Development and Validation of an In Vivo Candida albicans Biofilm Denture Model

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The most common form of oral candidiasis, denture-associated stomatitis, involves biofilm growth on an oral prosthetic surface. Cells in this unique environment are equipped to withstand host defenses and survive antifungal therapy. Studies of the biofilm process on dentures have primarily been limited to in vitro models. We developed a rodent acrylic denture model and characterized the Candida albicans and mixed oral bacterial flora biofilm formation, architecture, and drug resistance in vivo, using time course quantitative culture experiments, confocal microscopy, scanning electron microscopy, and antifungal susceptibility assays. We also examined the utility of the model for measurement of C. albicans gene expression and tested the impact of a specific gene product (Bcr1p) on biofilm formation. Finally, we assessed the mucosal host response to the denture biofilm and found the mucosal histopathology to be consistent with that of acute human denture stomatitis, demonstrating fungal invasion and neutrophil infiltration. This current oral denture model mimics human denture stomatitis and should be useful for testing the impact of gene disruption on biofilm formation, studying the impact of anti-infectives, examining the biology of mixed Candida-oral bacterial flora biofilm infections, and characterizing the host immunologic response to this disease process.

Candida spp. frequently establish biofilm lifestyles, proliferating as multicellular communities attached to surfaces, such as a medical device (21, 31). Adherent and encased in a polymeric extracellular matrix, biofilm cells are phenotypically distinct from free-floating planktonic cells. Not only are these organisms capable of proliferating in healthy hosts by surviving immune factors, they demonstrate increased resistance to common used antifungal drug therapies (1, 35, 39, 55). The majority of Candida biofilm infections are associated with placement of medical devices, such as a dentures, venous catheters, or urinary catheters (29). Treatment of these infections remains difficult due to the extraordinary drug resistance exhibited during biofilm growth. Although often costly and inconvenient, device removal is typically required for eradication of infection (48).

Oral candidiasis commonly occurs in those who are colonized with Candida and have risk factors for development of disease, such as HIV infection, chemotherapy treatment, or antibiotic exposure (63, 67). Local breaks in mucosa caused by mucositis, radiation, and trauma are also predisposing factors. Oral candidiasis may occur as an acute or chronic illness and can involve the tongue, buccal mucosa, or palate. The most common form of oral Candida infection, denture stomatitis, is prevalent in the elderly population and affects up to 70% of denture wearers (58, 66, 70). The condition begins with Candida biofilm growth on the denture-mucosa interface. Biofilm growth progresses over the denture surface, leading to inflammation of the denture-exposed palatal mucosa (54, 67). Clinical manifestations range from no symptoms to severe pain and difficulty swallowing (70). While Candida albicans is responsible for the majority of oral candidiasis (70 to 80%), infection by Candida glabrata, Candida tropicalis, and Candida parapsilosis has been reported less frequently (20, 63).

In vitro biofilm models have the advantage of lower cost and are ideal for high-throughput screening assays and antifungal testing (12, 56, 61, 71). These models have provided important phenotypic information regarding drug resistance in Candida biofilms. The models have also been valuable for characterizing the role of surface adherence properties, such as topography and hydrophobicity. However, it is difficult for in vitro models to account for all the factors which likely play a role during biofilm infection (38). For example, cells in the in vitro systems are not exposed to salivary immune components and proteins which may precondition, or coat, the surface and promote adherence. Although several of the host components which play a key role in promoting Candida adherence have been defined, the interaction is likely a complex phenomenon (42). In the denture biofilm niche, additional components hypothesized to impact this growth state include companion oral bacterial flora, contact between the device biofilm and the host mucosal surface, and salivary flow dynamics. Each of these conditions would be challenging to mimic in vitro.

