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Emergence of Resistance among USA300 Methicillin-Resistant *Staphylococcus aureus* Isolates Causing Invasive Disease in the United States[▽]

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USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates are usually resistant only to oxacillin, erythromycin, and, increasingly, levofloxacin. Of these, oxacillin and levofloxacin resistances are chromosomally encoded. Plasmid-mediated clindamycin, mupirocin, and/or tetracycline resistance has been observed among USA300 isolates, but these descriptions were limited to specific patient populations or isolated occurrences. We examined the antimicrobial susceptibilities of invasive MRSA isolates from a national surveillance population in order to identify USA300 isolates with unusual, possibly emerging, plasmid-mediated antimicrobial resistance. DNA from these isolates was assayed for the presence of resistance determinants and the presence of a pSK41-like conjugative plasmid. Of 823 USA300 isolates, 72 (9%) were tetracycline resistant; 69 of these were doxycycline susceptible and *tetK* positive, and 3 were doxycycline resistant and *tetM* positive. Fifty-one (6.2%) isolates were clindamycin resistant and *ermC* positive; 22 (2.7%) isolates were high-level mupirocin resistant (*mupA* positive); 5 (0.6%) isolates were trimethoprim-sulfamethoxazole (TMP-SMZ) resistant, of which 4 were *dfrA* positive; and 7 (0.9%) isolates were gentamicin resistant and *aac6′-aph2″* positive. Isolates with pSK41-like plasmids ($n = 24$) were positive for *mupA* ($n = 19$), *dfrA* ($n = 6$), *aac6′-aph2″* ($n = 6$), *tetM* ($n = 2$), and *ermC* ($n = 8$); 20 pSK41-positive isolates were positive for two or more resistance genes. Conjugative transfer of resistance was demonstrated between four gentamicin- and mupirocin-resistant and three gentamicin- and TMP-SMZ-resistant USA300 isolates; transconjugants harbored a single pSK41-like plasmid, which was PCR positive for *aac6′-aph2″* and either *mupA* and/or *dfrA*. USA300 and USA100 isolates from the same state with identical resistance profiles contained pSK41-like plasmids with indistinguishable restriction and Southern blot profiles, suggesting horizontal plasmid transfer between USA100 and USA300 isolates.

In the United States, a single pulsed-field type (PFT), USA300 (multilocus sequence type [MLST] 8), is the predominant cause of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections (26, 35). A subset of isolates with the pulsed-field gel electrophoresis (PFGE) pattern USA300-0114 and other closely related strains have disseminated clonally throughout the United States (25, 35) and typically carry Panton-Valentine leukocidin (PVL) toxin genes, the arginine catabolic mobile element (ACME), and staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa (13). Although PFT USA100 (MLST 5) is the most common PFT isolated in cases of health care-associated MRSA infections in the United States (23), USA300 isolates have now emerged as a cause of health care-associated infections (22, 23, 32). Whereas traditional health care-associated MRSA strains are usually resistant to multiple antimicrobial classes, USA300 isolates are typically susceptible to most classes of antimicrobial agents and are resistant only to oxacillin and erythromycin (12, 17, 35). However, resistance to other antimicrobial agents has been sporadically reported in USA300 isolates (i.e., resistance to fluoroquinolones, tetracycline, clindamycin, and mupirocin) (10, 16, 35). Treatment options for MRSA skin and soft-tissue infections include trimethoprim-sulfamethoxazole (TMP-SMZ), clindamycin, and tetracyclines (6, 9, 14); the emergence of resistance to these agents in USA300 strains will pose a challenge for treating both community- and health care-associated *S. aureus* infections.

Since 2001, when USA300 isolates were first recognized (25), these isolates have become increasingly resistant to the fluoroquinolones; 54% of the Active Bacterial Core surveillance (ABCs) isolates from 2005 to 2006 were resistant to levofloxacin (23). Fluoroquinolone resistance in *S. aureus* arises via chromosomal mutations in the DNA gyrase gene *gyrA* (18). Most other antimicrobial resistance in staphylococci emerges by acquisition of resistance determinants residing on plasmids and is often associated with transposons or insertion sequences (33). In previous analyses of USA300 isolates, several plasmids were identified. Among isolates with a typical susceptibility pattern (resistance to penicillin, oxacillin, and erythromycin), two plasmids were described: a small, 3.1-kb cryptic plasmid and a 27-kb mosaic plasmid that harbors several resistance determinants, including resistance to erythromycin (*msrA*) and penicillin (*blaZ*) (17, 35). Among USA300 isolates with resistance to tetracycline but susceptibility to doxycycline and minocycline, a 4.4-kb plasmid with the *tetK* determinant was reported (11, 30, 35), and isolates with clin-

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TABLE 1. PCR primers used in this study

