First Report of *cfr*-Encoding Plasmids in the Pandemic Sequence Type (ST) 22 Methicillin-Resistant *Staphylococcus aureus* Staphylococcal Cassette Chromosome *mec* Type-IV Clone

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ABSTRACT

Linezolid is often the drug of last resort for serious methicillin-resistant *Staphylococcus aure*us (MRSA) infections. Linezolid resistance is mediated by mutations in 23S rRNA and genes for ribosomal proteins, *cfr* encoding phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A ($PhLOPS_A$) resistance, its homolgue *cfr*(B) or *optrA* conferring oxazolidinone and phenicol resistance. Linezolid resistance is rare in *S. aureus*, and *cfr* even rarer. This study investigated the clonality and linezolid resistance mechanisms of two MRSA isolates from patients in separate Irish hospitals. Isolates were subjected to *cfr* PCR, $PhLOPS_A$ susceptibility testing, 23S rRNA PCR and sequencing, DNA microarray profiling, *spa* typing, pulsed-field gel electrophoresis (PFGE), plasmid curing and conjugative transfer. Whole-genome sequencing was used for single nucleotide variant (SNV) analysis, multilocus-sequence typing, L-protein mutation identification, *cfr*-plasmid sequence analysis and *optrA* and *cfr*(B) detection. Isolates M12/0145 and M13/0401 exhibited linezolid MICs of 64 and 16 mg/liter, respectively, and harbored identical 23S rRNA and L22 mutations, but M12/0145 exhibited the mutation in 2/6 23S rRNA alleles compared to 1/5 in M13/0401. Both isolates were ST22-MRSA-IV/t032, harbored *cfr,* exhibited the $PhLOPS_A$ phenotype and lacked *optrA* and *cfr*(B). They differed by five PFGE bands and 603 SNVs. Isolate M12/0145 harbored *cfr* and *fexA* on a 41-kb conjugative pSCFS3-type plasmid, whereas M13/0401 harbored *cfr* and *lsa*(B) on a novel 27-kb plasmid. This is the first report of *cfr* in the pandemic ST22-MRSA-IV clone. Different *cfr* plasmids and mutations associated with linezolid resistance in genotypically distinct ST22-MRSA-IV isolates highlights that prudent management of linezolid use is essential.

INTRODUCTION

The oxazolidinone antimicrobial agent linezolid was first introduced into clinical practice in 2000 and it quickly became the drug of last resort to treat skin and soft tissue infections and pneumonia caused by multidrug-resistant Gram-positive cocci, including methicillin-resistant *Staphylococcus aureus* (MRSA). Linezolid binds to the A site of the peptidyl transferase center in the V domain of the 23S rRNA component of the 50S subunit of the bacterial ribosome (1). Binding of linezolid interferes with the correct positioning of aminoacyl tRNA on the ribosome which prevents formation of the initiation complex and thus inhibits the initiation of protein synthesis (1).

Resistance to linezolid is predominantly mediated by (i) mutations in the drug target site (domain V of the six 23S rRNA alleles) or in the genes encoding the 50S ribosomal proteins (L3, L4 and L22) that have been speculated to result in the impairment of linezolid binding and/or (ii) acquisition of the transferable linezolid resistance gene *cfr* (2-4). The *cfr* gene encodes a methyltransferase that catalyzes the post-transcriptional methylation of adenosine at nucleotide position 2503 (*Escherichia coli* numbering) in 23S rRNA thus interfering with the binding of linezolid to its target (4, 5). However, due to overlapping binding sites, *cfr* methylation also affects the binding of four other classes of antimicrobial agents and results in the multiresistance PhLOPS$_A$ phenotype i.e. resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A compounds (6). Recently, a novel plasmid-located ABC transported gene *optrA*, conferring resistance to linezolid and phenicols, and a *cfr* homologue, *cfr*(B), have also been identified (7-9).

The *cfr* gene was first reported in bovine *Staphylococcus sciuri* isolated in 1997 and subsequently in many different staphylococcal species including methicillin-susceptible *S. aureus* (MSSA), MRSA and coagulase-negative and coagulase-variable (*Staphylococcus hyicus*)

74 staphylococci as well as in *Bacillus*, *Enterococcus*, *Streptococcus, Macrococcus*, *Jeotgalicoccus*,
75 *Proteus* and *Escherichia* species (10-12). It has been detected in isolates from humans, livestock,
76 meat products and the environment and has been identified on a variety of plasmids, although
77 chromosomal locations have also been reported (10, 13). In some instances different bacterial
78 species as well as a variety of animal and human hosts have been found to harbor similar *cfr*
79 plasmids or genetic environments highlighting the ability of *cfr* to spread (10). Specific insertion
80 sequences have been shown to play a role in *cfr* mobility and integration into different plasmid
81 types and *cfr* is often co-located with other resistance determinants, allowing for the co-selection
82 of *cfr* (10).

83 Reports of linezolid resistance remain relatively rare among *S. aureus* and *cfr* even more
84 so (14-16). The earliest reported *cfr*-mediated linezolid resistant *S. aureus* isolates were two ST5-
85 MRSA isolates recovered in 2005 from two patients in hospitals in Colombia and Indianapolis,
86 USA, respectively (17, 18). The *cfr* gene has subsequently been reported in a small number of
87 sporadically-occurring *S. aureus* isolates, predominantly MRSA, from both animals and humans
88 belonging to a range of genotypes including the multilocus sequence type (MLST) clonal
89 complex (CC) 5 (sequence types (ST) 627, 228, 5, 125 & 1788), CC/ST6, CC/ST8 (ST8-MRSA-
90 IV/USA300), CC9 (STs 9 & 63) and CC/ST398 as well as in association with an outbreak of an
91 unspecified MRSA clone in a Spanish hospital 2008 (17, 19-32). Although two studies localized
92 *cfr* to the *S. aureus* chromosome (one within the staphylococcal cassette chromosome *mec*
93 (SCC*mec*)-IVb J1 region (23) and one within the 23S rRNA allele 4 (18, 33)) it has
94 predominantly been reported on a diverse range of plasmids (10).

