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# A Novel Integrative Conjugative Element Mediates Genetic Transfer from Group G Streptococcus to Other β-Hemolytic Streptococci▽

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**Lateral gene transfer is a significant contributor to the ongoing evolution of many bacterial pathogens, including β-hemolytic streptococci. Here we provide the first characterization of a novel integrative conjugative element (ICE), ICE*Sde*3396, from *Streptococcus dysgalactiae* subsp. *equisimilis* (group G streptococcus [GGS]), a bacterium commonly found in the throat and skin of humans. ICE*Sde*3396 is 64 kb in size and encodes 66 putative open reading frames. ICE*Sde*3396 shares 38 open reading frames with a putative ICE from *Streptococcus agalactiae* (group B streptococcus [GBS]), ICE*Sa*2603. In addition to genes involves in conjugal processes, ICE*Sde*3396 also carries genes predicted to be involved in virulence and resistance to various metals. A major feature of ICE*Sde*3396 differentiating it from ICE*Sa*2603 is the presence of an 18-kb internal recombinogenic region containing four unique gene clusters, which appear to have been acquired from streptococcal and nonstreptococcal bacterial species. The four clusters include two cadmium resistance operons, an arsenic resistance operon, and genes with orthologues in a group A streptococcus (GAS) prophage. Streptococci that naturally harbor ICE*Sde*3396 have increased resistance to cadmium and arsenate, indicating the functionality of genes present in the 18-kb recombinogenic region. By marking ICE*Sde*3396 with a kanamycin resistance gene, we demonstrate that the ICE is transferable to other GGS isolates as well as GBS and GAS. To investigate the presence of the ICE in clinical streptococcal isolates, we screened 69 isolates (30 GGS, 19 GBS, and 20 GAS isolates) for the presence of three separate regions of ICE*Sde*3396. Eleven isolates possessed all three regions, suggesting they harbored ICE*Sde*3396-like elements. Another four isolates possessed ICE*Sa*2603-like elements. We propose that ICE*Sde*3396 is a mobile genetic element that is capable of acquiring DNA from multiple bacterial sources and is a vehicle for dissemination of this DNA through the wider β-hemolytic streptococcal population.**

Lateral gene transfer (LGT) plays a profound role in the generation of genetic diversity within bacterial pathogens (28, 35). LGT rapidly facilitates the adaptation of bacteria to novel environments and leads to the expansion of virulence determinants. Conjugative transposons are major mediators of LGT in prokaryotes and together with integrative plasmids are known as integrative conjugative elements (ICEs). ICEs often carry genes for auxiliary traits such as resistance to antibiotics and heavy metals and are widely implicated as the primary disseminator of such phenotypes (9).

The β-hemolytic streptococci constitute a group of human and animal pathogens that cause a wide variety of diseases in their respective hosts (24). The human pathogens include *Streptococcus pyogenes* (group A streptococcus [GAS]), *Streptococcus agalactiae* (group B streptococcus [GBS]), and *Streptococcus dysgalactiae* subsp. *equisimilis* (human group C and group G streptococcus [GGS]). Historically, GAS is associated with diseases such as pharyngitis, impetigo, scarlet fever, poststreptococcal glomerulonephritis, rheumatic disease, and rheumatic heart disease (16). In the 1980s, GAS emerged as a cause of a serious and potentially fatal invasive disease (15, 47) which is estimated to kill between 1,000 and 1,700 people each year in the United States (6). Similarly, GBS, a major veterinary pathogen, emerged as a leading cause of bacterial invasive disease in newborns in the 1970s (21). GGS has traditionally been considered a commensal organism found as part of the normal flora of the skin, throat, and other mucosal surfaces and caused only opportunistic infections in individuals with underlying risk factors (12). However, GGS is increasingly associated with a spectrum of disease in healthy individuals which overlaps that of GAS. These include epidemic pharyngitis, bacteremia, puerperal sepsis, peritonitis, cellulitis, septicemia, infective endocarditis, and glomerulonephritis (12, 14, 22, 23, 29, 30, 34, 49, 56). Additionally, GGS is also associated with serious streptococcal invasive diseases, including necrotizing fasciitis and toxic shock syndrome (57).

Changes in genome content, arising through mutations (e.g., allelic variation or gene duplication) or LGT have been hy-

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pothesized to contribute to changes in the disease association of GAS and GBS in the last few decades (1, 2, 5, 54, 58). Genome sequencing and other genetic studies have also reinforced the importance of mobile genetic elements (MGEs) in generating diversity within these species (5, 26, 37, 48, 51, 52). In fact, the pangenomes (representing the entire genetic repertoire of a species) of both GAS and GBS are considered to be open (51), implying that new genes continue to enter the population though ongoing interspecies LGT. In the absence of genomic sequences, our understanding of genomic variation in GGS is less clear. Given the close genetic relatedness between GAS, GBS, and GGS (24) and evidence that GGS genomes are more "chaotic" than GAS genomes (33), it is likely that the GGS pangenome is also open and is participating in interspecies LGT. In this regard, several studies have provided evidence for LGT involving GGS and GAS (27, 33). Our previous molecular epidemiological study also reported that intra- and interspecies LGT is likely to be occurring in environments where GGS and GAS are endemic (18, 19).

To date there has been little or no direct evidence for ICE mediated cross-species LGT between GGS and GAS or GGS and GBS. In the current study, we have provided the first detailed genetic and functional characterization of an ICE from GGS and demonstrate its conjugative transfer to both GAS and GBS. Significantly, ICE*Sde*3396 contains a large internal recombinogenic region that carries functional genes and operons derived from both streptococcal and nonstreptococcal species, implying that it is a vehicle for dissemination of novel genes through the wider β-hemolytic streptococcal population.

## MATERIALS AND METHODS

**Bacterial strains and molecular methods.** *S. dysgalactiae* subsp. *equisimilis* NS3396 was isolated from a patient presenting with pharyngitis and acute rheumatic fever (19). All other GGS, GAS, and GBS isolates (Table 1) were isolated from individuals presenting with streptococcal disease or as part of community surveys within Australia (17, 19, 20). The isolates used in this study are nonclonal, as determined by emm typing for GAS and GGS and serotyping for GBS. All streptococci were grown in Todd-Hewitt broth or on Todd-Hewitt agar supplemented with 2% horse blood. Growth medium was supplemented with kanamycin (500 μg/ml) and/or streptomycin (400 μg/ml) when appropriate.