Gene	Primer direction[a]	Primer sequence (5′ to 3′)	Fragment size (bp)	Source or reference for primer or sequence
mupA	F	GGG CCT TAA TTT CGG ATA GTG CTC	269	This study
	R	TAA TCT GGC TGC GGA AGT GAA ATC		11
traE	F	ACA AAT GCG TAC TAC AGA CCC TAA ACG A	317	This study
	R	GCC CTG CTG TTG CTG TAT CCA TAT T		11
traI	F	ACC GAT ATG AAT AAC ACC GTC	579	11
	R	AAA CCT TCA CAA GCA ATG GAC		
repA	F	TTT ATC GAA TGA CGC TAA AAT TGC TTA C	569	This study
	R	ATT AAT TCG GGG AGT TCT TGA AGT TCT		36
dfrA	F	TTC CCT TTT CTA CGC ACT A	420	This study
	R	AAT TAC CTT GGC ACT TAC C		36
aac6′-aph2″	F	GAG CAA TAA GGG CAT ACC AAA AAT C	504	19
	R	CCG TGC AAT TGT CTT AAA AAA CTG G		
ermC	F	GAT AAT ATC TTT GAA ATC GGC TCA	566	37
	R	CCT GCA TGT TTT AAG GAA TTG TTA		
ermA	F	TCT AAA AAG CAT GTA AAA GAA	645	34
	R	CTT CGA TAG TTT ATT AAT ATT AGT		
tetK	F	TAG GGG GAA TAA TAG CAC ATT	613	37
	R	AAT CCG CCC ATA ACA AAT A		
tetM	F	GAA CTC GAA CAA GAG GAA AGC	740	29
	R	ATG GAA GCC CAG AAA GGA T		
arcA	F	GAG CCA GAA GTA CGC GAG	724	11
	R	CAC GTA ACT TGC TAG AAC GAG		
lukS-PV	F	TCT CAT GAA AAA GGC TCA GGA	187	This study
	R	CAT GAG TTT TCC AGT TCA CTT CA		11

[a] F, forward; R, reverse.

damycin resistance carried a small, 2.6-kb plasmid harboring *ermC* (35). For USA300 isolates with both clindamycin and high-level mupirocin resistance, Diep et al. (10, 11) described a large conjugative plasmid, pUSA03, which carried two resistance determinants, *ermC* and *mupA*.

The pUSA03 plasmid is related to a plasmid family that was first detected in gentamicin-resistant staphylococcal strains isolated in the mid-1970s (1, 2) and includes pGO1, pSK41, and pLW1043 (4, 5, 36). These plasmids are defined in this study as "pSK41-like" plasmids. They contain a highly conserved, transfer-associated region consisting of 15 *tra* genes. The plasmids also typically have multiple copies of the insertion element IS*257*, which act as preferred sites for integration of mobile elements that can carry resistance genes (4, 5). Antimicrobial resistance determinants described on pSK41-like plasmids include *aac6′-aph2″*, which confers resistance to aminoglycosides (4, 5, 36, 37); *dfrA*, which confers high-level trimethoprim resistance (5); *mupA*, which confers high-level mupirocin resistance (11, 27); and *vanA*, which confers vancomycin resistance (36).

Previously, reports of USA300 isolates with plasmid-mediated resistance have been single events or restricted to specific geographical areas (10, 16). Here we describe USA300 isolates with unusual plasmid-mediated antimicrobial resistance patterns, including resistance to clindamycin, mupirocin (high level), gentamicin, trimethoprim (high level), and/or doxycycline. Outside of the ABCs collection (23), gentamicin and trimethoprim resistance have not been previously reported for USA300 isolates.

MATERIALS AND METHODS

Bacterial strains. Susceptibility results from 823 USA300 MRSA isolates, collected as part of the Centers for Disease Control and Prevention (CDC) Emerging Infections Program/ABCs for invasive MRSA between 2005 and 2008, were examined for the presence of resistance phenotypes that were likely plasmid mediated. We defined unusual phenotypes as typical USA300 isolates (PCR positive for *lukS-PV* and/or containing an SCC*mec*IVa element) resistant to gentamicin, clindamycin (both inducible and constitutive resistance), doxycycline (MIC by reference broth microdilution, ≥8 μg/ml) (7), TMP-SMZ, and mupirocin (MIC, ≥512 μg/ml) (7). All isolates had previously been identified, tested for antimicrobial susceptibility by reference broth microdilution, and typed using PFGE and SCC*mec* analysis as previously described (23). Subtyping of SCC*mec*IV was done with primers described by Okuma et al. (28). ABCs isolates were collected from eight surveillance sites: the state of Connecticut and the metropolitan areas of Atlanta, GA, San Francisco, CA, Denver, CO, Portland, OR, Rochester, NY, Nashville, TN, and St. Paul, MN (22). Also included in the study was the first gentamicin-resistant USA300 isolate identified in our laboratory, an isolate from a 2006 neonatal intensive care unit (NICU) outbreak demonstrating resistance to β-lactams, erythromycin, levofloxacin, gentamicin, and mupirocin.

