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Research paper

A novel multidrug-resistant PVL-negative CC1-MRSA-IV clone emerging in Ireland and Germany likely originated in South-Eastern Europe

Megan R. Earls[a], Anna C. Shore[a], Gráinne I. Brennan[b], Alexandra Simbeck[c], Wulf Schneider-Brachert[c], Teodora Vremeră[d], Olivia S. Dorneanu[d], Peter Slickers[e,f], Ralf Ehricht[f,g], Stefan Monecke[f,g,h], David C. Coleman[a,*]

[a] Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, Trinity College, University of Dublin, Dublin, Ireland
[b] National MRSA Reference Laboratory, St. James's Hospital, James's Street, Dublin, Ireland
[c] Institute for Clinical Microbiology and Hygiene, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
[d] Microbiology Unit, Department of Preventive and Interdisciplinary Medicine, University of Medicine & Pharmacy "Grigore T Popa", Iaşi, Romania
[e] Abbott (Alere Technologies GmbH), Jena, Germany
[f] InfectoGnostics Research Campus, Jena, Germany
[g] Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Straße 9, 07745 Jena, Germany
[h] Institute for Medical Microbiology and Hygiene, Medical Faculty "Carl Gustav Carus", Technical University of Dresden, Dresden, Germany

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ABSTRACT

This study investigated the recent emergence of multidrug-resistant Panton-Valentine leukocidin (PVL)-negative CC1-MRSA-IV in Ireland and Germany.

Ten CC1-MSSA and 139 CC1-MRSA isolates recovered in Ireland between 2004 and 2017 were investigated. These were compared to 21 German CC1-MRSA, 10 Romanian CC1-MSSA, five Romanian CC1-MRSA and two UAE CC1-MRSA, which were selected from an extensive global database, based on similar DNA microarray profiles to the Irish isolates. All isolates subsequently underwent whole-genome sequencing, core-genome single nucleotide polymorphism (cgSNP) analysis and enhanced SCC*mec* subtyping.

Two PVL-negative clades (A and B1) were identified among four main clades. Clade A included 20 German isolates, 119 Irish isolates, and all Romanian MRSA and MSSA isolates, the latter of which differed from clade A MRSA by 47–130 cgSNPs. Eighty-six Irish clade A isolates formed a tight subclade (A1) exhibiting 0–49 pairwise cgSNPs, 80 of which harboured a 46 kb conjugative plasmid carrying both *ileS2*, encoding high-level mupirocin resistance, and *qacA,* encoding chlorhexidine resistance. The resistance genes *aadE*, *aphA3* and *sat* were detected in all clade A MRSA and the majority (8/10) of clade A MSSA isolates. None of the clade A isolates harboured any enterotoxin genes other than *seh*, which is universally present in CC1. Clade B1 included the remaining German isolate, 17 Irish isolates and the two UAE isolates, all of which corresponded to the Western Australia MRSA-1 (WA MRSA-1) clone based on genotypic characteristics. MRSA within clades A and B1 differed by 188 cgSNPs and clade-specific SCC*mec* characteristics were identified, indicating independent acquisition of the SCC*mec* element.

This study demonstrated the existence of a European PVL-negative CC1-MRSA-IV clone that is distinctly different from the well-defined PVL-negative CC1-MRSA-IV clone, WA MRSA-1. Furthermore, cgSNP analysis revealed that this newly defined clone may have originated in South-Eastern Europe, before spreading to both Ireland and Germany.

Abbreviations: CC, clonal complex; cgSNP, core-genome single nucleotide polymorphism; MGE, mobile genetic element; MDR, multidrug-resistant; NJT, neighbour-joining tree; ST, sequence type; SMRT, single-molecule real-time; SCC*mec*, staphylococcal chromosomal cassette *mec*; WA, Western Australia; wgMLST, whole-genome multilocus sequence typing; WGS, whole-genome sequencing

* Corresponding author at: Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental University Hospital, University of Dublin, Trinity College Dublin, Dublin 2, Republic of Ireland.

E-mail addresses: megan.earls@dental.tcd.ie (M.R. Earls), anna.shore@dental.tcd.ie (A.C. Shore), GBrennan@STJAMES.IE (G.I. Brennan), Alexandra.Simbeck@klinik.uni-regensburg.de (A. Simbeck), Wulf.Schneider@klinik.uni-regensburg.de (W. Schneider-Brachert), doravre@yahoo.com (T. Vremeră), odorneanu@yahoo.com (O.S. Dorneanu), peter.slickers@alere.com (P. Slickers), Ralf.Ehricht@leibniz-ipht.de (R. Ehricht), monecke@rocketmail.com (S. Monecke), david.coleman@dental.tcd.ie (D.C. Coleman).

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1. Introduction

Staphylococcus aureus is both a major global pathogen and a prevalent commensal inhabitant of the skin and mucosal membranes of humans and animals (Aires-de-Sousa, 2017; Young et al., 2012). Approximately 25% of the *S. aureus* genome is composed of accessory genes, many of which are horizontally transferable between cells on mobile genetic elements (MGEs) such as plasmids, bacteriophages and pathogenicity islands (Carroll et al., 1995; Lindsay and Holden, 2004). The exact composition of the accessory genome is largely influenced by environmental factors and hence, *S. aureus* is capable of adapting to a wide variety of different hosts and stressful conditions (Lindsay and Holden, 2004; Malachowa and Deleo, 2010). Upon acquisition of the staphylococcal chromosomal cassette *mec* (SCC*mec*) MGE, which encodes either *mecA* or *mecC*, *S. aureus* develop into methicillin-resistant *S. aureus* (MRSA) (Katayama et al., 2000; Shore et al., 2011). MRSA exhibit resistance to almost all beta-lactam antibiotics and infection with multidrug-resistant (MDR) strains further limits treatment options (Assis et al., 2017; Hartman and Tomasz, 1984). Preventing the spread of MRSA is therefore of vital importance.

Global surveillance of MRSA is essential for the identification of international transmission routes and the subsequent development of effective infection prevention and control strategies (World Health Organisation, 2014). Surveillance investigations typically involve the categorisation of MRSA into presumptive clones (i.e. strains that have emerged from a single SCC*mec* acquisition event) and/or sub-clones. Traditionally, putative MRSA clones/sub-clones have been defined using conventional molecular typing techniques such as multilocus sequence typing (MLST), standard SCC*mec* typing, *spa* typing and PCRs for the detection of certain marker genes (Stefani et al., 2012; Coombs et al., 2012, 2014; Grundmann et al., 2014; Shore et al., 2014; Irish NMRSARL, 2016), including those encoding the Panton-Valentine leukocidin (PVL) (Panton and Valentine, 1932). Importantly, however, these methods involve the characterisation of only small subsections of the *S. aureus* genome and thus, their resolution is limited. More recently, whole-genome sequencing (WGS) has become increasingly widespread and the use of techniques such as single nucleotide polymorphism (SNP) analysis has enabled the quantitative comparison of strains with exceptionally high resolution (Earls et al., 2017; Kinnevey et al., 2016; Price et al., 2013). While WGS is generally considered the technical gold-standard during surveillance, a combination of DNA microarray profiling and enhanced SCC*mec* subtyping has also been used to accurately differentiate between clones (Earls et al., 2018; Monecke et al., 2011, 2016). As of 2017, approximately 300 different MRSA clones and 60 different SCC*mec* subtypes had been defined using these techniques (Monecke et al., 2016; *S. aureus* Genotyping Kit 2.0 Arraymate database (Abbott [Alere Technologies GmbH], Jena, Germany)). DNA microarray profiling and enhanced SCC*mec* subtyping can therefore be used to select relevant strains for WGS-based investigations.

