Comparative Genome Analysis of the Daptomycin-Resistant *Streptococcus anginosus* Strain J4206 Associated with Breakthrough Bacteremia

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Data deposition: This Whole Genome Shotgun project has been deposited at DNA Data Bank of Japan/EMBL/GenBank under the accession CP012719. The version described in this paper is the first version, CP012719. *Streptococcus anginosus* strain J4206 complete genome sequence is available at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/; last accessed October 6, 2016) under the accession number CP012719.

Abstract

Streptococcus anginosus is a member of the normal oral flora that can become a pathogen causing pyogenic infections in humans. The genome of daptomycin-resistant strain J4206, originally isolated from a patient suffering from breakthrough bacteremia and septic shock at the University of Texas Health Science Center at San Antonio, was determined. The circular genome is 2,001,352 bp long with a GC content of 38.62% and contains multiple mobile genetic elements, including the phage-like chromosomal island SanCI that mediates a mutator phenotype, transposons, and integrative conjugative elements. Daptomycin resistance involves multiple alterations in the cell membrane and cell wall, and unique features were identified in J4206 that may contribute to resistance. A cluster of capsular polysaccharide (CPS) genes for choline metabolism and transport are present that may help neutralize cell surface charges, destabilizing daptomycin binding. Further, unique *J4206* genes encoding sortases and LPXTG-target proteins that are involved in cell wall modification were present. The J4206 genome is phylogenetically closely related to the recently reported vancomycin-resistant SA1 strain; however, these genomes differ with SNPs in cardiolipin synthetase, histidine kinase *yycG*, teichoic acid modification genes, and other genes involved in cell surface modification. Transmission electron microscopy showed that the cell walls of both strains J4206 and SA1 were significantly thicker and more electron dense than daptomycin- and vancomycin-sensitive strain J4211. This comparative genomic study has identified unique genes as well as allelic variants in the J4206 genome that are involved in cell surface modification and thus might contribute to the acquisition of daptomycin resistance.

Key words: *Streptococcus anginosus*, daptomycin resistance, cell surface modification, genome analysis, mobile genetic elements.

Introduction

The *Streptococcus anginosus* group (SAG) is a subgroup of the viridans streptococci, consisting of three species: *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus* (Jensen et al. 2013; Asam and Spellerberg 2014). These streptococci are typically commensal human flora of the oral cavity, gastrointestinal, genital, and respiratory tracts (Whiley et al. 1992); however, they can cause pyogenic infections and are emerging pathogens in cystic fibrosis patients (Whiley et al. 1990; Parkins et al. 2008). *Streptococcus intermedius* and *S. constellatus* are mostly associated with brain, liver, and respiratory tract abscesses (Claridge et al. 2001). *Streptococcus anginosus* is isolated typically from blood cultures in most of the clinically relevant infections caused by this species (Whiley et al. 1992). Historically, the SAG has not been a well-studied

group, in part due to the lack of proper laboratory diagnostic techniques, which may have underestimated the frequency of these infections (Asam and Spellerberg 2014).

A number of reports have been published in the last decade describing SAG as bacterial pathogens. A population-based laboratory surveillance report in Canada showed the annual incidence rate of pyogenic streptococcal infection caused by SAG was 8.65/100,000 patients, which is higher than that of streptococcal groups A, B, G, and C (Laupland et al. 2006). Another report from Israel, which collected data from 245 patients over a 37-month period, showed that the annual incidence of SAG was 8.8/10,000 patients with *S. anginosus* being responsible for 82% of the total infections caused by this group (Siegman-Igra et al. 2012).

Recently, a daptomycin-resistant strain of *S. anginosus* J4206 was isolated from a 47 year-old male who was initially diagnosed with methicillin-resistant *S. aureus* (MRSA) with left trochanteric osteomyelitis, and consequently received daptomycin therapy (Palacio et al. 2011). At day 21 of his therapy, his deteriorating condition led to his admittance to the University of Texas Health Science Center at San Antonio with a diagnosis of septic shock. The daptomycin-resistant *S. anginosus* strain J4206 was isolated from his blood culture (Palacio et al. 2011).

Daptomycin is a lipopeptide antibiotic containing 13 amino acids and targets the bacterial cell membrane (Muraih et al. 2011). This antibiotic is anionic in charge and dependent on positively charged calcium ions to target the negatively charged membrane (Straus and Hancock 2006; Ho et al. 2007; Scott et al. 2007). The compromised cell membrane causes leakage of small ions from the cell and ultimately cell death (Jung et al. 2004; Straus and Hancock 2006; Muraih et al. 2011). Bacterial resistance to daptomycin is a complex process, and it is not yet known what factors specifically lead to daptomycin resistance. Although "daptomycin non-susceptibility" is sometimes the preferred term over "daptomycin resistance," in this report we will use the latter term (Bayer et al. 2013).

Daptomycin resistance has been associated with changes in bacterial cell surface including both the cell membrane and cell wall. In *S. aureus*, the most studied alleles associated with daptomycin resistance are in the multipeptide resistance factor gene *mprF* (Bayer et al. 2013). MprF is involved in formation of lysyl-phosphotidylglycerol (L-PG) by adding the amino acid lysine to PG (Ernst and Peschel 2011) and thus neutralizing the negative charge of the lipid molecule. MprF also plays a role in the transport of L-PG to cell membrane (Ernst et al. 2009). The *mprF* mutations associated with daptomycin resistance increase MprF activity, which results in higher levels of L-PG in the cell. This increase in L-PG makes the cell surface more positively charged and thus repels the calcium-daptomycin complex (Bayer et al. 2013). The other significant gene variants that have been associated with daptomycin resistance include staphylococcal genes *yycF* (response regulator), *yycG* (histidine kinase), *rpoB* (RNA polymerase subunit β), *rpoC* (RNA polymerase subunit β′), *grlA* (topoisomerase IV), and the enterococcal cardiolipin synthetase gene (Friedman et al. 2006; Palmer et al. 2011). Daptomycin resistance also has been associated with altered cell surface structure, reduced binding of daptomycin to the cell surface, and cell wall thickening (Bertsche et al. 2011; Bayer et al. 2013). However, many aspects of daptomycin resistance remain poorly understood, and there also are reports challenging both the charge repulsion and cell wall thickness hypotheses as explanations for daptomycin resistance (Kilelee et al. 2010; Yang et al. 2010).

In this report, we present the first complete genome sequence of a daptomycin-resistant member of the Streptococcaceae and compare it with the genomes of two previously reported daptomycin-susceptible *S. anginosus* strains, SA1 and J4211 (Srinivasan et al. 2014; Rahman et al. 2015). Strain J4211 was isolated from the same facility as J4206 while vancomycin-resistant strain SA1 was selected for comparison following BLAST search results that showed strong sequence homology to J4206. This comparative genomic study reveals several important, unique, elements such as LPXTG specific sortase enzymes, choline metabolism, and transport gene clusters and chromosomal island SanCI in the J4206 genome that might contribute to the acquisition of daptomycin resistance, which is thought to be complex and multifactorial. Additionally, the phylogenetic analysis of daptomycin-resistant strain J4206 and vancomycin-resistant strain SA1 shows that these strains of *S. anginosus* are very closely related at the genome level, and their common characteristics may provide insights into strains associated with both severe disease and antibiotic resistance.