Isolates were all recovered from normally sterile sites of patients in the surveillance areas, representing invasive infections (cases). Isolates were grouped into three epidemiologic categories based on case characteristics: (i) health care-associated, community-onset (HACO) infections (culture obtained from an outpatient or ≤3 calendar days after admission, with the first day of admission being day 1 for a patient with one or more of the following health care risk factors: presence of an invasive device at the time of admission, history of MRSA infection or colonization, or hospitalization, surgery, dialysis, or residence in a long-term care facility in the 12 months immediately preceding the culture date); (ii) hospital-onset (HO) infections (>3 calendar days after admission); and (iii) community-associated (CA) infections (community-onset cases without documented health care risk factors). Proportions of cases (patients) with specific characteristics were compared between those whose isolates did or did not show unusual resistance patterns by a chi-square test; a P value of <0.05 was considered statistically significant. All analyses were performed using SAS, version 9.1.3 (SAS Institute Inc., Cary, NC).

PCR assays. Oligonucleotide primers unique to this study were designed using Lasergene software (DNAStar, Inc., Madison, WI) from sequence data from published sequences (Table 1). Multiplex PCRs to identify antimicrobial resistance genes were performed using the Qiagen (Valencia, CA) multiplex PCR kit with DNA lysates prepared from isolates by using a modified sodium hydroxide-heat lysis procedure (8). USA300 isolates were further defined by the presence of ACME (11) and the PVL toxin genes by PCR for *arcA* and *lukS-PV*, respec-

tively. All isolates identified as having an unusual antimicrobial resistance phenotype were assayed for *traE*, *traI*, and *repA*, associated with the conjugative pSK41-like plasmid (4, 5), and for the resistance genes *ermC*, *ermA*, *mupA*, *dfrA*, and *aac6′-aac2″*. Isolates resistant to tetracycline and doxycycline were assayed for *tetK* and *tetM*. Multiplex PCR conditions were 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 90 s, and 72°C for 90 s; and a final extension at 72°C for 10 min.

Filter mating. A recipient strain, *S. aureus* RN4220-RF, used in filter matings, was derived from reference strain *S. aureus* RN4220 by sequentially selecting for resistance to rifampin and fusidic acid. Seven USA300 isolates (donors) resistant to gentamicin and either mupirocin (four isolates) or TMP-SMZ (three isolates), seven USA100 isolates (donors) resistant to gentamicin and either mupirocin (four isolates) or TMP-SMZ (three isolates), and *S. aureus* RN4220-RF (recipient) were grown in brain heart infusion (BHI) broth with gentamicin (15 μg/ml) (donor isolates) or fusidic acid (25 μg/ml) (recipient isolate) to maintain selective phenotypes. Donor isolates of the same PFT had different PFGE patterns and, where possible, USA100 donor isolates were chosen from the same geographical location as the USA300 donors. Individual cultures, grown in broth overnight, were diluted 1:10 and incubated at 37°C with shaking for 5 h. Cells from the mating mix (420 μl), consisting of a 20:1 ratio of donor to recipient cells, were collected on a Nalgene filter (pore size, 0.45 μm) under a vacuum. The filter was removed from the unit, placed on a BHI agar plate, and incubated at 37°C for 16 to 18 h. Cells were washed from the filter in 5 ml BHI broth, and direct and serial dilutions were plated on BHI agar containing 15 μg/ml of gentamicin, 25 μg/ml of fusidic acid, and 25 μg/ml of rifampin. Controls, consisting of donor or recipient cells alone, were treated similarly. The number of transconjugants was counted after 48 h of incubation at 37°C. The frequency of transfer was expressed as the number of resistant recipients per donor CFU at the end of the mating. The transconjugants were purified on media containing the selective drugs, picked onto drug-free agar, and examined for selected and unselected resistance genes by broth microdilution and PCR as described above.

Plasmid isolation and Southern hybridization analysis. *S. aureus* plasmids were isolated by following a modified protocol for the Midi-plasmid purification kit (Qiagen, Valencia, CA). Cultures were grown in 50 ml of BHI broth with 15 μg/ml of gentamicin to mid-log phase, harvested by centrifugation, suspended in Qiagen lysis buffer containing 50 μg/ml of lysostaphin, and incubated at 37°C for 30 min before beginning the manufacturer's protocol. HindIII (New England Biolabs, Beverly, MA)-restricted plasmid fragments were separated on a 0.75% agarose gel in Tris-borate-EDTA (TBE) buffer at 80 V for 5 h. The Trackit 1 Kb DNA ladder (Invitrogen, Carlsbad, CA) was used for sizing the linear fragments. Digoxigenin-labeled DNA probes were generated by PCR using the oligonucleotide primers shown in Table 1 and plasmid DNA isolated from the following control organisms: *S. aureus* FPR3757 (11) for *traE*, *traI*, *repA*, and *mupA*, and *S. aureus* HIP11714 MI-1 (vancomycin resistant *S. aureus* [VRSA-1]) (36) for *aac6′-aph2″* and *dfrA*. The DNA from duplicate agarose gels containing HindIII-restricted plasmid DNA from 14 RN4220-RF transconjugants and HindIII-restricted plasmid controls pUSA03 and pSK41 was transferred to Zeta-Probe membranes and hybridized individually using each of the six probes listed above.

