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Murine Vaginal Colonization Model for Investigating Asymptomatic Mucosal Carriage of *Streptococcus pyogenes*

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While many virulence factors promoting *Streptococcus pyogenes* invasive disease have been described, specific streptococcal factors and host properties influencing asymptomatic mucosal carriage remain uncertain. To address the need for a refined model of prolonged *S. pyogenes* asymptomatic mucosal colonization, we have adapted a preestrogenized murine vaginal colonization model for *S. pyogenes*. In this model, derivatives of strains HSC5, SF370, JRS4, NZ131, and MEW123 established a reproducible, asymptomatic colonization of the vaginal mucosa over a period of typically 3 to 4 weeks' duration at a relatively high colonization efficiency. Prior treatment with estradiol prolonged streptococcal colonization and was associated with reduced inflammation in the colonized vaginal epithelium as well as a decreased leukocyte presence in vaginal fluid compared to the levels of inflammation and leukocyte presence in non-estradiol-treated control mice. The utility of our model for investigating *S. pyogenes* factors contributing to mucosal carriage was verified, as a mutant with a mutation in the transcriptional regulator catabolite control protein A (CcpA) demonstrated significant impairment in vaginal colonization. An assessment of *in vivo* transcriptional activity in the CcpA⁻ strain for several known CcpA-regulated genes identified significantly elevated transcription of lactate oxidase (*lctO*) correlating with excessive generation of hydrogen peroxide to self-lethal levels. Deletion of *lctO* did not impair colonization, but deletion of *lctO* in a CcpA⁻ strain prolonged carriage, exceeding even that of the wild-type strain. Thus, while LctO is not essential for vaginal colonization, its dysregulation is deleterious, highlighting the critical role of CcpA in promoting mucosal colonization. The vaginal colonization model should prove effective for future analyses of *S. pyogenes* mucosal colonization.

The Gram-positive pathogen *Streptococcus pyogenes*, or group A streptococcus, is responsible for a wide variety of clinical manifestations ranging from the relatively benign and superficial otitis media, impetigo, and pharyngitis to less common and more invasive conditions, including necrotizing fasciitis and toxic shock syndrome, in addition to the postinfectious complications rheumatic fever and glomerulonephritis (1). The ability to produce such a diversity of infections in so many different tissue compartments is testament to the extensive plasticity of the *S. pyogenes* transcriptome and an abundance of secreted virulence factors (2). While not considered normal human flora, *S. pyogenes* can be identified as a colonizer of mucosal surfaces of the oropharynx, rectum, and vaginal mucosa, and a prolonged asymptomatic carriage state can develop (3–7). How *S. pyogenes* carriage exists on a mucosal surface without inducing disease is poorly understood, but the issue is significant in terms of gaining an improved understanding of host-pathogen interactions and the regulation of mucosal immunity. Furthermore, asymptomatic oropharyngeal carriage of *S. pyogenes* continues to confound the results of clinical testing for those with symptoms of pharyngitis, frequently, children with viral infections, in which the detection of carrier strains mistakenly results in inappropriate antibiotic exposures and expenses (8, 9).

Modeling of asymptomatic mucosal carriage in the human-restricted organism *S. pyogenes* has been challenging. Although murine and primate models of *S. pyogenes* oropharyngeal and nasopharyngeal colonization exist, their utility has several limitations, including limited access to the pharyngeal tissue site, the high cost and maintenance requirements of a primate colony, and the fact that many of these models are not capable of supporting more than a few specific strains of *S. pyogenes* for a limited period of time at a relatively low efficiency of colonization (10, 11). To overcome several of these obstacles, we have taken a novel approach by adapting a murine vaginal epithelial model to investigate *S. pyogenes* colonization and prolonged carriage.

Murine vaginal colonization models in preestrogenized mice have previously been used to investigate the mucosal biology of human pathogens, including *Candida albicans* (12), *Trichomonas vaginalis* (13), group B streptococcus (14), and *Neisseria gonorrhoeae* (15). The anatomy and physiology of the murine vaginal mucosa are quite similar to those of the human vaginal mucosa, and both respond similarly to estrogen cycling, with thickening and proliferation of the epithelial surface and accumulation of glycogen in the intermediate and superficial layers, although to a lesser degree in mice than in humans (16). While mice have a 10- to 25-fold lower level of serum estrogen, they respond dramatically to exogenous estradiol treatment, with enhanced glycogen deposition, intense epithelial proliferation, and increased epithe-

lial cell thickness, which overall presents a more human-like vaginal profile (17, 18). In addition, human and murine vaginal secretions have similar compositions, with similar concentrations of glucose (3.3 mM and 4.4 mM, respectively) and lactate (6.2 mM and 8.4 mM, respectively) (19).

Asymptomatic human vaginal carriage of *S. pyogenes* is typically detected at a low frequency among women of reproductive age, about 0.03% to 1% (3, 20). Group A streptococcus can be isolated from vaginal swab specimen cultures at a greater frequency from cases of vulvovaginitis and puerperal sepsis, often occurring with prior or concurrent pharyngitis (21–24). In particular, *S. pyogenes* serotype M28 isolates are overrepresented in cases of human female urogenital tract infection, puerperal sepsis, and neonatal infections (25–27). All M28 isolates and isolates of other serotypes associated with puerperal infections exhibit carriage of a 37.4-kb genomic island designated region of difference 2 (RD2), which is shared with group B streptococcus and which contains 7 putative proteins and potential virulence factors (28, 29). Hence, investigation of *S. pyogenes* vaginal colonization and invasive infections represents an opportunity to gain greater insight into bacterial pathogenesis, particularly with regard to interactions with the host mucosal immunity.

The regulation of metabolism and virulence factor expression among carriage isolates is an active field of investigation. Global regulators of bacterial gene transcription sensitive to environmental cues are increasingly recognized for their importance in pathogenesis (for a review, see references 30 and 31). Environmental carbohydrate concentrations have been determined to be a major environmental cue for *S. pyogenes* that is directly coupled to transcriptional regulation through the process of carbon catabolite repression (CCR) and the major CCR regulator catabolite control protein A (CcpA) (32–35). CcpA is a member of the LacI/GalR family of activator/repressor transcription factors and is widely conserved among low-G+C-content Gram-positive prokaryotes, including *S. pyogenes*. A detailed discussion of the biochemistry of CcpA-dependent CCR is beyond the scope of this article but has been discussed elsewhere (36). In short, the accumulation of glycolytic intermediates, including fructose-1,6-bisphosphate, from metabolism in a carbohydrate-rich environment results in a signal cascade activating CcpA. CcpA influences transcriptional expression by binding to operator sequences termed catabolite-responsive elements (cre), usually adjacent to or within promoter sequences of open reading frames regulated by CcpA, and regulating access of the RNA polymerase complex (36). Within *S. pyogenes*, carbon catabolite repression is central to global transcriptional regulation, not only by regulating carbohydrate metabolism pathways but also by functioning as a transcriptional activator or repressor of several important virulence factors, including the cysteine protease SpeB, the cytolysin streptolysin S, hyaluronic acid capsule, the extracellular DNase Spd, and the immunoglobulin-degrading EndoS (32–34, 37). Given the central role of CcpA in transcriptional regulation, it follows that loss of CcpA activity would be associated with dysregulation of gene expression at a detriment to pathogenesis. Supporting evidence is that *S. pyogenes* CcpA⁻ strains have been demonstrated to have reduced virulence in the murine skin ulcer model (34), reduced virulence in a murine intraperitoneal model of invasive infection, and impaired colonization in the murine oropharynx (32). While the mechanism of attenuated virulence in the CcpA⁻ strains has not been determined, we would hypothesize that the loss of CcpA-dependent

transcriptional regulation contributes to a loss of virulence through dysregulation of gene expression, with an inappropriate timing and/or magnitude of gene transcription impairing fitness.