Materials and Methods

Strains and Growth Conditions

The daptomycin-resistant *S. anginosus* strain J4206 was isolated from a 47-year old male in June, 2010 who received daptomycin therapy at 6 mg/kg/day. The patient was diagnosed with MRSA bacteremia and left trochanteric osteomyelitis (Palacio et al. 2011). Both daptomycin E-test and broth microdilution revealed daptomycin MIC of 4 μg/ml. The strain was susceptible to penicillin, cefotaxime, ceftriaxone, meropenem, levofloxacin, vancomycin, erythromycin, clindamycin, and tetracycline. The patient was treated successfully with ceftriaxone and vancomycin (Palacio et al. 2011). Strain J4211, which is susceptible to daptomycin, is another clinical strain isolated from the same hospital. The complete genome sequence of J4211 has been reported from our laboratory recently (Rahman et al. 2015). Strain SA1 was a urine isolate from a patient on vancomycin therapy following the insertion of ventriculoperitoneal shunt after a motor accident, and its genome sequence was recently reported (Srinivasan et al.

2014). For DNA isolation or electron microscopy, *S. anginosus* strains were grown overnight at 37 °C in Todd–Hewitt broth with 2% yeast extract (THY) broth and supplemented with 5% heat inactivated horse serum (THY-HS).

Genome Sequencing and Annotation

Chromosomal DNA was isolated as previously described (Pitcher et al. 1989; McShan et al. 1998). A DNA library was prepared using 50-ng of total genomic DNA according to Nextera DNA library kit protocols (Illumina, Inc.). Samples were indexed according to standard protocols so that they could be pooled together and sequenced simultaneously in a single run on the Illumina MiSeq using paired-end 250-bp sequencing with high coverage (600-fold) at the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research. Prior to sequencing, all libraries were run individually on the Agilent High Sensitivity DNA chip to confirm library quality and average insert size. Samples were pooled in equimolar amounts and 8pM of the pool was run on the sequencer. Per Illumina's recommendation, 10% phiX library was spiked into the library pool prior to loading for quality control purposes. A total of 30–40 million reads were collected for each run. Raw sequence data were aligned and further analyzed using CLC Genomics Workbench (CLC Bio.). DNA sequence assembly was done using the software package Geneious version 9.0.2 (Kearse et al. 2012). Sequence gaps were closed by PCR amplification using GoTaq DNA polymerase and master mixes, following the recommended protocol of the manufacturer (Promega, Madison, WI). Gene annotation was performed by the Rapid Annotation using Subsystem Technology (RAST) pipeline (Aziz et al. 2008; Overbeek et al. 2014). Genome annotation was completed using Artemis software (Rutherford et al. 2000) and BLAST search. The complete genome sequence of *S. anginosus* strain J4211 has been previously reported (Rahman et al. 2015) and is available at GenBank accession number no. CP012805.

Genome and Sequence Analysis

Genomic comparisons were done using CGView server (Grant and Stothard 2008), and genomic islands were compared using Geneious 9.0.2 (Kearse et al. 2012). Multiple DNA alignments were done using the Base by Base software package (Brodie et al. 2004). CRISPRfinder was used to identify CRISPR regions in J4206 genome (Grissa et al. 2007). The core genes of the three *S. anginosus* strains were analyzed and mapped for metabolic pathways using KEGG Automatic Annotation Server (KAAS) (Moriya et al. 2007) and visualized using the iPath v.2 (Yamada et al. 2011). A web-based tool, IslandViewer 3, was used to identify genomic islands present in the three strains (Langille and Brinkman 2009; Dhillon et al. 2013). The software package Parsnp was used for SNP analysis and to construct the phylogenetic tree for the core genes of the completed *S. anginosus* genomes (J4206, J4211, C1051, C238, SA1, and MAS624) (Treangen et al. 2014). The Parsnp generated results were visualized using Gingr (Treangen et al. 2014). The Venn-diagram identifying unique genes in each genome was constructed using the EDGAR database (Blom et al. 2009).

Electron Microscopy

Strains J4206, J4211, and SA1 were grown in 40 ml of THY-HS broth to an absorbance of 0.6 at 600 nm, harvested by centrifugation, and fixed in 0.1 M sodium cacodylate buffer containing 4% paraformaldehyde (EM grade) and 2% gluteraldehyde (EM grade) for 24 h at 4 °C. Samples were then post fixed for 90 min in 1% Osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate and rinsed three times for 5 min each in 0.1 M sodium cacodylate buffer. The samples were then dehydrated in a graded ethanol series as follows: 50%, 60%, 75%, 85%, 95%, and 100%. The samples were incubated in each concentration for 15 min on a rocker at room temperature. Then the bacteria had two 15-min treatments in 100% propylene oxide. Following dehydration, the samples were infiltrated in a graded Epon/Araldite (EMS) resin/propylene oxide series (1:3, 1:1, 3:1) for 60 min, 120 min, and overnight, respectively. The following day samples were further infiltrated with pure EMS resin for 45 min, 90 min, and then overnight. The bacterial samples were then embedded in resin plus BDMA (accelerator) and polymerized at 60 °C for 48 h. Ultrathin sections (100 nm) were collected on 400 mesh copper grids using a Leica EM Ultracut 6. Ultrathin sections were then stained with lead citrate and uranyl acetate before viewing on a Hitachi H7600 Transmission Electron Microscope at 80 kV and equipped with a 2k × 2k AMT digital camera. Microscopy was done at the Oklahoma Medical Research Foundation (ORMF) Imaging Core Facility. The thickness of cell walls was measured using the image processing program ImageJ (Schneider et al. 2012). One hundred and six measurements (8–10 measurements per cell) were done for each sample and data were analyzed by one-way analysis of variation (ANOVA) test using the software package PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Results and Discussion

General Features of J4206 Genome

The *S. anginosus* J4206 genome is 2,001,352 bp long with a GC content of 38.62%, which is similar to the values of other *S. anginosus* genomes sequenced to date. The probable number of genes is 1,912, covering 87.5% of the total genome and with a gene GC content of 39.28%. The J4206 genome contains integrative conjugative elements (ICE), a Tn916 like element, several phage-like elements, transposons, and a SanCI (*S. anginosus* phage-like chromosomal island) integrated into the DNA mismatch repair operon.

These mobile genetic elements, as well as INDELs associated with cell wall architecture, comprise the major differences between the J4206 genome and those of other recently described *S. anginosus* strains (fig. 1).

J4206 genome analysis showed the presence of genes encoding potential virulence factors for adhesion, invasion, and spreading, including a homolog of the pneumococcal surface adhesion protein (*psaA*), a fibronectin binding protein (*fbp*), a laminin-binding surface protein (*lmb*), pullulanase (*pulA*), and others (supplementary table S1, Supplementary Material online). The J4206 genome also possesses a typical anginosus group type II-A CRISPR system containing *cas9*, *cas1*, *cas2* and *csn2* genes upstream of the direct repeat and spacer sequences (supplementary table S2, Supplementary Material online) (Olson et al. 2013). The same CRISPR system is present in SA1 with 15 out of 26 spacers being identical. This extended region of identical CRISPR DNA suggests a recent ancestor for these two strains; however, enough evolutionary time has passed so that the remaining non-homologous spacers reflect the differing phages populations encountered by the two strains. Interestingly, this type II-A CRISPR system is absent in strain J4211 with only three direct repeats found as a remnant of the CRISPR. Metabolic analysis of the three *S. anginosus* strains using KAAS and iPath v.2 showed an overall similarity with some differences in the phosphotransferase systems (PTS). PTSs are membrane transport systems transferring phosphorus-containing sugar molecules across membranes. These

