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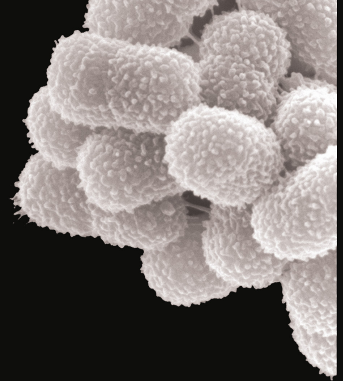

Chapter 7

Three-dimensional human skin equivalent as a tool to study *Acinetobacter baumannii* colonization

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Abstract

Acinetobacter baumannii can colonize body surfaces of hospitalized patients. From these sites, invasion into the host and spread to other patients and the hospital environment may occur. Eradication of the organism from the patient's skin is an important infection control strategy during epidemic and endemic episodes. In this study, a three-dimensional, air-exposed human epidermal skin equivalent was exploited to study *Acinetobacter* skin colonization. We characterized the adherence of *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T to and biofilm formation on the skin equivalent and the responses to these bacteria. Furthermore, we assessed the ability of the disinfectant chlorhexidine to decolonize the skin equivalents. Results revealed that both strains replicated on the stratum corneum up to 72 h, but did not invade the epidermis. *A. baumannii*, in contrast to *A. junii*, formed large biofilms on the stratum corneum. Bacterial colonization did not affect keratinocyte activation, proliferation or differentiation nor did it induce a strong inflammatory response. Disinfection with chlorhexidine solution resulted in complete eradication of *A. baumannii* from the skin without detrimental effects. This 3D model is a promising tool to study skin colonization and to evaluate the effects of novel disinfectant and antimicrobial strategies.

Introduction

Multidrug resistant (MDR) strains of *Acinetobacter baumannii* are notorious for their association with outbreaks of colonization and infection worldwide [6,23]. During such outbreaks, *A. baumannii* can colonize body surfaces of severely ill patients from which the organisms may invade the patient causing infection and/or spread to other patients and their environment. Thus, the skin is thought to constitute an important reservoir for *A. baumannii* during outbreaks and endemic episodes [1,17]. Insight into *Acinetobacter* skin colonization and the microbial ecology of the skin may result in novel strategies to prevent or interfere with skin colonization and thus contribute to eradication of the organisms from a ward.

Adherence and biofilm formation on plastic and adherence to human cells are widely used systems to study interactions of bacteria with abiotic and biotic surfaces. However, these systems may not adequately reflect the association of bacteria with the human skin, a process that takes place under relatively dry conditions [20]. Moreover, adherence and subsequent replication is strongly influenced by environmental conditions [19], including the physico-chemical barrier properties of the skin surface and its nutrient availability. Once bacteria have adhered to the skin, they may invade the epidermis and trigger an inflammatory response. To our knowledge little is known about the possible response of human skin cells to *Acinetobacter*.

Tissue-engineered, air-exposed human skin models are 3D systems that mimic the native skin to a high degree [10,11]. Such epidermal skin equivalents are generated by culturing primary keratinocytes at the air–liquid interface on cell-free matrices (e.g., inert filters or de-epidermized dermis). The keratinocytes will proliferate, migrate and differentiate during epidermal development resulting in skin equivalents that contain all layers of the native epidermis [11]. The skin equivalents also have barrier properties that show high similarities with the human skin [21]. These systems are interesting models for studying pathogen-skin interactions.

In the present study, we exploited a 3D human epidermal skin equivalent to study the adherence of an *A. baumannii* and an *A. junii* strain to the skin, subsequent biofilm formation and the skin's response to these bacteria. Furthermore, we explored the usefulness of this model to investigate the effects of a disinfectant on the bacteria and the human skin.

