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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: OD2030
2. Project title: Investigation of a novel Staphyloccus aureus isolate
3. Contractor organisation(s): University of Cambridge
4. Total Defra project costs (agreed fixed price): £ 52,707
5. Project: start date..... 01 September 2009
   end date..... 31 March 2010

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

This project was initiated following the discovery of a divergent *mecA* gene (a gene associated with methicillin resistant *Staphylococcus aureus*, MRSA) in *S. aureus* bacteria isolated from milk from dairy cows.

The project had two main objectives. Firstly to identify the familial relationships or phylogeny between the different isolates using multi-locus strain typing, spa typing and RAPD-PCR grouping. From this information a form of family tree (a dendrogram) was constructed. The second objective was to attempt to show that the divergent *mecA* gene was responsible for the antibiotic resistance of the bacteria that carried the gene by inserting it into a methicillin susceptible *S. aureus* (MSSA) using genetic engineering techniques and by deleting it to demonstrate the reversion to susceptibility.

The phylogenetic study showed that there were 5 separate branches in the dendrogram populated by *S. aureus* carrying the divergent *mecA* gene. The majority of these branches, or strain types, had already been associated with bovine adapted *S. aureus* isolates. No human associated *S. aureus* strain types were identified.

Initially it was planned to attempt to insert the gene by transduction of the divergent *mecA* locus from the bovine MRSA isolate into a susceptible *S. aureus* using phage transduction. During infection of bacteria by certain bacteriophages viral packaging mechanisms may incorporate bacterial genetic material into the new virion which can then be transferred to other bacteria during subsequent infections. Unfortunately the generalised transducing phage we use did not plaque well on the divergent *mecA* strain and it was not possible to make a good transducing lysate.

Attempts were then made to create a deletion mutant (or knockout) by homologous recombination. In order to achieve this 2Kb sequences of DNA upstream and downstream of the divergent *mecA* gene (but not including the *mecA* gene) were

amplified by PCR and flanked with AttB sites. These sequences were then combined with an erythromycin resistance gene. This construct was then inserted into a temperature sensitive plasmid possessing a chloramphenicol resistance gene and the plasmid used to transform *E. coli* in order to be grown up in volume. The plasmid was then used to transform *S. aureus* RN4220 (a MSSA) from which the plasmid, now habituated to *S. aureus*, was prepared for transfer to the divergent *mecA* bovine *S. aureus* isolate using electroporation. During cell division plasmid sequences will have been incorporated into the staphylococcal DNA and the transformed *S. aureus* was then cultured at 42°C to promote loss of the plasmid. Individual colonies were then subcultured for parallel growth on chloramphenicol and erythromycin. Parent colonies whose daughter colonies grew on erythromycin but not on chloramphicol would be expected to have lost their plasmids and incorporated the erythromycin resistance gene in place of their divergent *mecA* gene. Subsequent assessment of the β-lactam resistance of these colonies showed that they retained their MRSA phenotype. Although PCRs confirmed the insertion of the erythromycin resistance gene, a Southern blot showed that the divergent *mecA* gene was retained. This was probably due to an insertion of the construct by a single crossover event.

As an alternative experiment to demonstrate that presence of the divergent *mecA* was associated with β-lactam resistance, an attempt was made to insert a divergent *mecA* gene into a MSSA (RN4220) using the same technology as described above. In this experiment there was no increased β-lactam resistance of the recombinant *S. aureus* RN4220, compared to control *S. aureus* RN4220 without the recombinant divergent *mecA* gene. While a positive result would have represented good evidence for the association, a negative result may just mean that the background genome (RN4220), into which the divergent *mecA* gene was inserted, is incompatable with divergent *mecA*.

The main implications of these findings are that the divergent *mecA* is found in a variety of strain types (and over a wide geographical distribution) which implies that it confers some advantage to the bacteria. The divergent *mecA* gene is accompanied by other genes in a mobile genetic element which may confer antibiotic resistance in their own right, or in conjunction with the divergent *mecA* gene. Further research is required to investigate the mechanisms leading to antibiotic resistance equivalent to MRSA.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
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   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Background

