Genetic Manipulation of *Corynebacterium mastitidis* to Better Understand the Ocular Microbiome

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PURPOSE. *Corynebacterium* spp. are Gram-positive bacteria commonly associated with the ocular surface. *Corynebacterium mastitidis* was isolated from mouse eyes and was demonstrated to induce a beneficial immune response that can protect the eye from pathogenic infection. Because eye-relevant *Corynebacterium* spp. are not well described, we generated a *C. mast* transposon (Tn) mutant library to gain a better understanding of the nature of eye-colonizing bacteria.

METHODS. Tn mutagenesis was performed with a custom Tn5-based transposon that incorporated a promoterless gene for the fluorescent protein mCherry. We screened our library using flow cytometry and enzymatic assays to identify useful mutants that demonstrate the utility of our approach.

RESULTS. Fluorescence-activated cell sorting (FACS) of mCherry$^+$ bacteria allowed us to identify a highly fluorescent mutant that was detectable on the murine ocular surface using microscopy. We also identified a functional knockout that was unable to hydrolyze urea, Urease^{KO}. Although uric acid is an antimicrobial factor produced in tears, Urease^{KO} bacterium maintained an ability to colonize the eye, suggesting that urea hydrolysis is not required for colonization. In vitro and in vivo, both mutants maintained the potential to stimulate protective immunity as compared to wild-type *C. mast.*

CONCLUSIONS. In sum, we describe a method to genetically modify an eye-colonizing microbe, *C. mast.* Furthermore, the procedures outlined here will allow for the continued development of genetic tools for modifying ocular *Corynebacterium* spp., which will lead to a more complete understanding of the interactions between the microbiome and host immunity at the ocular surface.

Keywords: microbiome, fluorescent bacteria, transposon mutagenesis, host/microbe interaction

Despite the paucibacterial nature of the eye, the ocular surface harbors a distinct microbiome that is affected by age, sex, geographic region, and health status of the eye.[1–3] Critical for low-biomass sites such as the eye, refinement of advanced analytics of microbiome samples has allowed for a better characterization of the role that the microbiome plays in health and disease.[4] Specifically, patterns are emerging in ocular surface diseases, such as dry eye disease and keratitis, where a reduced bacterial diversity correlates with disease.[5,6] These results support the notion, first established in the gut, that a diverse ocular microbiome fosters ocular health.[4–7] Thus, we sought to directly show the presence of *Corynebacterium mastitidis* on the ocular surface, which would suggest that live, ocular microbes can colonize the ocular surface.

Traditionally, wild bacteria are historically difficult to genetically manipulate due to the endogenous bacterial mechanisms that can inhibit or prevent effectiveness of bacterial transformation through DNA recombination. Further, Gram-positive bacteria such as *C. mast* are more difficult to genetically engineer due to both a lack of available genetic tools and the complexity of the bacterial cell wall compared to Gram-negative bacteria such as *Escherichia coli*. Additionally, genetically modifying pathogenic bacteria, such as *Pseudomonas aeruginosa*, has been given greater attention than non-pathogenic corynebacteria. Moreover, the functions of *C. mast* genes in relation to ocular surface colonization and immunogenicity have yet to be described. For these reasons, we aimed to create a standard protocol that resulted in effective manipulation of the *C. mast* genome. Although many genetic tools exist for soil-inhabiting *Corynebacterium* (e.g., *C. glutamicum*), they did not translate well to human or murine commensal *Corynebacterium* spp. due to the diversity of genomes and growth conditions required by each microbe.[8,9] Therefore, we hypothesized that transposon mutagenesis would be an effective way to generate a library of *C. mast* mutants that would help us begin to better understand the roles of *C. mast* genes at the ocular surface. Therefore, the development

of a novel method to mutate the *C. mast* genome was required.

Even though there has been a sharp increase in studies that investigate the ocular microbiome, there remains a gap in knowledge in the functional relevance of fluctuations in the microbiome.[10] Specifically, limited data explicitly shows the consequences of non-pathogenic microbes colonizing the ocular surface.[11] Recently, *C. mast* was identified as a component of the ocular microbiome that can colonize the eye after birth, naturally or artificially through purposeful inoculation.[12] After application, *C. mast* remains at the ocular surface indefinitely and stimulates a protective immune response that relies on interleukin (IL)-17 production by conjunctival $\gamma\delta$ T cells.[11] Despite *C. mast* being detectable on ocular swabs, it was unclear whether the microbe was colonizing the cornea, conjunctiva, or surrounding skin tissue. Similarly, although *C. mast* DNA was detectable in conjunctival areas of the eye, whether these bacteria were living was not known.[11,13] Therefore, to address whether live *C. mast* does, indeed, colonize the eye, we used transposon mutagenesis to incorporate the gene for the fluorescent protein mCherry. The techniques used to create the mCherry *C. mast* mutant will form the foundation for future genetic modifications of *C. mast* to further describe the ocular microbiome and identify factors important for colonization and immune induction.

Conventionally, microbiome studies rely on gnotobiotic facilities and the monocolonization of mice with a specific microbe[14,15]; however, a benefit of *C. mast* and its relation to the ocular surface is that *C. mast* can colonize the eyes of mice housed in specific-pathogen-free conditions.[11] Although the community structure on human conjunctivae is significantly different than that of mice, the bacterial composition of human and mice eyes are quite similar.[16] Because of this, we used a murine model for our studies, with the knowledge that the human microbiome may be different. Furthermore, unlike the intestine, the eye is continually exposed to the outside environment and can be easily visualized without invasive procedures or sacrificing the animal. For these reasons, the relationship between *C. mast* and the ocular environment is an attractive avenue to pursue in ophthalmological microbiome studies. Therefore, we sought to genetically manipulate the *C. mast* genome to begin developing a toolbox of *C. mast* mutants, so that we can interrogate in more detail the relationship between the ocular microbiome and host immunity. Moreover, we needed to consider other factors for *C. mast*, including characteristics such as eye colonization and immune stimulation. Because the genes responsible for these characteristics have not been defined, we hypothesized that transposon mutagenesis would be the most effective way to generate a random library of mutants to allow us to assess the functionality of bacterial genes.

The utility of generating *C. mast* mutants that express fluorescent proteins is multifaceted. First, we hypothesized that fluorescent bacteria will be identifiable in vivo or directly ex vivo, so that conclusions about the location, abundance, and kinetics of ocular colonization could be made. Second, transposon delivery of genes of interest to the bacterial genome results in random insertions (in our case, it was the gene for mCherry) into the *C. mast* genome. Third, the random genome insertions can be used as promoter probes to identify genes that are actively expressed using our promoterless reporter gene for mCherry, because only transposon insertions downstream from an active promoter will be detectable by fluorescence. Fourth, transposons can be used for forward genetics by screening mutants that lose the ability to colonize the eye, thus allowing us to identify bacterial genes necessary for colonization. With a robust library of *C. mast* transposon (Tn) mutants, we can begin to further assess the relationship between *C. mast* and the ocular surface.

In this study, we describe a method to create a transposon library of *C. mast* mutants that harbor a cassette that includes the genes for kanamycin resistance (KanR) and mCherry. We used fluorescence-activated cell sorting (FACS) to screen the brightest mCherry$^+$ mutants and confirmed in vivo fluorescence detectability. In addition, we used a high-throughput enzymatic assay to identify bacteria that had a compromised ability to hydrolyze urea, which is normally toxic to bacteria. Both of these mutants confirmed our ability to mutate the *C. mast* genome without compromising the ability of this bacterium to colonize the eye and generate in vivo immunity. Therefore, our methods reveal new tools for studying the dynamics of the ocular microbiome and pave the way for further genetic manipulations of ocular bacteria.

METHODS

Animals

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in the animal facility at the University of Pittsburgh and were ocularly colonized with bacteria at 3 weeks of age. Mice were sacrificed by 7 weeks of age. The use of animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Bacteria and Culture Conditions

All *C. mast* and generated mutants were grown in Luria–Bertani (LB) liquid medium supplemented with 0.5% Tween 80 (LBT) and 100-µg/mL fosfomycin. For selection, and 50 µg/mL Kanamycin was added. In addition, LBT plates (1.5% agar) were used for streaking with the same amount of Tween 80, fosfomycin, and kanamycin. Liquid cultures were grown in shaking incubators, and bacteria were incubated at 37°C.

