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MAJOR ARTICLE

A Mobile Genetic Element Promotes the Association Between Serotype M28 Group A *Streptococcus* Isolates and Cases of Puerperal Sepsis

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Background. Bacterial infections following childbirth—so-called puerperal infections—cause morbidity in 5%–10% of all new mothers. At low frequency, the infection can spread to the blood, resulting in life-threatening sepsis known as puerperal sepsis. Pathogens causing puerperal sepsis include group A *Streptococcus* (GAS), and epidemiological analyses have identified isolates of a single serotype, M28, as being nonrandomly associated with cases of puerperal sepsis. The genomes of serotype M28 GAS isolates harbor a 36.3-kb mobile genetic element of apparent group B *Streptococcus* origin, termed region of difference 2 (RD2).

Methods. The phenotypic (determined via tissue culture and a vaginal colonization model) and regulatory (determined via RNA sequencing analysis) contributions of RD2 were assessed by comparing parental, RD2 deletion mutant, and complemented mutant serotype M28 GAS strains.

Results. RD2 affords serotype M28 isolates an enhanced ability to adhere to human vaginal epithelial cells and to colonize the female reproductive tract in a mouse model of infection. In addition, RD2 influences the abundance of messenger RNAs from >100 core chromosomal GAS genes.

Conclusions. The data are consistent with RD2 directly, via encoded virulence factors, and indirectly, via encoded regulatory proteins, modifying the virulence potential of GAS and contributing to the decades-old association of serotype M28 isolates with cases of puerperal sepsis.

Keywords. *Streptococcus pyogenes*; mobile genetic elements; phenotypic variation; puerperal sepsis; vaginal colonization; gene regulation.

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a human bacterial pathogen that causes diseases ranging from self-limiting pharyngitis, of which there are >600 million cases annually, to severely invasive necrotizing fasciitis, which has a mortality rate of 25%–45% [1]. Pregnant and postpartum women have a 20-fold increase in the incidence of invasive GAS infections, compared with nonpregnant women [2, 3], with a large portion of this difference being attributable to puerperal infections, which are infections that can initiate in the reproductive tract of women following childbirth [4]. Puerperal infections are the sixth-leading cause of death among new mothers globally [5]. While fatalities due to puerperal infections are rare in the United States, infection frequencies of up to 6% among new mothers have been reported [6, 7], and these can lead to major complications, such as infertility [8]. Puerperal infections can spread from the reproductive tract to the blood, resulting in life-threatening sepsis known as puerperal sepsis, which is responsible for approximately 75 000 maternal deaths globally per year [2, 9]. Several pathogens cause puerperal sepsis, with pathogens of the genus *Streptococcus* being prevalent [10]. While *Streptococcus agalactiae* (group B *Streptococcus* [GBS]) is the most common streptococcal species causing puerperal infections, GAS is the most common species causing severe puerperal infections [4].

GAS strains are divided into serotypes based on the sequence of the 5′ end of the *emm* gene [11]. That some GAS serotypes show nonrandom associations with particular disease manifestations has been known for >5 decades [12, 13]. Of importance to this work, serotype M28 strains are statistically significantly associated with cases of puerperal sepsis, as identified from multiple population-based case studies performed in the United States and Western Europe [14–17]. The molecular basis of this association is unknown [18]. However, comparative analysis of an M28 GAS genome sequence previously identified 2 foreign genetic elements, designated region of difference 1

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(RD1) and 2 (RD2) [19]. RD1 is 11.1 kb long and contains several hypothetical genes, none of which are predicted to encode virulence factors [19]. RD2 is 36.3 kb long, encodes 7 putative virulence factors, and shares significant homology to 2 chromosomal regions of multiple GBS isolates (Figure 1) [19, 20]. GBS organisms are constituents of the normal human vaginal microflora in approximately 25% of women [21] and are also a major cause of neonatal invasive infections [22]. Consequently, it was hypothesized more than a decade ago, but never tested owing to technical issues associated with the creation of an RD2 deletion mutant strain, that the acquisition of RD2 from GBS enhances the ability of M28 GAS isolates to cause puerperal sepsis.

Here, we describe an investigation of the phenotypic and regulatory consequences of RD2 acquisition by serotype M28 GAS. Our data are consistent with RD2 being a pathogenicity island that directly, via encoded virulence factors, and indirectly, via the transcriptional regulation of core chromosomally encoded genes, plays a key role in the association between serotype M28 GAS isolates and puerperal sepsis cases.

METHODS

Bacterial Strains and Growth Conditions

The GAS strains used in this study are summarized in Table 1. GAS were grown in Todd-Hewitt broth containing 0.2% yeast extract (hereafter, "THY broth"). When needed, spectinomycin or kanamycin were added to a final concentration of 150 μg/mL or 300 μg/mL, respectively.

Screening GAS Isolates for RD2

To test whether selected M11 and M28 GAS isolates harbored RD2, we performed polymerase chain reaction (PCR) analysis for detection of the RD2-encoded gene *r28*. PCR analyses included not only primers for the amplification of *r28* but also for the amplification of the core chromosomally encoded gene *ropB*.

Tissue Culture Assays of Adherence

These assays were performed using a protocol that we described previously [24].

