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Immunoproteomic Identification of *In Vivo*-Produced *Propionibacterium acnes* Proteins in a Rabbit Biofilm Infection Model

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***Propionibacterium acnes* is well-known as a human skin commensal but can also act as an invasive pathogen causing implant-associated infections. In order to resolve these types of *P. acnes* infections, the implants must be removed, due to the presence of an established biofilm that is recalcitrant to antibiotic therapy. In order to identify those *P. acnes* proteins produced *in vivo* during a biofilm infection, we established a rabbit model of implant-associated infection with this pathogen. *P. acnes* biofilms were anaerobically grown on dextran beads that were then inoculated into the left tibias of rabbits. At 4 weeks postinoculation, *P. acnes* infection was confirmed by radiograph, histology, culture, and PCR. *In vivo*-produced and immunogenic *P. acnes* proteins were detected on Western blot using serum samples from rabbits infected with *P. acnes* after these bacterial proteins were separated by two-dimensional gel electrophoresis. Those proteins that bound host antibodies were then isolated and identified by tandem mass spectrometry. Radiographs and histology demonstrated a disruption in the normal bone architecture and adherent biofilm communities in those animals with confirmed infections. A total of 24 immunogenic proteins were identified; 13 of these proteins were upregulated in both planktonic and biofilm modes, including an ABC transporter protein. We successfully adapted a rabbit model of implant-associated infection for *P. acnes* to identify *P. acnes* proteins produced during a chronic biofilm-mediated infection. Further studies are needed to evaluate the potential of these proteins for either a diagnostic test or a vaccine to prevent biofilm infections caused by *P. acnes*.**

Propionibacterium acnes is a Gram-positive facultative anaerobe that is a major colonizer and inhabitant of human skin (1) but that also causes invasive infections. Most *P. acnes* infections are chronic and include periodontitis, endodontic infections, endophthalmitis/keratitis, chronic rhinosinusitis, prostatitis, folliculitis associated with or without acne vulgaris, and implant-associated infections (2). With respect to implant-associated infections, *P. acnes* is frequently isolated from prosthetic shoulder joint infections, cerebrovascular device infections, fibrosis of breast implants, and infections of cardiovascular devices (2, 3). In all these diseases, biofilm formation is a major pathogenic strategy mediating the transition of *P. acnes* from a commensal to an opportunistic pathogen (2, 4, 5).

A biofilm is defined as a sessile community of microbial cells that (i) are attached to a substratum, interface, or each other; (ii) are embedded in a matrix of (at least partially self-produced) extracellular polymeric substances; and (iii) exhibit an altered phenotype with regard to growth, gene expression, and protein production compared to those in planktonic bacterial cells (6). The biofilm phenotype promotes the persistence of the infection, because microbes are embedded in the biofilm matrix and are recalcitrant to both antimicrobial treatment (7) and the host immune response (8). Compared to other bacterial species, there is a relative paucity of biofilm studies with *P. acnes*, since its pathogenic potential has only recently been recognized. Many putative virulence factors and strategies used against implant-associated infections caused by *P. acnes* are extrapolated from other pathogenic species, such as *Staphylococcus aureus* or *Pseudomonas aeruginosa* (2). A recent proteomic study analyzed the secretome of *P. acnes* during the planktonic mode of growth and identified several proteins that instigate the degradation of host tissue and inflammation (9). A comparison of the proteins produced in the biofilm mode of growth to those in the planktonic mode was beyond the scope of this study, but proteins produced in a biofilm mode are the critical phenotype in implant-associated infections. Biofilm bacteria presumably produce a different subset of proteins. Hence, we established a rabbit indwelling-device infection model caused by *P. acnes* to isolate serum for a biofilm immunoproteomic study of proteins that are produced *in vivo*. These proteins may be used in a vaccine formulation to prevent implant infections associated with *P. acnes* or as biomarkers for rapid diagnosis.

MATERIALS AND METHODS

Bacterial strains. All *in vitro* biofilm experiments were performed using the *P. acnes* American Type Culture Collection (ATCC) strain ATCC 11827, since it has been used in multiple biofilm studies (10, 11). For the rabbit implant model, we used a pathogenic clinical strain, *P. acnes* RMA 13884, isolated from a bone biopsy sample from a patient with spinal osteomyelitis at the University of California at Los Angeles, Los Angeles, CA. Bacterial stock cultures were stored at −80°C in 20% glycerol. The strains were grown anaerobically at 37°C from stock cultures on brain

Received 26 November 2014 **Returned for modification** 19 December 2014
Accepted 17 February 2015

Accepted manuscript posted online 18 February 2015

Citation Achermann Y, Tran B, Kang M, Harro JM, Shirtliff ME. 2015. Immunoproteomic identification of *in vivo*-produced *Propionibacterium acnes* proteins in a rabbit biofilm infection model. Clin Vaccine Immunol 22:467–476. doi:10.1128/CVI.00760-14.

Editor: H. F. Staats

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doi:10.1128/CVI.00760-14

heart infusion (BHI) agar plates (Difco) in a vinyl anaerobic gas chamber (Coy Laboratory Products, Grass Lake, MI).

Visualization of *P. acnes* ATCC 11827 and RMA 13884 *in vitro* biofilms on glass beads using scanning electron microscopy. We anaerobically incubated the *P. acnes* strains ATCC 11827 and RMA 13884 with soda-lime solid-glass beads (Walter Stern, Inc., Port Washington, NY) for 2 days at 37°C under static conditions. After isolating the glass beads with the attached biofilm, we fixed the biofilm by incubating the beads in 100% ethanol for 10 min, washed them with distilled water (dH_2O), and let the beads dry prior to performing scanning electron microscopy (SEM). SEM was performed using a Zeiss Supra 55VP field emission scanning electron microscope with a beam accelerating voltage of 1 kV and a working distance of 3 mm.