Nucleotide sequence determination and data analysis. Plasmid DNA for USA300 PAnicu was extracted using a magnetic bead suspension method previously described by Williams et al. (38) with a Plasmid-safe DNase treatment (Epicentre Biotechnologies, Madison, WI) prior to sequencing. Plasmid DNA for USA300 TN147and USA300 GA672 was extracted using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) with 200 μg/ml lysostaphin added to the cell pellet resuspension buffer P1. Purified DNA was submitted to the J. Craig Venter Institute (JCVI; formerly The Institute for Genomic Research) for sequencing. The annotations for the GenBank submission were generated using Plasmid-RAST (3) (http://cgat.mcs.anl.gov/plasmid-rast-dev/FIG/prast.cgi). The resulting GenBank-formatted annotation was uploaded into Clone Manager, version 9.0 (Scientific & Educational Software, Cary, NC), and the identity of each predicted protein was verified by BLASTP analysis.

Nucleotide sequence accession numbers. The complete nucleotide sequences are available under GenBank accession numbers GQ900432.1 (USA300 GA), GQ90433.1 (USA300 TN), GQ900434.1 (USA300 PAnicu).

RESULTS

USA300 isolate selection, antimicrobial susceptibility testing, and epidemiologic classification. Of the 2,670 MRSA isolates from the ABCs study that were submitted to the CDC between January, 2005 and April, 2008, 823 were identified as USA300 by PFGE analysis. Among these, resistance to penicillin and oxacillin (100%), erythromycin (85%), levofloxacin (45%), or tetracycline (8.4%) was not unusual (n = 746 [90.6%]). The prevalence of different combinations of resistance to these agents only and the likely molecular determinants conferring resistance are shown in Table 2. Seventy-one of 823 (8.6%) typical USA300 isolates were resistant to additional antimicrobial agents and were defined as having an unusual resistance phenotype. These 71 isolates, and a single USA300 MRSA isolate with mupirocin and gentamicin resistance recovered from an NICU patient in a hospital outside the ABCs program (Philadelphia, PA), were chosen for molecular analysis, since they demonstrated unusual resistance that was likely plasmid mediated (Table 3).

TABLE 2. Prevalence of usual USA300 antimicrobial resistance phenotypes that were not further evaluated

Phenotypic resistance[a]	No. (%) of isolates[b]	Expected resistance mechanism(s)[c]
OXA	30 (3.6)	*mec*
OXA, ERY	345 (42)	*mec*, *mrsA*
OXA, LEV	16 (2)	*mec*, gyrase gene mutations
OXA, ERY, LEV	355 (43)	*mec*, *msrA*, gyrase gene mutations
OXA, TET (± ERY, ± LEV)[d]	69 (8.4)	*mec*, *tetK*

[a] OXA, oxacillin; ERY, erythromycin; LEV, levofloxacin; TET, tetracycline.
[b] The total number of isolates was 746 (90.6% of the isolates in this study).
[c] The expected mechanisms are based on previous reports (11, 17, 18, 35).
[d] TET-resistant, doxycycline-susceptible isolates were resistant to ERY and/or LEV and were included in the total 746-isolate count.

Genotyping of USA300 isolates. Figure 1 shows a dendrogram demonstrating the relatedness of the USA300 PFGE patterns of the 72 study isolates and the number of isolates associated with each pattern. All isolates carried SCC*mec*IVa and, with one exception, were positive by PCR for the *arcA* locus of the ACME element. The exception was pattern USA300-0328 (3 isolates), which carried SCC*mec*IVa but was PCR negative for *arcA*. All isolates were positive for PVL, with the exception of one isolate with a USA300-0114 pattern that carried SCC*mec*IVa and was positive for *arcA* but negative for *lukS-PV*.

Molecular analysis of antimicrobial resistance mechanisms. Isolates identified as having unusual resistance were tested for the presence of resistance determinants and a pSK41-like plasmid by PCR. The results are shown in Table 3. USA300 isolates resistant to clindamycin (including inducible and constitutive resistance), mupirocin, or gentamicin were positive by PCR for *ermC*, *mupA*, and *aac6′-aph2″*, respectively. Twenty-five isolates positive for a pSK41-like plasmid were positive for the resistance genes *mupA* (20 isolates), *aac2′-aph6″* (7 isolates), and *dfrA* (6 isolates); 16 of these were positive for two or more resistance genes. USA300 isolates that were resistant only to clindamycin and positive only for *ermC* were negative by PCR for pSK41-like plasmid genes. Three isolates that were tetracycline resistant and had intermediate resistance to doxycycline were positive for *tetM*; one of these isolates was positive by PCR for both *tetM* and *tetK*. Of the five isolates that were resistant to TMP-SMZ, four were PCR positive for *dfrA* and one was PCR negative for *dfrA*. Since primers for *dfrA* were

