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Prevalence and characterization of *Staphylococcus aureus* in wastewater treatment plants by whole genomic sequencing

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ABSTRACT:

Infections with *Staphylococcus aureus* are being spread through contact with the community environment, but the role of wastewater treatment plants in the transmission routes is not defined. This study investigated the prevalence, types, genetic elements, and potential for transmission of *S. aureus* by these engineered systems. Synchronized sampling events at two wastewater treatment plants were conducted with isolates of *S. aureus* obtained by a selective enrichment method using acriflavine that suppressed *Staphylococcus epidermidis* growth. DNA was extracted from a subset of the *S. aureus* isolates, checked by PCR to assure the absence of *S. epidermidis*, and sequenced to determine the multilocus sequence type, *spa* type, and carriage of the methicillin resistance and Panton–Valentine leukocidin genetic elements. Sequences were analyzed for single nucleotide polymorphism differences in pairwise comparison of isolates. There were two dominant *S. aureus* clonal complexes identified in the isolates, one commonly identified as hospital-related (CC5) and one community-related (CC8). Both types of isolates were found at both treatment facilities, even though only one facility had significant hospital sewage inputs. The presence of *S. aureus* persisted through treatment, with some isolates recovered from the final processes showing genetic diversity. The presence of the Panton–Valentine leukocidin genetic element was greater than the 1–5% expected from global reports. Our results suggest that treatment provides an opportunity for genetic shift, while the persistence and release of evolved strains of *S. aureus* may provide an environmentally relevant pathway to new hosts in the environment.

1. Introduction

Staphylococcus aureus is one of a group of bacteria that has been shown to colonize the nose of approximately 30% of the human population (Chambers and Deleo, 2009), but infections by community acquired antibiotic resistant *S. aureus* are an emerging global epidemic (Grundmann et al., 2006). In the United States, methicillin-resistant *S. aureus* (MRSA) infections occurred at a rate of 18.8 per 100,000 people in 2015, were associated with serious complications like infective endocarditis, osteomyelitis, and sepsis, and a retrospective study of hospital costs resulting from MRSA infections averaged $34,657 per patient in the Minneapolis-area and $113,852 per patient in Rhode Island (Ortwine and Bhavan, 2018). *S. aureus* has developed rapid resistance to almost every antibiotic in use by acquiring mobile genetic elements from its surroundings and less frequently by gene mutation. Penicillin-resistant *S. aureus* was first isolated in the 1940s, and by the 1960s *S. aureus* had acquired the *mecA* gene and broad-spectrum resistance to all beta-lactam antibiotics, including penicillins, cabapenems, and methicillin. These methicillin-resistant *S. aureus* (MRSA) strains initially originated in the hospital setting (hospital-acquired MRSA or HA-MRSA) but increasingly, community-acquired MRSA (CA-MRSA) strains have emerged as the cause of skin and soft tissue infections.

Early methods for the classification of MRSA identified strains by their country (USA, UK, Sweden, etc.) or setting of origin (CA-MRSA or HA-MRSA). Further development in genetic analyses has allowed

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for classification based on distinct regions of the *S. aureus* chromosome. Multilocus sequence typing (MLST) compares partial sequences of seven housekeeping genes and is useful for measuring long term evolution of MRSA. *S. aureus* strains having identical sequences at all seven genes are grouped into the same sequence type (ST) while strains differing by a single nucleotide polymorphism (SNP) in less than three of the seven genes are grouped into a clonal complex (CC) (Enright et al., 2000). Strains can be further classified based on the presence/absence of the beta-lactam resistance gene, *mecA*, in MRSA or methicillin-sensitive *S. aureus* (MSSA), respectively. *Spa* typing is an inexpensive way to categorize *S. aureus* based on the number of tandem repeats and point mutations in a single locus of the *S. aureus* genome, the staphylococcal protein A or *spa* gene (Fenner et al., 2008). Discriminatory power of *spa*-type is quite high and the method is adapted to the short-term evolution of clinical isolates of *S. aureus*.

Epidemics of *S. aureus* infection have been described as waves of increasing resistance to antibiotics and the presence of virulence factors as a result of the uptake of mobile genetic elements (MGEs) from the environment (Chambers and Deleo, 2009). Current MRSA epidemics evolved from six different CCs; CC1, CC5, CC8, CC22, CC30 and CC45 (Cockfield et al., 2007). In the United States the most common HA-MRSA infections are caused by USA100 (CC5-ST5) whereas USA300 (CC8-ST8) causes the most CA-MRSA infections (Diep et al., 2004; Miller and Diep, 2008). USA300 strains frequently contain the virulence factor Panton-Valentine leukocidin (PVL), which likely enables CA-MRSA infections in otherwise healthy individuals (Kazakova et al., 2005).

The continual evolution of *S. aureus* makes the identification of clones and their lineage essential for categorizing isolated MRSA and MSSA strains. To date, the study of antibiotic resistant *S. aureus* has focused largely on the hospital setting, although the presence of antibiotic resistance elements in wastewaters, and their transfer between environmental reservoirs and human pathogens, has been observed and reported (Baquero et al., 2008; Miller et al., 2016; Munck et al., 2015; Schmieder and Edwards, 2012). The purpose of this study was to isolate and characterize *S. aureus* strains by whole genomic sequencing from two Wastewater Treatment Plants (WWTPs), one urban and one suburban, in order to better understand the impacts of sewershed sources on the types and distribution of *S. aureus*, determine if sewage treatment processes have an effect on the adaptation of *S. aureus*, and assess the potential for release of viable *S. aureus* in treated effluents.