In this study, we have adapted a preestrogenized murine vaginal mucosa model to investigate *S. pyogenes* colonization and carriage. We report that several *S. pyogenes* strains from diverse clinical backgrounds are capable of prolonged carriage at a relatively high degree of colonization efficiency. Estrogenization of mice was shown to promote a sustained and asymptomatic vaginal colonization analogous to that found in women of reproductive age with normal estrogen levels (3, 22, 23). Furthermore, we have validated the utility of our model for investigating streptococcal factors, in demonstrating that the absence of CcpA inhibits prolonged murine vaginal carriage through dysregulation of gene expression, including increased lactate oxidase (LctO) expression *in vivo*, likely limiting mucosal survival. This report adds a novel animal model to the field of *S. pyogenes* colonization and carriage and further contributes to our understanding of the role of global transcriptional regulators and their role in the biology of mucosal pathogens.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The principal strain used in this study was *S. pyogenes* HSC12, a spontaneous streptomycin-resistant derivative of the M14 strain HSC5 (38). Whole-genome sequencing has revealed that HSC12 contains a single nucleotide polymorphism at nucleotide position 1960 (AAA to ACA) in the 30S ribosomal subunit S12p (*rpsL*; SPy_0271) resulting in a missense mutation (K56T) conferring high-level streptomycin resistance (M. E. Watson, Jr., G. C. Port, and M. C. Caparon, unpublished data). Other strains used are listed in Table 1. Unless otherwise stated, all *S. pyogenes* strains had equivalent growth rates and yields in the media tested (data not shown). Routine culture of *S. pyogenes* was performed in Todd-Hewitt medium (Becton, Dickinson, Franklin Lakes, NJ) supplemented with 0.2% (wt/vol) yeast extract (Difco Laboratories, Detroit, MI) (THY medium). Where required, Bacto agar (Difco) was added to a final concentration of 1.4% (wt/vol) to produce solid medium. Experiments that required low glucose concentrations were performed in C medium (41). Incubation was performed at 37°C under anaerobic conditions (GasPak; Becton, Dickinson) for solid medium or in sealed tubes without agitation for liquid medium. Aerobic culture was conducted as described previously (33). For inoculation of mice, *S. pyogenes* was harvested from culture in THY broth at early logarithmic phase (optical density at 600 nm [OD_{600}], 0.2), washed once in phosphate-buffered saline (PBS), briefly sonicated on ice to disrupt long streptococcal chains, and resuspended in PBS to 10^8 CFU/ml. For cloning of recombinant DNA, *Escherichia coli* strain DH5α or TOP10 (Invitrogen, Grand Island, NY) was cultured in Luria-Bertani (LB) broth. When appropriate, antibiotics were added at the following concentrations: erythromycin, 500 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*; spectinomycin,100 μg/ml for both *E. coli* and *S. pyogenes*; and streptomycin, 1,000 μg/ml for *S. pyogenes* (all were obtained from Sigma Chemical Co., St. Louis, MO).

DNA manipulation and strain construction. Plasmid DNA was isolated by standard methods and used to transform *S. pyogenes* as previously described (42). Restriction endonucleases, ligases, and polymerases were used according to the manufacturer's recommendations (New England BioLabs, Ipswich, MA). The fidelity of all constructs derived by PCR was confirmed by DNA sequencing analysis performed by a commercial vendor (Integrated DNA Technologies, Inc., Coralville, IA). All references to genomic loci are based on the genome of *S. pyogenes* strain SF370 (43). In-frame deletion mutations were constructed using a routine method of allelic exchange (44) via *S. pyogenes* transformation by electroporation with derivatives of the *E. coli*-to-streptococcus temperature-sensitive shuttle vector pJRS233, previously reported as pCK195 for *ccpA*

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description (M type or phenotype)	Reference or source
Strains		
S. pyogenes		
JRS4	Spontaneous streptomycin-resistant derivative of strain D471 (M6)	50
SF370	Invasive isolate from wound infection (M1)	43
HSC12	Spontaneous streptomycin-resistant derivative of strain HSC5 *rpsL*$_{K56T}$[c] (M14)	38
MEW16	HSC12 with a *lctO* in-frame deletion produced by allelic exchange with pCK37 (LctO$^-$)	This study
MEW29	Spontaneous streptomycin-resistant derivative of strain SF370	This study
MEW41	HSC12 with a *ccpA* in-frame deletion produced using plasmid pCK195 (CcpA$^-$)[a]	This study
MEW47	MEW41 with a *lctO* in-frame deletion produced by using plasmid pCK37 (CcpA$^-$ LctO$^-$)[a]	This study
MEW106	MEW41 with full-length *ccpA* produced using plasmid pMEW74,[a] WT revertant (CcpA^r)	This study
MEW123	Streptomycin-resistant derivative of pediatric pharyngitis isolate (M28) produced by allelic exchange with pMEW96	This study
MEW132	Spontaneous streptomycin-resistant derivative of strain NZ131 from glomerulonephritis (M49)	J. Chang[b]
E. coli DH5α	F′ φ80d*lacZ*ΔM15 Δ(*lacIZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r$_k$$^-$ m$_k$$^+$) λ$^-$ *thi-1 gyrA96 relA1* Nal^r	Invitrogen
Plasmids		
pJRS233	6.0-kb *E. coli*-streptococcal temperature-restrictive shuttle vector for allelic exchange, erythromycin resistance marker	44
pCK37	pJRS233 with a *lctO* in-frame deletion allele	33
pCK195	pJRS233 with a *ccpA* in-frame deletion allele	33
pMEW74	pJRS233 with a *ccpA* full-length open reading frame for complementation	This study
pMEW96	pJRS233 with an *rpsL* point mutation allele (K56T) conferring streptomycin resistance after allelic exchange	This study

[a] The plasmid listed was used to replace the wild-type allele in the genome by a standard method (44).
[b] Original NZ131 streptomycin-resistant isolate derived by Jennifer Chang and Michael Federle (University of Illinois—Chicago).
[c] *rpsL*$_{K56T}$, the K-to-T change at position 56 encoded by *rpsL*.

(SPy_0514) and pCK37 for *lctO* (SPy_0414) (33). Generation of spontaneous streptomycin-resistant isolates was performed by plating a high density of *S. pyogenes* onto THY agar plates supplemented with 1 mg/ml streptomycin and incubation at 37°C overnight; streptomycin-resistant clones occurred in approximately 1 of 10^9 CFU plated. All mutants were confirmed by PCR, DNA sequencing, and phenotypic assays, when available. Descriptions of the resulting strains are listed in Table 1.

Reversion of the CcpA$^-$ strain to CcpA^r. Although several plasmid systems for *S. pyogenes* exist, none proved suitable for long-term *in vivo* stability in the absence of selective antibiotic pressure. Therefore, to reverse the *ccpA* in-frame deletion mutation, a strategy was implemented to restore the wild-type (WT) locus in the CcpA$^-$ mutant. A 2.2-kb fragment containing the entire *ccpA* open reading frame and flanking regions was amplified from HSC12 genomic DNA using primers CK214 (5′-CCCAT CGATGAGCGATGACGTCTGTTTCGGTAG-3′) and CK217 (5′-CCCG GATCCGGATCTGCTCCGTACTGCTCC-3′) (33), which were digested with ClaI and BamHI, respectively (sequences underlined in the primers), and inserted between the same sites of pJRS233 (44). The resulting plasmid (pMEW74) was then used to restore the wild-type *ccpA* locus as described above, creating strain MEW106 with restored CcpA (CcpA^r) (Table 1).

Murine vaginal colonization model. C57BL/6J and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were allowed to acclimatize for 1 week prior to manipulations. All mice were female and unless otherwise specified were used at 7 to 8 weeks of age. To synchronize estral cycles, mice were estrogenized by intraperitoneal injection of up to 0.5 mg β-estradiol 17-valerate (Sigma) dissolved in 0.1 ml sterile sesame oil (Sigma) 2 days prior to streptococcal inoculation and again on the day of inoculation (day 0). In some experiments, mice received intraperitoneal injections of 0.1 ml sterile sesame oil as a vector-only control; these mice are referred to as mock treated throughout this article. On day 0, mice were sedated by brief inhalation of 80% carbon

dioxide–20% oxygen gas, and the various inocula of *S. pyogenes* described in the text were instilled into the vaginal vault using a P20 micropipette (Gilson, Inc., Middleton, WI) in a total volume of 20 μl PBS. In some experiments, control mice received a vaginal instillation of phosphate-buffered saline alone; these mice are referred to as uncolonized throughout this article. At successive intervals over a 1-month period postinoculation, the vaginal vaults of sedated mice were gently washed with 50 μl PBS and serial dilutions in PBS were plated onto THY agar plates containing 1,000 μg/ml streptomycin to determine the numbers of viable CFU. This concentration of streptomycin suppressed the growth of the normal mouse vaginal flora but had no effect on the growth of the streptomycin-resistant *S. pyogenes* strains. The detection limit was mathematically calculated as the CFU density below which our drop-plating method may not detect the growth of viable streptococci. The drop-plating method involves making 10-fold serial dilutions of the vaginal wash specimens in phosphate-buffered saline and spotting 6-μl aliquots of the dilutions and an undiluted neat specimen onto agar plates in duplicate. The average number of colonies counted between duplicate spots from 6 μl was then adjusted for the dilution factor to calculate the number of CFU/ml or the number of CFU per 50-μl vaginal wash. The fewest number of colonies that could be identified was 1 colony between 2 duplicate spots, and when this average number (0.5) was adjusted for the dilution factor and plating volume (6 μl), the resulting number was approximately 84 CFU/ml. For colonization experiments, between 5 and 20 mice were tested per *S. pyogenes* strain, as indicated in the relevant figure legends. This experimental protocol (20100186) was approved by the Animal Studies Committee of the Washington University School of Medicine.

