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ORIGINAL PAPER

Characteristics and distribution of plasmids in a clonally diverse set of methicillin-resistant *Staphylococcus aureus* strains

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Abstract The aim of this study was to compare the plasmid contents of methicillin-resistant *Staphylococcus aureus* (MRSA) strains classified into different clonal clusters (CCs). The isolates were collected from 15 Czech hospitals in 2000–2008. Plasmid DNA was detected in 65 (89%) strains, and 33 of them harbored more than one plasmid type. Altogether 24 different types of plasmids were identified, ranging in size from 1.3 to 55 kb. Restriction endonuclease analysis, plasmid elimination, DNA hybridization, and sequencing were used for their further characterization. It has been found that the conjugative, erythromycin resistance and enterotoxin D encoding plasmids are harbored by strains from different CCs. On the other hand, chloramphenicol and tetracycline resistance plasmids, and most of the penicillinase and cryptic plasmids were only detected in certain CCs. Especially, the pUSA300-like plasmids were found exclusively in the USA300 clone strains. The high diversity in plasmid content detected in the study strains implies that plasmids play a major role in evolution of MRSA clonal lineages.

Keywords Methicillin-resistant *Staphylococcus aureus* · Plasmids · Genetic diversity · Antibiotic resistance · Molecular typing

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Introduction

Staphylococcus aureus is a major human pathogen that causes both nosocomial and community-acquired infections that range from superficial skin infection to severe life-threatening systemic diseases. MRSA strains whose defining feature is the staphylococcal cassette chromosome *mec* (SCC*mec*) conferring methicillin resistance have remained an important health problem worldwide. Apart from this cassette, MRSA strains contain other mobile genetic elements (MGEs) such as plasmids, prophages, transposons, and pathogenicity islands, which comprise about 15% of the genome (Lindsay et al. 2006). MGEs carry most of the genes through which staphylococcal strains vary from each other, such as resistance and virulence genes (Skov and Jensen 2009). There is evidence that the distribution of some MGEs between lineages is not random, as there are several natural barriers to horizontal transfer, such as mutual incompatibility of MGEs, varying ability of strains to participate in conjugation or transduction, and the presence of restriction modification systems (RMSs) (Cockfield et al. 2007; Waldron and Lindsay 2006). This is well demonstrated by the differences in the content of prophage- (Goerke et al. 2009), pathogenicity island-, and plasmid-borne virulence genes between *S. aureus* strains belonging to particular CC types (Booth

et al. 2001; Diep et al. 2006a; Holtfreter et al. 2007; Jarraud et al. 2002).

As for the antibiotic resistance genes carriage and dissemination, the most important MGEs are plasmids. Staphylococcal plasmids ranging in size from 1.2 kb to more than 100 kb are classified into three classes according to the size and replication mechanism (Novick 1987, 2008). Based on their mutual incompatibility, the staphylococcal plasmids have been divided into 15 Inc groups (Taylor et al. 2004). Many of the recently sequenced plasmids contain ORFs that have not been described so far, suggesting a wide variety of plasmid-borne genes in *S. aureus*.

The currently available data on the plasmid content of MRSA strains from European countries is limited. Coia et al. (1988) have reported that all the MRSA isolates obtained from UK hospitals harbor plasmids. In contrast, a much lower plasmid content has been detected by others, for example, 38.8% of MRSA strains isolated in a Portuguese hospital were plasmid free (Cristino and Pereira 1989) or, even more, multidrug-resistant hospital-acquired MRSA strains collected from a UK hospital were entirely devoid of plasmid DNA (Caddick et al. 2005).

As for the Central European region, recent studies of the plasmid content of MRSA are missing. So, the aim of this study was to analyze the plasmid content of MRSA strains representative of the general MRSA population currently found in the Czech Republic. We also attempted to determine whether or not there is a correlation between the presence of particular types of plasmids and the clonal lineages of the strains and to suggest possible routes of plasmid dissemination and its limits in the MRSA populations.

Materials and methods

Bacterial strains

More than 140 MRSA strains were collected from clinical microbiology laboratories of 15 hospitals in the Czech Republic between 2000 and 2008 and selected not to include duplicate isolates with identical pulsed-field gel electrophoresis (PFGE) patterns and with the same plasmid content. The final collection consisted of 73 isolates from both patients (66) and hospital environment (7). The strain *S. aureus* USA300 was obtained from Prof. F. Götz (University of Tübingen, Germany).

