Subtyping of ST22-MRSA-IV (Barnim epidemic MRSA strain) at a university clinic in Germany from 2002 to 2005

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Emergence of the meticillin-resistant *Staphylococcus aureus* (MRSA) Barnim epidemic strain (ST22-MRSA-IV) was demonstrated recently at University Hospital in Magdeburg, Germany. To aid the study of transmission events, it is important to have an epidemiological typing method with the ability to distinguish among MRSA isolates. The aim of this study was to determine the ability of phenotypic and genotypic methods to type ST22-MRSA-IV strains within a hospital for microevolution events. Forty-two ST22-MRSA-IV strains collected from 2002 to 2005 were analysed using antimicrobial testing, toxin gene analysis, PFGE, *spa* typing, fluorescent amplified fragment length polymorphism (fAFLP) and determination of staphylococcal interspersed repeat units (SIRUs). Four different antimicrobial patterns were observed. The majority of the isolates ($n=31$) were resistant towards erythromycin, ciprofloxacin and clindamycin, in addition to penicillin and oxacillin. All strains harboured the *sec* gene and showed a homogeneous profile of toxin genes. One isolate was typed as *spa* t022, two as *spa* t474 and the remainder belonged to *spa* type t032. PFGE yielded eight profiles and SIRU typing resulted in six different patterns. The fAFLP technique subdivided the individual PFGE profiles, but the grouping of isolates differed from that obtained by PFGE or SIRU typing. These results showed a diversity of ST22-MRSA-IV strains within a narrow clinical setting, indicating microevolution of the Barnim MRSA clone. The ability to distinguish among MRSA strains within an endemic setting will lead to a greater understanding of the transmission of MRSA and is necessary to be able to control the spread of various clones.

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INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA) emerged in the 1960s and is now commonly seen in hospitals, clinics and, since the mid-1990s, in the community. Epidemic MRSA strains in hospitals are usually clonal in origin. There are only a few pandemic MRSA clones, the so-called Iberian, Brazilian, Hungarian, New York/Japan, paediatric, and epidemic MRSA (EMRSA)-15 and -16 clones, named after the geographical area where they were first identified or

according to epidemiological characteristics. The strains of these successful lineages are responsible for most hospital-acquired MRSA infections all over the world today (Enright *et al.*, 2002; Robinson & Enright, 2003). In this regard, the Barnim EMRSA strain (ST22-MRSA-IV) in Germany is ancestral to the UK EMRSA-15 strain (Murchan *et al.*, 2003; Witte *et al.*, 2001).

In a previous study, we analysed 199 MRSA isolates from patients at University Hospital in Magdeburg, Germany, and the rehabilitation and chronic care facility (RCCF) in Flechtingen, Germany (Ghebremedhin *et al.*, 2005) and observed the emergence of the Barnim EMRSA ST22 strain. An understanding of the genetic microevolution and clonal spread of strains is necessary to understand the transmission of MRSA and to control its spread. Phenotypic typing methods are mostly restricted to the resistance pattern of the respective MRSA strains (Berger-Bächi, 2002). At present, there are numerous typing methods available, but there is no consensus regarding which method is the best to apply.

Abbreviations: CIP, ciprofloxacin; CLI, clindamycin; EMRSA, epidemic meticillin-resistant *Staphylococcus aureus*; ERY, erythromycin; fAFLP, fluorescent amplified fragment length polymorphism; FAM, 6-carboxy-fluorescein; FUS, fusidic acid; GEN, gentamicin; LIN, linezolid; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; OXA, oxacillin; PEN, penicillin; PVL, Panton-Valentine leukocidin; RCCF, rehabilitation and chronic care facility; RIF, rifampicin; SCCmec, staphylococcal cassette chromosome *mec*; SIRU, staphylococcal interspersed repeat unit; SXT, trimethoprim/sulfamethoxazole; TEI, teicoplanin; TET, tetracycline; VAN, vancomycin; VNTR, variable-number tandem repeat.

For epidemiological typing and for studying the dynamics of MRSA evolution in a hospital environment, knowledge of both strain identity and variability within the strain allow us to make judgements on whether direct cross-infection or independent acquisition has taken place. Thus, we need slowly [e.g. multilocus sequence typing (MLST), *agr*, SCC*mec* typing] as well as more rapidly [e.g. PFGE, fluorescent amplified fragment length polymorphisms (FAFLP), staphylococcal interspersed repeat units (SIRUs), *spa* typing] evolving genetic markers to study hospital-acquired MRSA. Methods that index neutral variation, which accumulates slowly in the genome, are better suited to answering global epidemiological questions.

PFGE is a highly discriminatory typing method that is well suited to the investigation of microevolution and recent transmission within a hospital and within a narrow time schedule. However, it has several limitations, including ease of reproducibility and interpretation of band patterns, even when using standardized methods (Murchan *et al.*, 2003). Guidelines and interpretative criteria for the analysis of discrete sets of isolates obtained during outbreaks have been proposed, but these are not appropriate for the analysis of large populations of organisms collected over extended periods. Somehow, PFGE is too discriminatory for studies assessing long-term epidemiology, as factors that may dramatically alter macrorestriction banding profiles (such as insertions, deletions and inversions) may obscure similarity among epidemiologically related isolates (Tenover *et al.*, 1995).