Transposon Mutagenesis of *C. mast*

Creation of the Transposome. We used an EZ-Tn5 Transposase (Lucigen, Middleton, WI, USA) and incorporated genes for mCherry and kanamycin resistance in order to genetically modify *C. mast*. This was accomplished by amplifying the genes for mCherry and aphA-3 by polymerase chain reaction (PCR) using pTony3, detailed below, as a template. Primers EZ-Tn50SacI-mCherry-F and -R were used to amplify a promoterless mCherry and kanamycin resistance mini-transposon. All DNA sequences are listed in the Table. The resulting amplicon was gel purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). We then created our transposome with our genes of interest by adding 2 µL of our transposon DNA with DNA containing the genes for mCherry and KanR and 4 µL of EZ-Tn5 Transposase. This mixture was incubated at 37°C for 2 hours. The reaction was then stopped by adding 1 µL of EZ-Tn5 10× Stop Solution and heating at 70°C for 10 minutes.

TABLE. DNA Oligomers and Synthetic DNA Sequences Used in This Study

Sequence Name or No.	Sequence (5′ to 3′)
EZ-Tn5-SacI-mCherry	phos-ctgtctcttatacacatctgagctctagaatttaccaggaggagaattc
EZ-Tn5-aphA-prom-R	phos-ctgtctcttatacacatctccttgaatatattgacaatactga
4600	gcaggaaattactgaactgaggggacaggcgagagacgatgccaaagagctacaccgacgagctggccgagtgggttgaatcccgcgcggccaagaagcgccggcgtgatgaggctgcggttgcgttcctggccggtgagggcggatgtcgatatgcgtaaggagaaaataccgcatcaggcggtcgactaaagggaacaactttgtggttatgtgcgttactactagtgtaaggttgagtcaggcaggctcaataggaggattttttatggaaaaaaaaggagttccgtgtgctcattaaatactgttttttgaaaggaaaaaataccgtagaagccaaaacctggttggacaatgaatttcccgactctgctcctggaaagagcacgatcattgattggtacgctaagttcaaacgcggagaaatgtccactgaggacggcgaacgcagcggacgcccaaaggaggtcgtcaccgatgagaatatcaagaaaattcacaagatgattcttaatgaccggaaaatgaagcttattgagattgcggaagctctcaaaatttccaaggagcgcgtaggccacatcatccatcagtaccttgatatgcggaagctttgtgccaaatgggtgcctcgtgagttgacgtttgatcaaaaacagcgccgcgtggacgattcaaaacgttgccttcagttgctcacccgcaataccccggagttctttcgccggtatgtgactatggatgagacttggttgcaccactacacccccggagtccaatcgccagtctgcggaatggaccgctacgggagagccgtccccgaagcgcggtaagactcaaaaatccgccggaaaagtgatggcttccgtattctgggacgcacacggaattattttttattgattatcttgagaaaggcaaaacgattaactcagactactacatggcactccttgaacgccttaaggtcgagattgccgcaaagcgtccgcatatgaagaaaaagaaagttctgttccatcaggataatgcgccatgtcataagtcgttcgtaccatggccaaaatccatgagttgggctttgagctcctgccgcacccaccttattcaccggatcttgcaccctcagatttctttctgttttcggatctcaagcgtatgcttgcgggcaaaaaattcggctgtaacgaggaagtcattgctgaaactgaagcttactttgaggcgaagcccaaggaatattaccagaatggaatcaagaaacttgaaggacggtacaatcgttgcatcgcactggagggtaattacgtcgaataaaaacgaattttgacaaaaaaatgtgttttttctttgttagaccggaatcctccaattcgccctatagtgagtcgtattacgcgcgggctttccccgtcaagctctaaatcgggggctccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggtgatggttcacttctttcctgcgttatccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcacgacagggtttcccgactggaaagcgggcagtgatagttctagctagagcggccgctggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataacccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaa
4601	cattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattggcaaaaaggccatccgtcaggatggccttctgcatgtgagcaaaaaggccagcaaaaggccaggaaccgtccgcataagatgatcttcttgagatcgttttggtctgcgcgtaatctcttgctctgaaaacgaaaaaaccgccttgcagggcggtttttcgaaggttctctgagctaccaactctttgaaccgaggtaactggcttggaggagcgcagtcaccaaaacttgtccttcagtttagccttaaccggcgcatgacttcaagactaactcctctaaatcaattaccagtggctgctgccagtggtgcttttgcatgtctttccgggttggactcaagacgatagttaccggataaggcgcagcggtcggactgaacggggggttcgtgcatacagtccagcttggagcgaactgcctacccggaactgagtgtcaggcgtggaatgagacaaacgcggccataacagcggaatgacaccggtaaaccgaaaggcaggaacaggagagcgcacgagggagccgccaggggaaacgcctggtatctttatagtcctgtcgggtttcgccaccactgatttgagcgtcagatttcgtgatgcttgtcaggggggcggagcctatggaaaaacggctttgccgcggccctctcacttcctgttaagtatcttcctggcatcttccaggaaatctccgccccgttcgtaagccatttccgctcgccgcagtcgaacgaccgagcgtagcgagtcagtgagcgaggaagcggaatatatcctgtatcacatattctgctgacgcaccggtgcagcctttttttctcctgccacatgaagcaagatcctttgatctttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaacacctagatacaggttgctgataagtccccggtctctagaatttaccaggaggagaattcatgctcagcaaaggagaggaagataatatggccatcatcaaagagtttatgcggttcaaggtccacatggaaggcagcgtgaacggccatgaattt
4062	aagatcctttgatctttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaacacctagatacaggttgctgataagtccccggtctctagaatttaccaggaggagaattcatgctcagcaaaggagaggaagataatatggccatcatcaaagagtttatgcggttcaaggtccacatggaaggcagcgtgaacggccatgaatttgagattgaaggagagggcgagggacgtccatacgagggaactcagactgcgaagctgaaggtgactaagggtggaccacttcctttcgcctgggatatcctgagccctcagtttatgtacggaagcaaagcatacgttaaacaccctgcggatatccctgactacctgaaactgtcatttcctgaaggttttaagtgggagcgtgtgatgaacttcgaagacggtggtgtcgtaaccgtgactcaggatagcagcttgcaagatggagaatttatctacaaagtaaaactgcgcggcacgaacttcccaagcgacggccccgttatgcaaaaaaaaacgatgggttgggaagcgtcttctgagcgcatgtatccagaggacggtgcgttgaaaggcgagatcaagcagcgccttaaactgaaggatggcggccattatgatgccgaagttaagaccacgtataaggcgaaaaagcctgttcagctccccggtgcatacaacgtcaacatcaagtgggacatcaccagccacaacgaagattataccatcgtggagcagtacgaacgcgctgagggacgccactcaacgggcggaatggatgagctgtacaagtaggaattccagataaaacgaaaggcccagtctttcgactgagccttcgttttatttgatgcctggcagttgaccatgacggtggattataaagatcatgatatcgattacaaggatgacgatgacaagcttgaagttcctattccgaagttcctattctctagaaagtataggaacttcggcgcgccgctctagacccctatagtgagtcccgggggtttaacggttgtggacaacaagccagggatgtaacgcactgagaagcccctagagcctctcaaagcaatttttcagtgacacaggaacacttaacggctgacagaatttttgaagacgaaagggcctcgtgatacgcctacccggggccccgacctgaatggaagcccgatacaaattcctcgtaggcgctcgggaccccctatctagcgaacttttagaaaagatataaaacatcagagtatggacagttgcggatgtacttcagaaaagaggtgattgattgagcaagctttatgcttgtaaaccgttttgtgaaaaaatttttaaaataaaaaaggggacctctagggtcccca attaattagtaatataatctattaaaggtcattcaaaaggtcatccaccggatca
4603	cccggggggtttaacggttgtggacaacaagccagggatgtaacgcactgagaagcccctagagcctctcaaagcaatttttcagtgacacaggaacacttaacggctgacagaatttttgaagacgaaagggcctcgtgatacgcctacccggggccccgacctgaatggaagcccgatacaaattcctcgtaggcgctcgggaccccctatctagcgaacttttagaaaagatataaaacatcagagtatggacagttgcggatgtacttcagaaaagaggtgattgattgagcaagctttatgcttgtaaaccgttttgtgaaaaaatttttaaaataaaaaaggggacctctagggtccccaattaattagtaatataatctattaaaggtcattcaaaaggtcatccaccggatcaatctaggtactaaaacagctcatccaacaagatatagtacttaatcttttcccagtcaggtttaattcccaggaggtcgaaaaacaatcgacgtactgttcttctccgatgtcctcgcggattgaccgggacacagaaagcaatatcgtaccatttatcggcgcgaccagaacgcccagatcgatgaaaccagacacttaccatcttttgacgaagatattggagtcacccaagtcaccgtggctaaatacaagctctcctcgggcttctcagttttaaggaaatcgtacagttcgcgcgggtccttgaatggctgcgtgtcttcctcccagttctcacagtctacgtctgcgaggtcattgttaagcaggtaatcaagctccgccagacgg

