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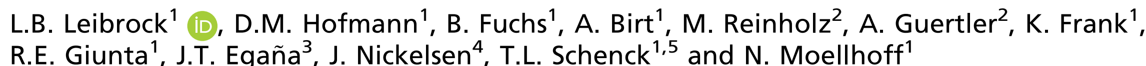
ORIGINAL ARTICLE

In vitro and *in vivo* detection of microbial gene expression in bioactivated scaffolds seeded with cyanobacteria



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Significance and Impact of the Study: Bioactivated dermal replacement materials using cyanobacteria have shown promising results in enhancing wound healing. Most importantly, the survival of the micro-organisms within the replacement material directly affects wound healing capacity. In this proof of principle study, we describe the development of a new approach that allows the detection of living cyanobacteria inside the scaffold in vitro and in vivo, based on real-time PCR.

Keywords
chronic wounds, cyanobacteria, photosynthesis, real-time PCR, wound healing.

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Abstract

Dermal replacement materials bioactivated with cyanobacteria have shown promising potential for wound regeneration. To date, extraction of cyanobacteria RNA from seeded scaffolds has not been described. The aim of this study was to develop a method to isolate total RNA from bioactivated scaffolds and to propose a new approach in determining living bacteria based on real-time PCR. Transgenic *Synechococcus* sp. PCC 7002 (tSyn7002) were seeded in liquid cultures or scaffolds for dermal regeneration *in vitro* and *in vivo* for 7 days. RNA was extracted with a 260/280 ratio of ≥2. The small subunit of the 30S ribosome in prokaryotes (*16S*) and RNAse P protein (*rnpA*) were validated as reference transcripts for PCR analysis. Gene expression patterns differed *in vitro* and *in vivo*. Expression of *16S* was significantly upregulated in scaffolds *in vitro*, as compared to liquid cultures, whilst *rnpA* expression was comparable. *In vivo*, both 16S and *rnpA* showed reduced expression compared to *in vitro* (*16S*: *in vivo* Ct value 13.21 ± 0.32, *in vitro* 12.44 ± 0.42; *rnpA in vivo* Ct value 19.87 ± 0.41, *in vitro* 17.75 ± 1.41). Overall, the results demonstrate *rnpA* and *16S* expression after 7 days of implantation *in vitro* and *in vivo*, proving the presence of living bacteria embedded in scaffolds using qPCR.

Introduction

In a society where chronic venous insufficiency, peripheral arterial disease, obesity and diabetes show a high prevalence throughout an ageing demographic, chronic wounds play a growing burden on the individual patient and healthcare systems worldwide. In the U.S. alone, over six million people suffer from chronic wounds, accounting for an annual healthcare cost of over US$25 billion (Sen *et al.* 2009). A recent report found a 6% prevalence of chronic wounds in Wales constituting up to 5.5% of all costs to the National Health Service (Phillips *et al.* 2016). Hence, there is great interest in improving treatment. Whilst different approaches have been explored, both conservative and surgical management of affected patients remains challenging.

Hypoxia, limited angiogenesis and a lack of regenerative factors are considered major pathophysiological

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mechanisms contributing to stalled wound healing and the development of chronic wounds (Sen *et al.* 2009; Nauta *et al.* 2014; Heyer *et al.* 2016; Han and Ceilley 2017; Coalson *et al.* 2019). As an innovative approach to target these pathological mechanisms, photosynthetic micro-organisms have been introduced into dermal replacement materials (Schenck *et al.* 2015; Chavez *et al.* 2020; Chavez *et al.* 2021). Recently, our study group demonstrated the ability of genetically modified cyanobacteria to release regenerative hyaluronic acid and provide a constant source of photosynthetic oxygen in bioactivated scaffolds *in vitro* (Chavez *et al.* 2021). Thus, potentially representing a useful tool to improve wound healing. However, in order to exert lasting effects on chronic wounds by reducing tissue hypoxia and increasing regenerative potential, cyanobacteria survival in skin scaffolds is crucial. A possible method to assess survival is real-time PCR, for which pure and high-quality bacterial RNA is required. In addition, real-time PCR analysis provides the basis for quantitative gene expression analysis that is needed to assess the molecular impact of bioactivated scaffold transplantation in future *in vivo* trials. To date, the extraction of RNA from scaffolds seeded with cyanobacteria has not been described. Therefore, the aim of this study was to develop a novel method to obtain bacterial RNA from bioactivated scaffolds and to identify stable housekeeping genes that allow the detection of living bacteria and future gene expression analysis *in vitro* and *in vivo*. By providing evidence of cyanobacteria survival within the seeded scaffolds, we lay the groundwork for potential future clinical applications.