TABLE 3. Unusual[a] antimicrobial resistance phenotypes and resistance genes identified in USA300 isolates

Resistance phenotype (drug or drug combination)[b]	No. of isolates[c]	No. of isolates with the following genetic determinant detected by PCR:						Source(s) of isolates
		ermC	*mupA*	*aac2′–aph6″*	*dfrA*	*tetM*	pSK41[d]	
CC	42	42	0	0	3	0	1	CA, CT, GA, NY, OR, TN
MUP	12	0	12	0	1	0	9	CA, GA, NY, OR
CC + MUP	5	5	5	0	0	0	5	CA, GA, OR
GM	1	0	0	1	1	0	0	CA
GM + TMP-SMZ	3	0	0	3	3	0	3	CA, TN
GM + MUP	4	0	4	4	1	0	4	GA, PA, TN
MUP + TMP-SMZ	1	0	1	0	0	0	1	OR
CC + TMP-SMZ	1	1	0	0	1	0	0	OR
CC + DOX	2	2	0	0	0	2	1	GA, OR
CC + MUP + DOX	1	1	1	0	0	1	1	TN
Total	**72**	**51**	**23**	**8**	**10**	**3**	**25**	

[a] Likely plasmid-mediated resistance to clindamycin, mupirocin, gentamicin, trimethoprim-sulfamethoxazole, and/or doxycycline.
[b] CC, clindamycin resistance (10 isolates had inducible resistance, and 41 had constitutive resistance); MUP, high-level mupirocin resistance; GM, gentamicin resistance; DOX, doxycycline resistance.
[c] The total number of isolates was 72 (8.7% of the isolates in this study).
[d] The presence of pSK41 was identified by positive PCR for *traE*, *traI*, and *repA*.

included in the multiplex assay used to test all isolates with unusual resistance, *dfrA* was also identified in six isolates which tested susceptible to TMP-SMZ. These isolates all demonstrated elevated but susceptible MICs (i.e., 1/19 to 2/38 μg/ml) to TMP-SMZ. Six of the 10 *dfrA*-positive isolates were also positive for genes associated with pSK41-like plasmids.

Epidemiological classification of USA300 infections. USA300 isolates with unusual resistance were more often recovered from patients with health care exposures (60 of 71 [84.5%]) than in cases without unusual resistance phenotypes (448 of 746 [60%]) (Table 4). More specifically, patients whose isolates showed unusual resistance were more likely to have documented prior MRSA infections (or colonization), recent hospitalization, surgery, or residence in long-term care settings than other cases (Table 4). In contrast, patients with unusual resistance were as likely as others to have undergone chronic dialysis or have infection onset during hospitalization (Table 4). Of note, 11 (15.5%) of the cases with unusual resistance were classified as community-associated infections.

Analysis of gentamicin resistance plasmids. Since gentamicin-resistant MRSA is typically associated with hospital acquisition (23, 24), it is possible that USA300 isolates acquired gentamicin resistance from health care-associated MRSA strains. To investigate this, we compared plasmids from USA300 and USA100 isolates with similar resistance phenotypes, collected as part of the ABCs study. Plasmids from four USA300 isolates resistant to both gentamicin and mupirocin were compared to plasmids from four gentamicin- and mupirocin-resistant USA100 isolates. In addition, plasmids from three gentamicin- and TMP-SMZ-resistant USA300 isolates were compared to plasmids from three gentamicin- and TMP-SMZ-resistant USA100 isolates.

All 14 isolates described above transferred gentamicin resistance to *S. aureus* RN4220-RF by conjugation. Mupirocin resistance was also transferred from the eight mupirocin-resistant isolates, and the six isolates with TMP-SMZ resistance transferred reduced susceptibility to TMP-SMZ. All transconjugants harbored a single plasmid that ranged from 41 kb to 55

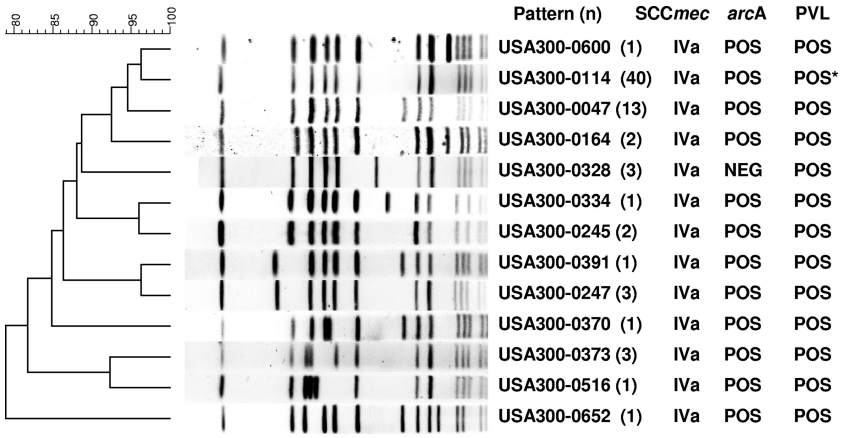

FIG. 1. Dendrogram of USA300 PFGE patterns of isolates with unusual antimicrobial susceptibility phenotypes, showing the number of isolates with each PFGE pattern (n), the SCC*mec* type, and the results of PCR for *arcA* and *lukS-PV*. POS, positive. The asterisk marks an exception to the positive PVL results for the USA300-0114 pattern: one isolate with this pattern that carried SCC*mec*IVa and was positive for *arcA* was negative for *lukS-PV*.