We recently discovered a divergent *mecA* gene carried by a novel SCCmec in a bovine MRSA. This strain (LGA251)[1-3] demonstrated the phenotypic characteristics of a heterogeneously resistant MRSA. Initial investigations revealed that it was resistant to cefoxitin and oxacillin by a disc diffusion assay for all *in vitro* conditions tested (different temperatures and salt concentrations). The strain tested negative for hyper-production of penicillinases by oxacillin and cefoxitin disc diffusion tests with adjacent amoxicillin-clavulanic acid discs (appropriate positive and negative control strains behaved as expected). The minimum inhibitory concentrations (MIC) values for cefoxitin and oxacillin determined by both agar dilution, and E-test methods, were 32 mg/L and 16 mg/L respectively. The isolate grew on MRSA ID agar, appearing as green colonies but PBP2a was not detected by the slide latex agglutination test (Mastalex). A multiplex PCR assay using primers designed to detect *mecA* and *femB* genes, following a published protocol[4] yielded negative results for the presence of the *mecA* gene and a positive result for *femB*. A further PCR designed to detect the SCC*mec-orfX* junction in MRSA, also produced a negative result[5]. To investigate the genetic basis for the β-lactam resistant phenotype, the entire genome of the strain was sequenced in collaboration with the Wellcome Trust Sanger Institute. A novel *mecA* gene and SCC*mec* operon were identified, together with regulatory genes (*mecI* and *mecR1*), genes for β-lactamase, an arsenical resistance operon, and site-specific recombination genes (*ccrA* and *ccrB*). LGA251 had a novel *spa* type and was characterised as ST425. Further bulk milk samples have been taken from the original discovery farm on a number of occasions since the original isolation in 2007, most recently in January 2010. On each occasion *S. aureus* carrying the divergent *mecA* gene were isolated from culture of bulk milk, demonstrating that MRSA can persist on dairy farms for many years.

CDS	"Top hits" in UniProt (matches)	Similarity %	Query position	Query length	Match position	Match length
blaZ	*Staphylococcus aureus* (USA300/TCH1516 strain) plasmid	68	1-279	283	1-279	281
mecA	*S. aureus* [BAB47623.1]	63	1-665	665	1-668	668
	S. epidermidis [AAW53314.1]	63	1-665	665	1-668	668
	S. sciuri [CAA73547.1]	63	1-665	665	1-668	668
	S. vitulinus [CAJ15578.2]	63	1-665	665	1-668	668
	S. kloosii [CAJ15579.2]	63	1-665	665	1-668	668
	S. pseudointermedius [CAP17722]	63	1-665	665	1-668	668
mecR1	*S. aureus* [CAG39069.1]	44	1-583	584	1-585	585
	S. sciuri [CAA73546.1]	44	1-583	584	1-585	585
	S. saprophyticus [BAG24378]	44	1-583	584	1-585	585
	S. pseudointermedius [CAP17721]	44	1-583	584	1-585	585
mecI	*S. pseudointermedius* [CAP17749]	67	1-123	124	1-123	123
	S. aureus [CAA74374.1]	66	1-123	124	1-123	123
	S. epidermidis [AAW53312.1]	66	1-123	124	1-123	123
ccrA	*S. saprophyticus* [BAG24390.1]	86	1-449	449	1-449	449
	S. aureus [AAW38691.1]	84	1-448	449	1-448	449
ccrB	*S. aureus* [BAB46972.1]	92	1-542	542	1-542	542
	S. pseudointermedius [CAP17737]	92	1-542	542	1-542	542

Table 1: Coding sequences (CDS) identified within the novel staphylococcal cassette chromosome mec, together with the "top hits" [EMBL number] retrieved from the Universal Protein Resource (UniProt) as a result of a BLAST search (protein level), and the similarity percentages between the query amino acid sequence and the "top hit".

A second, closely related strain possessing the divergent *mecA* gene has been isolated on the discovery farm. The second strain has a similar phenotype and the same MLST strain type but

possesses a different *spa* type and RAPD-PCR strain group. Unless otherwise stated all results refer to the LGA251 strain.

The SCC*mec* sequence data was submitted to Teruyo Ito, Keiichi Hiramatsu, Herminia de Lencastre and colleagues on the SCC*mec* nomenclature committee. They reviewed the divergent *mecA* data and agreed that it was a *mecA* allotype, although quite distant from the *mecA* normally associated with methicillin resistance. They suggested that as the *ccr* genes were a novel combination of *ccrAB* genes, *ccrA1ccrB3*, it should be regarded to be type-8 *ccr*. The SCC*mec* containing the divergent *mecA* gene is comparable to a previously unique *mec* gene complex *mecI-mecR1-mecA-blaZ*, which was first identified in a plasmid carried by a genome sequenced *Macrococcus caseolyticus*. It was suggested that its SCC*mec* type was also novel and was designated type XI.

