Engineered skin microbiome-assisted delivery to the pilosebaceous unit

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Abstract

Microbes are fascinating molecular machines which can be equipped with synthetic genetic programs that allow them to produce therapeutic molecules targeted on demand upon disease sensing. *Cutibacterium acnes* engraftment capacity and living habitat close to important pharmacological targets makes it an attractive chassis to create skin living therapeutics. Here, we report the engineering of this bacterium, the most abundant commensal of the human skin, to produce and secrete the therapeutic molecule neutrophil gelatinase-associated lipocalin thereby modulating sebum production.

Main

Engineering the human microbiome holds great potential for human health monitoring and maintenance. While many studies have targeted the human gut by engineering the probiotic organisms *E. coli* Nissle 1917 or *L. lacis* to treat various dysbiotic states or cancer\(^1\)–\(^9\), other microbiomes have moved in the focus of interest only recently.

The human skin microbiome contributes to skin protection from external factors such as pathogens\(^10\) but is also involved in other processes like wound healing or pH regulation\(^11\) and plays an active role in the interaction with immune cells\(^12\). Interestingly, the hair follicles and pilosebaceous units are inhabited almost exclusively by *Cutibacterium acnes*, a facultative anaerobic and gram-positive bacterium\(^13\). In this acidic niche, deep inside the skin, *C. acnes* feeds from sebum, especially triglycerides, produced by sebocytes\(^14,15\). The low environmental pH induced by the production of propionic acid impedes the growth of pathogens and contributes to skin homeostasis\(^16\). Additionally, it has been shown that *C. acnes* compositions can be applied successfully on human skin with a long lasting engraftment within human hair follicles\(^17\). Indeed, genomic studies observed a low turnover within the skin\(^18\) and it seems to occupy clonally each follicle\(^19\). Therefore, this species seems an attractive synthetic biology chassis for treating skin diseases due to its engraftment potential, specific niche, importance to skin homeostasis and close contact to relevant therapeutic targets\(^20,21\). Acne is a common skin condition caused by blockage or inflammation of the pilosebaceous follicles\(^22\), commonly treated with isotretinoin, a vitamin A derivative, which produces serious side-effects such as teratogenicity or extreme scaling of skin\(^23,24,25\). Isotretinoin treatment is associated with an upregulation of the LCN2 gene encoding NGAL (neutrophil gelatinase-associated lipocalin), a protein that contributes to acne symptoms reduction by inducing apoptosis in sebocytes\(^25–27\).

In this study, we evaluate the utility of *C. acnes* as a platform for the treatment of skin diseases. It has been key to create a new genetic engineering pipeline, to fully enable *C. acnes* as a chassis for synthetic microbiome-based therapies.

So far, the development of a genetic engineering toolbox for *C. acnes* was hampered by the intractability of *C. acnes*. Introduction of synthetic genetic circuits was until now non-efficient and labor intensive, with
low reproducibility. Therefore, we first tried to increase transformation efficiency by optimizing various steps from previously published protocols. We focussed on improving DNA delivery to the cell, DNA stability inside the cell and gene expression.

First, we developed an effective protocol to transform C. acnes. We improved electroporation protocol by screening various transformation buffer compositions and conditions, which were successfully used in other non-model organisms. Since electrotransformation efficiency seemed to be very low in general, we used a fluorescently labeled oligonucleotide to assess the entry and stability of DNA in C. acnes cells. DNA entry of the fluorescent oligo increased around 50-fold using a buffer containing 272 mM sucrose compared to other electroporation buffers tested (Fig. 1A). We observed a significant reduction in fluorescence after 1 h of recovery post-electroporation and concluded that the fluorescent oligo must have been partially degraded (Fig. S1A).