Multilocus sequence typing. We characterized both *P. acnes* strains with the expanded multilocus sequence typing (MLST) ($MLST_8$ genotyping) described in a study by McDowell et al. (12) (http://pubmlst.org/pacnes/) to identify six core housekeeping genes (*aroE*, *atpD*, *guaA*, *lepA*, *sodA*, and *gmk*) and two putative virulence genes (*camp2* and *tly*). Gene fragments for the eight genes were amplified from purified *P. acnes* DNA, as previously described and sequenced (12–15).

Implant-associated infection model in rabbits. We used a rabbit tibial implant infection model that was adapted from Shirtliff et al. (16) and Mader and Shirtliff (17) for other bacterial species, including *Streptococcus pyogenes* (18), *Staphylococcus epidermidis*, and *Acinetobacter baumannii* (our unpublished data).

The inoculum was prepared by inoculating 10 ml of BHI broth (Difco) grown on BHI plates and incubating the culture for 5 days in the anaerobic gas chamber, subculturing into 10 ml of fresh BHI medium containing 10 mg/ml dextran beads (Cytodex microcarrier beads; Sigma), which represents the foreign implantable material, and incubating for an additional 5 days.

New Zealand White female rabbits (Charles Rivers Laboratory, Wilmington, MA), 8 weeks of age and weighing 1.5 to 2.0 kg, were anesthetized, and a bacterial infection was introduced according to a previously described method (18). In short, an 18-gauge needle was inserted into the intramedullary cavity of the left tibial metaphysis, and then 0.1 ml of a 5% (wt/vol) solution of morrhuate sodium (Eli Lilly, Indianapolis, IN), 0.1 ml of the *P. acnes* mixture (inoculum described above), and 0.2 ml of sterile saline were sequentially injected. The animals were returned to their cage, and serum samples were collected prior to infection and 28 days postinfection.

After 4 weeks, radiographs were obtained for both tibias, and the animals were sacrificed. Both the infected left and uninfected right tibias were removed, dissected so as to be free of all soft tissue, and processed for bacterial culture and histology. The right uninfected tibia was the negative control for all diagnostic methods. A small representative bone sample of the infected tibia was fixed in 4% paraformaldehyde for further imaging. The remaining bone fragments were suspended in 3 ml of sterile phosphate-buffered saline (PBS) per g of tissue and vortexed for 5 min. All samples were serially diluted in PBS, plated on BHI agar, and incubated in the anaerobic chamber at 37°C for 10 days for bacterial count determination. All samples were also plated on tryptic soy agar (Fluka Analytical) and incubated aerobically to exclude contamination. The limit of detection with this culture method was 10 CFU/g of bone. In clinical cases of *P. acnes* infection, cultures can often be negative even when histology shows inflammation and PCR results are clearly positive for *P. acnes*. This negative culture can be the result of inadequate sonication to liberate and culture the biofilm bacteria or escape detection through intracellular persistence at upwards of 8 months (19–21). Therefore, *P. acnes*-specific 16S rRNA gene PCR was performed on all samples as a secondary method to detect *P. acnes* in the tibial samples. In short, bacterial genomic DNA was extracted by boiling 1 ml of the tibial bone suspension (including bone marrow) for 10 min following several washes with PBS. The suspension was centrifuged at 8,000 rpm for 10 min. The *P. acnes*-specific 16S rRNA gene was amplified by using the specific primers PA-F (5′-GGGTTGTAA ACCGCTTTCGCCT-3′) and PA-R (5′-GGCACACCCATCTCTGAGCA C-3′), and the conditions were as previously published (22). All PCR products were analyzed by electrophoresis.

Imaging of tibias. The tibia fragments were formalin fixed, decalcified in EDTA, paraffin embedded, and sectioned. The tissue sections were stained with hematoxylin and eosin (H&E) to identify the infiltrating immune cells. An acute infection was defined by the predominance of polymorphonuclear neutrophils in the infiltrate, and chronic osteomyelitis was defined by the predominance of plasma cells, lymphocytes, and macrophages in the infiltrate, dead bone (necrosis), or bone sequester, and fibrosis of the remaining tissue (23). We classified an infection as subacute if the criteria for both the acute and chronic osteomyelitis stages were fulfilled. Gram staining (modified Brown-Brenn staining) was performed to visualize the bacteria. Based on the Gram stain findings, bacterial morphology was examined using the propidium iodide reagent from the LIVE/DEAD BacLight viability kit (Invitrogen, Carlsbad, CA) on adjacent tissue sections, according to the manufacturer's protocol. Since fixation in formalin kills most bacteria, we used only the BacLight stain that is able to penetrate dead cells (propidium iodide) to visualize the cells within the biofilm. The slides were examined with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY).

Ethics statement. All animal procedures were approved and performed under the supervision of the Institutional Care and Animal Use Committee at the University of Maryland, Baltimore, MD.

Growth conditions for *in vitro* planktonic and biofilm samples. *P. acnes* strain ATCC 11827 was plated from the frozen stock onto BHI agar plates (Difco) and grown at 37°C under anaerobic conditions for 3 to 5 days. In order to prevent aberrant clonal isolate results that may be obtained by utilizing a single CFU, starter cultures were prepared by inoculating 10 ml of fresh BHI broth (Difco) with 8 to 10 single colonies of *P. acnes* (strain ATCC 11827) and incubated for 3 days at 37°C under anaerobic conditions.

Planktonic cultures were prepared by diluting the starter culture 1:100 in 50 ml of BHI and incubating for 3 days at 37°C under anaerobic conditions. On day 3, the planktonic cultures were centrifuged and resuspended in 500 μl of protein preservation solution (PBS supplemented with 0.02% sodium azide and cOmplete EDTA-free protease inhibitor tablets [Roche, Basel, Switzerland]). The bacterial suspensions were stored at −80°C until protein extraction.