To date, only one PVL-negative ST1-MRSA-IV clone has been well-defined in the literature (Monecke et al., 2011). Often referred to as Western Australia (WA) MRSA-1, this CC1 clone was first recovered in Australia in the late 1980s (Udo et al., 1993) and isolates indistinguishable from WA MRSA-1 by MLST, *spa* and array profiling have since been detected in the UAE, Egypt and Europe (Monecke et al., 2011). As suggested by its origin in the community, WA MRSA-1 is not typically associated with multidrug resistance. Specifically, this clone is generally associated with the penicillin resistance gene *blaZ*, the macrolide, lincosamide and streptogramin (MLS) resistance gene *erm*(C) and virulence-associated genes *sak*, *scn*, *sea*, *seh*, *sek* and *seq* (Coombs et al., 2011). The fusidic acid resistance gene, *fusC*, which is carried on a SCC element (SCC*fus*), is also common among WA MRSA-1. Furthermore, WA MRSA-57 and WA MRSA-45, the latter of which also harbours SCC*fus*, have been defined as sub-clones of WA MRSA-1 (Coombs et al., 2011). All three of these variants are typically associated with *spa* type t127 (Coombs et al., 2011). Interestingly, PVL-negative ST1-MRSA-IV-t127 have also been recovered from pigs, cattle, horses, rooks, companion animals and wild boars (Cuny et al., 2015; Loncaric et al., 2013, 2014; Porrero et al., 2013). Notably, however, these animal strains have not been investigated using WGS and their placement into a global context has not yet been established. A PVL-positive ST1-MRSA-IV clone known as USA400 has also been well-defined (Herold et al., 1998). Like WA MRSA-1/45/57, this clone is not typically associated with multidrug resistance.

A recent WGS-based study identified MDR PVL-negative CC1-MRSA-IV as the predominant CC1-MRSA clone among humans in Ireland (Earls et al., 2017). This study determined that a distinct variant of this specific CC1-MRSA-IV clone, which generally exhibited mupirocin resistance conferred by a conjugative *ileS2*-encoding plasmid (p140355), was responsible for a protracted hospital outbreak in Ireland (Earls et al., 2017). These findings were particularly alarming considering both the difficulty associated with treating MDR infections and the importance of mupirocin, which in many countries (including Ireland), is used in combination with chlorhexidine for nasal and body decolonisation, respectively (Irish Department of Health, 2013; Poovelikunnel et al., 2015). The aims of the present study relate to these recent findings. Firstly, this study investigated whether the MDR CC1-MRSA-IV clone identified in Ireland is present elsewhere. This involved comparing the whole-genome sequences and SCC*mec* elements of Irish MDR CC1-MRSA-IV and international isolates exhibiting similar genotypic characteristics to the Irish isolates, as determined by DNA microarray profiling. Secondly, considering that multidrug resistance is not typically associated with human PVL-negative CC1-MRSA-IV, this study investigated whether the MDR CC1-MRSA-IV clone identified in Ireland constitutes a sub-clone of WA MRSA-1/45/57 or a novel clone yet to be formally characterised. This involved (a) comparing the whole-genome sequences and SCC*mec* elements of MDR CC1-MRSA-IV and WA MRSA-1/45/57 and (b) comparing the whole-genome sequences of MDR CC1-MRSA-IV and MSSA isolates exhibiting similar genotypic characteristics to the MDR clone, as determined by DNA microarray profiling. Finally, considering the crucial role of mupirocin as a decolonising agent, this study aimed to further analyse the *ileS2*-encoding plasmid identified in the Irish outbreak variant in order to elucidate the factors driving its selection. To date, ten different *ileS2*-encoding plasmids have been described in staphylococci, six of which are members of the pSK41/pGO1 plasmid family and eight of which encode additional antimicrobial resistance genes (Ho et al., 2016; Pérez-Roth et al., 2010).

2. Materials and methods

2.1. Isolates

The present study included all 139 CC1-MRSA-IV isolates identified at the Irish National MRSA Reference Laboratory (NMRSARL) between 2007 and 2017 (Table 1). Eighty nine of these isolates (recovered between June 2013 and June 2016) underwent *spa* typing, DNA microarray profiling, WGS and MLST as part of the aforementioned study on a protracted hospital outbreak (Earls et al., 2017). During the present study, the remaining 50/139 isolates were identified as CC1-MRSA-IV based firstly, on their exhibiting *spa* types corresponding to CC1 and secondly, on their assignment to CC1-MRSA-IV following *in silico* DNA microarray profiling (see below). All available CC1 MSSA isolates (10 isolates recovered between 2004 and 2017) identified at the Irish NMRSARL based on *spa* typing, were included for comparison to the Irish CC1-MRSA-IV isolates (Table 1). The Irish *S. aureus* isolates included two PVL-positive MRSA and three PVL-positive MSSA isolates, which were included as potentially useful comparator isolates. Thirty-eight international *S. aureus* isolates were also investigated, including 21 MRSA isolates recovered in Germany between 2007 and 2018, five MRSA isolates recovered in Romania between 2010 and 2012, 10 MSSA isolates recovered in Romania between 2009 and 2012 and two MRSA

Table 1
Epidemiological and genotypic data associated with the 167 CC1-MRSA-IV isolates and 20 CC1-MSSA isolates investigated in the present study.

NJT position[a]	Country	MRSA/ MSSA	No. of isolates	Recovery period	Source(s)	Sequence type(s)[b]	*spa* type(s)[c]	SCC element subtypes
Subclade A1	Ireland	MRSA	86	2013–2017	10 hospitals/HCFs Community	ST1	t127 (n = 84) t922 (n = 2)	MW2-like SCC*mec* IVa with *dcs* insertion
Remaining clade A	Ireland	MRSA	32	2007–2017	11 hospitals/HCFs Community	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Germany	MRSA	20	2016–2018	3 hospitals Community	ST1(n = 19) ST4911 (n = 1)	t127	MW2-like SCC*mec* IVa with *dcs* insertion
	Romania	MRSA	5	2010–2012	1 hospital	ST1	t127	MW2-like SCC*mec* IVa with *dcs* insertion
		MSSA	10	2009–2012	1 hospital	ST1 (n = 8) ST4910 (n = 1) ST4912 (n = 1)	t127 (n = 8) t5633 (n = 1) t18248 (n = 1)	SCC-negative
Subclade B1	Ireland	MRSA	17	2012–2017	9 hospitals Community	ST1 (n = 15) ST4913 (n = 1) ST4914 (n = 1)	t127	MW2-like SCC*mec* IVa (n = 9) SCC*mec* IVa/SCC*fus*$_{476}$ (n = 8)
	UAE	MRSA	2	2009	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Germany	MRSA	1	2007	1 hospital	ST1	t127	MW2-like SCC*mec*
Remaining clade B	Ireland	MRSA	3	2014–2016	3 hospitals	ST1	t127	MW2-like SCC*mec* IVa (n = 1) SCC*mec* IVa/SCC*fus*$_{476}$ (n = 2)
		MSSA	6	2004–2017	4 hospitals	ST1	t127	SCC*fus*$_{476}$
Clade C	Ireland	MRSA	1	2016	1 hospital	ST1	t127	SCC*mec* IVa/SCC*fus*$_{476}$
	Ireland	MSSA	2	2013	2 hospitals	ST1	t127	SCC*fus*$_{476}$
Clade D	Ireland	MSSA	2	2017	1 hospital	ST1	t127	SCC-negative