Genomic DNA extraction, restriction enzyme digestions, ligations, PCR, and DNA sequencing all utilized standard procedures. Adapter PCR analysis was performed as previously described (18, 43). Briefly, genomic DNA was partially digested with a BclI and/or HindIII, purified, and ligated with a 5′ phosphorylated oligonucleotide adaptor containing BclI or HindIII overhangs, respectively. Standard PCR was then undertaken, using one primer complementary to the adapter sequence and another internal primer sequence specific for the DNA sequence of interest. The resulting products were purified after agarose gel electrophoresis and subject to DNA sequence analysis. The nucleotide (nt) sequence of adapter PCR primers is shown in Table 2.

**Determination of the ICE*Sde*3396 nucleotide sequence.** A combination of long PCR (11), primer walking, and adapter PCR was employed to determine the complete sequence of ICE*Sde*3396. Primers used for these activities were based on DNA fragments identified by the initial genomic subtraction of NS3396 (19) or on the sequence of the putative GBS conjugative transposon present in the GBS 2603V/R genome (52). Forward and reverse DNA strand contigs were assembled using the Staden package. Assignment of open reading frames (ORFs) was achieved using the bacterial annotation system, BASys (53). The complete ICE*Sde*3396 sequence is deposited in the GenBank database under accession number EU142041.

**PCR screening of streptococci.** The presence of ICE*Sde*3396 and ICE*Sa*2603 in GGS, GBS, and GAS was determined by PCR amplification of DNA representing three separate regions of ICE*Sde*3396 (Fig. 1). The presence of R1 in streptococcal genomes was determined by amplification of a 3.1-kb fragment spanning from ICE*Sde*3396_orf1 to ICE*Sde*3396_orf5. The 4.2-kb amplicon used to identify the presence of the R2 region extends from ICE*Sde*3396_orf17, within R1, to ICE*Sde*3396_orf23 in R2. We chose to amplify a region extending from R1 to R2 to ensure that these regions were adjacent to one another in the streptococcal genomes examined. The R3 region was identified by amplification of a 3.2-kb fragment (ICE*Sde*3396_orf55 to ICE*Sde*3396_orf61). The nucleotide

TABLE 1. Bacterial strains used in this study

| Isolate[a] | Emm type/serotype | Site of isolation | Source or reference |
|---|---|---|---|
| GGS | | | |
| NS3396 | STG480 | Throat | 19 |
| G120 | STG4831 | Throat | |
| G121 | STC74 | Unknown | 19 |
| G122 | STC74 | Unknown | 19 |
| GGS10 | STG6 | Unknown | 19 |
| GGS24 | STG6 | Unknown | 19 |
| GGS48 | STG4831 | Unknown | 19 |
| MD013 | STG10 | Skin | This study |
| MD048 | STG485 | Skin | 17 |
| MD077 | STG10 | Skin | 17 |
| MD128$^{GCS}$ | STG93464 | Throat | 17 |
| MD172 | STC74a | Skin | 17 |
| MD225 | STC74a | Peritoneum | 17 |
| MD263 | STC6979 | Sputum | This study |
| MD284 | STG11 | Skin | 17 |
| MD323$^{GCS}$ | STG62647 | Blood | 17 |
| MD378 | STG643 | Skin | This study |
| MD409 | STG10 | Skin | This study |
| MD448 | STG10 | Skin | 17 |
| MD543 | STG643 | Skin | 17 |
| MD581 | STC1400 | Skin | This study |
| MD699 | STG62647 | Skin | 17 |
| MD813 | STC74a | Blood | 17 |
| MD864 | STG166b | Skin | 17 |
| MD894$^{GCS}$ | STG62647 | Urine | 17 |
| MD896 | STG11 | Skin | 17 |
| NS1121 | STG4831 | Unknown | 19 |
| NS383 | New type | Blood | 19 |
| NS542 | STG652 | Blood | 19 |
| NS752 | STG6 | Blood | 19 |
| GBS | | | |
| RBH01 | III | Vagina | This study |
| RBH02 | Ib | Vagina | This study |
| RBH03 | III | Vagina | This study |
| RBH04 | 1a/V | Vagina | This study |
| RBH05 | V | Vagina | This study |
| RBH06 | II | Vagina | This study |
| RBH07 | Nontypeable | Vagina | This study |
| RBH08 | Ia | Vagina | This study |
| RBH09 | V | Vagina | This study |
| RBH10 | V | Vagina | This study |
| RBH11 | III | Vagina | This study |
| RBH12 | V | Vagina | This study |
| RBH13 | III | Vagina | This study |
| RBH14 | 1b | Vagina | This study |
| RBH15 | Nontypeable | Vagina | This study |
| RBH16 | III | Vagina | This study |
| B36PS | IV | Vagina | This study |
| B37PD | V | Vagina | This study |
| 50VD | 1a | Vagina | This study |
| GAS | | | |
| NS282 | st6030.1 | Skin | 20 |
| NS344 | 1 | Blood | 20 |
| NS351 | 58 | Skin | 20 |
| NS414 | 11 | Unknown | 20 |
| NS180 | 74 | Throat | 20 |
| NS195 | 19 | Blood | 20 |
| NS8 | 85 | Blood | 20 |
| NS14 | 102 | Skin | 20 |
| NS179 | 9.1 | Skin | 20 |
| NS20 | 75.1 | Blood | 20 |
| NS199 | 112 | Throat | 20 |
| NS204 | 2 | Blood | 20 |
| NS83 | stNS554 | Blood | 20 |
| NS205 | 56 | Blood | 20 |
| NS210 | 22 | Blood | 20 |
| NS225 | 99 | Blood | 20 |
| NS226 | 4.2 | Blood | 20 |
| NS235 | 24 | Blood | 20 |
| NS236 | 77 | Throat | 20 |
| NS240 | st2904 | Blood | 20 |

[a] GGS isolates possessing the group C carbohydrate are indicated by a superscript GCS.

TABLE 2. Primer combinations and expected amplicon sizes of the three ICE*Sde*3396 regions targeted in the PCR screening of clinical GGS, GBS, and GAS isolates[a]

| Primer | Nucleotide sequence (5′ to 3′) | Location[a] | Expected size (kb)[a] | Source or reference |
|---|---|---|---|---|
| R1F | CTACTTTTTGTTGCCATTTGG | Amplification of the R1 region (orf1 to orf5) in ICE*Sde*3396 | 3.1 | This study |
| R1R | GAGTCAGTGTCCAAACTTTTCG | | | |
| R2F | GTTTAAATCCCAGATAAGCTACG | Amplification of the R2 region (orf17 to orf23) | 4.2 | This study |
| R2R | ATCGATTATGATTTTGGTCAACG | | | |
| R3F | TTCCTACTTGTACCAATACTGC | Amplification of the R3 region (orf55 to orf61) | 3.2 | This study |
| R3R | GAGTGACTTAGTTATGAAGGACG | | | |
| Adapter1 | GATCCGCCTATAGTGAGTCGTATTAAC | Adapter PCR | | 18, 43 |
| Adapter2 | AGCTCGCCTATAGTGAGTCGTATTAAC | | | |

[a] ORFs from ICE*Sde*3396.

sequences of primers used for PCR screening of the streptococcal isolates for the presence of R1, R2, and R3 regions are shown in Table 2.