95 In Ireland, only one *cfr*-positive MRSA isolate has been reported to date (USA300/ST8-
96 MRSA-IVa) in which *cfr* was located on a novel plasmid (pSCFS7) together with a second
97 phenicol resistance gene *fexA* via integration of *cfr* into the *fexA*-carrying transposon Tn*558* (20).

Recently, *cfr* has also been reported in methicillin-resistant *Staphylococcus epidermidis* (MRSE) clinical isolates from Ireland, although the possible plasmid location of *cfr* in these isolates was not reported (34, 35).

ST22-MRSA-IV is a pandemic MRSA clone that is endemic in hospitals in Ireland and the UK and predominates among nosocomial MRSA in several other European countries, Asia and Australia (36-41). It has also been reported sporadically in the USA and South America (42, 43). Although mutational resistance to linezolid has been reported in ST22-MRSA-IV, *cfr* has not been reported (44). During 2012 and 2013, two epidemiologically unrelated linezolid-resistant MRSA isolates were recovered from two patients in two separate Irish hospitals and were submitted to the Irish National MRSA Reference Laboratory. The purpose of this study was to investigate the genetic basis of linezolid resistance and the genetic relatedness of these isolates. This study reports the first identification of *cfr* in association with two distinct *cfr* plasmids in two genetically distinct ST22-MRSA-IV isolates.

MATERIALS AND METHODS

Bacterial isolates. Two linezolid-resistant MRSA isolates recovered from patients in two separate Irish hospitals approximately 250 km apart, one in 2012 in Cork (M12/0145) and the other in 2013 in Dublin (M13/0401), were investigated. Isolate M12/0145 was recovered from a sputum sample and the patient had previously been treated with linezolid. Isolate M13/0401 was recovered from an abdominal wound swab and no data was available on linezolid treatment of this patient. Isolates were initially tentatively identified as *S. aureus* using the tube coagulase test as described previously (45) and as cefoxitin- and linezolid-resistant by disk diffusion using The European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology and interpretive criteria (46, 47). Definitive identification of isolates as *S. aureus* was determined by DNA microarry profiling (see below). The plasmid-free novobiocin-resistant *S. aureus* strain XU21 was used as a plasmid recipient in filter mating experiments (48). Isolates were stored at -80°C on Protect Bacterial Preservation System cryogenic beads in individual preserver vials (Technical Services Consultants Ltd., Heywood, UK).

Investigation of isolates for the PhLOPS$_A$ phenotype. The two linezolid-resistant MRSA isolates (M12/0145 and M13/0401), their respective *cfr* negative, plasmid-cured derivatives (M12/0145-C1 and M13/0401-C1) and the *cfr*-positive transconjugant derivative of XU21 (M12/0145/XU21-T1) generated following mating experiments between M12/0145 and the recipient strain XU21, were investigated for the PhLOPS$_A$ phenotype. Chloramphenicol, clindamycin and linezolid minimum inhibitory concentrations (MICs) were determined using the VITEK 2 (AST P580 panel, susceptibility tests for Gram-positive bacteria; bioMérieux, Basingstoke, Hampshire, UK) according to the manufacturer's instructions. Tiamulin MICs were determined using Etest strips ranging from 0.002 mg/liter to 32 mg/liter (Liofilchem, Roseto degli Abruzzi, Italy). Virginiamycin M$_1$ MICs were determined by broth microdilution (range 1

mg/liter to 256 mg/liter) using the Clinical and Laboratory Standards Institute (CLSI) methodology and virginiamycin M_1 powder (Sigma-Aldrich Chemical Co. Dublin, Ireland) (49). The absence of the $PhLOPS_A$ phenotype in the plasmid-free *S. aureus* recipient strain XU21 was determined as described previously (20).

Additional antimicrobial susceptibility testing. The two linezolid-resistant MRSA parental isolates, their cured and transconjugant derivatives and the recipient strain XU21 also underwent antimicrobial susceptibility testing against a panel of 23 antimicrobial agents and heavy metals according to EUCAST methodology (47) using previously described interpretive criteria and quality control strains (50). The 23 agents tested were amikacin, ampicillin, cadmium acetate, chloramphenicol, ciprofloxacin, erythromycin, ethidium bromide, fusidic acid, gentamicin, kanamycin, lincomycin, mercuric chloride, mupirocin, neomycin, phenyl mercuric acetate, rifampicin, spectinomycin, streptomycin, sulphonamide, tetracycline, tobramycin, trimethoprim, and vancomycin.

Genotyping. The two linezolid-resistant MRSA isolates and their cured derivatives underwent *spa* typing. Genomic DNA for *spa* typing was extracted from each isolate/derivative using enzymatic lysis and the DNeasy blood and tissue kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. PCRs were performed using GoTaq Flexi DNA polymerase (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions using the primers and thermal cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet, www.seqnet.org.) and a G-storm GS1 thermocycler (Applied Biosystems, Foster City, CA). PCR products were visualized by conventional agarose gel electrophoresis and were purified using the GenElute PCR clean-up kit (Sigma-Aldrich Ireland Ltd., Arklow, County Wicklow Ireland). Sequencing was performed commercially by Source Bioscience (Tramore, Waterford, Ireland) using an ABI

3730xl Sanger sequencing platform. The Ridom StaphType software version 1.3 (Ridom Gmbh, Würzburg, Germany) was used for *spa* sequence analysis and assignment of *spa* types (51). The two linezolid-resistant MRSA isolates also underwent pulsed-field gel electrophoresis (PFGE) using SmaI as described previously (52).

The StaphyType DNA microarray kit (Alere Technologies, Jena, Germany) was used for confirmation of isolates as *S. aureus*, for assigning isolates and derivatives to MLST STs and/or CCs and SCC*mec* types and for detecting antimicrobial resistance (including *cfr*) and virulence genes (53, 54). The DNA microarray procedures were performed according to the manufacturer's instructions and the primers, probes and protocols have been described previously in detail (53, 54). Genomic DNA for use with the DNA microarray was extracted from isolates and derivatives by enzymatic lysis using the buffers and solutions provided with the StaphyType kit and the Qiagen DNeasy Blood and Tissue kit (Qiagen, Crawley, West Sussex, UK). DNA microarray profiling of the plasmid-free *S. aureus* recipient strain XU21 was performed in a previous study (20).