Construction of the RD2 Deletion Mutant–Derivative Strain, M28ΔRD2

To assess the contribution of RD2 to the virulence characteristics of serotype M28 GAS isolates, we created an RD2 deletion mutant strain (M28ΔRD2) via a 2-step procedure. The first step, which created strain M28ΔRD2-Kn, replaced most of RD2 with a kanamycin resistance cassette. Strain M28ΔRD2-Kn was created using a plasmid construct in which a kanamycin resistance cassette was flanked by 1-kb regions complementary to the 5′ and 3′ ends of RD2. See Supplementary Table 2 for the PCR primers used to create the plasmid construct. The second step removed the remaining fragments of RD2 and the kanamycin resistance cassette from strain M28ΔRD2-Kn. The resultant strain, M28ΔRD2, is a markerless and scarless RD2 deletion mutant strain that harbors an intact RD2 integration site (and, hence, can be complemented by reintroduction of RD2). Strain M28ΔRD2 was created from strain M28ΔRD2-Kn by recombinational replacement using the suicide vector pBBL740, and the protocol was similar to that which we have previously described [25]. Strain M28ΔRD2 was verified by tiling PCR, targeted sequencing, and Western blot analyses.

Complementation of Strain M28ΔRD2

To facilitate complementation of M28ΔRD2, we first created strain M28.RD2^{SPEC}, which is a derivative of MGAS6180, in which a nonpolar spectinomycin resistance cassette was

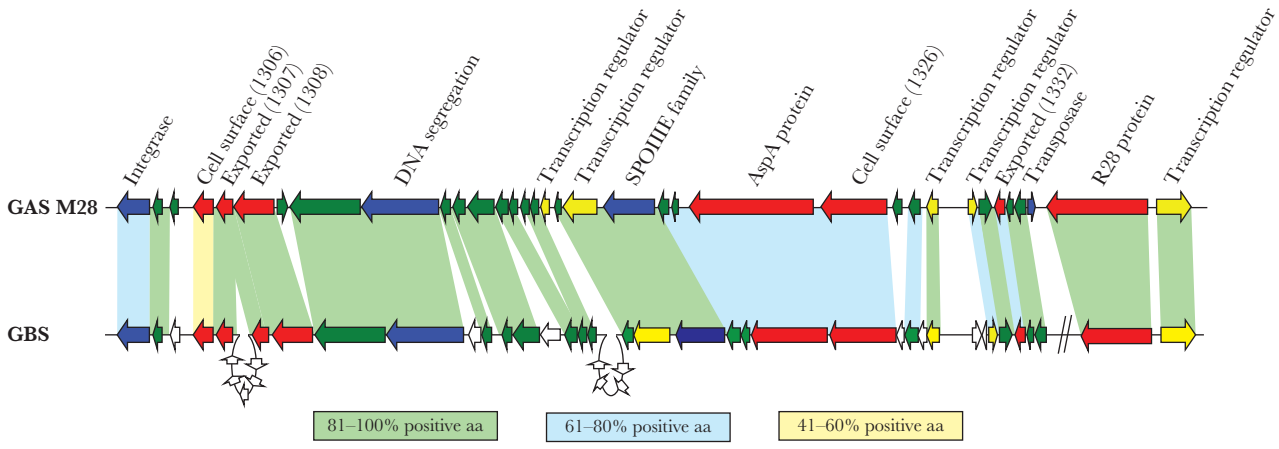

Figure 1. Comparison of region of difference 2 (RD2) from serotype M28 group A *Streptococcus* (GAS) to genes present in group B *Streptococcus* (GBS). Genes are represented by block arrows and are color coded on the basis of their putative open reading frame function or the type of protein they encode (blue, mobility; yellow, gene regulators; red, extracellular proteins; and green, hypothetical). Genes unique to GBS are colored white. Shading between GAS and GBS genes is based on homology at the protein level. Percentages of shared amino acid were calculated by adding the percentages of identical and strongly similar amino acids together for each of the compared protein pairs. The 2 diagonal lines on the GBS part of the figure signify that the 2 genes are not contiguous with the other genes in GBS but are contiguous with the other genes in GAS.

Table 1. Group A *Streptococcus* (GAS) Strains Used in This Study

Strain	Description	Reference
MGAS2221	A previously characterized serotype M1 GAS strain	[23]
PGAS951	M11 strain from the CDC; isolated in 2016 from a blood specimen of a patient in CT; the patient did not have puerperal sepsis	This study
PGAS953	M11 strain from the CDC; isolated in 2014 from a blood specimen of a patient in MN; the patient did not have puerperal sepsis	This study
PGAS955	M11 strain from the CDC; isolated in 2017 from a blood specimen of a patient with puerperal sepsis in MN	This study
PGAS956	M11 strain from the CDC; isolated in 2017 from a blood specimen of a patient with puerperal sepsis in NY	This study
PGAS958	M28 strain from the CDC; isolated in 2015 from a blood specimen of a patient in CA; the patient did not have puerperal sepsis	This study
PGAS959	M28 strain from the CDC; isolated in 2014 from a blood specimen of a patient in NY; the patient did not have puerperal sepsis	This study
PGAS961	M28 strain from the CDC; isolated in 2013 from a blood specimen of a patient with puerperal sepsis in OR	This study
PGAS962	M28 strain from the CDC; isolated in 2014 from a blood specimen of a patient with puerperal sepsis in NM	This study
MGAS6180	A previously characterized serotype M28 GAS strain	[19]
M28.RD2^{SPEC}	A derivative of MGAS6180 in which RD2 has been tagged with a spectinomycin-resistance cassette inserted between genes 1329 and 1330	This study
M28ΔRD2-Kn	A derivative of MGAS6180 in which all copies of RD2 have been deleted, with a kanamycin-resistance cassette replacing them	This study
M28ΔRD2	A derivative of M28ΔRD2-Kn in which the kanamycin resistance cassette was replaced with the natural RD2 integration site	This study
RD2^{Comp}	A derivative of M28ΔRD2 in which RD2 from strain M28.RD2^{SPEC} has been introduced	This study
M28ΔR28	An *r28* deletion mutant derivative of MGAS6180	This study