MLST is carried out by sequencing an internal fragment of seven unlinked housekeeping genes. Allelic profiles can be determined, and a clone is defined as a group of isolates having a strictly identical sequence for all seven genes (Enright *et al.*, 2000). MLST examines the slowly evolving genomic core and is a useful tool for defining chromosomal ancestry (Enright *et al.*, 2000). Although MLST is useful for defining evolutionary events, it does not provide a good marker for microevolutionary events within a hospital (Urwin & Maiden, 2003).

Variable-number tandem repeats (VNTRs) have been used as markers for the strain typing of various bacteria (Francois *et al.*, 2005; Hawkey *et al.*, 2003; Onteniente *et al.*, 2003; van Belkum, 1999). Determination of the number of repeats at each locus produces a digital profile, providing a highly portable typing method allowing comparisons between laboratories. Hardy *et al.* (2006) previously described the presence of VNTRs in staphylococci. Seven VNTRs, termed SIRUs, distributed around the genome are described, occurring in both unique and multiple sites and varying in length from 48 to 159 bp.

fAFLP is a DNA fingerprinting method based on restriction cutting of DNA and stringent PCR amplification of the resulting fragments. fAFLP analysis represents a highly discriminatory and reproducible tool for subtyping genetically homogeneous genomes and identifying outbreak

genotypes within bacterial genera (Desai *et al.*, 1998; Hookey *et al.*, 1999; Nair *et al.*, 2000; Thyssen *et al.*, 2000).

The *agr* locus regulates the production of most staphylococcal exoproteins, including exoenzymes, toxins, surface proteins and other virulence factors, by means of a density-dependent autoinducible signal transduction system driven by a short, post-translationally processed peptide (Ji *et al.*, 1995, 1997).

The *mecA* gene encoding the alternative penicillin-binding protein (PBP2a) is found on the chromosome in a genomic island designated staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.*, 2000). At least five major varieties of SCC*mec* exist and are defined by the specific *mec* locus present (composed of *mecA* and its two regulatory genes, *mecI* and *mecRI*), as well as the variety of site-specific recombinase genes present (either *ccrAB* or *ccrC*) (Katayama *et al.*, 2000; Ma *et al.*, 2002). SCC*mec* types I–III are larger elements (34–67 kb) that tend to contain resistance determinants in addition to *mecA* and are found more frequently in hospital-acquired MRSA strains (Katayama *et al.*, 2000; Ito *et al.*, 2004). In contrast, our ST22-MRSA strains harboured SCC*mec* type IV, which can be subtyped again into IVa–d (Okuma *et al.*, 2002; Oliveira & de Lencastre, 2002), now increasingly recognized as causative agents of community-associated disease in patients that are not affiliated with a hospital or healthcare environment (Zetola *et al.*, 2005).

In the present study, we conducted an analysis of ST22-MRSA-IV isolates to evaluate and compare widely used molecular techniques, namely, MLST, SCC*mec* typing and subtyping, fAFLP and PFGE, for their suitability and usefulness in the typing and fingerprinting of bacterial isolates and in tracking clonal evolution.

METHODS

Bacterial isolates, isolate identification and antibiotic susceptibility testing. MRSA strains were isolated and identified from various clinical specimens of patients from different departments of University Hospital in Magdeburg, Germany, and the RCCF in Flechtingen, Germany, obtained from August 2002 to April 2005. Identification and antibiotic susceptibility testing [penicillin (PEN), oxacillin (OXA), trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), erythromycin (ERY), clindamycin (CLI), moxifloxacin, gentamicin (GEN), vancomycin (VAN), teicoplanin (TEI), linezolid (LIN), quinopristin/dalfopristin, fosfomycin, fusidic acid (FUS), nitrofurantoin, norfloxacin, ciprofloxacin (CIP), levofloxacin, rifampicin (RIF) and tobramycin] were performed using an automated VITEK 2 system (bioMérieux). The results were interpreted in accordance with current National Committee for Clinical Laboratory Standards guidelines: breakpoints for oxacillin susceptibility used were an MIC of 2 mg l⁻¹ indicating susceptibility and an MIC of 4 mg l⁻¹ indicating resistance, as described previously (Ghebremedhin *et al.*, 2005).

DNA extraction. Strains were grown on brain heart infusion agar or in brain heart infusion broth at 37 °C overnight. Genomic DNA used as target for all molecular methods was extracted using a Qiagen DNA extraction kit according to the manufacturer's suggestions with

the modification that 30 µg lysostaphin ml⁻¹ and 1 mg lysozyme ml⁻¹ were added at the cell-lysis step. The concentration of DNA was estimated spectrophotometrically (Ghebremedhin *et al.*, 2005).

MLST. MLST was carried out as described by Enright *et al.* (2000). Analysis of the allelic profile of *S. aureus* isolates was performed using the database of the MLST home web page (<http://www.mlst.net>), where seven numbers depicting the allelic profile were assigned that defined the MLST type.

PFGE. All isolates were subjected to PFGE analysis of *Sma*I-digested chromosomal DNA, which was performed as described by Witte *et al.* (2004). Fragments of sizes in the range of the ladder (50–1000 kb) were included in the analysis. The criteria for PFGE pattern analysis state that up to three PFGE fragment differences compared with the prototype pattern B1 indicate a close relationship and thus that the isolate is probably a subclone of the prototype (Tenover *et al.*, 1995).