TABLE. Continued

Sequence Name or No.	Sequence (5′ to 3′)
	ctgtccaaagaattggtgtatgggcaatcgctgatatcgattgaatggaaaagacggatacactcggcgtaaagttcaatgatcttttctggagattgttc
	atcctcgtactcctcgctacacaggacaccgtccgcctccgacatgagcagattagaccatccgtcgtggcgttcgaaatgcaacaccttcgggacaggca
	gctttccttccagccacaacatcatgtcttttttcccgttcgacatcatacgtcgttcccttgtagcggctgtcggtcatcttgagatacaagtttttcattt
	tcaccgaccagcttatataccttcgcgggtgacataccttcggtatctttcacacaccgatacttctcgatcagcttcttcaactcaggagaaatgcgcat
	cttagccatttattatttccttcctcttttctacagtatttaaagatacccaagaagctaattataacaagacgaactccaattcactgttccttgcatt
	ctaaaaccttaaataccagaaaacagctttttcaaagttgttttgaaagttggcgtataacatagtatcgacggagccgattttgaaaccacaattatgat
	agaatttacaagctataaggttattgtcctgggtttcaagcattagtccatgcaagtttttatgctttgcccattctatagatatattgataagcgcgctg
	cctatgccttgccccctgaaatccttacatacggcgatatcttctatataaaagatatattatcttatcagtattgtcaatatattcaaggggatccgcgg
	gactctggggtacgcgtctaggattccgaaccggttctagagaccggggacttatcagccaacctgtctctcgaggttcaccgacaaacaaagtgggtaat
	aactgatataattaaattgaagctctaatttgtgagtttagtatacatgcatttacttataatacagtttttttagttttgctggccgcatcttctcaaata
	tgcttcccagcctgctttttctgtaacgttcaccctctaccttagcatcccttccctttgcaaatagtcctcttc
4604	agtgggtaataactgatataattaaattgaagctctaatttgtgt
4605	tgttccctttagtcgaccgcctgatgcggtattttctccttacg

A transposon delivery plasmid, pTony3, was generated for *C. mast* using yeast homologous recombineering as previously described.[9] Four synthetic double-stranded DNA fragments (Integrated DNA Technologies, Coralville, IA, USA) and one PCR amplicon with regions that direct recombination were recombined in a single step in Invitrogen *Saccharomyces cerevisiae* strain InvSc1 (Thermo Fisher Scientific), a ura3/ura3 mutant. Codon optimization for corynebacteria expression was done with online software at Integrated DNA Technologies. The first synthetic fragment, number 4600, contains an artificial promoter designed for expression in *Corynebacterium* (based on Patek et al.[17]) driving expression of the hyperactive C9 transposase.[18] The fragment also contains an ampicillin resistance cassette. The second fragment, number 4601, contains the p15a origin of replication for expression in *E. coli*. The third synthetic fragment, number 4602, contains a codon-optimized mCherry gene that is promoterless but has a ribosome binding site upstream of the start codon. The fourth fragment, number 4603, contains a codon-optimized kanamycin resistance gene *aphA-3* with the promoter region from the pHP45Ω-Km plasmid.[19] With the promoter region from the same plasmid, which originates from an *Enterococcus* species. The PCR amplicon contains an RP4-based origin of conjugal transfer, yeast centromere replicons CEN6 and ARSH4, and the *URA3* gene from pMQ132 using primers 4604 and 4605.[20] The resulting plasmid was verified by PCR and was partially sequenced at the University of Pittsburgh genomic core facility and by phenotype.

Electroporation of *C. mast*. To generate electrocompetent cells, *C. mast* was grown in a 1:1 mixture of non-capacitating medium (NCM)+ media and LBT until an optical density at 600 nm (OD_{600}) between 0.6 and 0.8 was achieved. NCM+ contains 17.4 g K_2HPO_4, 11.6 g NaCl, 5 g tryptone, 1 g yeast extract, 0.3 g trisodium citrate, 0.05 g $MgSO_4$–$7H_2O$, 91.1 g sorbitol, 1 mL Tween 80, and 20 g glycine.[21] When the culture was within that range, it was centrifuged at 4000*g* at 4°C for 5 to 10 minutes, followed by washing with cooled 10% glycerol three times. Spin cycles were done at 4000*g* for 5 to 10 minutes at 4°C. After the final wash, the bacterial pellet was resuspended in 10% glycerol and frozen at –80°C until use. We modified *C. mast* using the transposon generated above, and 1 µL of EZ-Tn5 reaction mixture was added to a 1-mm cuvette containing our electrocompetent bacteria. This mixture was then electroporated using a Gene Pulser Xcell Total System (Bio-Rad, Hercules, CA, USA) at 1.8 kV, 25 µF, and 200 ohms. After electroporation, the bacteria were then recovered in liquid LBT, placed in a shaking incubator for 1 to 2 hours at 37°C, and plated on agar plates containing kanamycin and fosfomycin. Colonies that displayed kanamycin resistance were then further screened under a fluorescent microscope to detect red fluorescence, which would indicate mCherry expression driven by a *C. mast* promoter.

Isolation of Fluorescent Mutants Using FACS

We pooled 1596 of our mCherry+ Tn mutant colonies into a single sample and diluted them in PBS and 2% fetal bovine serum (FBS). The top 2.5% of bacteria according to mCherry mean fluorescence intensity (MFI) were isolated using FACS and a Sony MA900 Cell Sorter (Sony, Tokyo, Japan). After isolation, sorted bacteria were grown on LBT agar plates with kanamycin (50 mg/mL), and fosfomycin (100 µg/mL). Individual colonies of bacteria were picked and expanded in liquid broth. Individual isolates were then run on a CytoFLEX LX Flow Cytometer (Beckman Coulter, Brea, CA, USA) to objectively identify the brightest mCherry+ isolate according to mCherry MFI (Yellow Laser 610 channel).

Urease Activity

The generated transposon mutant library was tested for its ability to hydrolyze urea into ammonia and carbon dioxide using a modified Stuart's Urea Broth with added 0.1% Tween 80.[22] Then, 1596 Tn mutants were pre-grown in 96-well plates with 200 µL of LBT in conditions mentioned previously. When they were grown, 10 µL of candidate culture was used to inoculate 200 µL of Stuart's Urea Broth in a 96-well plate. The 96-well plates were then incubated for 24 to 48 hours at 37°C at 3% CO_2 and were monitored for a color change (pink to yellow) and absorbance at 670 nm, indicating the hydrolysis of urea.

In Vitro Stimulation of Dendritic Cells

DC Differentiation. Wild-type (WT) female C57BL/6 mice purchased from The Jackson Laboratory were sacrificed, and femurs and tibias were extracted and placed in RPMI-1640/5% FBS. Bones were flushed into a petri dish using RPMI 1640/5% FBS media. Clumps were then

broken up by using a 30-gauge needle attached to a 5-mL syringe. After red blood cell lysis using $1\times$ ammonium–chloride–potassium (ACK) buffer, cells were counted, and 5×10^6 cells at a concentration of 1×10^6 cells per mL were plated in six-well tissue culture-treated plates with Dulbecco's Modified Eagle Medium + 10% FBS media supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Bone marrow–derived dendritic cells (BMDCs) were fed on day 4 with 2.5 mL per well of fresh media supplemented with GM-CSF and IL-4. On day 7, differentiated dendritic cells were harvested and cultured on 96-well tissue culture treated plates with 1×10^5 cells per well.

Upregulation of Costimulatory Molecules. To assess the upregulation of costimulatory molecules on DCs, 1×10^5 DCs were cultured with 1×10^5 CFU per well of bacteria and incubated at 37°C overnight. After the incubation period, cells were stained with a cocktail of fluorescent antibodies against CD11c Brilliant Violet (BV) 421 (1:250 dilution), CD11b PE Dazzle 594 (1:250 dilution), CD80 PerCP-Cy5 (1:250 dilution), CD86 PE (1:250 dilution), and a viability dye (Zombie UV, 1:1000 dilution) in a master mix with PBS + 2% FBS. Antibodies were purchased from BioLegend (San Diego, CA, USA). Viability dye was purchased from Thermo Fisher Scientific.