Abbreviations: CA, California; CDC, Centers for Disease Control and Prevention; CT, Connecticut; MN, Minnesota; NM, New Mexico; NY, New York; OR, Oregon.

inserted between the RD2-encoded genes M28_1329 and M28_1330 [26]. Verification that insertion of the spectinomycin resistance cassette did not alter the abundance of M28_1329 or M28_1330 transcripts was gained via quantitative reverse transcription (RT)–PCR (Supplementary Figure 1). To complement strain, M28ΔRD2 we recovered total DNA from strain M28.RD2^{SPEC} and used it to transform M28ΔRD2 via electroporation. Verification of the complemented strain, RD2^{Comp}, was performed by PCR, targeted sequencing, and Western blot analyses. In addition, quantitative PCR analysis was performed to assay RD2 copy number (Supplementary Figure 2).

Construction of the *r28* Deletion Mutant Strain M28△R28

To create an *r28* deletion mutant derivative of MGAS6180, we replaced the *r28* gene with a spectinomycin resistance cassette via a previously described protocol [26]. Verification of strain M28ΔR28 was performed by PCR, targeted sequencing, and Western blot analyses.

Tiling PCR Analysis

To assess for the presence or absence of RD2 in MGAS6180, M28ΔRD2, and M28^{Comp}, we performed an overlapping PCR analysis of this region. Fifteen pairs of PCR primers were designed that were arrayed across RD2 (Supplementary Table 2), with average PCR size of approximately 3 kb, of which approximately 1 kb overlapped with adjacent PCR products. PCR reactions were performed for each of the tested GAS strains, using genomic DNA from each strain as a template.

Isolation of GAS Cell Wall Proteins

Cell wall protein fractions were isolated from mid-exponential phase (OD_{600} 0.5) GAS cultures as previously described [24]. Proteins were separated on 7.5% Tris-HCl gels before they were transferred to membrane and analyzed by Western blot with a custom-made anti-R28 rabbit polyclonal antibody. After washing, Alexa Fluor 680 donkey anti-rabbit immunoglobulin G secondary antibody (Molecular Probes) was used (1:10 000 dilution), and the fluorescent signal was detected using a Li-Cor Odyssey Near-Infrared Imaging System. Note that the membrane was stained using the MemCode Reversible Proteins Stain Kit (Pierce) to serve as a loading control.

Quantitative RT-PCR Analyses

RNA samples from triplicate exponential phase cultures of each GAS strain under investigation were isolated, and converted into complementary DNA (cDNA), as previously described [23]. TaqMan primers and probes for the genes of interest, and the internal control gene *proS*, are shown in Supplementary Table 2.

Quantitative PCR Analyses

Total DNA samples from triplicate cultures of each GAS strain under investigation were isolated. The abundance of RD2 relative to that of MGAS6180 (which was previously shown to harbor approximately 8 copies of RD2 per genome [20]) was compared for each strain via use of primers and probes targeting the RD2-encoded gene *r28* and the non–RD2-encoded gene *proS*. Data were normalized to *proS* DNA levels.

Mouse Model of Vaginal Colonization

We followed slightly modified versions of 2 previously described protocols [27, 28]. Briefly, 2 days before streptococcal infection, female CD-1 mice (12 weeks old; Charles

River) were estrogenized by intraperitoneal injection of 0.5 mg of β-estradiol 17-valerate (Sigma-Aldrich) dissolved in 0.1 mL of sterile sesame oil (Sigma-Aldrich). On the day of inoculation (day 0), mice were inoculated in the vaginal lumen with 10 μL of GAS suspension that had been diluted to 1×10^6 colony-forming units (CFUs)/mL (hence, each mouse received 1×10^4 CFUs). Twenty mice were infected per GAS strain tested. Colonization levels were assayed on days 1, 3, 5, 7, and 10. To do so, the vaginal vaults of all mice were swabbed using FLOQSwabs (Copan Diagnostics). Each swab was subsequently transferred to a 1.5-mL microcentrifuge tube containing 500 μL of phosphate-buffered saline (PBS). The microcentrifuge tubes was thoroughly vortexed to release any GAS from the swab, and serial dilutions in PBS were plated onto Selective Strep Agar plates (Becton Dickinson) to enable determination of CFUs. This experiment was performed in accordance to the guidelines set forth in a protocol approved by the Institutional Animal Care and Use Committee of the University of Nevada–Reno.