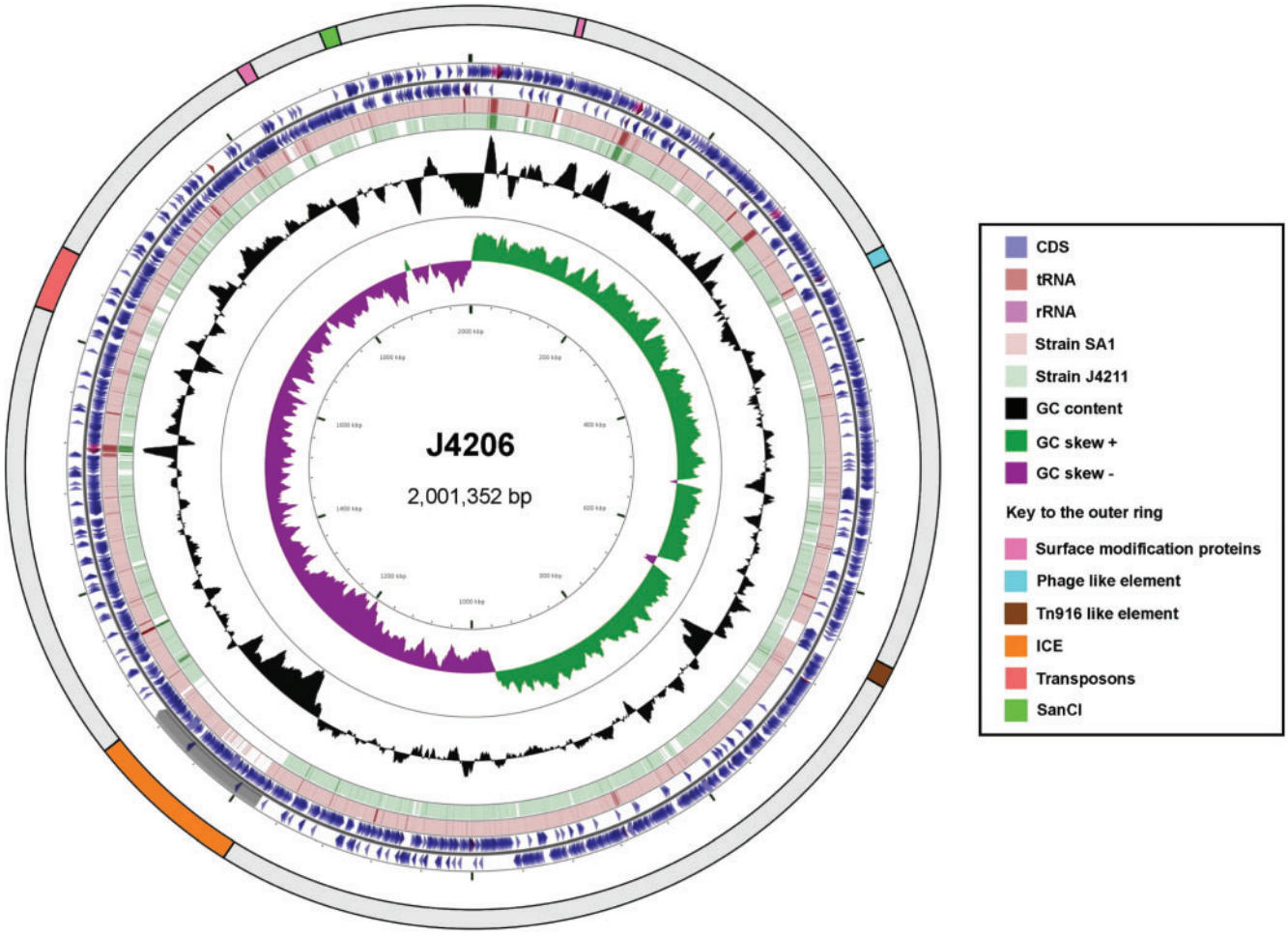

Fig. 1.—Circular representation of the *S. anginosus* strain J4206 genome and its comparison with strains, SA1 and J4211. The outer circle shows the mobile genetic elements found on the J4206 genome that are unique or significantly different from the two other strains; the color key is shown to the right of the map. The second and third circles show the predicted coding regions on J4206 genome on the forward (clockwise) and reverse (counterclockwise) strands, respectively. These two circles also include the tRNA and rRNA genes. The following two circles represent the BLASTN comparison of the SA1 genome (pink) and J4211 genome (green) to the J4206 genome. The colored areas in these comparisons show matches with minimum expectation values of $e = 0.00001$; uncolored areas failed to meet this level of stringency and are thus considered unique to strain J4206, The final two inner circles present the percent GC content and percent GC skew of the J4206 genome.

systems are involved in sensing outside environment for the bacteria and have regulatory functions (Postma et al. 1993); notably, both *S. anginosus* strains J4206 and J4211 encode more PTSs than does strain SA1 (supplementary Table S3, Supplementary Material online).

ICEs Targeting the RNA Methyltransferase *rumA1*

ICEs are mobile genetic elements capable of integration, excision and transfer via conjugation from one bacterium to another (Wozniak and Waldor 2010). These elements often contain antibiotic resistance elements and are important contributors to genomic diversity (Brochet et al. 2008; Wozniak and Waldor 2010). Strain SA1 contains the *vanG* element, conferring resistance to vancomycin, which integrates into the distal end of the RNA methyltransferase *rumA1* gene after base 1328 of the predicted ORF (Srinivasan et al. 2014); this insertion site also has been reported in the same position for other *vanG* elements found in some group B streptococcal strains (Srinivasan et al. 2014). The J4206 genome has a related ICE inserted just downstream of *rumA1* that does not include the *vanG* resistance cassette or the tetracycline-resistant element (fig. 2). Since integration is downstream of the gene rather than within the ORF, *rumA1* is present in full length with 1362 bases in J4206 genome similar to some other *S. anginosus* strains such as F0211 that was sequenced as part of the oral microbiome project (Chen et al. 2010). The J4206 genome contains a second ICE-like element downstream of the first ICE containing genes encoding conjugation, replication and restriction modification (RM) proteins. Except for the portion shared with the SA1 genome, the complete 109 kb long genomic island containing the two ICEs is unique (Srinivasan et al. 2014) and not present in either the daptomycin susceptible clinical isolate J4211 (fig. 1) or other currently available genomes on NCBI.

The evolution of ICEs is not clear but bioinformatic analysis suggests that there have been frequent recombination events between many ICEs and other mobile genetic elements like plasmids and phages, leading to genetic mosaicism (Wozniak and Waldor 2010). Such recombinational events undoubtedly contributed to the interspersed regions of similarity and dissimilarity between the ICEs found on the J4206 and SA1 genomes (fig. 2). The entire chromosomal island on the J4206 genome contains seven RM genes, including four RM genes that are shared by both J4206 and SA1 genomic region (fig. 2). RM systems contain one restriction enzyme and one modification methylase and mostly defend the bacteria from