Results and discussion

Seeding and RNA extraction of tSyn7002 embedded in scaffolds in vitro

Bioactivation of skin substitutes using photosynthetic, transgenic micro-organisms as a novel approach to treat chronic wounds is still in its infancy. A prerequisite for potential implementation into clinical practice is microbial survival over time, ensuring constant wound oxygenation and tissue regeneration. In addition, gene expression patterns of the microorganisms and host cells potentially infiltrating the scaffold provide insight into molecular and biological mechanisms associated with this approach. As a first step, it is crucial to extract RNA from the bioactivated scaffolds. Several protocols exist covering physical, chemical and thermal approaches for bacterial cell pellets (Pinto *et al.* 2009; Kim Tiam *et al.* 2019), RNA extraction from prokaryotes (Miskin *et al.* 1999; Sessitsch *et al.* 2002; Robbe-Saule *et al.* 2017; Zhang *et al.* 2021) as well as from cyanobacteria (Pinto *et al.* 2009; Hood *et al.* 2016; Kim Tiam *et al.* 2019). For eukaryotic cells, RNA extraction methods from different scaffolds types like hydrogels (Yu *et al.* 2013), polysaccharide scaffolds (Wang and Stegemann 2010) or alginate/gelatin scaffolds (Yu *et al.* 2019) are available. However, to our knowledge, no protocol exists describing the extraction of cyanobacterial RNA from dermal replacement materials. To close this gap and prior to evaluating the capacity of photosynthetic skin substitutes to improve wound regeneration *in vivo*, this study sets out to establish a method to extract bacterial RNA from bioactivated scaffolds. In addition, one objective was to prove the survival of bacteria in scaffolds as well as providing evidence of cyanobacterial gene expression *in vitro* and *in vivo*. As seen in Fig. 1 a,b, tSyn7002 were homogeneously distributed within the scaffold. To test whether RNA extraction from scaffolds seeded with tSyn7002 was feasible under optimal *in vitro* conditions, RNA was extracted using a modified protocol after 7 days of culture under constant illumination at 37°C. No difference in total RNA yield or RNA purity (ratio 260 nm/280 nm > 2) was detected between positive controls (tSyn7002 liquid cultures) and bioactivated scaffolds (Fig. 1c) demonstrating high and contamination-free RNA extraction. For negative control scaffolds, RNA concentration and purity significantly decreased to 7% and ~80%, respectively, compared to the positive control (Fig. 1c) as expected.

Housekeeping genes

Reference genes ought to be chosen carefully and their stability needs to be proven for the individual study setup. Based on data presented by Szekeres *et al.* (2014), real-time PCR was utilized to assess the gene expression of six different potential housekeeping genes in tSyn7002 liquid cultures, in order to determine the best fit for future gene expression analysis (Fig. 2a) (Szekeres *et al.* 2014). The housekeeping gene *16S* showed the highest expression with a Ct value of 11.4 ± 1.0 whilst all others were expressed similarly with Ct values ranging between 16.8 and 17.3 (Fig. 2b). Amongst these, *rnpA* showed the lowest Ct value with 16.8, which is why *16S* and *rnpA* were utilized as reference genes for the following experiments (Fig. 2b).

Gene expression of tSyn7002 embedded in scaffolds *in vitro*

To study bacterial survival, tSyn7002 were cultured in scaffolds for 7 days and real-time PCR analysis was conducted for *16S* and *rnpA* gene expression. *RnpA* expression did not differ significantly between liquid cultured cells and bioactivated scaffolds under constant light