PCR for SCCmec IV subtyping. Analysis of SCCmec type IV was performed as described by Oliveira & de Lencastre (2002). For differentiation between the SCCmec types IVa and IVb, we used the protocol of Okuma *et al.* (2002).

agr group-specific multiplex PCR, and Panton–Valentine leukocidin (PVL) gene and toxin gene detection. Extracted genomic DNA was used as a template to amplify specific *agr* alleles (GenBank accession nos X52543, AF001782, AF001783, AF288215, Z49220, AF346724 and AF346725). For multiplex PCR, one primer set was prepared to amplify the four specific *S. aureus agr* alleles using the primers described by Lina *et al.* (2003). Details are given in Ghebremedhin *et al.* (2005). The primers *agrB* and *agrC* were used to amplify the variable part of the *agr* operon (1060 bp) with primer annealing conducted at 50 °C (Strommenger *et al.*, 2004).

The genes *sea*–*see*, *seg*–*sej*, *tsst-1*, *eta*, *etb*, *hlgA*, *hlgC/B*, *lukE/D* and *lukS-lukF-PV* (*lukPV*) were detected by PCR, as described previously (Ghebremedhin *et al.*, 2005).

spa gene typing. The procedures described by Harmsen *et al.* (2003) were used for sequencing and analysis of the *spa* gene of all of the MRSA isolates.

fAFLP analysis. fAFLP analysis was performed as described by van den Braak *et al.* (2004). Briefly, 200 ng chromosomal DNA was digested with *Eco*RI and *Mse*I (both from New England Biolabs) at 37 °C. The DNA fragments were ligated using double-stranded *Eco*RI and *Mse*I adapters for 2 h at 37 °C. A pre-selective PCR with adapter-specific primers *Eco*RI (5'-GACTGCGTACCAGCTT-3') and *Mse*I (5'-GATGAGTCTGATCGC-3') was then performed. An aliquot was subjected to a selective PCR in which one of the primers contained additional nucleotides (primer *Mse*I+CC or primer *Mse*I+AC) at the 3' end, whilst primer *Eco*RI was labelled with 6-carboxyfluorescein (FAM) at the 5' end. PCR amplifications were performed with a Perkin-Elmer model 2400 thermocycler using the following protocol: an initial step of 72 °C for 2 min, 20 cycles of 94 °C for 1 s, 56 °C for 30 s and 72 °C for 2 min, and a final extension step at 72 °C for 7 min. PCR products were stored at –20 °C until analysis of the fAFLP using an automated gel electrophoresis and detection system.

fAFLP fragments were analysed on an ABI Prism 3100 DNA sequencer by adding 2 µl of the PCR product to 24 µl deionized formamide. To assess the size of the obtained fragments accurately, 1 µl GeneScan-500 marker (Applied Biosystems) labelled with the red fluorescent dye 6-carboxy-x-rhodamine was used as internal size standards.

The raw data from the GeneScan were converted to .ab1 format for analysis using BioNumerics software (version 3.0; Applied Maths).

Cluster analysis of the fAFLP patterns was performed with the Dice similarity coefficient using optimization at 1 % and position tolerance and change towards the end of the fingerprint at 1 %. Dendrograms were calculated using the unweighted pair-group method with arithmetic mean (UPGMA).

Strains were analysed individually by calculating the number of isolates that clustered together with the number of unique isolates to give a crude measure of the heterogeneity of strain populations.

Tandem repeat locus identification (SIRU typing). SIRU typing of the isolates was conducted according to the method of Hardy *et al.* (2004) and interpretation of the results was performed according to Hardy *et al.* (2004, 2006).

Statistical analysis. The Hunter (1990) discriminatory index was used to calculate the level of discrimination of each typing method.

RESULTS AND DISCUSSION

MLST analysis and distribution of ST22-MRSA among different departments at University Hospital

In a previous study, we detected a dominant MRSA clone, designated Barnim ST22-MRSA, at University Hospital, Magdeburg, and the RCCF, Flechtingen. The allelic profile for the sequence type ST22 was 7-6-1-5-8-8-6 (Ghebremedhin *et al.*, 2005). Meanwhile, the number of ST22-MRSA isolates detected increased to 42 within 24 months. ST22-MRSA isolates were most prevalent in the Department of Dermatology (45.2 %) and at the RCCF in Flechtingen (21.4 %), and 14.3 % of the MRSA isolates were found in distinct ICUs at University Hospital. The remaining strains were distributed among the different departments. The individual numbers of ST22-MRSA isolates for each department over the 24 month study period are given in Table 1.

MLST has been applied to define the evolutionary relationships of MRSA on a worldwide basis. Although it is useful for analysing evolutionary events, it does not provide a good marker for microevolution events within a hospital (Enright *et al.*, 2000). A combination of MLST with SCCmec typing permits the unambiguous assignment of collections of MRSA isolates to known or new MRSA clones (Enright *et al.*, 2000; Grundmann *et al.*, 2002). Interestingly, in our study, all analysed isolates carried SCCmec subtype IVa (see below), which defined this epidemic MRSA primarily as a regional clone (Shore *et al.*, 2005).