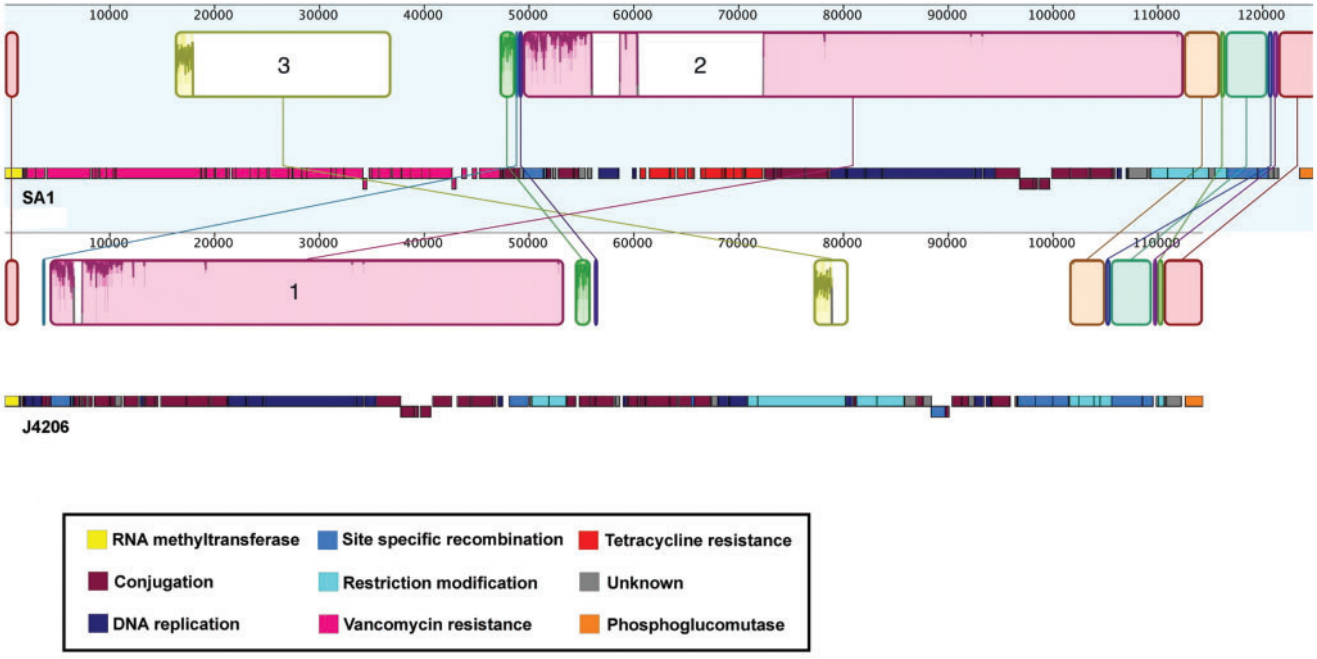

Fig. 2.—Integrative conjugative elements on *S. anginosus* J4206 and SA1 genomes. The ICE in the J4206 genome (bases 1180821–1290038) was compared with the related element in strain SA1 using a Mauve alignment to observe the evolutionary events such as rearrangements and inversions that have shaped the two regions. The region is 109 kb long on the J4206 genome and is flanked by a RNA methyltransferase gene on one end and the phosphoglucomutase gene on the other. The ORFs contained within these regions are shown for SA1 (above) and J4206 (below); their predicted function is indicated by the color code at the bottom of the figure. The pink block (1) above the J4206 genome shows an extended shared region with the SA1 ICE that contains many essential genes for conjugation and replication; however, an INDEL containing the tetracycline resistance module (2) is present in SA1 that is absent in J4206. The *vanG* vancomycin resistance element on SA1 is shown at 3.

unmodified invading DNA (Kobayashi 2001), which may be important in maintaining genome integrity in a naturally competent species like *S. anginosus* (Håvarstein et al. 1997). In a possible contrasting role, it has been suggested that RM systems may promote the appearance of INDELs and inversions in bacterial genomes such as those seen between the ICE genomic rearrangements observed between J4206 and SA1 genome (Kobayashi 2001), and selective pressures must preserve those arrangements that promote fitness of the host cell.

The Capsular Polysaccharide (CPS) Genomic Locus on the J4206 Genome

CPSs are thought to contribute to biofilm formation on tooth surfaces by oral streptococci (Cisar et al. 1995; Yang et al. 2009) and the *S. anginosus* CPS locus was first characterized in strain ATCC 33397 (Tsunashima et al. 2012). Since alterations in CPS expression or composition might promote biofilm formation in other body sites or provide enhanced protection from the immune system, the CPS loci of J4206, SA1, and J4211 were compared. In J4206, the CPS locus is 35 kbp long and contains INDELs representing an array of choline metabolism and transports genes not present in strains J4211 or ATCC 33397 (fig. 3). In figure 3, strain ATCC 33397 was used as a representative genome containing CPS locus (Tsunashima et al. 2012). Five phosphatidylcholine metabolism and transport genes (*SanJ4206_1762*, *1764*, *1765*, *1766*, and *1769*) are present including *licD* (phosphorylcholine metabolism) and *licC* (CTP-phosphocholine cytidylltransferase). A BLAST search of the available genomes on NCBI showed that these five choline metabolism genes as well as a few other genes on the CPS locus (SanJ4206_1771-75) are unique and present only in daptomycin-resistant strain J4206 and vancomycin-resistant strain SA1 (fig. 3). It remains to be determined whether this unique CPS operon contributes to antibiotic resistance, virulence, or both in these two *S. anginosus* strains; however, its common presence in these two strains is associated with invasive disease and antibiotic resistance could indicate that this CPS operon may be a characteristic of emerging virulent strains of this species. Further work is necessary in order to determine its role in daptomycin resistance.

Studies in *Clostridium perfringens* indicate that neutralization of anionic phosphatidylglycerol (PG) by the formation of either zwitterionic alanyl-PG (A-PG) or cationic lysyl-PG (L-PG) protects the bacterial cell membrane from daptomycin when the modified PG is translocated to the outside of the cell (Slavetinsky et al. 2012). Choline is a positively charged molecule that could neutralize the cell surface in a similar fashion. While the J4206 genome does not contain the L-PG forming protein MprF gene associated with resistance to antimicrobial peptides in *S. aureus* (Bayer et al. 2013), it does have the

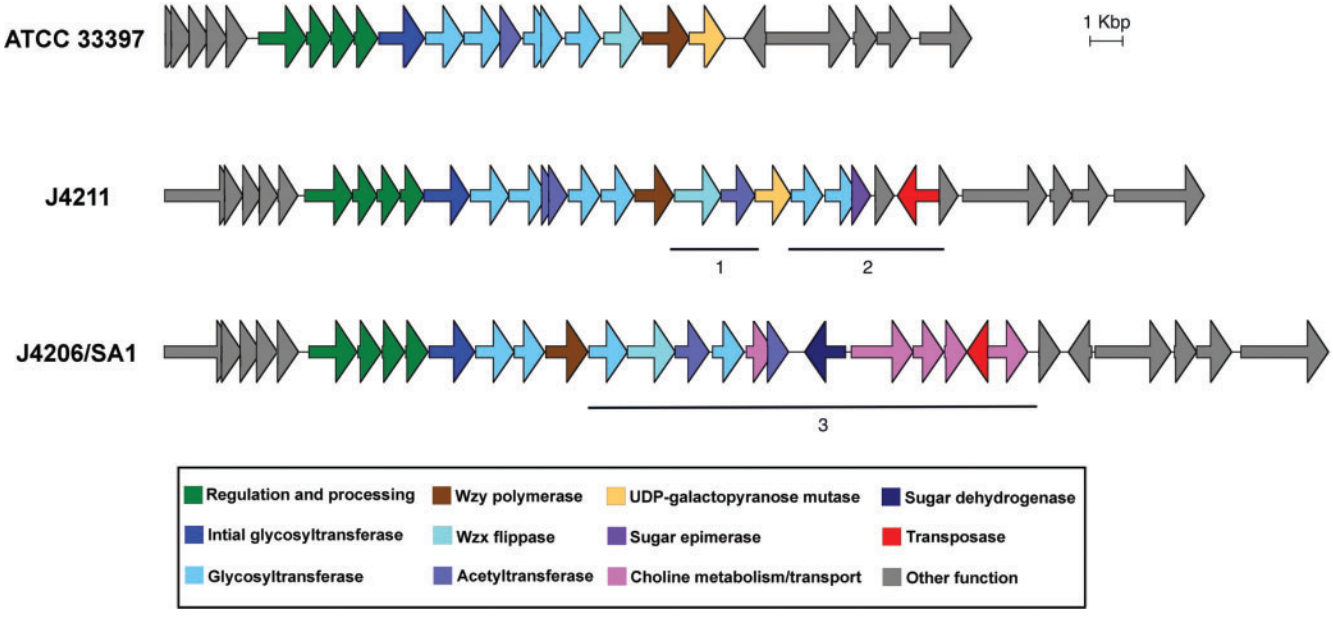

Fig. 3.—The capsular polysaccharide (CPS) gene clusters from *Streptococcus anginosus* strains ATCC 33397, J4211, and J4206. The CPS region of *S. anginosus* was first described in strain ATCC 33397 (Tsunashima et al. 2012), which was used as a baseline for comparison to the same cluster in strains J4211 and J4206. The J4206 CPS gene cluster shown in the figure is the region that includes genes SanJ4206_1756 through SanJ4206_1786 on the genome. The predicted function of each gene is indicated by the color shown in the legend. The numbered lines indicate notable INDELs that distinguish the regions of J4211 (numbers 1 and 2) and J4206 (number 3) from that of ATCC 33397. The J4206 INDEL contains choline metabolism and transport genes that may contribute to daptomycin resistance through cell surface modification.

