

# Resident Bacterial Flora in the Skin of C57BL/6 Mice Housed under SPF Conditions

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Research in cutaneous biology frequently involves models that use mice housed in SPF conditions. Little information is available concerning the species of bacteria that normally inhabit the skin of these mice. The aim of this study was to characterize the bacterial skin flora of mice housed under SPF conditions. Skin biopsies from C57BL/6 mice under normal and surgically prepped conditions were both cultured and analyzed by using DNA extraction and sequencing. The species isolated most commonly from culture were staphylococci. Coagulase-negative staphylococci were isolated more frequently than was *Staphylococcus aureus*. Molecular sequencing yielded several additional organisms not found by culture. Overall, culturing of isolates yielded 14 species of bacteria, and molecular sequencing identified another 6 species. Investigators conducting cutaneous research in mouse models should be aware of the cutaneous bacterial flora present on these mice.

**Abbreviations:** CNS, coagulase-negative staphylococci; DGGE, density gradient gel electrophoresis.

Mice are used frequently in cutaneous biology and wound healing research. Most mice studied in skin biology are housed in SPF facilities. However, many investigators are unclear about the functional definition of SPF. SPF conditions ensure that a predefined group of known pathogenic organisms are excluded from the environment where the animals are housed. Little is known about the bacterial species inhabiting the skin of normal mice housed under SPF conditions. Because bacterial colonization or infection by microorganisms can affect the outcome of studies,<sup>1</sup> identifying the bacterial flora colonizing murine skin under SPF conditions is important in understanding the effect of these organisms on cutaneous research in mice.

We use a mouse implant model to study skin interaction with biomaterials, with the goal of decreasing bacterial infections by restoring cutaneous barrier function.<sup>7</sup> Characterizing the bacterial flora of murine skin housed under SPF conditions establishes a critical baseline for studies such as ours, in which bacterial infection plays a key role.

## Materials and Methods

Animal experiments were conducted in accordance with protocols approved by the University of Washington Animal Care and Use Committee. Male C57BL/6 mice ( $n = 24$ ; strain code 027, Charles River, Wilmington, MA) were 6 wk old at the time of delivery to our institution and were housed in the SPF facility from 1 wk to 2 mo before their use in our experiments.

The University of Washington SPF facility is designed to maintain rodents in an environment free of infectious agents known to be pathogenic or capable of interfering with research objectives. The animals are maintained in a nonbarrier SPF facility. Sentinel animals are placed in each rack to monitor for specific infectious agents. Caging and associated equipment used

for housing and experimental equipment for SPF rodents are sterilized prior to use. Before their introduction into the facility, materials of biological origin (such as cells, tissues, serum, and cultures) must be tested by PCR or another acceptable method to verify that they are pathogen-free. Entry into the facility is limited and requires exchanging street clothes for dedicated scrubs, shoes, and hair bonnet. The mice are always handled by personnel wearing gloves, masks, and sleeves or gowns. Rodents from nonapproved vendors are quarantined for 5 to 9 wk before being housed in the SPF facility. Excluded agents are mouse hepatitis virus and parvovirus, minute virus of mice, reovirus 3, pneumonia virus of mice, epizootic diarrhea of infant mice, Theiler murine encephalomyelitis virus, lymphocytic choriomeningitis virus, ectromelia, Sendai virus, sialodacryoadenitis virus, rat parvoviruses, *Mycoplasma pulmonis*, pinworms, and fur mites.

Studies were carried out by using aseptic technique. According to standard protocol, the mice were isolated from direct human contact during their stay in the SPF facility. After the mice were euthanized, punch biopsies of dorsal trunk skin, the site typically used for biomaterial implantation studies, were obtained from skin unexposed to topical antiseptics, representing the natural skin condition, as well as from skin that had been shaved with an electric razor, cleared of hair by using depilatory cream (Nair, Church and Dwight, Princeton, NJ), and cleansed with povidone-iodine solution (Betadine, Purdue Pharma, Stamford, CT) and alcohol, representing skin prepped for implant surgery.