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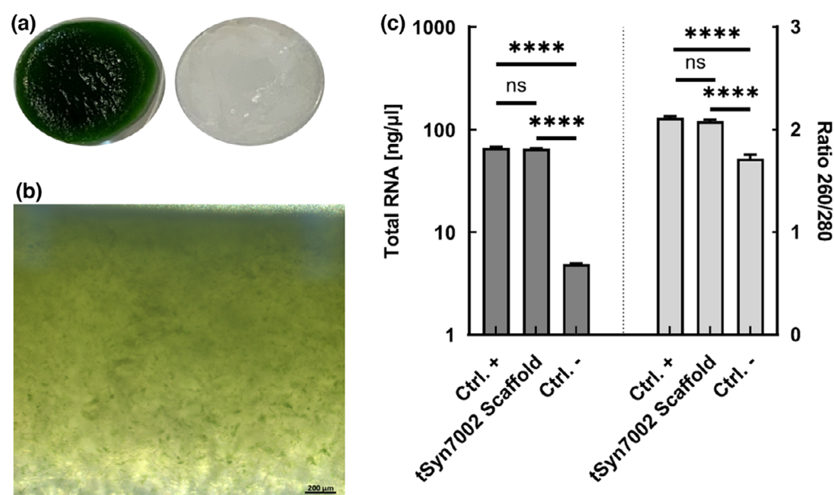

Figure 1 Scaffold seeding and RNA extraction. (a) Represents scaffolds seeded with (left) and without tSyn7002 (right). (b) Depicts a cross-section of a scaffold after seeding with tSyn7002. (c) Shows RNA concentration and RNA purity *in vitro*. ns = not significant, $****P \leq 0{\cdot}0001$. Ctrl.+ = liquid culture of 10^9 tSyn7002. Ctrl.− = Scaffold with 10^9 tSyn7002 cultured without illumination and under starvation conditions. The scale bar represents 200 µm. $n = 3$.

illumination whereas *16S* showed significantly higher expression (Fig. 2c,d). The proliferation of cyanobacteria during cultivation could be a possible explanation for this finding. However, RNA yield showed no elevation as would be expected if the absolute bacterial number had increased (Fig. 1c). Instead, gene expression could be altered by the scaffold itself, inducing mRNA transcription. Negative control scaffolds showed a decreased gene expression of more than 90% for *rnpA* and ~75% for *16S* as expected. Since the overall RNA yield was significantly lower in negative controls compared to liquid cultures and scaffolds that were seeded under optimal conditions, this points toward a reduction in the number of viable bacteria, rather than a downregulation of gene expression. Overall, these results provide evidence that real-time PCR is a helpful tool to analyse the presence of living bacteria embedded in scaffolds quantitatively. Providing evidence of cyanobacterial survival within the scaffold is a necessity for any future clinical application *in vivo*, as regenerative factors and oxygen can only be supplied by the living microorganisms.

RNA extraction and gene expression of tSyn7002 embedded in scaffolds *in vivo*

Recently, data have shown that tSyn7002 bioactivated scaffolds produce O_2 and release lymphoproliferative hyaluronic acid *in vitro* (Chavez *et al.* 2021). Before investigating the potential wound healing properties of this novel bioactivated scaffold in a murine full-skin defect model *in vivo*, evidence of living bacteria within the transplanted scaffold is required. Therefore, bilateral skin defects in mice were covered with bioactivated or negative control scaffolds for 7 days and bacterial survival was analysed by real-time PCR. RNA extraction from harvested scaffolds showed high concentration and purity (Fig. 3a).

The results clearly demonstrate the feasibility of the experimental approach, by successfully demonstrating *rnpA* and *16S* expression after 7 days of implantation *in vivo*. Whilst gene expression of *16S* was comparable to expression in liquid cultures, *rnpA* was significantly downregulated (Fig. 3b,c), despite high RNA yield and purity.

Interestingly, different gene expression patterns were found when comparing *in vitro* to *in vivo* experiments. *In vivo*, a decrease in gene expression was detected, in both 16S and rnpA compared to *in vitro* (Fig. 4a). This decrease is probably caused by suboptimal culture conditions when implanted in a living species (i.e. no constant illumination, nutrient deficiency, temperature) which results in a decreased number of viable bacteria.

However, Ct values were still within an acceptable range (*16S*: *in vivo* Ct value 13.21 ± 0.32, *in vitro* 12.44 ± 0.42; *rnpA in vivo* Ct value 19.87 ± 0.41, *in vitro* 17.75 ± 1.41) and in accordance with the literature reporting Ct values between 10–18 (Szekeres *et al.* 2014; Luo *et al.* 2019), thus still pointing towards substantial survival of cyanobacteria *in vivo*.