Abbreviations: HCFs, healthcare facilities; NJT, neighbour-joining tree; ST, sequence type.
[a] Based on core-genome single nucleotide polymorphism analysis.
[b] Multilocus sequence typing was performed using Ridom SeqSphere+ version 4.1 (Ridom GmbH). Allelic profiles: ST1, 1-1-1-1-1-1-1; ST4910, 1-1-1-1-40-1-1; ST4911, 1-1-1-1-1-1-649; ST4912, 1-731-1-1-1-1-1; ST4913, 1-1-1-1-1-1-648; ST4914, 1-1-663-1-1-1-1.
[c] *spa* typing was performed either using Ridom SeqSphere+ version 4.1, or as previously described (Earls et al., 2018). *spa* repeat successions: t127, 07-23-21-16-34-33-13; t922, 07-23-21-16-33-13; t5633, 15-13; t18248, 07-23-21-16-34-33-20.

isolates recovered in from the UAE in 2009 (Table 1). These international isolates were selected from a *S. aureus* isolate microarray profile database including approximately 25,000 strains recovered from humans and animals worldwide. Further details relating to this global database and the rationale used to select the international isolates are described below. All available epidemiological data associated with the 187 (149 Irish and 38 international) isolates investigated are detailed in Table S1. Isolates underwent species identification and methicillin resistance detection as previously described (Earls et al., 2018). All isolates were stored at −80 °C on individual Protect Bacterial Preservation System cryogenic beads (Technical Services Consultants Ltd., Heywood, United Kingdom).

2.2. *spa* typing

For the Irish isolates, *spa* typing was performed with genomic DNA extracted using the InstaGene matrix solution (BioRad, München, Germany), according to the manufacturer's instructions. Amplification of the variable X region in the *spa* gene, PCR clean-up, DNA sequencing and *spa* type assignment were performed as previously described (Earls et al., 2018). All international isolates underwent *spa* typing using Ridom SeqSphere+ version 4.1 (Ridom GmbH, Germany) following WGS (see below) (Bletz et al., 2015).

2.3. WGS

All isolates underwent WGS. Genomic DNA was extracted by enzymatic lysis using the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany) and the Qiagen DNeasy blood and tissue kit (Qiagen, West Sussex, United Kingdom). DNA quality was assessed and DNA dilutions were performed, as previously described (Earls et al., 2018). The Nextera XT DNA Library Preparation Kit (Illumina, Eindhoven, The Netherlands) was used according to the manufacturer's instructions and libraries underwent paired-end sequencing using the 500-cycle MiSeq Reagent Kit v2 (Illumina). Libraries were scaled to exhibit at least 50× coverage and the quality of each sequencing run was assured following cluster density and Q30 assessment. All reads were assembled into contigs using SPAdes v3.7.1 (Bankevich et al., 2012).

2.4. Genotyping and SCCmec subtyping using in silico DNA microarrays

The sequences of the hybridization probes utilised in *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany) DNA microarray and a previously described SCC*mec* subtyping array (Monecke et al., 2016) were mapped on the assembled genomes to generate hybridisation patterns comparable to real array hybridisation experiments, as previously described (Monecke et al., 2016). To compare the complement of antimicrobial and virulence-associated genes between groups of isolates, the two-tailed Fisher's exact test was performed using the GraphPad QuickCalcs website: https://www.graphpad.com/quickcalcs/contingency1/ (accessed July 2018). Results were considered statistically significant if $p < .05$. Specific SCC*mec*-related alleles of interest were compared using Clustal Omega (Sievers et al., 2011), while regions of interest within the SCC*mec* element were investigated using the publicly available National Centre for Biotechnology Information (NCBI) BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Details relating to the global S. aureus database

The database was generated using *in vitro* and/or *in silico* versions of the *S. aureus* Genotyping Kit 2.0 (Abbott [Alere Technologies GmbH], Jena, Germany), comprising experimental datasets for ca. 25,000 MRSA and MSSA isolates, as well as *in silico* re-analyses of ca. 3000 published genome sequences. In addition, the aforementioned SCC*mec* subtyping array (Monecke et al., 2016) was applied experimentally to a subset of ca. 2000 MRSA isolates and *in silico*, to all the genome sequences analysed. The global database includes 385 CC1-MRSA-IV isolates recovered in Europe, Australia, the Middle East, the USA and New

Zealand. The vast majority (382/385; 99.2%) of these CC1-MRSA-IV isolates exhibit one of four main genotypic patterns, while those remaining exhibit unusual characteristics (such as SCC pseudoelements or composite SCC elements) and possibly represent sporadic strains or variants/mutants. Three of the four main CC1-MRSA-IV patterns each match the description of previously defined clones (Monecke et al., 2011). These clones are (i) PVL-negative strains with SCC*mec* IV or SCC*mec* IV + SCC*fus* elements, referred to as WA MRSA-1/45/57, (ii) the PVL-positive "USA400" strain and (iii) a PVL-positive strain with a SCC*mec* IV + SCC*fus* composite element that appears to be restricted to Australia and New Zealand. A fourth pattern can be defined that differs from any well-defined CC1-MRSA-IV clones in several markers (see Results). This pattern corresponds to CC1-MRSA-IV previously identified in Ireland (Earls et al., 2017) and Romania (Monecke et al., 2014). Hereafter, this fourth genotypic pattern is referred to as the "undefined" pattern or clone.

2.6. Selection of international isolates

Following *in silico* genotyping, the Irish isolates could be compared to those represented in the global database. The vast majority of Irish MRSA isolates investigated exhibited either the WA MRSA-1/45/57 or the undefined genotypic pattern. Representative (based on country/location of isolation, genotypic variations and/or date of recovery) selections of international MRSA isolates exhibiting each of these two genotypic patterns were selected from the global database for further analysis using WGS. Specifically, one German and two UAE CC1-MRSA-IV isolates exhibiting the WA MRSA-1/45/57 genotypic pattern, and a further nineteen German and five Romanian CC1-MRSA-IV isolates exhibiting the undefined genotypic pattern were selected. A representative selection of international MSSA isolates exhibiting the undefined genotypic pattern (minus the SCC*mec* genes) was also selected from the global database for further analysis using WGS. This included 10 Romanian CC1-MSSA isolates. Importantly, it was noted that under-resourced countries were, despite all efforts, often poorly represented in the database.