Several in vivo models have been developed for study of oral candidiasis and denture stomatitis (58). The earliest studies utilizing acrylic plates fitted to the palates of primates found that Candida infection of these prostheses mimicked the histopathology of human denture stomatitis (8, 9). However, the cost and difficulty of maintaining primates prevented widespread use. An alternative model utilized rats with molded acrylic plates (47). Advantages of the rat model include reduced size and cost. The rat mucosal surface is also relatively easy to colonize with Candida, although maintenance of colonization often requires antibiotic administration (58). The rat
models have been integral for describing the basic pathologies of oral candidiasis and denture stomatitis. The investigations have primarily been directed toward the host response, and in-depth examination of the Candida biofilm phenotype has not been undertaken.

Here we adapt and test a rat model for Candida-associated denture stomatitis, with a focus on Candida biofilm formation. We chose to use a common acrylic denture material to form an oral device directly in the animal. One major advantage of the current device placement is the ability to form the denture without the costly and time-consuming ex vivo mold process. The model mimics denture stomatitis with respect to Candida biofilm formation and histology. The experimental system proved useful for testing of the impact of gene products and measurement of the response to treatment. It also demonstrated the value for study of mixed microbial biofilm interactions, comparisons with other models of biofilm niche infection, such as a venous catheter model, and examination of the host response to biofilm.

MATERIALS AND METHODS

Animals and denture maintenance. Specific-pathogen-free male Sprague-Dawley rats at weights of 350 g (Harlan Sprague-Dawley, Indianapolis, IN) were used for all studies. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Care criteria, and all studies were approved by the Institutional animal Care Committee. Rats were housed individually in an environmentally controlled room in metabolic cages to avoid oral contamination with bedding and animal waste. Animals were fed an Ensure liquid diet while dentures were in place to avoid contamination of biofilms with food particles. The animals were examined for signs of distress at 6 h after surgery and then every 12 h throughout the study. The oral mucosal area was examined twice daily for signs of inflammation or purulence.

Prior to placement of denture appliances, rats were immunosuppressed with a single dose of cortisone (200 mg/kg subcutaneously) on the day of infection to mimic a protocol commonly utilized for rodent oral candidiasis (49). For a subset of experiments, either ampicillin sodium-sulbactam sodium at 100 mg intraperitoneally (i.p.) twice a day (BID) or aztreonam at 50 mg/kg subcutaneously BID was administered 48 h prior to denture placement to reduce enteric bacterial colonization common to rodents due to coprophagy, or the consumption of feces. Dosing regimens were chosen based upon those previously shown to be effective in the treatment of rodent systemic bacterial infections (D. R. Andes, unpublished data).

Initial studies of the metabolic cages demonstrated that the rats continued to practice coprophagy. To address this, we modified a tube caging procedure that has been utilized in nutritional absorption studies (15). Specifically, an additional group of animals were housed in polyvinyl chloride (PVC) plastic tube caging in which the ability to turn and reach fecal material was eliminated. Animal monitoring during this additional housing restraint was increased to every 6 h throughout the study.

Denture placement. A 32-gauge stainless steel Babcock orthodontic wire (Miltex) was threaded across the hard palate and secured between cheek teeth of an anesthetized rat to serve as an anchor for the denture material (Fig. 1). Cheek teeth were etched using Uni-Etch 32% semiigel etchant, with benzalkonium chloride (Bisco, Inc., Schaumburg, IL) for micorentention. A metal spatula was placed over the hard palate to create an approximately 3- by 5-mm area across the palate and less than 1 mm of space between the hard palate and the denture material. This space was used for Candida inoculation. Cold-cure acrylic temporary crown and bridge material (Maxtemp HP; Henry Schein, Melville, NY) was applied over the check teeth and wire to create a surface approximately 8 by 10 mm in size and approximately 2-mm thick. Material was rapidly shaped with a small spatula to produce a rectangular denture, which was allowed to solidify for 5 min, after which the spatula was removed.

Organisms and inoculum. Candida albicans strains DAY185 and CN702 (ber1Δ/ber1Δ) were used for all studies (17, 43, 44). The strains were stored in 15% (vol/vol) glycerol stock at -80°C and maintained on yeast extract-peptone-dextrose (YPD) medium plus uridine (1% yeast extract, 2% peptone, 2% dextrose, and 80 μg/ml uridine) prior to experiments. Prior to denture inoculation, cells were grown at 30°C in YPD plus uridine liquid media with orbital shaking at 200 RPM overnight. To prepare the inoculum, cells were washed with phosphate-buffered saline (PBS), enumerated by hemocytometer counting, and resuspended in saline at 107 cells/ml. The final inoculum concentration was confirmed by microbiologic enumeration.