As depicted in Fig. 4, *16S* and *rnpA* did not show the same degree of relative gene expression decrease when comparing *in vivo* to *in vitro*. *16S* and *rnpA* are both considered as housekeeping genes. Hence, a similar decrease in gene expression would be expected, if the reduced

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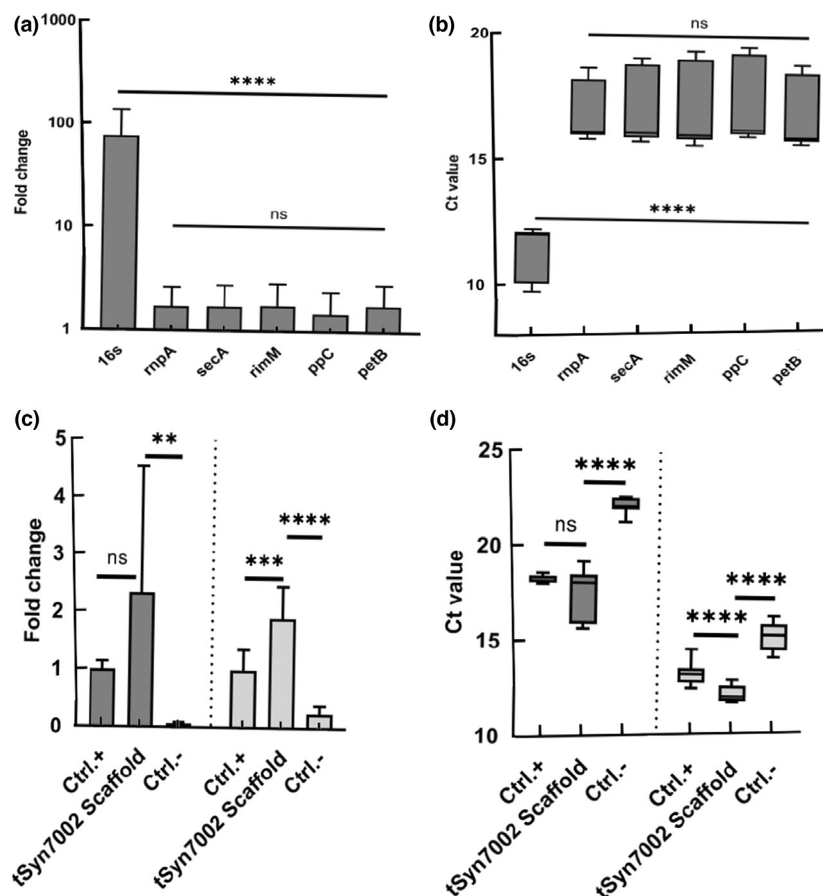

Figure 2 Housekeeping, *16S* and *rnpA* gene expression *in vitro*. (a) Relative gene expression of six tSyn7002 housekeeping genes in liquid culture. Gene expression was normalized to *ppC*. (b) Ct values for the different housekeeping genes are shown. Whiskers represents the minimum and maximum. The line indicates the median. Box shows the 95% confidence interval. ns = not significant, $****P \le 0.0001$. (c) Relative gene expression of *16S* and *rnpA* in tSyn7002 embedded scaffolds cultured for 7 days *in vitro*. Gene expression was normalized to positive control. (d) Represents the corresponding Ct values for *rnpA* and *16S*. Whiskers represents the minimum and maximum. The line indicates the median. Box shows the 95% confidence interval. ns = not significant, $**P \le 0.01$, $****P \le 0.0001$. Ctrl.+ = liquid culture of 10^9 tSyn7002. Ctrl.− = Scaffold with 10^9 tSyn7002 cultured without illumination and under starvation conditions. ■ = *rnpA*. ■ = *16S*.

quantity of microorganisms present within the scaffold were the only contributing factor. Therefore, the differences in gene expression between *in vivo* and *in vitro* samples are probably related to two factors, namely reduced bacterial numbers due to suboptimal culture conditions, as well as direct gene regulation, which is likely to be stress related (Szekeres *et al*. 2014, Luo *et al*. 2019).

Finally, in order to test whether the proposed real-time qPCR methodology truly only detected RNA from living microorganisms, excluding non-viable ones, we performed colony-forming units (CFUs) of cyanobacteria and extracted RNA after treatment with gentamycin (Fig. 5). The CFU assay indicated strong sensitivity of tSyn7002 towards gentamycin. Even the lowest concentration of 0.02% resulted in the death of tSyn7002. Subsequent RNA analysis of gentamycin treated cyanobacteria then revealed low RNA quantity, insufficient for qPCR analysis. Hence, the results support that the RNA isolated from seeded scaffolds and used for qPCR analysis belonged to living cyanobacteria predominantly. In addition, this supports the use of the proposed negative control (7 days of exposure in dark without nutrition) as a suitable experimental setup to distinguish between living and dead cyanobacteria, as RNA concentration was similarly reduced under these conditions.