In Vitro Stimulation of IL-17 From $\gamma\delta$ T Cells

First, 1×10^5 DCs were pulsed for 24 hours with 1×10^5 CFU of *C. mast* or Tn mutants at 37°C and 5% CO_2. The next day, $\gamma\delta$ T cells were isolated from the cervical and inguinal lymph nodes of WT B6 mice (The Jackson Laboratory) using FACS. About 1×10^5 $\gamma\delta$ T cells can be isolated from each WT B6 mouse. After isolation, 1×10^4 $\gamma\delta$ T cells were incubated with 1×10^5 DCs pulsed with *C. mast*. Then, 72 hours later, cells were harvested and stained with fluorescently labeled antibodies against the $\gamma\delta$ T-cell receptor (TCR) antibody Alexa Fluor 488, Vγ2 TCR allophycocyanin, CD11c BV 421, and the intracellular cytokines IL-17 BV 650 and IFNγ PE-Dazzle 594. Samples were analyzed on the CytoFLEX LX Flow Cytometer. Antibodies were purchased from BioLegend.

Colonizing Mice With Bacteria

WT C57BL/6 mice (The Jackson Laboratory) were used in this experiment because they lack *Corynebacterium* spp. in their ocular mucosa.[11] At 4 weeks, the tear film of mouse eyes was swabbed with a sterile cotton swab in order to disrupt the tear film. Eyes were inoculated three times every other day with 5×10^5 CFU of bacteria in 5 μL of PBS. One week after the final inoculation, mice were assessed for colonization by swabbing the conjunctiva with a cotton swab and plating on a LBT plate containing fosfomycin (to eliminate contaminating bacteria that were not *Corynebacterium* spp.) and a LBT plate containing fosfomycin and kanamycin to select for Tn mutants.

Immune Assessment of In Vivo Immunity

Mice were first colonized with bacteria as described above. Three weeks after the final inoculation, mice were sacrificed by cardiac perfusion to minimize blood cell contamination of tissues such as the conjunctiva. Conjunctiva were isolated by excising the eyelids and bulbar conjunctiva. Tissues from both eyes were pooled, minced, and incubated in a collagenase solution at 37°C while shaking for 1 hour. In parallel, cervical and submandibular lymph nodes were isolated, minced, and placed in a collagenase solution at 37°C while shaking for 30 minutes. After incubation, conjunctiva or lymph nodes were filtered through a 40-μm filter using the plunger from a 3-cc syringe plunger. After two washes with RPMI + 10% FBS, conjunctival cells were filtered again using a Corning Falcon Test Tube with Cell Strainer Snap Cap (352235; Corning Inc., Corning, NY, USA).

Either one-tenth of a lymph node sample or two-thirds of a conjunctival sample were directly stained with antibodies for immune cell neutrophil analysis or they were ex vivo stimulated with phorbol myristate acetate (PMA)/ionomycin in the presence of brefeldin A for 4 hours at 37°C. For ex vivo–stimulated samples, after the 4-hour incubation samples were stained with fluorescent antibodies against CD90.2, $\gamma\delta$ TCR, Vγ2 TCR, TCRβ, and the intracellular cytokines IL-17 and IFNγ. All flow cytometry samples were analyzed with the CytoFLEX LX Flow Cytometer, and the analysis software used was FlowJo 10.8.

Ex Vivo Imaging of Eye Colonizing Bacteria

Mice were colonized as described above. Two weeks after final inoculation, mice were sacrificed by cervical dislocation. Eyes were removed and placed into an RPMI + 10% FBS solution until imaging (up to 3 hours). Eyes were imaged with a SZX16 dissecting scope with an Olympus DP80 dual CCD camera dissecting microscope (Olympus, Tokyo, Japan) using a $10\times$ objective.

Statistical Analyses

All statistics were calculated using Prism 9 (GraphPad, San Diego, CA, USA). Statistical significance was determined using parametric *t*-tests for comparing two groups or ANOVA for comparing three or more groups. $P < 0.05$ was considered significant.

Whole-Genome Sequencing

Bacterial DNA was isolated using the NucleoSpin Tissue Kit with hard-to-lyse bacterial buffer (Macherey-Nagel, Düren, Germany). Isolated DNA was then sent to the Microbial Genome Sequencing Center (MiGS, Pittsburgh, PA, USA) for 200-Mbp whole-genome sequencing. Genomes were assembled using the PATRIC Bioinformatics Resource Center (Murfreesboro, TN, USA).[23]

RESULTS

Generation of mCherry⁺ *C. mast* Transposon Mutant

Recently, we identified *C. mast* as a bacterium that has a unique ability to colonize the ocular mucosa of mice. Detection and quantification of *C. mast* occurs by swabbing the eye and enumerating colonies on agar plates supplemented with the antibiotic fosfomycin, which limits the growth of contaminants but does not affect the growth of *C. mast* and other *Corynebacterium* spp.[24] Even though ocular swabs can accurately assess the *C. mast* colonization status of an eye, they cannot reliably reveal the location of *C. mast* within

the ocular surface environment, which includes the cornea, conjunctiva, lid margins, and lacrimal ducts, among others. In the interest of developing a system where we could assess bacteria in vivo, we sought to genetically modify *C. mast* to express the gene for the fluorescent protein mCherry that would allow for in vivo detection of *C. mast*.

To do this, we created a fragment of DNA that included a mCherry gene lacking a promoter directly after the Tn5 insertion site and a custom kanamycin resistance cassette (Fig. 1A). We then used the EZ-Tn5 system to make transposomes for mutagenesis of *C. mast*, which resulted in over 1000 mutant candidates that grew in the presence of the selective antibiotic kanamycin; however, not all colonies expressed visually detectable levels of mCherry. As per our design, only transposons that inserted in favorable locations would highly express mCherry, which allows us to identify strong active promoters in *C. mast*. Therefore, we used FACS to isolate the brightest 2.5% of genetically modified *C. mast* according to MFI (Fig. 1B). After FACS, bacteria were expanded on agar plates supplemented with kanamycin (50 mg/mL) to ensure preservation of the transgene after sorting.

Individual colonies were analyzed for fluorescence in an objective manner using flow cytometric analysis. We screened over 1000 isolates that expressed variable levels of mCherry fluorescence (Fig. 1B). Isolates 8.1 and 21.2 had similar fluorescence intensities, and isolates 10.1 and 13.2 had demonstrably less fluorescence on a per-cell level. Therefore, we decided to pursue only isolate 21.2 and refer to this isolate as the mCherry+ mutant. Whole-genome sequence analysis of this isolate revealed that the cassette was inserted into a dye-decolorizing peroxidase (DyP)-type peroxidase gene (Fig. 1C). We then used this candidate to further pursue in vitro and in vivo studies.