Under sterile technique, biopsies were placed either in brain–heart infusion culture broth (for anaerobic growth) or sterile culture cups containing sterile saline and gauze (aerobic), and transported immediately to the Microbiology Department at the University of Washington (Seattle, WA) for culture. Saline-stored samples were massaged automatically (Seward Stomacher 80, Brinkmann Instruments, Mississauga, Ontario, Canada) and seeded onto several plates (sheep blood agar, chocolate agar, and MacConkey agar) to maximize the number of bacterial species isolated. Skin samples placed in brain–heart infusion broth were

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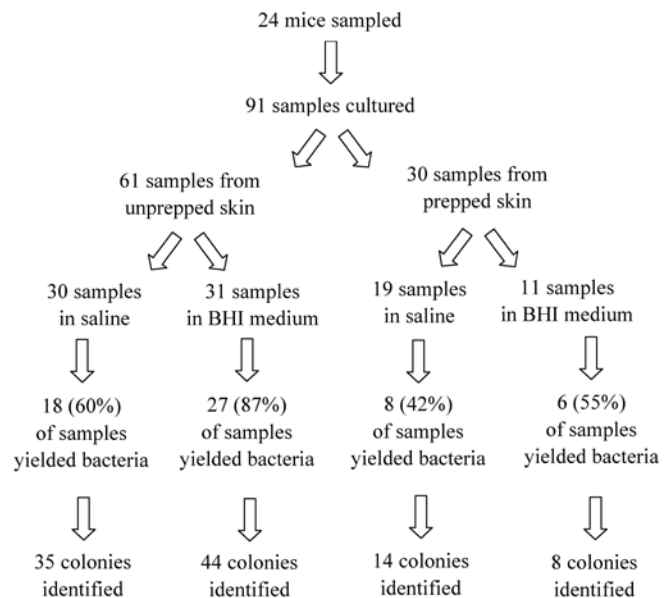
cultured at 35 °C and 3% CO<sub>2</sub> in the original container. Specimens that grew in brain–heart infusion broth were subcultured on the plates listed for saline-stored samples. In addition, an inoculated *Brucella* plate was incubated at 35 °C anaerobically, to recover any potential anaerobes from the broth. All culture results were read as final on day 7. Saline and broth containers without samples were cultured as controls. Organisms were identified by using Gram staining, catalase and coagulase characterization, automated identification and susceptibility testing (Vitek, Bio Merieux, Marcy L’Etoile, France), and 16S rRNA gene sequencing.

Cultured isolates were sent to the Center for Biofilm Engineering (Montana State University, Bozeman, MT) and further analyzed as follows. DNA was extracted (Microbial DNA Isolation Kit, Mo Bio Laboratories, Carlsbad, CA) from the colonies as well as known control organisms grown on agar plates. Sterile water was used as a negative control. PCR amplification was performed by using primers 8F (5’ AGA GTT TGA TCC TGG CTC AG 3’) and 1492R (5’ GGT TAC CTT GTT ACG ACT T 3’; Integrated DNA Technologies, Carlsbad, CA). Primer reactions and DNA amplification was performed by using a programmable thermocycler (PTC100, MJ Research, Waltham, MA) at the following parameters: 94 °C for 2 min; 15 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s; and a final extension step of 72 °C for 7 min. Verification of the presence of DNA was assessed in 1.5% agarose gels. Amplified DNA was cloned directly (TOPO Cloning Kit, Invitrogen, Carlsbad, CA), and plasmids were extracted (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA) from the clones. Plasmids were sent to Laragen (Los Angeles, CA) for sequencing, and sequenced results were returned by email. Sequence data were analyzed by using the BLAST program ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

Skin samples from 6 additional mice were frozen in saline and sent to the Center for Biofilm Engineering for further analysis. DNA was extracted from mouse skin biopsy samples, from positive controls (cultured isolates), and a negative control (sterile water) by using the DNeasy Blood and Tissue Kit (Qiagen). PCR amplification of mouse skin biopsy samples was performed by using primers 357F (5’ CCT ACG GGA GGC AGC AG 3’) and 518R+GC clamp (5’ CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CGT ATT ACC GCG GCTGCT GG 3’; Integrated DNA Technologies). Primer reactions, DNA amplification, and verification of the presence of DNA were performed as described earlier. The amplified DNA underwent density gradient gel electrophoresis (DGGE) by using a 40% to 60% denaturing gradient and a mutation detection system (DCode, BioRad Laboratories, Hercules, CA). The gel was run at 60 V and 60 °C with continuously stirred buffer for 16 h. Bands in the gel were visualized by using SYBR Gold (Invitrogen). DGGE bands of interest were cut directly from the gel and PCR-amplified as described earlier. Amplified DNA was cloned (TOPO Cloning Kit, Invitrogen), and plasmid extraction, sequencing, and data analysis were done as described earlier.