In contrast, *P. acnes* biofilm samples were generated using 0.8-mm soda-lime solid-glass beads (Walter Stern, Inc., NY) as an abiotic surface for biofilm growth. The biofilm cultures were generated by diluting the starter culture 1:100 into 100 ml of fresh BHI containing sterile glass beads and incubating the cultures at 37°C under anaerobic conditions. The biofilm samples were harvested at various time points postinoculation for early (day 3), maturing (day 5), and fully mature (day 21) biofilms, in which fully mature biofilms showed no further increase in depth. To isolate the biofilm sample, the bacterial culture was drained out, and the glass beads were washed once with BHI broth. The glass beads were immersed in 30 ml of protein preservation solution and homogenized at 25,000 rpm for 4 min with a Polytron PT1200 homogenizer (Kinematica, Lucerne, Switzerland) to remove the biofilm from the surface of the beads. Similar to the planktonic cultures, the biofilm samples were concentrated in 500 μl of protein preservation solution and stored at −80°C. All samplings were performed in triplicate.

Preparation of membrane- and cell wall-associated protein extracts. After thawing the frozen bacterial sample(s), 0.1-mm silica beads (BioSpec, Bartlesville, OK) were added to the cell suspension(s), and the cells were mechanically disrupted using a FastPrep instrument (Qbiogene, Irvine, CA) at 4 m/s for 45 s and then incubated on ice for 1 min. This cycle was repeated four more times. The bacterial lysates were centrifuged at 2,000 × *g* for 10 min at 4°C to remove large cell debris and beads. The supernatant was then separated into pellet (representing cell wall- and

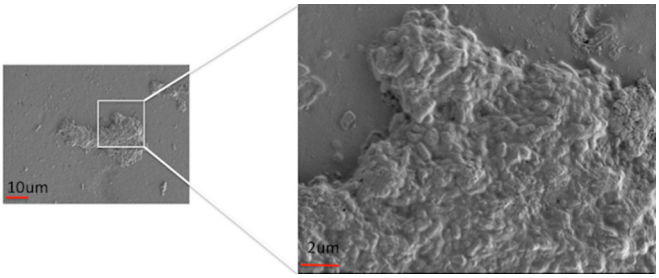

FIG 1 Scanning electron micrograph (SEM) of the *P. acnes* strain ATCC 11827 biofilm on solid glass beads (Walter Stern, Inc., Port Washington, NY) incubated for 2 days anaerobically at 37°C under static conditions (magnification, 2,000× [left panel] and 20,000× [right panel]), using a Zeiss Supra 55VP field emission scanning electron microscope with a beam accelerating voltage of 1 kV and a working distance of 3 mm.

TABLE 1 Characteristics of infected rabbits with *P. acnes* with two different bacterial inocula

Measurement	Characteristics for rabbit:			
	1	2	3	4
Infective dose (total CFU)	6×10^6	6×10^6	2×10^8	2×10^8
Macroscopic[a]	1+	1+	2+	2+
X ray[b]	1+	1+	3+	3+
CFU/g of bone	No growth	No growth	4×10^7	No growth
PCR specific for *P. acnes*	Negative	Negative	Positive	Positive
Histology[c]	Acute	Normal	Subacute	Chronic

[a] Clinical criteria per eye: 0, normal; 1+, no bone involvement, soft tissue swelling at proximal tibial metaphysis; 2+, soft tissue abscess, <15% widening of proximal tibial metaphysis; 3+, >15% widening of proximal metaphysis.
[b] Radiographic severity score: 0, normal; 1+, lytic changes around the needle stick, <5% disruption of the normal bone architecture; 2+, 5 to 15% disruption of normal bone architecture; 3+, 15 to 40% disruption of normal bone architecture; 4+, >40% disruption of normal bone architecture.
[c] Acute osteomyelitis was defined by a predominance of polymorphonuclear neutrophils in the infiltrate; chronic osteomyelitis was defined as an infection with predominance of plasma cells, lymphocytes, and macrophages, dead bone (necrosis) or bone sequester, and fibrosis of the remaining tissue (23). We classified an infection as subacute if the criteria from acute and chronic were fulfilled.

membrane-associated proteins) and cytosol (supernatant) fractions by centrifuging the sample at 15,000 × *g* and 4°C for 30 min (24). The pelleted fraction with the cell wall proteins from the planktonic or biofilm sample was resuspended, and the protein concentration was determined (25) using the Advanced protein assay reagent (Cytoskeleton, Inc., Denver, CO). Fifty-microgram aliquots of each protein sample were cleaned up and concentrated by tricarboxylic acid (TCA) precipitation. Subsequently, the 50-μg protein samples were used for the two-dimensional electrophoresis described below. The remainder of the supernatant was frozen at −80°C for future use.

Two-dimensional gel electrophoresis to visualize protein expression in the planktonic and biofilm phase and immunogenic protein identification. Cell wall- and membrane-associated proteins were separated by their intrinsic charge and size using two-dimensional gel electrophoresis (2DGE), completed according to the principles of O'Farrell (26) and Brady et al. (27). After electrophoresis, the gels were fixed and stained with Sypro Ruby stain. Immunoproteomic studies/Western blot analysis to detect immunogens produced in the planktonic and biofilm modes of growth were conducted, as described previously, using serial serum samples from infected rabbits (27). The blots from three biofilm and planktonic protein samples were also performed with naive rabbit serum isolated prior to challenge to exclude the possibility that the rabbit already had circulating antibodies against *P. acnes* or antibodies that were cross-reactive with *P. acnes* proteins.

The blots were performed in triplicate for the planktonic samples isolated at one time point and the biofilm samples at different maturation stages and compared. Immunoreactive spots detected on two out of three Western blots were selected and aligned with the proteins on the corresponding 2DGE gel. These spots were excised and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), as described previously (27).