**Conjugation of streptococci.** Filter mating experiments were carried out according to Smith and Guild (46). Prior to the mating, an antibiotically marked ICE was constructed by introducing a kanamycin resistance gene into ICE*Sde*3396. This was achieved by amplifying a 760-bp segment of ICE*Sde*3396 extending from nt 15061 to nt 15820 with flanking EcoRI and BamHI restriction sites. Another 638-bp segment extending from nt 17305 to nt 17942 in the ICE was also amplified with flanking BamHI and SpeHI restriction sites. The two fragments were ligated through their BamHI sites, and the product was cloned into pSF152. A kanamycin resistance gene was subsequently cloned into the internal BamHI site, creating pSF152-ICE:Km. pSF152-ICE:Km was transformed into NS3396 using standard procedures (41) and recombinants (NS3396 ICE*Sde*3396:Km) selected on Todd-Hewitt agar containing 500 μg/ml kanamycin. Integration of the plasmid into the NS3396 genome was confirmed via PCR and Southern hybridization.

For all conjugations, NS3396 ICE*Sde*3396:Km was used as the donor strain. Spontaneous streptomycin-resistant GGS, GBS, and GAS isolates were used as recipient strains. Three milliliters of an overnight culture containing recipient organisms was supplemented with $MgSO_4$ (10 mM), bovine serum albumin (2 mg/ml), and DNase I (200 U). The donor culture (300 μl) was then added, and the entire bacterial culture was filtered onto nitrocellulose. After filtering, the membrane was placed cell side up on Todd-Hewitt agar, overlaid with 5 mm of 1.5% Todd-Hewitt agar, and incubated at 37°C overnight. The next day, the filter paper was retrieved, placed into a 50-ml tube containing Todd-Hewitt broth, and vortexed for 10 s. The resulting suspension was serially diluted in phosphate-buffered saline and plated onto Todd-Hewitt agar containing kanamycin and streptomycin. The conjugation frequency was determined by dividing the average number of transconjugants by the average number of donors for each experiment.

**Resistance to cadmium, arsenite, arsenate, and copper.** Overnight cultures of GGS and GBS were resuspended in Todd-Hewitt broth to an optical density at 600 nm ($OD_{600}$) of 0.1. Aliquots of the cultures were supplemented with cadmium chloride (0 to 2 mM), sodium arsenate (0 to 7 mM), sodium arsenite (0 to 5 mM), or copper sulfate (0 to 7 mM) and grown at 37°C. The optical density of cultures was monitored at 600 nm. The $OD_{600}$ of cultures after 5 h of growth in the presence of arsenate, arsenite, or copper or 24 h of growth in the presence of cadmium are presented.

## RESULTS

**General features of ICE*Sde*3396.** We recently described genomic subtraction studies using pathovar (NS3396) and nonpathovar strains of GGS (19). A large proportion of the pathovar-specific DNA fragments identified in this study encoded partial genes with orthologues in GAS and/or GBS. While the majority of GAS-related DNA fragments were identified as being bacteriophage related (18, 19), the majority of the pathovar-specific GBS orthologues were related to an uncharacterized MGE found in GBS serotype V (2603V/R) (52). Through a combination of long PCR, primer walking, and adapter PCR, we determined the full nucleotide sequence of this putative MGE in NS3396. ICE*Sde*3396 was found to be approximately 64 kb in size and is predicted to contain 66 ORFs (Table 3). Only 38 of the ORFs are also found in the putative ICE in the GBS 2603V/R genome that we have designated ICE*Sa*2603 (Fig. 1). The shared ORFs encode proteins involved in conjugal processes and include a site-specific tyrosine-like recombinase, relaxases, bacterial mobilization proteins, and TraG.

ICE*Sde*3396 and ICE*Sa*2603 also share a number of genes predicted to encode putative virulence factors. These include the abortive infection genes *abiGI* and *abiGII* which have a role in lactococcal phage exclusion (13), a putative surface-associated agglutinin receptor which may modulate adherence of streptococci to oral sites through binding of salivary agglutinin (7, 52), and several genes predicted to encode proteins conferring resistance to cadmium and copper. Like their lactococcal homologues, the *abiGI* and *abiGII* genes of ICE*Sde*3396 and ICE*Sa*2603 exhibit a lower percent GC content (31% and 34.6%, respectively) than GGS and GBS genomes (and the respective surrounding DNA within the ICE), suggesting that they were acquired by the ICE through LGT (38).

**Mosaic structure of an 18-kb region unique to ICE*Sde*3396.** The defining difference between ICE*Sde*3396 and ICE*Sa*2603 is the presence of an 18-kb internal region within ICE*Sde*3396 (Fig. 1). The region encodes 21 ORFs (ICE*Sde*3396orf19 to -orf40) and is flanked by a small ORF with limited similarity to a transposase (Tn*1545*) at one end,and a second putative transposase associated with the insertion sequence IS*1216* at the other. When the genes in this region were compared to those in the GenBank database, it became apparent that this region consists of four clusters whose orthologues are found in unrelated bacterial species, including nonstreptococcal species (Fig. 1).

The first of these clusters contains two genes from a cadmium resistance operon, *cadC* (ICE*Sde*3396_orf20) and *cadD* (ICE*Sde*3396_orf21), which is found in all GAS genomes, in some but not all GBS genomes, and in *Streptococcus gordonii* and *Streptococcus parasanguinis*. *cadD* orthologues (but not *cadC*) are also found in *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The five genes in the second cluster are predicted to encode two putative recombinases, a truncated lysin, a cell