unique choline transporter gene (SanJ4206_1765) found in the J4206 CPS locus. J4206 may use zwitterionic phosphatidylcholine to alter its cell surface to achieve increased resistance to daptomycin. In addition to antimicrobial resistance, choline also may play a role in immune avoidance through host mimicry. Phosphatidylcholine is the most abundant eukaryotic membrane phospholipid, and this lipid molecule helps some bacteria to mimic host membranes and thus reduce the immune response towards the microbe (Clark and Weiser 2013). Thus, the novel choline metabolism genes may enhance strain J4206 survival by several mechanisms.

Phosphatidylcholine modification of the cell surface probably is not the only mechanism underlying daptomycin resistance. The daptomycin-susceptible strain J4211 does not contain these choline metabolism and transport genes, but the vancomycin-resistant, daptomycin-susceptible strain SA1 does. Therefore, while these genes may contribute to daptomycin resistance, it is probable that additional mechanisms must come in to play as has been observed in the identified non-MprF dependent staphylococcal mechanisms of daptomycin resistance (Bertsche et al. 2011). The degree to which these CPS locus genes contribute to antimicrobial resistance is an important area for future studies.

J4206 LPXTG Sortases and Their Target Proteins

Another set of notable INDELs in the J4206 genome contains two additional genes that encode proteins of the sortase family of enzymes (SanJ4206_1748 and 1749) and their predicted LPXTG target proteins (SanJ4206_0069 and 0070). Neither strains SA1 and J4211 have these genes (fig. 4), and the DNA sequences coding for the sortase enzymes (SanJ4206_1748 and SanJ4206_1749) appear unique, having little homology to available Genbank entries at the time of writing.

A sortase is a transpeptidase that cleaves a target protein between the threonine and the glycine of the LPXTG motif and anchors them to the cell wall peptidoglycan cross bridges. This process is mostly involved in anchoring bacterial proteins to the cell surface (Mazmanian et al. 2001; Marraffini et al. 2006). Sortase A can also anchor LPXTG motif proteins to lipid II which is a precursor in bacterial peptidoglycan synthesis (Ruzin et al. 2002). Lipid II possesses a pyrophosphate group

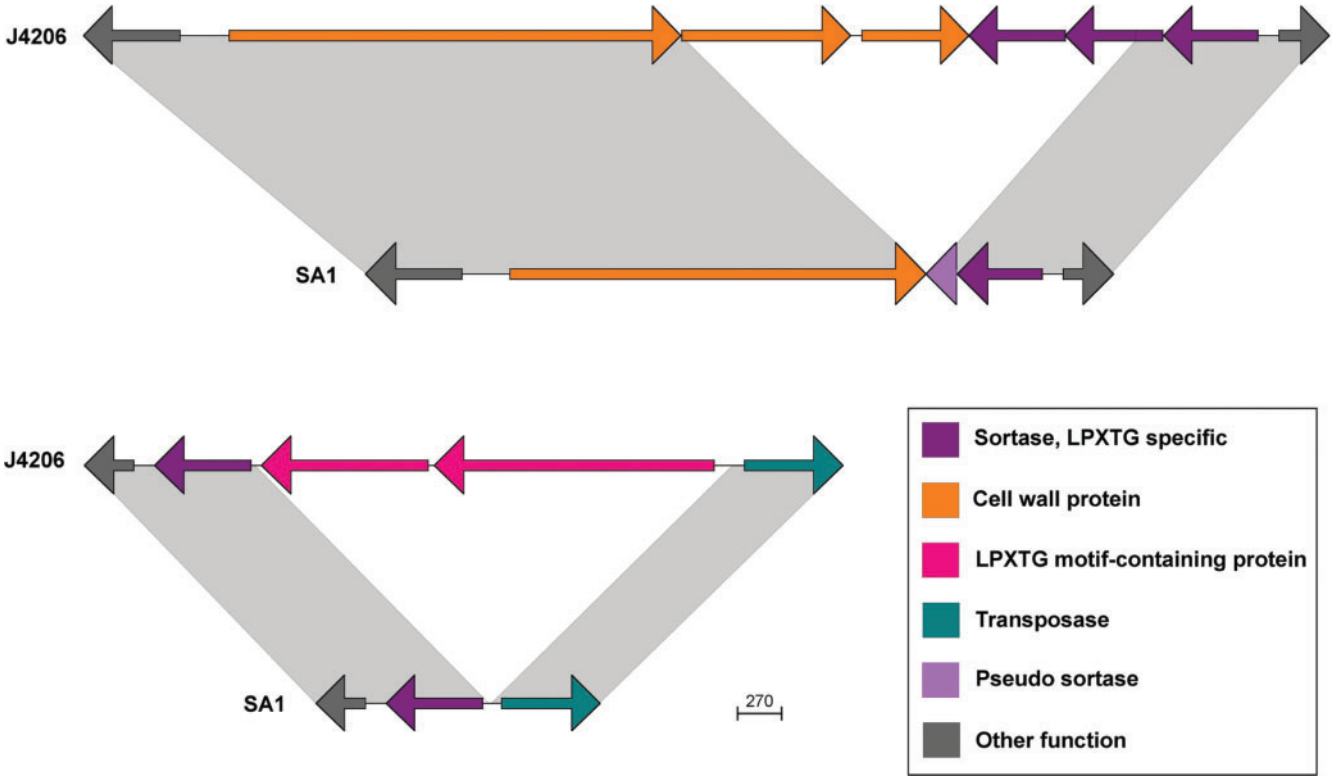

Fig. 4.—Strain J4206 contains novel LPXTG-specific sortases and target proteins. In the top of the figure, the region of the daptomycin-resistant J4206 genome encoding the unique LPXTG sortases (bases 1831473–1842376) is compared with the same region in vancomycin-resistant strain SA1. Similarly, the bottom of the figure shows the J4206 genome region (bases 75351–81990) containing the genes encoding the unique LPXTG motif-containing proteins that are the putative target of the sortases; again, this region is compared to the corresponding one in the SA1 genome. In both regions, the unique sortases and their targets appear to be INDELs acquired by J4206 that differentiate it from SA1. Color key is presented on the right. Maps were created using Gene Construction Kit (Textco BioSoftware, West Lebanon, NH).

and is a target for lantibiotics such as nisin which uses lipid II as a receptor to initiate pore formation on the bacterial surface, leading to death of the bacterium (Breukink and de Kruijff 2006). Daptomycin works as a complex with calcium and targets bacterial cell membrane based on charge properties. It is possible that daptomycin may also use lipid II as a receptor because of its anionic pyrophosphate group. The addition of a bulky group by sortase on lipid II will also mask the anionic pyrophosphate group in case of daptomycin non-susceptible bacteria. The J4206 sortases and LPXTG target proteins (fig. 4) that are absent on J4211 and SA1 genomes might lead to higher levels of sortase activity and target proteins, thus altering the bacterial surface structure to promote daptomycin resistance.

