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Genetic switch to hypervirulence reduces colonization phenotypes of the globally disseminated group A Streptococcus M1T1 clone

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Abstract
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Keywords
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Genetic Switch to Hypervirulence Reduces Colonization Phenotypes of the Globally Disseminated Group A *Streptococcus* M1T1 Clone

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**Background.** The recent resurgence of invasive group A streptococcal disease has been paralleled by the emergence of the M1T1 clone. Recently, invasive disease initiation has been linked to mutations in the *covR/S* 2-component regulator. We investigated whether a fitness cost is associated with the *covS* mutation that counter-balances hypervirulence.

**Methods.** Wild-type M1T1 group A *Streptococcus* and an isogenic *covS*-mutant strain derived from animal passage were compared for adherence to human laryngeal epithelial cells, human keratinocytes, or fibronectin; biofilm formation; and binding to intact mouse skin. Targeted mutagenesis of capsule expression of both strains was performed for analysis of its unique contribution to the observed phenotypes.

**Results.** The *covS*-mutant bacteria showed reduced capacity to bind to epithelial cell layers as a consequence of increased capsule expression. The *covS*-mutant strain also had reduced capacity to bind fibronectin and to form biofilms on plastic and epithelial cell layers. A defect in skin adherence of the *covS*-mutant strain was demonstrated in a murine model.

**Conclusion.** Reduced colonization capacity provides a potential explanation for why the *covS* mutation, which confers hypervirulence, has not become fixed in the globally disseminated M1T1 group A *Streptococcus* clone, but rather may arise anew under innate immune selection in individual patients.
cluding components of the extracellular matrix, cytokine precursors, immunoglobulins, and antimicrobial peptides [14–16], which could promote tissue damage or impair host immune functions. However, SpeB also cleaves some of the protein virulence factors of the bacterium, such as the fibrinogen-binding M1 protein [12, 17–19], various superantigens [20, 21], the secreted plasminogen activator streptokinase [22], and the deoxyribonuclease Sda1 [20], which thus attenuates key aspects of GAS pathogenicity.

CovR/S is an important global gene regulator that is responsible for regulating ∼10% of the GAS genome [4, 9]. It has been found that CovR acts mainly as a negative regulator, even in the absence of CovS, and that CovS inactivates the function of CovR [23]. Specific point mutations in covR/S and truncation mutations in covS result in considerable down-regulation of SpeB expression but concurrent up-regulation of many virulence factor genes, including those encoding Sda1, interleukin 8 protease SpyCEP, streptolysin O, streptococcal inhibitor of complement, and the hyaluronic acid capsule synthesis operon [9–12]. It has been hypothesized that increased expression of these virulence determinants, along with sparing them from SpeB degradation, promotes the proliferation and invasive spread of the covR/S-mutant strain [9, 10, 24]. Increased resistance to innate immune clearance, and in particular killing by neutrophils, may represent the major selection pressure that favors the covR/S mutation in vivo [10].

The routine occurrence of the covR/S mutation in M1T1 GAS and the dramatically increased animal virulence that follows this event raise the question of why this particular mutation has not become fixed during GAS evolution. We hypothesized that a counterbalancing selection pressure acts to maintain the wild-type genotype, and thus we sought to elucidate the fitness cost or costs of the covR/S mutation in the M1T1 GAS population.

METHODS

Bacterial strains, medium, and growth conditions. Well-characterized M1T1 clinical isolate 5448 and its mouse-passaged covS-mutant derivative 5448AP were used [10]. Correction of the covS mutation in the 5448AP strain restores wild-type phenotypes [25]. GAS strains were propagated using Todd–Hewitt broth (THB) or Todd–Hewitt agar (THA). Escherichia coli were grown using Luria–Bertani broth or Luria–Bertani agar. Erythromycin selection was used at 5 μg/mL for GAS and at 500 μg/mL for E. coli.

Growth curves and chain length assays. Overnight cultures of GAS bacteria were diluted into fresh THB, and the optical density at 600 nm (OD_{600}) was measured every 30 min to determine the growth curves. For chain length assays, GAS were centrifuged for 5 min at 3200 g to create a monolayer and viewed at 10−3 dilution by use of a Zeiss Axiovert 100 inverted microscope, and the chain length was calculated using 1 random field of view from 3 separate wells. Statistical significance was determined using 1-way analysis of variance (ANOVA) with the Tukey post hoc test.