Total RNA Isolation and RNA Sequencing (RNA-Seq) Analysis

Total RNA was isolated from duplicate THY broth cultures of MGAS6180 and M28RD2 in exponential phase, as previously described [23]. Ribosomal RNAs (rRNAs) were depleted using the Ribo-Zero Gram-Positive rRNA Removal Kit (Epicentre). The rRNA-depleted RNA was then used to generate cDNA libraries, using the ScriptSeq kit (Epicentre) [29]. To do this, RNA was fragmented, cDNA was synthesized using random hexamers containing a 5′ tagging sequence, RNA was hydrolyzed, and cDNA was tagged at the 3′ end. A limited number of PCR cycles (n = 14) were used to amplify the libraries via the 5′ and 3′ tags (the libraries were barcoded by using different primers), and the libraries were selected according to size (170–300 bp). The size-selected and barcoded libraries were run on an Illumina MiSeq flow cell system. Data were analyzed using CLC Genomics Workbench (Qiagen) and normalized to the overall sequencing depth, using total mapped read data. Statistical significance was tested using the Kal *Z* test with a false-discovery rate correction [30].

RNA-Seq data have been deposited in the Gene Expression Omnibus database at the National Center for Biotechnology Information (accession no. GSE116474; available at: http://www.ncbi.nlm.nih.gov/geo).

RESULTS

Serotype M28 GAS Isolates Show Greater Adherence to a Human Epithelial Cell Line Than Serotype M11 Isolates

We hypothesize that the overrepresentation of M28 GAS isolates among cases of puerperal sepsis is in part a consequence of the RD2 element conferring an enhanced ability to adhere to vaginal epithelial cells. As a first step in testing this hypothesis, we compared adherence between 4 serotype M11 GAS isolates and 4 serotype M28 isolates. Note that, for each serotype, 2 isolates were recovered from patients with puerperal sepsis, while 2 were from patients with non–puerperal sepsis invasive infections. We chose to compare M28 isolates to M11 isolates because several studies have identified M11 isolates as also being recovered, albeit at lower frequency than M28, from cases of puerperal sepsis [15, 16]. In addition, serotype M11 isolates are not known to harbor the RD2 element, while all M28 isolates do [19], and this was consistent with a PCR-based screen of the isolates used in our study (Figure 2A). To test adherence, we used a tissue culture–based model, using the human epithelial cell line A431 [31]. As a group, the serotype M28 isolates adhered at a 3.4-fold higher level than the serotype M11 isolates (Figure 2B). No differences in adherence were observed between the puerperal sepsis and non–puerperal sepsis isolates, consistent with there being little to no within-serotype differences between isolates causing puerperal sepsis and those causing other diseases. While the data do not reveal whether the higher M28 GAS adherence was due to the presence of the RD2 element, they are consistent with this hypothesis.

RD2 Can Be Deleted From M28 GAS

To facilitate investigation of whether RD2 modifies the virulence potential of GAS, we created strain M28ΔRD2, an RD2 deletion mutant derivative of the M28 isolate MGAS6180. Given the large size (36.3 kb), multicopy nature (n = approximately 8), and distribution of RD2 between both chromosomally integrated and extrachromosomal forms [20], the creation of M28ΔRD2 proved challenging, but we were ultimately successful. A complemented mutant derivative of M28ΔRD2, strain $RD2^{Comp}$, was also constructed, by introducing a modified RD2 element that was tagged with a spectinomycin resistance cassette. One of several methods that were used to verify the absence of the RD2 element in strain M28ΔRD2 and the presence of the RD2 element in strain $RD2^{Comp}$ was tiling PCR. A series of 15 primer pairs were created that spanned RD2, with Figure 3A showing the approximate locations of each of the 15 resultant PCR products with regard to the RD2 element. PCR products of the expected size were gained using genomic DNA from the parental isolate and the complemented mutant derivative, strain while, as expected, no PCR products were gained for the RD2 deletion mutant strain (Figure 3B). The expected results were also obtained from a Western blot analysis of the RD2-encoded cell surface protein R28, with the parental and complemented strains but not the RD2 deletion mutant or an isogenic *r28* deletion mutant strain (strain M28ΔR28) expressing this protein (Figure 3C).

RD2 Promotes GAS Adhesion to Human Epithelial Cells

Using our newly constructed MGAS6180 derivatives, we tested the hypothesis that RD2 promotes the ability of GAS to adhere to epithelial cells. To reduce the possibility of our data being confounded by cell line–specific interactions, 2 cell lines were