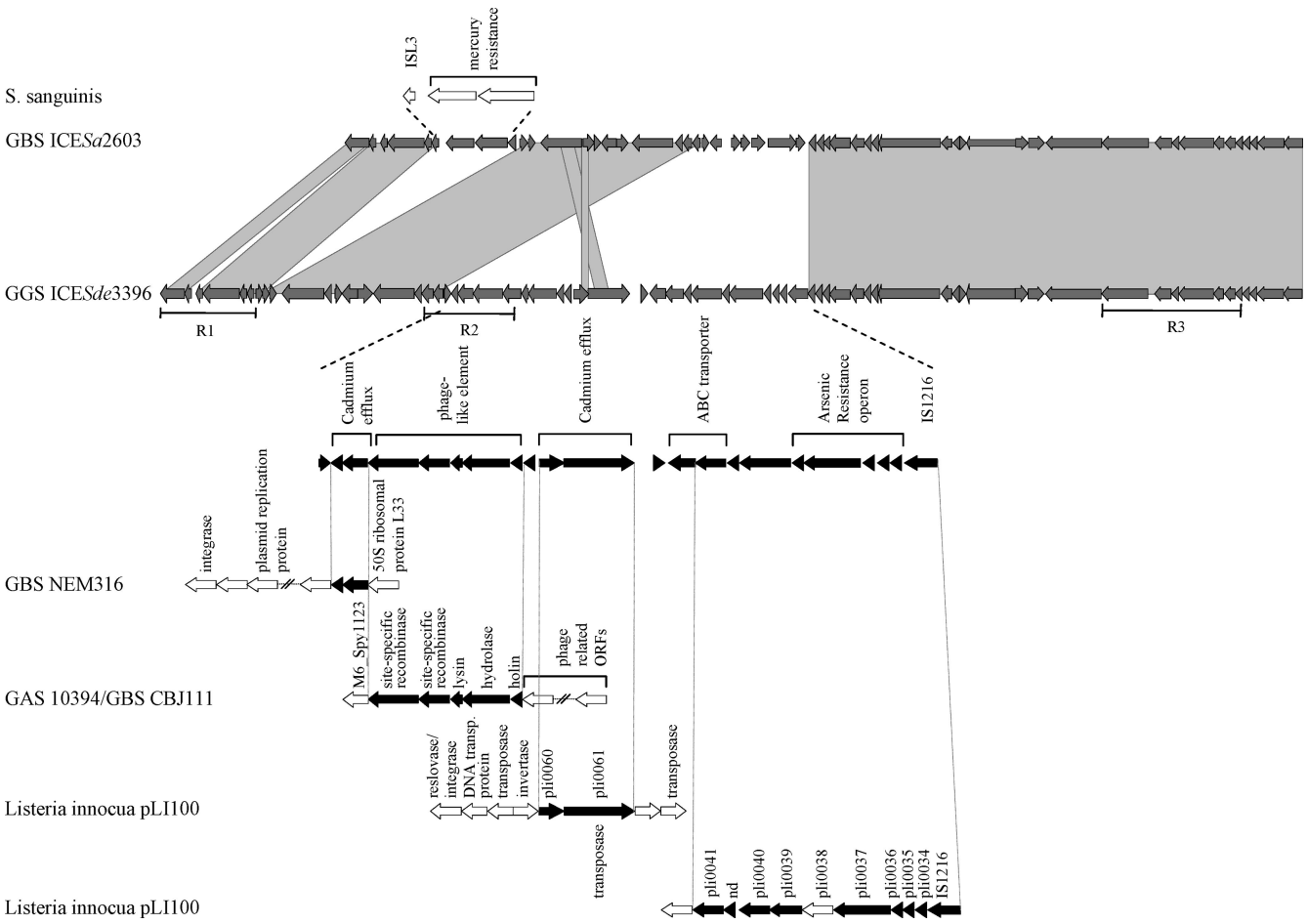


FIG. 1. Genetic organization and protein alignment of ICE*Sde*3396 with ICE*Sa*2603. Individual ORFs are represented by block arrows. Conserved proteins exhibiting greater than 45% amino acid identity between ICE*Sde*3396 and ICE*Sa*2603 are represented by the shading. The ICE*Sde*3396 genome was annotated using the bacterial annotation system BASys (53) and comparisons performed using the Artemis Comparison Tool (10). ORFs in the 18.1-kb recombinogenic region are depicted by block arrows. The four gene clusters in the 18-kb recombinogenic region and corresponding regions in other bacterial genomes are indicated by dashed lines. The R1, R2, and R3 regions are also shown.

wall hydrolase, and holin with similarity at the amino acid level to ORFs from a GAS bacteriophage (3, 4) and GBS MGE. The low amino acid sequence identity (<70%) and absence of significant nucleotide sequence homology suggest a distant evolutionary relationship between these orthologues. The third cluster (ICE*Sde*3396_orf28 and ICE*Sde*3396_orf29) encodes a second cadmium resistance operon lacking any sequence homology to the previously described cadmium resistance operon. The orthologues of this operon are found on plasmids from *Listeria innocua* (pLI100) and *Lactococcus lactis* (50) and a genomic island from *Streptococcus thermophilus* (40). The final cluster contains genes whose orthologues are found in a different region of the pLI100 plasmid. These include genes from a *yadG*- and *yadH*-like operon that also has orthologues in *Enterococcus faecalis*. The remaining seven genes of this cluster form a putative arsenic resistance operon. While the overall amino acid identity between the ORFs in this operon and their corresponding homologues from pLI100 is high (>98%), a truncation in the purported arsenic transporter (ArsB) and absence of an ArsC homologue suggests the operon is nonfunctional.

To provide a better understanding of how this recombinogenic region may have evolved, we undertook a genomic examination of each of the four clusters and surrounding DNA in their purported progenitor chromosomes. In each case, the cluster was situated in close proximity to MGE-associated genes, suggesting that incorporation of these elements into ICE*Sde*3396 has occurred through standard LGT (Fig. 1). The observation that the individual clusters (e.g., the two cadmium clusters) are also present in other unrelated bacterial species provides further support for the role of MGEs in their lateral dissemination.

**The chromosomal location of ICE*Sde*3396 is conserved.** In order to determine the flanking region of ICE*Sde*3396 in NS3396, adaptor PCRs using proximal ICE*Sde*3396-specific primers in conjunction with an adaptor-specific primer were performed (43). Sequencing of the PCR products flanking ICE*Sde*3396_orf66 identified two genes. The first of these genes encodes a small ORF with significant homology to a small hypothetical protein (SAG1300) adjacent to ICE*Sa*2603 in the 2603V/R genome (52). The second ORF was homologous to the 50s ribosomal gene L7/L12 and is also found in