Plasmid integrational mutagenesis. An intragenic fragment of hasA was amplified using forward primer hasA-F-BamHI (5′-GCAGGATCTTGGGACATCAACTTGGGAG-3′) and reverse primer hasA-R-XbaI (5′-GCATCTAGATTAATTCGATCTCTGTTGACCGC-3′), then cloned by BamHI/XbaI digestion into conditional vector pHY304. The resulting plasmid was transformed into the 5448 and 5448AP strains by means of electroporation, and erythromycin-resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover chromosomal insertions were selected by shifting to the nonpermissive temperature (37°C), which maintained erythromycin selection [26]. Integrational knockouts were confirmed unambiguously by means of polymerase chain reaction and were designated 5448ΔhasA and 5448APΔhasA.

Epithelial cell adherence assays. Assays were performed using HEp-2 cells (human laryngeal epithelial cells) and HaCaT cells (human keratinocyte cells) as described elsewhere [27]. Cells were plated at an inoculum of 2 × 10^5 cells/well and grown overnight at 37°C in 5% carbon dioxide. Midlogarithmic growth phase GAS were resuspended in Roswell Park Memorial Institute (RPMI) medium plus 2% fetal calf serum and added at a multiplicity of infection of 10:1. Plates were centrifuged for 10 min at 500 g and incubated for 30 min at 37°C in 5% carbon dioxide, then washed 5 times with phosphate-buffered saline (PBS) to remove nonadherent bacteria. One hundred microliters of trypsin was added to release cells, which were lysed with 0.02% Triton X-100. Bacteria were serially diluted and plated on THA for enumeration. Bacterial adherence was calculated as a percentage of the initial inoculum. More than 95% cell viability of HEp-2 and HaCaT cells was documented in all assays, as determined by means of trypan blue staining. Bacterial strains grew equally in RPMI medium plus 2% fetal calf serum for the 30-min duration of the experiment. Statistical significance was determined using 1-way ANOVA with the Tukey post hoc test.

Competition adherence assays were performed identically, but with the addition of 2 × 10^6 colony-forming units (CFUs) per well of an equal mix of the 5448 and 5448AP strains or an equal mix of the 5448ΔhasA and 5448APΔhasA strains to cell monolayers. After serial dilutions were plated on THA overnight, 50 individual colonies from each condition (5448 and 5448AP strains or 5448ΔhasA and 5448APΔhasA strains) were selected. The proportion of the 5448 strain to the 5448AP strain was determined by the proportion of SpeB-positive colonies to SpeB-negative colonies, respectively, by means of methods that are detailed elsewhere [28]. Assays were performed in triplicate,

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and statistical significance was determined using an unpaired t test.

**Hyaluronic acid capsule assay.** Bacterial cultures were grown to midlogarithmic phase in THB. Five milliliters of culture (OD₆₀₀, 0.4) was centrifuged and resuspended in 500 μL of deionized water. Serial dilutions of bacterial suspension were plated to confirm the equivalent number of CFUs. Four hundred microliters of the bacterial suspension was placed in a 2-mL screw-cap tube with 1 mL of chloroform. Tubes were shaken for 5 min in a Mini-BeadBever-8 (Biospec Products), then centrifuged at ~13,000 g for 10 min. The level of hyaluronic acid in aqueous phase was determined using a hyaluronic acid test kit (Corgenix) according to the manufacturer’s instructions.

**Fibronectin-binding assays.** Fibronectin was bound to 96-well plates (Costar) as described elsewhere [29]. Bacteria were grown to midlogarithmic phase, washed in PBS, and resuspended to a concentration of 2 × 10⁶ CFUs/mL. Plates were washed 3 times with sterile PBS, and 100 μL (2 × 10⁵ CFUs) of bacterial solution was added to each well. Plates were centrifuged at 500 g for 10 min, incubated at 37°C for 1 h, then washed 5 times with PBS to remove nonadherent bacteria. Adherent bacteria were released by use of 100 μL of 0.25% trypsin/1 mmol/L ethylenediaminetetraacetic acid (Gibco) for 10 min at 37°C. Bacteria were serially diluted in PBS and plated onto THA for enumeration of CFUs. Bacterial adherence was calculated as a percentage of the initial inoculum. Assays were performed in triplicate, and statistical significance was determined using 1-way ANOVA with the Tukey post hoc test.