2. Materials and methods

2.1. Sampling sites

Sewage samples were collected from two, activated sludge based, WWTPs in Lexington, Kentucky. Town Branch (TB) WWTP collects urban sewage from high density apartments, several hospitals, a prison, an airport, the University of Kentucky, and multiple industrial companies located centrally and to the north of the city. TB has an average daily flow of 19.8 million gallons per day (MGD) and design capacity of 30 MGD. The total resident population feeding to TB is (about) 121,836, but due to the multiple major hospitals in this sewershed, sewage is also generated from a transitory population inhabiting 1,646 beds for a total of 363,837 inpatient days per year (Kentucky Health Facts, 2011). West Hickman (WH) WWTP has a design capacity of 33.9 MGD with an average daily flow of 20.4 MGD from the expanding suburban sprawl in south part of the city. The total residential population supplying WH is about 164,940, with an additional transitory population inhabiting 152 beds for a total of 30,576 inpatient days per year (Kentucky Health Facts, 2011). Grab samples were collected from these two WWTPs from the untreated influents, the effluents of the sedimentation tank (final clarifier after activated sludge treatment), and the final chlorinated effluents. Samples were taken in the morning (9:00–10:00 a.m.) on the same days to provide a snapshot in time. Samples were taken on 6 days between February and June, but 2 sampling events did not produce presumptive *S. aureus* isolates from any of the samples collected.

2.2. Selective enrichment and isolation of Staphylococcus aureus

One liter of samples from three different sample sites within the WWTPs were transferred on ice to the Environmental Research and Training Laboratories (ERTL) at the University of Kentucky and processed within 12 h of collection. Samples were passed through sterile, stainless steel, tea-strainers to remove large particles. Then, 200 mL of the strained sample was centrifuged at 3,700×*g* for 10 min to sediment bacteria. The supernatant was discarded and the pellet enriched with an equal volume of 2× mannitol salt broth (MSB) that had been augmented to a final strength of 0.0165 mg/mL acriflavine (Acros Organics), 75 IU/mL polymyxin B (OXOID), and 3.5% potassium tellurite (OXOID). Acriflavine was added to suppress co-growing *Staphylococcus epidermidis* (Davis et al., 2006). Polymyxin B was added to suppress the growth of Gram-negative bacteria. Potassium tellurite was added to enhance *S. aureus* respiration and provide a visual growth signal (black precipitant). The pellet-broth mixture was vortexed and incubated at 37 °C for 48 h. Cultures with a black color were centrifuged at 3,700×*g* for 10 min, the bulk of the supernatant discarded, and the pellet spread onto petri dishes containing mannitol salt agar (MSA) augmented with 0.0165 mg/mL acriflavine. These pellet spread plates were transferred back to the incubator (37 °C) and incubated for 48 h. Yellow colonies were considered to be presumptive *S. aureus*, and at least 10 colonies, or up to 10%, of all presumptive colonies were re-streaked onto new acriflavine-augmented MSA petri dishes to create single colony clones for further testing. The COAGULASE CRYO™ (Hardy Diagnostics) test was used to verify colonies as coagulase positive. Coagulase positive colonies were subjected to ELISA testing (MRSA latex test, Denka Seiken, Japan) to verify the presence of the penicillin binding protein responsible for beta lactamines resistance. A positive ELISA confirmed the selected *S. aureus* isolate as presumptive MRSA while a negative ELISA result was classified as presumptive MSSA. A subset of isolates from different days, and from different processes was selected for regrowth prior to PCR and whole genomic sequencing. All selected *S. aureus* isolates were inoculated into acriflavine-augmented MSB, incubated at 37 °C for 48 h, and frozen at −80 °C until DNA extraction. Genomic DNA obtained from broth-cultured colony isolates was purified using the DNeasy kit (Qiagen, Courtaboeuf, France). DNA content of 50 µl extract aliquots was measured by Nano Drop, then extracts were shipped on ice to the University of Geneva Genomic Research Laboratory in Switzerland for whole genome sequencing.

2.3. Bacterial isolate strain characterization

A molecular assay previously developed and validated was used to complete the characterization of collected *S. aureus* isolates. A triplex, quantitative PCR assay targeting species-specific genes allowed for the detection of *S. epidermidis* or *S. aureus* as well, as the presence of the *mecA* gene (Francois et al., 2003) in extracts, and was used to confirm the *in silico* determination obtained from whole genome sequencing information. The genetic information was used to type the isolates by application of two standardized methods. Both methods relied upon identification of specific DNA sequences for specified gene fragments. Two typing approaches

were applied; MLST and *spa* sub-typing to the *S. aureus* isolate DNA. MLST consisted of amplification and sequencing parts of 7 essential house-keeping genes with known mutation frequencies. Each sequence was compared to a widely available sequence database containing all known alleles that correspond to a specific number. The resulting 7-digits number was then reduced to a single number that represents a sequence type. Strains with similar MLST sequence types are considered to share general genetic content of the conserved genome (Enright et al., 2000). Single locus *spa* typing used the sequence of the repeated region of the gene coding for staphylococcal protein A (*spa*). The sequence and order of specific repeats in this region of the *spa* gene were matched with unique numeric codes and used to classify the *spa*-type of all *S. aureus* strains (Strommenger et al., 2008). Presence of *pvl* was confirmed using a PCR assay already validated (Francois et al., 2004). Multi-locus sequence typing analysis was performed using annotated genomes and submitted to the Center for Genomic Epidemiology database available at (http://cge.cbs.dtu.dk/services/MLST).

2.4. Genome sequencing and analysis

DNA was subjected to whole genome sequencing on the Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) using 100 bases reads with paired-ends according to the Truseq protocol (Illumina), following the manufacturer's recommendations. The quality of sequence reads was assessed with the Fastqc program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were quality filtered using the fastq-mcf program (Ea-utils: https://github.com/ExpressionAnalysis/ea-utils/blob/wiki/FastqMcf.md). Genome assembly was performed using the Edena v3 assembler (Hernandez et al., 2013) with parameters as follows; minimum read overlapping of 60-bp and minimum contig size retained of 500-bp. Assembled genomes were annotated using the Prokka v1.10 program (Seemann, 2014) with default values for BlastP analysis, and the e-value threshold set to 10e-6 for accurate BlastP analysis. The "Get_homologues.pl" script was used for core proteome comparisons (Contreras-Moreira and Vinuesa, 2013). The CVTree3 Web Server was used to perform the phylogenetic analysis (Xu and Hao, 2009). Prokka annotation and blastP analysis were performed to identify specific genes involved in the phenotype, evolution, and virulence of the strain.