Generation of Urease[KO] *C. mast* Transposon Mutant

Due to the high concentration of urea found in tears, we hypothesized that a *C. mast* mutant lacking an ability to hydrolyze urea would not survive the harsh environment at the ocular surface and the elevated levels of urea in tears. In addition, urease activity is also conserved in other corynebacteria, such as *C. glutamicum*, *C. bovis*, and *C. propinquum*,[25–27] which also provides a convenient method to test our Tn mutant library for the ability to screen for knockouts of interest. To screen for urease activity, we
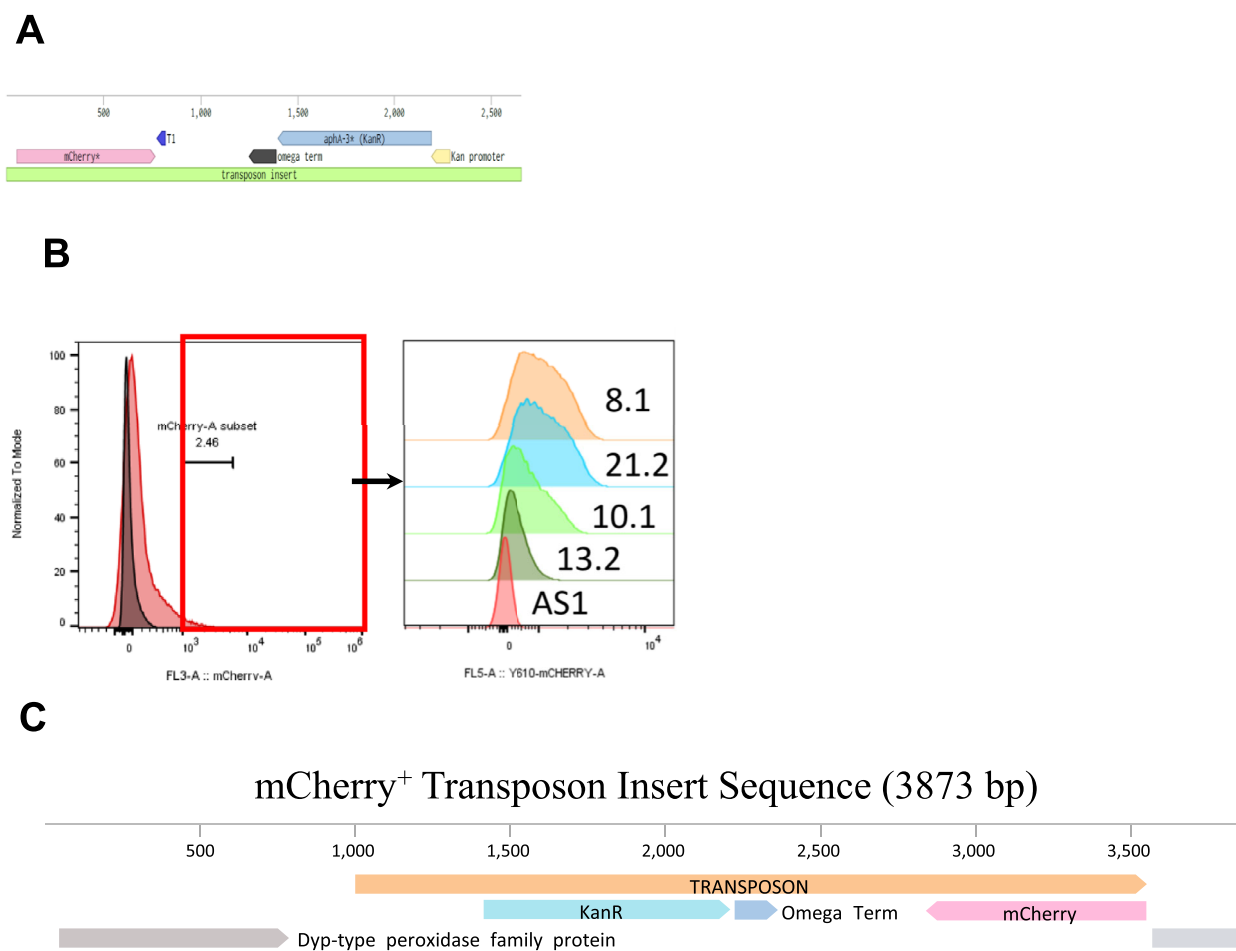

FIGURE 1. Mutagenesis of *C. mast* to incorporate mCherry. (**A**) Configurations of transposon, mCherry, and KanR genes in the EZ-TN5. These were used to genetically modify *C. mast*. (**B**) The *left* flow plot portrays the sorting strategy where the top 2.5% of fluorescent bacteria were sorted and used for further study. The *right* flow plot shows assessment of individual colonies from the sorted bacteria. Numbers indicate individual isolates of bacteria. (**C**) Genome insertion site for mutant mCherry+. Insertion happened in a DyP-type peroxidase family protein.
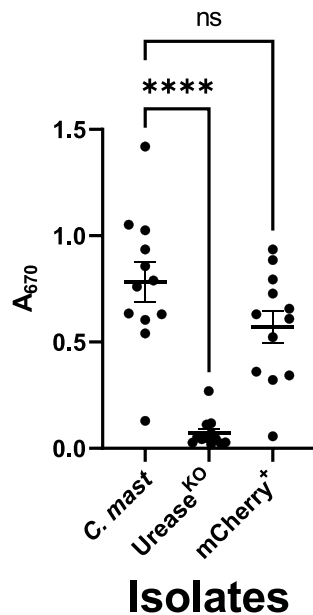
A

B

C
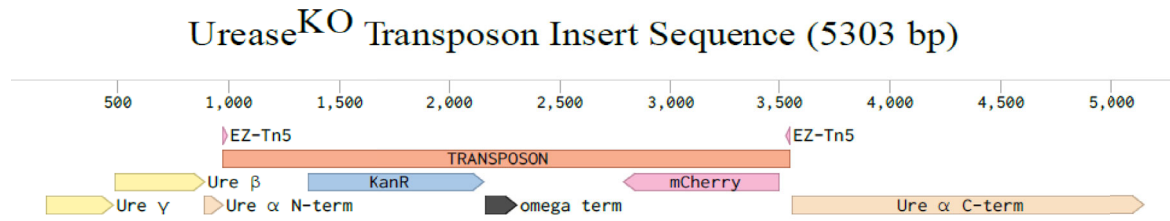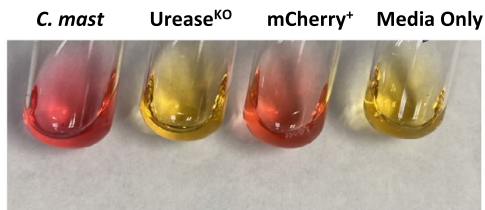
FIGURE 2. Interruption of urea alpha subunit. (**A**) Genome insertion site for mutant Urease^{KO}. The gene insertion occurred in the urease a subunit gene. (**B**) Color change in WT *C. mast* and mCherry$^+$ signifying that urea has been hydrolyzed. Urease^{KO} displays no color change, as does media only (negative control), signifying that no urea has been hydrolyzed. (**C**) Absorption values at 670 nm for *C. mast*, Urease^{KO}, and mCherry$^+$. Symbols represent individual colonies from three independent experiments. *Bars* represent mean ± SEM 670-nm absorbance. Significance was determined using an ANOVA Kruskal–Wallis test (****$P < 0.0001$).