TABLE 3. Features and predicted ORF functions of ICE*Sde*3396

| ORF | Position (bp) | | No. of aa | Direction of DNA strand | % G/C content | % aa identity | Species and gene no./locus[f] | Predicted function |
|---|---|---|---|---|---|---|---|---|
| | Start | Stop | | | | | | |
| 1 | 1212 | 1 | 403 | − | 32.34 | 100 | SAG1247 | Site-specific recombinase (tyrosine-like) |
| 2 | 1450 | 1223 | 75 | − | 34.65 | 100 | SAG1248 | Hypothetical |
| 3 | 1953 | 1729 | 74 | − | 33.78 | 100 | SAG1249 | Transcriptional regulator, Cro/CI family |
| 4 | 3999 | 2134 | 621 | − | 32.05 | 99 | SAG1250 | Relaxase |
| 5 | 4351 | 3986 | 121 | − | 34.43 | 100 | SAG1251 | Bacterial mobilization protein (MobC) |
| 6 | 4720 | 4358 | 120 | − | 34.71 | 100 | SAG1252 | Hypothetical |
| 7 | 5043 | 5150 | 35 | + | 26.85 | 100 | SAJ_1275 | Hypothetical |
| 8 | 5211 | 5693 | 160 | + | 39.54 | 100 | SAJ_1276 | Hypothetical |
| 9 | 5534 | 5875 | 113 | + | 38.60 | 93[a] | *S. suis* 89/1591[c] | Transposase/integrase |
| 10 | 8279 | 6150 | 709 | − | 37.61 | 100 | SAG1257 | Probable cadmium efflux ATPase CadA |
| 11 | 8640 | 8272 | 122 | − | 33.60 | 99 | SAG1258 | Cadmium efflux system accessory protein CadC |
| 12 | 8843 | 9142 | 99 | + | 36.33 | 100 | SAG1259 | Hypothetical |
| 13 | 9984 | 9196 | 262 | − | 30.29 | 100 | SAG1260 | Hypothetical |
| 14 | 9990 | 10589 | 199 | + | 42.50 | 100 | SAG1261 | Hypothetical membrane-spanning protein |
| 15 | 12850 | 10763 | 695 | − | 43.92 | 98 | SAG1262 | Probable copper exporting ATPase TcrB-like |
| 16 | 13195 | 12989 | 68 | − | 36.23 | 98 | GBS 2603V/R[c,d] | Copper-transporting ATPase TcrZ-like |
| 17 | 13819 | 13343 | 158 | − | 42.77 | 96[a] | SAG1263 | Copper-transporting ATPase TcrA-like |
| 18 | 14252 | 13806 | 148 | − | 36.24 | 92 | SAG1264 | Transcriptional repressor TcrY-like |
| 19 | 14460 | 14552 | 30 | + | 40.86 | | | ICE*Sde*3396_orf19 |
| 20 | 15048 | 14710 | 112 | − | 32.74 | 99 | SAI2183[c] | Cadmium efflux system accessory protein CadC |
| 21 | 15717 | 15061 | 218 | − | 33.49 | 99 | SAI2182[c] | Cadmium resistance protein CadD |
| 22 | 17311 | 15749 | 520 | − | 37.56 | 71 | M6_Spy1124 | Site-specific recombinase |
| 23 | 18222 | 17311 | 303 | − | 37.50 | 67 | M6_Spy1125 | Site-specific recombinase |
| 24 | 18499 | 18338 | 53 | − | 30.86 | 58 | Tn*1207*.3orf55 | Phage lysin |
| 25 | 20077 | 18608 | 489 | − | 43.06 | 59 | SAM_0643 | Amidase/phage cell wall hydrolase |
| 26 | 20479 | 20084 | 131 | − | 34.09 | 66[a] | SAM_0642 | Holin |
| 27 | 20663 | 20457 | 68 | − | 36.71 | | | ICE*Sde*3396_orf27 |
| 28 | 21300 | 21659 | 119 | + | 30.83 | 99 | *L. innocua* pli0060[c] | Cadmium efflux system accessory protein CadC |
| 29 | 21656 | 23773 | 705 | + | 37.11 | 100 | *L. innocua* pli0061[c] | Cadmium efflux ATPase CadA |
| 30 | 24476 | 24706 | 76 | + | 32.47 | | | ICE*Sde*3396_orf30 |
| 31 | 25589 | 24819 | 256 | − | 32.94 | 90 | *E. faecalis* V583 EF3208[c] | Inner membrane transport permease YadH-like |
| 32 | 26517 | 25582 | 311 | − | 35.69 | 97[a] | *L. innocua* pli0041 | ABC transporter, ATP subunit YadG-like |
| 33 | 26782 | 26672 | 36 | − | 35.14 | 99[d] | *L. innocua* pLI100 | ND[b] |
| 34 | 28480 | 26807 | 557 | − | 37.51 | 99 | *L. innocua* pli0040 | Coenzyme A disulfide reductase LpdA |
| 35 | 28713 | 28501 | 70 | − | 37.09 | 100[a] | *L. innocua* pli0039 | Arsenical resistance protein ACR3-like (ArsB) |
| 36 | 30510 | 28768 | 580 | − | 37.81 | 99 | *L. innocua* pli0037 | Arsenical pump-driving ATPase ArsA |
| 37 | 30893 | 30546 | 115 | − | 36.21 | 98 | *L. innocua* pli0036 | Arsenical resistance repressor ArsR |
| 38 | 31398 | 31027 | 123 | − | 38.98 | 98 | *L. innocua* pli0035 | Arsenical resistance trans-acting repressor ArsD |
| 39 | 31666 | 31400 | 88 | − | 32.96 | 98 | *L. innocua* pli0034 | Arsenical resistance repressor ArsR |
| 40 | 32558 | 31875 | 227 | − | 37.43 | 98 | *L. innocua* pli0032 | Transposase (IS*1216*) |
| 41 | 33200 | 32841 | 119 | − | 34.17 | 77 | SAG1273 | Hypothetical |
| 42 | 33576 | 33187 | 129 | − | 36.15 | 97 | SAG1274 | Hypothetical |
| 43 | 33800 | 33573 | 75 | − | 38.60 | 96 | SAG1275 | Hypothetical |
| 44 | 34930 | 33854 | 358 | − | 38.25 | 91 | SAG1276 | Hypothetical zinc finger protein |
| 45 | 35608 | 34970 | 212 | − | 39.44 | 95 | SAI1373 | Hypothetical |
| 46 | 35994 | 35704 | 96 | − | 32.30 | 91 | SAG1278 | Hypothetical |
| 47 | 36307 | 36008 | 99 | − | 42.33 | 84 | SAG1279 | Hypothetical |
| 48 | 43214 | 36378 | 2278 | − | 41.00 | 99 | SAG1280 | SNF2-related helicase |
| 49 | 43802 | 43251 | 183 | − | 38.04 | 100 | SAG1281 | Hypothetical |
| 50 | 43977 | 43786 | 63 | − | 42.71 | 100 | SAG1282 | Calcium-binding protein |
| 51 | 46772 | 46882 | 36 | + | 41.44 | 100[d] | GBS 2603V/R | ND |
| 52 | 48873 | 43978 | 1631 | − | 41.71 | 99 | SAG1283 | Agglutinin receptor precursor |
| 53 | 49140 | 49742 | 200 | + | 34.66 | 100 | SAG1284 | Abortive infection protein AbiGI |
| 54 | 49739 | 50584 | 281 | + | 31.80 | 100 | SAG1285 | Abortive infection protein AbiGII |
| 55 | 53471 | 50670 | 933 | − | 42.68 | 93 | SAG1286 | *N*-acetylmuramoyl-L-alanine amidase |
| 56 | 55824 | 53473 | 783 | − | 40.43 | 99 | SAG1287 | Hypothetical |
| 57 | 57050 | 56196 | 284 | − | 41.75 | 99 | SAG1289 | Hypothetical |
| 58 | 57323 | 57069 | 84 | − | 44.31 | 100 | SAG1290 | Hypothetical |
| 59 | 59146 | 57329 | 605 | − | 40.98 | 99 | SAG1291 | Conjugal transfer protein TraG |
| 60 | 59670 | 59146 | 174 | − | 39.24 | 99 | SAG1292 | Hypothetical |
| 61 | 60301 | 59711 | 196 | − | 39.26 | 100 | SAG1293 | Putative protease |
| 62 | 60537 | 60298 | 79 | − | 35.42 | 100 | SAG1294 | Hypothetical |
| 63 | 60929 | 60540 | 129 | − | 41.03 | 100 | SAG1295 | Hypothetical (potential arsenate reductase) |
| 64 | 61362 | 60934 | 142 | − | 38.46 | 100 | SAG1296 | Hypothetical |
| 65 | 62701 | 61346 | 451 | − | 44.32 | 99 | SAG1297 | DNA methylase |
| 66 | 63668 | 62849 | 272 | − | 40.42 | 100 | SAG1299 | Replication initiator |