After a first attempt, a minority of strains showed poor assembly results; typically, >1000 contigs with genome size of 4 megabases (instead of 2.6–2.9 MB), due to the presence of contaminating DNA from *Proteus mirabilis,* a Gram-negative bacteria frequently identified in environmental samples. This observation followed blast results of resulting contigs for samples showing unusual number of contigs after *de novo* assembly. The genome of 4, *P. mirabilis* strains, identified from different locations and publicly available, were used ensuring a sufficient representability of the species (accession numbers: CP021694.1, CP015347.1, CP004022.1 and CP017085.1) to subtract reads from the contaminant organisms by using CLARK (v.1.2.3.2 using default parameters) (Ounit et al., 2015). Resulting cleaned datasets were then subjected to a new analytical assembly attempts that showed improvement of results and a decrease in the number of contigs.

The phylogenetic relationship of isolates was investigated by genomic, SNP–based analysis using *S. aureus* core genome in the Parsnp v1.0 program (Treangen et al., 2014). Briefly, all SNPs identified in the common core genome of our strain collection were considered. The BlastP analysis was used to investigate the presence of specific genes involved in the phenotype, evolution, and virulence of the isolates. Additionally, all published sets of sequencing reads from *S. aureus* ST8 isolates (Von Dach et al., 2015) were retrieved from a previous study, enriched with some genomes of *S. aureus* USA300 from American origin (Planet et al., 2013), and included in the genome comparisons. A cutoff value of 23 SNPs from >2.5 nucleotides was used for establishing an epidemiological link between the isolates as previously established (Von Dach et al., 2015). Isolate genomes have been deposited at ENA (https://www.ebi.ac.uk/ena) under the following name: PRJEB29126.

2.5. Naming protocol for selected isolates

The 18 selected isolates were sent for genomic analysis to a research lab in Switzerland with only a number as an identifier in order to provide blind samples without reference to place, date, or sample type. The genetic results were reported back with SA added to the original number (SA1) to identify them as *S. aureus*, isolate 1. For the creation of easily interpretable spreadsheets and tables (Tables 4–6), the SA# was followed by initials for the WWTP (TB, WH) and sampling site (IN, ST, EFF for influent, sedimentation tank and effluent, respectively).

3. Results

3.1. Enrichment and isolation of clean colonies of S. aureus from sewage samples

Key to the goals of this research was the ability to effectively recover clean colonies of methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) from environmental samples without the presence of the commonly, co-cultured bacteria *S. epidermidis*. Acriflavine acted as a growth suppressant for all laboratory strains of *Staphylococcus* when added to mannitol salt agar, but was markedly more effective on reducing growth of *S. epidermidis* (ATCC29887) than the strains of MSSA (ATCC25923) and MRSA (BAA2420) tested. Results from replicate bacterial stock titers plated on MSA, with and without acriflavine, showed a significant reduction in the average number of colonies cultured for *S. epidermidis* titer (65%), with smaller reductions in methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) of 46% and 17%, respectively. Pairwise multiple comparison between the strains by Tukey testing showed significant differences only in the growth suppression of *S. epidermidis* versus ($P < 0.05$) the MRSA strain (Table 1). The effectiveness of acriflavine suppression was further demonstrated in the absence of *S. epidermidis femA* genes in DNA extracts from the 18 selected *S. aureus* isolates sent for sequencing. Quantitative PCR results produced on the isolates were negative for the *femA* gene in all 18 selected clones (Francois et al., 2003). The absence of co-growing *S. epidermidis* in the isolates supports the use of acriflavine augmented enrichment media for selective enrichment and isolation of *S. aureus* from complex sewage matrices.

However, as was seen upon sequencing of the DNA extracts obtained, suppression was not complete as some bacteria were still capable of co-growth with *S. aureus* in suppressive media. *Proteus mirabilis* grew in the acriflavine augmented media, appearing in 6

Table 1
Comparative growth inhibition of *S. epidermidis* versus *S. aureus* laboratory stocks on mannitol salt agar augmented with acriflavine with statistical significance.

Bacterial Stock	Average percent titer reduction (n = 5)	Tukey pairwise multiple comparison			Statistical Difference $P < 0.05$
S. epidermidis	65 ± 3	*S. epidermidis*	*vs*	MRSA	YES
MSSA	46 ± 11	*S. epidermidis*	*vs*	MSSA	NO
MRSA	17 ± 13	MSSA	*vs*	MRSA	NO

Table 2
S. aureus DNA extracts subjected to *in silico* removal of *P. mirabilis*: 6 from a total of 18 isolates.

Isolate	Sample Date	WWTP	Sample Site
SA3	2.10.2017	Town Branch	Influent
SA5	2.10.2017	West Hickman	Influent
SA10	2.24.2017	West Hickman	Influent
SA13	6.26.2017	Town Branch	Influent
SA17[a]	6.26.2017	West Hickman	After sedimentation tank
SA18	6.26.2017	West Hickman	Effluent

[a] Extract DNA sequence unrecoverable after *in silico* removal of *P. mirabilis* sequences.

of the 18 selected isolates. The genetic information for *Proteus mirabilis* was removed *in silico* and a new assembly was performed; however, the sequence for one DNA extract (SA17) could not be reassembled, leaving only 17 of the 18 isolates for typing and comparison. As shown in Table 2, *Proteus* was detected in isolates obtained from both sewage treatment plants and suggests the need for further refinement of salt-based suppressive media for isolation of *S. aureus* from environmental samples.

3.2. Typing of S. aureus sewage isolates

There were three different clonal complexes (CC) and several MLST sequence types (ST) identified for the sewage isolates obtained from the local WWTPs based on the results of BLASTP analyses of whole genome sequencing for specific sequences, and MLST of 7 housekeeping genes (Fig. 1 and Table 3.). Fig. 1 shows only one isolate (SA2) was typed as MLST CC59-ST59, related to Asian strains (Li et al., 2016), and was found at the more urban Town Branch WWTP whose sewershed includes an airport, multiple hospitals, and a large university while the other 16 isolates had very close similarity and fell into two major MLST CCs; CC5 and CC8. The MLST CC59-ST59 varied in its core genome sequence greatly from all other isolates (>14,000 SNP) and was not found to carry antibiotic resistance (*mecA*) and Panton–Valentine leukocidin (PVL) mobile genetic elements (Table 3, Fig. 2). The MLST CC59-ST59 MSSA isolate type was not found at the more suburban West Hickman WWTP sewershed, or at any other site during the time of study.