Infection of denture. After removal of the spatula, the hard palate beneath the acrylic device was inoculated with 107 C. albicans cells/ml (0.1 ml). Animals were sacrificed after 6, 24, 48, or 72 h for histopathology or device removal and study. For treatment studies, antifungals were applied to mature biofilms after 48 h of biofilm growth.

Fungal and bacterial qualitative and quantitative cultures from dentures. To determine the viable burden of Candida and the accompanying oral flora, microbiologic counting was performed on dentures. Devices were placed in 2 ml 0.15 M NaCl, sonicated for 10 min, and vortexed. For Candida microbiology, 1:10 dilutions were plated on Sabouraud dextrose agar (SAB) plus 2.5 μg/ml chloramphenicol. For determination of aerobic bacteria, dilutions were plated on Mueller-Hinton agar plus 2.5 μg/ml amphotericin B. For anaerobic cultures, specimens were passed into an anaerobic chamber (80% N2, 10% H2, and 10% CO2; Coy Laboratory Products, Grass Lake, MI), where dilutions were made in prereduced anaerobically sterilized PY (peptone yeast extract; Anaerobe Systems, Morgan Hill, CA) broth and placed on reduced plates of LKV (laked blood with kanamycin and vancomycin), PEA (phenylethyl alcohol agar), and EYA (egg yolk agar) that were incubated in the chamber at 35°C for 5 days (media from Remel, Lenexa, KS). Dilutions were passed out of the chamber, plated, and incubated in 5 to 7% CO2 for 1 day. Organisms were identified by Gram stain morphology and rapid testing, e.g., catalase, coagulase, PYR (pyrrolidonyl ami-nopeptidase), and streptococcal grouping (13). Experiments were performed on two or three separate occasions.

SEM. Acrylic devices were processed for scanning electron microscopy (SEM) as previously described for venous catheters (3). After removal of each denture, it was washed with phosphate-buffered saline (PBS) and placed in fixative (1% [vol/vol] glutaraldehyde and 4% [vol/vol] formaldehyde in PBS) overnight. The samples were rinsed with PBS, placed in 1% osmium tetroxide for 30 min, and rinsed with PBS. The samples were subsequently dehydrated in a series of ethanol washes (30% for 10 min, 50% for 10 min, 70% for 10 min, 95% for 10 min, and 100% for 10 min). Final desiccation was accomplished by critical-point
were obtained at 200°C. Sections were stained with either hematoxylin and eosin. After animals were sacrificed and the dentures were removed, the tongues were used for mucosal biopsy and histopathology at the end of the study period (48 h).

To discern if the model mimicked denture stomatitis, we performed mucosal biopsy and measured the following. The biofilms were imaged on an Axiovert 200 with excitation and emission filters at 490/528 nm and 490/617 nm for FUN-1 and ConA, respectively, as fluorescence within intravacuolar structures. ConA binds to glucose and mannose residues of cell wall polysaccharides and is visualized as green fluorescence. Metabolically active cells process this dye, which results in a shift to red fluorescence within intravacuolar structures. ConA binds to glucose and mannose residues of cell wall polysaccharides and is visualized as green fluorescence. Co-localization of fluorescent dyes (FUN-1 and ConA) in metabolically active cells results in a yellow to orange fluorescence during multichannel image capture.

Organisms were dislodged by scraping the surface with a plastic loop, vortexing, and sonicating. Cells were flash frozen with liquid nitrogen in AE buffer (50 mM sodium acetate at pH 5.2, 10 mM EDTA), and RNA was collected using the hot method as previously described for venous catheters (3).