Such an early onset degradation could be due to exonucleases but also due to functional restriction-methylation systems (R-M) which would hamper DNA delivery. The C. acnes strain KPA171202 has been described to have one functional R-M system harboring a IIIB methylase. To overcome the prokaryotic R-M system, we created a Δdam Δdcm ΔhsdMS E. coli strain harboring the C. acnes IIIB methylase. This strain, dubbed EC-24, produces DNA that lacks E. coli-specific methylation but instead mimics the methylation pattern of C. acnes KPA171202. Thus, shuttling plasmids through EC-24 before delivery into C. acnes should contribute to R-M evasion. Indeed, the methylation of recognized patterns showed to be a limiting factor in transforming plasmids into C. acnes. While we rarely obtained transformants in the absence of C. acnes-specific methylation, transformation efficiency increased up to 200-fold when plasmid methylation mimicked that of the host bacterium (Fig. 1B).

Other factors affecting transformation efficiency were optical density (OD) of cells at the point of competent cell preparation and the transformed DNA amounts. Lower amounts of DNA and interestingly higher OD were favorable for the outcome (Fig. S1B and S1C). Curiously, freezing the competent cells improved transformation efficiency up to 12-fold over the freshly prepared ones (Fig. S1D). We hypothesize that the freezing and thawing may be affecting membrane integrity (as seen for other microorganisms) and therefore contributing to DNA internalization.

To further increase transformation efficiency we looked into cell-wall-weakening agents that have been shown to improve transformation in gram-positive bacteria. We tested L-glycine, L-threonine and penicillinG, supplemented with sucrose for osmotic stability. L-threonine treated cells showed no improvement but a decrease in transformation efficiency compared to untreated cells (data not shown). L-glycine pre-treatment increased efficiency 60-fold, while penicillinG pre-treatment between 2–10 ug/mL increased transformation efficiency up to 1200-fold compared to non pre-treated controls (Fig. 1C).

Creating gene knock-outs in C. acnes by homologous recombination has been shown in a handful of cases. To increase efficiencies of gene knock-ins (KIs) and knock-outs (KOs) in C. acnes we investigated the use of minicircles (MC). We used MC vectors purified from E. coli strains lacking
endogenous R-M systems (Δdam, Δdcm, ΔhsdMS) and compared them to the original suicide vector pMW535. We observed that MCs from strains without any R-M system (JMC3 strain: Δdam, Δdcm, ΔhsdMS) increased the number of transformants over those containing one or more endogenous R-M systems (JMC2 strain: dam+, Δdcm, ΔhsdMS; ZYCY strain: dam+, dcm+, hsdMs+). Also, an increase in efficiency was observed when the ermE resistance cassette in our vectors was re-coded to avoid AGCAGY motif recognized by the C. acnes R-M IIIIB system (Fig. S1E).

Ideally, real-world applications of engineered microbes should avoid the presence of antibiotic resistance cassettes for safety and regulatory concerns. To create a C. acnes strain that no longer uses an antibiotic resistance marker for selection of KOs or KIs in C. acnes, we tested the feasibility of using a dual selection based on the KO of the thymidine kinase (tdk) gene. Strains which are deficient to tdk will not metabolize 5′-fluoro-2′-deoxyuridine (FUDR) into a toxic compound and therefore do not block RNA and DNA synthesis. The tdk KO can be used as a dual selection and therefore serves as an potential auxotrophy as described by Norville et al. 2016. Since the insert contained an ermE cassette, transformants could be obtained either through negative selection (growth of tdk KO clones in the presence of FUDR) or through positive selection (growth of ermE KI clones in the presence of erythromycin).

We verified the correct knock-out of the thymidine kinase gene by junction PCR and functional testing (Fig. 1D). We selected for surviving clones on the inhibitor FUDR or on ermE and tested the potential break out rate of the wild type. C. acnes wild type was unable to grow in the presence of the inhibitor at concentrations of 50 μg/mL while the KO strain was able to grow at all concentrations tested (Fig. S1F).

After improving transformation efficiency in C. acnes we wanted to test the feasibility of producing and secreting proteins of therapeutic interest. We created a knock-in strain that was able to produce and secrete the therapeutic molecule NGAL, a protein that is upregulated in severe patients treated with the acne drug isotretinoin. This protein showed mediating effects of triggering apoptosis in sebocytes which in turn led to the reduction of sebum and therefore C. acnes count.