Determination of leukocyte density in vaginal wash specimens. Aliquots of vaginal wash specimens (20 μl) were spotted onto glass microscope slides (Fisher Scientific, Pittsburg, PA) without dilution and allowed to air dry. Specimens were heat fixed and then stained using a commercially available, modified Wright-Giemsa stain (Kwik-Diff;

Thermo Fisher Scientific, Waltham, MA). Stained slides were examined under a bright-field microscope at ×10 to ×40 magnification using a Leica DM100 microscope (Leica Microsystems Inc., Buffalo Grove, IL), and the proportions of nucleated epithelial cells, anucleated cornified epithelial cells, and leukocytes were determined (40). Data presented are the means and standard errors of the means derived from at least 3 independent experiments, where each individual experimental point was analyzed by assessment of vaginal wash specimens from at least 3 mice per group. For each assessment of leukocyte density, multiple fields of view were examined.

Histologic examination of mouse vaginal tissue. In selected experiments mice were euthanized and the vaginal canal was removed and bisected longitudinally for analysis as follows: one half of the vagina was immediately placed into 4% paraformaldehyde in PBS for fixation, and the other half was immediately homogenized for recovery of whole-tissue numbers of CFU or RNA (see below). Following overnight fixation, the tissues were placed in ethanol and then encased in paraffin, sectioned, and stained with Gram stain, hematoxylin-eosin, and periodic acid-Schiff (PAS) by the histology core facility at the Washington University School of Medicine. Assessment of epithelial growth was performed by measuring the height of the epithelial layer from the basal lamina to the apical surface using ×40 magnification and the microscope's ocular micrometer (45).

Transcript analysis by real-time reverse transcription-PCR (RT-PCR). Following collection, RNA in vaginal wash fluid was stabilized by addition of 10 volumes of RNAlater solution (Qiagen, Valencia, CA), and samples were immediately processed or frozen at −80°C for later use. Total RNA was isolated from vaginal wash fluid or homogenized vaginal tissue by shaking with 0.1-mm silica spheres using Lysing Matrix B FastRNA tubes and a FastPrep high-speed reciprocating shaking device (QBioGene, Carlsbad, CA). RNA was further purified by use of an RNeasy minikit (Qiagen). Genomic DNA was removed by digestion during isolation (RNase-free DNase set; Qiagen) and by treatment with DNase I (amplification grade; Invitrogen) following spin-column purification. The integrity of the RNA was assessed by electrophoresis, and purity was measured by determination of the A_{260}/A_{280} ratio (purity was acceptable if it was >1.8). Synthesis of cDNA was performed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time amplification of select genes was performed using an iCycler thermocycler (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). The primers used are listed in Table S1 in the supplemental material. Relative transcript levels were determined using the $2^{-\Delta\Delta CT}$ method (46). Transcript levels for *recA* (SPy_2116) were used to normalize expression levels for each gene of interest, as the level of *recA* transcription is stable using the indicated experimental conditions (47). The specificity of the primers for *S. pyogenes*-specific cDNA was evaluated using RNA samples prepared from estradiol-treated but non-colonized mice; under these conditions, the primer sets either failed to produce DNA amplification products or produced amplicons that were distinctly different by melting point analysis (data not shown). Values represent the means and standard errors of the means of three independent experiments, each analyzed in duplicate (vaginal washes from six mice used per group).

H₂O₂ production and sensitivity to H₂O₂. Production of hydrogen peroxide (H₂O₂) was measured following growth in liquid C medium as previously reported (48). Data reported are the means and standard errors of the means normalized to the results for strain HSC12 in C medium (set to 100%) from at least three independent measurements from separate days. All of the experimental comparisons were performed in parallel, and similar trends were observed with shorter periods of incubation (harvesting of the medium at earlier time points in stationary phase), although peroxide accumulation was maximal in our studies after overnight growth (late stationary phase), and therefore, this point was chosen for these experiments. For determination of the sensitivity of strains to exogenous peroxide, 5 μl of overnight growth in THY broth was inoculated into 200 μl of fresh THY medium supplemented with up to 5 mM hydrogen per-

oxide (Sigma), cultured in 96-well plates sealed with plastic tape, and incubated overnight at 37°C. The indicated peroxide concentration is the concentration prior to inoculation and growth. The final growth density was measured by determination of the absorbance (OD_{600}) using a microplate reader (Infinite 200 PRO series; Tecan US, Inc., Durham, NC). Shown are mean and standard error of the mean values; each point is representative of 2 independent experiments, each performed in triplicate.

Statistical analyses. Differences in the duration of *S. pyogenes* vaginal carriage were tested for significance using a log-rank analysis of Kaplan-Meier product limit estimates. Differences between groups for recovery of CFU in vaginal washes and in leukocyte counts from vaginal smears over time were tested using a repeated-measures analysis of variance. Differences in relative transcript levels, peroxide production, and sensitivity were tested for significance with a two-tailed paired *t* test. For all tests, the null hypothesis was rejected for *P* values of <0.05. For statistical computation, the resources available in GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) or the VassarStats Site for Statistical Computation by Richard Lowry (http://faculty.vassar.edu/lowry/VassarStats .html) were used.

RESULTS

Colonization of murine vaginal mucosa by *S. pyogenes* is enhanced by pretreatment with estradiol. For many pathogens, colonization of the murine vaginal mucosa is enhanced by pretreatment with estradiol and is more efficient during the estrus phase of the estral cycle for reasons that have not been fully established (12–15, 49). To determine if *S. pyogenes* vaginal carriage responded similarly, C57BL/6J or BALB/c mice received intraperitoneal injections of 17β-estradiol at 48 h prior to inoculation and again on the day of inoculation (day 0). Approximately 1×10^6 CFU of *S. pyogenes* was then deposited into the vaginal vault in 20 μl of PBS, and colonization was assessed by determination of the number of *S. pyogenes* CFU recovered from a single 50-μl wash. Individual samples were collected intermittently over a period of 34 days. In C57BL/6J mice treated with vehicle alone (sesame oil with no estradiol), inoculation with wild-type strain HSC12 exhibited a median duration of colonization of 13 days (range, 2 to 22 days; Fig. 1A). Mice treated with injections of 0.005 mg of estradiol had a median duration of colonization of 14 days (range, 12 to 22 days; P = 0.477). However, higher doses resulted in significantly enhanced colonization, as treatment with 0.05 or 0.5 mg of estradiol resulted in median durations of colonization of 22 days (range, 18 to up to 34 days; P = 0.0207) and 32 days (range, 26 to up to 34 days; P < 0.0001), respectively (Fig. 1A). Estradiol-treated mice maintained a higher *S. pyogenes* bacterial density over time, which for pretreatment with 0.5 mg estradiol remained consistently between 10^6 and 10^7 CFU recovered in washes over the first 18 to 22 days (Fig. 1B). In contrast, for mice treated with vehicle alone, the decline in the numbers of recoverable *S. pyogenes* CFU was significantly more rapid (Fig. 1B). Thus, colonization was sensitive to the amount of estradiol, with increasing doses correlating with a concomitant increase in recovery of *S. pyogenes* from washes (data not shown for 0.005 and 0.05 mg estradiol-treated groups). However, colonization was not sensitive to a higher inoculum, as infection by 1×10^8 CFU did not result in significantly higher levels of carriage following day 4 (data not shown). Colonization capacity and the numbers of recoverable CFU were similar in response to estradiol supplementation in BALB/c mice, suggesting that this model could potentially be applied to several mouse strains (data not shown). Remarkably, colonized mice were asymptomatic and demonstrated no overt signs