Plasmid analysis and whole-genome sequencing. Plasmid curing and filter mating conjugative transfer experiments were performed as described previously (48, 55, 56). The two linezolid-resistant parental MRSA isolates underwent whole-genome sequencing (WGS) in order to (i) determine the genetic organization of *cfr* and its surrounding regions in these isolates and to compare these to each other and to those previously described; (ii) determine the number of single-nucleotide variants (SNVs) between the two linezolid-resistant MRSA isolates; (iii) assign the two linezolid-resistant MRSA isolates to MLST STs as the DNA microarray only assigned these isolates to MLST CCs; (iv) identify any possible linezolid resistance-associated ribosomal target site mutations in the *rplC* (L3), *rplD* (L4) and *rplV* (L22) genes in the two *cfr*-positive

194 MRSA isolates and (v) to detect *optrA and cfr*(B). The 23S rRNA alleles were amplified by PCR
195 as described previously (57) and sequencing reactions were performed by Source Bioscience.
196 For both isolates WGS was performed using a MiSeq desktop sequencer (Illumina, Essex,
197 UK) and, for M13/0401 only, WGS was also performed using a PacBio RS sequencing system
198 (Pacific Biosciences, California, USA) with subsequent Hierarchal Genome Assembly Process
199 (HGAP.3; The Genome Analysis Centre (TGAC), Norwich, UK), to confirm the genetic
200 organization of the novel *cfr* plasmid identified. Genomic DNA for WGS was extracted from
201 both isolates using the Qiagen DNeasy Blood and Tissue kit. For the MiSeq WGS libraries were
202 prepared using the Nextera XT library preparation reagents (Illumina). Reads generated using the
203 MiSeq were checked for quality, trimmed and assembled into contigs using the Velvet *de novo*
204 assembler which is incorporated in to SeqSphere software version 2.3
205 (http://ridom.com/seqsphere). For PacBio WGS genomic DNA was checked for quality and
206 concentration according to TGAC guidelines. Contigs generated from both WGS methods were
207 analyzed seperately using the BioNumerics Genome analysis tool (GAT) plugin (version 7.5,
208 Applied Maths, Sint-Martens-Latem, Belgium), the Artemis genome browser and annotation tool
209 (58) and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Open reading frames (ORFs)
210 were predicted using the BioNumerics annotation tool and BLAST software packages. ORFs
211 were aligned with best fitting matches in GenBank and the location of start and stop codons were
212 checked for consistency and modified if required. Any gaps identified in the *cfr* region in the
213 isolates, were closed by PCR and sequencing using primers based on the surrounding contigs
214 followed by amplimer sequencing at Source Bioscience. Data were analyzed and overlapping
215 sequences were assembled using BioNumerics. The genetic organizaton of the *cfr* region in each
216 isolate was confirmed by PCR and primers listed in supplemental Table S1. For M12/0145 this
217 was done for the Δ*tnpA-fexA* region encompassing *cfr* and not the entire *cfr*-encoding plasmid in

this isolate due to its high similiarity to a previously described *cfr* plasmid. For M13/0401, this was done for the entire plasmid as it was distinct from those described previously.

Mi-Seq WGS data for M13/0401 was also resequenced against the *de-novo* Mi-Seq assembly of isolate M12/0145 followed by alignment and SNVs were identified and confirmed if they exhibited ≥ 40x coverage i.e. each SNV was covered by at least 40 reads, thereby avoiding ambiguous SNVs and increasing confidence in SNV validity. All synonymous and non-synonymous mutations were included. Insertions and deletions (indels) and repetitive regions were excluded.

Nucleotide accession numbers. The following nucleotide sequences from M12/0145 and M13/0401 have been deposited in GenBank as follows: *cfr*-encoding plasmids (M12/0145, KU521355 and M13/0401, KU510528), 23S rRNA V domain (M12/0145: allele 1, KU510534; allele 2, KU510535; allele 3, KU510536; allele 4, KU510537; allele 5, KU510538; allele 6, KU510539; M13/0401: allele 1, KU510529; allele 2, KU510530; allele 3, KU510531; allele 4, KU510532; allele 5, KU510533) and *rplV*/L22 (M12/0145, KU510541 and M13/0401, KU510540).

RESULTS

Phenotypic and genotypic characteristics of linezolid-resistant MRSA. Both isolates (M12/0145 and M13/0401) were assigned to ST22-MRSA-IV and *spa* type t032. Each isolate exhibited the $PhLOPS_A$ phenotype with linezolid MICs of 64 mg/liter (M12/0145) and 16 mg/liter (M13/0401) (Table 1). Both isolates lacked *optrA* and *cfr*(B) but harbored *cfr* and one isolate (M12/0145) also harbored the phenicol exporter gene, *fexA* (Table 1). The isolates differed by five bands in PFGE analysis and 603 SNVs following WGS analysis (MiSeq coverage of 131x and 170x for M12/0145 and M13/0401, respectively). Both *cfr*-positive MRSA isolates also exhibited resistance to ampicillin, erythromycin, lincomycin, ciprofloxacin and fusidic acid and carried the resistance genes *blaZ* and *erm*(C). Isolate (M13/0401) was also resistant to rifampicin (Table 1). Both isolates harbored the enterotoxin C gene *sec* and the enterotoxin gene cluster *egc* but differed by the presence of immune evasion complex (IEC) genes in isolate M12/0145 (Table 1).

Characterization of the genetic environment of *cfr* in ST22-MRSA-IV. Whole-genome sequence analysis as well as results from plasmid curing experiments indicated that *cfr* was plasmid located in both ST22-MRSA-IV isolates. The *cfr*-positive isolate M13/0401 was successfully cured of *cfr*, whereas the *cfr*- and *fexA*-positive isolate M12/0145 was successfully cured of both genes (Table 1). Cured derivatives of both isolates (M12/0145-C1 and M13/0401-C1) lacked the $PhLOPS_A$ phenotype but were otherwise indistinguishable from their respective parental isolates in terms of antimicrobial resistance phenotype, antimicrobial resistance and virulence genes detected using the DNA microarray and MLST-SCC*mec* and *spa* types (Table 1). While the *cfr*-negative cured derivative M13/0401-C1 was linezolid susceptible, the *cfr*- and *fexA*-negative cured derivative M12/0145-C1 exhibited linezolid resistance, with a linezolid MIC

of 8 mg/liter (Table 1), but this was lower than the corresponding linezolid MIC exhibited by its *cfr*-positive parental isolate (M12/0145 linezolid MIC of 64 mg/liter, Table 1).