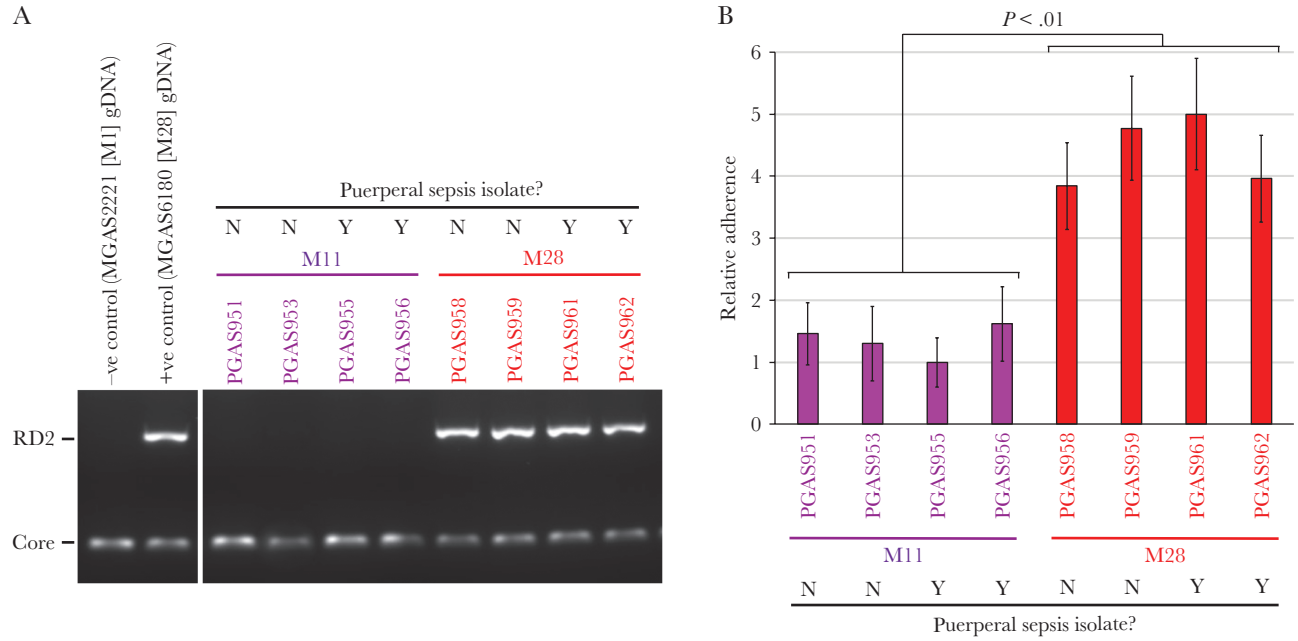

Figure 2. Serotype M28 group A *Streptococcus* (GAS) isolates have enhanced abilities to adhere to human epithelial cells, relative to M11 isolates. Four serotype M11 and 4 serotype M28 GAS isolates were compared. *A,* Polymerase chain reaction (PCR) analysis for detection of the region of difference 2 (RD2)–encoded gene *r28* (RD2) revealed that the RD2 element is present in the tested M28 isolates but not in the tested M11 isolates. Amplification of a core chromosomally encoded gene, *ropB* (Core), served as a PCR control. *B,* A tissue culture–based adherence assay, using the human epithelial cell line A431. Data are presented relative to findings for the lowest adhering strain. The experiment was performed on 4 occasions, mean values (± standard errors of the mean) shown. Statistical significance was tested via a *t* test. Neg, negative; Pos, positive.

used: A431 and the human vaginal epithelial cell line VK2/E6E7 [32]. In each case, the parental and complemented isolates adhered at a higher level (by 3–4-fold) than the RD2 deletion mutant strain (Figure 4A and 4B). Thus, the RD2 element increased the ability of GAS to adhere to host cells. That the enhanced adherence phenotype was not simply attributable to the RD2-encoded R28 protein, which was previously shown to serve as an adhesin [33], was identified by including an *r28* deletion mutant strain in our adherence assays. Strain M28ΔR28 showed levels of adherence similar to that of the parental strain (Figure 4A and 4B). Hence, although R28 may have a contributory role in the ability of RD2 to enhance GAS adherence, other RD2-encoded factors appear to play more significant roles.

RD2 Enhances Colonization in a Murine Model of Vaginal Colonization

Given the adherence phenotype associated with RD2 and the association of serotype M28 isolates with puerperal sepsis, we hypothesized that the gain of RD2 by M28 isolates increases their ability to colonize the female reproductive tract. To test this hypothesis, we used a murine model of vaginal colonization [27, 28]. The parental, RD2 deletion mutant, and complemented strains were used to infect female mice that were pretreated with estradiol to synchronize their estrous cycle. Infections occurred by instilling 10 μL of PBS containing 1×10^4 GAS CFUs into the vaginal lumen. Colonization levels were calculated by swabbing the vaginal lumen of infected animals over time and determining the number of GAS CFUs on each swab. We identified 2–6-fold higher levels of CFUs after day 1 for the parental and complemented mutant strains, relative to the RD2 deletion mutant strain (Figure 5). Thus, the RD2 element confers onto serotype M28 GAS isolates an enhanced ability to colonize the female reproductive tract.

RD2 Remodels the GAS Transcriptome

The RD2 element encodes 5 putative transcription regulators (Figure 1). While examples are rare, some studies have identified pathogenicity island–encoded regulators that have core chromosomal genes as regulatory targets [34–36]. To assess whether genes located outside of RD2 are differentially regulated between the presence and absence of this element, we performed an RNA-Seq–based transcriptome analysis. MGAS6180 and derivative M28ΔRD2 were compared from exponential phase THY broth cultures. Transcripts from 108 core chromosomal genes were identified as being differentially regulated, using a cutoff of ≥2-fold (Figure 6A and Supplementary Table 1). Transcripts that were altered in abundance in the presence of RD2 included several that encode virulence factors, such as the immunomodulatory endoglycosidase EndoS [37]. Verification of differential *endoS* mRNA levels between the presence and absence of RD2 was gained via quantitative RT-PCR and included use of the complemented strain $RD2^{Comp}$ (Figure 6B). Increasing and decreasing the abundance of different virulence factors, resulting in the generation of new virulence factor profiles, is key to the ability of GAS to cause distinct diseases [38–40]. Thus, the