2.7. Neighbour-joining tree analysis

The WGS data were analysed using BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium). All isolate genomes underwent whole-genome MLST (wgMLST) using both assembly-free and assembly-based algorithms, as previously described (Earls et al., 2018). This wgMLST scheme consists of 3904 wgMLST loci (Roisin et al., 2016), 1861 of which are core genes (Leopold et al., 2014). A multiple sequence alignment of the concatenated core genes was generated and a core-genome (cg) SNP analysis was performed. cgSNPs were called exclusively in positions shared by all samples. Only cgSNPs with at least 5× read coverage (including 1× coverage in each direction) were considered. Potentially indel-related cgSNPs, occurring within 12 bp of each other, were removed. Positions with ambiguous base calls and cgSNPs in repetitive regions were excluded. Two separate neighbour-joining trees (NJTs) were generated with permutation resampling (1000 replicates) based on the cgSNP analysis. The first NJT included all MRSA isolates only (Fig. S1). This tree was used to confirm the clonality of the isolates exhibiting the undefined genotypic pattern (Fig. S1). The second NJT included all MRSA and MSSA isolates (Fig. 1). This tree was used to determine whether any of the MSSA isolates investigated were closely related to the undefined clone. A distance matrix based on cgSNP differences was also generated. The quality statistics window in BioNumerics was used to assess the quality of the sequence read sets, de novo assemblies, and cgMLST allele calls. Traditional STs were assigned following MLST analysis using Ridom SeqSphere+ version 4.1 (Ridom GmbH).

2.8. Plasmid sequence analysis

A conjugative *ileS2*-encoding plasmid, p140355 (GenBank accession number: KY465818) was previously detected in 50 mupirocin-resistant CC1-MRSA-IV isolates from Ireland, all of which were also included in the present study (Earls et al., 2017). In this previous study, a single mupirocin-resistant CC1-MRSA-IV isolate underwent single-molecule real-time (SMRT) sequencing and the entire *ileS2*-encoding plasmid (p140355) was obtained on a single contig. The sequence reads of the remaining 49 mupirocin-resistant CC1-MRSA-IV isolates from that study were then aligned to the SMRT sequence. In the present study, the sequence reads of an additional 32 *ileS2*-encoding CC1-MRSA-IV isolates were mapped against the p140355 sequence using the Burrows-Wheeler aligner (BWA-mem; http://arxiv.org/abs/1303.3997). The Artemis sequence viewer (https://www.sanger.ac.uk/science/tools/artemis) was used to visually assess the mapping of reads. The genetic organisation of the SMRT-derived p140355 sequence was confirmed by PCR using either Go Taq DNA polymerase (Promega, Madison, Wisconsin, USA) or the Expand Long Template PCR system (Roche Products Ireland Limited, Dublin, Ireland), according to the manufacturers' instructions. The primers used are detailed in Table S2. SnapGene v4.1.9 was used to construct a genetic map of the plasmid (Fig. 2). All open reading frames (ORFs) comprising at least 30 codons were identified and annotated, if possible, using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This database was also used to compare the p140355 sequence with other plasmid sequences of relevance.

2.9. Accession numbers

The contigs of a representative isolate (clade A; A_01; undefined CC1-MRSA-IV genotypic pattern) were submitted to GenBank (accession number: RBVO00000000.1). The sequence read sets of all isolates investigated were submitted to the NCBI Sequence Read Archive database (accession number: PRJNA494507).

3. Results

3.1. Identification of four distinct clades using cgSNP analysis

A total of 2891 core-genome sequence positions exhibited polymorphisms that fulfilled all filtering criteria. Neighbour-joining tree analysis based on these 2891 positions revealed that the 187 isolates investigated grouped into one of four main clades (A, B, C and D; Fig. 1). Clade A included 143 MRSA isolates, 86 of which formed a tight subclade termed subclade A1, and 10 MSSA isolates. Clade B included 23 MRSA isolates, 20 of which formed a loose subclade termed subclade B1, and six MSSA isolates. Clade C included one MRSA and two MSSA isolates, while clade D included two MSSA isolates only.

The clade A MRSA isolates exhibited the undefined CC1-MRSA-IV genotypic pattern, while the subclade B1 isolates exhibited the WA MRSA-1/45/57 genotypic pattern. The clade A MRSA isolates were therefore characterised in detail and compared to the subclade B1 isolates. A single centrally-located subclade A1 isolate was selected as a subclade representative for all analyses involving clade A, while subclade A1 was examined independently. Hereafter, clade A is therefore described as including 58 MRSA isolates and 10 MSSA isolates. The remaining isolates in clade B and those in clades C and D were examined separately.

3.2. Identification of clade specific SCCmec characteristics

Clades A and B1 were differentiated by 171 cgSNPs, while clade A MRSA specifically were differentiated from clade B1 by 188 cgSNPs. All clade A MRSA isolates harboured a SCC*mec* type IVa element similar to that identified in the MW2 (USA400) MRSA strain (GenBank accession

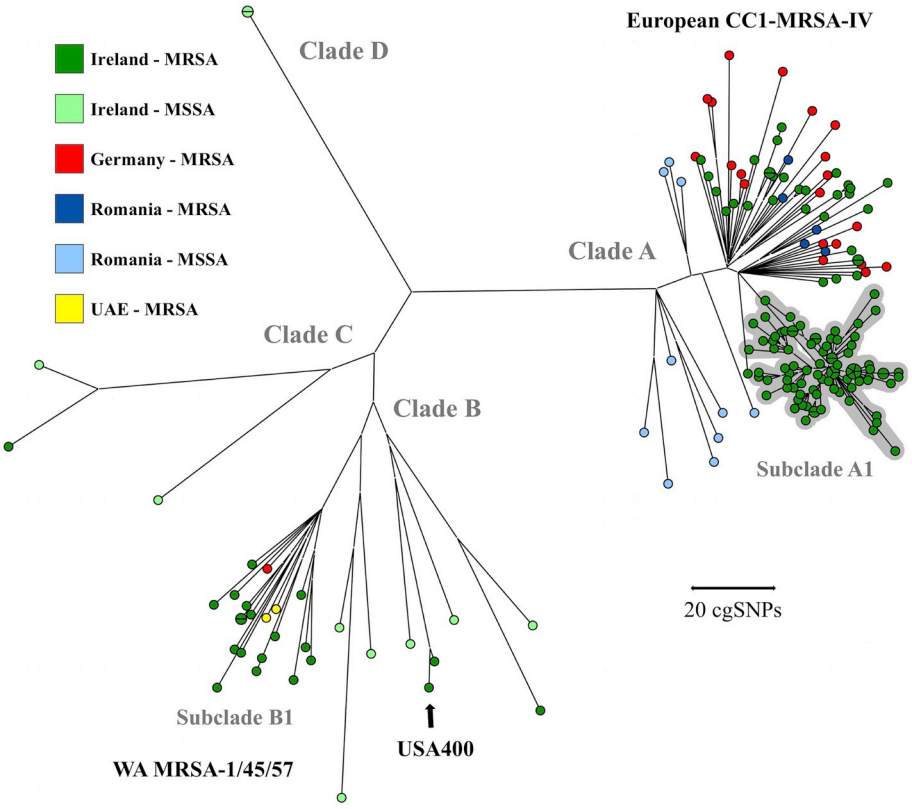

Fig. 1. A neighbour-joining tree based on a cgSNP analysis of 167 CC1-MRSA-IV and 20 CC1-MSSA isolates. The countries in which the isolates were recovered are indicated in the colour legend. Subclade A1 is shaded in grey. All branches yielded 100% permutation resampling support. The WA MRSA-1/45/57 isolates and the Irish clade B isolate marked as USA400 were identified based on genotypic characteristics. Abbreviation: cgSNPs, core-genome single nucleotide polymorphisms.