The second dye set was selected for simultaneous imaging of yeast, hyphae, and bacteria within the mixed-species denture biofilm. This set included SYTO 9 dye (Molecular Probes, Eugene, OR) to visualize bacteria in the mixed biofilm, as previously described (23). Calcofluor white was selected for its ability to bind to Candida hyphal cell walls, and FUN-1 was chosen for imaging of yeast. Dentures were stained with calcofluor white (22.5 μg/ml), SYTO 9 (5 μM), and FUN-1 (50 μM) in PBS with 2% glucose for 30 min at 30°C. Both live and dead yeast cells were stained with the FUN-1 dye and visualized with a diffusely distributed yellow-green fluorescence.

The biofilm, cells were resuspended for antifungal susceptibility testing to amphotericin B, micafungin, and fluconazole using NCCLS methods (37). Comparisons were made to susceptibility testing with planktonic cells using the standard NCCLS protocol and identical inocula (10⁶ CFU/ml).

RNA collection and quantitative reverse transcription-PCR (RT-PCR). Biofilm cells were dislodged from dentures by scraping, vortexing, and sonicating. Cells were flash frozen with liquid nitrogen in AE buffer (50 mM sodium acetate at pH 5.2, 10 mM EDTA), and RNA was collected using the hot method as previously described (3, 40). The RNA integrity was assessed using an Agilent Bioanalyzer 2100 with an RNA Nano chip before use.

Quantitative real-time reverse transcription-PCR (RT-PCR). This method was used to quantify the mRNA abundances of a subset of transcripts (CDR1, CDR2, ERG25, ALS1, SAP5, GDB1, and BGL2). Transcripts for study were chosen based on differential expression in the central venous catheter biofilm model and included those involved in drug resistance, biofilm adhesion, and glucan modification (40, 45). The TaqMan probe and primer sets were designed using Primer Express (Applied Biosystems, Foster City, CA) (Table 1). The Quantitect probe RT-PCR kit (Qiagen, Valencia, CA) was used in an ABI Prism 7700 version 1.7 sequence detection system (Applied Biosystems) as previously described (3, 40). Reactions were performed in triplicate and analyzed using the threshold cycle (2⁻⁴⁻) method (34). The comparative expression method generated data as transcript fold changes normalized to a constitutive reference gene transcript (ACT1) and relative to log-phase planktonic cells grown in RPMI at 37°C on an orbital shaker set to 200 RPM.

RESULTS

Denture placement and animal well-being. Following oral device placement, the animals did not appear ill throughout the duration of the study.

*d FAM, 6-carboxyfluorescein; TAMSp, 6-carboxytetramethylrhodamine.
the course of the experiments (72 h) and continued normal intake of the food and water. The oral cavity did not appear visibly inflamed, and no purulence was noted.

**Time course experiment analysis.** Time course experiments showing viable counts from the dentures demonstrated an increasing viable burden of both *Candida* spp. and bacteria from 6 to 48 h after denture placement and inoculation (Fig. 2A). At the 6-h time point, <2 log$_{10}$ CFU/device of viable *Candida* spp. and bacteria was adherent to the device surface. By the final time point (48 h), there were approximately 6 log$_{10}$ CFU/device of viable *Candida* spp. and 8 log$_{10}$ CFU/device of viable bacteria present on each denture, based on microbiologic plate counts. Classification of bacterial organisms suggested the presence of a polymicrobial biofilm consisting of both aerobic and anaerobic organisms (Fig. 2B). The aerobic Gram-positive coccus species included viridans group *Streptococcus* spp., *Enterococcus* spp., and *Streptococcus agalactiae*. In early studies, *Escherichia coli* was a prominent species identified. We felt this was likely related to the rodent practice of coprophagy. We attempted to address enteric oral colonization in two ways. First, we administered a broad-spectrum antibiotic (ampicillin-sulbactam) prior to denture placement. Antibiotic treatment resulted in a 10-fold decrease in the number of biofilm-associated bacteria and a 100-fold increase in *C. albicans* bacteria (Fig. 2C). For the next experiment, we housed the animals so that coprophagy was not possible and administered an antibiotic agent with a narrow aerobic Gram-negative bacterial spectrum of activity, aztreonam. Both conditions were undertaken 2 days prior to denture placement, and the housing continued throughout the denture biofilm study. These conditions resulted in a >1,000-fold reduction in aerobic Gram-negative rods (3.5 log$_{10}$ CFU/device compared to 7 log$_{10}$ CFU/device) (data not shown).