SCCmec typing and subtyping

To study additional differences among the isolates according to SCCmec IV, subtype analysis was performed by PCR. SCCmec subtypes could be determined for all 42 ST22-MRSA strains by PCR and were found to be type IVa.

spa typing

The *spa* gene of *S. aureus* encodes protein A and was used for typing of the 42 ST22-MRSA-IV isolates using Ridom

Table 1. Characterization of ST22-MRSA strains by analysis of antimicrobial resistance profile, toxin gene profile, *spa* type, SIRU profiles and fAFLP fingerprints

Strain	Lab.	No.	Date	Clinic	Antibiotyp [*]	<i>spa</i>	PFGE	SIRU profile	fAFLP + AC	fAFLP + CC	Toxin profile [†]
23	VA	22489	29.08.02	Endocrinology	Type II	t032	B1	1403h-2	I	II	C
1	BK	6474	30.12.02	Cardiothoracic surgery	Type I	t032	B1	1403h-2	III	I	C
15	VA	171	03.01.03	Dermatology	Type I	t032	B1	1303h-2	III	I	A
20	VA	766	10.01.03	Dermatology	Type I	t032	B1	1403h-2	I	I	A
7	VA	2326	28.01.03	Dermatology	Type I	t032	B1	1403h-2	II	II	A
8	VA	2501	30.01.03	Dermatology	Type I	t032	B1	1403h-2	II	II	A
2	VA	2722	03.02.03	General surgery	Type I	t032	B1	1403h-2	III	II	A
14	VA	3415	11.02.03	Dermatology	Type I	t032	A1	1403h-2	III	I	A
37	VA	3507	12.02.03	Flechtingen	Type I	t032	B1	1403h-2	III	II	A
33	VA	3668	13.02.03	Anaesthesiology ICU	Type III	t474	B2	1403b-2	II	I	A
36	UR	1611	14.02.03	Anaesthesiology ICU	Type III	t474	B2	1403b-2	II	III	A
10	VA	4055	19.02.03	Dermatology	Type I	t032	A1	1303h-2	III	IV	A
3	VA	4393	23.02.03	General surgery	Type I	t032	B1	1403h-2	II	I	B
38	BK	1061	24.02.03	Flechtingen	Type I	t032	B3	1403h-2	I	II	A
19	VA	4668	26.02.03	Dermatology	Type I	t032	A1	1303h-2	III	I	A
11	UR	2233	04.03.03	Dermatology	Type I	t032	B1	1403h-2	II	I	A
18	VA	5349	05.03.03	Dermatology	Type I	t032	Unique P10	1403h-2	II	I	A
12	VA	6005	13.03.03	Dermatology	Type I	t032	A1	1303h-2	II	I	A
16	VA	6073	13.03.03	Dermatology	Type I	t032	B1	1403h-2	III	III	A
21	VA	6170	14.03.03	Dermatology	Type I	t032	B1	1403h-2	III	III	A
4	VA	6194	15.03.03	General surgery ICU	Type I	t032	B1	1403h-2	IV	II	A
17	VA	6321	17.03.03	Dermatology	Type I	t032	B1	1403h-2	I	I	A
9	VA	6402	18.03.03	Dermatology	Type I	t032	B1	1403h-2	I	II	A
13	VA	6861	24.03.03	Dermatology	Type I	t032	B1	1403h-2	III	II	C
35	BK	2742	22.05.03	Flechtingen	Type II	t032	B1	1303h-2	III	II	A
26	VA	13140	05.06.03	Dermatology	Type I	t032	B1	1403h-2	II	III	A
24	VA	13844	16.06.03	Dermatology	Type III	t032	B1	1403h-2	I	II	D
25	VA	14172	19.06.03	Dermatology	Type I	t032	B1	1403h-2	I	II	A
27	VA	14829	27.06.03	Otorhinolaryngology	Type I	t032	B5	1603h-2	I	II	A
28	VA	18312	11.08.03	Internal medicine ICU	Type I	t032	B6	1401h-2	III	II	A
29	VA	19482	26.08.03	Internal medicine ICU	Type I	t032	B1	1403h-2	I	II	D
31	VA	20591	08.09.03	Neurology	Type III	t032	B1	1403h-2	II	I	A
34	VA	20537	09.09.03	Flechtingen	Type II	t032	B3	1403h-2	I	II	A
32	VA	20728	10.09.03	Cardiology	Type III	t032	B1	1403h-2	III	III	C
5	VA	24851	02.11.03	Flechtingen	Type I	t032	B1	1403h-2	II	I	A
30	VA	25089	05.11.03	Anaesthesiology ICU	Type III	t032	B7	1403h-2	III	I	B
6	BK	5955	14.11.03	Flechtingen	Type I	t032	B1	1403h-2	III	III	B
22	VA	27735	05.12.03	Dermatology	Type I	t032	B1	1403h-2	I	II	B
39	BK	825	24.02.05	General surgery	Type I	t032	B1	1403h-2	II	II	A
40	BK	860	26.02.05	Flechtingen	Type I	t022	B1	1403g-2	I	II	A
41	VA	6266	31.03.05	Flechtingen	Type I	t032	B1	1403h-2	III	II	A
42	VA	6928	11.04.05	Flechtingen	Type I	t032	B1	1403h-2	III	I	C

*I, PEN-OXA-CIP-CLI-ERY; II, PEN-OXA-CIP-ERY; III, PEN-OXA-CIP.