Materials and methods

Generation of epidermal skin equivalents

Human keratinocytes were isolated from fresh plastic surgery surplus skin as previously described [10]. Briefly, the epidermis and dermis were enzymatically and mechanically separated and each layer subsequently digested to obtain single cell suspensions. Keratinocytes were cultured in Dermalife medium (Lifeline cell technology) supplemented with penicillin (10000 U) and streptomycin (10 mg/ml). Epidermal skin models were generated as described [11,21]. In short, approximately $2x10^5$ keratinocytes from a secondary culture were seeded onto a filter insert (12 mm in diameter, Costar, Corning) in 12-wells plates in Dermalife medium. Three days after seeding, cells were put air-exposed by aspirating the apical medium. The basal medium was replaced with CnT-02-3D medium (CellTec) supplemented with 2.4×10^{-2} µM bovine serum albumin, 25 µM palmitic acid, 15 µM linoleic acid and 7 µM arachidonic acid. Prior to bacterial inoculation, the medium was replaced by keratinocyte medium without penicillin and streptomycin. Experiments were performed using 7 days air-exposed cultures.

Preparation of bacterial inoculum

A. baumannii type strain ATCC19606^T and *A. junii* type strain ATCC17908^T (=RUH2228^T) were used. Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at -80°C. Inocula from frozen cultures were grown overnight at 37°C on sheep blood agar plates (BioMerieux). Bacteria were cultured for 2.5 h at 37°C in Luria Bertani (LB) medium [10 g of Bactotryptone, 5 g of Yeast extract (both from BD, Sparks) and 5 g of sodium chloride (Merck) in 1 l of distilled water] under vigorous shaking. This suspension was diluted in phosphate buffered saline (PBS; pH 7.4) to a concentration of approximately $3x10^5$ colony forming units (CFU)/ml as calculated from the absorbance of the suspension at 600 nm, and verified afterwards using standard vital counts.

Colonization of epidermal skin equivalents

Skin equivalents were incubated with 300 µl of the bacterial suspension at 37°C / 7.3% CO_2. After 1 h, the bacterial suspension was aspirated to remove non-adherent bacteria. At different intervals after inoculation, the number of viable detachable and adherent bacteria was assessed microbiologically. Briefly, 600 µl of PBS were applied to the skin and the detachable bacteria were collected, serially diluted and plated onto diagnostic sensitivity test (DST) agar plates to determine the number of CFU. To assess the number of adherent bacteria, two biopsies (each 4 mm in diameter) were taken from the skin, homogenized in PBS using a glass Potter-Elvehjem tissue homogenizer and the

homogenates were subsequently serially diluted. The lower limit of detection of detachable and adherent bacteria was 12 and 115 CFU/skin equivalent, respectively. The number of adherent bacteria per skin equivalent (113.04 mm^2) was calculated by multiplying the number of adherent bacteria in two biopsies (25.12 mm^2) by 4.5. The total number of bacteria per skin equivalent was calculated by adding up the number of detachable bacteria per skin equivalent and the number of adherent bacteria per skin equivalent.

Microscopic analysis of bacterial replication on skin equivalents

To visualize bacterial colonization of the skin equivalents, one biopsy of each skin equivalent was fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Next, paraffin blocks were cut into 5 µm sections, deparaffinized and rehydrated and then stained with Alcian-blue in combination with Periodic acid-Schiff (PAS, both from Merck) to detect polysaccharides.

Immunohistochemical analysis of keratinocyte response

For analysis of the effects of colonization of the skin equivalents, the levels of keratin (K) 16, a marker for keratinocyte activation or stress; Ki67, a marker for keratinocyte proliferation; and K10, a marker for early keratinocyte differentiation were determined by immunohistochemical analysis. In short, standard antigen retrieval of deparaffinised and rehydrated paraffin sections was performed by immersing sections in 0.01 M citrate buffer (pH 6.0) for 30 min at 90°C followed by slow cooling down to room temperature for at least three h prior to staining of the sections. Sections were incubated overnight at 4°C with the primary mouse antibodies directed against human K16 (AbD serotec, clone LL025, 5x diluted), Ki67 (DAKO, clone MIB-1, 75x diluted), and K10 (Abcam, clone DE-k10, 50x diluted). Thereafter, sections were incubated for 60 min with secondary biotinylated goat-anti-mouse antibodies (DAKO) and subsequently 30 min with streptavidin-biotinylated horseradish peroxidase (DAKO). Chromogen 3-amino-9-ethyl-carbozole (AEC) solution was used as substrate solution. Sections were washed three times with PBS between subsequent incubations and finally counterstained with haematoxylin.