## Results

Control biopsy containers appropriately demonstrated no growth. From the 91 skin samples sent for culture, a total of 101 bacterial colonies were preliminarily identified by the Microbiology Department (Figure 1). The identities of 85 random isolates were confirmed by sequencing 16S rRNA. For most isolates, the bacterial identities determined by 16S sequencing were the same as those determined by using phenotypic tests. However, several isolates labeled phenotypically as *S. aureus* were identified as *S. saprophyticus* by 16S sequencing. The organisms identified



**Figure 1.** Overview of the cultured mouse skin samples, showing the numbers and percentages of samples yielding bacteria. The number of C57BL/6 mouse skin samples cultured in each condition (unprepped versus prepped skin), culture vessel (saline versus brain–heart infusion broth), the numbers and percentages of cultured samples that yielded bacteria, and the total number of colonies isolated in each group.

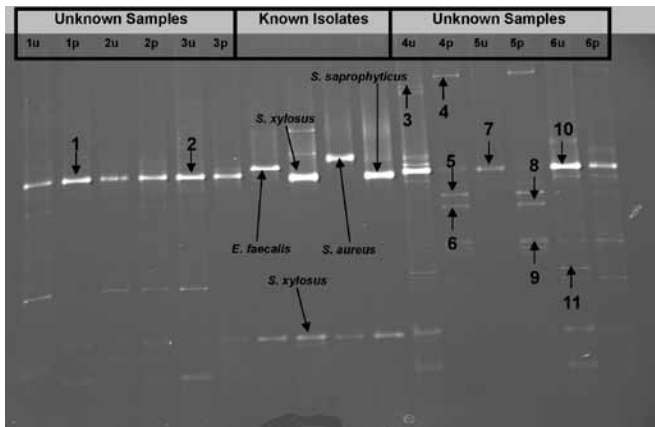
by sequencing of 16S DNA from cultured isolates were *S. saprophyticus* ( $n = 19$ ), *S. aureus* ( $n = 17$ ), *S. lentus* ( $n = 16$ ), *S. cohnii* ( $n = 9$ ), *S. xyloso* ( $n = 9$ ), *Enterococcus faecalis* ( $n = 5$ ), *S. nepalensis* ( $n = 3$ ), and *Stenotrophomonas maltophilia*, *Microbacteria testaceum*, *Aerococcus viridans*, *Bacillus licheniformis*, *B. anthracis*, *B. subtilis*, and *B. circulans* ( $n = 1$  each).

The samples used to identify organisms by DGGE yielded some bands that correlated with known bacteria from cultured isolates, as well as several unique bands that differed from known bacteria and were removed for sequencing. The additional organisms identified by this technique were bacteria not found by culture alone. Figure 2 shows the DGGE gel demonstrating the additional bacteria that were not identified by culture alone.

Of the samples collected into sterile saline, 34 of 49 (69%) cultured isolates were coagulase-negative staphylococci (CNS), 3 (6%) were *S. aureus*, and 12 (25%) were nonstaphylococcal species. Of the samples collected in brain–heart infusion broth, 18 of 52 (35%) cultured isolates were CNS, 26 (50%) were *S. aureus*, and 8 (15%) were nonstaphylococcal species (Figure 1). Of the samples of skin that was prepped prior to culture, 16 of 22 (73%) of cultured isolates were CNS, while 0 (0%) were *S. aureus*, and 6 (27%) represented nonstaphylococcal species. In unprepped skin samples, 36 of 79 (45%) cultured isolates were CNS, 29 (37%) were *S. aureus*, and 14 (18%) were nonstaphylococcal species.

## Discussion

Based on bacterial culture results, skin samples collected in saline were more likely to yield CNS, whereas samples collected in brain–heart infusion broth were more likely to yield *S. aureus*. Although this study was not designed to determine the effect of skin prepping on bacterial colonization, mouse skin that was prepped prior to culture yielded fewer organisms overall but relatively more CNS and nonstaphylococcal