The other isolates were classified into two clonal complexes; MLST CC8 (USA 300 and USA700 lineage) and MLST CC5 (USA 100 lineage). The length of the genomes from the isolates varied from 2,730,977 to 3,044,920 base pairs (Table 3). There were large differences (>7,000 single nucleotide polymorphisms) in the core genome sequence between CC5 and CC8 isolates. However, when comparing isolates within a single clonal complex against each other, the SNP differences were less than 1,000, with the exception of isolate SA10, whose MLST designation was CC8-ST72 (USA700 lineage). This ST72 MRSA isolate core genome sequence was at least 7,000 SNP different from any other isolate, even within the CC8 group (Fig. 2). This isolate was also a singular event, only occurring once during the period of sampling in West Hickman WWTP influent.

The two MLST CC groupings (CC8 and CC5) were found at both WWTPs, and some isolates carried the staphylococcal cassette chromosome associated with antibiotic resistance (*mecA*) and PVL mobile genetic elements (Tables 4 and 5). In both CC5 and CC8, there were methicillin resistant isolates (MRSA) and methicillin susceptible isolates (MSSA) based upon the carriage or lack of the *mecA* gene. However, the CC8 isolates had greater *mecA* and PVL carriage than CC5. The number of *S. aureus* isolates recoverable from sewage from the suburban-community sewershed of West Hickman WWTP was greater than the urban, hospital-dominated sewershed of Town Branch WWTP. The prevalence of the mobile genetic elements that encode for PVL in influent isolates was higher (41%) than the reported prevalence in the global population of *S. aureus* strains (1–5%) (Tristan et al., 2007).

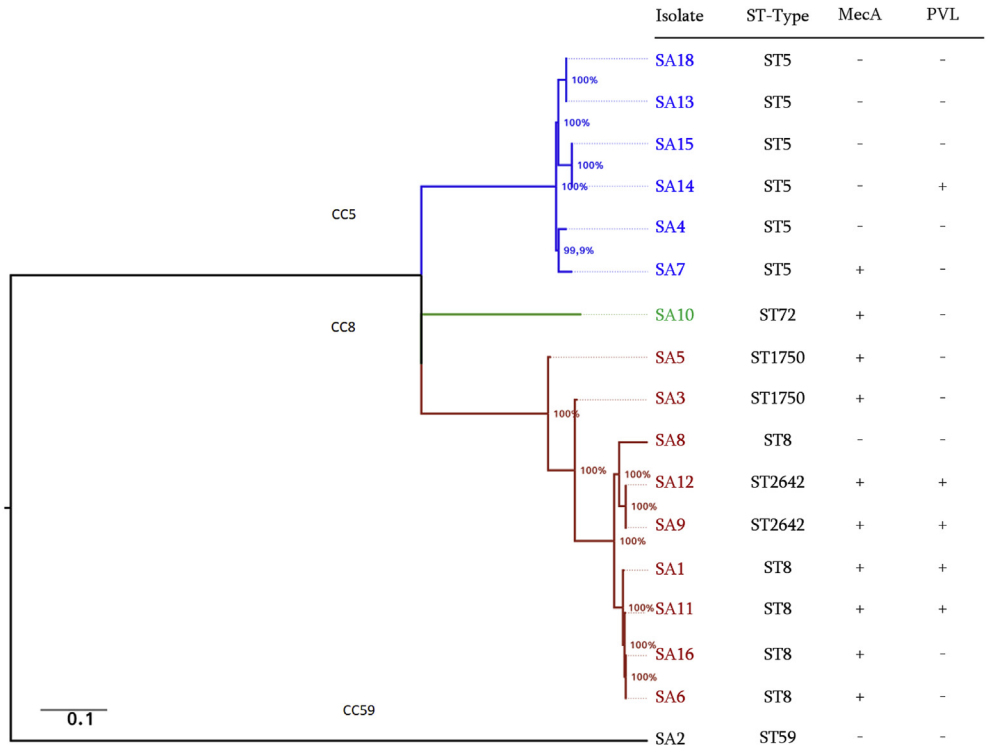

Fig. 1. Phylogenic tree denoting interrelations between isolates of *S. aureus* derived from WWTPs with select MGE carriage indicated.

Table 3
MLST typing of selected sewage derived *S. aureus* isolates based on the allelic profile of seven housekeeping genes.

Isolate	Genome size	MLST ST-Type	Alleles at the seven MLST loci						
			arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*
SA1	2869764	ST8[a]	3	1	1	1	4	4	3
SA2	2738216	ST59	19	23	15	2	19	20	15
SA3	2989750	ST1750[a]	3	3	1	1	4	4	183
SA4	2730977	ST5	1	4	1	4	12	1	10
SA5	3011809	ST1750[a]	3	3	1	1	4	4	183
SA6	2877021	ST8	3	3	1	1	4	4	3
SA7	2830652	ST5	1	4	1	4	12	1	10
SA8	2807634	ST8[a]	3	3	1	201	4	4	3
SA9	2786085	ST2642[a]	3	3	1	1	4	4	305
SA10	2950959	ST72	1	4	1	8	4	4	3
SA11	2891576	ST8	3	3	1	1	4	4	3
SA12	2786169	ST2642[a]	3	3	1	1	4	4	305
SA13	3044920	ST5	1	4	1	4	12	1	10
SA14	2879664	ST5	99.78% to allele 1	4	1	4	12	1	10
SA15	2842038	ST5	99.78% to allele 1	4	1	4	12	1	10
SA16	2875847	ST8	3	3	1	1	4	4	3
SA18	2960154	ST5	1	4	1	4	12	1	10

[a] Single Locus Variant of ST8.