TABLE 4. Epidemiologic classification and patient characteristics among 823 cases of invasive USA300 MRSA infections with unusual[a] resistance phenotype statuses, Active Bacterial Core surveillance, 2005 to 2008

Epidemiologic classification (patient characteristic)	No. (%) of USA300 isolates with:		P (χ^2 test)
	No unusual resistance (n = 746)	Unusual resistance (n = 71)	
Health care-associated, community-onset infections[b]	353 (47.3)	50 (70.4)	0.0002
History of MRSA infection or colonization	139 (18.6)	28 (36.4)	0.0001
Hospitalization (prior year)	314 (42.1)	49 (63.6)	0.0005
Surgery (prior year)	168 (22.5)	28 (36.4)	0.0039
Dialysis (prior year)	67 (9.0)	9 (11.5)	0.4577
History of residence in a long-term care facility	72 (9.7)	15 (19.2)	0.0088
Community-associated infections	278 (37.3)	11 (15.5)	<0.0001
Hospital-onset infections	95 (12.7)	10 (14.1)	0.3275
Classification undetermined	20 (2.7)	0	0.1432

[a] Likely plasmid-mediated resistance to clindamycin, mupirocin, gentamicin, trimethoprim-sulfamethoxazole, and/or doxycycline.
[b] Characteristics are not mutually exclusive.

kb; transconjugants were positive for pSK41-like plasmid genes *traE*, *traI*, and *repA*, as well as *aac6′-aph2″*, and either *mupA* or *dfrA*. Also, *dfrA* was identified in the transconjugants from two donors in which reduced susceptibility to TMP-SMZ was not previously recognized (Fig. 2, lanes 2 and 3). The transfer frequency of donor to transconjugant cells ranged from 1×10^{-5} to 4×10^{-8}.

HindIII restriction and Southern hybridization of plasmid DNA from the 14 transconjugants revealed 10 different patterns (Fig. 2). Plasmid-specific markers *traE* and *traI* hybridized with a 9.3-kb fragment, and *repA* hybridized with a 5.8-kb fragment, of each plasmid. Similarly, *aac6′-aph2″* hybridized with a 2.5-kb fragment of each plasmid. Probes for *mupA* and *dfrA* hybridized with fragments of various sizes.