**Denture biofilm imaging.** (i) SEM. We used SEM to assess biofilm formation and architecture over the 48-h period following denture placement and inoculation. The choice of the imaging time course experiment was based upon the identification of architecturally early and mature biofilms in previous studies (3, 11, 24, 33, 55, 56). Imaging of the denture demonstrated adherent yeast cells after 6 h (Fig. 3). After 24 h, a confluent layer of yeast cells had covered the denture surface, and an extracellular matrix became apparent. Over the course of 48 h, a mature biofilm composed of two fungal morphologies (yeast and hyphae) and host cells encased in extracellular matrix was visualized. In addition, bacteria were embedded in the biofilm. These bacilli and cocci were found growing in groups associated with *Candida* biofilm cells and matrix material.

(ii) Confocal microscopy. Two sets of fluorescent dyes were used to examine biofilm formation on the denture surfaces. First, imaging with the Live/Dead FUN-1 dye (red) confirmed the presence of viable yeast in a mature biofilm attached to the denture surface after 48 h (Fig. 4A). The extracellular matrix surrounding the cells was visualized using ConA carbohydrate staining and appears green in the image capture.

The second set of dyes attempted to image both the *Candida* spp. and associated bacteria simultaneously. We utilized SYTO 9 staining to image the bacteria attached to the denture and embedded in the biofilm. The utility of the combination of SYTO 9 and calcofluor white in the imaging of mixed-species biofilms has previously been demonstrated for *Candida* and *Staphylococcus aureus* biofilms (23). In addition to these dyes, we included FUN-1 to visualize live yeast cells. In the multichannel image, bacteria appear yellow, and the predominant form was coccus (Fig. 4B). The clusters were found attached to calcofluor white-stained hyphae and yeast cells, which appear blue.

**Comparison of the reference strain and the biofilm-defective mutant.** We next sought to test the ability of the model to detect the phenotype of a *Candida* strain previously shown to produce poor biofilms. We chose the bcr1Δ/bcr1Δ strain, which lacks a zinc finger transcription factor and produces minimal biofilms in vitro and in a central venous catheter in vivo biofilm model (43, 44). We hypothesized that the mutant would also exhibit a biofilm defect in the rat denture model. Compared to an otherwise isogenic reference strain, the bcr1Δ/bcr1Δ infection of the rat denture resulted in 4-fold-fewer adherent cells (bcr1Δ/bcr1Δ, 2 × 10$^5$ *Candida* CFU/device; reference strain, 8.7 × 10$^5$ *Candida* CFU/device). Interestingly, a 50%-higher burden of bacteria was found within the bcr1Δ/bcr1Δ biofilm.
(1.5 × 10^3) compared to that associated with the reference strain biofilm (1 × 10^4).

**Antifungal drug susceptibility.** Similar to reports from *in vitro* biofilm studies and the central venous catheter *in vivo* biofilm model, the triazole, fluconazole, did not effectively reduce the burden of viable *Candida* in this denture model (3, 6, 11, 28, 30, 32, 35, 53). The devices of control animals contained approximately 5.3 log_{10} CFU/device, while fluconazole treatment decreased the burden by less than 0.5 log_{10} CFU/device (to 4.5 and 4.8 log_{10} CFU/ml for topical and systemic therapies, respectively). Although previous *in vitro* and *in vivo* catheter biofilm studies have shown echinocandins to success-

![FIG. 3. Scanning electron microscopy (SEM) images of a C. albicans rat denture biofilm. (A) Rat dentures were harvested after 6, 24, or 48 h of growth, processed for SEM, and imaged. Scale bars for images at 50× and 1,000× magnification represent 600 μM and 30 μM, respectively. (B) Hyphal elements and bacteria are visualized in a denture biofilm imaged at 2,000× magnification.](image)
fully treat these infections, both systemic and topical echinocandin therapies were fairly ineffective in the denture model. Over the $5.3 \log_{10}$ CFU/device growth in control animals, treatment with 48 h of topical or systemic micafungin therapies reduced the viable *Candida* to $4.8$ and $4.7 \log_{10}$ CFU/device, respectively. The lack of effect of the systemic micafungin may be a result of reduced drug accumulation in the oral mucosa (69).