A transconjugant derivative of the *S. aureus* recipient strain XU21 (M12/0145/XU21-T1, Table 1) was obtained using MRSA isolate M12/0145 as the donor; it exhibited the $PhLOPS_A$ phenotype and was otherwise indistinguishable from XU21 apart from the presence of *cfr* and *fexA* (Table 1). Several separate attempts to generate a transconjugant derivative of XU21 using M13/0401 as donor were unsuccessful. In contrast, isolates M05/0060 (a *cfr*-positive ST8-MRSA-IVa isolate and the only previously described *cfr*-positive MRSA from Ireland) (20) and M12/0145 (*cfr*-positive ST22-MRSA-IV, this study), shown to harbor conjugative *cfr* plasmids, consistently yielded *cfr*-positive transconjugants when used as positive controls.

Based on the whole-genome sequence, the *cfr* plasmids in M12/0145 and M13/0401 were found to differ substantially from each other (Fig. 1(a) and (e)) and were identified on four and two contigs, respectively, following Mi-Seq WGS, and, for M13/0401 only, on one contig following Pac-Bio sequencing (Pac-Bio coverage for M13/0401 of 100x). For isolate M12/0145, the *cfr*-encoding plasmid was 41,587-bp in size and it was most similiar in size and genetic organization to the previously reported 39-kb *cfr*-carrying plasmid pSA737 in MRSA ST239 (Genbank accession no. KC206006; 94% DNA sequence homology). In fact, the genetic organization of the *cfr* region in M12/0145 was very similiar to that previously described for pSA737/pSCFS3-like *cfr* plasmids from a diverse range of staphylococcal species from a variety of human and animal hosts (Supplemental Table S2). The region surrounding *cfr* in all of these plasmids, and in the present study in M12/0145, consists of an IS*21*-like element (IS*21-558*) and *cfr* inserted into the *fexA*-carrying transposon Tn*558* resulting in a truncation of the Tn*558*-transposase genes *tnpA* and *tnpB* (Fig. 1 (a)–(d)). The transposase genes *ΔtnpB* and *tnpC*, *orf138* (encoding a putative oxidoreductase) and *fexA* are located downstream of *cfr* and *orf2,* IS*21-558*

(consisting of two overlapping ORFs encoding *istA* and *istB)* and *ΔtnpA* are located upstream of *cfr* (Fig.1 (a) & (b)). The DNA sequence of the *cfr* region in M12/0145 and the *cfr* region in pSA737 differed only by a deletion of a thymine (T) nucleotide base in the intergenic region between *orf2* and *cfr* in M12/0145. However, beyond the *cfr* region the only difference identified was a 2,326 bp region in M12/0145, located ca. 8 kb downstream of *cfr*, that is not present in pSA737. This region in M12/0145 consisted of a transposase gene and a *istB*-like gene with 48% DNA sequence homology to *istB* that may be involved in transposition.

The *cfr* region in M12/0145 was also compared to the corresponding region in the ST8-MRSA-IVa isolate M05/0060 carrying pSCFS7, the only previously described MRSA isolate recovered in Ireland found to carry *cfr* (Fig. 1(d)). Although both *cfr* plasmids carried *fexA* and appeared to be derivatives of the insertion of IS*21-558* and *cfr* into Tn*558* they differed mainly due to the insertion site of the IS element and *cfr* (Fig. 1(a) & (d)). In pSCFS7, the integration of the IS*21-558-cfr* region within Tn*558* resulted in a truncation of the IS element and *tnpB* while in M12/0145, both *tnpA* and *tnpB* are truncated but the IS*21-558* element is intact (Fig. 1 (a) & (d)).

For isolate M13/0401 the *cfr*-encoding plasmid was 27,502 bp in size and the region immediately upstream of *cfr* was similar to that in M12/0145 and consisted of *orf2* and IS*21-558* (Fig. 1(a) & (e)). The DNA sequences of these genes were 100% identical to that found in M12/0145. The *cfr* gene differed by one nucleotide base only, at position 983 between the two isolates (T in M12/0145, G in M13/0401), resulting in a different amino acid in M12/0145 (serine) and M13/0401 (arginine). In contrast to the *cfr* region in M12/0145, the ABC transporter gene *lsa*(B) encoding low-level lincosamide resistance was also detected upstream of *cfr* in M13/0401 (Fig.1(e)). This ABC transporter gene has previously been reported in *cfr* plasmids p12-03322 (ST2 MRSE, Fig. 1(f)) (59), pSCFS6 (*Staphylococcus warneri*) (60) and pSCFS1 (*Staphylococcus sciuri*) (11). However, these latter two plasmids (pSCFS6 and pSCFS1), differ

substantially from the *cfr*-containing region identified in M13/0401 with pSCFS6 also containing *fexA* and pSCFS1 harboring the spectinomycin resistance gene *spc* and the macrolide-lincosamide streptogramin B resistance gene *erm*(33), but lacking IS*21-558*. The genetic organization of the *cfr* region in M13/0401 showed highest overall similarity to p12-00322 (Fig. 1(e) & (f)). However, in p12-00322, the *cfr* region is flanked by IS*257* elements, which were not identified in M13/0401. Similar to M12/0145, the region downstream of *cfr* in M13/0401 contained a transposase gene and an *istB*-like gene with 48% DNA sequence homology to *istB* but these were not identified in p12-00322 (Fig. 1(e) & (f)).