**Figure 2.** DGGE gel of DNA extracted from mouse skin samples. Un-prepped skin is denoted with 'u,' whereas prepped skin is denoted with 'p.' Bands 1u/1p through 6u/6p represent DNA extracted from skin samples of 6 different mice. The center 5 bands represent DNA from known bacterial isolates previously cultured. Several unknown bands (numbers 1 to 11) were removed, amplified, and sequenced, and the following bacteria were identified from these bands: 1) *Staphylococcus aureus*, 2) *Staphylococcus saprophyticus*, 3) *Porphyromonas gingivalis*, 4) *Flavobacteria symbiont* 3, 5) *Burkholderia cepacia*, 6) *Burkholderia cepacia*, 7) *Staphylococcus lentus*, 8) *Burkholderia cepacia*, 9) *Brevundimonas nasdae*, 10) *Staphylococcus lentus*, and 11) *Microbacteriaceae bacterium*.

species than skin that was left unprepped before samples were obtained.

Our results show that staphylococci were the predominant species identified, and similar to findings for human skin<sup>10</sup>, CNS outnumbered coagulase-positive *S. aureus* in mouse skin samples. However, key differences exist between mouse and human skin flora. Most notably, *S. epidermidis*, the predominant bacterial species occupying normal human skin,<sup>8,10</sup> was not identified in mouse skin. Combining skin culture and DNA analysis techniques, *S. saprophyticus*, *S. aureus*, *S. lentus*, *S. xylosum*, and *S. cohnii* were the 5 most predominant bacteria identified from murine skin.

CNS are important pathogens in infections associated with percutaneous devices.<sup>9,14</sup> In immunocompromised mice with dermatitis, *S. xylosum* was isolated from skin lesions.<sup>15</sup> Although some investigators have not detected *S. aureus* in normal (hairless) mouse skin,<sup>3</sup> others have.<sup>2,10</sup>

Two recent reports have described the composition of microbial flora of ear skin biopsies from C57BL6/J mice,<sup>5,11</sup> in which the organisms typically were *Proteobacteria* and primarily pseudomonads. In addition, these previous studies<sup>5,11</sup> used small numbers (3 and 4) of mice. In contrast to the previous results, the predominant bacteria in the current study were staphylococci of the division *Firmicutes*; we did not detect pseudomonads in the specimens from our mice by using either culture or molecular microbiology methods. These differences in results may reflect differences in mouse microflora among different geographic or anatomic locations.

*Bacillus anthracis* is a member of the *B. cereus* group. Although these organisms are phenotypically heterogeneous, the phenotypic differences are primarily due to genes carried on plasmids, and the genomes of *B. anthracis* and *B. cereus* are very similar.<sup>6</sup> *B. anthracis* has been suggested to be a lineage of *B. cereus*.<sup>6</sup> Therefore, these 2 species may be indistinguishable based on 16S sequence. Bacteria in the *B. cereus* group are common microorganisms, and some are considered to be ubiquitous because their spores are incredibly durable. Although the mouse skin sample may have harbored *B. anthracis*, this

organism was more likely to actually be another member of the *B. cereus* group.

Animal health reports from the vendor document occasional *S. aureus* colonization in the mice shipped, determined by nasopharyngeal sampling. The presence of *S. aureus* on mouse skin may be particularly important because this species is one of the most common agents causing primary cutaneous infections in humans.<sup>4</sup> Our techniques identified not only this organism but also *Enterococcus faecalis* and several other species. *Enterococcus*, a gut bacterium, has been cultured from mouse skin by others.<sup>13</sup>

Although DGGE separation and sequencing techniques cannot replace culture as a method of identifying and quantifying bacterial flora, this technique did identify several species of bacteria that were not found by culture alone. This result is not surprising, because estimates are that only about 1% of bacterial species have been cultured.<sup>12</sup> Our findings indicate that neither method should stand alone, and culture plus molecular methods allowed identification of both common and rare bacterial species present on murine skin. Molecular analysis of cultured isolates yielded 14 different species and 6 genera of microorganisms. Direct molecular analysis identified an additional 6 species and 5 genera of bacteria that were not found by culture.

Bacterial skin flora not only serve as potential pathogens in disrupted skin but also include commensal organisms that protect the host from more pathogenic bacteria.<sup>4</sup> Some of the organisms we identified may have been the result of contamination from human contact or the facility, although the lack of *S. epidermidis* suggests that this is not the case.