The phenotype of the biofilm-dispersed *Candida* observed was similar to that described using other biofilm models (3, 53). The suspension of biofilm cells remained viable in the presence of concentrations of amphotericin B, fluconazole, and micafungin that far exceeded the planktonic MIC (amphotericin B, 0.03 µg/ml; fluconazole, 0.5 µg/ml; micafungin, 0.015 µg/ml).

The biofilm MIC, which was defined as the concentration necessary to produce a 50% reduction in the number of viable dislodged cells, was >256 µg/ml for each compound.

**Transcriptional profile of biofilm-associated cells.** To test the ability to utilize the model for examination of *Candida* biofilm-associated gene expression, we compared the transcriptional profile of the denture-associated biofilm cells to that of free-floating planktonic cells. Over 1.5 g of total RNA was isolated from a single denture. We chose a subset of genes which we had previously found to have increased expression during biofilm formation in an *in vivo* central venous catheter biofilm model relative to planktonic growth (40). For each of
the selected genes, transcripts were more abundant for den-
ture-associated biofilm cells than for planktonic cells (Table 2).
While the venous catheter and denture expression studies
were not undertaken at the same time, it is interesting that the fold
change in expression of this subset of genes during biofilm
growth varied by as much as 10-fold between the two
in vivo
biofilm models.

**Histopathology.** H&E staining of the rat oral mucosal sur-
face demonstrated inflammation, marked by infiltration of
d polymorphonuclear cells in the keratinized and deeper layers
of the epithelium (Fig. 5). GMS staining confirmed tissue in-
vasion by *Candida* hyphae, consistent with the findings of
pseudomembranous candidiasis. Hyphae were also observed in
the superficial keratinized layer of the palate by GMS staining.
The constellation of fungal invasion and neutrophilic inflam-
mation is consistent with acute denture stomatitis (47, 58).
Unlike models with prolonged infection, epithelial atrophy was
not found (47).

**DISCUSSION**

*Candida*, one of the most common human fungal pathogens,
adopts a biofilm lifestyle, often growing attached to a prosthe-
sis or medical device (19, 22, 29). Many devices have been
demonstrated to serve as substrates for biofilm growth and
infection, including oral prostheses, catheters (venous or uri-
nary), vascular stents, pacemakers, and joint implants (29).
Advancing age and a number of pathological conditions of the
head and neck often result in a decline in oral health and the
need for prosthetic devices (70). *Candida* spp. and oral bacte-
rial flora can grow as a biofilm on the surface of an oral device,
resulting in denture stomatitis and often device malfunction.
The device biofilm and associated mucosal inflammation neg-
atively impact a patient’s ability to eat and speak. In addition,
these mixed-species biofilms harbor pathogenic bacterial or-
ganisms which commonly cause dental caries and less fre-
cently cause more severe disease, such as pneumonia or en-
docarditis (14, 60). Biofilm infections in general, including
those associated with dentures, are notoriously difficult to
eradicate due to associated resistance to both host defenses
and anti-infective therapy (12, 25, 55).

