies the difference between JABs24 and DSM10 in terms of PdegQ promoter activity. The varying DegQ-mediated activation of the DegU regulon was also observed by the detection of the lipopeptides surfactin and plipastatin as well as the formation of secretory proteases. Accordingly, a natively expressed degQ version reduced surfactin but increased plipastatin production, while a significantly higher extracellular protease activity was detected in the presence of the non-mutated degQ promoter version.

While surfactin production is negatively affected by DegQ-associated DegU regulation, increased plipastatin biosynthesis is achieved in the presence of native degQ expression (Miras & Dubnau, 2016; Vahidinasab et al., 2020). This opposing regulatory mechanism was transferable to both JABs24 and DSM10. Accordingly, after integration of a natively expressed degQ version in JABs24, the resulted strain BCKN1 produced only approx. 75% of surfactin but eightfold increased plipastatin titers, while the elimination of degQ in DSM10 (strain BCKN2) showed a threefold increase in surfactin concentration and a fivefold reduction in plipastatin formation. In summary, DegQ can be considered as a regulatory decision point for DegU-mediated production of either surfactin or plipastatin. Accordingly, lipopeptide-producing derivative strains of B. subtilis 168, encoding silenced degQ expression, appear to be predestinated for surfactin formation, whereas DSM10 and other B. subtilis wild-type strains show more effective plipastatin or fengycin production.
Besides the biotechnologically useful production of lipopeptides, another aspect is the DegU-associated formation of secretory proteases. In this study, the comparison of extracellular protease activities between JABs24 and DSM10\textsuperscript{T} showed the effect of silenced degQ gene expression. Thus, DSM10\textsuperscript{T} was found to have fivefold higher protease activity compared to JABs24. Notably, both surfactin and plipastatin showed no decrease in their concentrations during the cultivation process, although secretory protease activity differed significantly between both strains, suggesting that lipopeptides are less targeted by native extracellular proteases. Subsequent integration of a natively expressed degQ version in JABs24 (strain BCKN1) increased extracellular protease production twofold, whereas a sevenfold decrease was observed after deletion of degQ in DSM10\textsuperscript{T} (BCKN2). Altogether, evidence for a quantitative effect of degQ expression on the production of extracellular proteases is documented.

**FIGURE 3** Comparison of lipopeptide production and extracellular protease activity during the time course of shake flask cultivation with 8 g/L glucose. Production parameters were determined for (a) JABs24 (168 sfp\textsuperscript{+}), (b) DSM10\textsuperscript{T}, (c) BCKN1 (JABs24 amyE::P\textsuperscript{degQ}-degQ from DSM10\textsuperscript{T}), and (d) BCKN2 (DSM10\textsuperscript{T} degQ::erm). Gray bars indicate the extracellular protease activity, dashed lines represent the cell dry weight (CDW) and green dots display the surfactin, blue dots represent the plipastatin concentration over cultivation time. The data points represent a mean of at least two biological replicates. The error bars show the standard deviation of calculated values.

**TABLE 3** Summary of parameters of cultivation with JABs24 and DSM10\textsuperscript{T} wild-type strains and their inversed degQ mutant strains BCKN1 and BCKN2.

| \textit{B. subtilis} strains | End of exponential phase | Cultivation time [h] | CDW [g/L] | surfactin conc. [mg/L] | \(Y_{P/X,\text{surfactin}}\) [mg/g] | plipastatin conc. [mg/L] | \(Y_{P/X,\text{plipastatin}}\) [mg/g] | secretory protease activity [\(\Delta A/\text{h·mL}\)] |
|-----------------------------|--------------------------|----------------------|-----------|------------------------|---------------------------------|-------------------------|---------------------------------|---------------------------------|
| JABs24                      | 20                       | 1.58                 | 898.7     | 568.8                  | 0.5                             | 0.32                    | 5.7                             |
| DSM10\textsuperscript{T}    | 16                       | 2.73                 | 306.9     | 112.4                  | 18.6                            | 6.81                    | 42.8                            |
| BCKN1                       | 16                       | 1.56                 | 488.0     | 312.8                  | 4.2                             | 2.69                    | 9.8                             |
| BCKN2                       | 20                       | 1.33                 | 1290.0    | 969.9                  | 3.2                             | 2.41                    | 4.7                             |
5 | CONCLUSIONS

The degQ loci of the lipopeptide-producing strains DSM10\textsuperscript{T} and JABs24 differ by a single point mutation that leads to a drastic reduction of degQ gene expression in JABs24. Based on opposing regulatory mechanisms related to the DegU regulator, the presented strains show beneficial yields in surfactin or plipastatin production, which was confirmed by constructed degQ mutant strains. An additional negative effect of silenced degQ expression in JABs24 was furthermore quantitatively examined on the formation of extracellular proteases. Although a lipopeptide degradation cannot be excluded, different signal strengths of the protease activities measured during the cultivation processes did not lead to a decrease in lipopeptide concentration.

ETHICS STATEMENT

None required.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Lars Lilge: Conceptualization—Lead, Project administration—Lead, Investigation—Equal, Supervision—Lead, Data curation—Equal, Formal analysis—Equal, Methodology—Equal, Writing-original draft—Equal, Writing-review & editing—Equal. Maliheh Vahidinasab: Visualization—Leading, Data curation—Equal, Formal analysis—Equal, Methodology—Equal, Writing-original draft—Equal, Writing-review & editing—Equal. Isabel Adiek, Philipp Becker and Chanthiya Kuppusamy Nesamani: Methodology—Equal, Data curation—Equal, Formal analysis—Equal, Chantal Treinen: Formal analysis—Equal, Methodology—Equal, Writing-review & editing—Equal. Mareen Hoffmann and Kambiz Morabbi Heravi: Formal analysis—Equal, Writing-review & editing—Equal. Marius Henkel: Formal analysis—Equal, Data curation—Equal, Writing-review & editing—Equal. Rudolf Hausmann: Funding acquisition—Leading, Formal analysis—Equal, Writing-review & editing—Equal.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. An overview of the collected data is available in Zenodo at https://doi.org/10.5281/zenodo.5511929

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