Material and methods

Cyanobacteria strain and culture conditions

Transgenic *Synechococcus* sp. PCC 7002 (tSyn7002) was used for all experiments (Zhang *et al*. 2019). This transgenic strain overexpresses the *Pasteurella multocida* hyaluronic acid synthase, which enables hyaluronic acid

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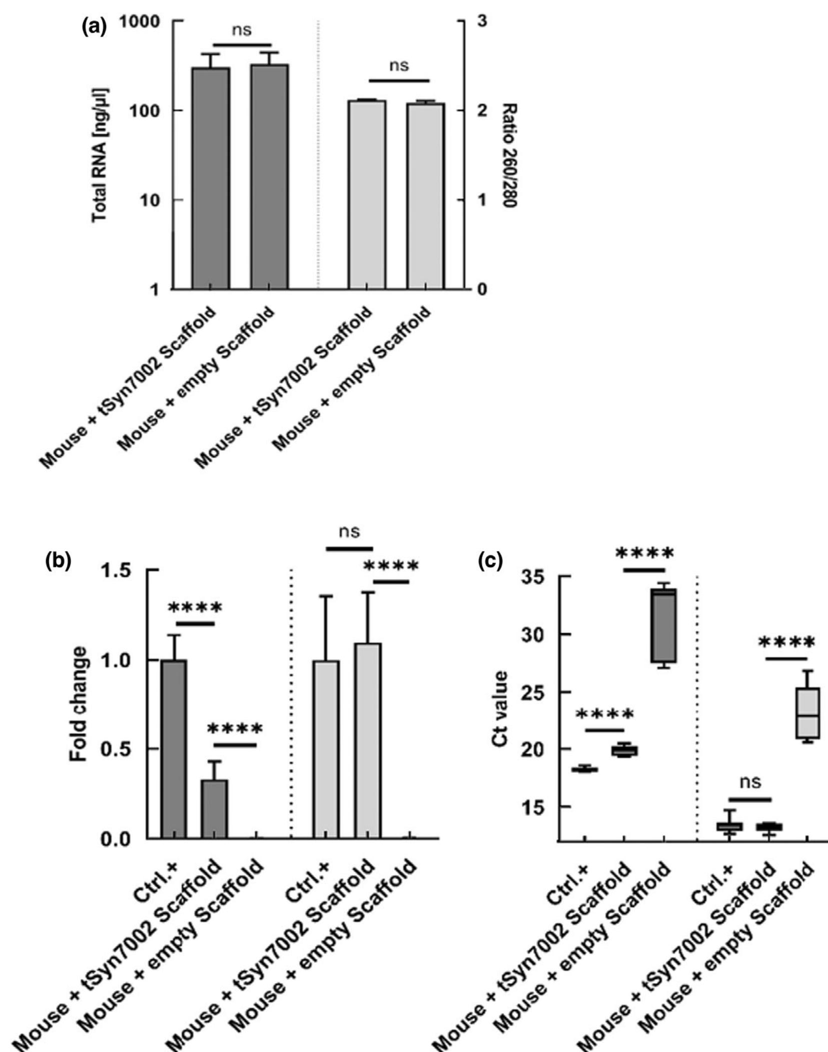

Figure 3 Expression of *16S* and *rnpA in vivo*: (a) depicts the RNA concentration and RNA purity extracted from *in vivo* samples. (b) Gene expression of *16S* and *rnpA* from mouse-explanted scaffolds embedded with 10^9 tSyn7002 after 7 days of implantation is shown. Gene expression was normalized to positive control. (c) Displays the corresponding Ct values for *rnpA* and *16S*. Whiskers represents the minimum and maximum. The line indicates the median. Box shows the 95% confidence interval. ns = not significant, ****$P \leq 0.0001$. Ctrl.+ = liquid culture of 10^9 tSyn7002. ■ = *rnpA*. ■ = *16S*. $n = 3$ with three technical replicates.

production and secretion when induced with the compound isopropyl β-D-1-thiogalactopyranoside. Detailed culture conditions have been described previously (Chavez *et al.* 2021). tSyn7002 were cultured in A-D7 medium supplemented with 1 g l^{-1} glucose at 37°C under constant illumination with light intensity being 32.5 µE*m^{-1}*s^{-1} (LED, Sebson, Dortmund, Germany). Cell numbers were determined using light-microscopy (Primover; Zeiss, Oberkochen, Germany) and a Neubauer cell chamber.