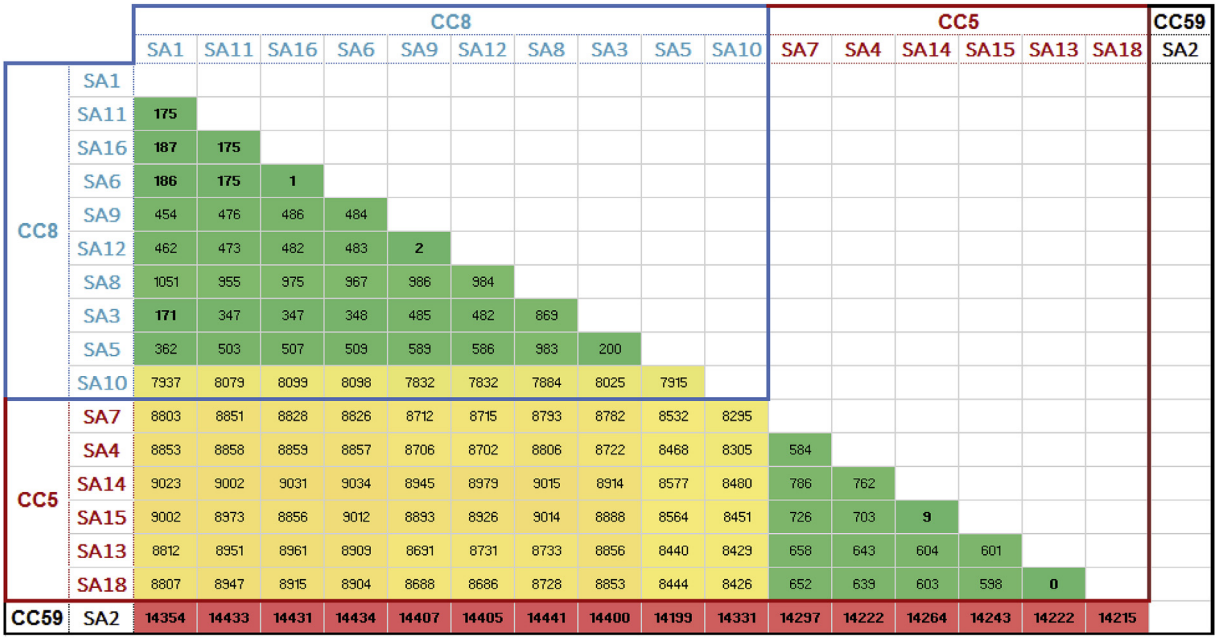

Fig. 2. Isolate pairwise comparison matrix for SNP differences observed for common core genome organized by clonal colony type with color infill indicating degree of difference from minimal (green) to moderate (yellow) to maximum (red).

Table 4
Characterization of CC8 isolates from WWTP samples by MLST type, spa type, and presence of select mobile genetic elements.

CC8								
Date	Location		Isolate	Typing		Select MGE		
	WWTP	Site	ID	MLST	*spa*	*mecA* presence	*mecA* Type	PVL presence
02.10.2017	TB	Influent	SA1	ST8	t1578	(+)	IVa	(+)
	TB	Influent	SA3	ST1750	t008	(+)	IVa	
	WH	Influent	SA5	ST1750	?	(+)	IVa	
	WH	Influent	SA6	ST8	?	(+)	IVa	
02.16.2017	WH	Sedimentation tank	SA8	ST8	t6671			
02.24.2017	WH	Influent	SA9	ST2642	t024	(+)	IVb	(+)
	WH	Influent	SA12	ST2642	t008	(+)	IV	(+)
	WH	Influent	SA10	ST72	?	(+)	IVa	
	WH	Influent	SA11	ST8	t008	(+)	IVa	(+)
06.26.2017	WH	Sedimentation tank	SA16	ST8	?	(+)	IVa	

? = undefined type.

Table 5
Characterization of CC5 isolates from WWTP samples by MLST type, spa type, and presence of select mobile genetic elements.

CC5								
Date	Location		Isolate	Typing		Select MGE		
	WWTP	Site	ID	MLST	*spa*	*mecA* presence	*mecA* Type	PVL presence
02.10.2017	WH	Influent	SA4	ST5	t002			
02.16.2017	WH	Sedimentation tank	SA7	ST5	T888	(+)	II	
06.26.2017	TB	Influent	SA13	ST5	t002			
	WH	Influent	SA14	ST5	t002			(+)
	WH	Influent	SA15	ST5	t002			
	WH	Influent	SA18	ST5	t002			

3.3. Comparisons of S. aureus sewage isolates to illuminate differences and similarities in presence, persistence, and genetic diversity

Differences in the prevalence for recoverable *S. aureus* sewage isolates in the WWTPs influent was observed. As can be seen in Tables 4 and 5, only a few isolates were found in the inlet to the urban Town Branch WWTP (3 of 17 recoverable) over the 4-month sampling period, and on only 2 of the 4 sampling days were any presumptive *S. aureus* isolates enriched from this urban WWTP. In contrast, the suburban West Hickman WWTP always yielded isolates, and from multiple points across the process.

Isolates of the CC5 and CC8 lineage appeared at both WWTPs (Tables 4 and 5) with little difference in their core genetic sequences (Fig. 2) and housekeeping genes (Table 3), but with some diversity in their *spa* types, and the carriage of the mobile genetic elements *mecA* and PVL (Tables 4 and 5). Select comparisons of isolate characteristics are presented in Table 6. Three characteristics are compared: between isolates; similarity in sources, persistence of a source over the time of study, persistence of an isolate type through the treatment process, and diversity of isolates with respect to *spa* type, *mecA* carriage and type, and PVL carriage.

To determine if the sources impacting the two WWTPs were similar, 5 isolate pair comparisons were considered (Table 6). All pairings included an isolate from Town Branch WWTP compared against an isolate from West Hickman WWTP, that were collected on the same day of study. All 5 isolate pairs had <610 SNP differences between their core genomes, and one pairing (SA13 and SA18) had no SNP differences, suggesting that this pair of isolates were strictly clonal, or may have an epidemiological link as defined by prior study (SNPs below 23 of >2.5 million nucleotides) (Von Dach et al., 2015). This is indicative of an identical source that is spread across the city and collected in the separate sewersheds. All 5 pairs of isolates had the same MLST type. All identifiable *spa* types were the same for the 5 paired isolates, with the same results for *mecA* presence and the type of cassette present. The only differences seen in 3 of the 5 pairings was the additional carriage of the PVL gene. From these results it appears that similar sources are feeding into both sewersheds and are spread across the city regardless of land use, population density, or hospital placement.