To determine the proliferation index, the number of Ki67 positive nuclei from the total number of basal cells was used. A minimum of 100 basal cells was counted in sections of three different samples at a magnification of 200x.

Determination of cytokine and chemokine levels

The levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, and TNFα in culture media were determined by ELISA (all from Biosource, Invitrogen) according to manufacturer's instructions. The lower

limit of detection was 1 pg/ml for IL-1α and IL-1β, 15 pg/ml for IL-6, 7 pg/ml for IL-8 and 25 pg/ml for IL-10 and TNFα.

Determination of gene expression levels

Total RNA was extracted from homogenized skin biopsies using the RNeasy Mini kit (Qiagen), followed by a treatment with DNase I (Qiagen). cDNA synthesis was performed on 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-RAD) following manufacturer's instructions. For each sample, a control for genomic DNA contamination was included by adding sterile water instead of reverse transcriptase. Real-time quantitative PCR was performed in an ICycler IQ (Bio-RAD) in a final volume of 25 µl comprising 1x IQ SYBR Green Supermix (Bio-RAD), 10 pmol of each primer (Table 1) and 5 µl of 10x diluted cDNA. PCR conditions consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56.5°C (for Ki67), 57.8°C (for LL-37), 60.8°C (for GAPDH and IL-1β), 61.7°C (for TNFα) or 62°C (for β2M, IL-1α, IL-6, IL-8, hBD-2, hBD-3, K16 and K10) for 15 s, and elongation at 72°C for 20 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-2 microglobulin (β2M) were used for standardization. Experiments were performed in duplicate. Data were acquired and analyzed using ICycler IQ Optical System software (Bio-RAD) with automatic adjustment of the baseline and threshold parameters.

Table 1. Primer sequences

	Forward	**Reverse**
β2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
GAPDH	AAGGTCGGAGTCAACGGATTT	ACCAGAGTTAAAAGCAGCCCTG
IL-1α	CGCCAATGACTCAGAGGAAGA	AGGGCGTCATTCAGGATGAA
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-8	GCCAGGAAGAAACCACCGGAAGG	GGCTGCCAAGAGAGCCACGG
TNFα	CCTGTGAGGAGGACGAACAT	GGTTGAGGGTGTCTGAAGGA
hBD-2	TGATGCCTCTTCCAGGTGTTT	GGATGACATATGGCTCCACTCTTA
hBD-3	TTATTGCAGAGTCAGAGGCGG	CGAGCACTTGCCGATCTGTT
LL-37	ATTTCTCAGAGCCCAGAAGC	CGGAATCTTGTACCCAGGAC
K16	GAGATGCGTGACCAGTACGA	TTGTTCAGCTCCTCGGTCTT
K10	AGCATGGCAACTCACATCAG	TGTCGATCTGAAGCAGGATG
Ki67	AATTCAGACTCCATGTGCCTGAG	CTTGACACACACATTGTCCTCAGC

Decontamination of colonized skin equivalents

Two days after inoculation with *A. baumannii* ATCC19606^{T}, 0.5% chlorhexidine solution in 70% ethanol or as a control PBS was applied to the skin equivalents using a sterile cotton swab. The numbers of detachable and adherent bacteria were assessed as described above 24 h after application of the disinfectant.

Statistical analysis

Results are expressed as mean ± standard errors of the mean unless stated otherwise. Data were analyzed for statistical significance using the Wilcoxon rank sum test (SPSS 17.0). P values ≤0.05 were considered significant.

Results

Replication of *Acinetobacter* on skin equivalents

To compare the persistence of *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T on the skin, human epidermal skin equivalents were inoculated with approximately $1x10^5$ CFU suspended in 300 µl of PBS. The suspensions were aspirated from the skin equivalents after 1 h to allow air-exposure again. The total number of bacteria on the skin equivalent increased during the first 12 h from $7x10^3 \pm 6x10^3$ to $9x10^7 \pm 3x10^6$ CFU for the *A. baumannii* strain and from $2x10^4 \pm 1x10^4$ to $1x10^8 \pm 1x10^7$ CFU for the *A. junii* strain and thereafter remained stable for the duration of the experiment (Figure 1).