†A, *sec*, *seg*, *sei*, *hlgA*, *hlgC/B*, *lukD/E*; B, profile A + *sea*; C, profile A + *lukPV*; D, A + *sed*.

StaphType software. The majority of the ST22 isolates were typed as *spa* t032 ($n=39$; 92.9%) with the repeat succession 26-23-23-13-23-31-29-17-31-29-17-25-17-25-16-28, two isolates were typed as *spa* t474 (with the repeat succession 26-23-13-23-31-05-17-25-16-28) and one isolate from the

RCCF in Flechtingen was typed as *spa* t022 with the repeat succession 26-23-13-23-31-29-17-31-29-17-25-17-25-16-28 (Table 1). The *spa* type t022 had one deletion in the repeat succession compared with the prototype t032, whereas t474 differed from t032 by one insertion and seven deletions. *spa*

type t474 was reflected by the PFGE profile B2, whilst *spa* type t022 belonged to PFGE prototype B1.

spa typing has been shown to be discriminatory enough for outbreak investigation but also to be efficient for global epidemiological studies. The isolates that belonged to *spa* type t032 revealed a heterogeneous resistance profile, whilst the two isolates with *spa* type t474 shared the same resistance phenotype (antibiotype III) (see Table 1). From this result, we concluded that the ST22-MRSA clone may indeed be heterogeneous. Our data support the previous findings that sequence information applied as MLST or *spa* types is useful for both evolutionary studies and global epidemiological analysis, and to a lesser extent for short-term or local epidemiological analyses (Tang *et al.*, 2000). However, *spa* typing is more discriminatory compared with MLST data.

PFGE pattern analysis

PGFE analysis of the 42 ST22-MRSA strains revealed two different restriction patterns, A and B (distinguishable by four to five fragment bands), with most of the isolates exhibiting pattern B ($n=37$; 88.1 %) (Fig. 1). Within pattern A ($n=4$), we detected no variations. Within the major pattern B, minor variations (up to three bands) allowed their differentiation into the prototype pattern B1 and the variant patterns B2, B3, B5, B6 and B7 according to O'Neill *et al.* (2001). One strain showed a pattern variation not described by O'Neill *et al.* (2001), but fitted PFGE pattern P10 as described by Grady *et al.* (1999). This 'new' pattern type differed by four bands from pattern B1. The prototype B1 was isolated over the time of the study from different departments at University Hospital, whilst A1

occurred exclusively in the Department of Dermatology between February and March 2003. The profile B2 ($n=2$) was isolated from two different patients within 2 days in the Anaesthesiology ICU. Strains exhibiting the profile B3 ($n=2$) were detected at different time points in two specimens that originated from the RCCF in Flechtingen. The additional profiles B5 ($n=1$), B6 ($n=1$) and B7 ($n=1$) were isolated from different departments at University Hospital only once during the study period.

PFGE indicates variations throughout the chromosome and is the most common genotypic method used in reference and clinical laboratories for typing MRSA. As PFGE analyses genetic markers that undergo rapid evolutionary change, it seems a suitable method to monitor microevolutionary events (Bannermann *et al.*, 1995; Peacock *et al.*, 2002). In our study, we used the high discriminatory power of PFGE for subtyping ST22-MRSA-IV isolates from University Hospital, collected over a time period of 24 months. In total, seven different PFGE patterns were detected. The demonstration of different PFGE profiles within the epidemic Barnim MRSA clone in our study illustrates the role that genetic events such as point mutations, insertions and deletions may play in altering PFGE patterns of closely related isolates. The majority of isolates within the B cluster differed by one genetic event from the prototype B1 (B2, B3 and B5). The PFGE pattern B1 is known to be the dominant type strain of ST22-MRSA-IV. For epidemiological typing, knowledge of both strain identity and variability within the strain allows us to make judgements on whether direct cross-infection or independent acquisition has taken place. However, interpretation of PFGE data obtained from a widespread strain such as Barnim MRSA can be problematic with regard to strain variability.

Fig. 1. PFGE of chromosomal DNA *Smal* restriction of the 42 ST22-MRSA-IV isolates. The spectrum of restriction fragments shown ranges from 10 to 700 kb. Strains were classified into eight pulsotypes (see Table 1); the abundant PFGE pattern represents prototype B1 of ST22-MRSA, whereas A1 is represented by strains 10, 12, 14 and 19. Strain 18 belongs to the unique pattern P10, strain 30 to B7, and strains 33 and 36 to B2. Strain 27 represents B5 and strain 28 represents B6, whilst B3 is represented by strains 34 and 38.

Table 2. Correlation among the various typing methods used for subtyping ST22-MRSA-IV isolates

PFGE pattern (<i>n</i>)	Antibiotype* (<i>n</i>)	<i>spa</i> type (<i>n</i>)	SIRU profile (<i>n</i>)
B1 (30)	I (25), II (2), III (3)	t032 (29), t022 (1)	1403h-2 (27), 1303h-2 (2), 1403g-2 (1)
B2 (2)	III (2)	t474 (2)	1403b-2 (2)
B3 (2)	I (1), II (1)	t032 (2)	1403h-2 (2)
B5 (1)	I (1)	t032 (1)	1603h-2 (1)
B6 (1)	I (1)	t032 (1)	1401h-2 (1)
B7 (1)	III (1)	t032 (1)	1403h-2 (1)
P10 (1)	I (1)	t032 (1)	1403h-2 (1)
A1 (4)	I (4)	t032 (4)	1303h-2 (3), 1403h-2 (1)

*I, PEN-OXA-CIP-CLI-ERY; II, PEN-OXA-CIP-ERY; III, PEN-OXA-CIP.