Scaffold generation and embedding of tSyn7002

Integra bilayer matrix wound dressing (IM; Integra Life Science Corporation, Plainsboro, NJ) was used as a scaffold. Bacterial seeding was performed as described previously (Schenck *et al.* 2014, 2015; Chavez *et al.* 2021). In brief, for all experiments, scaffolds with a diameter of 12 mm were used. The scaffolds were cut using a biopsy punch (Acuderm Inc., Fort Lauderdale, FL). For bacteria seeding into the scaffolds, 1×10^9 tSyn7002 bacteria were transferred into a 50 ml tube and centrifuged at 4000 ***g*** for 5 min. Thereafter, the supernatant was discarded and bacteria were washed with 50 ml PBS and the suspension was centrifuged at 4000 ***g*** for 5 min. Subsequently, the pellet was resuspended in 100 µl medium. Next, 50 µl Fibrin (Baxter Duplojet Combi, ref No. 3400667 Baxter Healthcare SA, Switzerland) were added, and the solution was pipetted

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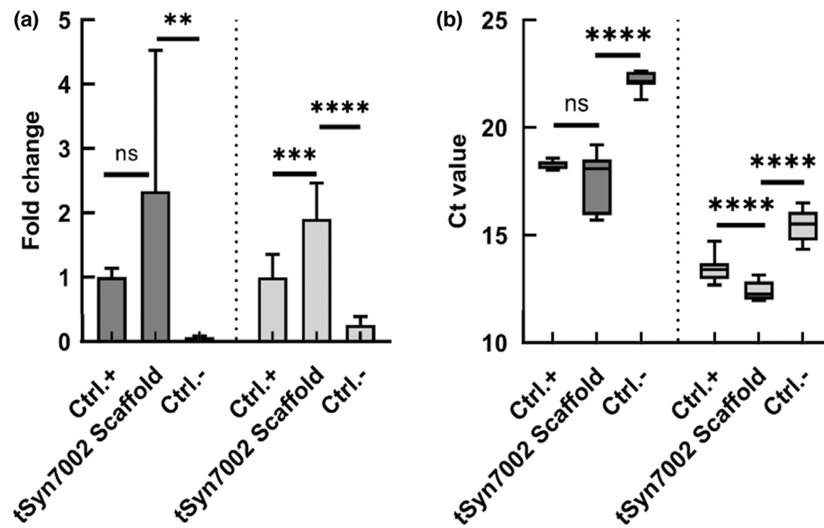

Figure 4 Gene expression comparison between *in vitro* and *in vivo*. (a) Comparison of *rnpA* and *16S* Ct values between *in vitro* and *in vivo* conditions after 7 days culture time relative to positive control. (b) Displays the corresponding Ct values. Whiskers represents the minimum and maximum. The line indicates the median. Box shows the 95% confidence interval. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. ■ = *rnpA*. ■ = *16S*. $n = 3$ with three technical replicates.