Here, we adapted a rat denture model to mimic human
*Candida* denture stomatitis, the most common form of oral
*Candida* adhesion is complex (18, 41, 42). Saliva has a

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<th>Transcript</th>
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<th>RT fold change in cells (biofilm/planktonic)</th>
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<tr>
<td>CDR1</td>
<td>Multidrug transport</td>
<td>2.5</td>
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<tr>
<td>CDR2</td>
<td>Multidrug transport</td>
<td>19</td>
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<td>BGL2</td>
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**FIG. 5.** Histopathology of rat oral mucosa in the denture stomatitis model. Rat dentures were infected with *C. albicans*. After 48 h, animals were sacrificed, and dissected samples were fixed. Sections were stained with either hematoxylin and eosin (H&E) or Gomori’s methenamine silver (GMS) for *C. albicans*. Images were obtained at 200× magnification.
physical cleansing effect and innate immune defense components, including lysozyme, histatin, lactoferrin, and IgA, which can act to decrease colonization and adherence (18, 50). However, saliva proteins, such as mucins, may also facilitate adherence by absorbing to Candida or coating the oral device. Full incorporation of the impact of the numerous and complex component makeup of saliva on biofilm formation is difficult to accomplish without use of an in vivo model.

Compared to other commonly used laboratory animals, we found several advantages to employing a rat for the denture stomatitis model. Unlike smaller animals, such as mice, rats have a larger oral cavity, easing denture placement. However, rats are much less expensive and easier to maintain than larger animals, such as primates (58). The current model has the advantage of not requiring a mold for manufacture of a preformed device (47, 58). With this technique, the device can be constructed directly in the oral cavity and has the benefit of a well-fitted apparatus. One limitation of this method of placement is a small amount of variability (less than 10%) in size among the devices.

Using this model, we found histopathologic changes to be consistent with candidiasis and acute denture stomatitis. Similar to previously described mucosal models, we observed hyphal invasion of the epithelial layer and inflammation marked by polymorphonuclear cells (47, 58). Unlike models with prolonged mucosal or device infection, epithelial atrophy was not identified, perhaps due to the relatively short duration of the current studies (47). Future investigation, including a prolonged time course experiment, may be of interest for examination of the histopathology of chronic denture stomatitis. Given the animal tolerance to the denture model, longer studies should be feasible. In addition, this model should be useful for examination of the host response to biofilm infection through histopathological comparisons of animals with mixed-species biofilm infections and those with single-species biofilm infections or by study of the response to genetically manipulated strains.

Microscopic examination of the denture surface demonstrated a C. albicans biofilm. The biofilm consisted of adherent yeast and hyphae embedded in an extracellular matrix. In addition, host cells and bacteria were identified in the biofilm. This biofilm architecture was similar to that described for a rat venous catheter biofilm (3). The main difference was the presence of a mixed microbial biofilm infection of the denture model. Confocal microscopy confirmed the presence of adherent bacteria on the surfaces of hyphae. These biofilm cells were resistant to several antifungal drug classes, a characteristic typical of C. albicans biofilm cells, described in the rat venous catheter model, and mimicking the therapeutic recalcitrance of denture stomatitis observed in patients (1, 3, 7, 16, 35, 39, 46, 55).

The current denture model provides an additional tool for examining the impact of specific gene products on biofilm formation, architecture, and drug resistance in vivo. Using this model, we were able to examine the role of Bcr1p, a transcription factor implicated in biofilm formation, and corroborated its importance in this niche as well (43, 44). A difference in the requirement of an adhesin, Als3p, for biofilm formation in vitro and in vivo has previously been demonstrated using a venous catheter model (43). Similarly, we anticipate that biofilm formation in the oral cavity, subjected to saliva and specific oral host defenses, may require a unique set of gene products. Potential features may include the requirement of gene products involved in quorum sensing, bacterial interactions, or adherence to epithelial cells. The current studies indicate the utility of the denture model for investigation of these questions.

One method of determining the gene products required for growth and proliferation in an environmental niche is through gene expression profiling. This model provides a means to examine the transcriptome of the C. albicans denture-associated biofilm cells. In current experiments, transcript abundances of a subset of biofilm-associated genes were compared directly between in vivo denture biofilm conditions and planktonic conditions (Table 2) (35, 40, 53, 59, 68). Biofilm cells from both the denture and catheter in vivo models exhibited a direction of expression similar to that of planktonic growth profiles with transcriptional abundance for each of the chosen genes. However, the magnitude of expression varied between the two models. For example, the transcript abundance of SAP5, a member of the extracellular aspartyl proteinase gene family known to function in pathogenicity by degrading tissue barriers, destroying host defense molecules, or digesting proteins for nutrient supply, was higher for the denture biofilm model (>250-fold increase versus 30-fold increase) (36, 57, 64). This finding is consistent with prior studies implicating the gene product in mucosal candidiasis pathogenicity (36, 57, 64).