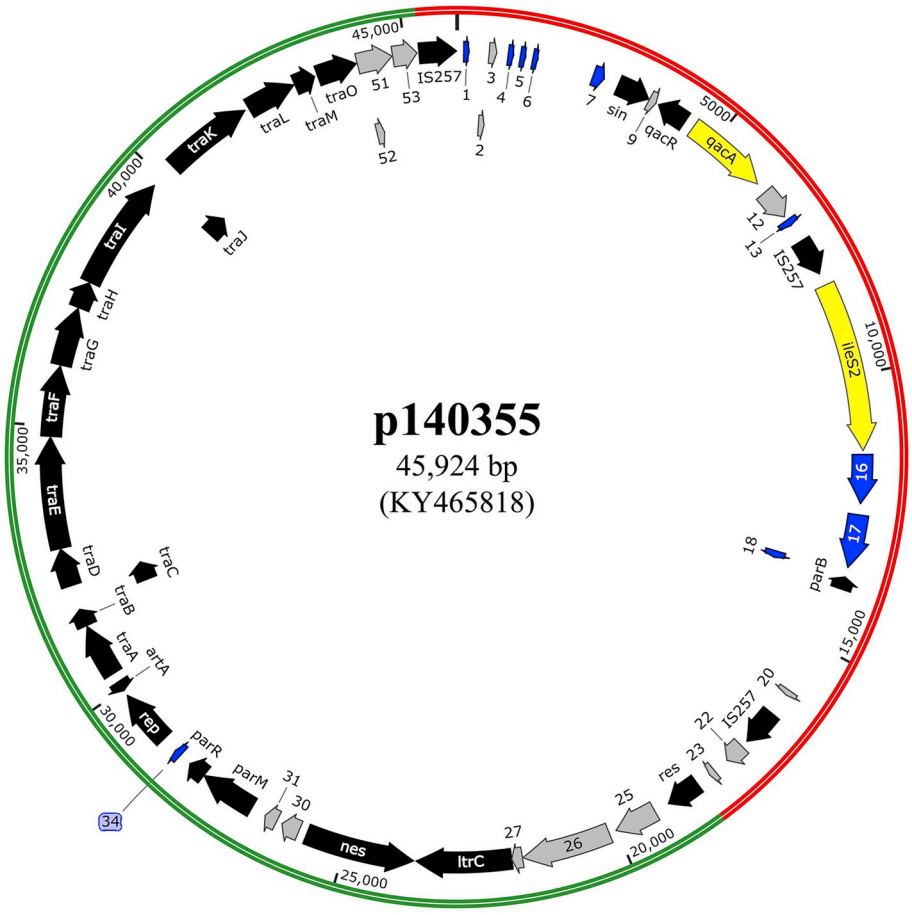

Fig. 2. Genetic map of *S. aureus* multi-resistance plasmid, p140355 (GenBank accession number: KY465818.1). The plasmid backbone is shown in green, while the accessory region is depicted in red. All known genes are shown in black except for the antimicrobial resistance genes, which are highlighted in yellow. Genes which have undergone National Centre for Biotechnology Information prediction are shown in blue: 1, MobA/MobL family protein; 4, Fst family toxin; 5, quinone reductase; 6, quinone reductase; 7, MarR family transcriptional regulator; 13, haloacid dehalogenase-like hydrolase family protein; 16, fructosamine kinase family protein; 17, major facilitator superfamily protein; 18, DDE transposase superfamily protein; 34, XRE family transcriptional regulator. Genes encoding hypothetical proteins are shown in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number: BA000033.2). In contrast to MW2-like SCC*mec* IVa, however, the clade A SCC*mec* element harboured a 4710 nucleotide insertion in the downstream constant segment (*dcs*) adjacent to *orfX*. This sequence encodes five different hypothetical proteins and corresponds to nucleotide positions 280,690–285,400 of GenBank entry RBVO000005.1. This gene cluster has also been detected in the SCC*mec* elements of several *S. aureus* (e.g. GenBank accession number: CP007672.1) and *Staphylococcus epidermidis* (e.g. LT571449.1) strains and one *Staphylococcus capitis* strain (CP007601.1). The subclade B1 isolates harboured MW2-like SCC*mec* IVa (without the *dcs* insertion). Half (10/20) of the subclade B1 isolates also carried a SCC*fus* element that included the *tirS* marker gene, *ccrA1* and *ccrB1* (i.e. SCC*fus*$_{476}$, as described in GenBank entry BX571857.1).

All clade A MRSA isolates harboured the same allelic variants of the cassette chromosome recombinase genes, *ccrA2* (1350 bp) and *ccrB2* (1629 bp). All subclade B1 isolates harboured the same *ccrA2* allele and the vast majority (16/20) harboured the same *ccrB2* allele, while those remaining harboured an allele which differed from the predominant *ccrB2* allele by 1 SNP. The *ccrA2* alleles in the SCC*mec* elements of clades A and B1 differed by 23 SNPs. The *ccrB2* allele in the clade A SCC*mec* element differed from the predominant *ccrB2* allele in the subclade B1 SCC*mec* element by 51 SNPs.

3.3. Clade A MRSA

The 58 clade A MRSA isolates were recovered in Ireland (n = 33), Germany (n = 20) and Romania (n = 5), and exhibited 0–109 (average 62.6, standard deviation [SD] 13.8) pairwise cgSNPs (Fig. 1; Table 1). All isolates were identified as ST1-MRSA-IVa-t127, with the exception of a single ST4911-MRSA-IVa-t127 isolate from Germany (Table 1). The 33 Irish MRSA isolates were recovered in 11 different hospitals/healthcare facilities (HCFs) and the community between 2007 and 2017 (Table 1) (Earls et al., 2017). Notably, the first and second clade A MRSA isolates identified in Ireland (which differed by 52 cgSNPs) were recovered in the community in 2007 and 2008, respectively, while the third such isolate was recovered in a hospital in 2012 (Table S1). The German isolates included 1/5 isolates (i.e. one of five isolates in the global database exhibiting the undefined genotypic pattern) recovered in a hospital in the Saxon city of Dresden in 2016, and 19/37 isolates recovered between 2016 and 2018 in two different hospitals and the community in the Bavarian city of Regensburg. The Romanian isolates included 5/40 isolates recovered in a hospital in the North-Eastern city of Iaşi between 2008 and 2012. Interestingly, no phylogenetic subgrouping of isolates recovered in the same country was observed (Fig. 1). Indeed, the Irish isolates exhibited 1–102 (average 69.0, SD 14.0) pairwise cgSNPs, the German isolates exhibited 12–115 (average 79.0, SD 16.2) pairwise cgSNPs and the Romanian isolates exhibited 53–71 (average 59.0, SD 6.8) pairwise cgSNPs.