To test whether the reduction in C. acnes counts could be a consequence of increased levels of NGAL, we assessed the potential toxicity of purified human NGAL on C. acnes cells. We did not observe any toxic, nor growth-inhibiting effect on C. acnes incubated with different concentrations of NGAL and concluded that we could use C. acnes as a potential shuttle for NGAL to reduce sebum through its mediating effect on human sebocytes (Fig. 1G).

To create a strain which was able to secrete NGAL we chose secretion signals of highly secreted endogenous proteins in C. acnes. To reduce dependency on strain and growth conditions we tested a set of three different secretion signals carried by strongly secreted proteins: camp1 (PPA1340), camp2 (PPA0687) and roxP (PPA1939). We fused the LCN2 gene encoding NGAL-his to the secretion signals and expressed it from a C. acnes strong constitutive promoter (camp1 promoter); an erythromycin resistance cassette in the same construct enabled the positive selection of transformants (Fig. 1E).
Constructs were stably inserted in the genome by homologous recombination targeting the tdk gene. We tested expression and secretion levels of NGAL by Ni-affinity protein purification of His-tagged proteins followed by SDS-PAGE and western blot (Fig. 1F). We assessed both the intracellular NGAL and the NGAL secreted to the supernatant. *C. acnes* with the PPA1340 or PPA1939 secretion peptide had the highest secretion levels of NGAL followed by PPA0687 (Fig. S2A).

To test the functionality of the protein in decreasing sebum in an arachidonic acid stimulated inflammatory *in vitro* sebocytes assay, we incubated NGAL produced recombinantly in *E. coli*, human (HEK293) and *C. acnes* cells at a concentration of 50 ng/mL and compared the levels of sebum reduction to 10 𝜇M isotretinoin. Arachidonic acid treatment increased sebum production around 190-fold compared to vehicle treated sebocytes. We showed that the *C. acnes* pBR13 protein decreased significantly sebum, around 2-fold after 48h, in sebocytes similarly to the control and reference compound isotretinoin (Fig. 1H). Interestingly, also *E. coli* produced NGAL was able to decrease sebum production (1.7-fold) in contrast to previously published results where the authors did not observe a decrease in SEB-1 sebocytes viability and concluded that the human NGAL undergoes post translational modifications which in turn provided the functionality of the protein 44. *C. acnes* produced NGAL seemed to have undergone post translational modification, given by its increased molecular weight compared to *E. coli* produced NGAL. Indeed, the molecular weight of *C. acnes* is closer to the human one (Fig. 1F).

We also tested potential cytotoxicity of NGAL protein produced in different hosts on SZ95 sebocytes or PCi-SEB_Cau and did not observe any cytotoxic effect (Fig. 1G). No apoptotic effect was observed when measured by TUNEL staining (Fig. S2B).

Finally, we wanted to test if our sebum-modulating strain could engraft efficiently on skin. Therefore, we applied both the wild type and the engineered *C. acnes* on the back of mice for three consecutive days and continued sampling until day 7 (Fig. 2A). We followed engraftment by junction PCR and confirmed the presence of the engineered strain until assay endpoint at day 7 (Fig. 2B). We conducted gram-staining of cryosections of the skin and observed an increase in gram-positive bacteria on the skin and in the hair follicles of treated mice. The gram-staining observed in the control mice, which was quantitatively much less abundant than in treated mice, probably reveals the natural flora (Fig. 2C).

Since bacteria seemed to colonize on murine skin, *in vivo* protein production was assessed. qPCR analysis revealed increased expression of NGAL in samples treated with the engineered bacteria whereas wild type and vehicle-treated murine skin showed limited expression (Fig. 2D). To investigate potential inflammation derived from *C. acnes* treatment, we assessed expression levels of inflammatory cytokines IL1-β, IL-6 and TNFα. No differential expression of inflammatory cytokines was observed when comparing treated and untreated samples (Fig. 2E).