Genotyping of the strains

The *spa* typing was performed as described previously (Shopsin et al. 1999). The *spa* type determination was carried out using the Ridom SpaType Software v.2.0.3 (Ridom). PFGE was done for all strains using *Sma*I restriction endonuclease (Roche Diagnostics) as described previously (Pantucek et al. 1996). Multi Locus Sequence Type (MLST) CCs were deduced from the Based Upon Repeat Pattern (BURP) grouping of *spa* types (Strommenger et al. 2006) and by comparison with the PFGE patterns using the in-house PFGE database and Bionumerics v6.5 software (Applied Maths). According to the PCR detection of the *sau1hsdS* gene profiles designed by Cockfield et al. (2007), the occurrence of SauI type RMS was determined. Strains were analyzed for the SCC*mec* types (I to VI) by the PCR method as described previously (Milheirico et al. 2007). To assign the strains to the USA300 clone, PCR screening for the arginine catabolic mobile element (ACME) and *lukF*-PV–*lukS*-PV genes was performed as described previously (Diep et al. 2006b). To describe the resulting genotype, the following pattern was used: *spa* type/SCC*mec* type/clone name.

Plasmid analysis

Plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche Diagnostics) according to the manufacturer's protocol, with the exception that bacteria were lysed by lysostaphin (30 μg/ml, 20 min/37°C; Dr. Petry Genmedics, Reutlingen, Germany). Plasmid DNA was digested with restriction endonucleases *Alu*I, *Eco*RI, *Hin*dII, and *Hin*dIII (Roche Diagnostics) according to the manufacturer's recommendations and analyzed by electrophoresis in 1.2% agarose gel (Serva) in 1% TAE buffer at 4.5 V/cm. The Supercoiled Ladder (Sigma-Aldrich) and 2-Log DNA Ladder (New England Biolabs) were used as DNA molecular weight markers. Ethidium bromide staining and UV-irradiation were employed for DNA visualization.

Plasmid size was determined on the basis of restriction fragment length as the average of three independent measurements obtained from three different agarose gel electrophoreses. If there was more than one plasmid type in a strain, we used one of the following approaches (1) One of the plasmids was eliminated and then the remaining plasmid DNA was isolated and cleaved by the appropriate endonuclease; (2) Undigested target plasmid DNA was recovered from the SeaKem GTG agarose gel (Lonza) and then cleaved by the appropriate endonuclease; (3) The complete sequence of one of the small plasmids present in the given strain was determined.

Curing of plasmids

Plasmid curing was done by treating the strains with 0.004% SDS or by incubation at 45°C for 24 h and single colony screening by a replica plate method as described

previously (Sonstein and Baldwin 1972). Loss of plasmids was verified by agarose gel electrophoresis and by PCR using primers specific for the relevant plasmid-borne genes as described below.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were done by the disk diffusion method using the following concentrations of antibiotics per disk (Oxoid): penicillin (10 IU), oxacillin (1 μg), ampicillin (10 μg), erythromycin (15 μg), ciprofloxacin (5 μg), gentamicin (10 μg), clindamycin (2 μg), tetracycline (30 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), and rifampicin (5 μg). The interpretation of results was performed according to the Guidelines of the Clinical and Laboratory Standards Institute (CLSI 2010).