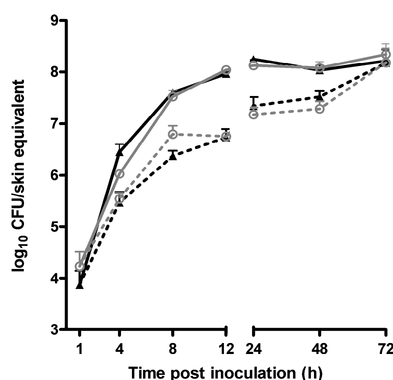

Figure 1. *A. baumannii* and *A. junii* replication on skin equivalents. Human epidermal skin equivalents were inoculated with approximately $1x10^5$ CFU of *A. baumannii* ATCC19606^T (triangles) or *A. junii* RUH2228^T (circles). After 1 h, the non-adhered bacteria were removed. At different intervals thereafter, the numbers of detachable and adherent bacteria were determined microbiologically. Results are expressed as mean number of adherent (dotted lines) and total (solid lines) colony forming units (CFU) per skin equivalent ± standard errors of the mean of three independent experiments.

At 48 and 72 h after infection, the number of adhered *A. baumannii* ATCC19606^T was significantly greater than the number of detachable bacteria, indicating that the proportion of bacteria that adhered increased over time (Figure 1). For the *A. junii* strain,

the number of adherent bacteria exceeded that of detachable bacteria at 72 h after infection.

Acinetobacter biofilm formation on skin equivalents

Microscopic analysis revealed that bacteria of both strains persisted on the stratum corneum and did not invade the epidermis (Figure 2). Although there was no significant difference in the number of adherent bacteria between *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T (Figure 1), Alcian blue-PAS staining revealed large biofilm structures by the *A. baumannii* but not by the *A. junii* strain on the stratum corneum at 48 and 72 h (Figure 2).

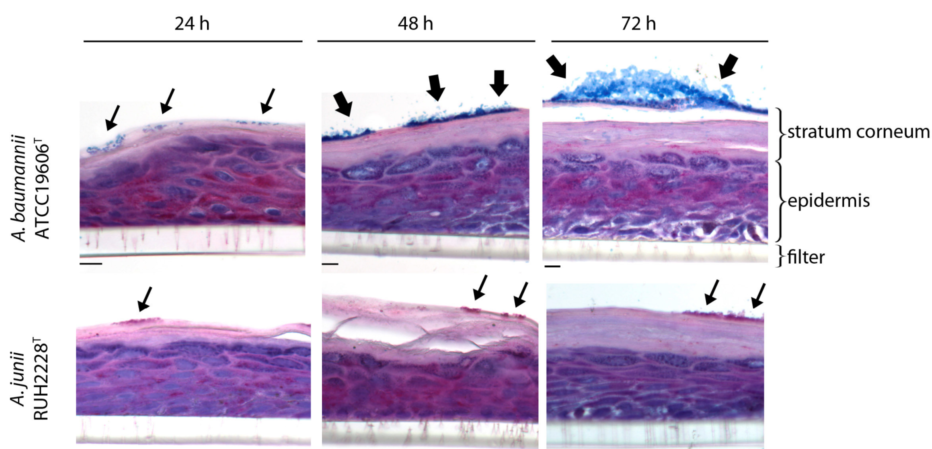

Figure 2. Biofilm formation on skin equivalents. Skin equivalents were exposed to *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T up to 72 h. Thereafter, 5 µm sections of paraffin-embedded skin were stained with Alcian blue PAS and biofilm formation was analysed by light microscopy. Small arrows indicate clusters of bacteria, large arrows indicate bacteria within a biofilm matrix. Scale bars, 20 µm.

Response of skin equivalents to *Acinetobacter*

Subsequently, the effect of bacterial colonization on keratinocyte differentiation, proliferation and cytokine and antimicrobial peptide production was evaluated. Results revealed that exposure of the skin equivalents to *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T did not affect the mRNA expression of Ki67, K10 and K16 in keratinocytes (Figure 3A). In agreement, no difference was seen in protein expression of the activation/stress marker K16 between colonized and PBS-exposed skin equivalents (Figure 3B).