The MRSA isolates within clade A harboured a broader range of antimicrobial resistance genes than those in clade B (Table 2). Specifically, *aphA3*, encoding neomycin and kanamycin resistance, *aadE*, encoding aminoglycoside resistance and *sat*, encoding streptothricin resistance, were detected in all clade A MRSA but were universally absent from clade B. Furthermore, *tet*(K), encoding tetracycline resistance, and *erm*(C) were more common in MRSA in clade A than in clade B1 (Table 2). The majority (47/58) of clade A MRSA isolates harboured the immune evasion cluster (IEC) type E (*sak* and *scn*), while those remaining harboured an undisrupted *hlb* gene. None of the clade A MRSA isolates harboured any enterotoxin genes other than *seh*, which is universally present in CC1.

3.4. Subclade A1

The 86 Irish MRSA isolates within subclade A1 exhibited 0–49 (average 17.4, SD 8.6) pairwise cgSNPs, and were recovered in 10 different hospitals/HCFs and the community, between 2013 and 2017 (Table 1) (Earls et al., 2017). The vast majority (84/86) of these isolates were characterised as ST1-MRSA-IVa-t127, while those remaining were identified as ST1-MRSA-IVa-t922. The majority (60/86; 70%) of subclade A1 isolates were recovered in a single hospital (Table S1).

The *ileS2* gene was markedly prevalent among subclade A1 isolates, but absent from (non-subclade A1) clade A MRSA (Table 2). Almost all (80/82) *ileS2*-encoding isolates also harboured the general efflux pump-encoding gene, *qacA*, which confers resistance to chlorhexidine among other compounds (Table 2). A ~7 kb insertion encoding *qacA*, was identified upstream of the *ileS2* region (Fig. 2). The only two other *ileS2* and *qacA*-encoding plasmid sequences (GenBank accessions numbers: KU882683 and KU882684) in the NCBI database correspond to plasmids unrelated to p140355 (50% and 24% query cover, respectively) that were previously recovered from *Staphylococcus lugdunensis*. The p140355 plasmid was characterised as a 45,924 bp circular plasmid of the pSK41/pGO1 family, exhibiting a GC content of 28.6% (Fig. 2). The plasmid backbone accounted for approximately 27.5 kb of the p140355 sequence, while the remaining 18.5 kb comprised the accessory region (Fig. 2). A total of 54 ORFs were identified, 29 of which encode known genes, 10 of which have undergone NCBI prediction and 15 of which encode hypothetical proteins (Fig. 2). The sequence reads of the 80 *ileS2* and *qacA*-encoding isolates mapped well to the p140355 sequence (Earls et al., 2017). Correspondingly, the sequence reads of the two *ileS2*-encoding isolates which lacked *qacA* (A1_01 and A1_61) failed to map to the *qacA* region but mapped well to the remainder of the plasmid sequence. The *tet*(K) gene and IEC genes, *sak* and *scn*, were also more common in subclade A1 than in clade A MRSA (Table 2).

3.5. Clade A MSSA

Considering that clade A included MRSA isolates recovered in Ireland and Romania as early as 2007 and 2009, respectively (Fig. 1; Table 1), CC1 MSSA from both of these countries were considered potential precursors to clade A MRSA. Forty Romanian CC1-MSSA isolates exhibiting the undefined CC1-MRSA-IV genotypic pattern (excluding genes typically located in SCC*mec*) were identified in the global database. Ten of these isolates were selected for WGS and subsequent cgSNP analysis. They grouped into clade A, differing from the MRSA isolates within this clade by 47–130 cgSNPs (Fig. 1). The Romanian MSSA isolates were recovered in the same hospital as the Romanian ST1-MRSA-IV isolates. The majority (8/10) of Romanian MSSA isolates were characterised as ST1 MSSA, while two isolates were identified as ST4910 and ST4912 (Table 1). Similarly, the majority (8/10) of Romanian MSSA isolates were identified as *spa* type t127, while the ST4910 isolate was identified as *spa* type t5633 and one ST1 isolate was assigned *spa* type t18248 (Table 1). Similar to clade A MRSA, 8/10 clade A MSSA isolates carried *aphA3*, *aadE* and *sat*. The *erm*(C) gene was also detected in 8/10 isolates, while 9/10 isolates harboured *tet*(K). Furthermore, clade A MSSA did not carry any enterotoxin genes other than *seh*, and either harboured IEC type E (8/10) or lacked IEC associated genes (2/10). Importantly, none of the 10 Irish MSSA isolates investigated grouped in close proximity to the clade A MRSA isolates (Fig. 1).

3.6. Subclade B1 (MRSA only)

The subclade B1 isolates were recovered in Ireland (n = 17), the UAE (n = 2) and Germany (n = 1), and differed by 0–116 (average 77.9, SD 17.0) pairwise cgSNPs. All subclade B1 isolates were identified as t127 (Table 1). The Irish isolates were recovered in nine different hospitals and the community between 2012 and 2017 (Table 1). Eight of the Irish isolates were identified as ST1-MRSA-IVa/SCC*fus*$_{476}$, while seven were identified as ST1-MRSA-IVa and the remaining two were characterised as ST4913-MRSA-IVa and ST4914-MRSA-IVa. The UAE isolates included 2/4 isolates recovered in a hospital in Abu Dhabi in 2009, both of which were characterised as ST1-MRSA-IVa/SCC*fus*$_{476}$.

Table 2
Differences in resistance and virulence-associated gene carriage between CC1-MRSA-IV in clade A and subclade B1, and in subclade A1 and the remainder of clade A.

Gene	Clade A[a]		Subclade B1[a]		*p* value[a]	Subclade A1[a]		Remaining clade A		*p* value[a]
	n	%	*n*	%		*n*	%	*n*	%	
Resistance										
aadD	0	0	**3**	**15**	**0.02**	1	1	0	0	1
aadE	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
aacA-aphD	2	3	0	0	1	0	0	2	4	0.16
aphA3	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
blaZ	58	100	20	100	1	86	100	57	100	1
erm(A)	0	0	**4**	**20**	**0.0034**	0	0	0	0	NA
erm(C)	**57**	**98**	3	15	**< 0.0001**	86	100	57	98	1
fusB	0	0	0	0	NA	5	6	0	0	0.16
fusC	0	0	**10**	**50**	**< 0.0001**	0	0	0	0	NA
ileS2	1	2	0	0	1	**82**	**95**	0	0	**< 0.0001**
lnu(A)	0	0	0	0	NA	2	2	0	0	0.52
mecA	58	100	20	100	1	86	100	57	100	1
qacA	2	3	0	0	1	**80**	**93**	1	2	**< 0.0001**
sat	**58**	**100**	0	0	**< 0.0001**	86	100	57	100	1
tet(K)	**53**	**91**	0	0	**< 0.0001**	**85**	**99**	52	91	**0.04**
Virulence										
hlb[b]	11	19	0	0	0.06	2	2	**11**	**19**	**0.002**
sak	47	81	20	100	0.06	**84**	**98**	46	81	**0.002**
scn	47	81	20	100	0.06	**84**	**98**	46	81	**0.002**
sea	0	0	**19**	**95**	**< 0.0001**	0	0	0	0	NA
seh	58	100	20	100	1	86	100	57	100	1
sek	0	0	**19**	**95**	**< 0.0001**	0	0	0	0	NA
seq	0	0	**19**	**95**	**< 0.0001**	1	1	0	0	1

[a] Statistically significant results are shown in bold.
[b] Undisrupted-*hlb*.