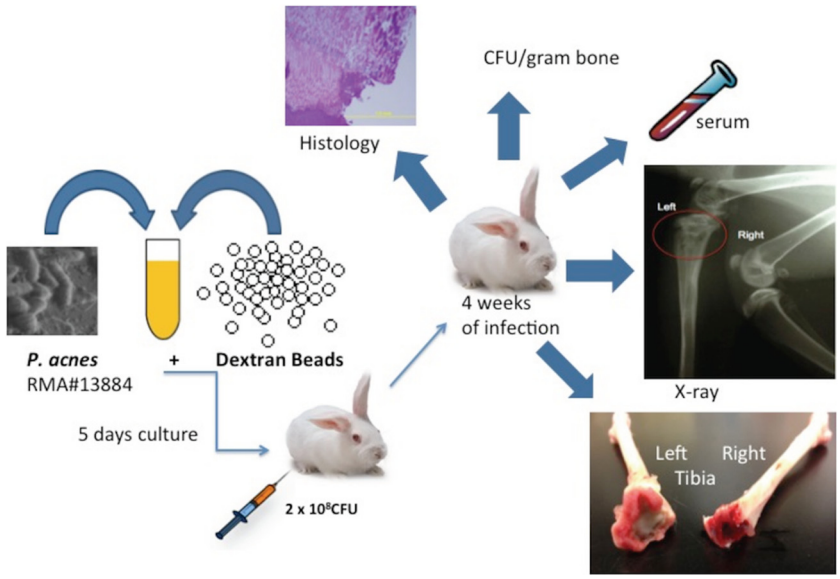

FIG 2 Schematic flow chart of the implant-associated osteomyelitis model in rabbits. After a 5-day incubation of the *P. acnes* strain RMA 13884 with dextran beads, rabbits were infected with a *P. acnes* inoculum in the left tibia. After 4 weeks of infection, blood serum was isolated, and X rays of both tibias were performed. The tibias were macroscopically and histologically examined, and the bacterial loads were quantified and reported as CFU per g of bone.

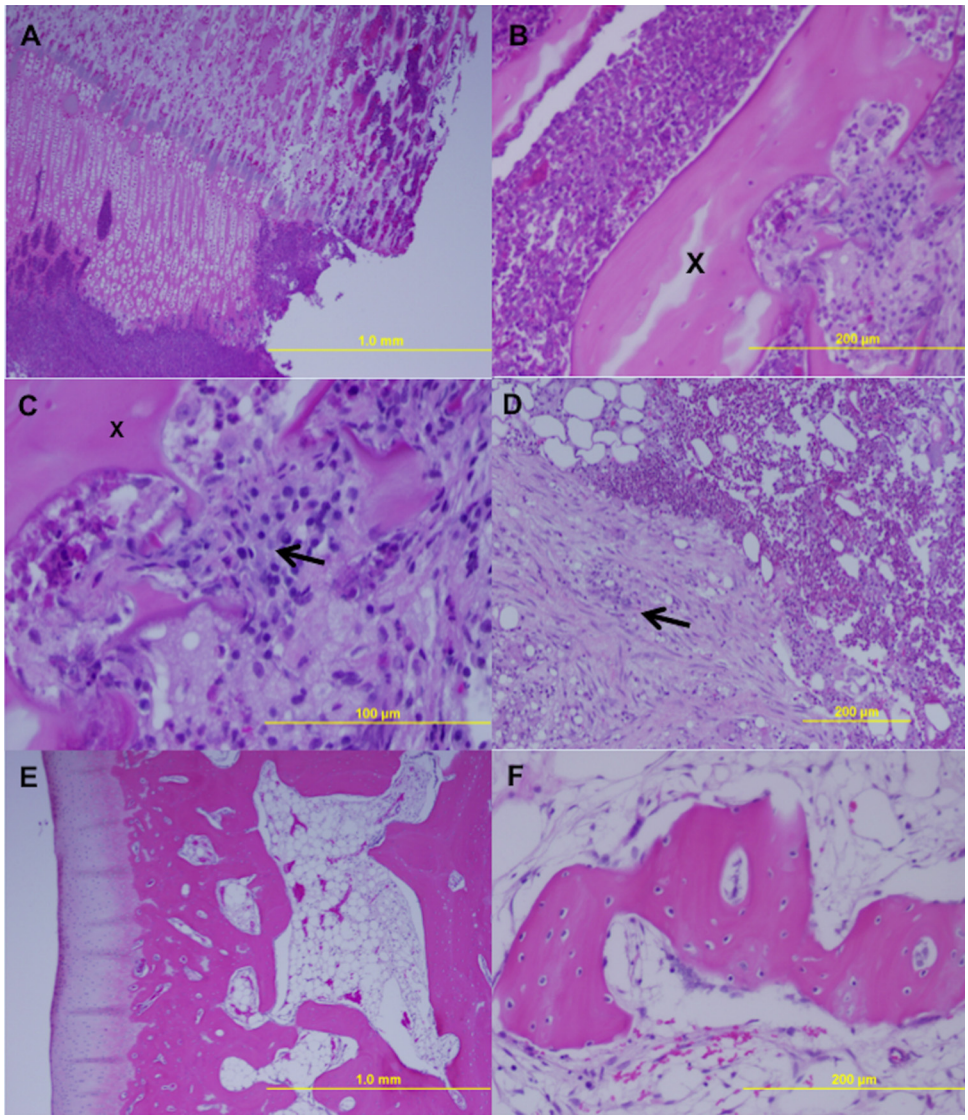

FIG 3 H&E-stained histologic sections from infected rabbit 3 (A to C) and rabbit 4 (D) showing subacute (rabbit 3) and more chronic (rabbit 4) osteomyelitis after challenge with *P. acnes* 2×10^8 CFU (classification adapted from that of Gaida et al. [23]). (A) Overview of inflammation of bone (cortex and medulla) and cartilage, with migration into the knee joint (4× magnification). (B and C) Inflammation with granulocytes with bone sequester (X) and osteoclasts (arrow) (magnification, 20× [B] and 40× [C]). (D) Chronic inflammation with fibrosis (arrow) of the medulla (10× magnification). (E and F) Noninfected tibia with an overview of the cartilage, bone cortex, and medulla as a control (magnification, 4× [E] and 40× [F]).

RESULTS

Strain characterization. *P. acnes* ATCC 11827 used for the *in vitro* planktonic and biofilm sampling was characterized with the expanded MLST (13, 14, 28) as belonging to sequence type 103 (ST103) and clonal complex 1 (CC1) (lineage type IA1), and the clinical strain RMA 13884 used for the *in vivo P. acnes* infections was characterized as ST105 (IA1). This ST was previously found to link CC1 and CC4 into a much larger CC, with ST1 as the founder genotype and ST4 as the subfounder. We utilized both strains in this study, since antigen discovery using the serum infected by one strain of *P. acnes* being used to probe antigens produced by another well-characterized strain would strengthen our results of finding common immunogenic *P. acnes* proteins.