Cell surface modification is a major cause of daptomycin resistance for *Staphylococcus* species (Bertsche et al. 2011; Bayer et al. 2013). The presence of phosphatidylcholine metabolism and transport genes in the CPS locus (fig. 3) and the additional LPXTG specific sortase enzymes and target proteins (fig. 4) represent some of the most striking features of the J4206 genome that could contribute to bacterial surface modification. These genetic systems may represent potential targets for novel antimicrobial drug development and their role in daptomycin resistance in *S. anginosus* needs to be explored further.

SanCI and Other Chromosomal Islands

Multiple mobile genetic elements are present on the J4206 genome, including a phage-like chromosomal island (*S. anginosus* chromosomal island (SanCI) J4206), which is integrated into the 5′ end of DNA mismatch repair (MMR) gene *mutL* (Nguyen and McShan 2014). SanCI J4206 is a member of a large family of related phage-like chromosomal islands found in many streptococcal species; the best characterized one of these are the SpyCI of *S. pyogenes* that use the same target site for integration, mediate a growth-dependent mutator phenotype, and alter global transcription patterns of many virulence genes (Scott et al. 2008; Scott et al. 2012; Hendrickson et al. 2015). The presence of a SanCI in the J4206 MMR operon suggests that this strain would exhibit a mutator phenotype, which may have played a role in the acquisition of daptomycin resistance. Inactivation of MMR can contribute to the genome changes in two important ways: through increasing the appearance of point mutations and through removing a barrier to non-homologous recombination. Previous studies have shown that a mutator phenotype induced daptomycin resistance in staphylococcal and enterococcal strains under laboratory conditions (Friedman et al. 2006; Palmer et al. 2011), and the diminished MMR activity predicted for strain J4206 may account for some of the changes that would promote resistance to daptomycin discussed below. Further, inactivation of MMR has been demonstrated to remove barriers to recombination between divergent DNA sequences (Rayssiguier et al. 1989; Matic et al. 2000), and the presence of unique genes in J4206 also described below may have resulted in non-homologous recombination events allowed by a MMR deficient cellular environment.

Phage-like chromosomal islands integrated into *mutL* are frequently present in the genomes of the anginosus group: *S. anginosus* strains whileyi MAS624, whileyi CCUG39159, C238, 1505, F0211 and SA1 harbor closely related SanCI, and *S. intermedius* strains ATCC 27335, JTH08, SK54, F0413 all have less related SinCI present. Strain J4211, by contrast, lacks one of these elements in its genome (Nguyen and McShan 2014). These islands share a common genetic plan (fig. 5), and variations between the SanCI are from base substitutions rather than INDELs (insertions and deletions). The sequences of SanCI J4206 and SanCI SA1 are nearly identical except for DNA substitutions in the putative toxin-antitoxin region at the distal end of the island. As might be predicted from the common and conserved bacterial attachment site (*attB*), the integrase gene is the most conserved region in all these islands except for SinCI JTH08 (fig. 5). SinCI F0413 shows evidence of horizontal transfer between *S. anginosus* and *S. intermedius*, having an integrase gene that is more related to the SanCI than to SinCI JTH08 and the other SinCI (Nguyen and McShan 2014). Interestingly, this difference is more evident at the DNA level where SinCI JTH08 and F0413 only share 17% nucleotide identity than at the amino acid level where the two encoded integrases have 74% identity (not shown). The overall conservation of the SanCI and SinCI integrase proteins probably reflects the limitations imposed by targeting a conserved DNA sequence for site-specific integration.

In total, 11 putative genomic islands (supplementary table S4, Supplementary Material online) are present in the J4206 genome. One lambdoid prophage-like element is present in J4206 (location: 344231–355982) but not found in either strains SA1 and J4211; this element encodes some identifiable phage proteins as well as one LPXTG target protein (SanJ4206_0315). The DNA sequence encoding the LPXTG protein is novel and shares only 2% sequence identity with some *Bacillus* species DNA sequences present in Genbank. Also missing from strain SA1 but present in the J4206 genome is a Tn916-like transposon (location: 641632–658307) that contains multiple Tn916-associated ORFs including an integrase and excisionase. Multiple other transposons are present on the J4206 genome (location: 1609950–1674533) that are missing in the J4211 genome. The presence of multiple mobile genetic elements suggests a high rate of horizontal gene transfer for strain J4206, which may have contributed to the acquisition of the unique genes whose products modify the cell surface discussed above.

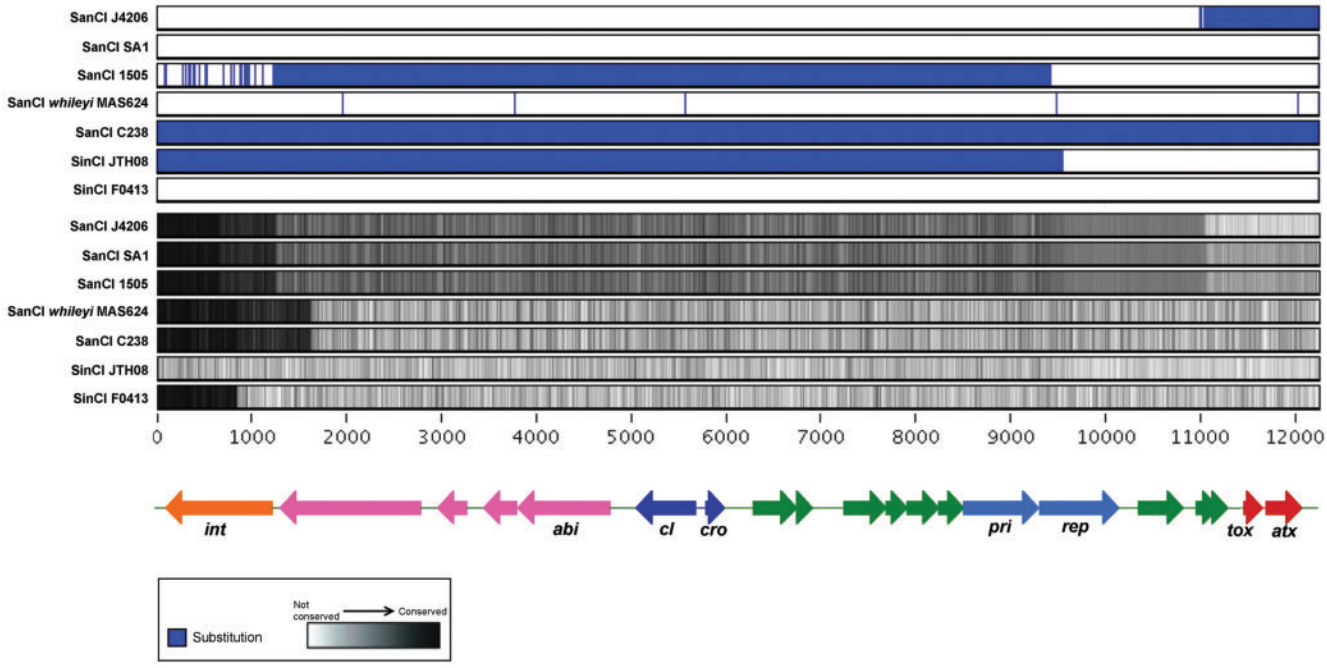

Fig. 5.—Comparison of *S. anginosus* chromosomal islands (SanCI) integrated into the DNA Mismatch Repair (MMR) operon from sequenced SAG genomes. The ~12 kbp J4206 SanCI genome with predicted ORFs is presented with the multiple alignment of this region with the corresponding islands from other genome strains shown above. The alignment is presented by percent similarity (grayscale) and by identifying substitutions (blue). Chromosomal islands with significant differences have been selected for comparison. Color key of predicted gene functions: Green: genes of unknown function; red: possible toxin-antitoxin maintenance genes; light blue: DNA replication; dark blue: control of lysogeny; pink: INDELs; orange: site-specific integrase. Known genes are indicated next to their ORF.