Detection of plasmid-borne genes

Resistance genes were detected by PCR using plasmid DNA as the template. The primers for the detection of the penicillin resistance gene *blaZ* (Martineau et al. 2000), tetracycline resistance gene *tetK* (Ng et al. 2001), aminoglycoside resistance gene *aac(6′)–aph(2″)* (Strommenger et al. 2003), and macrolide-lincosamide resistance gene *ermC* (Martineau et al. 2000) were adopted. The other primers were designed in this study as follows: cat-F (GCGACGGAGAGTTAGGTT) and cat-R (GCCTATCTG ACAATTCCTGA) (positions 1,477–1,890 in NC_002013) for the chloramphenicol resistance gene *cat*; cadA-F (TTATTGGTAAATGGGCAGAGG), cadA-R (ACGAA TGCTTGGGCTGGAG) (positions 9,176–9,629 in GQ900378), cadDX-F (GGATATTAGGTTTATTGGG TT) and cadDX-R (CGCCACAACTTGCTATCGTA) (positions 4,702–4,863 in NC_010063) for the cadmium resistance genes *cadA* and *cadDX*; arsB-F (CCTTTGT GATTTGGCAACC) and arsB-R (CTTGACCATATG TATCGCAGAC) (positions 14,461–14,678 in NC_00 3140) for the arsenite resistance gene *arsB*; and traK-F (ACCCTAGTGGCGAAGTTTA) and traK-R (GATATT TGGCCATTCGTCTAGT) (positions 34,271–34,977 in NC_005024) for the *traK* gene to detect the *tra* region of conjugative plasmids. Each reaction mixture (25 μl) contained 0.2 mM dNTP (Roche Diagnostics), 1.5 mM $MgCl_2$, 1× PCR buffer, 1 U *Taq* DNA-polymerase (Invitrogen Life Technologies), 200 nM of each primer, and 10 ng of template DNA. An initial denaturation of DNA at 94°C for 5 min was followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 45 s), ending with a final extension at 72°C for 4 min. PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

DNA hybridization experiments

To determine whether the *ermC*, *tetK*, and *cat* genes were located on plasmids, undigested electrophoresed plasmid DNA was blotted onto a nylon membrane (Roche Diagnostics) by Southern capillary blotting. The probes used for the detection of these genes were prepared by PCR amplification using the gene-specific primers listed above and plasmid DNA as template. The probe labeling, hybridization experiments and visualization were performed by Dig DNA Labeling and Detection Kit (Roche Diagnostics) according to the suppliers' recommendations.

β-Lactamase assay

The *β*-lactamase production was tested by the nitrocefin disk assay according to the manufacturer's protocol (ERBA-Lachema, Brno, Czech Republic).

Virulence gene analysis and toxin production assay

The most often described plasmid-borne staphylococcal virulence genes, that is, the enterotoxin D gene *sed* and exfoliative toxin B gene *etb*, were determined by PCR as described previously (Johnson et al. 1991; Růžičková et al. 2005). The production of exfoliative toxin B and enterotoxin D was assayed by using the Reverse Passive Latex Agglutination Kits EXT-RPLA and SET-RPLA (Denka Seiken). The leukocidin genes *lukS-PV* and *lukF-PV* were detected by PCR as described previously (Lina et al. 1999).

DNA sequencing

Plasmid DNA was cloned in the vector pBluescript SK(−) (Stratagene). The complete sequencing of three small plasmids was done by the Eurofins MWG Operon Sequencing Department (Ebersberg, Germany). The GenBank/EMBL/DDBJ accession numbers for the new sequence data for plasmids pDLK1, pDLK2, and pDLK3 are GU562624-GU562626.

Results

Genotypic characterization of strains

The genotype characteristics of the plasmids and their respective host strains are provided in Table 1. From the BURP clustering and comparison of the PFGE patterns (data not shown), it was deduced that 17 of 21 *spa* types were clustered into eight clonal clusters, that is, CC1, CC5, CC8, CC22, CC30, CC45, CC80, and ST88, and four were