FIG 2 Estradiol treatment promotes vaginal mucosal thickening and decreased inflammation in *S. pyogenes*-colonized vaginal tissue. Estradiol-treated (0.5 mg) and mock-treated mice were left uncolonized or were colonized by vaginal inoculation of 1×10^6 CFU of *S. pyogenes* HSC12. Shown are cross sections of vaginal walls from organs harvested at 8 days postinoculation that were stained with hematoxylin-eosin and imaged. Anatomy landmarks are the vaginal lumen (VL), epithelium (E), and lamina propria (LP). (A) Mock treated, uncolonized; (B) mock treated, colonized; (C) estradiol treated, uncolonized; (D) estradiol treated, colonized. Note the thickening of the epithelium in panels C and D following estradiol treatment. For colonized tissue, note the decreased numbers of leukocytes (arrow in panel B) in the epithelium of estradiol-treated (D) versus non-estradiol-treated (B) mice. Magnifications, ×40.
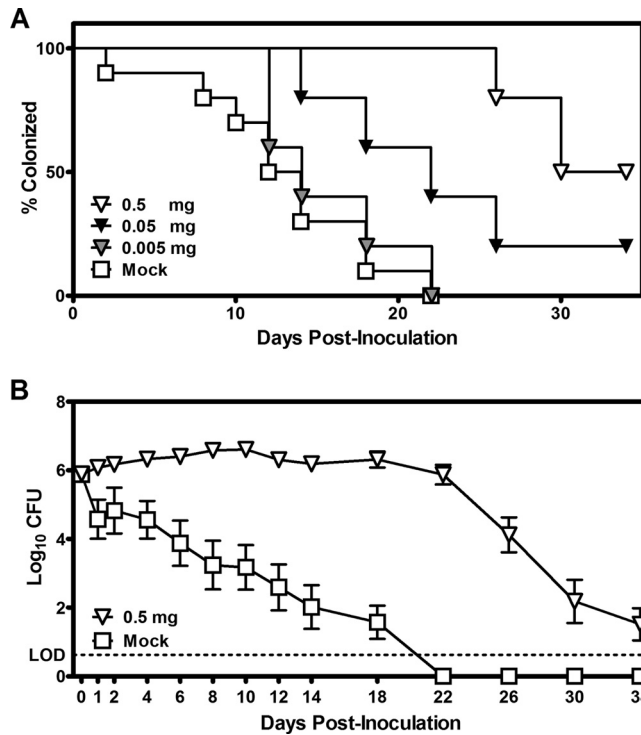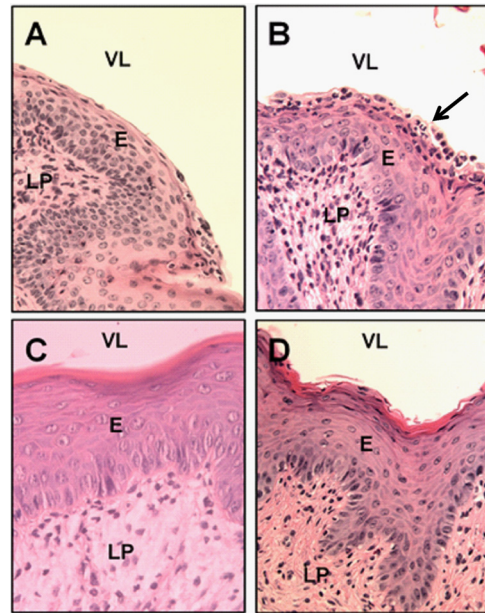
FIG 1 Estrogenization promotes *S. pyogenes* vaginal colonization. Groups of 5 C57BL/6J mice each were pretreated with the indicated concentrations of estradiol or with sterile sesame oil only (mock). Mice received treatments at day −2 and just prior to vaginal inoculation (day 0). Mice were then challenged with 1×10^6 CFU of *S. pyogenes* HSC12, and the numbers of CFU were determined at the time points indicated by washing the vaginal vault with 50 μl of PBS and plating the washes on medium containing streptomycin. Data for mice treated with 0.5 mg of estradiol and mock-treated mice are pooled from 3 independent experiments, representing a total of 15 mice each; data for mice treated with 0.005 mg and 0.05 mg of estradiol are from a single experiment, representing 5 mice each. (A) Kaplan-Meier analysis to compare the number of mice from each group that remained colonized. A log-rank analysis indicated that treatment with 0.5 mg ($P < 0.001$) and 0.05 mg ($P < 0.05$) estradiol promoted a significantly longer period of colonization than mock treatment. (B) Comparison of the number of *S. pyogenes* CFU recovered in vaginal washes. Shown are the mean and standard error of the mean of the number of CFU recovered in 50-μl washes at each time point. An individual mouse was considered colonized when any streptomycin-resistant *S. pyogenes* isolate was recovered in culture at a level above the limit of detection (LOD), calculated to be 84 CFU/ml. A repeated-measures analysis of variance test comparing the number of CFU recovered over the time points assayed indicated that the group treated with 0.5 mg estradiol exhibited significantly greater numbers of CFU recovered than the mock-treated group ($P < 0.001$).

of illness, including fur ruffling, lethargy, weight loss, ulcer formation, or purulent vaginal discharge. Since pretreatment with 0.5 mg estradiol followed by inoculation with 1×10^6 CFU of *S. pyogenes* supported the longest carriage duration and the highest bacterial load in the absence of any adverse effects of estradiol supplementation, such as overgrowth of normal vaginal flora contaminating our selective medium containing streptomycin or otherwise causing illness in the mice, this regimen was chosen for use in subsequent experiments, unless otherwise stated.

Estradiol supplementation is associated with decreased inflammation in *S. pyogenes*-colonized murine vaginal tissue. Estradiol is known to promote the estrus phase of the murine estrous cycle, a period associated with proliferation and thickening of the

vaginal epithelium, production of glycogen, and a reduction in the presence of inflammatory cells (15). To characterize the changes elicited by the treatment regimen used here and by *S. pyogenes* colonization, a histological analysis was conducted. Following treatment with vehicle alone, the normal and healthy appearance of the vaginal tissue of C57BL/6J mice in the absence of colonization (Fig. 2A) changed following infection, with the presence of an increased number of inflammatory cells, particularly at the epithelial surface, as noted by day 8 postinoculation (Fig. 2B, arrow). Preestrogenized mice exhibited a significant thickening of the vaginal epithelium (Fig. 2C), with an associated significant increase in the quantified height from the basal lamina to the apical surface (9.3 ± 0.9 units versus 16.6 ± 1.7 units; $P < 0.001$). In addition, staining of sections from pretreated mice with the periodic acid-Schiff (PAS) reagent, used for glycogen staining, showed an increased amount of staining, suggestive of increased glycogen deposition (data not shown). In contrast to non-estradiol-supplemented mice, supplemented mice did not exhibit any apparent increase in the presence of inflammatory cells following colonization with *S. pyogenes* at day 8 (compare Fig. 2C and D), and the epithelium appeared intact and healthy.

Estradiol supplementation suppresses vaginal fluid leukocyte concentration. The analysis presented above suggested that a decreased presence of inflammatory cells correlated with the ability of estradiol to enhance *S. pyogenes* colonization. To examine this in greater detail, the composition of vaginal cells in smears of
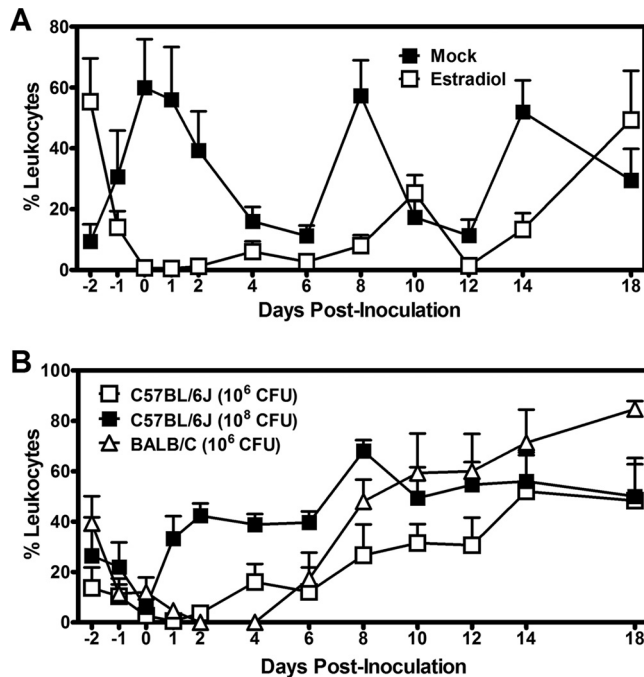
FIG 3 Leukocyte concentration in vaginal wash specimens. Vaginal washes from estradiol-treated (0.5 mg) and mock-treated mice were collected at the indicated time points following challenge with *S. pyogenes* HSC12. Samples were stained with a modified Wright-Giemsa stain for determination of the number of leukocytes by microscopic examination. Leukocyte concentrations are expressed as the percentage of leukocytes relative to the total number of cells recovered. Each symbol represents the mean and standard error of the mean derived from 6 mice at each time point. Differences between mean values over time were tested for significance using a repeated-measures analysis of variance. (A) C57BL/6J mice were treated as indicated, and vaginal washes were analyzed for leukocyte influx. Leukocyte concentrations were significantly lower in estradiol-treated mice over the period of observation ($P <$ 0.01). (B) BALB/c or C57BL/6J mice were estradiol treated and challenged with *S. pyogenes* HSC12 at the inoculum densities indicated. Over the period of observation, a dose of 1×10^8 CFU and colonization of BALB/c mice were both associated with significantly higher leukocyte concentrations relative to those in C57BL/6J mice challenged with 1×10^6 CFU ($P < 0.01$).