[a] Proteins with partial homology to the GenBank database that may represent truncated proteins through a frameshift or deletion events.
[b] ORFs with less than 35% amino acid identity to the GenBank database are defined as having no database homologue (ND).
[c] These ORFs had equal identity to multiple ORFs from the same/different isolate(s).
[d] Homology is based on BlastN analysis and represents a nonannotated ORF in the GenBank database.
[e] aa, amino acid(s).
[f] Streptococcal species unless indicated otherwise.

proximity to ICE*Sa*2603. Subsequent PCR analysis confirmed that the 50s ribosomal L7/L12 gene flanked the ICEs in all ICE-positive GGS and GBS strains examined in this study (data not shown). The similar flanking sequences suggested that integration occurs via a specific mechanism common to the tyrosine family of recombinases (8).

**Distribution of ICE*Sde*3396 and ICE*Sa*2603 in β-hemolytic streptococci.** The presence of ICE*Sde*3396-like and ICE*Sa*2603-

TABLE 4. Distribution of R1, R2, and R3 regions in GGS, GBS, and GAS isolates

| Regions[a] | GGS ($n$ = 30) | GBS ($n$ = 19) | GAS ($n$ = 20) |
|---|---|---|---|
| $R1^-$ $R2^-$ $R3^-$ | 15 | 10 | 20 |
| $R1^+$ $R2^+$ $R3^+$ | 4 | 7 | 0 |
| $R1^+$ $R2^-$ $R3^+$ | 2 | 1 | 0 |
| $R1^+$ $R2^+$ $R3^V$ | 2 | 0 | 0 |
| $R1^-$ $R2^+$ $R3^+$ | 2 | 0 | 0 |
| $R1^-$ $R2^-$ $R3^+$ | 4 | 0 | 0 |
| $R1^+$ $R2^+$ $R3^-$ | 1 | 0 | 0 |
| $R1^+$ $R2^V$ $R3^-$ | 0 | 1 | 0 |

[a] $^V$, a PCR product was generated for this region. However, the size of this product differed from that observed for the corresponding fragment in ICE*Sde*3396.

like elements in other GGS, GBS, and GAS strains was examined using PCR that targeted three independent regions of ICE*Sde*3396 (Fig. 1). The first product (R1) extended from ICE*Sde*3396_orf1 to ICE*Sde*3396_orf5. The second product (R2) extended from ICE*Sde*3396_orf17 to ICE*Sde*3396_orf23 and includes DNA common to both ICE*Sde*3396 and ICE*Sa* 2603, as well as DNA unique to ICE*Sde*3396 (i.e., the 18-kb chimeric region). Amplification of this fragment enabled this differentiation between strains harboring ICE*Sde*3396-like and ICE*Sa*2603-like MGEs and also confirmed the proximal locations of these regions in ICE*Sde*3396-like ICEs. The third fragment (R3) spanned ICE*Sde*3396_orf55 to ICE*Sde*3396_orf61.

Of the 69 β-hemolytic isolates screened, 11 were positive for all three regions (i.e., $R1^+$ $R2^+$ $R3^+$), implying that they possessed ICE*Sde*3396-like elements (Table 4). Four of the 11 positive isolates were GGS, and the remainder were GBS. Another four isolates (three GGS and one GBS) possessed both the R1 and R3 regions but lacked the R2 region (i.e., $R1^+$ $R2^-$ $R3^+$), suggesting that they harbored ICE*Sa*2603-like elements. The difference in the proportion of strains that were $R1^+$ $R2^+$ $R3^+$ or $R1^+$ $R2^-$ $R3^+$ was not found to be statistically significant, as determined by Fisher's exact test. The diagnostic PCR also identified nine GGS isolates, and one GBS isolate possessed at least one of the three regions used to determine the presence of the ICEs but did not conform to the PCR profile used to define ICE*Sde*3396-like ($R1^+$ $R2^+$ $R3^+$) or ICE*Sa* 2603-like ($R1^+$ $R2^-$ $R3^+$) elements. Of note, none of the GAS isolates screened were positive for any of the three regions.

**ICE*Sde*3396 confers resistance to cadmium and arsenate.** To test whether the metal resistance operons identified within ICE*Sde*3396 were functional, NS3396 ($R1^+$ $R2^+$ $R3^+$), GGS10 ($R1^-$ $R2^-$ $R3^-$), GBS RBH01 ($R1^+$ $R2^+$ $R3^+$), and GBS RBH03 ($R1^-$ $R2^-$ $R3^-$) were grown in Todd-Hewitt broth supplemented with increasing concentrations of sodium(III) arsenite, sodium(V) arsenate, copper chloride, or cadmium chloride, and optical density of the cultures was measured at regular intervals. Whereas growth of GGS10 was inhibited by 1 mM arsenate, both NS3396 and GBS RBH01 showed little or no growth inhibition in 5 mM arsenate. In contrast to the ICE-lacking strains, both ICE-positive strains possessed an increased tolerance to cadmium at concentrations below 1 mM (Fig. 2). No association between the presence of ICE3396-like elements and arsenite or copper resistance was observed.