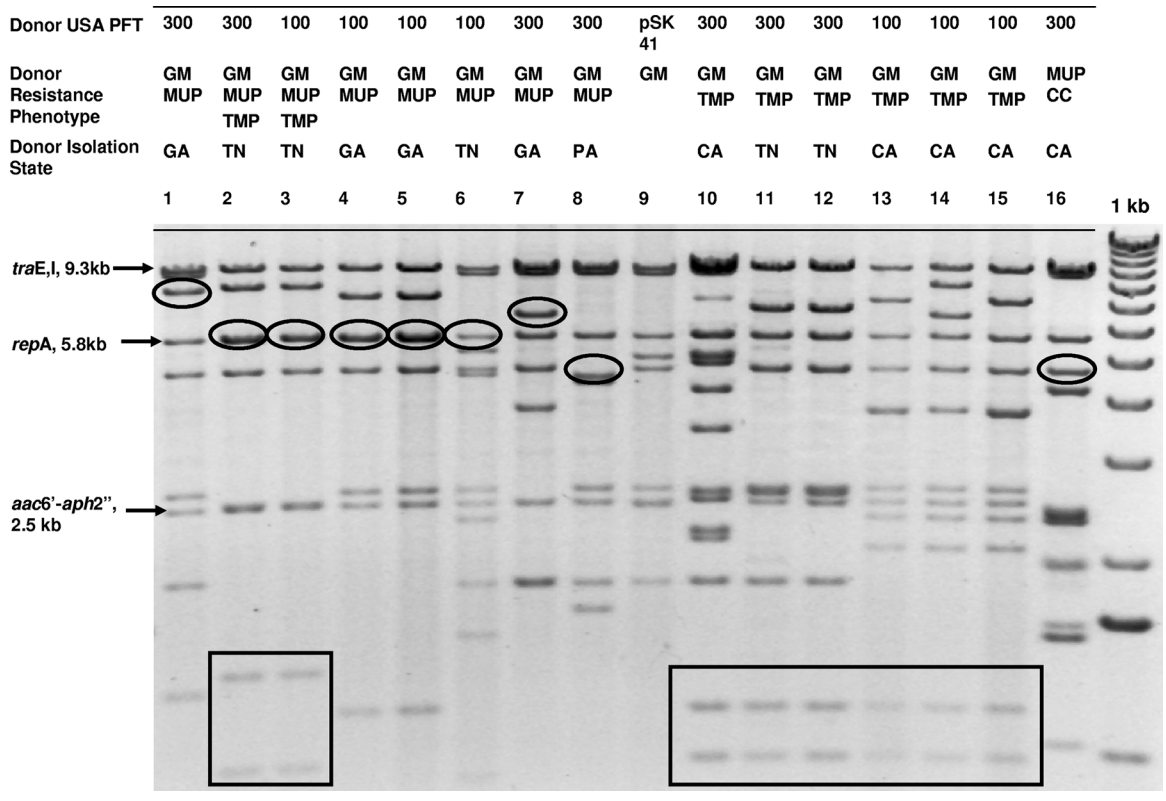

FIG. 2. Gel showing a summary of Southern hybridization data from six gels of HindIII-restricted RN4220-RF transconjugant plasmid DNA, pSK41 (lane 9), and pUSA03 and pUSA02 (lane 16). Four probes each hybridized with a unique HindIII fragment in all plasmid DNA containing the following genes: *traE* and *traI* (9.3-kb HindIII fragment), *repA* (5.8-kb HindIII fragment), and *aac6′-aph2″* (2.5-kb HindIII fragment). Circled HindIII fragments are those with which the *mupA* probe hybridized, and boxed HindIII fragments are those with which the *dfrA* probe hybridized. The donor pulsed-field types (USA100 or USA300), donor antimicrobial resistance phenotypes (GM, gentamicin; MUP, mupirocin; TMP, trimethoprim; CC, clindamycin), and states where donors were isolated are shown at the top of the figure.

TABLE 5. Resistance genes and phenotypes of sequenced pSK41-like plasmids

Gene(s)	Phenotypic resistance	Presence[a] in the following plasmid:							
		pSK41 (46.4 kb)	pGO1 (54 kb)	pLW043 (57.9 kb)	pUSA03 (37.1 kb)	pV030-8 (39 kb)	pTN147 (43.9 kb)	pGA672 (47.3 kb)	pPAnicu (44.1 kb)
aac6′-aph2″	Gentamicin, tobramycin, kanamycin	X	X	X			X	X	X
aadD	Neomycin	X	X				X	X	X
qacA, *qacB*, *smr*	Quaternary ammonium compounds	X	X	X				X	X
dfrA	Trimethoprim		X	X			X		
vanA	Vancomycin			X					
mupA	Mupirocin				X	X	X	X	X
ermC	Macrolides-lincosamides-streptogramins				X				
blaZ	β-Lactams			X					
bleO	Bleomycin	X	X				X		

[a] An X indicates the presence of a gene. The source or reference for each plasmid is given in parentheses as follows: pSK41 (4), pGO1 (5), pLW043 (36), pUSA03 (11), pV030-8 (GenBank accession number NC_010279), pTN147 (this study; GenBank accession number GQ900433.1), pGA672 (this study; GenBank accession number GQ900432.1), and pPAnicu (this study; GenBank accession number GQ900434.1).

Transconjugant plasmids with indistinguishable restriction and Southern hybridization profiles were identified from a USA100 and a USA300 donor isolate from Tennessee (Fig. 2, lanes 2 and 3). Several indistinguishable transconjugant plasmids were also identified from donors of the same PFT with the same resistance phenotypes, but with different PFGE patterns (Fig. 2, lanes 4 and 5 [USA100], 11 and 12 [USA300], and 13 and 15 [USA100]).

Plasmids from three isolates (Fig. 2, lanes 1, 2, and 8) were selected for sequence analysis based on their divergent HindIII restriction profiles and antibiotic resistance markers. All three plasmids were pSK41-like plasmids based on homology of the conjugative and replication gene sequences. The resistance determinants of these USA300 plasmids (pTN147, pGA672, and pPAnicu) were compared to those identified in five previously sequenced pSK41-like plasmids (Table 5).

DISCUSSION

In this study, we reported unusual plasmid-mediated antimicrobial resistance among USA300 isolates causing invasive disease. The frequency of isolates with unusual resistance was low, ranging from 6.2% (i.e., clindamycin resistance) to less than 1% (i.e., gentamicin resistance, TMP-SMZ resistance, and doxycycline resistance). In the past, USA300 isolates were predominantly from community-associated infections, but data from the ABCs MRSA project and other studies have demonstrated that isolates of this lineage are found with increasing frequency in health care-associated infections (21, 23, 32). It has been hypothesized that as these isolates move into the health care setting, they will acquire resistance determinants more commonly seen in health care-associated MRSA strains, such as USA100 (23, 25). In support of this hypothesis, our data indicate that a majority (84.5%) of invasive USA300 isolates with unusual resistance phenotypes were isolated from patients who had one or several contacts with the health care system. While only 15.5% of isolates with unusual resistance were from community-associated infections, the presentation of these isolates in the community could complicate appropriate empirical therapy for potential community-associated MRSA infections.