Streptococcus anginosus Strains Genomic Diversity and SNPs Analysis

A phylogenetic tree was constructed using single nucleotide polymorphism (SNP) analysis of the six complete *S. anginosus* genome strains (fig. 6A; the pink lines represent SNPs). *Streptococcus anginosus* strain J4206 is closely related to SA1, together forming a separate branch of the tree, while the other four genomes occupy a different branch. Closely linked strains J4206 and SA1 shared the common characteristics of antibiotic resistance associated with cell surface changes and being associated with severe patient disease, and thus potentially may be representatives of an emerging group of virulent *S. anginosus* strains. However, in spite of its relatedness to strain SA1, strain J4206 shows unique features not found in the other *S. anginosus* genomes analyzed so far. In figure 6B, a Venn-diagram compares strains J4206, J4211, and SA1, showing that 147 unique genes are present in strain J4206 which are absent on the other two genomes. These unique genes include the transposases, LPXTG specific sortase and target proteins, and conjugative element proteins discussed above as well as genes associated with metabolism and genes of unknown function (supplementary table S5, Supplementary Material online), requiring further future study.

Previous studies in laboratory induced daptomycin resistance in staphylococci and entercocci have identified SNPs in a number of genes, including *mprF*, the *yycFG* operon (also termed as *vicRK* and *walRK*), *rpoB*, *rpoC*, *grlA* (*parC*), and cardiolipin synthetase, as potentially contributing to this phenotype (Friedman et al. 2006; Palmer et al. 2011). Additionally, expression analysis of daptomycin-resistant *S. aureus* strains revealed differential expression for a number of genes including *tagA* (wall teichoic acid biosynthesis gene), and the *dltABCD* operon (which adds D-alanine esters to teichoic acids) (Bertsche et al. 2011; Song et al. 2013). SNP analysis of strains J4206 and SA1 revealed a variety of SNPs present in a number of these genes as well as in other genes involved in cell surface modification (table 1); a number of these SNPs lead to amino acid changes and are identified in the table. SNPs were also detected in the promoter region of *dltABCD* operon, which may cause it to have differential expression in J4206 as compared with SA1. One of the non-MprF-dependent staphylococcal daptomycin resistance mechanisms involves overexpression of the *tagA* and *dltA* genes leading to increased production and D-alanylation of cell-wall teichoic acid, respectively; this process maintains the positive surface charge that repels the daptomycin-calcium complex (Bertsche et al. 2011). While neither genes *mprF* or

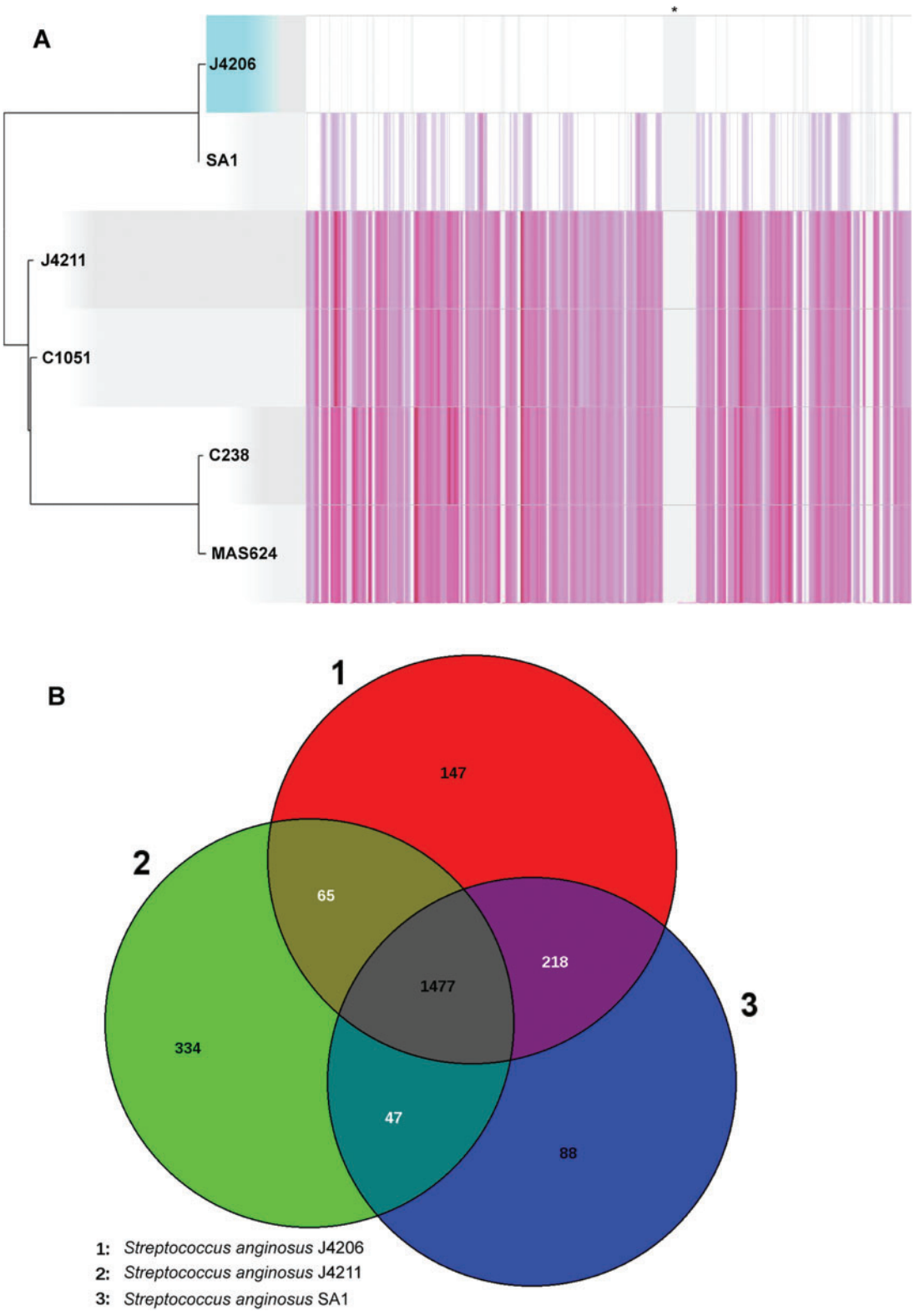
A
J4206
SA1
J4211
C1051
C238
MAS624
B
1
2
3
147
65
218
1477
334
47
88
1: Streptococcus anginosus J4206
2: Streptococcus anginosus J4211
3: Streptococcus anginosus SA1

Table 1

SNPs Leading to Amino Acid Changes in Protein Sequences Associated with Cell Wall or Cell Surface Changes that might Contribute to Daptomycin Resistance in *Streptococcus anginosus* Strain J4206 Compared with SA1 are Presented

	ORF	Gene	Annotated protein function	Variation	Amino acid change
Cell surface modification proteins	SanJ4206_0106		LPXTG domain protein	SNPs, nucleotide deletion	T1779A, S1800G, S1823A, K1862T, T1887K, T1891N, K1892N, R1916H, G1926D, G1965E, Q1966D, D1967S, D1968Q, V1970I, A1971L, I1982V
	SanJ4206_1766		Choline Kinase	15 SNPs	S119C, N130T, I143R, V156I, S174A
	SanJ4206_1779	*cpsC*	Capsular polysaccharide biosynthesis protein	4 SNPs	V93I, S159A
Proteins associated with daptomycin resistance in previous studies	SanJ4206_0667	*yycG*	Histidine kinase	Multiple SNPs	V9I, D55N, V59I, M62V, H412N
	SanJ4206_1789		Cardiolipin synthetase	SNP	D297Y
	SanJ4206_1861	*dltB*	D-Alanyl-lipoteichoic acid biosynthesis protein	Multiple SNPs	A87T, R88G, G90S, L107F, M111T, I137M, Q193K, Q196H, L212F, V225L, I230W, V241I
	SanJ4206_1862	*dltA*	D-Alanine-poly(phosphoribitol) ligase	Multiple SNPs	R15Y, A32V, T120N, V126L, T127A, T128A, V131T, A149D, A190I
	SanJ4206_1863	*dltX*	D-Ala-teichoic acid biosynthesis protein	1 SNP	F11L

NOTE.—Each ORF is identified by its locus in the J4206 Annotated Genome, a gene name if available, and the predicted protein function. The amino acid substitutions are listed using standard nomenclature.

tagA, which were identified in the staphylococcal studies, are present on the J4206 genome (or in SA1), the variant *dltA* may fulfill a similar function in J4206.