**Fluorescence microscopy.** Glass coverslips were coated with fibronectin by incubating them overnight at 4°C in a 50 μg/mL solution in PBS. Slides were then washed with PBS and blocked overnight at 4°C with PBS plus 1% bovine serum albumin. Bacteria were grown to midlogarithmic phase (OD₆₀₀ 0.4), centrifuged, and resuspended in PBS to a value of OD₆₀₀ of 1.0. Fluorescein isothiocyanate (FITC) was added to a final concentration of 100 μg/mL and incubated on ice for 30 min. FITC-labeled bacteria were pelleted, washed twice with sterile PBS, and then resuspended to a value of OD₆₀₀ of 0.4 in PBS. Five hundred microliters of FITC-labeled bacterial solution was added to glass coverslips in the bottom of 24-well tissue culture plates. Plates were centrifuged for 10 min at 500 g, then incubated for 1 h at 37°C. Coverslips were washed 5 times with PBS, fixed with 4% paraformaldehyde overnight at 4°C, washed again, and mounted on microscopes slides with ProLong Gold (Invitrogen). Slides were visualized using a DeltaVision RT Deconvolution microscope (University of California, San Diego, Neuroscience Microscopy Shared Facility).

**Static biofilm formation on polystyrene.** Determination of biofilm formation on polystyrene was performed using a modified O’Toole-Kolter crystal violet stain assay [30]. Briefly, 8 individual wells of a black-sided, clear-bottomed, 96-well microtiter plate (Greiner Cellstar; catalog no. 655090) were inoculated with 150 μL of overnight culture diluted 1:100 in THB supplemented with 1% (weight per volume) yeast extract for each strain studied. Plates were sealed with Aerasal breathable film (Excel Scientific) and incubated for 24 h at 37°C. Plates were washed, and cells were fixed with 4% paraformaldehyde. Six wells were stained with 0.2% crystal violet, extracted in acetone and ethanol, and assayed for crystal violet absorbance at 595 nm for biofilm biomass quantification. The remaining 2 wells for each strain were stained with SYTO 9 nucleic acid stain (Invitrogen), and biofilms were visualized using a Nikon A1 laser scanning confocal microscope. Images were reconstructed from Z sections and rendered for 3-dimensional visualization by use of NIS-Elements software (version 3.0; Nikon). The assay was performed 4 times, and statistical significance was determined using 1-way ANOVA with the Tukey post hoc test.

**Static biofilm formation on epithelial cells.** The visualization of biofilm formation by GAS strains on epithelial cells was modified from the method of Manetti et al [31]. HaCaT keratinocytes were seeded in RPMI medium into 35-mm tissue culture dishes with a 10-mm diameter glass coverslip insert (Fluorodish; World Precision Instruments) and cultured for 48 h. HaCaT cells were then washed, and 200 μL of 1:10 dilutions of overnight cultures of GAS strains in RPMI medium were added to each dish. After 15 min, HaCaT cells were washed to remove unattached bacteria. Pilot experiments revealed that 8-h incubation was sufficient for wild-type GAS (5448) to form a robust biofilm on HaCaT cells without extensive cell loss and/or death. After 8 h, dishes were fixed with 4% paraformaldehyde, blocked, and blotted with mouse anti–GAS-M1 polyclonal antibody to allow fluorescent visualization. The HaCaT cells remaining 2 wells for each strain were stained with SYTO 9 nucleic acid stain (Invitrogen), and biofilms were visualized using a Nikon A1 laser scanning confocal microscope, and rendered 3-dimensionally as described above.