Table 1 Characteristics of plasmids and their host strains

Plasmid	Size (kb)	Clonal cluster (CC), strain description (number of strains)	Strain antibiogram[i]	Plasmid-borne genes	Similar plasmids (GenBank accession no. and reference)
Penicillinase plasmids	20	CC1, t127/IV/clone (3)	O A	*blaZ*	Not found
	26	CC45, t026/IV/Berlin clone (3)	O E Cli Cip G Cmp	*blaZ, cadDX*, *aac(6′)–aph(2″)*	Not found
	27	CC8, t008/IV/USA300 clone (10)	O E Cip A	*blaZ, cadDX*	pUSA-HOU-MR-like (CP000731; Highlander et al. 2007)
	28	t437/V/clone (2)	O E Cli A T Cmp	*blaZ*	Not found
	30	CC80, t044/IV/clone (1)	O A T	*blaZ, cadA, tetK*	Not found
	30	CC80, t044/IV/clone (2)	O E Cli Cip A T	*blaZ, cadA, tetK*	Not found
	31	CC8, t008/IV/USA300 clone (1)	O E Cip A	*blaZ, cadDX*	pUSA-HOU-MR-like (CP000731; Highlander et al. 2007)
	36	CC22, t223/IV/clone (1)	O A Cot	*blaZ, arsB*	Not found
Conjugative plasmids	38	CC8, t211/IA/Iberian clone (3)	O E Cli Rif Cip G A T	*traK, blaZ, cadDX*	Not found
	38	CC5, t041/I/South German clone (5)	O E Cli Cip G A	*traK, blaZ, cadDX*	Not found
Enterotoxin D plasmids	27.6	CC5, t002/II/clone (1)	O E Cli Cip A	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
	27.6	CC5, t002/V/clone (1)	O A	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
	27.6	CC5, t002/IV/clone (1)	O E Cip A	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
	27.6	CC5, t105/II/clone (1)	O E Cli Cip A	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
	27.6	t003/V (1)	O E Cli Cip A	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
	27.6	t186/IV (1)	O E Cli Cip A T	*blaZ, cadDX*, *sed*	pIB485-like (Omoe et al. 2003)
Tetracycline resistance plasmid	4[a]	CC5, t041/I/South German clone (1)	O E Cli Cip G A T	*tetK*	pT181-like (NC_001393; Khan and Novick 1983)
Erythromycin resistance plasmids	2.4	CC8, t030/IIIA/Czech clone (10)	O E Cli Rif Cip G A T	*ermC*	pDLK1 (GU562624; this study), pT48-like (NC_001395; Catchpole et al. 1988)
	2.4[b]	CC45, t026/IV/Berlin clone (4)	O E Cli Cip G Cmp	*ermC*	pT48-like (NC_001395; Catchpole et al. 1988)
	2.4[c]	CC5, t002/II/clone (1)	O E Cli Cip A	*ermC*	pT48-like (NC_001395; Catchpole et al. 1988)
	2.4[d]	CC5, t002/IV/clone (1)	O E Cip A	*ermC*	pT48-like (NC_001395; Catchpole et al. 1988)
Chloramphenicol resistance plasmid	2.9[b]	CC45, t026/IV/Berlin clone (4)	O E Cli Cip G Cmp	*cat*	pDLK2 (GU562625; this study), pC194 (NC_002013; Horinouchi and Weisblum 1982)
Cryptic plasmids	1.3[e]	CC1, t127/IV/clone (3)	O A	–	pDLK3 (GU562626; this study)
	1.5[f]	CC80, t044/IV/clone (1)	O E Cli Cip A T	–	Not found
	2	CC8, t030/IIIA/Czech clone (4)	O E Cli Rif Cip G A T	–	Not found
	2.2[g]	CC8, t030/IIIA/Czech clone (16)	O E Cli Rif Cip G A T	–	Not found
	3[h]	CC8, t008/IV/USA300 clone (8)	O E Cip A	–	pUSA01-like (NC_007790; Highlander et al. 2007)
	3	CC8, t211/IV/USA300 clone (1)	O E Cip A	–	pUSA01-like (NC_007790; Highlander et al. 2007)
	3.5	CC8, t024/IV/clone (1)	O E T	–	Not found

[a] 4-kb tetracycline resistance plasmid was harbored together with the conjugative plasmid by one of the CC5 strains

[b] 2.4-kb erythromycin resistance plasmid was harbored together with the 2.9-kb chloramphenicol resistance and/or 26-kb penicillinase plasmids by the CC45 strains

[c] 2.4-kb erythromycin resistance plasmid was harbored together with the 27.6-kb enterotoxin D plasmid and 3-kb cryptic plasmid by the CC5 strain

[d] 2.4-kb erythromycin resistance plasmid was harbored together with the 27.6-kb enterotoxin D plasmid by the CC5 strain

[e] 1.3-kb cryptic plasmid was harbored together with the 20-kb penicillinase plasmid

[f] 1.5-kb cryptic plasmid was harbored together with the 30-kb penicillinase plasmid

[g] 2.2-kb cryptic plasmid was harbored together with the 2.4-kb erythromycin resistance plasmid by the CC8 strains

[h] 3-kb cryptic plasmid was harbored together with the 27-kb penicillinase plasmid by the CC8 (t008) strains

[i] *O* oxacillin, *A* ampicillin, *E* erythromycin, *Cli* clindamycin, *Rif* rifampicin, *Cip* ciprofloxacin, *G* gentamicin, *T* tetracycline, *Cmp* chloramphenicol, *Cot* co-trimoxazole

singletons. The most abundant clone was CC8 (*spa* types t008, t024, t030, and t211).