Since a recent study indicated that hemolytic *P. acnes* strains are more virulent than are nonhemolytic strains (29), we examined the hemolytic phenotypes of both our strains using Columbia agar with 5% sheep blood, and both strains were found to possess *in vitro* hemolytic activity. In addition, the two *P. acnes* strains demonstrated no relevant differences in protein expression in an investigation of the banding patterns at identical time points for multiple phases of growth (data not shown).

Biofilm formation confirmed by scanning electron microscopy. SEM studies confirmed biofilm formation in both *P. acnes* strains (ATCC 11827 and RMA 13884) incubated with soda-lime solid-glass beads for 2 days at 37°C under static conditions. Figure 1 shows a young *P. acnes* (ATCC 11827 strain) biofilm with embedded cells within an exopolymeric matrix that appears similar to the glycocalyx of the biofilms of staphylococci.

Implant-associated infection model in rabbits. We established a novel implant-associated infection model in rabbits to

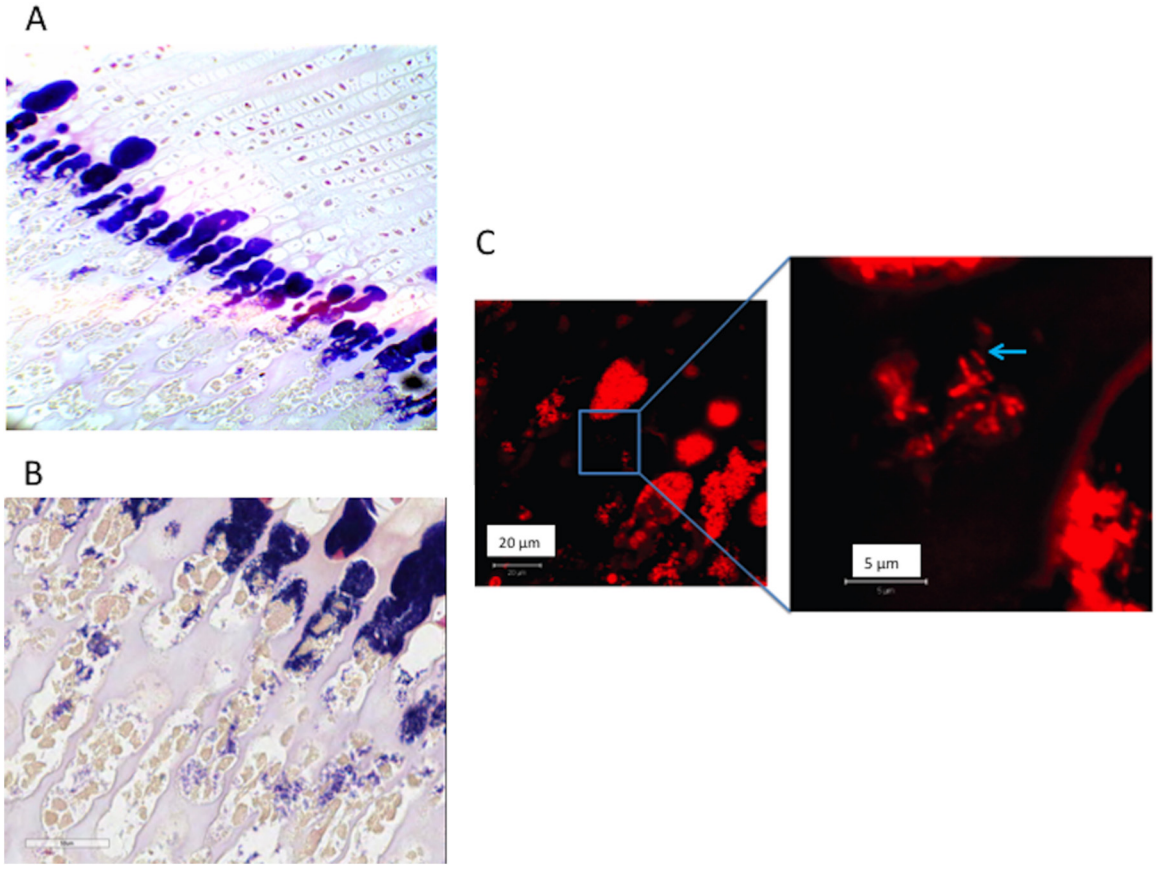

FIG 4 Formalin-fixed histology sections of osteomyelitis in rabbit 3 with positive Gram staining (blue) at the transition cartilage to bone (magnification, 20× [A] and 40× [B]) and confirmation of the rod shape of *P. acnes* (blue arrow) visualized with propidium iodide (LIVE/DEAD BacLight system) using confocal microscopy (C).

evaluate a *P. acnes*-mediated chronic infection and protein expression *in vivo*. We used rabbits because of their relative reproducibility and ease of diagnosis through presacrifice radiographs. In addition, this model has similar clinical and physiological properties of infection to those in humans and provides for ample serum samples to be obtained for subsequent Western blot (17). Based on previous literature with this rabbit model (17, 18), we chose 2 different infection doses. The rabbits infected with 6×10^6 CFU had slight macroscopic and radiological changes, but the tibial samples tested negative upon anaerobic culture and 16S rRNA PCR for *P. acnes*. Therefore, we surmised that this lower dose could be cleared by the host immune response in this model. In comparison, the macroscopic evaluation of the left tibia from rabbits infected with 2×10^8 CFU showed inflammation, periosteal elevation, and bone thickening (Fig. 2 and Table 1). Subsequent evaluation of the tibial samples from those rabbits confirmed *P. acnes* infection by bacterial culture and/or PCR testing. Histological sections of these infected rabbits showed either subacute or chronic osteomyelitis (histological classification adapted from that of Gaida et al. [23], defined in Materials and Methods), confirming the successful establishment of a chronic infection (Fig. 3). We further confirmed the presence of *P. acnes* and rod-shaped bacteria in histological sections with Gram stain and propidium iodide staining, respectively (Fig. 4).