**Murine skin adherence assay.** Bacterial cultures were grown to midlogarithmic phase (OD₆₀₀ 0.4) and washed with sterile PBS. Bacteria were diluted to 2 × 10⁶ CFUs/mL. Ten microliters of bacterial solution (2 × 10⁵ CFUs) was spotted onto prewarmed THA plates. Once the droplets had dried, agar disks containing the bacteria were excised using an 8-mm biopsy punch. Shaved CD1 mice were anaesthetized with ketamine and xylazine, and bacterial agar disks were affixed with Tegaderm transparent wound dressing (3M). A total of 4 mice, each with 2 disks of the wild-type 5448 bacteria on their left flank and 2 disks of the covS-mutant 5448AP bacteria on their
right flank, were used. After 1 h, the mice were euthanized with isoflurane. The skin under the bacterial disks was excised and placed into 2-mL screw-cap tubes containing 1 mL of PBS. The tubes were shaken in a Mini-BeadBeater-8 (Biospec Products) on mix setting for 2 min to remove nonadherent bacteria. Skin was then transferred to a fresh screw-cap tube containing PBS, shaken for 2 min, and transferred to a fresh 2-mL tube containing 1 mL of PBS and 1-mm silica-zirconia beads (Biospec Products). Tissue was homogenized by shaking twice with the Mini-BeadBeater-8 at full for speed for 1 min, with placement on ice between shakings. The homogenate was serially diluted in sterile PBS and plated on THA for enumeration. Bacterial adherence was calculated as a percentage of the initial inoculum. Statistical significance was determined using an unpaired t test.

**Histological analysis.** The in vivo adherence assay was performed as described above using 2 × 10^7 CFUs/spot. Excised skin was placed in Formalde-Fresh solution (Fisher) overnight, then sectioned and Gram stained at the University of California, San Diego, Histopathology Core Facility (Nissi Varki, director).

**LL-37 resistance assays.** Bacteria were grown to mid-logarithmic phase and resuspended in PBS plus 20% THB at 1 × 10^9 CFUs/mL. Ninety microliters of bacteria was then added to 10 μL of varying concentrations of human cathelicidin LL-37 or the murine cathelicidin CRAMP in a 96-well plate.

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**Figure 1.** Increased capsule expression and reduced capacity to adhere to epithelial cells as a result of the covS mutation in group A Streptococcus. A, Adherence to HEp-2 cells (human pharyngeal epithelial cells) and HaCaT cells (human keratinocytes) of clinical isolate 5448 and its mouse-passaged covS-mutant derivative 5448AP. B, Competitive adherence to HEp-2 and HaCaT cells. C, Hyaluronic acid capsule levels. The strains 5448ΔhasA and 5448APΔhasA are capsule-deficient mutant strains. D, Adherence of the 5448ΔhasA and 5448APΔhasA strains to HEp-2 and HaCaT cells. Values shown in all panels are means ± standard deviations.
After 24 h, 5 μL of suspension from each well was plated on THA and incubated overnight. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of LL-37 or CRAMP that yielded no detectable bacterial growth when the sample was plated on THA. For experiments in which there was variance in the MIC between replicates, the values are shown as a range.

**Ethics approvals.** Permission to obtain human blood samples and undertake animal experiments was obtained from the University of California, San Diego, and University of Wollongong human and animal subject protection committees. Human volunteers provided informed consent before blood samples were obtained.

**RESULTS**

**Reduction of adherence to epithelial cells in hypervirulent covS-mutant M1T1 GAS.** The well-characterized M1T1 clinical isolate 5448 and the isogenic covS-mutant derivative 5448AP were used in this comparative analysis. To investigate the ability of wild-type and covS-mutant bacteria to adhere to epithelial cells, we utilized 2 human cell lines: HEp-2 laryngeal cells and HaCaT keratinocytes, which represent the throat and skin focal points of GAS colonization and mucosal infection. The 5448AP strain had a marked decrease in adherence, compared with the 5448 strain (P < .001), in both HEp-2 and HaCaT cells (Figure 1A). In a competition binding assay, the 5448 strain outperformed the 5448AP strain in adherence to both HEp-2 and HaCaT cells (Figure 1B). The observation that the adherence of the 5448AP strain was not rescued by coinfection with the 5448 strain suggests that a cell surface factor, not a secreted factor, was responsible for this defect in epithelial cell binding.