The German isolate represented a group of four isolates recovered in the aforementioned Dresden hospital in 2007 and was identified as ST1-MRSA-IVa. A large group of Australian isolates ($n = 46$; recovered between 2001 and 2009) exhibiting WA MRSA-1/45/57 genotypic patterns were also identified in the global database. However, these Australian isolates did not undergo WGS as part of this study.

Apart from *fusC*, *aadD* (encoding aminoglycoside resistance) and *erm*(A) (encoding MLS resistance) were the only resistance genes that were more common in subclade B1 than in clade A MRSA (Table 2). However, the subclade B1 isolates harboured a wider range of virulence-associated genes than clade A MRSA (Table 2). Specifically, in addition to the CC1-associated *seh* gene, the enterotoxin genes *sea*, *sek* and *seq* were significantly common in clade B (Table 2). The majority (19/20) of subclade B1 isolates harboured IEC type D (*sea*, *sak* and *scn*), while IEC type E (*sak* and *scn*) was detected in a single instance.

3.7. Remaining isolates

All remaining isolates were recovered in Ireland (Fig. 1). The nine remaining (non-subclade B1) clade B isolates differed from those in subclade B1 by 83–158 cgSNPs. These isolates included two closely related (10 cgSNPs) PVL-positive ST1-MRSA-IVa-t127 isolates, one of which was identified as USA400 based on its genotypic pattern and the other of which harboured SCC*fus*$_{476}$ (Fig. 1). Clade B also included six ST1-MSSA-t127 isolates, all of which harboured SCC*fus*$_{476}$ and half (3/6) of which were PVL-positive. Clade C included one ST1-MRSA-IVa-t127 and two ST1-MSSA-t127 isolates, all three of which were PVL-negative and harboured SCC*fus*$_{476}$. The clade C MRSA isolate was relatively closely related (38 cgSNPs) to one of the MSSA isolates, while the remaining clade C MSSA isolate differed from its closest neighbour by 119 cgSNPs. Clade D included two PVL- and SCC-negative ST1-MSSA-t127 isolates which were indistinguishable using cgSNP analysis.

4. Discussion

This study confirmed the existence of a previously undefined PVL-negative CC1-MRSA-IV clone that may have emerged in Romania or neighbouring regions and has become prevalent in both Ireland and Bavaria, Germany. This clone is distinctly different to the well-characterised PVL-negative CC1-MRSA-IV clone known as WA MRSA-1 (and closely related WA MRSA-57 and WA MRSA-45), which was first identified in Australia. Although indistinguishable using a combination of MLST, SCC*mec* typing and *spa* typing, cgSNP and SCC*mec* analysis revealed a clear distinction between these two clones. Both clones were identified among PVL-negative CC1-MRSA-IV recovered in Ireland. A mupirocin-resistant variant of the newly defined European CC1-MRSA-IV clone harbouring a conjugative *ileS2*-encoding plasmid first came to notice following a protracted hospital outbreak in Ireland (Earls et al., 2017). In the present study, this plasmid was shown to harbour both *ileS2* and *qacA*, encoding resistance to mupirocin and chlorhexidine, respectively, which are commonly recommended as a treatment combination for MRSA decolonisation (Poovelikunnel et al., 2015).

Analysis of CC1-MRSA-IV isolates from Ireland, Germany, Romania and the UAE identified two main PVL-negative CC1-MRSA-IV clades (A and B1) which were differentiated by 188 cgSNPs (Fig. 1). Clade-specific SCC*mec* characteristics identified in the *ccr* genes and *dcs* indicated that the clones represented by these clades likely evolved from MSSA following separate SCC*mec* acquisition events. Furthermore, a potential marker sequence of the clone represented by clade A was identified as an insertion within *dcs*. Genotypic evidence strongly suggests that clade B1 represents the WA MRSA-1/45/57 clone. Indeed, clade B1 isolates generally harboured *blaZ*, *sak*, *scn*, *sea*, *seh*, *sek* and *seq* (Table 2), all of which are characteristic features associated with WA MRSA-1/57/45 (Coombs et al., 2011). Interestingly, the clade B1 isolates which carried SCC*fus*$_{476}$ (i.e. the isolates matching the WA-45 description) were interspersed between those which did not harbour SCC*fus*$_{476}$ (i.e. those matching the WA-57 description). Furthermore, a high degree of relatedness (10 cgSNPs) was noted between two PVL-positive MRSA isolates in clade B, only one of which carried SCC*fus*$_{476}$. These observations bring into question the stability of the SCC*fus*$_{476}$ element, although further studies are warranted. There is limited information on fusidic acid consumption in Ireland and Germany, however, it is often

used in the community in Europe (Dobie and Gray, 2004; Mason and Howard, 2004). In contrast to clade B1, clade A isolates exhibited genotypic characteristics which differed substantially from those associated with WA MRSA-1/45/57. Specifically, clade A isolates generally harboured *aphA3*, *aadE*, *sat* and *tet*(K), none of which are associated with WA MRSA-1/45/57, and lacked *sea*, *sek* and *seq*, all of which are associated with WA MRSA-1/45/57 (Coombs et al., 2011) (Table 2). Notably, the genotypic characteristics exhibited by clade A isolates did not correspond with USA400 either (Côrtes et al., 2017), suggesting it unlikely that the clade A clone derived from USA400 by loss of the genes encoding PVL. Indeed, microarray genotyping identified one of the clade B isolates as USA400 (Fig. 1).

A 2014 study used microarray genotyping to identify PVL-negative CC1-MRSA-IV as the predominant MRSA clonal group in a Romanian hospital between 2008 and 2012 (Monecke et al., 2014). Distinct genotypic differences were noted between this CC1-MRSA-IV clone and WA MRSA-1/45/57 (Monecke et al., 2014). It was hypothesised in the Romanian study that this highly prevalent CC1-MRSA-IV clone may have emerged locally due to marked similarities between the microarray profiles of CC1-MRSA-IV and CC1-MSSA isolates recovered in the same region (Monecke et al., 2014). Significantly, the Romanian isolates investigated in the present study were also included in the 2014 study. The results of the present study strongly support the conclusions of the 2014 Romanian study. Firstly, the existence of a PVL-negative CC1-MRSA-IV clone in Romania that is distinct from WA MRSA-1/45/57 was confirmed and secondly, the close relatedness of the Romanian CC1-MRSA-IV and Romanian CC1 MSSA (which differed by as few as 47 cgSNPs) was verified (Fig. 1). The available evidence suggests it is likely that this novel CC1-MRSA-IV clone originated in Romania based on the following facts: (i) CC1 MSSA are common in Iaşi, Romania (Monecke et al., 2014), (ii) the CC1-MRSA-IV clone was predominant in Iaşi by 2008 (Monecke et al., 2014), at which time it had been detected in only two patients in Ireland and (iii) none of the Irish CC1-MSSA isolates investigated grouped in close proximity to clade A. However, the lack of published studies on MRSA and/or MSSA from neighbouring countries means the possibility of this clone having originated elsewhere in South-Eastern Europe cannot currently be ruled out.