The existence of this new *mecA* gene raises important questions about the potential transfer of resistance genes between pools of human and animal pathogens, and the development of antibiotic resistance in farm animals. The Veterinary Laboratories Agency (VLA) recently completed a study for the Department for Environment Food and Rural Affairs (Defra) which reported a zero prevalence of MRSA isolated from dairy cattle in England and Wales as determined by detection of the *mecA* gene by PCR (Report OD2020[6]). The study included a survey of antibiotic resistance in *S. aureus* isolates collected from cattle in 2006/7 looking at a total of 940 confirmed *S. aureus* isolates, recovered from bovine clinical mastitis samples from 465 herds. These were tested to determine the antimicrobial susceptibility pattern and presence of the *mecA* gene. All of these isolates were *mecA*-negative by conventional PCR. Of these, 2.6% (n=24 isolates) presented oxacillin MICs exceeding 2 mg/L, of which 1.1% (11 isolates) were resistant to cefoxitin by disc diffusion[6]. When the 24 isolates were examined for the presence of the divergent *mecA,* over 50% (13 isolates) were shown, by PCR and sequencing, to possess the divergent *mecA* gene.

We recently tested an isolate cultured by the HPA laboratory from a human patient in Cambridge that demonstrated phenotypic MRSA-like resistance but tested negative for *mecA* by PCR using conventional primers. Using primers for the divergent *mecA* this strain tested positive; sequencing of the PCR product revealed an identical sequence for the *mecA* gene in this isolate to the sequence found in the discovery strain. This human isolate came from an anonymous panel of 500 MRSA that was assembled to validate a *mecA* PCR assay. It is not known if its isolation was as a result of carriage or infection or whether the person had been in contact with livestock.

Scientific Objectives.

The proposed work attempted to complete two tasks: 1. The strain typing of up to 24 phenotypically resistant isolates (including the 13 isolates that carry the divergent *mecA* gene) together with phylogenetic analysis, and 2. The production and phenotypic testing of a laboratory generated mutant of *S. aureus* from which the divergent *mecA* has been deleted. In addition to the contracted work *spa* typing was undertaken on all isolates carrying the divergent *mecA* gene.

Details of the Methods Used, Results Obtained and the Extent to which Objectives have been met.

1. Strain typing of divergent mecA isolates and phylogenetic analysis

Multi-locus sequence typing (MLST) was performed on the VLA isolates together with Random Amplified Polymorphic DNA (RAPD)-PCR.

(a) MLST typing methodology

Typing of isolates by MLST consisted of two distinct steps: amplification of internal fragments of seven housekeeping genes for *S. aureus* by PCR (amplicon length ~500bp); and sequencing of approximately 450bp fragments of each of the amplified genes on both strands[7].

The seven housekeeping genes and the primer sequences used are described in the table below. One base change (G to A) on the sequence of the original *gmk*-Up primer was introduced to achieve an exact match with the corresponding region on published *S. aureus* strains.

Genes	Full name	Primer	Sequence (5' to 3')
arcC	Carbamate kinase	*arcC*-Up	TTG ATT CAC CAG CGC GTA TTG TC
		arcC-Dn	AGG TAT CTG CTT CAA TCA GCG
aroE	Shikimate dehydrogenase	*aroE*-Up	ATC GGA AAT CCT ATT TCA CAT TC
		aroE-Dn	GGT GTT GTA TTA ATA ACG ATA TC
glpF	Glycerol kinase	*glpF*-Up	CTA GGA ACT GCA ATC TTA ATC C
		glpF-Dn	TGG TAA AAT CGC ATG TCC AAT TC
gmk	Guanylate kinase	*gmk-Up'*	ATC GTT TTA TC[A] GGA CCA TC
		gmk-Dn	TCA TTA ACT ACA ACG TAA TCG TA
pta	Phosphate acetyltransferase	*pta*-Up	GTT AAA ATC GTA TTA CCT GAA GG
		pta-Dn	GAC CCT TTT GTT GAA AAG CTT AA
tpi	Triosephosphate isomerase	*tpi*-Up	TCG TTC ATT CTG AAC GTC GTG AA
		tpi-Dn	TTT GCA CCT TCT AAC AAT TGT AC
yqiL	Acetyl CoA acetyl-transferase	*yqiL*-Up	CAG CAT ACA GGA CAC CTA TTG GC
		yqiL-Dn	CGT TGA GGA ATC GAT ACT GGA AC

Table 2: Primer sequences used for the MLST of *S. aureus* isolates.

The melting temperature (Tm) of the primers ranges from 56ºC to 65ºC. The optimal PCR annealing temperature in our laboratory was determined experimentally to be 59ºC.

A negative control with no target DNA and a positive control with DNA from a *S. aureus* type colony was included in each PCR run.