vaginal fluid was examined since this predictably fluctuates throughout the estrous cycle and can be used to determine the stages of individual mice and monitor mucosal inflammation (40). As expected for mice cycling with natural levels of endogenous hormones, C57BL/6J mice injected with sterile sesame oil alone and inoculated with PBS exhibited only a fluctuating density of leukocytes that peaked every 4 to 6 days (Fig. 3A). In contrast, estradiol-treated mice had a significant blunting of leukocyte density that appeared within 24 h and persisted for about 10 to 14 days (Fig. 3A). When estradiol-treated mice were colonized with 10^6 CFU HSC12, there was not a statistically significant increase in the vaginal fluid leukocyte presence compared to that for estradiol-treated but uncolonized mice (compare Fig. 3B to A), replicating a relatively asymptomatic carriage observed in women of reproductive age. Despite estradiol supplementation, the vaginal mucosa was still capable of mounting an inflammatory response, as a higher HSC12 inoculum (1×10^8 CFU) elicited a significant increase in vaginal fluid leukocyte density (Fig. 3B). Higher leukocyte densities developed at the later time points and were temporally associated with the beginning of a decline in viable *S. pyogenes*

HSC12 recovered from vaginal washes (Fig. 3B; compare to Fig. 1B). As mentioned above, there was no significant difference in *S. pyogenes* colonization frequency and the number of CFU recovered from vaginal washes between C57BL/6J and BALB/c mouse strains; however, estradiol-treated BALB/c mice showed significantly elevated numbers of leukocytes in vaginal washes compared to C57BL/6J mice (Fig. 3B). Therefore, leukocyte influx into vaginal fluid did not correlate exactly with the clearance of *S. pyogenes* from vaginal carriage, as colonization persisted through periods of high vaginal fluid leukocyte density and eventually declined over time. Monitoring of leukocyte influx following colonization may better serve as a marker of inflammation rather than an indicator of *S. pyogenes* clearance.

Estradiol treatment promotes colonization of multiple *S. pyogenes* strains. In order to assess the compatibility of the preestrogenized murine vaginal carriage model with different *S. pyogenes* strains, five diverse *S. pyogenes* strains with distinct M types were tested, including HSC12 (M14), MEW29 (M1), JRS4 (M6), MEW123 (M28), and MEW132 (M49) (listed in Table 1) (38, 43, 50). In particular, note that MEW123 is an M28 isolate, and members of this group are overrepresented in cases of human female urogenital tract infection, puerperal sepsis, and neonatal infections (25–27). All strains were able to establish long-term asymptomatic colonization in estradiol-pretreated mice (Fig. 4). There was no significant difference in the kinetics of clearance between MEW29, MEW123, and HSC12, and all three strains had similar median durations of colonization (range, 6 to 34 days) over the 34-day period of observation. However, despite similar median durations of colonization, analysis of clearance kinetics revealed that carriage by JRS4 and MEW132 was significantly prolonged compared to that by HSC12, MEW29, and MEW123 (Fig. 4). When colonization densities were compared, the numbers of CFU of HSC12, MEW123, and MEW132 recovered in vaginal washes were consistently and significantly higher over the first 22 days than the numbers of CFU of either JRS4 or MEW29 (Fig. 4). Thus, the preestrogenized murine vaginal carriage model is robust in its ability to support colonization by multiple diverse strains, although the different strains produce varied patterns of colonization.

***S. pyogenes* CcpA promotes mucosal carriage.** Given the proximity of glycogen and other complex carbohydrate substrates in vaginal epithelial tissue, the role of CcpA, a central regulator of gene expression in response to carbohydrates, was examined. Since strain HSC12 consistently showed stable high-level colonization over a period of 3 to 4 weeks, this strain was chosen for this analysis and is referred to as the wild type (WT). When a mutant of HSC12 containing an in-frame deletion of *ccpA* (SPy_0514) was inoculated into preestrogenized C57BL/6J mice, it was found to be significantly attenuated for carriage at the vaginal mucosa, having a median duration of 8 days (range, 2 to 14 days) versus the 30 days (range, 26 to up to 34 days) obtained for the WT strain (Fig. 5A). Restoration of the wild-type *ccpA* locus in the mutant background also restored the ability of the resulting CcpA^r strain (MEW106) to colonize at levels equivalent to those for the wild-type strain with a median duration of up to 34 days (range, 26 to 34 days; Fig. 5A), and the numbers of recoverable CFU from vaginal washes were not significantly different from those for the wild-type strain (Fig. 5B). Determination of the numbers of viable CFU showed a rapid decline in the recovery of the CcpA$^-$ strain from vaginal washes compared to the levels of recovery of the CcpA^r and wild-type
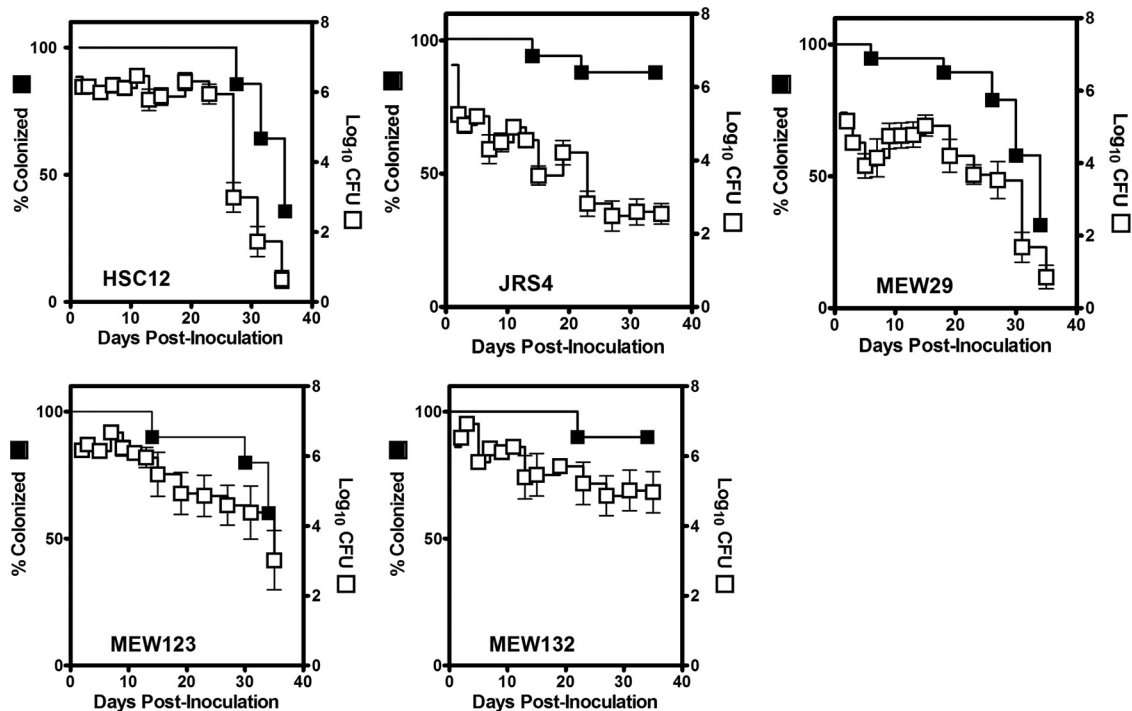
FIG 4 Several strains of *S. pyogenes* are capable of colonizing the murine vagina. Estradiol-treated mice (0.5 mg) were vaginally challenged with 1×10^6 CFU of *S. pyogenes* strains HSC12, JRS4, MEW29, MEW123, and MEW132. The left axis of each panel shows the results of a Kaplan-Meier analysis of the percentage of mice that remained colonized at the indicated time points. The right axis shows the mean number of CFU recovered in vaginal washes, where each symbol represents the mean and standard error of the mean derived from 14 (HSC12), 18 (JRS4), 19 (MEW29), 10 (MEW123), and 10 (MEW132) mice challenged with the indicated strains. A log-rank analysis indicated that strains HSC12, MEW29, and MEW123 had significantly shorter durations of colonization than strain JRS4 or MEW132 ($P < 0.01$). A repeated-measures analysis indicated that strain HSC12 had a significantly higher number of CFU over the period of observation than strains JRS4 ($P < 0.001$), MEW29 ($P < 0.001$), and MEW132 ($P < 0.01$).