A well-described mechanism of resistance transfer in *S. aureus* is the pSK41-like conjugative plasmid, and USA300 isolates with pSK41 plasmid-mediated resistance were reported previously (11). These plasmids carried *ermC*-mediated clindamycin resistance and *mupA*-mediated mupirocin resistance, and they were identified in limited geographical areas (i.e., San Francisco, CA, and Boston, MA) (10, 16). In this study, unusual resistance determinants in USA300 were frequently associated with pSK41-specific genes *traE*, *traI*, and *repA*. Specifically, pSK41-like genes were identified in 25 (35.2%) of the USA300 isolates with unusual resistance, particularly in those isolates that were positive for resistance determinants *aac6′-aph2″*, *mupA*, and *dfrA*, conferring resistance to gentamicin, mupirocin, and trimethoprim, respectively. Conjugation studies confirmed the location of these resistance determinants on a pSK41-like plasmid. These results suggest that pSK41 is an important vector for the dissemination of resistant determinants in USA300. Also, evidence of a pSK41-like plasmid was found in USA300 isolates from five of eight states where surveillance was conducted, indicating that these plasmids are more geographically widespread than was previously recognized.

We looked for evidence of identical pSK41-like plasmids in USA300 and USA100 isolates with similar resistance profiles from the same geographical location. This analysis was limited to gentamicin-resistant isolates, since gentamicin resistance in *S. aureus* is usually associated with hospital acquisition. Indistinguishable plasmids were identified both in USA100 isolates with different PFGE patterns and in USA300 isolates with different PFGE patterns in the same geographical location. In addition, indistinguishable plasmids were identified in a USA100 isolate and a USA300 isolate from the same geographical location. These results suggest horizontal plasmid transfer between isolates of the same lineage and between isolates of different lineages.

Not all of the unusual plasmid-mediated resistance determinants identified in this study were associated with pSK41-like plasmids. No pSK41-like plasmid was present in USA300 isolates for which the only unusual resistance was clindamycin. These isolates were positive for *ermC* by PCR, and the gene was most likely carried on a small plasmid, as had been determined previously in clindamycin-resistant USA300 isolates (35). Three USA300 isolates from health care-associated infections were doxycycline intermediate, tetracycline resistant,

and *tetM* positive. Two of these isolates were associated with pSK41-like plasmids and other resistance determinants, and one isolate was negative for pSK41-like genes. The location of *tetM* was not investigated.

This is the first report of TMP-SMZ resistance in USA300 isolates. This is significant because TMP-SMZ therapy has been recommended for empirical treatment of infections originating in the community when coverage for MRSA is desired (6, 9, 14, 20). The mechanism and epidemiology of TMP-SMZ resistance were interesting. A known *S. aureus* resistance determinant has been identified only for trimethoprim, *dfrA*. No resistance determinant is known for resistance to sulfamethoxazole, but the mechanism is likely due to mutations in the chromosomal dihydropteroate synthase, which inhibits the modification of *p*-aminobenzoic acid to dihydrofolate, blocking folic acid synthesis (15). Several TMP-SMZ-susceptible isolates in this study were positive for *dfrA*, a resistance gene carried by pSK41-like plasmids. This is of concern, because it signals the emergence of transferable trimethoprim resistance in a MRSA lineage that has proven to be a successful pathogen.

As the CDC database of MRSA strains has expanded, we have identified isolates with similarities to both USA300 and USA500. Five TMP-SMZ-resistant isolates, originally identified as USA300 by PFGE, were excluded from the study because they were PVL negative and did not contain SCC*mec*IVa. In addition to being related to USA500 isolates by PFGE analysis, these isolates were also *arcA* negative and *dfrA* negative. USA500 isolates, primarily seen in health care-associated infections, are characteristically resistant to TMP-SMZ (23, 25) and generally negative for *dfrA*, suggesting another mechanism of trimethoprim resistance.

In summary, this is the first report describing gentamicin, TMP-SMZ, and doxycycline resistance in USA300 isolates, although there have been reports of sporadic cases of gentamicin and doxycycline resistance in USA300 isolates (10, 31). We also describe USA300 isolates with resistance to clindamycin and mupirocin in more diverse geographical regions than have previously been reported (10, 16). These isolates are epidemiologically associated with health care exposures; as USA300 isolates emerge in health care settings, these isolates may acquire additional antimicrobial resistance, with pSK41-like plasmids being an important vector for transmitting resistance. If USA300 isolates become more resistant, as described here, empirical therapy for community-associated MRSA infections may become more complicated. These findings highlight the need for ongoing assessment of phenotypes as they relate to empirical treatment guidelines, as well as the need for evaluation of methods to minimize cross-transmission of resistance elements.

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