Sequencing of the fragments of the amplified housekeeping genes was performed using a protocol provided by Dr Keith Jolley (Department of Zoology, Oxford University, UK; Personal communication). Cleaned pellets of amplified DNA on plates were sequenced using facilities at the University of Cambridge.

Sequence data was processed using the Staden Bioinformatics Package. The *S. aureus* MLST database (http://saureus.mlst.net/) was interrogated to obtain the allelic profiles and sequence types of the isolate.

(b) RAPD-PCR methodology

In this PCR strain typing method, segments of DNA are randomly amplified using several arbitrary, short primers (9-10 nucleotides), against a large template of genomic DNA. Gel electrophoresis is then used to examine the resulting PCR products. The resulting patterns of bands on the gels provide distinct strain profiles.

Primer sequences (shown below) and optimised cycling parameters were established in this laboratory based on a technique described by Pereira et al.[8]. Three different primers are used to increase the discriminatory power.

Primer	Sequences (5' to 3')
786	GCG ATC CCC A
797	AGC GTC ACT G
798	TGA CCC GCC

During previous work with *S. aureus* strains isolated from bulk milk work in Cambridge using these three primers was found to be more discriminatory than MLST.

(c) Phylogenetic Analysis methodology

Phylogenetic analysis was used to identify the relationships between *S. aureus* strains carrying the divergent *mecA* and other human and animal strains based on their MLST ST and their RAPD strain groupings using MEGA (Molecular Evolutionary Genetics analysis) version 4.0[9]. An unrooted phylograms was constructed using the Neighboor Joining distance method[10] with the evolutionary distances computed using the Maximum Composite Likelihood model[11]. The nucleotide sequences for each MLST locus were aligned. The aligned sequences for the seven loci were then concatenated in the order arcC, aroE, glp, gmk, pta, tpi and yqiL. A 3,198-bp sequence was produced for each ST to provide a basis for the analysis.

(d) Strain typing and phylogeny results

Typing of the 24 bovine isolates by MLST revealed the presence of different STs and clones among this group of isolates (results shown in Table 3 below). The thirteen divergent *mecA*-positive *S. aureus* isolates were characterised as ST130 (n=1), ST151 (n=1), ST425 (n=1), ST1526 (n=1), and ST1245 (n=9). These bovine strains were identified from samples submitted to different VLA regional laboratories, which were widely distributed across the country (Figure 1, map showing ST distribution). *Spa* typing also identified a variety of strains within this collection of bovine isolates (shown in table 4). The remaining eleven bovine isolates, which were *mecA*-negative, were typed as ST97 (n=5), ST118 (n=1), ST151 (n=3), ST1074 (n=1) and ST1527 (n=1). RAPD-PCR analysis revealed 7 different RAPD groups. RAPD and *spa* typing were only performed on divergent *mecA* positive isolates.

One of the VLA *mecA* positive isolates was the same strain type as the discovery strain (LGA251, ST425), and shared a *spa* type with a second divergent *mecA* positive strain isolated from the discovery farm (LGA254, t6292). However the VLA ST425 strain C02 937 could be distinguished from LGA254 by RAPD-PCR grouping. Interestingly the VLA strain was submitted to Langford VLA which is the nearest VLA location to the discovery farm.

The human divergent *mecA* isolate (found in Cambridge), strain type (ST130), had the same strain type as a divergent *mecA* positive isolate submitted to VLA Bury St Edmunds. However the two isolates had different *spa* types and RAPD groups.

One strain type, ST151, a relatively common bovine lineage, was represented in both the *mecA* positive and negative isolates.

As expected both RAPD-PCR and spa typing were more discriminatory than MLST.