Persistence of the isolate types can be noted in the city over time, and through the water treatment processes at one WWTP, West Hickman. Table 6 presents a comparison between two MRSA isolates (SA6 and SA16) found on different days, over 4 months

Table 6
Pairwise isolate comparisons for select conditions based on MLST and spa typing, and carriage and type of MGEs.

Comparison Condition	Isolate Pair by ID #s	Location		Core genome SNP difference	MLST Type	*spa*Type		Similarity in Select MGE Carriage and Type Yes = (+) in both isolates. No = (+) in only one isolate			
		WWTP	Sample Site			Same Type	Type	*mecA* (+)	*mecA* Type	PVL presence	
Similar sources	SA13	TB	IN	0	ST5	Yes	t002				
	SA18	WH	EF								
	SA1	TB	IN	186	ST8	NA	t1578	Yes	IVa	No	(+)
	SA6	WH	IN				?				(−)
	SA3	TB	IN	200	ST1750[a]	NA	t008	Yes	IVa		
	SA5	WH	IN				?				
	SA13	TB	IN	601	ST5	Yes	t002				
	SA15	WH	IN								
	SA13	TB	IN	604	ST5	Yes	t002			No	(−)
	SA14	WH	IN								(+)
persistence over time	SA6	WH	IN	1	ST8	NA	?	Yes	IVa		
	SA16	WH	ST				?				
persistence in WWTP	SA15	WH	IN	598	ST5	Yes	t002				
	SA18	WH	EF								
Diversity	SA9	WH	IN	2	ST2642[a]	No	t024	Yes	IVb	Yes	(+)
	SA12	WH	IN				t008		IV		(+)
	SA14	WH	IN	9	ST5	Yes	t002			No	(+)
	SA15	WH	IN								(−)
	SA4	WH	IN	584	ST5	No	t888	No	(−)		
	SA7	WH	ST				t002		II		

? = undefined type.
[a] Single Locus Variant CC8-ST8.

apart in time, at the same treatment plant, West Hickman. There is only 1 SNP difference between the core genomes of SA6 and SA16, and their MLST classification, *mec*A presence and type, and PVL carriage are the same. These two isolates are homologous and are considered to have originated from the same progenitor cell type. Although separated by 4 months of time in the same sewershed, and present at different points in the WWTP process, one from the influent and one from the final sedimentation tank effluent, this finding suggests that the original progenitor cell type persists, either circulating in the population of the sewershed, or in the treatment plant processes.

Table 6 also shows a comparison between isolates found on the same day, but before and after treatment (SA15 and SA18). While the MLST allelic profile, *spa*, and absence of *mec*A and PVL are consistent between these isolates found at the influent and final effluent of the West Hickman WWTP, there is a difference in the core genome of 598 SNPs, so these MSSA isolates would not be considered cloned from the same progenitor cell. However, the presence of such a similar CC5-ST5 isolate in only 200 mLs of both the influent (raw untreated sewage) and in the fully treated final effluent (after chlorination, at point of release into natural waters) is suggestive of the ability of this type of *S. aureus* to resist treatment, even chlorination, and persist outside of its original host. It is possible that the isolates came from different hosts within the community and the relative point of isolation in the WWTP is not indicative of growth and change by passage through the treatment process. However, persistence in either the community hosts, or the WWTP sludges, would allow for related but diverging strains to circulate through the sewersheds, WWTPs, and upon release, the environment.

Table 6 also presents comparisons that highlight the diversity in the carriage of mobile genetic elements, *spa* types, or SNP differences between *S. aureus* isolates found in the sewersheds and WWTPs. Two MSSA CC5-ST5 isolates were detected in West Hickman influent samples on the same day (SA14 and SA15) with the same *spa* type protein (t002). These two isolates had only 9 SNP differences in their core genome and they were considered to be cloned from the same progenitor cell and typed as MLST CC5-ST5. One isolate (SA14) contained the mobile genetic element PVL while the other isolate (SA15) did not. The presence of these two homologous isolates from the same sample point, isolated at the same time on the same day, with differences in PVL presence, suggests a potential for transference of mobile genetic elements in the population, or in the collection systems of the sewershed where they come in contact with other bacteria carrying these elements. Another isolate comparison (SA9 and SA12) with even less difference between the two core genomes (2 SNP) shows a different type of genetic diversity. The two isolates classified as CC8-ST2642 were found in West Hickman influent on the same day, contained PVL and *mec*A, but had different types of *spa* proteins (t024 and t008). Again, in the influent, on two separate days, homologous clones were isolated that had differences in *spa* protein type denoting diversity in genetic makeup. Another comparison of less closely related CC5-ST5 isolates SA4 and SA7 (584 SNP difference) obtained from the same day of sampling, but from two different WWTP sites, displays even greater genetic diversity, with differences in the *spa* protein type and *mec*A presence, supporting that diversity can be found. Clearly, there is the potential for both MRSA and MSSA to evolve, either in the population hosts, upon passage through the sewershed, or passage through WWTP processes.

There were two occurrences where the *mec*A presence in isolates coming off of the West Hickman sedimentation tank was inconsistent with that of the majority of other influent isolates of that MLST CC group. SA7 was the only member of the MLST CC5-ST5 group of isolates obtained across the February to June sampling period that contained *mec*A and was classified as MRSA instead of MSSA. All other MLST CC5-ST5 isolates were MSSA from the USA100 lineage. SA7 had more than 584 SNP difference when compared against any other MLST CC5-ST5 isolate. The MLST CC5 isolate group was very consistent in *mec*A absence and t002 *spa* type, with the exception of the single SA7 isolate obtained from sampling after the activated sludge treatment processes. The MLST CC8 group of isolates in general was more diverse than the CC5 group, and while most of the CC8 group carried *mec*A, the SA8 isolate from the sedimentation tank effluent did not contain *mec*A, and was the only CC8 isolate classified as MSSA. The SA8 isolate had more differences in the core genome when compared against the other CC8 isolates varying from 869 to 1051 SNP and *spa* type (t6671). Both of these isolates (SA7 and SA8) from the varying CC groups had unique *spa* types when compared to the other group members, adding to the genetic diversity that was seen for these *S. aureus* isolates from sewage that had undergone treatment in the West Hickman plant. The difference in carriage of mobile genetic elements could potentially have happened while passing through the treatment plant, especially in conventional activated sludge processes where the mean cell residence time can range from 3 to 15 days and the microbial mass is dense, replete with bacteriophage, and contains multiple species of bacteria.