In conclusion, this study presents evidence for the controlled use of the engineered skin commensal *in vivo* through a dual selection marker and its sebum reducing properties by secreting the therapeutic
molecule NGAL \textit{in situ}. The basis of this strain will support future clinical studies and its use in humans while providing the basis for regulatory measures.

Effective delivery of genetic circuits to the skin, opens very important avenues including sensing, sebum and immune modulation, and the development of smart microbes with advanced properties.

\textbf{Methods}

\textbf{Bacterial Culture}

\textit{C.\ acnes} KPA171202 was grown on Brucella agar plates (Condalab) from glycerol and incubated for 3 days, 37\textdegree C anaerobically using GasPak EZ anaerobe pouch system (BD). When grown in liquid, \textit{C.\ acnes} KPA171202 was inoculated to an OD\textsubscript{600} of 0.1 and grown in Brain-Heart Infusion broth (BHI) (Sigma) at 37\textdegree C, 110 rpm, anaerobically using AnaeroGen system (Thermo Scientific), until reaching the desired optical density. \textit{E.\ coli} DH5a (NZYTech) was used for routinely cloning and \textit{E.\ coli} dam-dcm- (New England Biolabs) to create a \textit{C.\ acnes} methylase proficient shuttle strain. \textit{E.\ coli} strains were grown in LB medium either supplemented with Ampicillin 50 \mu g/mL or Kanamycin 50 \mu g/mL and grown at 37\textdegree C at 225 rpm.

\textbf{Cloning of plasmids}

Plasmids for homologous recombination were cloned as previously described \textsuperscript{35}. In short, homology arms were amplified from genomic DNA including restriction sites for Ncol, SpeI and Acc65I. Homology arms were digested with Acc65I and ligated and finally cloned into the pGEM-T-easy vector by restriction-enzyme-mediated cloning. In a second step the gene of interest was cloned between the homology arms in a second step. Replicative plasmids are based on the \textit{P. freudenreichii} vector pBRESP36A \textsuperscript{45} and were optimized by Lood et al. for protein production in \textit{C.\ acnes} \textsuperscript{46}. Selected secretion peptides and LCN2 genes were cloned downstream of the p1340 promoter using NheI and SpeI restriction sites.

\textbf{Minicircle production}

Minicircle strains ZYCY10P3S2T \textsuperscript{47} (System Biosciences), JMC2 and JMC3 \textsuperscript{48} were used to produce minicircles being methylated or lacking endogenous methylation. Gene flanking homology arms were cloned into the parental plasmid pMC BESPx MCS1 (Systems Biosciences) and transformed into ZYCY10P3S2T (ZYCY) (Systems Biosciences), JMC2 or JMC3 minicircle Production Strains. MC production was performed according to the manufacturer’s protocol.

Creating a MIIIB proficient dam- dcm- hdsMS- \textit{E.\ coli} strain

To create an \textit{E.\ coli} strain that produces plasmids with the \textit{C.\ acnes} methylation pattern, lambda-Red recombineering was used as described by Datsenko & Wanner \textsuperscript{49}. Briefly, a recipient \textit{dam-/dcm-} strain (NEB) was first transformed with pKD46 plasmid \textsuperscript{49} and selected with 100 \mu g/ml ampicillin at 30\textdegree C.
Transformants were grown at 30°C and induced with 0.4% arabinose for 1h before transformation with the linear DNA fragment to be recombined into the chromosome (see below). Transformants were selected at 37°C with 25 µg/ml kanamycin, and successful recombineering was verified by colony PCR and Sanger sequencing. The temperature-dependent loss of pKD46 was confirmed through the absence of growth in Amp 100 µg/ml plates.