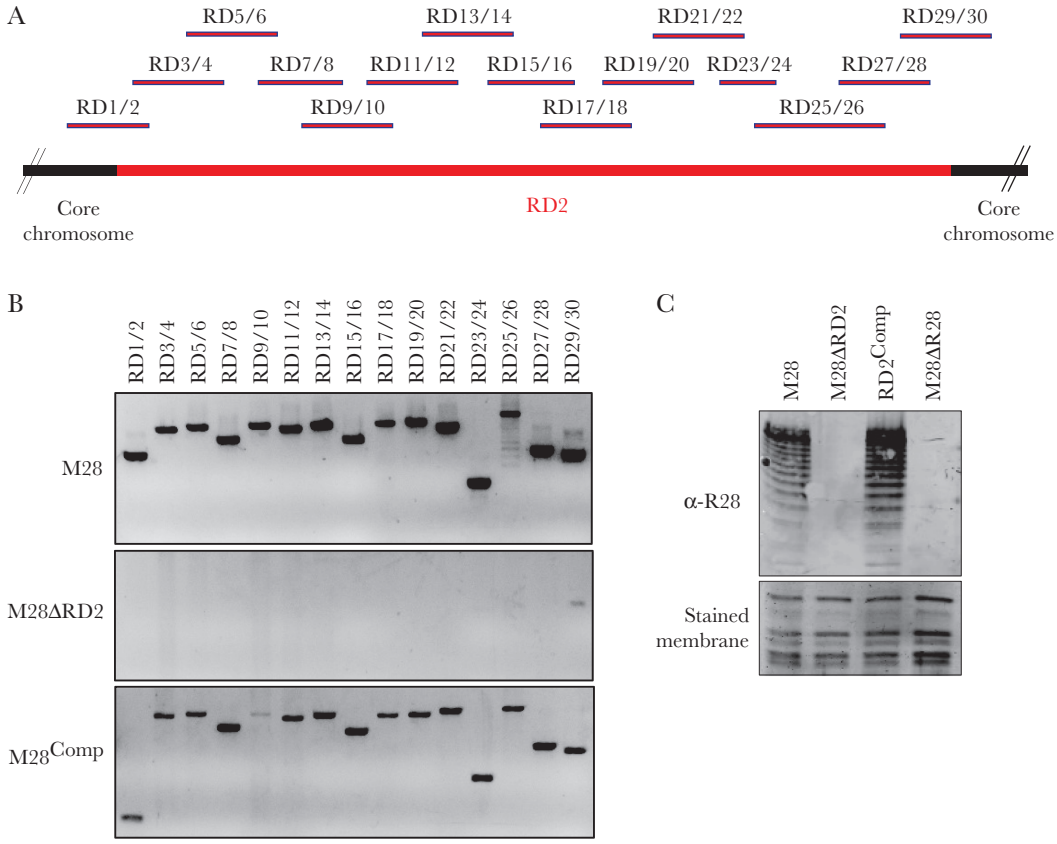

Figure 3. Verification of region of difference 2 (RD2) deletion and complementation in strains M28ΔRD2 and RD2^{Comp}, respectively. Total DNA from the parental (M28), RD2 deletion mutant (M28ΔRD2), and complemented mutant (RD2^{Comp}) strains was isolated and used in polymerase chain reaction (PCR) analysis with 15 pairs of primers (RD1 to RD30). *A*, Distribution of the primers and expected PCR products across RD2. Note that primers RD1 and RD30 are located outside of RD2. *B*, Generated PCR products for the 3 tested strains. Note that there is a 1-kb size difference between the RD1/2 PCR product of the parental and RD2^{Comp} strains, and this is due to the presence of a 1-kb insertion sequence that flanks RD2 in MGAS6180 but not in the complemented strain. *C*, Western blot analysis of cell wall–associated R28 expression in the 4 indicated GAS strains. Note the characteristic ladder pattern of R28 [33]. The membrane was stained prior to Western blot analysis, to serve as a loading control.