The remainder of the *cfr*-carrying plasmid in M13/0401 was also distinct from p12-00322. While 14 additional ORFs were identified in the *cfr* plasmid in M13/0401 it lacked the putative conjugation machinery (*tra*), encompassing the majority of the remainder of p12-00322 (59). A gene (*ssaA*) encoding a SsaA-like transposon-related protein was detected 5,648 bp upstream of *lsa*(B) in M13/0401. The *ssaA* gene exhibited 63.3% DNA sequence homology to *ssaA* present on the *Staphylococcus cohnii cfr*-containing plasmid pHK01 (61) and 48.8% DNA sequence homology to *ssaA* on plasmids pSK73 (*S. aureus*; Genbank accession no. GQ915269.1) and p12-02300 (ST2 MRSE) (59). A BLAST search of the amino acid sequences of other predicted ORFs identified within the DNA sequence of the *cfr*-carrying plasmid in M13/0401 indicated that, although the percentage homology was low (30-40%), a number of these exhibited amino acid identity to proteins involved in DNA transfer including a variety of proteins from bacilli and staphylococci involved in conjugation (Supplemental Table S3). The remaining predicted ORFs exhibited similarity to hypothetical proteins only.

Characterization of ribosomal mutations associated with linezolid resistance. The same two mutations were detected in multiple 23S rRNA alleles and in L22 of both *cfr*-positive ST22-MRSA-IV isolates. These included a change from guanine to thymine at nucleotide

position 2603 (in 2/6 alleles in M12/0145 and 1/5 alleles in M13/0401) in the V domain of the 23S rRNA gene and an amino acid change from alanine to valine at position 29 in L22. No amino acid changes were detected in the L3 or L4 proteins in either isolate.

DISCUSSION

ST22-MRSA-IV is a pandemic nosocomial MRSA clone and previous studies have revealed the ability of this clone to adapt to the introduction of different antimicrobial agents into the healthcare environment (38). In the present study we report another step in the evolution of this MRSA clone, with the first report of the transferable multidrug resistance gene *cfr* in two independent ST22-MRSA-IV isolates. Although both isolates were from patients in Irish hospitals, they were epidemiologically unrelated i.e. from two geographically disparate hospitals. In addition, although both isolates were assigned to *spa* type t032 and only a single difference was detected in their antimicrobial resistance phenotypes (rifampicin resistance in one isolate only), DNA microarray profiling revealed some differences in terms of an additional antimicrobial resistance gene (*fexA*) and virulence gene complex (IEC) in one isolate. Whole-genome sequence analysis ultimately provided the definitive evidence that these two ST22-MRSA-IV isolates were genotypically as well as epidemiologically distinct, due to the large numbers of SNVs identified (603 SNVs).

Detailed plasmid analysis of the two ST22-MRSA-IV isolates revealed that *cfr* has been introduced on two distinct plasmids into ST22-MRSA-IV. In the ST22-MRSA isolate M12/0145 *cfr* and *fexA* were co-located on a conjugative plasmid that was very similar to pSA737 (29, 30) previously described in isolates of other MRSA genotypes and in a variety of CoNS species from both animals and humans (Supplemental Table S3). Plasmid pSA737 is a pSCFS3-type plasmid, one of the most common types of *cfr*-containing plasmids. While the genetic environment of *cfr* in the second ST22-MRSA-IV isolate (M13/0401) revealed some similarities to that in M12//0145 in terms of the presence and location of *orf2* and the IS*21-558* transposase genes, *istAS* and *istBS*, it was otherwise distinct from the plasmid in M12/0145. In fact, the *cfr* region in M13/0401 showed most similarity to that in the MRSE plasmid p12-00322 with both harboring

lsa(B), but both *cfr* regions were carried on otherwise distinct plasmids. Genes with homology to those involved in mobility were identified in M13/0401 but the *tra* genes of p12-00322 were absent. Despite repeated attempts, filter mating experiments using M13/0401 as a donor failed to yield any transconjugants suggesting that the *cfr*-carrying plasmid present in M13/0401 was non-conjugative, at least under the conditions tested.

Anecdotal data on two additional linezolid-resistant ST22-MRSA-IV isolates recovered from two other patients in the same hospital as M13/0401, and within three months of the isolation of M13/0401, indicated that these two isolates were indistinguishable from M13/0401 based on antimicrobial susceptibility testing, *spa* typing and DNA microarray data (data not shown). Although these two isolates were originally phenotypically linezolid-resistant and *cfr*-positive by PCR, they were subsequently found to be linezolid susceptible and lacked *cfr* following storage and subculturing indicating the instability of the *cfr*-carrying plasmid in these isolates. However, the recovery of three genotypically indistinguishable *cfr*-positive isolates from patients in the same hospital in a similar timeframe does suggest the ability of this *cfr*-positive ST22-MRSA-IV strain to spread between patients. The patient from whom M12/0145 was recovered was also found to harbor an indistinguishable ST22-MRSA-IV strain based on DNA microarray profiling and *spa* typing that was linezolid-susceptible and lacked *cfr* and the $PhLOPS_A$ phenotype (data not shown). Furthermore, the patient from whom M12/0145 was recovered had been treated previously with linezolid. This isolate may represent a precursor to the *cfr*-positive ST22-MRSA-IV isolate identified in the present study or an example of the loss of *cfr* in this strain.

The origin of the *cfr*-carrying plasmids in these ST22-MRSA-IV isolates is as yet unknown. Both plasmids were distinct from a previously reported *cfr*-encoding plasmid characterized in Ireland from a ST8-MRSA-IV isolate (20). The *cfr*-carrying plasmid in

409 M12/0145 may have spread from other staphylococci, either *S. aureus* or CoNS, as the same
410 plasmid types have been reported elsewhere, in both human and animal staphylococcal isolates.
411 The *cfr*-carrying plasmid from M13/0401 is distinct from those described previously but
412 similarities to those in MRSE suggest CoNS as a possible source. Recent reports of *cfr*-harboring
413 MRSE in Ireland raise the possibility that MRSE may be the source of these *cfr* plasmids,
414 although analysis of the *cfr* region in these MRSE has not yet been reported so that a comparison
415 is not possible (34, 35). Enterococci could also be the source of *cfr* in the ST22-MRSA-IV
416 isolates as linezolid resistance appears to be more common among enterococci. Only a single *cfr*-
417 positive linezolid-resistant enterococcal isolate has been reported from Ireland with no detailed
418 plasmid analysis (62). Detailed systematic analysis of additional staphylococcal and enterococcal
419 isolates from both animals and humans in Ireland for *cfr* is necessary to determine the source of
420 these *cfr* plasmids and to prevent further spread.