The linear DNA fragment to be recombined into the *E. coli* chromosome was prepared as follows. First, the MIIIIB methylase gene was amplified from the genome of *C. acnes* KPA171202, and it was cloned under Bba_J23100 promoter and Bba_B0034 RBS in a pJET1.2 vector, together with the Tn5 neomycin phosphotransferase (KanR) gene amplified from pKD13. The region containing the MIIIIB methylase and the KanR genes was then amplified with primers PP-19 and PP20, which provide 50 bp homology regions to the *E. coli* genome. In particular, primers were designed so that recombination occurs in the hsdMS locus, thereby inactivating the hsdMS methylation system upon insertion of the *C. acnes* methylase gene. To ensure that only the linear PCR product (and not the template plasmid) was transformed, the template plasmid was digested with XhoI and AatII for 1h at 37°C prior to the PCR, and then the PCR product was treated with DpnI for 1h at 37°C and the correct band was purified from a 1% agarose gel. The template-free, purified PCR product was transformed into dam-/dcm- cells carrying pKD46 as described above.

**Transformation of *C. acnes***

*C. acnes* KPA171202 was grown to an OD$_{600}$ of 0.4, 0.7, 1.0 and 1.8. Cells were spun down at 1700 x g for 10 min at 4°C and washed with equal volume of EPB (272 mM sucrose, sterile filtered) followed by a second spin under the same conditions. Cells were resuspended in 1 mL EPB and washed another 5 times, 9400 x g, 1 min, 4°C. Then the pellet was resuspended in residual liquid. Competent cells were diluted 1:4 in EPB to a final volume of 50 ul and 500–8000 ng of DNA were added to each sample. Cells were transferred to a pre-cooled 0.1 cm Electroporation cuvette (BioRad) and electroporated 1.5 kV, 400 Ω and 25 uF. Cells were recovered in 100 µl BHI media and plated on a Brucella agar plate. After 24 h anaerobic incubation at 37°C, cells were resuspended in 1 mL of BHI to remove remaining non incorporated DNA, spun down 5 min at 1700 x g and resuspended in 200 ul BHI. 50 µl of resuspended cells were then plated on 4x Brucella agar plates supplemented with 10 µg/mL erythromycin per sample. Transformants were obtained following anaerobic incubation at 37°C for 7 days.

To improve electroporation efficiency different pre-treatments were tested. For glycine of penicillinG pre-treatment, cells were grown in presence or absence of 1.25% of glycine or 10 ug/mL PenicillinG, 4 h prior to electroporation. For osmotic stabilization 0.4 M of sucrose was added also 4 h prior.

**Thymidine kinase knock-in functional testing**

The tdk knock-in was created as previously described in Sörensen et al. 2010 targeting the thymidine kinase locus and cloning the pGEM-T-easy plasmid with homologous arms to the region. For simplicity the antibiotic resistance cassette, coding for erythromycin (ermE) was cloned together with the LCN2
gene between the homology arms to replace the tdk gene with an ermE cassette. To evaluate the functionality of the tdk dual selection marker \(^{41}\), we tested the knock-in in sensitivity to FUDR (50 ug/mL) and erythromycin (10 ug/mL) and compared it to the wild type. Therefore, we plated \textit{C. acnes} KPA171202 wild type bacteria and knock-in on Brucella agar plates either containing ery (10 ug/mL), FUDR (50 ug/mL) or neither of the components. Only the knock-in strain could grow on both erythromycin and FUDR-containing plates, while the wild type was sensitive to both.

TCA precipitation and protein purification

Recombinant \textit{C. acnes} strains were inoculated in 25 mL BHI media supplemented with 10 ug/mL erythromycin to a starting OD\(_{600}\) of 0.1 and grown for 48 h at 37\(^\circ\)C, anaerobically, 110 rpm. Cells were spun down and the supernatant and pellet were treated separately. The supernatant was used for protein purification or trichloroacetic acid (TCA) precipitation while the pellet was subjected to lysis by a FastPrep FP120. Protein was precipitated as previously described \(^{50}\) and deoxycholate (DOC)-trichloroacetic acid precipitation was followed.

For protein purification, cell-free supernatants were prepared by centrifugation and directly loaded onto a 5-mL HisTrap FF column (Cytiva). After washing the column with binding buffer A (50 mM Tris-HCl pH7.4, 500mM NaCl, 5mM Imidazole, 10% Glycerol), protein was eluted with elution buffer (Buffer A with 300mM Imidazole). The fractions containing NGAL protein were pooled and dialyzed with a storage buffer (50mM TRIS-HCl pH7.4, 500 mM NaCl and 10% Glycerol). Pooled fraction was analyzed by Western blotting with anti 6xHIS Tag antibody. Protein concentration was quantified by Qubit Protein Assay Kit (Thermo Fisher Scientific).