	arc	*aro*	*glp*	*gmk*	*pta*	*tpi*	*yqi*	**ST**
VLA strains								
C02 467: divergent *mecA* +ve	6	57	45	2	7	58	52	**130**
C02 937: divergent *mecA* +ve	18	33	6	20	7	50	48	**425**
C03 125: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 362: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 363: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 364: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 365: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 366: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 367: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 370: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C03 371: divergent *mecA* +ve	6	193	45	2	7	58	52	**1245**
C04 288: divergent *mecA* +ve	6	229*	45	2	7	58	52	**1526***
C04 831: divergent *mecA* +ve	6	72	12	43	49	67	59	**151**
C02 737: divergent *mecA* -ve	3	1	208*	1	1	5	3	**1527***
C02 936: divergent *mecA* -ve	3	1	1	1	1	5	3	**97**
C03 124: divergent *mecA* -ve	3	1	1	1	1	60	3	**118**
C03 134: divergent *mecA* -ve	6	72	145	43	49	67	59	**1074**
C03 189: divergent *mecA* -ve	6	72	12	43	49	67	59	**151**
C03 611: divergent *mecA* -ve	3	1	1	1	1	5	3	**97**
C04 164: divergent *mecA* -ve	3	1	1	1	1	5	3	**97**
C04 824: divergent *mecA* -ve	3	1	1	1	1	5	3	**97**
C04 847: divergent *mecA* -ve	6	72	12	43	49	67	59	**151**
C04 859: divergent *mecA* -ve	3	1	1	1	1	5	3	**97**
C05 232: divergent *mecA* -ve	6	72	12	43	49	67	59	**151**
Human Isolate								
SA227: divergent *mecA* +ve	6	57	45	2	7	58	52	**130**
Discovery Isolates								
LGA251: divergent *mecA* +ve	18	33	6	20	7	50	48	**425**
LGS254: divergent *mecA* +ve	18	33	6	20	7	50	48	**425**

Table 3: Results of multi-locus sequence typing of 24 phenotypically oxacillin resistant *S. aureus* strains collected by the VLA together with three other isolates also found to carry the divergent *mecA* gene. Where the allele or sequence type is a new addition to the database it is indicated by an asterisk (*).

VLA strains	spa type	spa repeats
C02 467: divergent mecA-positive	t6220	r04-r82-r17-r25-r17-r25-r25-r17
C02 937: divergent mecA-positive	t6292*	r14-r44-r12-r17-r23-r18-r110-r17-r17-r17-r23-r24
C03 125: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 362: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 363: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 364: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 365: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 366: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 367: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 370: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C03 371: divergent mecA-positive	t843	r04-r82-r17-r25-r17-r25-r25-r16-r17
C04 288: divergent mecA-positive	t6293*	r04-r82-r17-r24-r25-r25-r16-r17
C04 831: divergent mecA-positive	t529	r04-r34
Human Isolate		
SA227: divergent mecA-positive	t1736	r04-r82-r17-r25-r16-r17
Discovery Isolates		
LGA251: divergent mecA-positive	t6300*	r14-r44-r12-r17-r23-r18-r110-r17-r17-r23-r24
LGS254: divergent mecA-positive	t6292*	r14-r44-r12-r17-r23-r18-r110-r17-r17-r17-r23-r24

Table 4: Results of *spa* typing of the 13 *S. aureus* strains carrying divergent *mecA* gene collected by the VLA together with three other isolates also found to carry the divergent *mecA* gene. Where the *spa* type is a new addition to the database it is indicated by an asterisk (*).

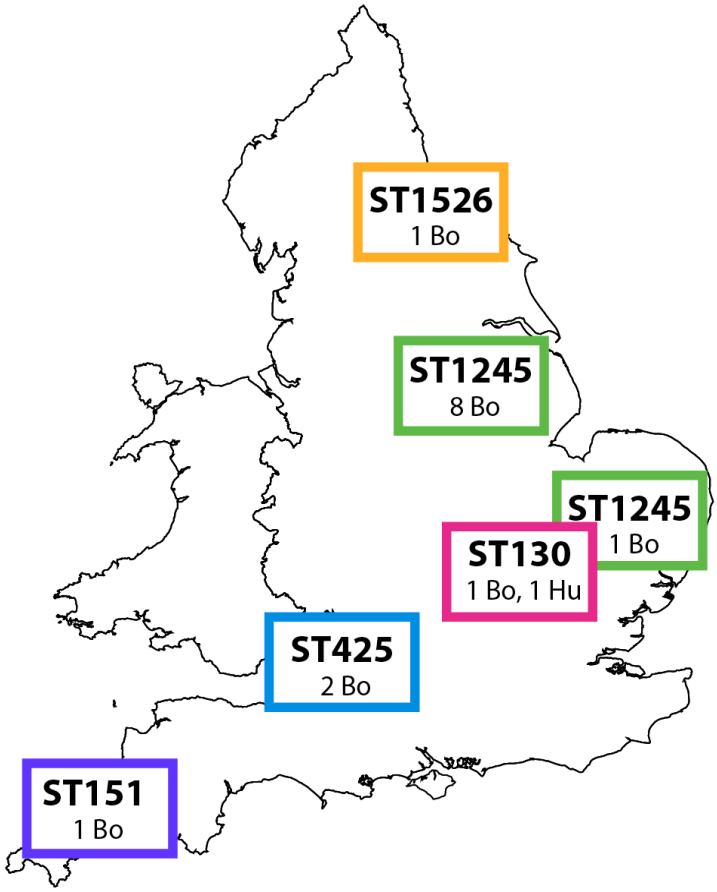

Figure 1. Map of the UK indicating the approximate locations from which different *S. aureus* sequence types possessing divergent *mecA* originated (Bo=bovine, Hu=human).