SIRU typing and correlation with PFGE patterns

PCR was performed on the ST22-MRSA isolates to analyse the discriminatory power of the different loci, as described in Methods. Among the 42 isolates, 6 different SIRU profiles (1303h-2, 1401h-2, 1403b-2, 1403g-2, 1403h-2 and 1603h-2) were detected (Tables 1 and 2). The SIRU profile 1403h-2 was the predominant type ($n=32$; 76.2%). Of these 32 strains, 27 belonged to PFGE profile B1, 2 strains belonged to PFGE profile B3 and 1 strain to PFGE profile A1. The B7 strain and the 'unique' (P10) strain also belonged to SIRU type 1403h-2. The SIRU type 1603h-2 ($n=1$) correlated with PFGE profile B5 ($n=1$), SIRU type 1403b-2 ($n=2$) was associated with both strains of PFGE profile B2 exhibiting the *spa* type t474, and SIRU type 1401h-2 ($n=1$) correlated with PFGE profile B6 ($n=1$). SIRU type 1403g-2 ($n=1$) was found to belong to the pulsotype B1 strain but with *spa* type t022. Surprisingly, SIRU profile 1303h-2 ($n=5$) was found within the two major PFGE restriction patterns, B1 ($n=2$) and A1 ($n=3$) (Table 2).

According to Hardy *et al.* (2004), SIRU may be a more appropriate evolutionary marker for studying transmission events compared with PFGE. They showed that the clustering of epidemic strains was the same by SIRU typing and PFGE, but their results indicated that subtyping of EMRSA differed between the two (Hardy *et al.*, 2004, 2006). We have supported and extended their results for the EMRSA-15-derived German epidemic Barnim MRSA clone, ST22-MRSA-IV. The *spa* gene, encoding protein A, contains a tandem repeat unit VNTR sequence, which was identified as SIRU locus 21. Thus, using SIRU analysis we detected three profiles, 1403h-2, 1403g-2 and 1403b-2, which differed from each other only within SIRU locus 21. In our study, variation in copy number and DNA sequence within the repeat units of *spa* was also used as a separate genetic marker (see above). SIRU type 1403g-2 was congruent with *spa* type t022 and SIRU type 1403b-2 was reflected by *spa* type 474. *spa* type t032 was reflected by five different SIRU profiles (1403g-2, 1403h-2, 1303h-2, 1603h-2 and 1401h-2). In accordance with Malachowa *et al.* (2005), we concluded that SIRU analysis provides new perspectives on the variability of

the *S. aureus* genome and may contribute to a better understanding of the genetic relationships among clones. In this regard, it was interesting to compare the SIRU profiles between EMRSA-15 isolates and isolates belonging to the EMRSA-15-derived Barnim clone. Surprisingly, the collection of EMRSA-15 strains described by Hardy *et al.* (2004) and the collection of ST22-MRSA-IV strains from our study shared only three SIRU types, 1303h-2, 1403g-2 and 1403h-2. Moreover, the most dominant SIRU type, 1203h-2, among the EMRSA-15 strains (44.3%) was not present in our study isolates. In contrast, 76.2% of our study isolates showed the SIRU type 1403h-2 in comparison with 25.8% of the EMRSA-15 isolates. Although subtyping of the ST22-MRSA strains led to similar results using PFGE and SIRU analysis, SIRU typing was able to distinguish among isolates with the same PFGE profile and vice versa. This has been described previously for bacteria other than *S. aureus*: Noller *et al.* (2003) demonstrated the ability of VNTRs to distinguish among isolates with the same PFGE profile during an investigation of an *Escherichia coli* O157:H7 outbreak. Isolates previously thought to be part of an outbreak according to PFGE were redefined as being sporadic isolates when investigated using VNTRs. Thus, our data add more information on the microevolution of the ancestral EMRSA-15 from the UK.

Cluster analysis of faFLP data

faFLP was used to subtype isolates of the epidemic ST22-MRSA. The optimum combination of enzymes and primers for analysis of ST22-MRSA had been predicted by modelling faFLP using different primer combinations. The primer combinations that were tested initially (*EcoRI* + A and *MseI*, *EcoRI* + C and *MseI*, *EcoRI* + AT and *MseI*, *EcoRI* and *MseI* + C, *EcoRI* and *MseI* + AC, and *EcoRI* and *MseI* + CC) generated fragments ranging in size from 80 to 400 bp. We decided to conduct faFLP using the selective primers *MseI* + AC or *MseI* + CC and FAM-labelled *EcoRI* due to the discriminatory power revealed by these primer combinations. The presence and absence of fragments in each profile were scored. For reasons of reproducibility, the DNA was reextracted and the samples were again subjected to faFLP.

The fragment profiles from the different DNA extracts of the same isolate were shown to be nearly identical (data not shown).