Western blot

NuPAGE LDS sample buffer (4x) containing \(\beta\)-mercaptoethanol was added to samples to a final concentration of 1x. Proteins were denatured for 10 min at 95\(^\circ\)C and loaded on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and run for 1.5 h, 120 V in 1x MOPS SDS running buffer (Invitrogen). Proteins were transferred to an activated PVDF membrane (Brand) in NuPAGE 1x transfer buffer supplemented with methanol (10%) for 1 h, 20 V. Proteins were blocked with 4% milk TBST (1x) for 1 h at RT and then incubated o/n at 4 degrees with a mouse anti-histidine primary antibody (1:800) (MCA1396; BioRad). Membrane was washed three times with 1xTBST buffer and incubated with secondary anti-mouse IgGk BP-HRP (1:1000) (sc-516102; Santa Cruz Biotechnology) for 1 h RT. After three washes in TBST (1x), the membrane was developed using Pierce™ ECL Western Blotting Substrate (Thermo Fisher). This reaction emits light at 428 nm and this light signal was captured by a digital imager (BioRad).

Sebocyte culture

Immortalized human sebocytes, SZ95 \(^{51}\) were seeded in a density of 2x10^5 cells/well and maintained in DMEM/F12 (Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 U/mL
Pen/Strep, 1mM CaCl$_2$ and 5 ng/ml human epidermal growth factor (EGF; Sigma-Aldrich). Cells were incubated at 37ºC in a humidified 5% CO2 incubator.

PCI-SEB_Cau (Phenocell) were seeded and maintained following manufacturer protocols in PhenoCULT-SEB basal medium supplemented with Supplement A (1/1000) for 3 days prior to exposure and starting the assays.

Sebum modulation assay

PCI-SEB_Cau human iPSC derived sebocytes (Phenocell) were seeded in a 24 well plate at a cell density of 25000 viable cells/cm$^2$ in PhenoCULT-SEB basal medium supplemented with 1/1000 Supplement A. Cells were grown in a humidified incubator (37 ºC, 5% CO2) for 3 days before being exposed and starting the assay. Experiment was performed in triplicates being treated with either vehicle, 5 uM arachidonic acid (AA) (Sigma), 5 uM AA + 10 uM ISO (13-cis retinoic acid)(Sigma), 5 uM AA + 50 ng/mL rhNGAL E. coli, 5 uM AA + 50 ng/mL C. acnes pBR13,14 or 16 NGAL. Cells were exposed for 48 h and then cell lipid content stained with the fluorescent marker BODIPY 493/503 (Sigma Aldrich) following manufacturer's recommendations. The nucleus was stained with DAPI. Samples were analyzed by confocal fluorescence microscopy and fluorescence measured and normalized.

MTT cytotoxicity assay

Human SZ95 sebocytes were seeded in a 12-well plate to a density of 2x10^5 cells/well and incubated in a humidified atmosphere (37ºC, 5% CO2) until 80% confluence in DMEM/F12 (Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 U/mL Pen/Strep, 1mM CaCl$_2$ and 5 ng/ml human epidermal growth factor (EGF; Sigma-Aldrich Co.). PCI-SEB_Cau (Phenocell) were seeded to 2x10^5 cells/well incubated in a humidified atmosphere (37ºC, 5% CO2) until 80% confluence in PhenoCULT-SEB basal media supplemented with 1/1000 Supplement A for 3 days prior to treatment. Then, SZ95 and PCI-SEB_Cau were incubated with purified NGAL protein in four replicates, produced in E. coli or in HEK293 (LC2-H5222, ACROBiosystems), with concentration 0, 100, 1000 ng/mL for 96 h and then subsequently incubated with fresh medium and 10% MTT (5 mg/mL in phosphate buffered saline) for 2 h at 37ºC. Afterwards, the medium was removed carefully and 500 µL of dimethyl sulfoxide (DMSO) 99% purity was added to lysate the cells and dissolve the purple insoluble crystals of MTT. The cell lysate was transferred to a new 96-well plate and then the absorbance was read using a Microplate Autoreader at excitation/emission of 540/630 nm (Tecan). Absorbance values were considered directly proportional to cell viability.