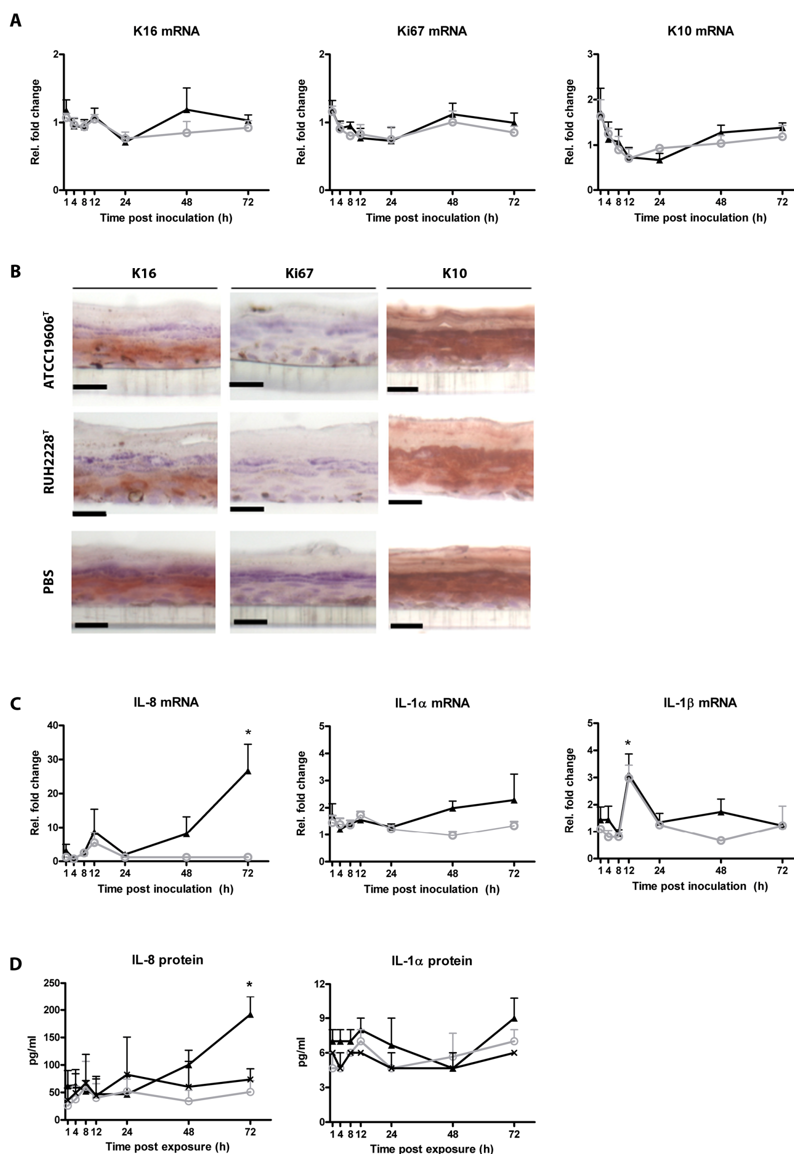

Figure 3. Response of keratinocytes to *Acinetobacter.* Skin equivalents were exposed to *A. baumannii* ATCC19606^T (triangles), *A. junii* RUH2228^T (circles) or as a control PBS (crosses). At various intervals thereafter, mRNA expression (A) and at 48 h thereafter, protein levels (B) of keratinocyte activation/stress marker K16, proliferation marker Ki67 and differentiation marker K10 were determined using qPCR and immunohistochemistry, respectively. Scale bars, 20 µm. In addition, the mRNA expression (C) and protein (D) levels of the chemokine IL-8 and the pro-inflammatory cytokine interleukin (IL)-1α and IL-1β at different intervals were determined using qPCR and ELISA, respectively. Results are expressed as mean fold change in mRNA expression relative to PBS-exposed skin equivalents (A and C) or mean cytokine level in pg/ml (D) ± standard errors of the mean of three independent experiments. *, significantly different from PBS.