VLA strains	786	797	798	**Grouping**
C02 467: divergent mecA-positive	1	1	1	**1**
C02 937: divergent mecA-positive	2	2	2	**2**
C03 125: divergent mecA-positive	3	1	2	**3**
C03 362: divergent mecA-positive	3	1	2	**3**
C03 363: divergent mecA-positive	3	1	2	**3**
C03 364: divergent mecA-positive	3	1	2	**3**
C03 365: divergent mecA-positive	3	1	2	**3**
C03 366: divergent mecA-positive	4	1	3	**4**
C03 367: divergent mecA-positive	4	1	3	**4**
C03 370: divergent mecA-positive	3	1	2	**3**
C03 371: divergent mecA-positive	3	1	2	**3**
C04 288: divergent mecA-positive	6	1	2	**5**
C04 831: divergent mecA-positive	5	3	4	**6**
Human Isolate				
SA227: divergent mecA-positive	2	2	5	**7**
Discovery Isolates				
LGA251: divergent mecA-positive	1	1	2	**8**
LGA254: divergent mecA-positive	2*	1*	2*	**9***

Table 4: Results of RAPD-PCR grouping of the 13 *S. aureus* strains carrying divergent *mecA* gene collected by the VLA together with three other isolates also found to carry the divergent *mecA* gene. For each primer (786, 797 and 798) a band pattern is evident; the combination of patterns for the three primers allows the grouping of each isolate to be established (its strain group). *The RAPD-PCR grouping of LGA254 was performed from a separate set of gels not including the VLA strains but with the same control strains.

It is of interest to note that three strain types 130, 1245 and 1526 differed only in their *aroE* alleles, and therefore all belong to the same clonal complex. STs 130 and 1245 have previously been identified as bovine associated STs. The human divergent *mecA* isolate was also in this group (ST130). Geographically all these isolates were found on the eastern side of England (Yorkshire, Nottinghamshire, Suffolk and Cambridgeshire). Interestingly the nearest related strain type in the database is ST398 which is responsible for zoonotic MRSA disease in continental Europe and has been found in a variety of species including mastitic dairy cows.