4. Discussion

The consistent presence of *S. aureus* in sewage samples from two local wastewater treatment plants in Lexington, Kentucky confirmed the presence of MSSA and MRSA in sewage and effluents from treatment processes in agreement with findings reported by prior studies (Börjesson et al., 2009, 2010; Goldstein et al., 2012; Thompson et al., 2013). However, methicillin resistant *S. aureus* prevalence was lower than expected from direct PCR results of prior study where 68% of pellets from sewage samples across a treatment plant were positive for *mecA* (Börjesson et al., 2009). Of the clones isolated in this study from the 32, 200-mL, sewage samples collected at multiple sites across both WWTPs, and enriched prior to pelleting, streaking, and coagulase and Elisa typing, only 7 were positive for presumptive MRSA (22%) and the majority of these were in the influent samples. Utilizing only influent samples where 5 of 12, 200-mL volumes were positive for presumptive MRSA growth, a Most Probable Number (MPN) of viable presumptive MRSA was estimated by Thomas's formula as 3 organisms/L. The prevalence of MSSA in our study was greater (20 positive of 32, prevalence rate of >62%). Utilizing only influent samples where 8 of 12, 200-mL volumes were positive for growth, the estimated MPN of viable presumptive MSSA in influent was 6 organisms/L. For the chlorinated effluent samples, the estimated MPN of viable presumptive MSSA was 0.4 organisms/L. There was a 93.3% removal of MSSA across the WWTP.

In part, this difference in prevalence was likely due to our method of isolation and enrichment of viable *S. aureus* from liquid sewage samples prior to genetic probing, the suppression of *S. epidermidis* during enrichment, and our use of whole genomic sequencing in combination with PCR for the identification of potential MGEs originating from the co-growth of *S. epidermidis* and other bacteria. Others have investigated bacteria in natural waters with multiplex PCR and found the *mec*A gene present in *P. vulgaris*, *M. morganii*, and *E. faecalis*, and all of these organisms had been grown on selective Baird-Parker agar and had presented appropriate colony phenotypes (Kassem et al., 2008). Our initial evaluation of commercially available selective broths and agars agreed with this study; most of the bacteria that grew in Baird-Parker broth and agar presenting appropriate colony phenotype, were not coagulase positive, and were not presumptive *S. aureus*. The

addition of acriflavine to mannitol salt agar and broth was an improvement, resulting in many fewer total isolates from sewage samples, but more that were presumptive *S. aureus*. The presence of *S. epidermidis* was suppressed in the acriflavine modified broths and agars, which is critical for sequencing. Although *P. mirabilis* was still able to co-grow with *S. aureus* in some of the isolates, with suppression of *S. epidermidis*, the presence of the *mecA* gene was more reliably able to differentiate MRSA from MSSA in environmental samples. Our study showed a need to evaluate reported densities of MRSA with respect to the potential of the methods applied to generate false positives to avoid overestimation of MRSA prevalence.

Recent investigations into hundreds of isolates have documented that *S. aureus* and *S. epidermidis* not only grow together in the same niches on the body, they share over 1,400 genes, an amount that corresponds to over 50% of the average genome complement for either bacteria (Méric et al., 2015). Due to this overlap, and the rapidity with which *S. aureus* evolves and adapts to environmental pressures, it is critical to design genetic detection and classification strategies that avoid utilizing mobile and highly variable regions of the genome like *mecA* (Francois and Schrenzel, 2008), and to utilize more of the genome to corroborate PCR findings. It has been recommended that presumptive *S. aureus* isolate extracts be scanned for the presence of the *femA* gene from *S. epidermidis* to allow accurate discrimination between the two species of bacteria (Francois et al., 2003; Von Dach et al., 2015). It is our advice to other researchers to screen by PCR for *femA*, or another *S. epidermidis* specific marker, prior to further genomic investigation of environmental isolates. In this way one can preclude misclassification and false positive MRSA prevalence that would be associated when classifying an isolate where MSSA is co-growing with *S. epidermidis* containing *mec*A.

The predominant *S. aureus* types isolated were related to the reported types known to circulate in the USA, and were related to the USA300 (MLST-CC-8) and USA100 (MLST-CC5) lineages. Isolate types with very similar genomic makeup were detected in influent throughout the time of study, simultaneously at both WWTPs. This is suggestive of an endemic presence of similar sources of *S. aureus* across the whole city feeding into the WWTPs influent. The presence of the mobile genetic element PVL was only found in influent samples, and its prevalence was greater in the USA300 lineage MLST-CC8 than the USA100 lineage MLST-CC5, which is the same as reported for 366 clinical isolates by others (O'Hara et al., 2016). The PVL prevalence in our USA100 lineage MLST-CC5 influent isolates was greater than reported for clinical samples (25% *vs* 0%) and less in our influent isolates for the USA300 lineage MLST-CC8 (50% *vs* >95%) (O'Hara et al., 2016). This is suggestive of the continuing evolution of these bacteria.

There did not appear to be any positive correlation between the number of hospitals in the sewersheds and *S. aureus* prevalence. Indeed, there appeared to be a negative correlation with the Town Branch sewershed reporting 10 times the number of inpatient days/year, yet having the lowest prevalence of *S. aureus* isolation. It is unknown why WWTPs of similar size, but with dissimilar amounts of waste input from hospital sources, would have a difference in *S. aureus* prevalence, but it is speculated that hospital cleaning products and protocols may be reducing the presence of sewage microbes more effectively than in residential/business areas of the city. The even distribution of the strain types between the sewersheds may be correlated with the large number of hospital associated employees in Lexington; a city of 318,449 people, with a reported ratio of 2.7 acute care hospital beds for every 1000 people, which is above the national average for a regional health center in a sparsely populated regions (2.0 beds for every 1000 people) (Healthcare TDA, 2016). For our area of study, with large numbers of hospital workers travelling between the two sewersheds, presumably carrying different strains of *S. aureus* on or in their person, there appears to be no meaning to the terms hospital- or community-acquired *S. aureus* carriage with respect to the presence of the CC5 and CC8 clonal types of *S. aureus* in sewage.