Daptomycin Resistance and Increased Cell Wall Thickness

Several studies have found a correlation between previous vancomycin exposures prior to the appearance of daptomycin resistance (Patel et al. 2006; Sakoulas et al. 2006; Mwangi et al. 2007; Pillai et al. 2007). Further, the development of intermediate vancomycin resistance in *S. aureus* (MICs of 4–8 μg/ml) is associated with bacterial cell-wall thickening which ultimately was found to impact daptomycin susceptibility (Cui et al. 2006). In the case of strain J4206, the patient from whom it was isolated had been treated with vancomycin before appearance of the daptomycin-resistant strain after initiation of daptomycin therapy (Palacio et al. 2011). This patient had multiple episodes of MRSA infections since 2006 and received multiple 6–8 week long treatments with vancomycin without any *Streptococcus* isolated during this period. In 2010, he received daptomycin therapy for MRSA bacteremia that led to the emergence of daptomycin-resistant *S. anginosus* J4206 (Palacio et al. 2011). Therefore, it was of interest to examine the cell wall of J4206 to observe if any differences might exist between it and daptomycin-sensitive strains SA1 and J4211.

TEM analysis of antibiotic-resistant strains J4206 and SA1 showed that their cell walls were significantly thicker (25.3 ± 5.4 nm and 28.8 ± 4.6 nm, respectively) than that of J4211 (19.9 ± 4.3 nm) with a P value of <0.0001 (fig. 7). The cell walls of strains J4206 and SA1 were also more electron dense than the J4211 cell wall suggesting a change in charge density in the cell wall that might be due to the activity of the novel phosphatidylcholine choline biosynthesis genes present in both J4206 and SA1 genomes or to altered profiles of membrane-associated proteins (fig. 7). Since both vancomycin resistance and daptomycin resistance involve the structure of the cell surface, it is not surprising that both these strains show similar structure under TEM. The previous vancomycin exposure by strain J4206 prior to daptomycin treatment may have increased its cell-wall thickness and set the stage for the development of daptomycin resistance. Such increases in cell-wall thickness and density correlates with previous studies

FIG. 6.—(A) Phylogeny and SNPs of the completed *S. anginosus* strains. The phylogenetic tree presented was constructed using concatenated sequences of the six aligned *S. anginosus* genomes. Each SNP showing where the other five strains differ from J4206 is presented by a pink line. The asterisk (*) indicates the location of the 109 kb long ICE region on J4206 genome. Strains J4206 and SA1 that share the traits of antibiotic resistance and severe disease association form a distinct phylogenetic branch. The graph was constructed by using the command line tool Parsnp (Treangen et al. 2014). (B) Genes unique to strains J4206, J4211, and SA1. The Venn diagram shows the number of shared and unique genes for each strain. Severe disease strains J4206 and SA1 share 218 genes that are not present in strain J4211 while having 147 and 88 unique genes, respectively. See supplementary table S5 (Supplementary Material online) for details. The graph was constructed by using the EDGAR database (Blom et al. 2009).

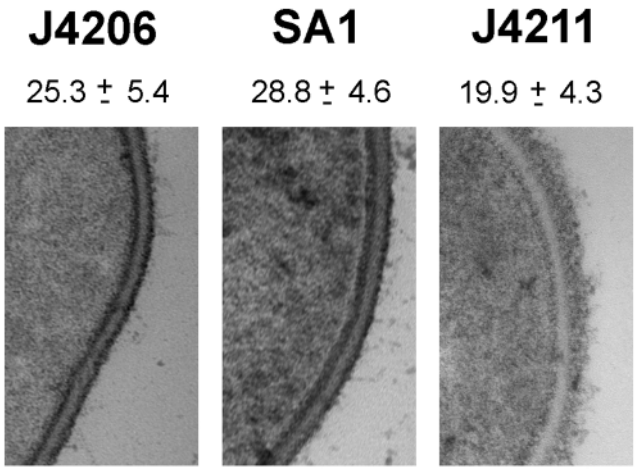

Fig. 7.—Resistance to daptomycin and vancomycin is associated with increased cell wall thickness and electron density. TEM analyses of the daptomycin resistant strain (J4206), the vancomycin resistant strain (SA1), and the daptomycin susceptible strain (J4211) showed that the two antibiotic-resistant strains had increased cell wall thickness and increased electron density following staining for microscopy. The thickness of cell walls (in nanometers) was measured at ×50,000 magnification, and 106 separate measurements were done to determine the average cell wall thickness of each strain. Data are presented as mean ± SD. One-way ANOVA was used for statistical analysis.

showing a similar phenomenon in unrelated daptomycin-resistant strains (Bayer et al. 2013; Bertsche et al. 2011).

Conclusions

The sequencing and analysis of the genome of daptomycin-resistant *S. anginosus* strain J4206 and its phylogenetic comparison with daptomycin susceptible *S. anginosus* strains led to several important findings. As was seen with previously described daptomycin-resistant bacteria, the J4206 genome probably does not contain a specific resistance element for this antibiotic, and so it is likely that multiple factors contribute towards this resistance as has been postulated in other bacteria (Bayer et al. 2013). Several novel genetic features revealed by this study may collectively contribute to daptomycin resistance, including increased cell-wall thickness, LPXTG-specific sortase enzymes, choline metabolism, and transport gene clusters, SNPs in the genes for cell surface modification proteins, and a predicted mutator phenotype mediated by the SanCI integrated into the DNA mismatch repair operon. Daptomycin is becoming one of the alternative antibiotics of choice with the increasing incidence of multiple drug-resistant bacteria including vancomycin-resistant strains (Bayer et al. 2013). In strain J4206, daptomycin resistance is a likely multidimensional process involving mutations, INDELs, increased cell-wall thickness, and probable altered patterns of expression for multiple genes. The exact contribution of each of these factors in daptomycin resistance, both separately and in combination, remains to be determined. Specific gene knockouts, transposon mutagenesis and other means of global gene inactivation will be useful in finding genes specifically linked to resistance, and these studies will need to be combined with global transcriptional studies to understand the role they play in metabolism in both the presence and absence of antibiotic. The phylogenetic relatedness of daptomycin-resistant strain J4206 and vancomycin-resistant strain SA1 suggests that emerging strains of *S. anginosus* might exist, being characterized by cell wall changes that promote antibiotic resistance and by severe disease association. At present, the sample size is too small to reach any definitive conclusion, but the appearance of closely related strains associated with antimicrobial resistance from geographically separated locations is intriguing. It is one of the ironies of the fight against bacterial infectious diseases that antibiotic use has opened the door for such emerging pathogens that were little noticed in decades past. For example, 30 years ago *Acinetobacter baumanii* was considered a member of a genus associated with soil while now it is a major cause of antibiotic-resistant nosocomial infections (Antunes et al. 2014). Thus, the J4206 genome will be an important tool for understanding the nature of daptomycin resistance and virulence in streptococci, providing us with a platform for future studies that will uncover the molecular mechanisms underlying this means of escape from a member of our antibiotic arsenal.

Supplementary Material

Supplementary tables S1–S5 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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