421 Both *cfr*-positive ST22-MRSA-IV isolates also harbored a mutation in 23S rRNA
422 (G2603T) and this mutation has been shown previously to confer linezolid resistance in *S. aureus*
423 and *S. epidermidis* (32, 63). Isolate M12/0145 exhibited a linezolid MIC of 64 mg/L and
424 harbored mutations in two 23S rRNA alleles while isolate M13/0401 exhibited a linezolid MIC
425 of 16 mg/L and harbored mutations in one 23S rRNA allele suggesting a possible relationship
426 between the number of mutated alleles and the linezolid MIC. Furthermore, while curing both
427 isolates of their *cfr*-carrying plasmids resulted in a reduction in their respective linezolid MICs,
428 the cured derivative of M13/0401 was linezolid susceptible (linezolid MIC of 2 mg/liter) while
429 that of M12/0145 (which had the two mutated 23S rRNA alleles) remained borderline linezolid
430 resistant (linezolid MIC of 8 mg/liter). Mutations were also detected in the gene for the L22
431 protein which resulted in the amino acid substitution A29V in both isolates. Little is known about
432 the effects, if any, of L22 mutations on linezolid resistance, although it is assumed that L22 plays

433 a role due to its close proximity to the linezolid binding site (64). The presence of distinct *cfr*-
434 carrying plasmids in two ST22-MRSA-IV isolates indicates independent acquisition, and this,
435 combined with mutation-mediated linezolid resistance suggests exposure to linezolid may have
436 played a role in their emergence. Alternatively, since *cfr* encodes resistance to multiple
437 antimicrobial agents, and the co-location of *cfr* on plasmids with other resistance genes in these
438 isolates i.e. *fexA* and *lsa*(B), other antimicrobial agents may provide the selective pressure for the
439 emergence of *cfr*.

440 The identification of *cfr* in two distinct ST22-MRSA-IV strains is alarming. The distinct
441 plasmids identified highlight the ability of *cfr* to spread and to complicate treatment options.
442 Prudent management of linezolid usage is essential to prevent linezolid resistance becoming more
443 widespread.

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Table 1. Phenotypic and genotypic characteristics of the parental, cured and transcojugant derivatives of linezolid resistant ST22-MRSA-IV isolates

Isolate or derivative[a]	CC/ST-SCC*mec* type	*spa* type	*cfr* & *fexA* carriage	$PhLOPS_A$ phenotype[c]	$PhLOPS_A$ agent MICs (mg/liter)[c]							
					LZD	CHL	CLI	TIA	VIR	Resistance to other antimicrobial agents[d]	Other resistance genes[e]	Virulence genes
M12/0145	CC/ST22-MRSA-IV	t032	*cfr* & *fexA*	Yes	64	128	2	>32	>256	AMP, CIP, ERM, FUC, LIN	*blaZ*, *erm*(C), *fexA*	*sec, egc*, IEC (*sak*, *chp* & *scn)*
M12/0145 -C1	CC/ST22-MRSA-IV	t032	None	No	8	0.25	0.5	1	8	AMP, CIP, ERM, FUC, LIN	*blaZ*, *erm*(C), *fexA*	*sec, egc*, IEC (*sak*, *chp* & *scn)*
M12/0145 /XU21-T1	CC8-MSSA	ND	*cfr* & *fexA*	Yes	8	>256	>256	>32	>256	None	*fosB, sdrM*	none
XU21[b]	CC8-MSSA	ND	None	No	1	8	0.25	1	1	None	*fosB, sdrM*	none
M13/0401	CC/ST22-MRSA-IV	t032	*cfr*	Yes	16	>256	>256	>32	>256	AMP, CIP, ERM, FUC, LIN, RIF	*blaZ*, *erm*(C), [*lsa*(B)]	*sec, egc*
M13/0401 -C1	CC/ST22-MRSA-IV	t032	None	No	2	4	0.12	2	8	AMP, CIP, ERM, FUC, LIN, RIF	*blaZ*, *erm*(C)	*sec, egc*

[a]M12/0145 and M13/0401 are the *cfr*-positive parental isolates. Cured derivatives are indicated with "C1" after the parental isolate numbers. The *cfr*- and *fexA*-positive transconjugant derivative M12/0145/XU21-T1 was generated by filter mating using M12/0145 as the plasmid donor and XU21 as the plasmid recipient. XU21 was the plasmid-free recipient strain used in conjugation experiments.

[b]The phenotypic and genotypic characteristics (apart from resistance to antimicrobial agents outside of the $PhLOPS_A$ phenotype) of the plasmid-free *S. aureus* recipient strain XU21 were determined in a previous study (20).

[c]Resistance to phenicols (CHL, chloramphenicol), lincosamides (CLI, clindamycin), oxazolidinones (LNZ, linezolid), pleuromutulins (TIA, tiamulin) and streptogramin A compounds (VIR, virginiamycin) is indicative of the $PhLOPS_A$ phenotype.

[d]The resistance of each isolate was also determined to the following antimicrobial agents: amikacin; AMP, ampicillin; cadmium acetate; CIP, ciprofloxacin; ethidium bromide; ERM, erythromycin; gentamicin; kanamycin; LIN, lincomycin; mercuric chloride; mupirocin; neomycin; phenyl mercuric acetate; RIF, rifampicin; sulphonamide; tetracycline; tobramycin; trimethoprim; vancomycin.

[e]All resistance genes, apart from *lsa*(B) which is indicated in square brackets, were detected by DNA microarray profiling using the StaphyType Kit (Alere). *lsa*(B) was detected in isolate M13/0401 in close proximity to *cfr* from the whole-genome sequence.

Other abbreviations: CC, MLST clonal complex; ST, sequence type; SCC*mec*, staphylococcal cassette chromsome *mec*; IEC, immune evasion complex; ND, not determined.