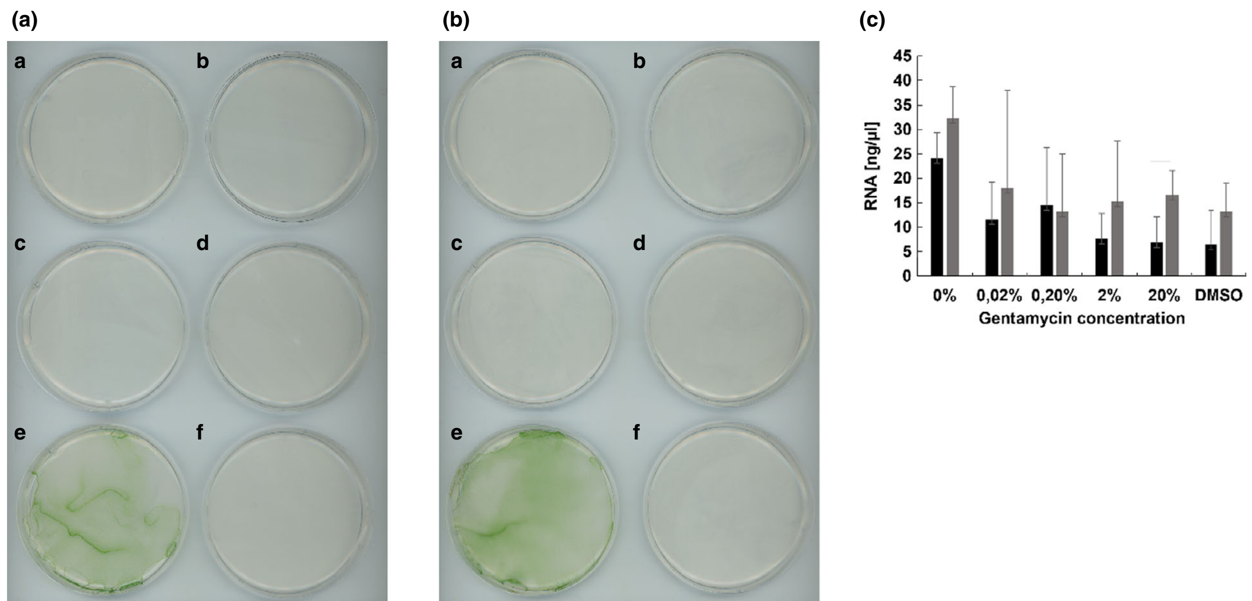

Figure 5 CFU assay and RNA isolation: CFU assay of tSyn7002 treated with different gentamycin concentrations (a = 20%, b = 2%, c = 0.2%, d = 0.02%, e = 0%, f = 10% DMSO) for 1 h (a) and 24 h (b). (c) shows the RNA amount of tSyn7002 treated with gentamycin at 1 h (■) and 24 h (■).

onto the scaffold. Lastly, to ensure fixation of seeded bacteria within the scaffold, 50 µl of Thrombin (Baxter Duplojet Combi, Ref No. 3400667 Baxter Healthcare SA) were added onto the scaffold to seal the bacteria within it. Afterwards, scaffolds were cultured at 37°C in A-D7 medium supplemented with 1 g l^{-1} glucose under constant illumination. As a negative *in vitro* control group, we used scaffolds seeded with 10^9 tSyn7002 bacteria cultured under starvation conditions (room temperature without illumination and without medium supply) for 7 days. Liquid-cultured bacteria under constant illumination were used as positive controls *in vitro*. Empty scaffolds (i.e. without integration of tSyn7002) served as negative controls for *in vivo* experiments.

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Implantation and excision of scaffolds in mice

The experimental procedure of the present study was approved by the District Government of Upper Bavaria (ROB-55.2-2 532.Vet_02–19-96). Experiments were performed on female SKH1 hairless mice aged 6–8 weeks and with body weights between 20 and 25 g (Charles River, Sulzfeld, Germany). Mice were kept in the Central Animal Facility at the Medical Faculty of the Ludwig-Maximilian-University Munich under 12 h light/dark cycles, with constant temperature, in individually ventilated cages. To allow photosynthetic growth, illumination was enforced using LED light sources (light intensity 32.5 $\mu E * m^{-1} * s^{-1}$, LED; Sebson) fixed around cages. Scaffold implantation in mice was previously published in detail (Schenck *et al.* 2014, 2015). At 7 days after implantation, mice were euthanized by cervical vertebrae dislocation. The skin from the back, including the scaffolds, was excised for further analysis. Subsequently, scaffolds were fast frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA extraction

RNA from liquid cultures and scaffolds seeded with tSyn7002 was extracted using Roche high pure RNA isolation Kit (Cat. No. 11828665001). Extraction is described in detail, as it was modified from the manufacturer's protocol.