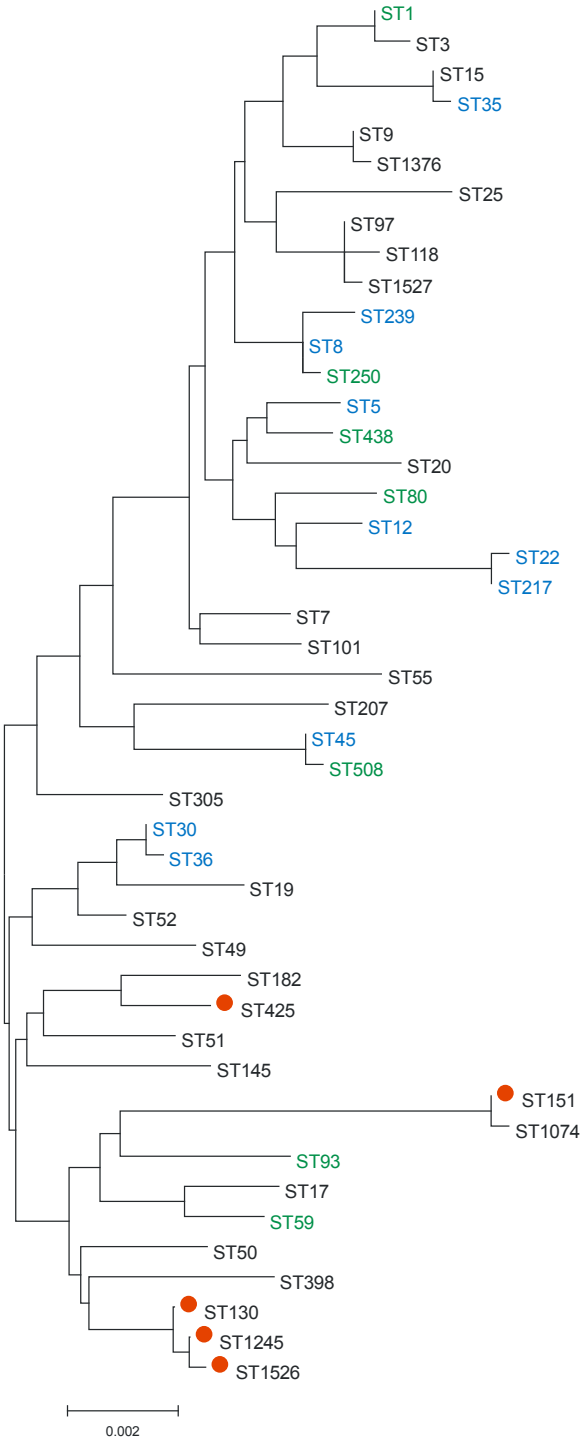

Figure 2. Evolutionary relationships of 46 *S. aureus* strains based on their MLST sequence types. The tree was constructed using the Neighbour-Joining method[10]; it was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method[12], and are in the units of the number of base substitutions per site. Orange spots indicate STs in which divergent *mecA* has been found. Green text indicates STs associated with CA-MRSA strains; blue text indicates STs associated with HA-MRSA strains.

2. Production of a laboratory generated mutant of *S. aureus* from which the divergent *mecA* has been deleted.

This laboratory work was performed at Nottingham University under the supervision of Dr. Phil Hill.

Initially it was planned to attempt to insert the gene by transduction of the divergent *mecA* locus from the bovine MRSA isolate into a susceptible *S. aureus* using phage transduction. During infection of bacteria by certain bacteriophages viral packaging mechanisms may incorporate bacterial genetic material into the new virion which can then be transferred to other bacteria during subsequent infections. Unfortunately the generalised transducing phage we use did not plaque well on the divergent *mecA* strain and it was not possible to make a good transducing lysate. Therefore two further approaches were used to see if the methicillin resistance of this strain was due to the presence of the divergent *mecA* gene:

(a) We attempted to create a deletion mutant (or knockout) by homologous recombination. In order to achieve this 2Kb sequences of DNA upstream and downstream of the *mecA* gene (but not including the *mecA* gene) were amplified by PCR and flanked with AttB sites. These sequences were then combined with an erythromycin resistance gene. This construct was then inserted into a temperature sensitive plasmid possessing a chloramphenicol resistance gene and the plasmid used to transform *E. coli* in order to be grown up in volume. The plasmid was then used to transform *S. aureus* RN4220 (a MSSA) from which the plasmid, now habituated to S. aureus, was prepared for transfer to the divergent *mecA* bovine S. aureus isolate using electroporation. During cell division plasmid sequences will have been incorporated into the staphylococcal DNA and the transformed *S. aureus* was then cultured at 42°C to promote loss of the plasmid. Individual colonies were then subcultured for parallel growth on chloramphenicol and erythromycin. Parent colonies whose daughter colonies grew on erythromycin but not on chloramphicol would be expected to have lost their plasmids but incorporated the erythromycin resistance gene in place of their *mecA* gene. Subsequent assessment of the β-lactam resistance of these colonies showed that they retained their MRSA phenotype. Although PCRs confirmed the insertion of the erythromycin resistance gene, a Southern blot showed that the divergent *mecA* gene was retained. This was probably due to an insertion of the construct by a single crossover event.

A schematic representation of this experiment is shown in Figure 2 to show the effect of a single crossover (an unintended outcome) and a double crossover (the expected outcome). Although the single crossover would have incorporated a single copy of the chloramphenicol resistance gene this would still have appeared to be chloramphenicol sensitive compared to bacteria retaining the plasmid, which would have possessed multiple copies of the gene.