Identification of immunogenic proteins in *P. acnes* biofilm and/or planktonic growth phases. We separated *P. acnes* proteins into cell wall/membrane and cytosol protein fractions. In order to find those proteins that would serve as a diagnostic or potential vaccine candidate, we targeted the cell wall/membrane protein fraction for further analysis with the Western blot technique using the immune serum from a culture-confirmed tibial infection in a rabbit (four weeks postchallenge). Immunogenic protein spots appearing on at least two blots were excised and identified by MALDI-TOF tandem mass spectrometry (MS/MS). We found 24 proteins that were immunogenic in either planktonic, biofilm, or both modes of growth. Table 2 lists the protein identities and provides the molecular mass, isoelectric point, protein function, and expression pattern (biofilm, planktonic, or both growth modes) of each immunogen. Representative sets of both the 2DGE gel and Western blot for a late-stage biofilm (21 days) and a planktonic sample (3 days) are shown in Fig. 5.

DISCUSSION

P. acnes has not historically been associated with indwelling medical device infections, since cultures would often require incubation periods of up to 13 days (30). In addition, the presence of *P. acnes* in a biopsy specimen has often been interpreted as a contaminant in many clinical laboratories. However, improved pre-

TABLE 2 Identities and characteristics of immunogenic antigens of *P. acnes*, listed by expression profile and molecular mass

Protein no.	Full name of protein[a]	Protein name[b]	Best protein accession no.[a]	Function[a]	pI[c]	Molecular mass (kDa)	Expression (biofilm, planktonic, or both)
1	Methylmalonyl-CoA mutase	MutA	E4DBY8	Lactate fermentation to propionate and acetate	4.698	69.111	Both
2	Chaperone protein DnaK	DnaK	W4TZS5	Heat shock protein, ATP binding	4.476	59.03	Both
3	60-kDa chaperonin 1	GroL1	P0CY97	Heat shock protein, protein misfolding, ATP binding	4.613	56.840	Both
4	Pyruvate kinase	PykA	G5EZ02	Glycolysis	5.01	51.091	Both
5	Phosphoglycerate kinase	Pgk1	Q6A9J3	Carbohydrate degradation, glycolysis	4.752	42.218	Both
6	DivIVA domain protein	DivIVA	E4D8W2	Cell division	5.016	40,124	Both
7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	G7U8Y4	Glucose metabolic process	5.485	35.95	Both
8	Malate dehydrogenase	Mdh	Q6A6Z5	TCA cycle	4.831	34.686	Both
9	Carbamate kinase	ArcC	D4HCA4	Arginine metabolism	4.641	31.977	Both
10	Translation elongation factor	Tef	W4TZY5	Protein biosynthesis	4.599	29,648	Both
11	ABC transporter, ATP-binding protein	Abc1	D4HAH2	ATB binding	6.18	26.002	Both
12	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	BgmA	Q6AAU8	Glycolysis	5.981	27.991	Both
13	Methylmalonyl-CoA epimerase	Mce	D4HDR2	Amino acid metabolism, isomerase	5.593	16.84	Both
14	Translation elongation factor G, partial	FusA	F9NNW3	Protein biosynthesis	4.789	76.605	Biofilm
15	Succinate dehydrogenase or fumarate reductase	SdhA	E4E2R8	Oxidoreductase in carbohydrate metabolism, TCA cycle	5.898	75.667	Biofilm
16	Oxaloacetate decarboxylase	OxdC	G7U7Q6	Glycolysis, oxaloacetate metabolism	5.23	54,690	Biofilm
17	Putative tryptophan 2,3-dioxygenase	Ptd1	E4D811	Aromatic amino acid family metabolic process	5.011	50.782	Biofilm
18	Fumarate hydratase class II	FumC	G7U975	TCA cycle, fumarate metabolic process	5.512	50.689	Biofilm
19	Elongation factor	Tuf	Q6A6L7	Protein biosynthesis	5.214	44.14	Biofilm
20	RecA protein	RecA	Q5IV56	DNA repair	5.258	37.153	Biofilm
21	Fructose-biphosphate aldolase class II	FbaA	E4D532	TCA cycle, fumarate metabolic process	5.037	36,771	Biofilm
22	Succinyl-CoA ligase	SucD	E4D6H5	Ligase (e.g., ATP binding)	5.708	30.566	Biofilm
23	Enolase	Eno	Q6AAB8	Glycolysis (cell attachment)	4.418	45.53	Planktonic
24	Pyridoxal 5′-phosphate lyase	PdxS	G8V9H3	Pyridoxal phosphate biosynthetic process (vitamin B_6)	4.788	31.530	Planktonic

[a] Identification and function of the proteins were obtained from the UniProt database of *P. acnes* complete proteome (ID 1.81) (www.uniprot.org). CoA, coenzyme A; TCA cycle, tricarboxylic acid cycle.
[b] The protein names were used for labeling the protein spots in Fig. 5.
[c] Average values derived from isoelectric point calculator (http://isoelectric.ovh.org).

paratory procedures, including the sonication of explanted implants and tissue biopsy specimens for bacterial culture, as well as molecular methods, such as specific PCR, have increased the diagnosis rates for *P. acnes* (2, 31). Even with these advances, diagnosis during early onset of the infection is still difficult, because patient complaints of an infected shoulder arthroplasty are limited to pain and stiffness only (32), thereby allowing the infection to develop into a mature biofilm infection. These types of infections are recalcitrant to antibiotics alone and require extensive surgical debridement combined with antibiotic therapy for infection resolution. In the present study, we successfully developed a rabbit model for chronic *P. acnes* indwelling medical device infections and generated convalescent-phase serum for further immunoproteomic studies. In the subsequent immunoproteomic analyses of planktonic- and biofilm-derived samples, we identified a total of 23 immunogenic cell wall proteins and found that 13 proteins were upregulated in both the planktonic and biofilm modes of growth.