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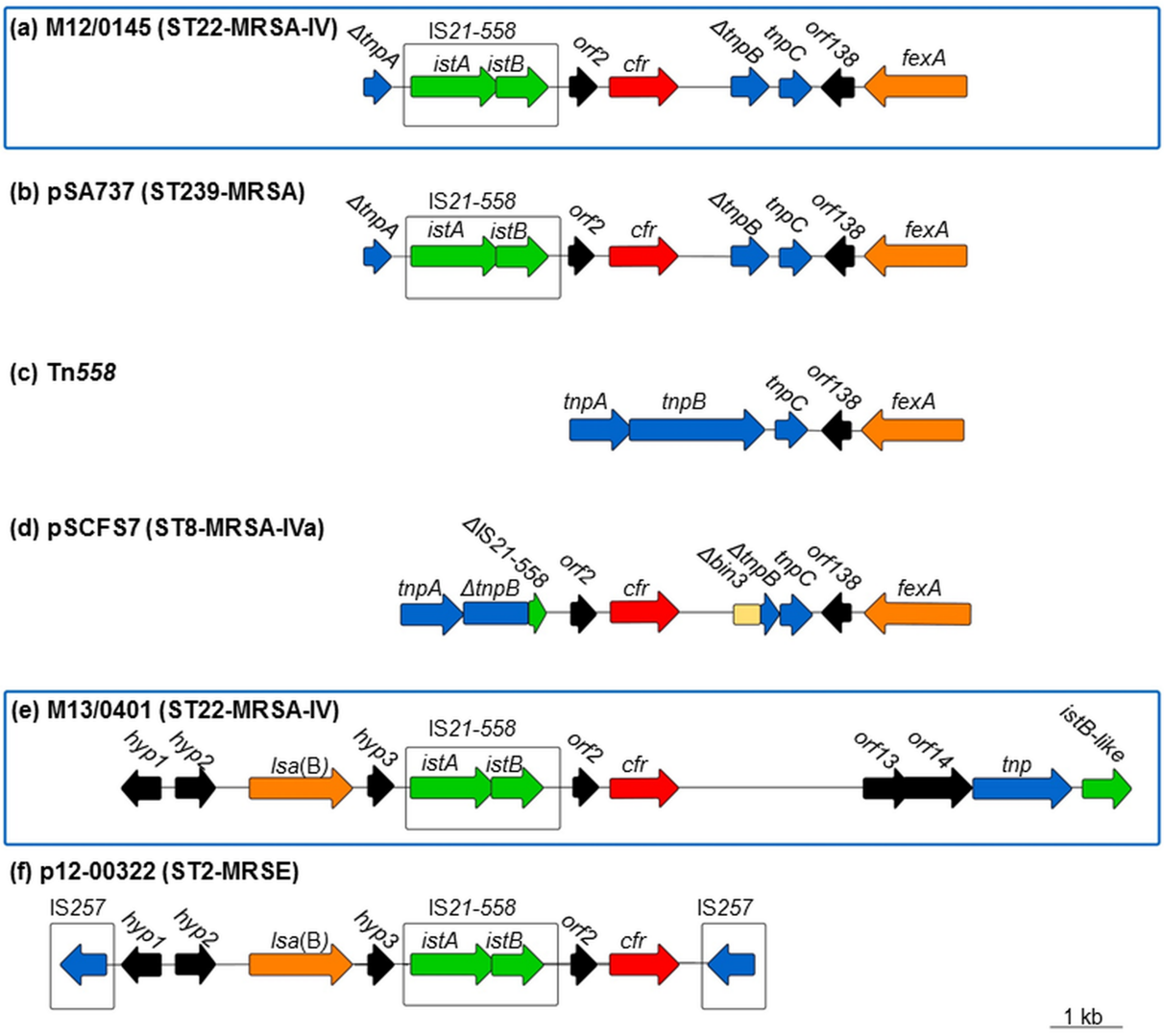
FIGURE LEGEND

Figure 1. Schematic representation of the *cfr*-containing regions of the ST22-MRSA-IV isolates (a) M12/0145 and (e) M13/0401 identified in the present study (surrounded by a box (blue outline)) and previously described comparator plasmids and transposons (b) pSA737 (Genbank accession number KC206006) (29, 30), (c) Tn*558* (AJ715531) (4), (d) pSCFS7 (FN995111, FN995110 and FR675942) (20) and (f) p12-00322 (KM521836) (59). Arrows indicate the direction of transcription of each open reading frame (ORF). Insertion sequence elements are surrounded by a box (black outline). Each gene or group of genes are represented by a different color shading i.e. red, *cfr*; orange, antibiotic resistance genes other than *cfr*; green, IS*21-558* transposition genes; blue, other transposition genes; black, genes encoding hypothetical proteins. Horizontal lines between ORFs indicate intergenic regions.

Figure 1


(a) M12/0145 (ST22-MRSA-IV)
ΔtnpA
IS21-558
istA
istB
orf2
cfr
ΔtnpB
tnpC
orf138
fexA
(b) pSA737 (ST239-MRSA)
ΔtnpA
IS21-558
istA
istB
orf2
cfr
ΔtnpB
tnpC
orf138
fexA
(c) Tn558
tnpA
tnpB
tnpC
orf138
fexA
(d) pSCFS7 (ST8-MRSA-IVa)
tnpA
ΔtnpB
ΔIS21-558
orf2
cfr
ΔtnpB
Δbin3
tnpC
orf138
fexA
(e) M13/0401 (ST22-MRSA-IV)
hyp1
hyp2
lsa(B)
hyp3
IS21-558
istA
istB
orf2
cfr
orf13
orf14
tnp
istB-like
(f) p12-00322 (ST2-MRSE)
IS257
hyp1
hyp2
lsa(B)
hyp3
IS21-558
istA
istB
orf2
cfr
IS257
1 kb

Supplemental Table S1. Primers used to confirm the genetic organization and orientation of the *cfr* region in the ST22-MRSA-IV isolates M12/0145 and M13/0401