grew transposon mutants in a modified Stuart's Urea Broth. Urease activity in this media will change the pH indicator from yellow to pink. After screening the transposon library, we found a single mutant that had completely deficient urease activity. Whole-genome sequencing revealed that the transposon cassette was inserted into the urease α subunit gene of *C. mast* (Fig. 2A). Although WT *C. mast* and the mCherry$^+$ mutant caused a color change in the Stuart's Urea Broth, there was no observable color change in the Urease^{KO} or media-only samples (Fig. 2B). We quantified these differences by measuring the absorbance at 670 nm (Fig. 2C).
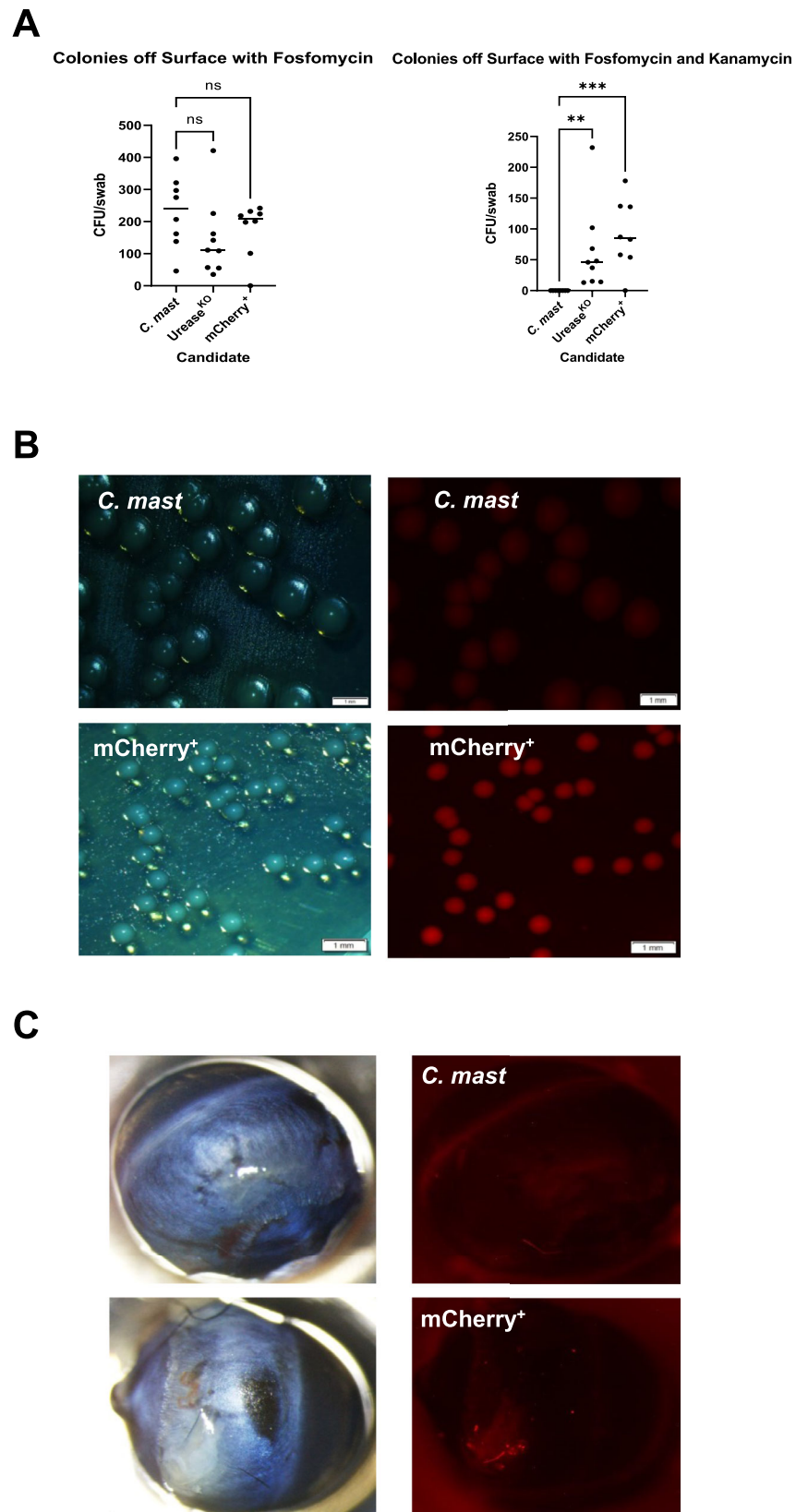
FIGURE 3. Genetically modified *C. mast* colonized the ocular mucosa. (**A**) Three-week-old mice were inoculated three times with WT *C. mast* or the Tn mutants over a span of a week. Two weeks after the final inoculation, the eyes of colonized mice were swabbed and streaked on agar plates that did not include (*left*) or included (*right*) the selective antibiotic kanamycin. Graphs display the numbers of CFU per swab. *Symbols* represent data from individual mice from three independent experiments. Significance was determined using an ANOVA Kruskal–Wallis test (***$P = 0.0005$, **$P = 0.056$). *Lines* represent median values for each isolate. (**B**) Representative images of *C. mast* and mCherry$^+$ colonies after swabbing mouse eyes 2 weeks after the final inoculation. (**C**) Representative pictures of eyes colonized with *C. mast* and mCherry$^+$ 2 weeks after the final inoculation. In (**B**) and (**C**), the data are representative of three independent inoculation experiments.
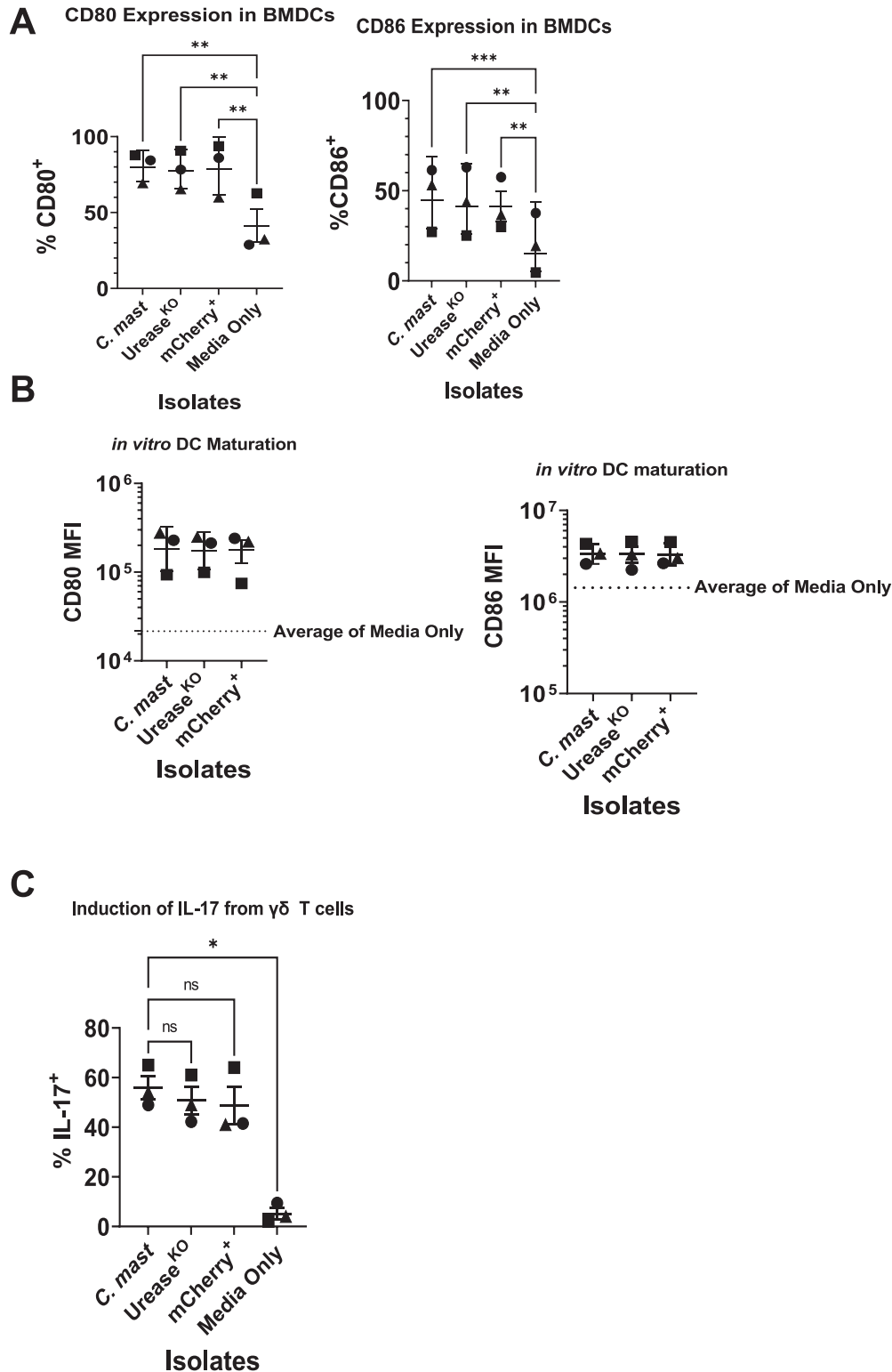
FIGURE 4. Tn mutants maintained immunogenicity in vitro similar to WT *C. mast*. (**A**, **B**) DCs were differentiated from the bone marrow of C57BL/6 mice for 6 days, and 1×10^5 BMDCs were then incubated with 1×10^5 of live bacteria 24 hours after introduction to bacteria. BMDCs were stained with fluorescent antibodies against CD80 and CD86. Stimulations were performed in triplicate, and values were averaged to achieve a single *n* value. *Symbols* in graphs represent an average value for (**A**) CD80/86% or (**B**) CD80/86 MFI from an individual experiment. Data are pooled from three independent experiments, and like symbols between groups were from the same experiment. (**C**) The 1×10^4 $\gamma\delta$ T cells were isolated and added to BMDC$^+$ bacteria cell cultures 24 hours after bacteria were added. Then, 72 hours later, $\gamma\delta$ T cells were assayed for IL-17 production. Data represent the percentage of $\gamma\delta$ T cells that were producing IL-17 after incubation with bacteria. In all graphs, *bars* represent mean $\pm$ SEM. *Symbols* represent individual experiments. Significance was determined using an ANOVA Kruskal–Wallis test (***$P = 0.007$, **$P < 0.0032$, *$P = 0.0382$).