According to the fAFLP *MseI* + AC and FAM-labelled *EcoRI* fingerprints, analysis of the banding patterns with 1 % optimization and 1 % position tolerance revealed three major clusters and one outlier cluster (Fig. 2). Cluster I ($n=12$) contained the major relatively homogeneous group of isolates and consisted exclusively of proto-pulsotypes. At 95 % similarity, the cluster could be further subdivided into three subclusters, Ia ($n=10$), Ib ($n=1$) and Ic ($n=1$). Cluster II ($n=12$) contained the type strain of the prototype B1 ($n=8$), and A1 ($n=1$), P10 ($n=1$) and B2 ($n=2$) strains, which were obtained from patients from different departments and in different isolation periods. The B2 strains that originated from the Anaesthesiology ICU also grouped in this cluster.

The strains of cluster III ($n=17$), with 66 % similarity, included the majority of the isolates and consisted of prototype B1 ($n=12$), A1 ($n=3$), B6 ($n=1$) and B7 ($n=1$).

Cluster IV ($n=1$) included only one B1 strain. Cluster IV joined the other major clusters at 75.7 % similarity. Thus, A1 strains belonged to two main clusters. In this regard, three isolates of A1 were grouped into cluster III and the remaining one into cluster II.

Using the primer combination *MseI* + CC and FAM-labelled *EcoRI* in fAFLP fingerprints, and using 1 % optimization and 1 % position tolerance, three major clusters and one outlier were detected (Fig. 3). Cluster I ($n=15$) consisted of members of the proto-pulsotype B1 ($n=9$), A1 ($n=3$), B2 ($n=1$), B7 ($n=1$) and unique P10 ($n=1$), whereas the other isolates of B2 grouped into cluster III ($n=6$), which also included the proto-pulsotype B1 isolates ($n=5$). Cluster II ($n=20$) also included the proto-pulsotype B1 isolates ($n=16$), B3 ($n=2$), B5 ($n=1$) and B6 ($n=1$). Thus, the two isolates that were typed as *spa* type t474 with SIRU profile 1403b-2 did not cluster in one group. Within the different clusters, further subdivisions could be performed for the isolates of cluster II with a similarity of 88.2 %. The outlier isolate joined the other clusters with a similarity of 64.7 %.

Grady *et al.* (1999) described fAFLP analysis as a potent method for subtyping MRSA for the purpose of hospital infection control. In the present study, we asked whether fAFLP was applicable to the subtyping of the major endemic MRSA of MLST type ST22. Indeed, fAFLP was able to discriminate the main PFGE patterns. Furthermore, the additional PFGE patterns also grouped in different clusters of the fAFLP fingerprints. Thus, the discriminatory power of fAFLP, as demonstrated in other studies (Desai *et al.*, 1998; Grady *et al.*, 1999; Harmsen *et al.*, 2003; Tang *et al.*, 2000), was supported in our study and indeed seemed to be superior to PFGE. However, both methods were not concordant in terms of discerning clusters of related isolates.

Fig. 2. Dendrogram showing cluster analysis (UPGMA) of the 42 ST22-MRSA-IV isolates based on fingerprints using primers *EcoRI* and *MseI* + AC. Results were generated using BioNumerics software with 1 % optimization and 1 % position tolerance.

Thus, fAFLP analysis can be expected to yield insights into the evolution of MRSA as a species. The fAFLP technique samples the whole genome sequence in a predictable and rigorous fashion by monitoring base substitutions

Fig. 3. Dendrogram generated using BioNumerics software showing cluster analysis of the 42 ST22-MRSA-IV isolates based on fingerprints using primers *EcoRI* and *MseI*+CC, with 1 % optimization and 1 % position tolerance.

accumulating throughout the genome, rather than being based on the fingerprints of a mobile genetic element. This could provide a valuable measure of microevolutionary change, e.g. within one clone such as ST22-MRSA-IV. The discriminatory power of fAFLP is determined by the choice

of restriction enzymes and by the selectivity of the primers used. In case of the two isolates of pulsotype B2, the fAFLP *MseI*+CC and *EcoRI* fingerprints distinguished between these isolates, whereas the fAFLP *MseI*+AC and *EcoRI* pattern banding analysis put both isolates of pulsotype B2 in the same cluster II, which was supported by the SIRU profile pattern and *spa* typing. In summary, genome-sequence-derived fAFLP was broadly congruent with PFGE for typing MRSA. Its resolving power generally appeared to be superior to the other techniques used in this study (Table 3). This is particularly the case where strains contain few genetic discriminatory elements and where fAFLP might be capable of revealing chains of transmission not demonstrable by PFGE.

Analysis of toxin genes and the presence of PVL

We analysed all 42 ST22 strains for the presence of *tsst-1*, *sea-see*, *seg-sej*, the erythrogenic toxin A and B genes *eta* and *etb*, the leukocidin genes *hlgA*, *hlgC/B* and *lukE/D*, and the PVL gene. The results of the toxin gene analysis are given in Table 1. None of the MRSA strains expressed the *eta*, *etb* or *tsst-1* gene. Five ST22 isolates carried the PVL gene. All strains possessed the genes for *sec*, *seg*, *sei*, *hlgA*, *hlgC/B*, and *lukE/D*. In addition to *seg* and *sei*, the ST22-MRSA isolates were found to be positive for one or more additional *se* genes. Thus, in addition to *sec*, four strains were positive for *sea* and two for *sed* (Table 1).