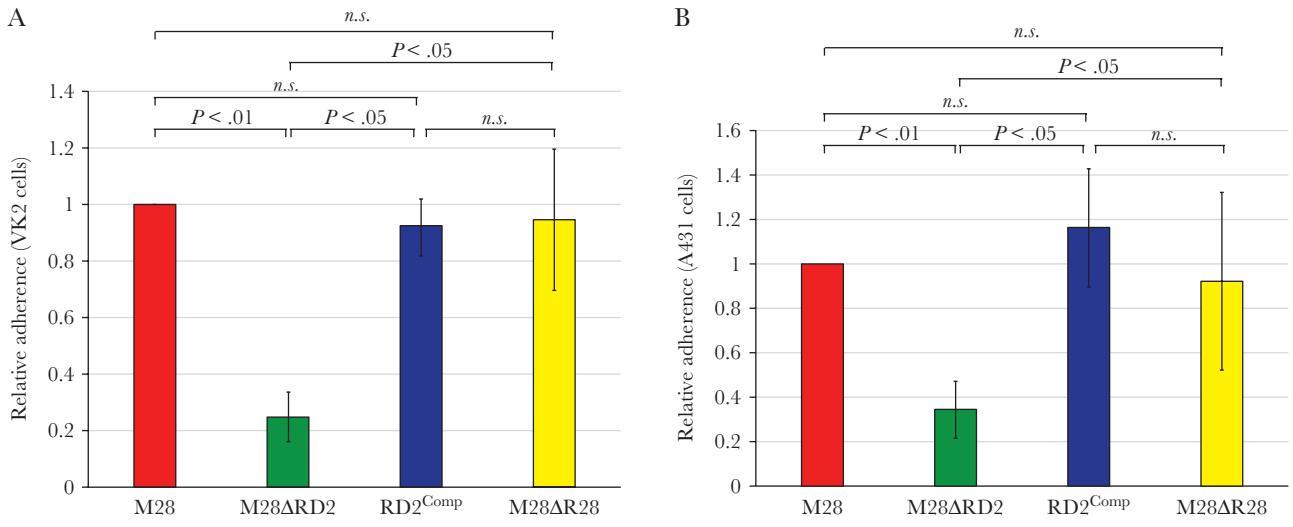

Figure 4. The region of difference 2 (RD2) element enhances the ability of serotype M28 group A *Streptococcus* (GAS) to adhere to human epithelial cells. The indicated strains were compared in tissue culture-based assays of adherence, using VK2/E6E7 (*A*) and A431 (*B*) human cell lines. Each experiment was performed on 4 occasions, with mean values (± standard errors of the mean) shown. For both data sets (*A* and *B*), $P < .001$ (via overall analysis of variance). Pair-wise strain comparisons were investigated using the Tukey multiple comparisons test, with *P* values shown. NS, not significant.

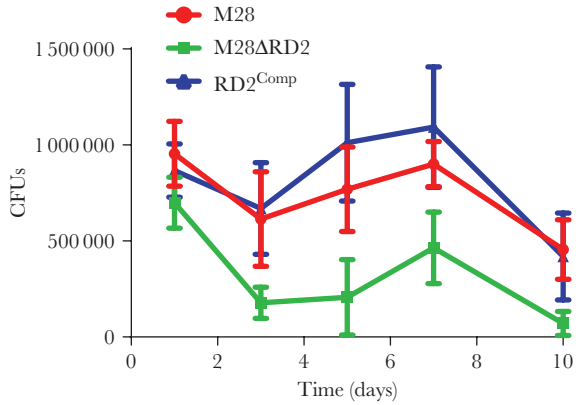

Figure 5. Region of difference 2 (RD2) promotes the ability of serotype M28 group A *Streptococcus* (GAS) isolates to colonize the murine vagina. Mice treated with estradiol (0.5 mg) were vaginally challenged with 1×10^4 colony-forming units (CFUs) of the indicated GAS strains. Shown is the mean number (± the standard error of the mean) of CFUs recovered from vaginal swabs over time. A repeated-measure analysis indicated that a statistically significantly lower number of CFUs was recovered over time for the RD2 deletion mutant strain, relative to the parental and complemented mutant strains ($P < .01$).

data are consistent with the phenotypic consequences of RD2 acquisition, which at a minimum is enhancement of vaginal colonization (Figure 5), in part being attributable to the RD2-mediated regulation of genes located outside of this element.

DISCUSSION

Phenotypic heterogeneity between isolates of a given bacterial species is a commonly observed phenomenon [41, 42]. In some instances, this heterogeneity is a consequence of the isolate-specific presence or absence of virulence factor–encoding mobile genetic elements. For example, cases of staphylococcal scaled skin syndrome are caused by only a subset of *Staphylococcus aureus* isolates—those that harbor a bacteriophage or plasmid that encode an exfoliative toxin [43]. Here, we investigated whether the presence of the RD2 genomic island in serotype M28 GAS isolates contributes to their nonrandom association with cases of puerperal sepsis. We identified that RD2 enhanced the ability of GAS to adhere to human epithelial cell lines and to colonize in a murine model of vaginal colonization (Figures 4 and 5) and, hence, that RD2 can appropriately be classified as a pathogenicity island. We also discovered that RD2 regulates the abundance of mRNAs from core chromosomally encoded genes (Figure 6), consistent with this element modifying the virulence potential of GAS through both direct and indirect mechanisms.

The RD2 pathogenicity island is distributed in the GAS population mostly along serotype-specific lines. All serotype M28 and most serotype M2 isolates tested to date contain RD2 [19]. Of note, serotype M2 GAS strains are associated with cases of vaginitis and urinary tract infections [14, 19], which we hypothesize may in part be due to RD2 modifying the disease potential of these isolates, similar to that described here for M28 GAS isolates and puerperal sepsis. RD2 has also been detected at low frequency in select isolates of other serotypes (ie, M4, M48, M53, M61, M77, and M87) [19]. A molecular explanation for the distribution of RD2 in the GAS population (ie, what are the barriers to the transfer and acquisition of this element?) is currently under investigation.