A

Conjunctival Vγ2⁺ γδ T Cells   **Conjunctival Vγ2⁻ γδ T Cells**

B

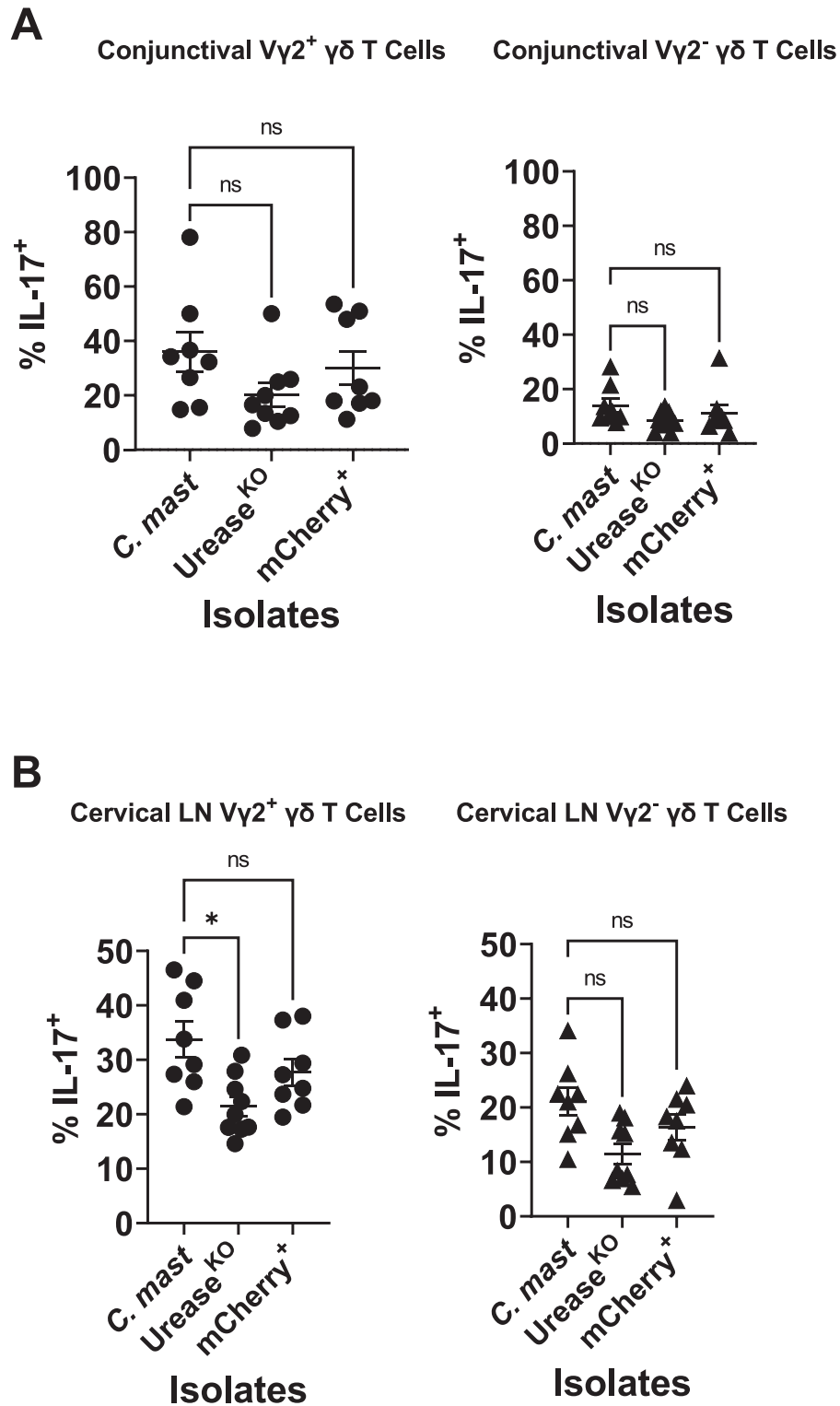**Cervical LN Vγ2⁺ γδ T Cells**   **Cervical LN Vγ2⁻ γδ T Cells**

FIGURE 5. Tn mutants maintained immunogenicity in vivo similar to WT *C. mast*. (**A**, **B**) C57BL/6 mice were inoculated with individual Tn mutants or WT *C. mast*. Two weeks after the final inoculation, mice were harvested, single-cell suspensions were stimulated with PMA/ionomycin, and intracellular IL-17 was monitored by flow cytometry. Graphs display the percentage of Vγ2⁺ (*left*) or Vγ2⁻ (*right*) that produced IL-17 after stimulation in the (**A**) conjunctiva or (**B**) cervical lymph nodes. *Symbols* represent data for individual mice from three independent experiments. *Bars* represent average ± SEM. Differences were determined to be non-significant using an ANOVA Kruskal–Wallis test.

Transposon *C. mast* Mutants Can Stably Colonize the Ocular Mucosa

Due to the random nature of transposon insertion into the *C. mast* genome, we wanted to (1) confirm that the mCherry[+] mutant retained an ability to colonize the eye, and (2) assess whether hydrolyzation of urea was critical for eye colonization. Therefore, we sought to compare the ability of Tn mutants to colonize the ocular surface with WT *C. mast*. To do this, we employed the approach where we disrupt the tear film and inoculate the eye with the bacteria of interest.[11] Two weeks after the final inoculation, we measured colonization quantitatively by enumerating colonies grown from conjunctival swabs and qualitatively by imaging fluorescent bacteria on the eye. We observed no statistical difference in the pattern of colonization between the Tn mutants and WT *C. mast* as determined by conjunctival swabs. Notably, bacteria grown from swabs of Tn mutants maintained antibiotic resistance (Fig. 3A). Further, the mCherry[+] mutant maintained mCherry expression after isolation from the conjunctiva of mice (Fig. 3B). Colonized eyes were enucleated and imaged directly ex vivo to reveal localized areas of mCherry fluorescence in the conjunctival region, but there was no detectable fluorescence at the corneas of these mice. The presence of fluorescence directly ex vivo suggests that the promoter in the mCherry[+] isolate remains active in vivo. Additionally, these data reveal that Tn mutants and likely WT *C. mast* remain associated with the conjunctiva and are not routinely found on the cornea during steady-state homeostasis.

Tn Mutants Stimulate In Vitro Immunity Similarly to WT *C. mast*

In vitro, *C. mast* induces the production of IL-17 from $\gamma\delta$ T cells co-cultured with BMDCs through the detection of bacterial antigen on non-classical major histocompatibility complex molecules and the stimulation of innate cytokines such as IL-1β.[11] We were interested in identifying whether *C. mast* Tn mutants stimulated BMDCs similarly to WT *C. mast*. Therefore, we wanted to assess DCs for (1) maturation after incubation with *C. mast* mutants, and (2) their ability to stimulate $\gamma\delta$ T cells to produce IL-17 in culture. To measure DC maturation, we co-incubated BMDCs with WT *C. mast* or the *C. mast* Tn mutants for 48 hours and used flow cytometry to measure the upregulation of co-stimulatory molecules CD80 and CD86. There were no statistically significant differences in the frequency of cells that upregulated CD80 and CD86 and the mean fluorescence of co-stimulatory molecule expression between WT *C. mast* and the *C. mast* Tn mutants (Figs. 4A, 4B). We next wanted to test whether *C. mast* Tn mutants maintained an ability to induce IL-17 production from $\gamma\delta$ T cells. For this, we incubated BMDCs with either Tn mutant or WT *C. mast* for 24 hours. This allowed for maturation of DCs and the initiation of IL-1β secretion, which is critical for the production of IL-17 in this model.[11] After 24 hours, FACS-isolated $\gamma\delta$ T cells were added to the DC:bacteria cultures, and co-cultures were incubated an additional 72 hours. There were no statistical differences in the frequency of IL-17^{+} $\gamma\delta$ T cells between the groups, which suggests that these Tn mutants maintain in vitro immunogenicity despite the interruption of the DyP-type peroxidase or urease 1A gene (Fig. 4C).

Tn *C. mast* Mutants Stimulate In Vivo Immunity

Vendor-purchased mice are generally devoid of *C. mast* and have a limited number of $\gamma\delta$ T cells within conjunctival tissue.[11] When *C. mast* colonizes the eye, a marked increase in $\gamma\delta$ T cells within the conjunctiva occurs, and these $\gamma\delta$ T cells produce IL-17, which is a factor that influences neutrophil recruitment to the conjunctiva and leads to protection of the ocular surface from infection. We next asked if mutations in the DyP-type peroxidase or urease 1A gene prevented mice from generating an immune response toward *C. mast*. Therefore, we colonized mice with *C. mast* Tn mutants, mCherry[+] or Urease[KO], or WT *C. mast* and waited 3 weeks to allow for the generation of in vivo immunity. We observed no significant differences in the $\gamma\delta$ T cell response within the conjunctiva or draining lymph nodes with regard to IL-17 production after stimulation with PMA/ionomycin (Figs. 5A, 5B). Moreover, about 38% of $\gamma\delta$ T cells within the conjunctiva expressed the Vγ2 TCR (data not shown), which is known to recognize *C. mast* antigens.[11] This suggests that the presumed *C. mast* $\gamma\delta$ TCR stimulant is retained in both Tn mutants. Together, these data suggest that these Tn mutants maintain their ability to stimulate protective immunity despite the genes that are disrupted by the transposon insertions. Moreover, we can conclude from these data that the interrupted genes in the tested mutants do not play a role in immune recognition of *C. mast*.