Classical EMRSA-15 is characterized by the production of enterotoxin C (*sec*), although enterotoxin C-negative variants of EMRSA-15 have been reported (O'Neill *et al.*, 2001). In our study, all ST22-MRSA isolates carried the gene for enterotoxin C and showed a homogeneous profile of toxin genes. We observed acquisition of the *sea* gene in four MRSA strains and acquisition of the PVL gene in five cases. It is known that enterotoxin genes are located on mobile elements. In this regard, the staphylococcal enterotoxin A gene is encoded by phage (Betley & Mekalanos, 1985). The additional presence of the *sea* or PVL gene could not be correlated with a specific PFGE pattern profile. All PVL- and *sea*-positive strains belonged to PFGE type B1 with the exception of one *sea*-positive strain, which showed PFGE profile B7. The acquisition of toxin genes on mobile elements can go in parallel with extended antibiotic resistance. In our study, the presence of the *sea* or PVL gene did not change the resistance pattern compared with *sea*- or PVL-negative strains. As expected, the presence of *sea* or the PVL gene did not change the SIRU profile or the respective *spa* type. The fAFLP pattern also was not specifically affected by the acquired toxin genes. In summary, neither of the typing techniques indicated a linkage between genotype and virulence (Hacker *et al.*, 1997).

agr group specificity and *agrB-D-C* sequencing

On the basis of the *agrC* 5' end sequence, all isolates in this study could be grouped into *agr* group I. Sequencing the

Table 3. Discriminatory power of the typing methods used in this study

	Method					
	Antibiotype	PFGE	<i>spa</i> typing	SIRU	AFLP + AC	AFLP + CC
No. of types	3	8	3	6	4	4
Discriminatory power	0.37	0.49	0.14	0.41	0.69	0.64

variable part (*agrB*-D-C) of the *agr* locus revealed no nucleotide changes within this region for any of the ST22-MRSA isolates (data not shown).

Antimicrobial resistance phenotypes of the ST22-MRSA isolates

Initially, we examined the antibiotic resistance phenotypes of all of the ST22-MRSA-IV isolates. All were uniformly sensitive to VAN, TEI and FUS. We found four different resistance phenotypes using a panel of antibiotics (PEN, OXA, SXT, TET, ERY, CLI, GEN, VAN, TEI, LIN, FUS, CIP and RIF). The most common phenotype, PEN-OXA-CIP-ERY-CLI, named antibiotype I, was present in 78.6 % ($n=33$) of the ST22 isolates under study. The resistance pattern PEN-OXA-CIP-ERY, named antibiotype II, was detected in 7.1 % ($n=3$) of the isolates, whereas the resistance pattern PEN-OXA-CIP (antibiotype III) was involved in 14.3 % ($n=6$) of the isolates. As is apparent from Table 1, the most prominent resistance phenotype, PEN-OXA-CIP-ERY-CLI, was predominant in the Department of Dermatology, University Hospital.

Antibiotype I was observed throughout the study period, whereas antibiotype II was only found in the ST22-MRSA isolates in February 2003. Antibiotype III was characteristic of the ST22-MRSA isolates that were obtained between mid-June and November 2003. However, there was no correlation with PFGE pattern, SIRU typing or fAFLP profile, or with toxin profile (Table 2).

With regard to the strains under study, we detected three different antibiotypes, PEN-OXA-CIP, PEN-OXA-CIP-ERY, PEN-OXA-CIP-ERY-CLI. In 1996, the Barnim epidemic MRSA first appeared in German hospitals with the resistance phenotype PEN-OXA-ERY-CIP-CLI. The majority of the studied strains with PFGE profile B1 (80 %) and all with profile A1, the major restriction patterns of ST22-MRSA-IV, showed the original resistance pattern. Thus, 20 % of PFGE prototype B1 ($n=6$) and some variants of prototype B1, namely B2, B3 and B7, showed a narrowed resistance pattern compared with the main Barnim MRSA strains. Thus, we did not see a strict concordance between antibiotic susceptibility pattern and PFGE pattern. Changes in DNA content due to deleted resistance genes may be too marginal to change the pulsotype (Lipsitch, 2001). This also applies to SIRU typing, *spa* typing and fAFLP analysis.

Discriminatory power of the typing methods

Reproducibility was satisfactory with all phenotypic and genotypic typing methods. In accordance with Hunter (1990), a calculator (<http://insilico.ehu.es/>) was used to determine the discriminatory power of each typing technique used in the study. The most discriminatory methods seemed to be PFGE and fAFLP, followed by SIRU typing and antibiotic resistance phenotyping. The results are given in Table 3. Although there was concordance in general between the different typing methods, it seemed that the more methods used, the more confusion there was in terms of typing. Due to the fact that each method of typing looked at different genetic markers, no single typing technique was really able to decide which was an outbreak and which was an endemic MRSA strain. Nonetheless, to identify microevolutionary events, we propose a combination of two partially contradictory approaches, classification using the most stable markers by MLST and *spa* and SIRU typing, and subtyping of the bacterial organism by PFGE and fAFLP, in order to yield maximal information about bacterial dissemination.

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