To provide additional support for a correlation between the tolerance to cadmium and arsenate and the presence of ICE*Sde*3396-like elements, additional GGS and GBS strains that were $R1^+$ $R2^+$ $R3^+$, $R1^+$ $R2^-$ $R3^+$, or $R1^-$ $R2^-$ $R3^-$ were grown in the presence of 3 mM arsenate or 0.4 mM cadmium chloride (Fig. 3). Four of the five $R1^+$ $R2^+$ $R3^+$ isolates were capable of growing in 3 mM arsenate. In contrast, none of the $R1^+$ $R2^-$ $R3^+$ isolates were able to grow in the presence of arsenate. With the exception of a single strain, all ICE-harboring isolates were also able to grow in the presence of cadmium. None of the six ICE-negative strains grew in the presence of arsenate or cadmium.

**ICE*Sde*3396 is transferable to other β-hemolytic streptococci.** As the nucleotide sequence data suggested that ICE*Sde*3396 has the full complement of genes necessary for conjugation, we next investigated whether we could transfer this element to other β-hemolytic streptococci. To do so we marked ICE*Sde*3396 by replacing a 1.5-kb segment within the 18-kb recombinogenic region with a gene conferring kanamycin resistance. We reasoned that as the18-kb recombinogenic region was absent in ICE*Sa*2603, it would not contain any genes necessary for conjugal processes. After filter mating between NS3396 ICE*Sde*3396:Km and GGS10, doubly antibiotic-

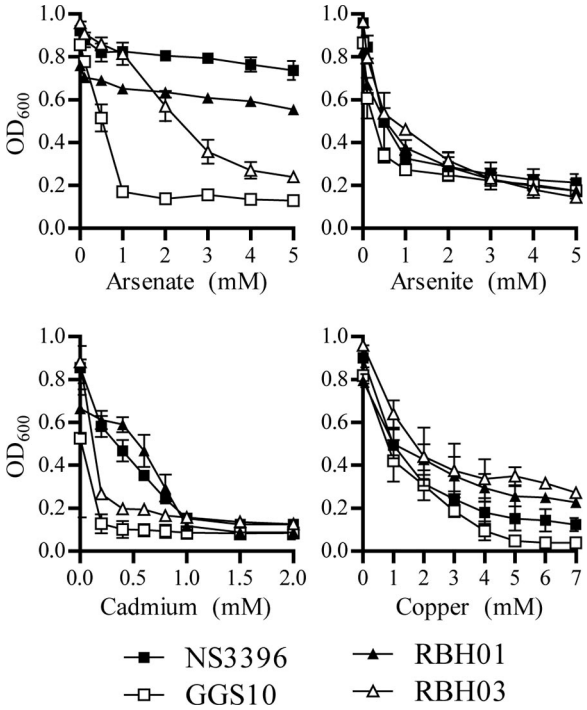


FIG. 2. Growth of GGS and GBS in the presence of arsenate, arsenite, cadmium, and copper. GGS strains are represented by squares and GBS strains by triangles. Closed symbols represent isolates containing ICE*Sde*3396 or ICE ICE*Sde*3396-like elements (i.e., $R1^+$ $R2^+$ $R3^+$). Open symbols represent ICE*Sde*3396-negative strains (i.e., $R1^-$ $R2^-$ $R3^-$). Strains were grown overnight in Todd-Hewitt broth, harvested, and resuspended medium containing increasing concentrations of sodium arsenate, sodium arsenite, cadmium chloride, or copper chloride. The $OD_{600}$ of cultures was measured at regular intervals during the growth cycle. Data is presented is the $OD_{600}$ after 24 h of growth in the presence of cadmium and 5 h of growth in the presence of arsenite, arsenate, and copper.

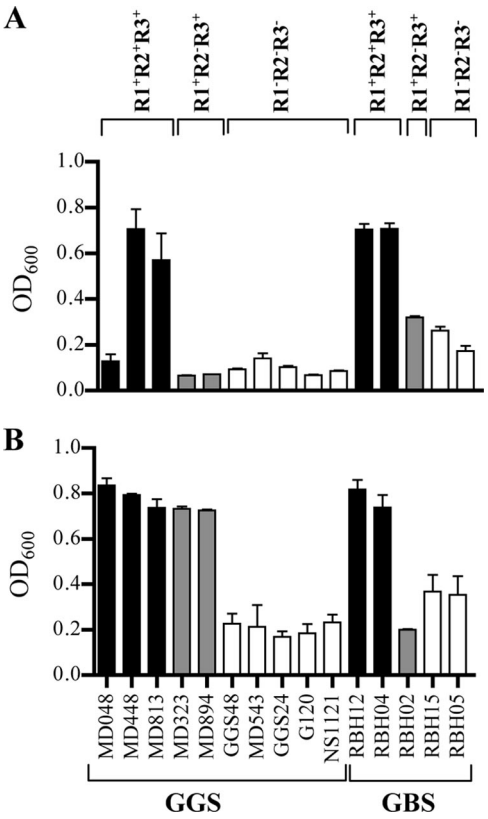


FIG. 3. ICE*Sde*3396 is associated with increased resistance to arsenate and cadmium. GGS and GBS were grown in the presence of 3 mM sodium arsenate (A) or 0.4 mM cadmium chloride. (B) Black bars represent isolates containing ICE*Sde*3396-like elements (i.e., $R1^+$ $R2^+$ $R3^+$). Gray bars represent isolates containing ICE*Sa2603*-like elements (i.e., $R1^+$ $R2^+$ $R3^+$). Open bars represent ICE*Sde*3396-negative strains (i.e., $R1^-$ $R2^-$ $R3^-$). Data is presented as the $OD_{600}$ after 5 h of growth in the presence of arsenate and 24 h of growth in the presence of cadmium.