Up-regulation of capsule expression has previously been shown in covS-mutant GAS [9, 11]. We hypothesized that this up-regulation of capsule in the 5448AP strain was an important contributor to the phenotype of decreased epithelial cell binding. With the exclusion of differences in growth characteristics that may affect subsequent assays, we showed that the capsule-deficient mutant strains, 5448ΔhasA and 5448APΔhasA, exhibited similar growth to their respective parent strains in THB and formed chains of similar length (data not shown). The 5448AP strain was found to have statistically significantly more hyaluronic acid capsule than the 5448 strain, whereas the mutant strains 5448ΔhasA and 5448APΔhasA were found to have no capsule (Figure 1C). Upon a genetically defined disruption of the capsule biosynthesis gene hasA, a difference in adherence between the wild-type strain and the covS-mutant strain to either epithelial cell line was no longer observed (Figure 1D). Although there was no difference observed between the 5448ΔhasA and 5448APΔhasA strains, both of these strains displayed less binding than the wild-type 5448 strain. This find-

![Figure 2](image_url) Reduced binding of group A Streptococcus (GAS) to fibronectin as a result of the covS mutation. **A,** Binding of the 5448 and 5448AP strains and the capsule-deficient mutants 5448ΔhasA and 5448APΔhasA to immobilized fibronectin. Values shown are means ± standard deviations. **B,** Fluorescence microscopic analysis of fluorescein isothiocyanate–labeled GAS to fibronectin-coated glass coverslips. White scale bars, 20 μm.
...ing supports a model in which in low level capsule expression can contribute to epithelial cell adherence, in particular through hyaluronic acid binding to host CD44 receptors [32, 33], whereas marked hyperencapsulation impairs adherence, likely through cloaking of higher-affinity GAS adhesins and extracellular matrix binding proteins. It is important to note that the M1T1 genome sequence [34] lacks most of the well-characterized GAS surface proteins known to bind fibronectin or other extracellular matrix proteins, including Sfb1, PrfF, PrfFII, SOF, and SfbX. The 2 known GAS fibronectin-binding proteins encoded in the GAS genome sequence (FBP54 and FbaA) are not differentially regulated upon the in vivo selection of the covR/S mutation [9]. Thus, the relative contribution of capsule to adherence in the covR/S-intact wild-type M1T1 strain may be greater than in other serotype backgrounds. Nevertheless, our data suggest that up-regulation of capsule expression following covS mutation is the principal reason for the reduced binding to epithelial cells that was observed in the 5448AP strain.

**Reduced capacity of hypervirulent covS-mutant M1T1 GAS to bind fibronectin.** Fibronectin is an important component of the extracellular matrix that acts as a target for GAS adhesins; thus, fibronectin binding represents an important initial step in the colonization process [35, 36]. The covS-mutant strain 5448AP has a statistically significantly reduced capacity to bind fibronectin (P<.01), compared with the wild-type parental strain (Figure 2A). The capsule-deficient strain 5448ΔhasA exhibited similar binding capacity to the wild-type 5448 strain, whereas strain 5448APΔhasA showed increased binding compared with the 5448AP strain (Figure 2A). Fluorescence microscopic analysis of FITC-labeled bacteria corroborated these findings (Figure 2B). Reduced capacity to bind extracellular matrix components may affect the ability of covS-mutant GAS to colonize the host. Moreover, increased binding by the 5448APΔhasA strain compared with the wild-type 5448 strain suggests that the dramatic capsule up-regulation effectively masks binding increases that would otherwise result from gene-expression changes that are linked to the covS mutation.