Current animal models for *P. acnes* bone infections associated with an implant are limited to a hematogenous infection model of total knee arthroplasties (33), an implant-associated osteomyelitis model in rabbits (34), and a bone implant model in BALB/c mice (35). These studies examined the capability of *P. acnes* to establish chronic infections but did not evaluate the *in vivo* protein expression of *P. acnes* and/or the host humoral response. We chose to develop a comparatively easy and representative model of musculoskeletal infection and used this model to examine the protein expression of *P. acnes* during a chronic infection. Our model was a rabbit osteomyelitis model established for *S. aureus* by Mader and Shirtliff (17) and was successfully adapted for *A. baumannii* (our unpublished data) and *S. pyogenes* (18) by using an intramedullary injection of *P. acnes*-infected dextran beads to provide an abiotic surface for colonization and biofilm growth.

While other bacterial species (e.g., *S. aureus* [17]) require a low bacterial inoculum, 10^8 CFU was needed for *P. acnes* to establish a chronic infection, most likely due to the reduced virulence associated with this bacterial species and the resulting indolent infection that it produces. This observation is also similar to the findings of the two nonhematogenous studies that required a high inoculum of 10^7 to 10^8 to achieve chronic infection (34, 35).

The clinical strain RMA 13884 in this study was chosen since (i) it was relevant and recently isolated from a clinical case of spinal osteomyelitis with hardware, and (ii) it matched the 1A lineage with the *P. acnes* laboratory strain from the ATCC that has

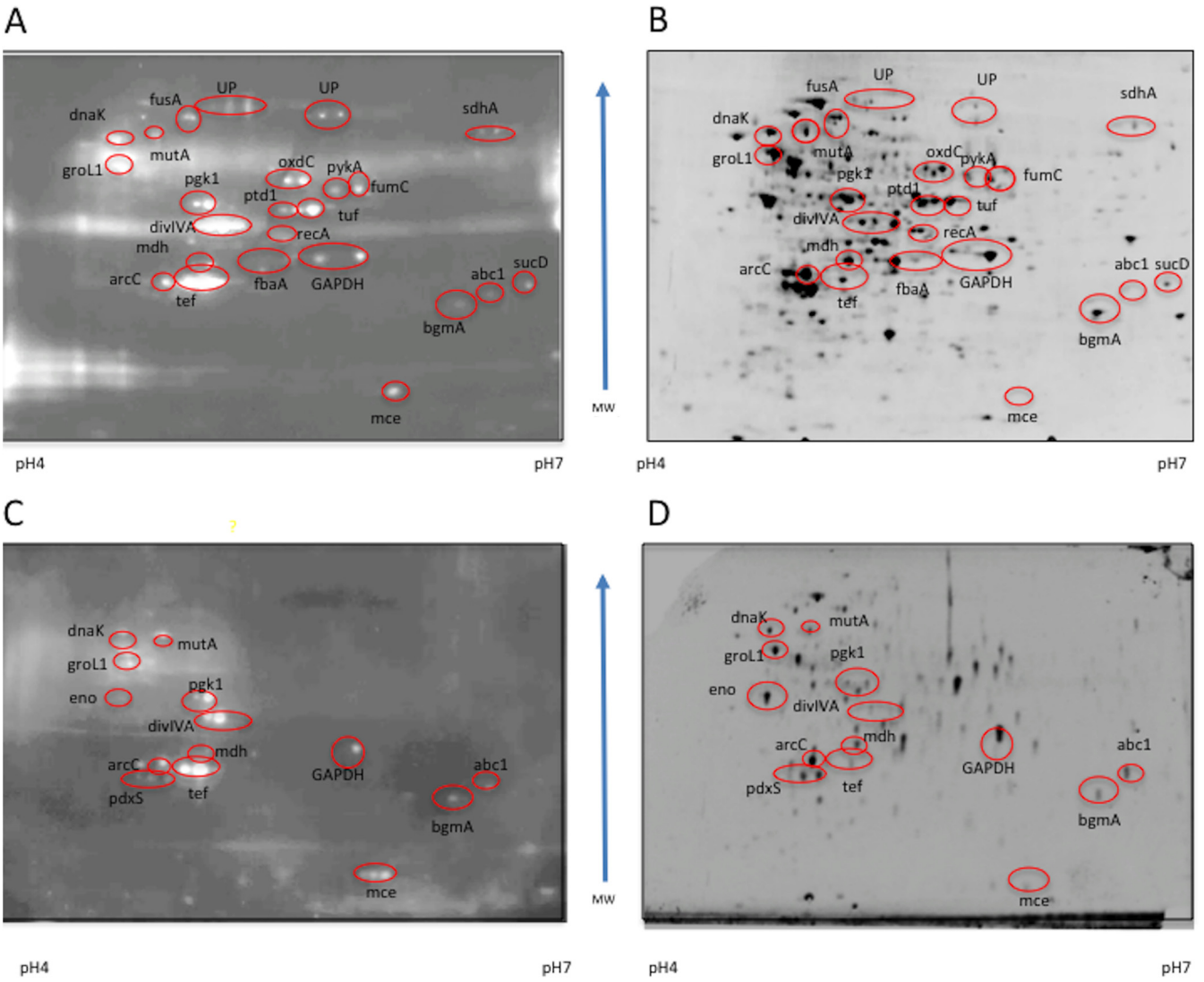

FIG 5 Western blot (A and C) and corresponding 2DGE gels (B and D) with identification of immunogenic proteins of *P. acnes* in a biofilm of 21 days (A and B) and in a planktonic phase of 3 days (C and D). For the Western blot, rabbit serum after 4 weeks of infection was used to identify immunogenic proteins. pH levels are indicated at the bottom and the molecular weights (MW) of the proteins (in thousands) in the middle of the panels. These protein spots were visually identified on Sypro-stained gels and excised for MALDI-TOF MS analysis. The abbreviated protein names are explained in Table 2. Note that some spots listed in Table 2 are not present in these figures, as not all spots were visible in every sample at every time point. UP, uncharacterized protein.

been used in multiple *in vitro* studies (10, 11). Although some *P. acnes* strains are more frequently associated with implant-associated infections (e.g., 1B isolates) (12, 36, 37), the 1A lineage types used in this study are also capable of causing these chronic infections, as shown by the isolation of a 1A lineage strain from an infected patient and its ability to induce a chronic infection in this study. Future studies will include a comparison of the infectious doses between the arrays of lineages but that were beyond the scope of the present study.