Tunel assay

SZ95 and PCI-SEB_Cau sebocytes were cultured in 96-well plates as described above. Wells were rinsed with PBS and treated in triplicates with vehicle control, 50 ng/ml, 100 ng/ml, 1 µg/ml of NGAL (E.coli, HEK293 or C. acnes) protein for 24 hours. Samples were prepared by manufacturer's instructions for In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics). DNase I treatment of positive and negative
controls (without terminal transferase) were included as assay controls according to manufacturer’s
instructions. Digital images from Operetta HCS fluorescence microscopy experiments were processed
using the Harmony high content analysis version 4.9 software package (Perkin-Elmer). When performed,
image processing was restricted to changes in brightness and/or contrast and was applied equally
across the entire image and different images of the same experiment, including controls. For image
analysis, at least three fields per biological replicate were randomly chosen. To measure staining
intensity, the Harmony high content analysis software package was used with custom algorithms. The
DAPI channel was used to detect nuclei. Cytoplasmic area was determined as a ring of constant size
around each nucleus and was used to measure the mean fluorescence intensity of the staining of
interest.

Bacteria engraftment on murine skin

Bacteria (WT and KI) was grown in 20 mL to a OD$_{600}$ of 1, spun down at 1700 x g, 10 min and washed 3
times with 0.5% of peptone/PBS and resuspended in 0.5% peptone/PBS to a final volume of 500 ul. Mice
(C57BL/6J) were shaved on the back at an area of 2cm x 5cm. Wild type, knock out or vehicle were
applied with a cotton swab on the shaved areas and repeated for consecutive 3 days. Every day before
application a sample was taken with a moistened cotton swab and dissolved in 0.5 mL QuickExtract™
DNA Extraction Solution (Lucigen) and treated for 6 min at 65ºC followed by 2 min at 95ºC with 15
seconds vortexing between steps. Samples were taken every 24 h during the full assay period of 7 days. 5
µl DNA extract was used for amplification of the SLST region or 16S V3-V4 region linked to illumina
adaptors using KAPA HiFi HotStart Readymix (Roche) (Initial denaturation for 5 min at 95°C followed by
35 cycles of 98°C for 20 s, 62°C for 25 s, and 72°C 30 s; and a final elongation for 1 min at 72°C).

SLST_illumina_fwd:

5’-TCGCCACAGTCGAGATGTATAAGAGACAGTTGCTCGCAACTGCAAGCA-3’ and

SLST_illumina_rev:

5’-GTCTCGTGGGCTCGGAGATGTATAAGAGACAGCCGCTGGCAATGAGCAT-3’

16S_illumina_fwd:

5’-TCGCCACAGTCGAGATGTATAAGAGACAGCCTACGGGNGGCWGCAG-3’ and

16S_illumina_rev:

5’-GTCTCGTGGGCTCGGAGATGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3’.

Junctions were PCR amplified using KAPA HiFi HotStart Readymix (Roche) (Initial denaturation for 3 min
at 95°C followed by 25 cycles of 98°C for 20 s, 62°C for 15 s, and 72°C 30 s; and a final elongation for 1
min at 72°C) using primer pairs Junction1_fwd and rev and Junction2_fwd and rev (see Supplementary Table 1).

PCR products were loaded in a 1% agarose gel and visualized by a transilluminator.

Skin biopsies

Bacteria was applied to shaved back of mice for 3 consecutive days. 24h after the last application mice were sacrificed and skin biopsies were taken. Samples for RNA extraction were stored in RNAlater solution while samples for cryosections were frozen embedded in OCD solution.