The *sau1hsdS* gene profiles of the strains were estimated to check the assignment of strains to clonal lineages. It was found that the CC types identified based on the *sau1hsdS* gene variants were congruent with those determined according to the BURP clustering. In eight strains, the occurrence of SauI type RMS was not confirmed and thus the strains were considered as non-typeable.

Plasmid content and characteristics of the plasmid types

Plasmid DNA was detected in 89% (65/73) of the strains. The analysis of undigested as well as digested plasmid DNA profiles revealed that 44% (32/73) of strains contained a single plasmid while 45% (33/73) of isolates contained more than one plasmid type (Supplemental Fig. 1a). To distinguish between plasmid types, the *Hin*dIII restriction endonuclease analysis of plasmids (REAP) was performed. For the plasmids that could not be cleaved by *Hin*dIII, the enzymes *Eco*RI, *Alu*I, and *Hin*dII were used (data not shown). According to the REAP patterns, 24 different plasmid DNA profiles were revealed in plasmid-positive strains (Supplemental Fig. 1b), which represented combinations of compatible plasmids. We determined 24 different types of plasmids ranging in size from 1.3 to 55 kb. Eighteen different plasmids characterized in more detail are listed in Table 1.

Penicillinase plasmids

More than half (39/73) of the strains under study contained large plasmids ranging in size from 20 to 55 kb. In 19 strains, large plasmids were present separately, while in another 20 strains, they were found in combination with small plasmids. In all strains containing large plasmids, the presence of the *blaZ* gene coding for β-lactamase was proved and all but five isolates were also producers of β-lactamase.

Five different *blaZ*-positive plasmids were described: (1) A 38-kb conjugative plasmid found in 11% (8/73) of strains, carrying, in addition to the *blaZ* gene, the gene for cadmium resistance *cadD* and the *traK* gene essential for conjugative plasmid transfer. (2) A 27.6-kb enterotoxin D plasmid detected in 8% (6/73) of strains, carrying the *blaZ* and *cadD* genes and also the *sed* gene for enterotoxin D. All six *sed* gene-positive strains were producers of this enterotoxin. The *Eco*RI analysis showed that this plasmid was identical to the typical staphylococcal enterotoxin D plasmid pIB485 described previously (Omoe et al. 2003). (3) A 26-kb penicillinase plasmid found in 4% (3/73) of the strains, harboring, apart from the *blaZ* and *cadD* genes, the *aacA-aphD* gene for gentamicin resistance. (4) A 30-kb penicillinase plasmid present in 4% (3/73) of the isolates, containing, in addition to the *blaZ* and *cadA* genes, the *tetK* gene for tetracycline resistance. (5) A 27-kb penicillinase plasmid detected in 13% (10/73) of the strains, carrying the *blaZ* and *cadD* genes. The in silico comparison of the *Hin*dIII and *Eco*RI REAP patterns showed the similarity of this plasmid to the penicillinase plasmid pUSA-HOU-MR (CP000731). One isolate 07/759 contained some variant of this penicillinase plasmid enlarged by about 4 kb in size. When comparing the resistance genes content and antibiograms of the strains harboring a pUSA-HOU-MR-like plasmid with those containing its variant, no difference was found.

In the derivatives obtained by the elimination of the 38-kb conjugative, 27.6-kb enterotoxin D, 26-kb penicillinase, and 30-kb penicillinase plasmids, changes in the sensitivity to antibiotics and loss of β-lactamase production were revealed. For a 27.6-kb enterotoxin D plasmid-cured derivative, the loss of enterotoxin D production was confirmed by the RPLA test.

Plasmid conferring erythromycin resistance

The *ermC* gene was detected in 31% (17/55) of strains resistant to erythromycin that carried a plasmid. For detailed characteristics, we determined the complete sequence of the erythromycin resistance plasmid from one of the Czech MRSA clone strains, designated pDLK1 (2,402 bp). Searching nucleotide databases using the Blast tools, we found two regions for staphylococcal-specific recombination RSA and RSB on this plasmid in addition to the resistance (*ermC*) and replication genes. Based on the 95% nucleotide sequence homology (100% in the replication region), the plasmid belongs to the group of pT48-like (NC_001395) erythromycin resistance plasmids from incompatibility group Inc12.