Most of the immunogenic proteins identified in this study (Table 2) are known to have a metabolic function in bacteria. This phenomenon of proteins with metabolic functions having antigenic potential has been observed in other immunoproteomic studies with *P. acnes* (9, 38) and other Gram-positive pathogenic strains, such as *S. aureus* (27) and *S. pyogenes* (18). We focused our immunoproteomic analysis on cell wall- and membrane-associated proteins of *P. acnes*, since previous studies have shown that these proteins may be able to be used as novel diagnostic biomarkers or vaccine candidates (39). Our study might exclude secreted and/or cytosolic proteins (9) that are also important for biofilm pathogenesis and for the development of vaccine candidates. One protein of particular interest to our laboratory was an ABC transporter protein (accession no. D4HAH2, Table 2). We previously identified an ABC transporter in *S. aureus* that was highly immunogenic in infected rabbits (27, 39). Furthermore, patients with wound infections, bacteremia and sepsis, pneumonia, arthritis, urinary tract infections, catheter-related bloodstream infections, and peritonitis due to *S. aureus* have a similar response, with high IgG levels against the ABC transporter (40, 41).

The secretome for planktonic *P. acnes* was already published by Holland et al. (9). This study found that secreted proteins, such as glycoside hydrolases, esterases, and proteases, were mainly in-

volved in degradation, but it also identified unique proteins, such as Christie-Atkins-Munch-Petersen (CAMP) factors, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypothetical proteins. Our immunoproteomic study identified GAPDH as an immunogenic protein as well, which suggests that GAPDH is associated with the cell wall, in addition to being secreted into the extracellular milieu. This observation confirms the findings in studies of other Gram-positive bacteria, such as streptococci (18, 42); in these, GAPDH is described as a multifunctional protein, which is anchorless, found on the surface of many Gram-positive pathogens, and it contributes to adhesion and virulence. Based on its ability to perform dual or multiple mechanistically distinct functions, it belongs to a group of moonlighting proteins (43). We found other moonlighting proteins in the cell wall fraction, such as elongation factors, 10-kDa chaperonin, and enzymes involved in glycolysis, pyruvate metabolism, and the tricarboxylic acid cycle (Table 2).

Of note, we found the protein enolase to be upregulated in all stages of *in vitro* planktonic and biofilm growth, but we did not detect immunoreactive spots corresponding to this protein on the biofilm blots, which has to be interpreted as a false-negative result. The enolase protein converts 2-phosphoglycerate to phosphoenolpyruvate during glycolysis but might also mediate bacterial attachment to the abiotic and biotic surfaces. In *Streptococcus pneumoniae*, the protein enolase serves as a pneumococcal plasminogen receptor as a surface-displaced protein and is therefore a key factor in attachment (44). To date, there are no studies describing the function of enolase in *P. acnes*.

The results of our immunoproteomic study are robust, since we focused only on immunoreactive spots and identified the proteins that were seen in at least two of three 2DGE gels. Since the protein concentration of samples excised from the 2DGE gel significantly influences the quality of MALDI spectra (45), we were not able to identify low-abundance protein spots (labeled as unknown proteins [UP] in Fig. 5). The *P. acnes* strains we used were not sequenced yet; therefore, some of the unidentified proteins by MALDI spectra might also be strain specific. Another limitation of our experimental design is the use of rabbit instead of human serum to investigate the humoral immune response. Differential protein expression of *P. acnes* in a host or differences in the immune responses might influence whether a vaccine candidate in one host will have efficacy against *P. acnes* in a second host species. However, an ethical agreement for the use of human serum is very strict, and the Helsinki Declaration demands preliminary results to be determined in animal studies first (46), which we present in this study. Therefore, further studies with human serum are now possible to conduct in order to verify our study results. Another limitation of the study is that the number of rabbits used was relatively low, and only two isolates of a single clonal type of *P. acnes* were used. Although this study demonstrates that the rabbit osteomyelitis model can be used to study *P. acnes* indwelling medical device infections, care must be taken to draw conclusions until an expanded study using more animals and strains can be performed.

The rates of indwelling medical device infections associated with *P. acnes* have long been underestimated. Due to the problematic diagnosis and indolent nature of these infections, they often progress to a full mature biofilm infection that relies on surgical intervention for resolution. Therefore, the early diagnosis and/or prevention of these infections will minimize surgical procedures and improve clinical outcomes. In addition, while a number of studies have evaluated the potential for active and passive vaccines to prevent the chronic skin disease acne vulgaris, which is also caused by *P. acnes* (47–49), no subsequent studies have evaluated the efficacy of this vaccine(s) against implant-associated infections. To identify effective vaccine candidates for *P. acnes* implant-associated infections, it is necessary to find key *P. acnes* virulence factors and/or proteins that are produced *in vivo*, recognized by the host immune system, and immunogenic. In addition, both planktonic and biofilm immunogenic cell wall proteins have to be taken into account, since targeting both phenotypes might be crucial to the generation of an effective vaccine against *P. acnes*-mediated implant infections.

Taken together, we found 13 upregulated proteins of *P. acnes* mainly associated with metabolic functions in both planktonic and biofilm growth stages, which need to be further analyzed as vaccine candidates within immunization protection studies or as biomarkers in diagnostic tests for implant-associated infections.

ACKNOWLEDGMENTS

We thank Ellie J. C. Goldstein for providing the *P. acnes* clinical strains used in this study and A. McDowell for helping with the MLST identification.

We have no commercial or other association that might pose a conflict of interest.

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health grant (R01 AI69568-01A2), a 3-year fellowship grant by the Swiss National Science Foundation (SNF) (Switzerland) (grant PBZHP3_141483), and a grant from the Swiss Foundation for Medical-Biological Grants (SSMBS) (Switzerland) (grant P3MP3_148362/1).

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