RNA extraction and qPCR of skin biopsies

Total RNA was extracted from skin (NucleoSpin RNA extraction kit, Macherey-Nagel) according to the manufacturer instructions. RNA was then used for cDNA synthesis (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific). Quantitative PCR amplification reactions were performed in the QuantStudio 7 Pro Real-Time PCR System (Applied Biosystems) using TaqMan and Sybr Green Gene Expression Master Mix. Values were normalized to Sdha. The following TaqMan assays (Thermo Fisher Scientific) were used to quantify mRNA expression of mouse Sdha (Mm01352366_m1), Il-1β (Mm00434228_m1), Il-6 (Mm00446190_m1) and Tnfα (Mm00443258_m1) and primers LCN_qPCR_fwd and rev for detection of human NGAL.

Gram staining of skin sections

Cryosections from frozen tissue embedded in OCT (Optimal Cutting Temperature (OCT) compound Tissue-Tek) were cut at 7um thickness with cryotome. Frozen skin sections were air-dried at room temperature for 15 minutes, fixed with 4% paraformaldehyde for 15 minutes and stained with Gram following standard procedures. Crystal violet was applied for 1 minute following water washing, then iodine for 1 minute then water wash, rapid decolorization was achieved with acetone and a final 1 minute incubation with Safranin with subsequent washes were applied. After drying the samples DPX (Sigma Aldrich) mounting media was applied. Conventional images were captured using a Zeiss apotome microscope.

Declarations

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References


**Figures**

**Figure 1**

Optimization of electrotransformation in *C. acnes* and NGAL expression A) Fluorescence measurement of competent cells prepared with different electroporation buffers (EPB) and transformed with fluorescently labeled Cy3 oligonucleotide. DNA internalization is measured by fluorescence intensity. EPB-1: 0.5 M

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**Figure 1**

Optimization of electrotransformation in *C. acnes* and NGAL expression A) Fluorescence measurement of competent cells prepared with different electroporation buffers (EPB) and transformed with fluorescently labeled Cy3 oligonucleotide. DNA internalization is measured by fluorescence intensity. EPB-1: 0.5 M.
sucrose, 1mM KOAc, pH 5.8, EPB-2: 30 % PEB6000, 6.6 % sucrose, EPB-3: 272 mM sucrose, 7 mM NaPi, 1 mM MgCl₂, EPB-4: 272 mM sucrose, EPB-5: H₂O. B) Transformants obtained after mimicking C. acnes R-M system compared to E. coli dam-dcm- cells. C) Fold change in transformation efficiency comparing various cell-wall-weakening agents in comparison to no pre-treatment. Results represent three independent experiments. D) Representative graphics of tdk gene replacement by genes encoding NGAL and an erythromycin resistance cassette. Primers amplifying junctions are represented as red arrows. Junction PCR of successful replacement of tdk gene by genes coding for LCN2 and ermE. E) Variants of NGAL fused to endogenous secretion peptides. F) Western blot of NGAL protein produced in either HEK293, E. coli or C. acnes. HEK293 produced NGAL is glycosylated and has therefore a higher molecular weight than the E. coli produced protein. G) Viability of C. acnes incubated, SZ95 or PCi-SEB_Cau treated with HEK293 produced NGAL. H) Lipid staining of PCi-SEB_Cau sebocytes inflamed with arachidonic acid (5 μM) and treated with either isotretinoin (10 μM) or NGAL protein (C. acnes or E. coli at 50 ng/mL).

Figure 2

Engraftment of NGAL producing C. acnes strain on mice skin A) Experimental set-up of bacterial application and sampling. B) Agarose gel of skin samples taken by swabbing followed by DNA extraction and PCR. Junction PCR is shown and compared to control (vehicle) or wild type KPA171202. Only modified strain shows amplification. C) Gram-staining of cryosection of control mice vs. C. acnes colonized mice. D) qPCR of LCN2 gene of skin biopsies compared to control and wild type treated skin (control and wild type samples were taken together in plot), E) qPCR targeting inflammatory markers TNFα, IL-1β and IL6 of skin biopsies compared to control, wild type or engineered C. acnes treated skin.
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