For liquid cultures, 1×10^9 bacteria were resuspended in 200 µl 10 mmol Tris (pH = 8). 4 µl of 50 mg ml^{-1} lysozyme (Merck KGaA, Darmstadt, Germany; Cat. No. L6876) was added to disturb the bacterial cell wall. After incubation at 37°C for 10 min on a thermoshaker, 400 µl lysis buffer was added. The sample was transferred to a high pure filter tube and centrifuged for 15 s at 8000 ***g***. Subsequently, the flowthrough liquid in the collection tube was discarded, and 100 µl DNA-DNase buffer (1/10) was pipetted onto the filter tube and incubated for 60 min at room temperature. Then, 500 µl of wash buffer I was added to the filter tube and the sample was centrifuged for 15 s at 8000 ***g***. The flow-through liquid was discarded, and a second wash step was performed by adding 500 µl wash buffer II followed by centrifugation for 2 min at 12000 ***g***. Finally, the flow-through liquid and the collection tube was discarded, and the filter tube was placed in a clean sterile 1.5 ml microcentrifuge tube. The RNA was eluted by adding 30 µl elution buffer to the filter tube followed by two centrifugation steps for 1 min at 8000 and 12000 ***g***, respectively. Subsequently, the eluate was transferred into a sterile LoBind 1.5 ml microcentrifuge tube. RNA samples were stored at −80°C until further analysis.

For RNA extraction of tSyn7002 cultured in scaffolds, the scaffold was halved, and the silicon layer was carefully removed. Next, the scaffold was dissected into smaller units and transferred to a 2 ml microcentrifuge tube with 200 µl 10 mmol Tris (pH = 8). Subsequently, a 7 mm stainless steel bead (Qiagen, cat. no. 69990, Venlo, the Netherlands) was added to the microcentrifuge tube and the scaffold was crushed using a Tissue Lyser (Qiagen, LT). Afterwards, the sample was transferred into a 1.5 ml microcentrifuge tube and centrifuged for 1 min at 12000 ***g***. The supernatant was transferred into a new 1.5 ml microcentrifuge tube and 4 µl lysozyme (50 mg ml^{-1}) was added. Further RNA isolation steps were performed as described for liquid cultures.

RNA yield and purity

The amount and purity of RNA were determined photometrically by measuring the ratio at 260/280 nm using a Tecan Infinite TM plate reader equipped with a NanoQuant plate (Tecan, Tecan Group Ltd., Maennedorf, Switzerland).

cDNA synthesis

Total RNA, 500 ng, was used for cDNA synthesis, which was conducted according to the manufacturer's protocol (Roche, Transcriptor First Strand cDNA Synthesis Kit, Cat. No. 04897030001, Basel, Switzerland).

Real-time PCR

All primers were purchased from Eurofins (Eurofins Genomics Germany GmbH, Ebersberg, Germany). Real-time PCR conditions are described in Table S1. The sequences are provided in Table S2. In addition, the innuMix real-time PCR DsGreen Standard kit (Analytik Jena, Jena, Germany) containing dNTP's, hot-start DNA polymerase, and SYBR Green was used for analysis. To confirm that *in vivo* samples contain mice RNA, β-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression were investigated. β-actin expression was normalized to GAPDH expression (data not presented).

Normalization to a positive control

CT values were transformed into fold change using the Eq. 2^(-CT value). From this data, a mean control value was calculated for *16S* and *rnpA*. Afterwards, the following equation was used to normalize gene expression:

$$\frac{\text{Fold change value of measured gene}}{\text{Fold change value of control gene}} * 1$$

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CFU assay

tSyn7002 were resuspended in A-D7 medium to a final concentration of 1×10^6 bacteria per ml. 1 ml of this bacteria solution was seeded per Agar plate and exposed to different gentamycin concentrations for 1 h and 24 h (0.02; 0.2, 2, 20, 10%, DMSO). Subsequently, RNA isolation was performed as described above.

Statistical analysis

All data are shown as mean $\pm$ standard deviation. All experiments were performed in biological and technical triplicates. Differences between groups were analysed using Student's *t*-test. Analyses were performed using GraphPad Prism 9.1.1 software (GraphPad Software, LLC, San Diego, CA) and differences were considered statistically significant at a probability level of $P \leq 0.05$.

Author contributions

Lars B. Leibrock, Daniel M. Hofmann, Alexandra Birt and Nicholas Moellhoff conceived the study design. Daniel Maria Hofmann, Benedikt Fuchs and Alexandra Birt conducted the experiments. Lars B. Leibrock performed statistical analyses and first manuscript writing. Nicholas Moellhoff, Thilo L. Schenck, Konstantin Frank, Anne Guertler, Jörg Nickelsen, José Tomás Egaña and Markus Reinholz and Riccardo E. Giunta executed manuscript revision and editing. Nicholas Moellhoff, Thilo L. Schenck and Riccardo E. Giunta supervised the project.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Real-time PCR conditions.
Table S2. Real-time PCR primer sequences.

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