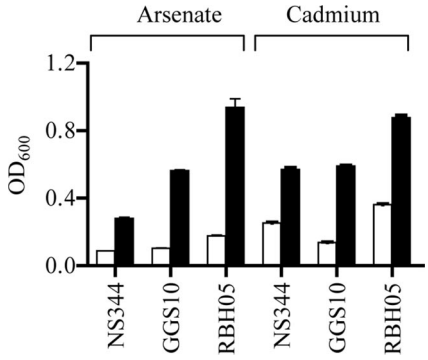


FIG. 4. Growth of ICE3396-negative and ICE3396-positive transconjugants in the presence of 0.4 mM cadmium chloride and 3 mM sodium arsenate. Parental strains are represented by the open bars. Transconjugants harboring ICE*Sde*3396 are represented by black bars. Data is presented as the mean $OD_{600}$ of cultures grown for 5 h in the presence of arsenate and 24 h in the presence of cadmium.

resistant GGS recipients (i.e., kanamycin and streptomycin resistant) were observed on Todd-Hewitt agar, suggesting that transfer of the ICE had occurred. Subsequently, we successfully transferred the ICE to GAS NS344 and GBS RBH05. The frequency of mobilization of ICE*Sde*3396:Km into GGS, GBS, and GAS was found to be $2 \times 10^{-4}$, $6 \times 10^{-6}$, and $1.5 \times 10^{-3}$, respectively. Compared to their parental strains, all transconjugants were also found to have increased tolerance to arsenate and cadmium (Fig. 4). As the kanamycin resistance marker was incorporated into the 18-kb recombinogenic region, it was conceivable that this region alone may be incorporated into recipient strains through the resident IS*1216* transposase. To test for this possibility, five GAS, GBS, and GGS transconjugants were chosen at random, and PCR analysis using the diagnostic primers specific for the genes in the R1, R2, and R3 regions was undertaken. All transconjugants tested possessed all three regions (data not shown).

## DISCUSSION

MGEs enable the nonvertical transfer of DNA between bacterial species. As such, they provide vehicles for rapid microbial adaptation and evolution. Previous studies have demonstrated MGEs are present in GAS, GBS, and GGS genomes and that many virulence factors are associated also with MGEs in these species (25, 45, 52). Bacteriophages are the most common MGE found in GAS genomes. All GAS strains analyzed to date have at least one prophage sequence, and most are polylysogenic (25). In contrast, bacteriophages appear to be much rarer in GBS and ICEs more prevalent. Without a genomic sequence, our understanding of the factors that influence genetic variation of GGS is less clear. Our previous study (18) identified a bacteriophage in NS3396 with significant homology to a bacteriophage from GAS. In the current study, we identified an ICE within the same GGS isolate that shares significant homology with a putative ICE in GBS. Thus, GGS appears capable of receiving and/or donating DNA to both these pathogenic species.

ICE*Sde*3396 carries genes whose orthologues can be found in numerous bacterial species, including GBS, GAS, *S. parasanguinis*, *Streptococcus suis*, *Enterococcus* spp., *Lactococcus* spp., *Listeria* spp., and *Neisseria* spp. Genetic analysis of the hypothesized progenitor chromosomes of the four independent clusters in the 18-kb recombinogenic region of ICE*Sde*3396 demonstrated that each was flanked by MGE-associated genes, indicating that the accumulation of these clusters within ICE*Sde* 3396 occurs through typical LGT events. Additional transposase/integrases are also found in other parts of ICE*Sde*3396 and ICE*Sa*2603. An earlier study by Tettelin et al. suggested that both the GAS and GBS pangenomes were open (51). We hypothesize that as GGS is closely related to these species, its genome is also open, and that ICE*Sde*3396 is one element that contributes to the open genomes of β-hemolytic streptococci. The ICE serves as a repository for genes from diverse bacterial origins; incorporation of these genes within the ICE also prevents disruption of chromosomally encoded genes whose functions may be critical to bacterial fitness. Additionally, the ICE is a vehicle that enables dissemination of these newly acquired genes through the β-hemolytic population.

Our functional studies demonstrated that four of the five isolates harboring ICE*Sde*3396-like elements possessed an increased resistance to arsenate. Similarly, six of seven isolates harboring the ICE*Sde*3396-like or ICE*Sa*2603-like element

had increased tolerances to cadmium. The association between the presence of the ICEs and tolerance to these metals and the fact that these tolerances were transferred to recipient strains provides strong evidence that these phenotypic properties are carried on the MGEs. We attribute the lack of cadmium resistance in GBS RBH02 and arsenate resistance in GGS MD048 to either ongoing modular evolution of the ICE or mutations resulting in a loss of phenotype. Modular evolution is a hallmark of all MGEs (28), and the cadmium resistance operon common to ICE*Sde*3396 and ICE*Sa*2603 lies adjacent to a putative transposase/integrase. Transposition events involving this element may have resulted in the loss or mutation of the cadmium operon region in GGS MD048. This is supported by the data demonstrating that the locus was amplifiable from ICE*Sde*3396 in NS3396 but not the ICE in MD048. The archetypal arsenic resistance operons contain three genes, *arsR*, *arsB*, and *arsC* (44). As the *ars* operon in the ICE*Sde*3396 includes a truncated *arsB* and lacks an *arsC* homologue, we were surprised to find the operon to be functional. As there is no apparent advantage to possession of this operon, further mutations accumulating in this region (deletions or point mutations) may eventually render the operon nonfunctional.

Although ICE*Sde*3396 does not carry antibiotic resistance genes, it does contain several genes whose homologues are found in other MGEs that do harbor antibiotic-resistant determinants, suggesting that transfer of DNA between these elements can occur. As an example, ICE*Sde*3396 contains homologues of genes found in a *mefA* carrying MGE in GAS 10394. More strikingly, a transposase associated with IS*1216* is present at one end of the 18-kb chimeric region. IS*1216* is part of a promiscuous insertion element that in other bacterial species carries genes conferring resistance to vancomycin (32, 39), erythromycin (32), and chloramphenicol (55). An IS*1216* element from *Enterococcus hirae* also carries genes encoding a low affinity penicillin binding protein (42). Penicillin has remained the drug of choice for treatment and prevention of β-hemolytic infections for over 50 years. Unlike related bacteria, the β-hemolytic streptococci have not developed resistance to penicillin; the reasons for this remain unknown (31). As penicillin resistance has not arisen naturally in β-hemolytic streptococci, even in the face of long-term penicillin treatment, mutation of existing genes seems to be an unlikely source of future resistance. Rather, we speculate that transfer of penicillin resistance genes via an ICE is more likely to provide a mechanism for the acquisition of penicillin (or other antibiotic) resistance phenotypes. In this regard, tetracycline resistance genes linked to IS*1216* have recently been reported in GGS (36). As antibiotic resistance increases in other bacterial species found in the same environmental niches occupied by β-hemolytic streptococci, the probability of transfer of antibiotic resistance determinants can only increase.

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## ADDENDUM IN PROOF

After the manuscript was accepted, we found that RBH02 (Fig. 3) did not yield PCR products of any size. Consequently, its expected genotype is $R1^{-}$ $R2^{-}$ $R3^{-}$, which in fact correlates with its resistance phenotype. This change does not affect our conclusions.

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