Even the most rigorous description of SPF status conveys no information other than the organisms that the animals in question do not have. The composition of the flora (microbiota) present remains unknown and likely varies among different SPF populations due to such factors as differences in the methods of establishing SPF status (fostering on SPF mothers, cesarean derivation, derivation by embryo transfer), flora of the stock from which the foster mothers were derived, number of generations since derivation, the different environments in which a stock may have been maintained since derivation, rigorosity of SPF protocols implemented in facilities in which the stock may have been maintained, genotype of the animals, diets the animals have been fed, and so on. Therefore, the results of the current study are applicable only to the strain and facility from which the data were obtained and cannot be applied broadly without additional data establishing whether common patterns exist.

Although only the skin flora of one strain of mice from a single vendor was investigated, this study presents important information about the potential variety of bacterial organisms present on the skin of research mice in SPF facilities. These organisms may colonize mice in SPF facilities frequently but remain undetected because they are not screened for in SPF protocols. Investigators need to be aware of the possible presence of these bacteria living on murine skin, as identified in this study. These observations may be important in understanding the role of host microflora in cutaneous biology and wound healing research conducted in mice.

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## References

1. **Baker DG.** 1998. Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. *Clin Microbiol Rev* **11**:231–266.
2. **Besch-Williford C, Franklin CL.** 2007. Aerobic gram-positive organisms, p 389–406. In: Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, Smith AL, editors. *The mouse in biomedical research*. Amsterdam (the Netherlands): Elsevier.
3. **Bibel DJ, Miller SJ, Brown BE, Pandey BB, Elias PM, Shinefield HR, Aly R.** 1989. Antimicrobial activity of stratum corneum lipids from normal and essential-fatty-acid-deficient mice. *J Invest Dermatol* **92**:632–638.
4. **Chiller K, Selkin BA, Murakawa GJ.** 2001. Skin microflora and bacterial infections of the skin. *J Invest Dermatol Symp Proc* **6**:170–174.
5. **Grice EA, Kong HH, Renaud G, Young AC; NISC Comparative Sequencing Program, Bouffard GG, Blakesley RW, Wolfsberg TG, Turner ML, Segre JA.** 2008. A diversity profile of the human skin microbiota. *Genome Res* **18**:1043–1050.
6. **Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolstø AB.** *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. 2000. *Appl Environ Microbiol* **66**:2627–2630.
7. **Isenhath SN, Fukano Y, Usui ML, Underwood RA, Irvin CA, Marshall AJ, Hauch KD, Ratner BD, Fleckman P, Olerud JE.** 2007. A mouse model to evaluate the interface between skin and a percutaneous device. *J Biomed Mater Res A* **83**:915–922.
8. **Kloos WE, Bannerman TL.** 1994. Update on clinical significance of coagulase-negative staphylococci. *Clin Microbiol Rev* **7**:117–140.
9. **Martin MA, Pfaller MA, Wenzel RP.** 1989. Coagulase-negative staphylococcal bacteremia. Mortality and hospital stay. *Ann Intern Med* **110**:9–16.
10. **Nagase N, Sasaki A, Yamashita K, Shimizu A, Wakita Y, Kitai S, Kawano J.** 2002. Isolation and species distribution of staphylococci from animal and human skin. *J Vet Med Sci* **64**:245–250.
11. **Scharschmidt TC, List K, Grice EA, Szabo R, NISC Comparative Sequencing Program, Renaud G, Lee CC, Wolfsberg TG, Bugge TH, Segre JA.** Matriptase-deficient mice exhibit ichthyotic skin with a selective shift in skin microbiota. 2009. *J Invest Dermatol* **129**:2435–2442.
12. **Staley JT, Konopka A.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu Rev Microbiol* **39**:321–346.
13. **van der Waaij D, Sturm CA.** 1971. The production of 'bacteria-free' mice. Relationship between fecal flora and bacterial population of the skin. *Antonie van Leeuwenhoek* **37**:139–151.
14. **Weinstein MP, Mirrett S, Van Pelt L, McKinnon M, Zimmer BL, Kloos W, Reller LB.** 1998. Clinical importance of identifying coagulase-negative staphylococci isolated from blood cultures: evaluation of MicroScan rapid and dried-overnight gram-positive panels versus a conventional reference method. *J Clin Microbiol* **36**:2089–2092.
15. **Won YS, Kwon HJ, Oh GT, Kim BH, Lee CH, Park YH, Hyun BH, Choi YK.** 2002. Identification of *Staphylococcus xylosum* isolated from C57BL/6J *Nos2<sup>tm1Lau</sup>* mice with dermatitis. *Microbiol Immunol* **46**:629–632.