Isolate no.	Gene/region amplified	Primer name	Nucleotide sequence (5'-3')	Nucleotide coordinates[a]	Amplimer size (bp)
M12/0145	*ΔtnpA - cfr*	tnpAF	GGTTCAGAAAGTAATTGTGGAGGA	31961-31984	4400
		cfrR	CCTATAATTGACCACAAGC	36343- 36361	
	cfr - tnpC	cfrF	GACTTTCGGCACCGGTAAT	35243-35261	2887
		tnpCR	GTTCATTCTCTTCTTCTAAGGCCTT	38106-38130	
	tnpC - fexA	tnpCF	CAGCTAGCTAAAGACAAGTCGGA	37842-37864	2781
		fexAR	GAGAACCGAATCTTTAATCA	40604-40623	
M13/0401	*orf1 – orf7*	orf1F	CAGTCATAGGCACACAAAC	776-794	8350
		orf7R	GCAACCAGTCAACAAGATC	9108-9126	
	ssaA - intergenic region between *ssaA* & *hyp1*	ssaAF	GGTAACTATGACAGACGGTTATAGC	9013-9037	3098
		intssaAR	GCTATATTGTGGCTC TGC	12096-12111	
	Intergenic region between *ssaA* & *hyp1* - *hyp1*	intssaAF	GGACAATTGCCATTAACG	11813-11830	3155
		0401hyp1R	CCTTTTGCATATCCCTAC	14951-14968	
	hyp1 – intergenic region between *hyp* and *istA*	HypF	CCAGCTGTTTAATTGGTTG	14801-14819	2530
		IntR	CGATATATTTGGATACGTG	17313-17331	

Intergenic region between *hyp* and *istA* - *istB*	BPF	GGAAAACGAGGAGTGATTACG	17205-17225	2238
	istBSR	CGATTTATGCGTCAAGC	19427-19443	
istB - *cfr*	istBSF	CCTCAACCATTATTACGAGC	19340-19359	1746
	cfrR	CCTATAATTGACCACAAGC	20168-21086	
cfr – o*rf1*	cfrF1	GACTTTCGGCACCGGTAAT	19967-19985	8539
	CR2	CCTTTATTCGCTCTTACATCACG	982-1004	

[a]Nucleotide coordinates based on the nucleotide sequence of the *cfr* region (*ΔtnpA – fexA*) in M12/0145 and the entire plasmid in M13/0401 (Genbank accession no. X and X, respectively; accession numbers pending).

Supplemental Table S2. *cfr*-containing pSA737- and pSCFS3-type plasmids previously identified in staphylococci from animals and humans showing a similar genetic organization to the *cfr* region identified in M12/0145 in the present study[a]

Plasmid name	Region sequenced	Nucleotide sequence identity to other *cfr* regions	Staphylococcal species	Genotype (*n*)[a]	Host	Year of isolation	Country of origin	Genbank accession no.	Reference
pSA737	Entire plasmid	99.7% to pSCFS3	MRSA	ST239-t037	Human clinical	2007	USA	KC206006	(1, 2)
p2823634	5.5 kb IS21-558 to Δ*tnpB*	100% to pSA737	MRSA	USA300	Human clinical	2011	USA	KJ819951	(3)
p2823586	5.5 kb IS21-558 to Δ*tnpB*	100% to pSA737	MRSA	USA300	Human clinical	2011	USA	KJ819952	(3)
p2823605	5.5 kb IS21-558 to Δ*tnpB*	100% to pSA737	MRSA	USA300	Human clinical	2011	USA	KJ819953	(3)
pSCFS3	9.5 kb Δ*tnpA* to *fexA*	99.7% to pSA737	*Staphylococcus aureus*	NA	Porcine respiratory tract infection	2000	Germany	AM086211	(4)
pSCFS3-type	*cfr*-containing BglII fragments	ND (similar *cfr* region to pSCFS3)	MRSA	ST398-t034	Porcine nares	2007	Germany	NA	(5)
pSCFS3-type	*cfr*-containing BglII fragments	ND (similar *cfr* region to pSCFS3)	MSSA	ST9-t3198	Porcine nares	2007	Germany	NA	(5)
pSEPI8573/pSE1243	Entire plasmid	100% to pSA737	MRSE	ND	Human clinical	2008-‘09	USA	KC222021	(2)
pHNTLD18	5.7 kb EcoRI *cfr* fragment	100% to pSA737	*Staphylococcus equorum*	NA	Retail meat	2012	China	KF751702	(6)
pSS-02	14 kb *cfr* region	99.8% to pSCFS3	*Staphylococcus saprophyticus* & *Staphylococcus sciuri*	NA	Porcine nares	2010	China	JF834910	(7)
pSS-02-type	14 kb *cfr* region	100% to pSS-02	*Staphylooccus haemolyticus* & *Staphylococcis cohnii*	NA	Human clinical blood culture	2009-‘10	China	JX827253	(8)

pHNCR35	10 kb *radC* to *fexA*	ND	*Staphylococcus simulans*	NA	Human hog market worker	NA	China	KF861983	Unpublished Genbank accession no. KF861983.1
pSS-02-type	*cfr* flanking regions	ND (similar *cfr* region to pSCFS3)	MRSA	ST627-t002-dt12w-IVb (3); ST6-t304-dt12w-IVb (2); ST63-MRSA-t899-dt12v-IVb (1)	Porcine nares & lungs	2012/13	China	NA	(9)

[a]The genetic organization of pSA737- and pSCFS3-type plasmids consists of **Δ***tnpA*-IS21-558 (*istAS* & *isaBS*)-*cfr*-**Δ***tnpB-tnpC-orf138-fexA*.
[b]Where available, multilocus sequence types, *spa* types and *dru* types are indicated with the prefixes ST, t and dt, respectively. USA300 genotype was determined by pulsed-field gel electrophoresis. Where available SCC*mec* types are indicated with roman numerals and subtypes with alphabetic designations. *n*, number of isolates and is only indicated where more than one isolate was identified.
NA, not applicable; ND, not determined.

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Supplemental Table S3. Details of predicted open reading frames (ORFs) identified within the *cfr* plasmid of M13/0145 exhibiting amino acid identity to proteins involved in horizontal gene transfer

ORFs	Closest similiarity (Genbank accession no.)	% amino acid identity (query coverage)	Conserved protein domain family	Function
1	TraG bacilli(WP_021038275.1)	38% (98%)	SXT_TraD	Conjugal transfer protein
2B	SAPIG1862 staphylococci (WP_031882362.1)	32% (87%)	TcpC	Conjugative transposon protein
3	pGIAK1_5 bacilli (AGQ45426.1)	37% (96%)	TcpE	Putative conjugative transposon membrane protein
4	VirB4 family protein bacilli (WP_021038260.1)	39% (97%)	MYSc_Myo14	Conjugal transfer and type IV secretion systems
10	Ssb *Staphylococcus aureus* (WP_012818034.1)	36% (84%)	ssb	Binding of single stranded DNA