HSC12 strains (Fig. 5B). Analysis of homogenates of vaginal tissue revealed that no CFU were recovered from mice that had cleared the CcpA⁻ strain, as assessed by recovery in vaginal washes, showing that the mutant did not continue to persist in an intracellular reservoir (data not shown). A histological analysis showed that infection by the CcpA⁻ strain did not produce inflammation and there was no significant influx of leukocytes into the vaginal fluid during the clearance phase of the infection (data not shown). Taken together, these results demonstrate that CcpA plays a critical role in promoting mucosal colonization.

Dysregulation of gene expression in the CcpA⁻ mutant contributes to impaired mucosal carriage. In order to investigate the molecular basis for the attenuation of the CcpA⁻ strain, gene transcript levels were analyzed following recovery of streptococcal RNA from vaginal wash specimens. The specific transcripts measured (Table 2) were chosen on the basis of regulation by CcpA both during *in vitro* growth and during infection of murine subcutaneous tissue (33, 34) and were analyzed by real-time RT-PCR at 48 h and 96 h postinoculation. Surprisingly, relatively few transcripts were found to differ in expression between the CcpA⁻ strain and the WT strain in this model system (Table 2). Exceptions were a decreased expression of *speB*, which encodes a cysteine protease, and a significantly enhanced expression of *lctO*, which encodes lactate oxidase, a peroxide-producing enzyme (Table 2). The latter observation is consistent with the findings of prior studies that show that CcpA binds to a catabolite-responsive element site with the *lctO* promoter and functions as a repressor of

lctO expression (33). Furthermore, derepression of *lctO* is associated with self-intoxication caused by the overproduction of H_2O_2 in the presence of oxygen (33). To determine if excess LctO expression was limiting vaginal mucosa carriage, a previously characterized in-frame deletion mutation (33) was introduced into the WT and CcpA⁻ strains. As expected, inactivation of *ccpA* in the HSC12 background was associated with a significant increase in H_2O_2 production that was insensitive to repression by glucose (Fig. 6A). Interestingly, the CcpA⁻ mutant was found to have increased susceptibility to H_2O_2, as revealed by a lower growth yield in the presence of 2 mM H_2O_2 supplemented into the growth medium (Fig. 6B); we suspect that a small amount of H_2O_2 produced in the absence of LctO repression by CcpA in this system added to the 2 mM H_2O_2 supplemented in the medium and exceeded a threshold level where the CcpA⁻ strain was differentially inhibited. Also as expected, the inactivation of *lctO* by itself or in the CcpA⁻ strain background (a double CcpA⁻ LctO⁻ mutant strain) abrogated both peroxide production in aerated cultures (Fig. 6A) and the CcpA⁻ strain's increased sensitivity to 2 mM H_2O_2 (Fig. 6B). When analyzed in preestrogenized mice, the double CcpA⁻ LctO⁻ mutant strain was not attenuated compared to the defective CcpA⁻ strain, and its phenotype was essentially identical to that of the WT strain in both the median duration of carriage (Fig. 5A) and bacterial burden (Fig. 5B). The fact that the LctO⁻ strain also did not demonstrate any attenuation of carriage (Fig. 7A and B) shows that the suppressive effect of this mutation was specific to the CcpA⁻ background and was likely due to its
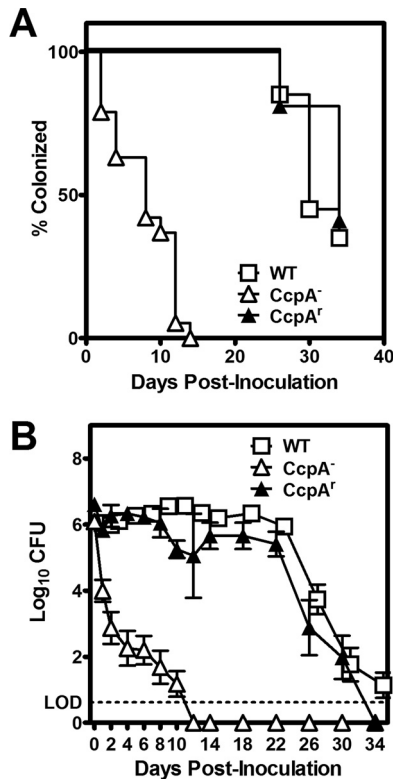
FIG 5 Deletion of CcpA limits the duration of mucosal carriage. Estrogen-treated (0.5 mg) C57BL/6J mice were vaginally challenged with 1×10^6 CFU of *S. pyogenes* strains HSC12 (WT) and MEW41 (CcpA⁻) and revertant strain MEW106 (CcpAʳ). Vaginal washes were collected at the time points indicated and processed for determination of the number of CFU. (A) Kaplan-Meier analysis of the percentage of mice that remained colonized. Log-rank analysis indicated that the CcpA⁻ mutant was significantly attenuated compared to the WT and CcpAʳ strains ($P < 0.001$). (B) Numbers of CFU recovered in vaginal washes. Each symbol represents the mean and standard error of the mean derived from 20 (WT), 19 (CcpA⁻), and 5 (CcpAʳ) mice challenged with the indicated strains. LOD, limit of detection, calculated to be 84 CFU/ml. A repeated-measures analysis indicated that the CcpA⁻ mutant was significantly attenuated compared to the WT and CcpAʳ strains ($P < 0.001$).

ability to alleviate overexpression of LctO. Thus, these data demonstrate that CcpA makes a critical contribution to mucosal carriage by its ability to downregulate a gene whose overexpression decreases fitness in the mucosal environment.

DISCUSSION

Investigation of factors critical to mucosal colonization and carriage in *S. pyogenes* has been hampered by the fact that it is a human-restricted pathogen with no known animal or environmental reservoir. Previous animal models for *S. pyogenes* have suffered from the fact that the number of strains which can function in those models and/or reproduce the disease phenotype was limited (10, 51, 53, 54). This work presents a novel animal model of *S. pyogenes* mucosal colonization and carriage which will potentially advance the field of streptococcal host-pathogen interactions at the mucosal surface. Importantly, the preestrogenized murine vaginal colonization model is capable of supporting at least five diverse *S. pyogenes* strains, including the M14 HSC12, M6 JRS4, M1 SF370, and M49 NZ131 strains and an M28 isolate, MEW123, and this will facilitate investigation into factors distin-

guishing carriage ability between different strains. There are likely multiple differences between these strains in terms of expression of various adhesins, exotoxins, and other potential virulence factors that could account for their variable phenotypes in vaginal mucosa carriage. As just one example, strains HSC12 and SF370 display oxygen-induced expression of the fibronectin-binding adhesin protein F (*prtF*) and do not express this protein under anaerobic conditions; in contrast, JRS4 has a mutation in the regulatory gene *rofA* which permits constitutive expression of protein F, even anaerobically (39).