DISCUSSION

In this study, we developed a system for genetically modifying the ocular bacterium *C. mast*. Specifically, this study relied on transposon mutagenesis to incorporate genes encoding mCherry and kanamycin resistance into the *C. mast* genome. Through a series of selections that included antibiotic resistance and objective measurement of fluorescence using flow cytometry we were able to identify a mCherry[+] *C. mast* mutant that is maintained and detectable on the ocular surface using fluorescence microscopy. In addition, we were able to use a method of high-throughput screening to identify a *C. mast* mutant, Urease[KO], that lacked an ability to hydrolyze urea but maintained an ability to colonize the eye similar to WT *C. mast*. Together, these data reveal that *C. mast* can be genetically manipulated to express proteins of interest while retaining an ability to influence the ocular microenvironment. Through this study, we developed a method to generate novel, bacteria-based tools that can provide a better understanding of the ocular microenvironment and has the potential to influence the environment during health and disease.

C. mast and other *Corynebacterium* spp. are Gram-positive bacteria; the thick peptidoglycan layer in the cell wall makes genetic engineering of the bacterial genome difficult.[28,29] Despite this, the methods to genetically engineer *C. glutamicum* are particularly well developed due to its use in the industrial production of amino acids.[30] Unfortunately, the phenotypic and genotypic differences between *C.* glutamicum and *C. mast* are vast, such that new methods were needed for the genetic manipulation of *C. mast*. Specifically, growth conditions for *C. mast* are more restrictive than those for *C. glutamicum*, in that *C. mast* grows at a slower rate and at a higher temperature and requires a lipid source for optimal growth.[31] Additionally, *C. mast* is less receptive to foreign DNA than other bacteria, resulting in low transposon mutagenesis efficiencies and marked differences in

electroporation conditions. Moreover, for the purposes of *C. mast*, we had to consider additional factors such as the ability to colonize the inhospitable environment of the eye, maintaining fluorescence (mCherry$^+$ mutant) in vivo, and the ability to stimulate protective immunity. Because of these factors, we reasoned that the random nature of insertions associated with transposon mutagenesis was an ideal method to incorporate the mCherry gene and identify the brightest candidate for in vivo tracking.

In this study, we used two methods to screen the Tn mutant library: (1) objective measurement of mCherry fluorescence, and (2) urease hydrolysis. Due to the random nature of insertion into the *C. mast* genome, it was possible that the gene for mCherry incorporated downstream from a promoter was selectively active, which may have translated into inconsistent fluorescence when the bacteria were grown in broth, on plates, and/or in vivo. Therefore, we used flow cytometry to objectively identify the brightest mCherry mutants, and we used imaging to ensure stability of fluorescence in vivo. We found that the mCherry$^+$ mutant maintained high fluorescence throughout several types of in vitro and in vivo growing conditions. Whole-genome sequencing of the mCherry$^+$ mutant revealed that the mCherry insertion occurred directly downstream of the promoter for the DyP-type peroxidase. DyPs are a haem class of peroxidases that have varied functionalities in addition to peroxidase activity, including hydrolase of anthraquinone dye, uptake of iron without degrading heme, and oxidation of β-carotene independent of peroxides like H_2O_2.[32,33] Although these characteristics of DyP-type peroxidases have been defined in organisms such as *Bjerkandera adusta*, *Escherichia coli*, *Marasmius scorodonius*, *Staphylococcus aureus*, and *Streptomyces lividans*, the functionality of DyP-type peroxidases has not yet been defined in *Corynebacterium* spp.[32] In our study, we revealed through observable mCherry fluorescence that the promoter of DyP-type peroxidase in *C. mast* is active when *C. mast* colonizes the ocular surface. Despite the apparent in vivo activity of the DyP-type peroxidase promoter, our data also reveal that DyP-type peroxidase is not required for ocular colonization due to interruption of the gene by the transposon insertion.

Our data also rule out hydrolysis of urea as a requirement for a bacterium to colonize the ocular mucosa. It is known that urea is present at high concentrations in mammalian tears.[34] Because of this, we hypothesized that urease activity may be a feature of eye-colonizing bacteria. Therefore, we performed a high-throughput screen of our Tn mutant *C. mast* library to identify candidates that lacked urease activity. Notably, the mutant that lacked urease activity maintained an ability to colonize the eye and stimulate host immunity.

Before this study, it was apparent that select *Corynebacterium* spp. had an ability to remain at the ocular surface indefinitely, and it was likely that these bacteria were colonizing the conjunctiva. Our original studies used fluorescence in situ hybridization to identify the locale of *Corynebacterium* spp. in the ocular microenvironment, and these data were supported by other studies.[35,36] In these studies, *C. mast* exhibited a filamentous form suggestive of defined colonization and adherence to ocular tissue. Here, we were able to use live in vivo imaging to conclude that mCherry$^+$ *C. mast* does not reside in the cornea or the limbal region of the eye. In support of previous studies, *C. mast* appears to remain well into the conjunctival region and does not possess an ability to remain at the central region of the eye long term. Similar to previous studies, *C. mast* was not

uniformly distributed within the conjunctiva but existed in pockets, which supports a notion that *C. mast* and potentially other eye-colonizing bacteria form pockets of biofilms to survive at the ocular surface. Additionally, our inoculation procedures likely allow the conjunctiva to harbor larger loads of bacteria, and naturally inoculated hosts may have smaller pockets of bacteria associated with the conjunctiva. Future studies are required to better understand the kinetics of colonization throughout various ages and states of inflammation.

The purpose of this study was to genetically engineer *C. mast* to express a fluorescent protein that would allow for longitudinal analysis of the ocular microbiome. Because of the ability of *C. mast* to colonize the ocular surface, we posit that this and similar bacteria would be attractive candidates to act as long-term drug delivery vehicles for inflammatory ocular surface diseases. Specifically, bacteria can be engineered to express proteins of interest, such as murine (m)IL-10, which was incorporated into *Lactobacillus lactis*. After colonization of the intestine, mIL-10 *L. lactis* was able to reduce the disease severity of colitis in mice by continually producing and secreting mIL-10.[37] This same concept may apply to diseases at the ocular surface. Applying this example to the eye, it is known that IL-10 can increase the rate of corneal wound healing.[38] Similarly, IL-10 deficiency leads to persistent corneal inflammation that can be resolved with the administration of exogenous IL-10.[39] Therefore, there may be utility in an eye-colonizing bacterium that can produce and secrete IL-10 to alleviate ocular surface inflammation. Here, we have outlined methods to genetically engineer *C. mast* and have effectively laid the framework for developing genetically engineered therapies that can be used to treat ocular surface disease.

There are limitations with the model used in this study. Specifically, our laboratory uses *C. mast* that was originally isolated from sheep but has been found in humans.[12,40] More recently, *C. mast* was isolated from the skin and eyes of mice.[11,41–43] Whereas *C. mast* seems to be prevalent in mice, a recent analysis shows that *C. mast* is present at the ocular surface of 2% of humans.[44] This would suggest that there may be differences in the ability of microbes to colonize the eyes of mice and humans. This may be attributable to environmental differences between mice and humans. Additionally, mucins, lipids, epithelial surface proteins, and/or other host factors may make the ocular surface environment favorable to specific microbes. More work is required to better understand these differences and how they affect the composition of the ocular surface microbiome and the associated host immune responses. We expect that the procedures outlined here will provide a road map to generating *C. mast* mutants to assess the microbial genes responsible for colonization of the human ocular surface. That said, in vitro models of colonization using cornea/conjunctival epithelial cells and/or organoid systems will require development to optimally test how this and other microbes may colonize the human eye.

In summary, to the best of our knowledge, this study is the first of its kind in the eye where an ocular commensal was genetically modified to express genes of interest. In addition, we have shown that even with our genetic modifications, the bacterium is still able to stably colonize the ocular mucosa while stimulating a beneficial immune response similar to WT *C. mast.* This has revealed a new tool to study the dynamics of the ocular microbiome in real time while paving the way for future genetic manipulations

for the purpose of identifying genes responsible for ocular colonization and creating a long-term drug delivery vehicle for inflammatory ocular surface diseases.

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