Moreover, the expression of K10 in the suprabasal viable cell layers of the exposed and non-exposed skin equivalents confirms that the early differentiation program was not altered by the *Acinetobacter* strains. In addition, the number of proliferating cells in the basal layers was not influenced by the bacteria, i.e. 20±2 versus 18±1 cells positive for Ki67/100 basal cells in *A. baumannii*-colonized skin versus PBS-exposed skin.

Furthermore, we assessed the production of the pro-inflammatory cytokines TNFα, IL-1α, IL-1β, IL-6, and IL-8 and that of the anti-inflammatory cytokine IL-10 by the skin equivalent in response to both *Acinetobacter* strains. During the first 48 h of infection, skin equivalents expressed similar levels of IL-1α and IL-8 after exposure to the two *Acinetobacter* strains as to PBS. However, after 72 h mRNA and protein expression levels of IL-8, but not of IL-1α, were slightly but significantly ($p<0.05$) higher in skin equivalents colonized by the *A. baumannii* strain as compared to those colonized by the *A. junii* strain and/or exposed to PBS (Figure 3C,D). In addition to IL-8 and IL-1α, IL-1β mRNA expression levels were significantly ($p<0.05$) higher in skin equivalents exposed to the *Acinetobacter* strains as compared to PBS-exposed skin equivalents, but only at 12 h of infection (Figure 3C). IL-1β protein was, however, not detectable. Moreover, TNFα, IL-6 and IL-10 mRNA and proteins were not detectable in skin equivalents.

The skin equivalents constitutively expressed the antimicrobial peptides human beta-defensin (hBD)-2 and -3, but no LL-37. Infection of skin equivalents with the *Acinetobacter* strains did not induce enhanced expression of these peptides (data not shown).

Eradication of *A. baumannii* from skin equivalent

Finally, we examined the effect of chlorhexidine on *A. baumannii* ATCC19606^T colonization and on the epidermis. Swapping of skin equivalents colonized by the *A. baumannii* strain for 48 h with 0.5% chlorhexidine solution in 70% ethanol resulted in undetectable levels of *A. baumannii* on the skin (Figure 4A). In addition, this chlorhexidine solution did not affect skin morphology (Figure 4B) and mRNA expression levels of K16, IL-1α and IL-8, but enhanced the expression levels of Ki67 and K10 11-fold and 4-fold, respectively (Figure 4C). This indicates that chlorhexidine enhanced keratinocyte proliferation and differentiation but did not induce epidermal activation/stress or inflammation in the skin (Figure 4C).

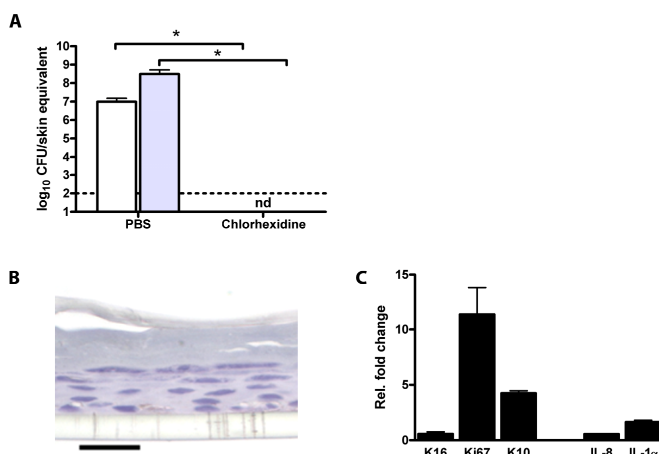

Figure 4. Treatment of infected skin equivalents with chlorhexidine. Skin equivalents were infected with *A. baumannii* ATCC19606^{T} for 48 h. Thereafter, chlorhexidine or PBS was applied to the skin equivalents. A, Twenty-four hours after treatment, the numbers of detachable and adherent bacteria were determined microbiologically. Results are expressed as mean number of adherent (white bars) and total (gray bars) colony forming units (CFU) per skin equivalent ± standard errors of the mean of three independent experiments. The dotted line represents the lower limit of detection. nd, not detectable; *, significant ($p<0.05$) difference. B, Light micrograph of section of chlorhexidine-treated skin stained with Alcian blue PAS. Scale bar, 20 µm. C, Twenty-four hours after treatment, mRNA expression levels of the keratinocyte activation/stress (K16), proliferation (Ki67) and differentiation (K10) markers as well as the inflammatory mediators IL-8 and IL-1α were determined using qPCR. Results are expressed as mean mRNA expression levels relative to PBS-treated skin ± standard deviations of four measurements.