Another advantage of using a murine vaginal model to study *S. pyogenes* colonization was the relative ease of access to the tissues to monitor the mucosal immune response. In this model, an influx of vaginal leukocytes was found to be a marker of inflammation, with higher leukocyte percentages being noted in vaginal washes from BALB/c mice than those from C57BL/6J mice. A previous study (55) also reported a significantly higher vaginal fluid leukocyte response from BALB/c mice than C57BL/6J mice, when both were similarly colonized with *N. gonorrhoeae*, supporting a difference between the two mouse strains in the innate immune response that may be common to several mucosal pathogens. Therefore, the vaginal carriage model may be useful in investigating other bacterial factors that interact with the innate response to mucosal pathogens, and using BALB/c mice as the host in this model may help highlight factors affecting inflammation. Furthermore, this model may help explain the inverse relationship between estrogen availability and symptomatic *S. pyogenes* vaginal disease in humans. For unclear reasons, vulvovaginitis tends to be more common in prepubertal girls, postmenopausal women, and women with other hypoestrogen conditions (20, 22–24). In our model, estradiol supplementation was associated with asymptomatic carriage, which seems to mimic the situation in women of reproductive age vaginally colonized with *S. pyogenes*. Estradiol supplementation is known to promote the estrus phase of the murine estrous cycle, a time associated with vaginal epithelium proliferation and thickening, production of glycogen, and a minimal presence of inflammatory cells (15). Es-

TABLE 2 Only a subset of CcpA-regulated genes is dysregulated in the CcpA⁻ mutant during vaginal carriage

Transcript[b]	Relative transcript expression[a]			
	48 h	*P* value	96 h	*P* value
speB	0.07 ± 0.06	0.010	0.48 ± 0.35	0.116
lctO	5.76 ± 2.18	0.001	4.58 ± 1.09	0.053
hasA	0.94 ± 0.10	0.545	0.40 ± 0.05	0.203
sagA	0.62 ± 0.12	0.140	0.72 ± 0.24	0.303
malM	1.04 ± 0.27	0.873	1.13 ± 0.53	0.772
cfa	1.59 ± 0.72	0.122	0.43 ± 0.30	0.077
ackA	0.85 ± 0.22	0.200	0.77 ± 0.70	0.463
arcA	1.82 ± 1.70	0.506	2.51 ± 3.04	0.267

[a] Relative transcript expression is expressed as expression for the CcpA⁻ strain/expression for HSC12, with the $2^{-\Delta\Delta CT}$ value normalized using the transcript level of *recA* (46). Values represent means ± standard errors of the means of three independent experiments, each analyzed in duplicate. Samples are from 48 h and 96 h postinoculation. For each experimental group (WT and CcpA⁻) vaginal washes from six mice were included in the analysis.
[b] The *recA* (SPy_2116), *speB* (SPy_2039), *lctO* (SPy_0414), *hasA* (SPy_2200), *sagA* (SPy_0738), *malM* (SPy_1292), *cfa* (SPy_1273), *ackA* (SPy_0109), and *arcA* (SPy_1543) transcripts were analyzed on the basis of previous work showing that these genes are CcpA regulated (32, 33, 34).
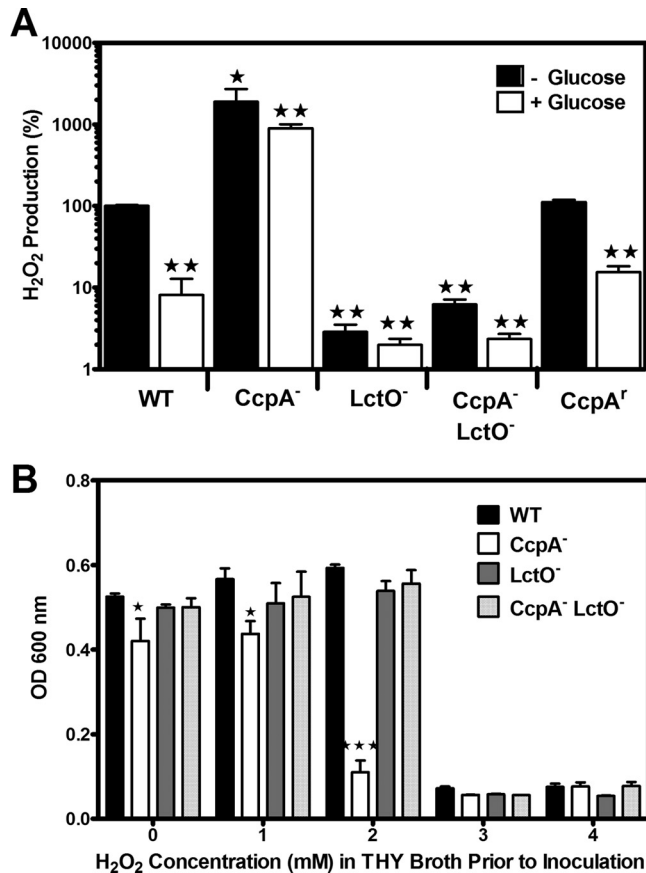
FIG 6 Hydrogen peroxide production and sensitivity of CcpA⁻ mutants. (A) H₂O₂ production following overnight culture in aerated C medium with (+) and without (−) supplemented glucose (5 mM). H₂O₂ levels are shown relative to those in the WT strain. Data presented are the means and standard errors of the means derived from a minimum of 3 independent experiments. (B) H₂O₂ sensitivity. Strains were cultured in THY medium supplemented with the indicated concentrations of H₂O₂ prior to inoculation. Culture density was assessed after overnight static culture by determination of the OD₆₀₀. Data represent the means and standard errors of the means derived from 2 independent experiments, each performed in triplicate. The strains tested are HSC12 (WT), MEW41 (CcpA⁻), MEW16 (LctO⁻), MEW47 (CcpA⁻ LctO⁻), and MEW106 (CcpAʳ). Comparisons between HSC12 and mutant within each H₂O₂ concentration utilized a two-tailed, paired t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

FIG 7 Deletion of LctO rescues a CcpA⁻ strain. Estrogen-treated (0.5 mg) C57BL/6J mice were vaginally challenged with 1×10^6 CFU of S. pyogenes strains HSC12 (WT) and MEW16 (LctO⁻) and the double mutant MEW47 (CcpA⁻ LctO⁻). Vaginal washes were collected at the time points indicated and processed for determination of the numbers of CFU. (A) Kaplan-Meier analysis of the percentage of mice that remained colonized. A log-rank analysis indicated that both the LctO⁻ single mutant and CcpA⁻ LctO⁻ double mutant exhibited significantly longer durations of carriage than the WT (P < 0.0001). (B) Numbers of CFU recovered in vaginal washes. Each symbol represents the mean and standard error of the mean derived from 20 (WT), 17 (LctO⁻), and 18 (CcpA⁻ LctO⁻) mice challenged with the indicated strain. LOD, limit of detection, calculated to be 84 CFU/ml. A repeated-measures analysis comparing the numbers of CFU recovered over the entire 34-day time frame of the experiment between the WT, the LctO⁻ mutant, and the CcpA⁻ LctO⁻ double mutants did not meet statistical significance. A subset repeated-measures analysis comparing the numbers of CFU recovered over days 22 through 34 of the experiment between the three groups showed that both the LctO⁻ mutant and the CcpA⁻ LctO⁻ double mutant exhibited significantly higher numbers of CFU recovered from vaginal washes than the WT (P < 0.0001).

tradiol exerts activity through two distinct estrogen receptors (estrogen receptor α [ERα] and ERβ) that populate different tissues and cell lines (56). Estrogen receptor β has previously been shown to have anti-inflammatory activity with repression of multiple cytokines in immune cells, whereas ERα is more associated with anabolic activity in the female mammary gland and reproductive tract (57). Which receptor and which pathway are more important for producing asymptomatic S. pyogenes carriage are unknown, but the murine vaginal carriage model could provide valuable insight into estradiol and its link to carriage and inflammation.