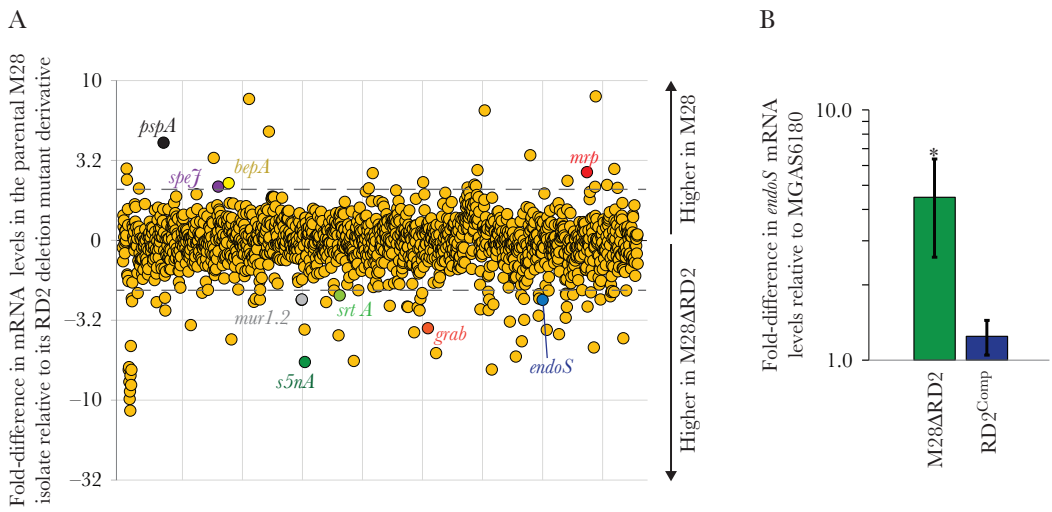

Figure 6. Acquisition of the region of difference 2 (RD2) element alters the group A *Streptococcus* (GAS) transcriptome. *A*, Results of RNA sequencing analysis comparing the parental M28 strain MGAS6180 and its RD2-deletion mutant derivative (M28ΔRD2) during exponential phase growth in Todd-Hewitt broth containing 0.2% yeast extract. Each circle represents messenger RNA (mRNA) from a single gene. Genes are arranged in the same order as they appear in the chromosome. Select virulence factor–encoding mRNAs are colored and labeled. Dashed lines indicate ±2-fold. *B*, Findings of TaqMan-based quantitative reverse transcription polymerase chain reaction analysis. The abundance of *endoS* mRNA was determined from triplicate exponential phase GAS cultures of each indicated strain, run in duplicate. Shown is the mean (± standard deviation). $P < .001$ (via overall analysis of variance). Pair-wise strain comparisons with the parental isolate were investigated using the Tukey multiple comparisons test. *$P < .05$.

Our data are consistent with RD2 enhancing the adherence and colonization of M28 GAS isolates to the female reproductive tract (Figures 4 and 5), a precursor to the development of puerperal sepsis. Thus, we hypothesize that the presence of RD2 is a contributing factor in the enhanced frequency of M28 GAS recovery from cases of puerperal sepsis. Note that, although the development of puerperal sepsis may be enhanced by RD2, this element is not required for it, as evident from data showing that the non–serotype M28 puerperal sepsis isolates lack RD2 (Figure 2A) [44–46].

We hypothesize that the enhanced adherence phenotype conveyed by RD2 is, at least in part, a culmination of the activity of the 4 putative cell wall–anchored proteins encoded by this element (R28, AspA, M28_1306, and M28_1326; Figure 1). The R28 protein has previously been shown to function as an adhesin [33], although we saw no phenotypic consequences to deleting the R28-encoding gene in our tissue culture–based adherence assays (Figure 4A and 4B). Differences in the cell lines (ME180 vs VK2/E6E7 and A431) and protocols (eg, the length of time GAS organisms were incubated with the tissue culture cells before washing) may account for the disparate data with regard to the adhesin activity of R28. AspA also likely contributes to our observed adhesin phenotype, given previous data showing a role for this protein in binding to respiratory epithelial cells [47]. While the M28_1306 and M28_1326 proteins have not been studied in detail, they are both expressed during infection, as assessed by convalescent-phase serum but not acute-phase serum from infected patients harboring antibodies against these proteins [19]. Both proteins have LPXTG motifs toward their carboxy terminus, consistent with them being covalently anchored to the GAS cell wall. Note that, while the 4 proteins described above are the most obvious candidate proteins encoded within RD2 that promote adherence, we cannot rule out other RD2-encoded proteins directly or indirectly influencing this activity.

We identified that genes located outside of the RD2 element were differentially regulated in the presence of RD2 (Figure 6A). A molecular explanation for this regulatory phenotype remains to be investigated. However, our hypothesis is that RD2-encoded regulators target the transcription of core chromosomally encoded genes, and we aim to follow up on this hypothesis by creating and using M28 derivatives with mutations in one or more of the regulator-encoding genes. Consistent with this are data from a recent study that identified that enhancing transcription of the RD2-encoded regulator Spy1337 alters global transcript profiles and strain virulence [48].

In conclusion, we have identified that RD2 is a pathogenicity island conferring onto GAS an increased ability to colonize in a murine model of vaginal colonization. This enhanced colonization phenotype likely contributes to the association of RD2-containing serotypes, M2 and M28, with infections of the female urinary and/or reproductive tracts. Our findings expand insight into the importance of horizontal gene transfer as a mechanism driving strain emergence and phenotypic heterogeneity in a prevalent gram-positive pathogen.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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