Discussion

The main conclusion from the present findings is that the 3D human skin equivalent is a promising model to study skin colonization by *Acinetobacter* strains and to evaluate the effects of disinfectants and other antimicrobial agents. This is of importance as *A. baumannii* is able to colonize the skin [2,7,8,17,24], which can be a source for infection and spread to other patients and the environment. Recent papers have emphasized the benefits of skin disinfection as a tool to reduce colonization pressure in a ward [2,12,22]. Our main conclusion is based on the following findings. First, both *A. baumannii* ATCC19606^{T} and RUH2228^{T}, which belongs to *A. junii,* a species that occurs on the human skin, adhered to and replicated on the stratum corneum up to 72 h without invading the epidermis. An important difference between these two strains was the ability of the *A. baumannii* strain, but not the *A. junii* strain, to form a large biofilm on the skin equivalents. Secondly, bacterial colonization did not affect keratinocyte activation,

proliferation or differentiation nor did it induce a strong inflammatory response. Thirdly, disinfection with chlorhexidine solution resulted in complete eradication of the *A. baumannii* strain from the skin without detrimental effects.

We have previously shown that both *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T were able to form large biofilms on plastic [5]. On the present human skin model, however, only *A. baumannii* ATCC19606^T formed large biofilms. A possible explanation for the latter observation could be that *A. baumannii*, but not *A. junii*, can utilize the nutrients of the stratum corneum for biofilm formation. In agreement with this suggestion it has been shown that the metabolic versatility of *A. baumannii* is considerably greater than that of *A. junii* [3,18]. The ability of *Acinetobacter* to form biofilms appears to depend on the surface to which the bacteria adhere, as suggested earlier by Gaddy et al [13]. Although more strains should be examined before a definitive conclusion can be drawn, we hypothesize that the ability of *A. baumannii* to form a biofilm on human skin plays an important role in its persistence on the skin.

We have previously shown that human bronchial epithelial cells and cultured human macrophages produce considerable amounts of inflammatory mediators in response to these *Acinetobacter* strains [5]. Using this skin equivalent model, however, *A. baumannii* ATCC19606^T and *A. junii* RUH2228^T induced a poor inflammatory response. This may be explained as follows. In our previous study, the cells were cultured in monolayers and infected with bacteria, enabling direct contact between the bacteria and the cells. However, in the current experiments the bacteria did not invade the epidermis, and thus, did not come in direct contact with live keratinocytes. These results underline the importance of the stratum corneum as a protective barrier against infections, as also shown by Duckney et al, who demonstrated that *Staphylococcus epidermidis* and *Propionibacterium acnes* induced an inflammatory response in a reconstructed human epidermis model only when applied subcutaneously in the culture medium and not when applied topically on the stratum corneum [9]. Moreover, it emphasizes that results from studies into pathogen-host interactions using keratinocyte monolayers (lacking a stratum corneum) will not reflect the in vivo situation.

With the emergence of antibiotic resistance, there has been a re-appraisal of the use of antiseptics for skin disinfection [12,15]. Chlorhexidine is widely used as an antiseptic against many microorganisms, including *A. baumannii* [12]. However, several studies have shown that chlorhexidine may have toxic effects on human fibroblasts [14] and be detrimental to wound healing [14,16], making its use controversial in particular situations. Although the *A. baumannii* type strain used in this study was fully susceptible to treatment with chlorhexidine, a MDR *A. baumannii* isolate that was resistant to chlorhexidine concentrations up to 1% has already been reported [4]. In this light, it is

necessary to develop novel disinfectant strategies to eradicate *A. baumannii* from the colonized skin.

In summary, we have described a novel model for *Acinetobacter* skin colonization using 3D human epidermal skin equivalents. This model will be beneficial in characterizing bacterial growth kinetics and the interactions of different bacterial species on a biotic surface. Moreover, this model may also be advantageous for identifying and evaluating new targets for disinfection and antimicrobial strategies.

Acknowledgements

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