The utility of the murine vaginal model in identifying streptococcal factors contributing to carriage was demonstrated in this work by demonstrating the critical role of the transcriptional regulator CcpA. Previous investigations have identified virulence defects in CcpA⁻ strains of several species of Staphylococcus, Strep-

tococcus, and Clostridium, among others (32, 33, 52, 58–62). The abundance of data linking CcpA's regulatory activity to virulence confirms the importance of CcpA, among other global transcriptional regulators, in a central role for disease pathogenesis. The results of the present study add to the growing body of knowledge linking CcpA and virulence through mucosal colonization ability and demonstrate that dysregulated expression of LctO, a CcpA-regulated metabolic enzyme, is responsible for limiting survival in a CcpA⁻ strain. Importantly, this study illustrates that dysregulation of transcriptional expression can negatively impact an organism's ability to maintain colonization, an observation that is likely broadly applicable to many mucosal pathogens.
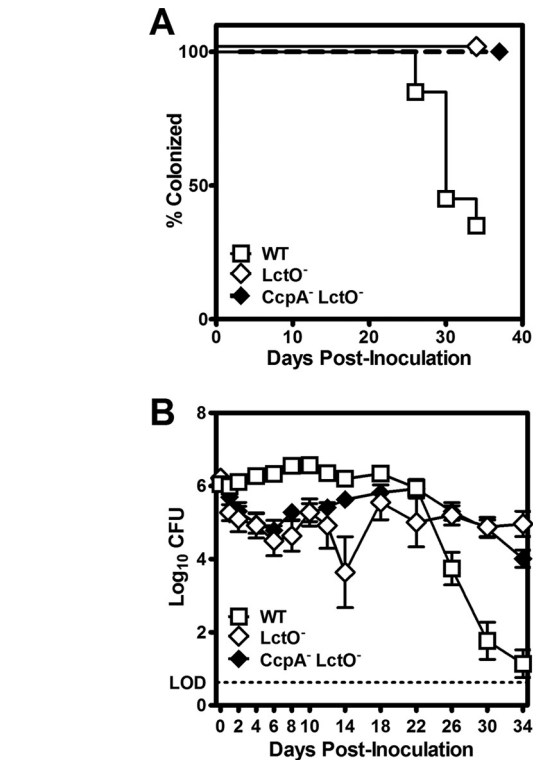
Carbon catabolite repression by CcpA is a complex process that allows a pathogen to regulate metabolism and virulence factor expression in response to changing environmental conditions following infection (such as the depletion of available carbohy-

drates) or upon encountering a new environment or stage in the infectious process. In support of this, *S. pyogenes* CcpA⁻ strains recovered from the relatively confined environment of the murine skin ulcer model showed that dysregulation was not a static all-on or all-off process, but even among genes that were strongly regulated by CcpA *in vitro*, there were rather dynamic fluctuations in the magnitude of differences between gene expression patterns between wild-type and CcpA⁻ strains (34). Some of these fluctuations may have been due to changing carbohydrate availability in the ulcer tissue bed; others may be due to the influence of additional factors, including LacD.1, which function as coregulatory proteins along with CcpA for some gene transcripts, including the cysteine protease SpeB (33, 34).

The present study identifies an increase in the transcription of LctO from vaginal washes colonized by CcpA⁻ strains; however, beyond LctO there were relatively few differences in transcript expression identified from vaginal washes of mice colonized with the CcpA⁻ strain and mice colonized with the HSC12 parent strain at the time points examined (Table 2). This could be due to several reasons. *S. pyogenes* organisms colonizing the murine vaginal epithelium could be relatively metabolically inactive with a low level of transcriptional activity that could make finding comparative differences difficult. Alternatively, the little to no difference in transcript expression between CcpA⁻ strains and the HSC12 parent strain could suggest that available carbohydrate levels in the murine vaginal mucosa are not sufficient to signal CcpA-mediated regulation to a significant degree to promote differences. It has previously been determined that estradiol-treated murine and human vaginal secretions both have glucose concentrations of 3 to 4 mM, which calculates to about 0.072% (wt/vol) (19). Prior experiments by our group showed a significant reduction in *lctO* transcription *in vitro* with ≥0.1% glucose in C medium (33); thus, it is possible that in the murine vaginal model the glucose concentrations were too low to show significant differences for many genes normally regulated by CcpA. *S. pyogenes* HSC12 can utilize a variety of carbohydrate sources, most of which are ultimately converted to glycolytic intermediates which activate CcpA-mediated repression of LctO. Vaginal secretions contain monosaccharides, including glucose, mannose, and glucosamine, in addition to oligosaccharides, including maltose, maltotriose, and maltotetraose (63, 64). Carbon catabolite repression in strain HSC12 can be induced by glucose, maltose, maltotriose, pullulan, galactose, and mannose (data not shown); strain HSC12 cannot directly utilize glycogen or lactose, although other bacterial species present in the vaginal flora may degrade these sugars into forms that *S. pyogenes* strain HSC12 can utilize for carbon catabolite repression. Another possibility is that in the murine vaginal environment, there are perhaps additional coregulatory proteins (LacD.1, among other possibilities) that could be acting to help coordinate gene expression in the absence of input from CcpA. In our experiments, however, even in a CcpA⁻ strain, dysregulation of gene expression on a global scale did not sufficiently explain the attenuation in carriage more so than dysregulation of a single gene, as observed when inactivation of LctO in the CcpA⁻ background prolonged carriage of the double mutant strain.

When in the presence of glycolytic intermediates, CcpA binds to the *lctO* promoter within a catabolite-responsive element sequence located upstream of the *lctO* ATG translation start site, blocking *lctO* transcription. In the CcpA⁻ strain, LctO transcrip-

tion is largely unchecked, producing abundant amounts of peroxide that accumulate to lethal concentrations (33). Inactivation of LctO in the CcpA⁻ strain prevented hydrogen peroxide production, and this resulted in a prolonged *in vivo* duration of carriage of the CcpA⁻ LctO⁻ strain. In *S. pyogenes*, lactate oxidase is the principal enzyme producing significant levels of hydrogen peroxide; the enzyme NADH oxidase also contributes a small and relatively insignificant amount of detectable peroxide (65); this fact may explain why H_2O_2 production in the LctO⁻ and CcpA⁻ LctO⁻ strains was low but not undetectable (Fig. 6A). Among both clinical isolates and lab-generated mutants, inactivation of LctO activity and reduced hydrogen peroxide production have previously been demonstrated to prolong stationary-phase survival under aerobic conditions (33, 65, 66). It was surprising to find such a strong phenotype related to peroxide production in the murine vaginal compartment, as traditional thought is that the vaginal environment is of relatively low oxygen tension (67). However, in humans, vaginal lactobacillus species producing hydrogen peroxide are thought to be associated with vaginal health and a reduced risk of acquiring disease, such as bacterial vaginitis (68–70). By this, the vaginal compartment presumably has sufficient available oxygen for lactobacillus and, likewise, *S. pyogenes* to have the capacity to produce peroxide. Alternatively, there may be differences in the oxygen tension present in the murine vaginal environment and that present in the human vaginal environment. Oxygen may have been further introduced into the murine vaginal compartment in our model via repeat vaginal specimen collection for culture; mechanical insertion of contraceptive diaphragms or tampons has previously been shown to transiently increase the vaginal oxygen content in humans (67, 71).

The present study is not the first to find that CcpA⁻ strains are attenuated in mucosal colonization and carriage. CcpA⁻ strains of *Streptococcus pneumoniae* were previously demonstrated to be attenuated for carriage in the murine nasopharynx (62), just as *S. pyogenes* was attenuated for carriage in the murine oropharynx (32). Neither of these prior studies identified specific defects that could complement the CcpA⁻ strain phenotype like the double Δ*ccpA* Δ*lctO* mutations were capable of doing in this study. In *S. pneumoniae*, H_2O_2 production under aerobic conditions is mostly due to the activity of pyruvate oxidase (SpxB); under glucose limitation, the pneumococcal LctO also shows peroxide-generating activity. In *S. pneumoniae*, LctO was also found to be upregulated in CcpA⁻ strains; however, SpxB was not influenced by *ccpA* deletion, as determined by microarray analysis (62, 72). Pneumococcal stains producing peroxide have competitive advantages within mixed bacterial populations, presumably by inhibiting the growth of peroxide-susceptible species (73, 74). However, *S. pneumoniae* SpxB mutants were found to be defective in animal models of nasopharyngeal colonization, pneumonia, and sepsis, although factors in addition to peroxide generation alone may have contributed to this phenotype (74–77). In our model of *S. pyogenes* vaginal colonization, we noted no significant defect in carriage of strains with a mutation of *lctO*. Altogether, these results suggest that regulated hydrogen peroxide generation by several streptococcal species is critical for mucosal colonization and virulence.

Asymptomatic mucosal carriage with *S. pyogenes* is a concept that remains poorly understood, in terms of both the basic science underlying the microbiology and immunology occurring at the mucosal surface and the clinical science on how to manage pa-

tients who are carriers of group A streptococcus. As shown here, the vaginal colonization model is effective for investigating traits critical for *S. pyogenes* mucosal carriage. This model should prove beneficial in future investigations of *S. pyogenes* mucosal colonization and carriage and investigation of the role of innate and adaptive host immune properties in response to carriage, an understanding of which will be key to the development of anti-*S. pyogenes* vaccines effective at the mucosal surface.

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