**Reduction of biofilm formation in hypervirulent covS-mutant M1T1 GAS.** Biofilm formation has been proposed to play a role in GAS colonization, as well as in the persistence and recurrence of GAS infection [31, 37]. We found that the covS-mutant 5448AP strain exhibits statistically significantly less biofilm formation than the wild-type 5448 strain (P<.001) (Figure 3A). The 5448ΔhasA strain exhibited similar biofilm formation to the wild-type strain, whereas the 5448APΔhasA strain produced greater biofilms than either the wild-type or 5448ΔhasA strains. Taken together, these data illustrate that although further gene regulation differences may affect biofilms, capsule up-regulation in covS-mutant GAS is the major factor that limits biofilm formation and effectively negates any potential positive contribution of up-regulated genes in the covS-mutant strain. Confocal microscopy was used to visualize biofilm formation on both polystyrene (Figure 3B) and epithelial cell layers (Figure 3C). Impaired biofilm formation may contribute to a reduced ability of covS-mutant M1T1 GAS to colonize and persist in new hosts.
Reduced capacity of hypervirulent covS-mutant M1T1 GAS to bind murine skin. A mouse model of skin colonization was used to investigate the comparative capacity of the 5448 and 5448AP strains to colonize the host at a relevant infection site. The covS-mutant 5448AP strain was found to have a statistically significantly reduced ability to adhere to live mouse skin, compared with the 5448 strain \((P < .001)\) (Figure 4A), as further illustrated by the lack of adherent bacteria visible in Gram-stained skin sections (Figure 4B). Reduced survival of GAS on live skin may relate to sensitivity to cathelicidin antimicrobial peptides [38, 39]. However, no statistically significant difference between the wild-type strain 5448 and the covS-mutant strain 5448AP was noted in resistance to killing by the human cathelicidin LL-37 (MIC for both strains, 14–16 \(\mu\)mol/L) or the murine cathelicidin CRAMP (MIC for both strains, 4 \(\mu\)mol/L). Thus, the colonization defect of the covS-mutant strain is likely related to reduced adherence and/or biofilm phenotypes and not increased susceptibility to these cutaneous antimicrobials.

DISCUSSION

M1T1 GAS is the most common cause of streptococcal infections in several Western countries [1, 5, 6]. An inverse relationship between SpeB expression in clinical isolates of the M1T1 strain and disease severity indicates that inactivation of SpeB through mutation in the covR/S regulator facilitates invasive disease initiation [8–10]. Although the covS mutation in M1T1 GAS results in improved neutrophil resistance and propensity for bacterial dissemination [9, 10], here we identify potential counterbalancing fitness costs associated with the covS mutation in the realm of GAS fibronectin binding, epithelial adherence, and biofilm formation.

We recovered statistically significantly more of the 5448 strain than we did of the 5448AP strain from the mouse skin adherence model, which shows that the covS mutation and its associated phenotypes, in particular up-regulation of capsule biosynthesis, confer a colonization defect in M1T1 GAS despite the dramatic increase in virulence at subsequent stages of infection. These data are supported by a recently published finding of an inverse correlation between the ability to adhere to host cells and GAS virulence [40]. Recently, it was also shown that wild-type GAS outcompete covS-mutant strains in human saliva [41]—a finding that supports a model in which such mutations result in increased systemic virulence but come at a fitness cost for other stages of the infection process.

Differential optimization of GAS phenotypic characteristics for survival at different stages of disease pathogenesis is evident in this study. Epithelial cell binding via the extracellular matrix and biofilm formation can promote GAS colonization of the pharynx or skin in the face of competition from the normal resident microflora. However, such close interactions with host cells could be disadvantageous in systemic or bloodstream infection, in which phagocytes of the innate immune system seek to eradicate the pathogen. Hence, there is selective pressure for mutations in covR/S with up-regulation of capsule and other neutrophil and serum resistance factors, including SpyCEP [42], streptolysin O [43], Sda1 [9, 10], and streptococcal inhibitor of complement [44]. Analogous patterns of in vivo evolution have been recently described in relation to persistent Pseudomonas aeruginosa infection in patients with cystic fibrosis, with positive selection of mutations allowing for genetic changes that are advantageous to life within the host [45].

The global dissemination and persistence over decades of the
M1T1 GAS clone as the prevalent disease-associated strain not only indicates robust colonization properties but also the propensity to mutate to an immunoresistant phenotype that is capable of systemic dissemination. Our data indicate that this phenotype does not become fixed, because the cost of the covS mutation, and in particular hyperencapsulation, makes the mutant strain less capable of epithelial colonization than the parent phenotype. It is conceivable that similar paradigms exist for many leading human bacterial pathogens, for which relatively uncommon invasive disease events occur in certain individuals against a much larger backdrop of asymptomatic colonization or self-limited mucosal infection.

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