Following its emergence in South-Eastern Europe, putatively in Romania, the European CC1-MRSA-IV clone spread to Ireland (Fig. 1; Table 1). While this clone was present in the Irish community by 2007, it was not detected in the Irish healthcare system until 2012 and did not become prevalent in Ireland until 2013 (Table S1). These findings support the previously outlined suggestion that consideration of risk-factors relating to the acquisition of MRSA in the community (and not only in healthcare settings e.g. previous hospitalisation, non-intact skin) may be appropriate during targeted MRSA screening in Irish hospitals (Earls et al., 2017; Irish Department of Health, 2013). Interestingly, the Irish clade A isolates (which were recovered between 2007 and 2017) exhibited a relatively high level of genotypic diversity (1–102 cgSNPs), suggesting that the European CC1-MRSA-IV clone may have been introduced into Ireland on several occasions. Indeed, the first two European CC1-MRSA-IV isolates identified in Ireland were recovered just 26 days apart (Table S1) and differed by 52 cgSNPs. Correspondingly, the rate of migration from Romania to Ireland rose dramatically in 2007 and was consistently high until 2017 (Irish National Central Statistics Office, 2009, 2017). Phylogenetic analysis also indicated that the European CC1-MRSA-IV clone spread to Germany (Fig. 1; Table 1). Although only sporadically encountered in Dresden, this clone became prevalent in Regensburg between 2016 and 2018, as demonstrated by its regular detection in the community and in two different hospitals. Remarkably, considering their recovery during a period of approximately two years, the German clade A isolates exhibited a particularly high level of genotypic diversity (12–115 cgSNPs), indicating that the European CC1-MRSA-IV clone may have been introduced into Germany on multiple occasions. Indeed, a 2014 German study demonstrated that 7/51 (14%) Romanian healthcare workers recently employed in a hospital in Aachen were colonised with an unusual MRSA-t127 strain (Scheithauer et al., 2014). In 2015, Romanians represented the second largest group (213,000 people) of foreign nationals living in Germany (Federal Office for Migration and Refugees, Germany, 2016), with a particularly large community living in the Regensburg region.

As previously described, a largely mupirocin-resistant variant of the European CC1-MRSA-IV clone (subclade A1) was responsible for a protracted outbreak in an Irish hospital between 2013 and 2016 (Earls et al., 2017). Isolates of this variant were also recovered from several other hospitals and the community (Earls et al., 2017). As part of the present study, cgSNP analysis revealed that the outbreak continued into 2017, and spread to three additional hospitals (Fig. 1; Table 1). Importantly, this study also revealed that *ileS2*-encoding plasmid (p140355) previously identified among the outbreak isolates also encodes *qacA* and therefore constitutes the first reported plasmid in *S. aureus* to encode resistance to the two antimicrobial agents (i.e. mupirocin and chlorhexidine) commonly used for *S. aureus* decolonisation (Poovelikunnel et al., 2015). While successful read alignment of the 80 *ileS2* and *qacA*-encoding outbreak isolates against the p140355 sequence suggested that all such isolates harboured this plasmid, two outbreak isolates appeared to harbour a variant of this plasmid which did not encode *qacA*. Notably, however, this *qacA*-negative plasmid did not disseminate with the same success as p140355. Therefore, despite the general lack of emphasis on antiseptic-resistance in the literature and public domain, it is possible that the combination of mupirocin and chlorhexidine resistance may have driven the selection of p140355-harbouring MRSA in Ireland.

It is highly likely that the CC1-MRSA-IV clone defined in this study is present in other European countries in addition to Romania, Ireland and Germany. Indeed, PVL-negative ST1-MRSA-IV-t127 have been recovered from humans, cattle, pigs, cow's milk and goat's milk in Italy, and from rooks in Austria (Alba et al., 2015; Basanisi et al., 2017; Cortimiglia et al., 2015; Loncaric et al., 2013; Monaco et al., 2013; Normanno et al., 2015). Furthermore, where detailed, the genotypic characteristics of these PVL-negative ST1-MRSA-IV-t127 correspond to those associated with the European CC1-MRSA-IV clone defined in this study. For example, between 2009 and 2011, PVL-negative ST1-MRSA-IV-t127 which generally harboured *aphA3*, *blaZ*, *sat* and *tet*(K), while exhibiting variable *erm*(C), *sak* and *scn* carriage, were isolated from dairy cows, humans and pigs in Italy (Alba et al., 2015). Indeed, 19% of the non-outbreak (i.e. non-subclade A1) European CC1-MRSA-IV isolates identified during this study did not harbour any human immune evasion genes (i.e. *chp*, *sak* or *scn*), indicating that this clone may be also be prevalent in animals (Table 2). Furthermore, while this study was under review, an Italian study (which included one of the Irish clade A1 isolates investigated in the present study) confirmed the presence of the European CC1-MRSA-IV clone in a paediatric hospital in Florence (Manara et al., 2018). Moreover, in line with the findings of the present investigation, this Italian study estimated that the novel CC1-MRSA-IV clone diverged 6–28 years ago.

The present study has demonstrated the existence of a European PVL-negative CC1-MRSA-IV clone that is distinctly different from the previously characterised and well-defined PVL-negative CC1-MRSA-IV clone, WA MRSA-1/45/57. Furthermore, cgSNP analysis revealed that this MDR clone may have originated in South-Eastern Europe, before spreading to both Ireland and Germany. Finally, the *ileS2*-encoding plasmid which facilitated the spread of this clone in Ireland was determined to also encode *qacA*, highlighting the increasing importance of effective mupirocin and chlorhexidine alternatives for *S. aureus* decolonisation.

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Declaration of interest

SM, RE and PS are employees of Abbott (Alere Technologies GmbH). The other authors declare no conflicts of interest.

Author contributions

MRE conceived the study, performed the WGS data analysis and drafted the manuscript. ACS conceived the study and assisted with WGS data analysis and writing the manuscript. DCC conceived the study, purchased the required materials, assisted with data analysis and drafted the manuscript. GB conceived the study assisted with data analysis and drafted the manuscript. AS and WS-B conceived the study, assisted with data analysis and drafted the manuscript. TS and OD conceived the study assisted with data analysis and drafted the manuscript. PS, RE and SM conceived the study, assisted with bioinformatics analysis, microarray analysis data analysis and drafted the manuscript. All authors read and approved the final manuscript.

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