Plasmid conferring chloramphenicol resistance

The *cat* gene, which is the most common plasmid-borne determinant of chloramphenicol resistance in *S. aureus*, was detected in the same plasmid type in four strains. To characterize this plasmid type in detail, its complete sequence was determined and was designated pDLK2 (2,908 bp). Based on the 99% nucleotide sequence similarity, the plasmid belongs to the group of pC194-like (NC_002013) chloramphenicol resistance plasmids from incompatibility group Inc8.

Plasmid conferring tetracycline resistance

The typical plasmid-borne gene *tetK* coding for tetracycline resistance was detected on a 4-kb plasmid present in a

single isolate. Based on the high similarity of the *Hin*dIII REAP pattern, this tetracycline resistance plasmid was assigned to the group of pT181-like staphylococcal plasmids.

Cryptic plasmids

In addition to the plasmids described above, 48% (35/73) of MRSA strains contained numerous small plasmids whose phenotypic expression was not recognized. Neither PCR nor subsequent hybridization showed the presence of the *ermC*, *cat*, or *tetK* resistance genes on these plasmids. The size of these plasmids ranged from 1.3 to 3.5 kb. They were the only plasmids in 12 strains, while in the others, they were present along with other plasmids. One of the cryptic plasmids was completely sequenced and named pDLK3 (1,365 bp). A comparative analysis using Blastn showed that the plasmid carries two ORFs (Rep_2 pfam01719, RHH_1 pfam01402) involved in replication and an *oriT* sequence with a transfer function. The replication region has been found homologous to that of the pA1 plasmid (*Lactococcus plantarum*), and the sequence of the single-strand origin *sso* showed a 100% homology with that of the cryptic plasmids pSK3/6 (*S. aureus*). Furthermore, the *nic* site of the *oriT* sequence of pDLK3 was identical to that of the mobilizable *S. aureus* plasmid pC223 (NC_005243). The nucleotide sequence analysis described above revealed that pDLK3 appears to be a mosaic plasmid, which, on account of its own *oriT*, can play a certain role in horizontal plasmid transfer.

Strain genotypes versus plasmid distribution

Furthermore, our study compared the plasmid contents of strains classified into different CCs. Table 1 illustrates that some plasmids were detected in isolates from various *spa*-defined clusters, while others were contained exclusively in strains of a specific CC type, as demonstrated by the following examples.

The 38-kb conjugative plasmid occurred in strains from two different CCs, that is, CC5 (t041/I/South German clone) and CC8 (t211/IA/Iberian clone). All these strains originated from a hospital burn unit where they were isolated in 2000 through 2003.

Similarly, the 27.6-kb enterotoxin D plasmid was detected in strains differing in *spa* type, antibiogram, and PFGE profile. These MRSA strains originated from three different hospitals and belonged to the lineages defined as CC5 (t002/IV, V/clones and t105, t002/II/clone), t003/V/clone, and t186/V/ST88 clone. The above-mentioned data support the possibility of plasmid transfer between MRSA strains belonging to different *spa*-defined clusters, especially between various isolates from CC5.

The small erythromycin resistance plasmid pDLK1 also occurred in strains from three different lineages: CC8 (t030/IIIA/Czech clone), CC45 (t026/IV/Berlin clone), and CC5 (t002/IV, II/clones). The plasmid pDLK1 was always detected together with other plasmids: (1) with cryptic plasmids in isolates classified into CC8, (2) with chloramphenicol resistance (pDLK2) and penicillinase plasmids in isolates from CC45, and (3) with enterotoxin D and cryptic plasmids in isolates belonging to CC5.

On the other hand, the presence of some plasmids was associated with specific clonal lineages. It was revealed that the 27-kb penicillinase and 3-kb cryptic plasmids of strains belonging to USA300 clone are similar to those from the whole genome-sequenced strain USA300-HOU-MR (CP000732), but their distribution varied between USA300 isolates. These strains carried either pUSA-HOU-MR-like penicillinase or cryptic plasmids separately, or both of them together. Similarly, the chloramphenicol resistance plasmid pDLK2 occurred only in strains of CC45 (t026/IV/Berlin clone), which originated from the same hospital. All plasmids mentioned above were unique to particular clones, which indicated their vertical transfer within a clonal lineage.

The strain collection contained eight isolates without extrachromosomal plasmid DNA. The isolates were characterized by unique *spa* types not detected in any plasmid-positive strain. Most of the strains belonged to t037/IIIA/Czech clone.