Based on our findings, it is suggested that researchers utilize more of the *S. aureus* genome, and multiple typing methods to fully capture the changes that occur to *S. aureus* when it is transported through the environment, especially for the purpose of investigating potential evolution in engineered systems like a WWTP. Many typing methods, like PCR, use a small fraction of the genome, yet a more robust characterization can be obtained by looking at the entire genome, and combining *spa* typing with MLST typing, SNP changes of a core genome, and the presence and types of mobile genetic elements. In comparing isolates with high similarity in their core genomes (SNP), we observed diversity in the types of protein A and mobile genetic elements (*mecA*, PVL) present. In contrast to O'Hara et al. (2016), who proposed replacing MLST with *spa* typing due to their overlapping CC typing results, we found *spa* typing combined with MLST and SNP analysis of the core genome useful in highlighting genetic divergence. In some cases, the core genome had limited SNP differences (0–600), and the MLST CC was the same, but changes in *spa* type and carriage of mobile genetic elements outside of the focus regions of MLST and core genome analysis revealed genetic diversity in strain types. This robust approach may be useful to track evolution in the population, sewershed, and WWTP environments.

Based on our results, we propose the use of a combined scheme that incorporates core genome comparison, MLST, *spa* typing, and identification of select mobile genetic elements to investigate environmental evolution in WWTPs. The system we envision applying would consider isolates of *S. aureus* from the inlet of the WWTP and across bioactive processes like activated sludge where biological and bacteriophage density is high, to evaluate genomic changes with an understanding of the relative rates of change for the selected elements in *S. aureus*. Changes in the *S. aureus* core genome and resultant MLST type should change slowly while *spa* and other MGEs will change more quickly in response to environmental pressures. If comparison of *S. aureus* isolates sharing similarity in their core genome and MLST type repeatedly demonstrates changes in the faster evolving genomic elements of these isolates after biological treatment, support for *S. aureus* evolution in the WWTP can be garnered. Utilizing the most commonly isolated MLST-CC type from the influent, and obtaining isolates after biological treatment with similar core genome and MLST types will enable comparison of the change in pathogenic state reflected in changes in *spa* type, and MGEs such as *mec*A and PVL. Detecting alteration in these evolving MGEs among *S. aureus* isolates of the same MLST type could assess if co-culture with other organisms in the WWTP environs results in altered virulence of *S. aureus*. Ramsey et al. (2016) found that co-culture of *S. aureus* with *Corynebacterium* species reduced *S. aureus* virulence. We found two isolates (SA7 and SA8) of the dominant clonal complexes (MLST-CC5 and MLST-CC8) after treatment in activated sludge that exhibited atypical *spa* types (t888 and t6671 respectively) and had more than 500-SNP difference in core genome when compared to the predominant influent isolates, which suggests the evolution of these isolate through biological treatment. However, much more research needs to be done to determine if genetic changes in isolates trend towards a gain or loss of virulence as they move through the treatment process. In our data, isolates recovered after activated sludge treatment differed in their carriage of virulent MGEs when compared to influent isolates of the same clonal colony. Isolate SA7 (CC5) was different from the predominant influent CC5 isolates in *mecA* carriage. Isolate SA8 (CC8) no longer carried *mecA* and PVL

MGEs when compared to WWTP CC8 influent isolates.

There is ample reason to continue to evaluate the impact of biological treatment processes for potential changes in the virulence of pathogenic organisms input into sewersheds, WWTPs, and the role these facilities play in the transmission of evolved pathogens in the environment. Wastewater effluent has allowable numbers of Gram-negative coliforms established by permitting processes, and chlorine-based disinfection processes that are designed to reduce coliforms in wastewater effluents are not as effective against Gram-positive bacteria like *S. aureus*. Therefore, WWTPs can be an anticipated source of *S. aureus* in the environment. The presence of viable *S. aureus* in a small volume (200 mL) of effluent could be extrapolated to the release of 5.5×10^8 organisms each day into the receiving stream assuming a consistent concentration and a single bacterium in the sample volume. In the environment, *S. aureus* has been shown to persist in waters, and sediments longer than coliforms (Levin-Edens et al., 2012), so their presence in the environment could be undetected. In the sediments of the receiving streams where conditions enhance survival, contact could provide transmission to new hosts.

5. Conclusions

It is paramount that we understand the true prevalence, fate, and transport of *S. aureus* through our sewersheds, treatment processes, and streams so that treatment strategies are optimized to protect people and other potential hosts from needless infection and disease. With current information it is difficult to evaluate the exact dimensions of the potential health risks related to engineered sewer systems, especially with methods that do not prevent the growth of closely related bacteria that share the MGEs used to identify MRSA. Our results support the need for more coordinated investigations, based on analyses that provide a robust degree of resolution, prevent false positives, and utilize more of the epidemiological approaches and genetic typing methods than have been applied to these systems in the past. This study has provided new methodology for the enrichment and isolation of *S. aureus* from complex environmental samples that resulted in the identification of two dominant clonal complexes (CC5-hospital related and CC8-community related) in the sewersheds and WWTPs of Lexington, KY. The presence of multiple hospitals feeding into one of the WWTPs did not have an impact on the presence, or type of isolate recovered, as both clonal lineages were isolated from both WWTPs. The presence of both isolates persisted across treatment, although greater than one log removal was observed between the influent and sedimentation tank effluent. For two isolates obtained after treatment, there was genetic diversity observed in the spa type and carriage of MGEs, suggesting that treatment provides an opportunity for evolution and genetic shifts. The isolation of viable *S. aureus* at all sites across a treatment plant, and in fully treated effluent, suggests that WWTPs may well serve as reservoirs, and continuous sources, for the introduction of potentially pathogenic *S. aureus* into our environment.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.04.035.

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