Variability in gene expression between the sites likely reflects the differences in environmental factors, including temperature, nutrient availability, oxygen level, and surface adherence properties. Further exploration for potential biofilm niche similarities and differences in global gene expression may provide important pathogenesis and therapeutic insights into these difficult-to-manage infections.

Of the biofilm-associated phenotypes, the profound drug resistance characteristic of biofilm cells is of specific interest due to the difficulty in eradication of these organisms (1, 35, 39, 55). Using the current model, we were able to test the susceptibility of denture-associated C. albicans cells both in vivo and ex vivo. Similar to previous investigations, the biofilms cells were extraordinarily resistant to the triazole, fluconazole, when tested on disrupted biofilm cells or following in vivo systemic or topical treatment (2, 35, 53, 62). Unlike previous investigations, micafungin, an echinocandin, did not demonstrate significant anti-Candida biofilm activity using these methods (4, 30). The reason for this apparent discrepancy is unclear but may reflect C. albicans growth in a mixed-species biofilm at this distinct site. The model may prove to be beneficial as an additional environmental condition for testing of potential antifungal or anti-Candida biofilm drugs.

One unique feature of the Candida denture biofilm relative to most systemic biofilms is the mixed-species nature of the process in the former. Although Candida spp., including C. albicans, C. glabrata, and C. tropicalis, are predominant organisms, many types of bacterial organisms may be present. The most common bacterial organisms include Streptococcus spp., Staphylococcus spp., and anaerobes, but molecular analysis of denture biofilms obtained from patients with stomatitis identified over 50 bacterial species involved in the process (10, 52). Interestingly, the bacterial species found on the dentures of
patients with stomatitis appear distinct from those adherent to the dentures of healthy patients. The impact of bacterial microflora during development of denture stomatitis has not been fully explored, but investigations have begun to examine the interaction between Candida spp. and bacteria in mixed biofilms. Candida has been reported to form mixed biofilms with both Staphylococcus and Streptococcus during denture and catheter infection (5, 65). These bacterial species have been shown to interact with Candida and, in some instances, impact adhesion and antimicrobial susceptibility (1, 23). The current denture model provides ideal tools for examination of mixed biofilms.

The intricacies of the bacterial-fungal interaction highlight the importance of the oral microflora during denture placement. One potentially important difference in the oral cavities of rodents and humans is the higher burden of enteric flora present in the mouths of rodents due to coprophagy. We found the rat oral cavity to harbor many Gram-negative organisms, presumably linked to this behavior. To inhibit growth of these organisms and more closely mimic human denture stomatitis, we treated the animals with a broad-spectrum antibiotic prior to denture placement and housed animals in metabolic cages with wire cage floors. In a subset of experiments, animals were treated with an antibiotic specific to Gram-negative organisms and were housed in a tube which did not allow the animal to turn in place (15). These conditions greatly decreased coprophagy and the burden of Gram-negative organisms. We considered the possibility of using gnotobiotic, germ-free animals and colonizing a subset of animals with pathogens typical of human denture stomatitis. However, we currently do not have a gnotobiotic facility, and at this time, the cost would be prohibitive.

In conclusion, these studies indicate the utility of the denture model for testing the impact of gene products, studying biofilm architecture, and examining biofilm drug resistance. Furthermore, the model is potentially valuable for the investigation of mixed-species biofilms, bacterial-fungal interactions, and the impact of individual host factors, such as salivary proteins and immune components. Future investigations may include gnotobiotic animals with defined microflora to examine the impact of specific bacteria on C. albicans biofilm formation and drug resistance. To better understand the role of the host response in this unique process, studies may include animals with genetically defined immune deficiencies or with reduced salivation. Comparisons to other biofilm infection models promise to identify fungal and host elements distinct for the oral biofilm environment.

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