Discussion

We found that 89% of the MRSA strains analyzed contained one or more extrachromosomal plasmids. As 24 different types of plasmids were detected, a representative set of plasmids of *S. aureus* was obtained. From the available genomic data on *S. aureus* (http://www.ncbi.nlm.nih.gov/genomes/lproks.cg), it follows that 11 (61%) of the 18 MRSA genomes contain one or more extrachromosomal plasmids. Similar data have been reported by Cristino and Pereira (1989) who detected the presence of plasmids in 61% of MRSA isolates. The proportion of MRSA strains containing plasmids found in our study is higher and thus closer to the data of Coia et al. (1988) who have reported 100% of MRSA isolates containing plasmids. Brady et al. (2007) noticed a synergy between successful clones and their plasmid profiles. They suggested that both the genetic background and compatible plasmids contribute to the fitness of *S. aureus* clones. It is surprising that even though the *S. aureus* (MRSA) population dynamically evolves and one clone is replaced by another, many plasmids circulate in the population for dozens of years, as evidenced by sequence analysis of the plasmids pDLK1 and pDLK2 or

analysis of the restriction profiles of the enterotoxin D plasmid, which is suggestive of structural stability and evolutionary significance of these MGEs.

Drug resistance conferred by plasmids mostly corresponded to the antibiograms of the particular isolates. On the other hand, strains from a multiresistant Czech clone (t037/IIIA) (Melter et al. 2003) whose antibiograms showed resistance to a number of antibiotics, only harbored cryptic plasmids or, in 10 strains, a cryptic plasmid together with the erythromycin plasmid. In accordance with the data from the *S. aureus* genome sequencing projects, we assume that the relevant genes are carried on transposons or plasmids integrated in the bacterial chromosome (Holden et al. 2004; Oliveira et al. 2000; Rolain et al. 2009). Curing of the penicillinase plasmids resulted in the loss of β-lactamase production by the strains, but this was not accompanied by a change in their oxacillin and penicillin resistance. Their resistance to β-lactamase antibiotics is therefore probably caused by the *mecA* gene on the SCC*mec* cassette (Hiramatsu et al. 1990).

Surprisingly, about half of the strains carried small plasmids whose phenotype remained unclear. These most likely cryptic plasmids were present either alone or in combination with other plasmids. To date, many staphylococcal cryptic plasmids have been characterized in nucleotide databases. However, there is a lack of information on the role these plasmids play in nature as well as on the biological advantages they provide to the bacteria. Therefore, further studies are needed to better understand their function.

As far as the correlation between the plasmid content and CC type is concerned, Waldron and Lindsay (2006) have reported that *S. aureus* strains from different lineages exchange DNA at lower rates than do strains of the same lineage, mainly due to the presence of restriction modification systems. This is in accordance with the fact that some of plasmid types in our study were detected in particular CC only. Nevertheless, Corvaglia et al. (2010) in their study of the SauI RMS have pointed out the variability in the HsdS protein sequence between CCs that enable, for instance, plasmid transfer from CC8 strains to CC5 strains but not to CC30 strains. This is consistent with our finding that all three plasmid types occurring across the lineages were first isolated from CC8 strains to be also detected in CC5 strains 2 years later.

Moreover, this study provides evidence that the plasmid content of genotypically related strains may change within relatively short periods of time, as can be inferred from the variability in the plasmids detected in strains of the Czech-, Berlin-, and USA300 clones. The differences in the plasmid content observed between strains of the USA300 clone support the assumption of a rapid evolution of this invasive clone in line with the study of Highlander et al. (2007). Our observations concerning the USA300 clone plasmid content are also consistent with the findings of Kennedy et al. (2010) who have reported that several USA300 isolates contained essentially identical plasmids that support the theory of the recent clonal emergence of the epidemic USA300 strains.

In conclusion, here we showed that plasmids represent a significant part of the MRSA genomes that contribute considerably to the resistance to various clinically used antibiotics and also to the virulence of the strains via the *sed* gene dissemination. We can also conclude that isolates from various CCs and their individual subclones currently found in the Czech Republic clearly differ in the plasmid content and that certain types of the plasmids were associated with particular CC types. However, there are also plasmids that occur across the lineages. The presence of so many different plasmids in various combinations in MRSA strains implies that they play an important role in the evolution of MRSA clonal lineages and their genotypic diversification.

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