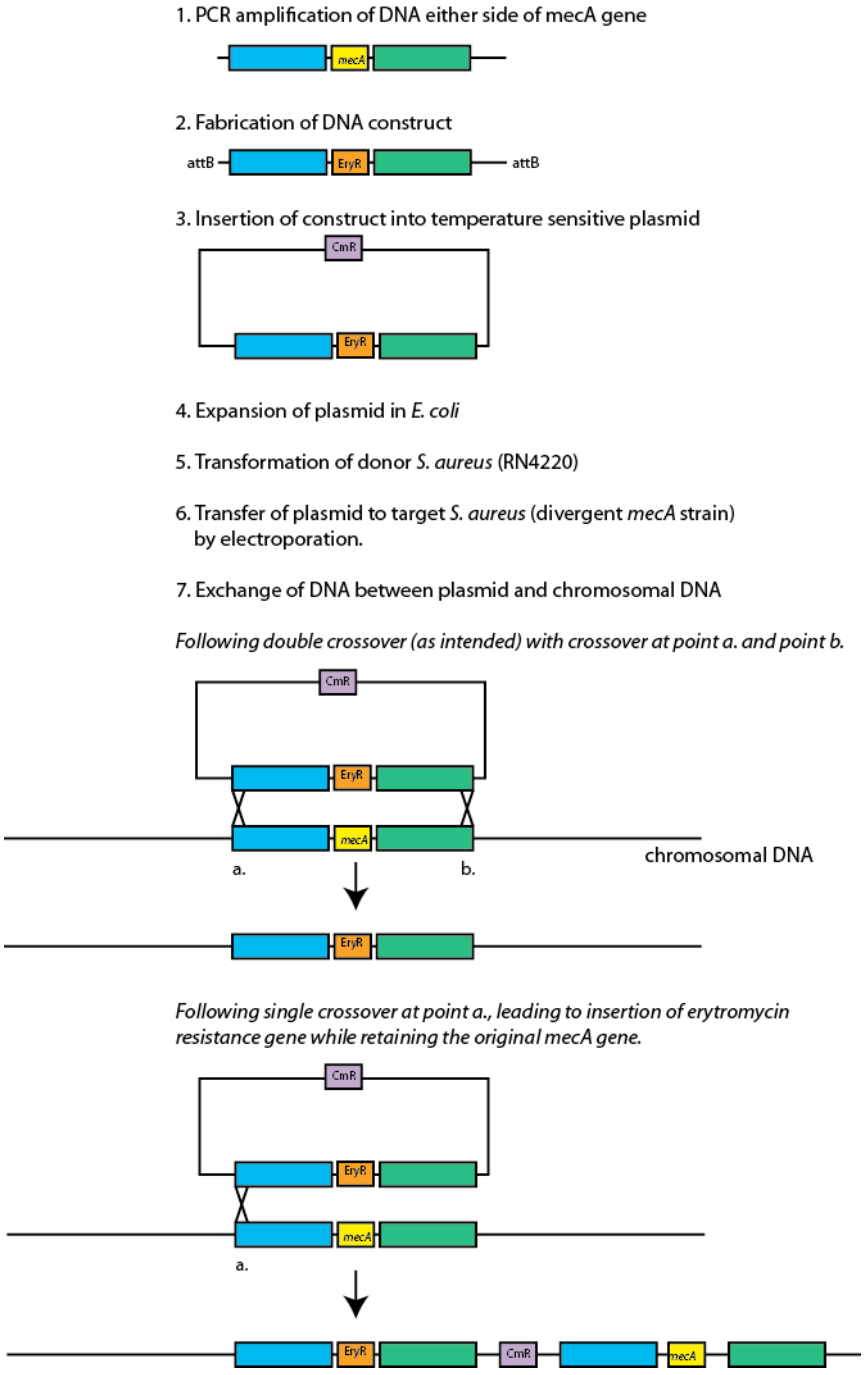

Figure 2: A schematic representation of the attempt to delete the divergent mecA gene by homologous substitution showing the effect of both a single and a double crossover event during the final insertion into chromosomal DNA.

(b) As an alternative experiment to demonstrate that presence of the divergent *mecA* was associated with beta-lactam resistance, an attempt was made to insert a divergent *mecA* gene into a MSSA (RN4220) background. The divergent *mecA* open reading frame was amplified by PCR and inserted into a xylose regulatable expression vector conferring erythromycin resistance. The plasmid was used to transform *S. aureus* RN4220 which was selected on erythromycin plates and recombinants subjected to phenotypic analysis by disk diffusion assay on Columbia agar + 2% NaCl supplemented with xylose (to induce *mecA* expression), or with glucose (to repress *mecA* expression), together with methicillin, oxacillin or cefoxitin disks. The data obtained indicated no increased resistance of recombinant *S. aureus* RN4220 in the presence or absence of xylose, and no difference between the recombinant *S. aureus* RN4220 compared with control *S. aureus* RN4220 without the recombinant *mecA* gene. The control experiment of expressing conventional *mecA* in the same MSSA background was not performed.

These data therefore do not support the hypothesis that the methicillin resistant phenotype of *S. aureus* LGA251 is due to the divergent *mecA* gene under study. We would emphasize that this conclusion does not mean that these results constitute evidence that methicillin resistance is not caused by the divergent *mecA* gene.

Implications of the findings & possible future work

The strain typing and phylogenetic studies indicate that the SCC*mec* element containing the divergent *mecA* gene was identified in 3 separate lineages (MLST clonal complexes 130 and 151 plus ST425). The fact that the mobile genetic element containing the divergent *mecA* has been conserved and/or acquired among a range of strain types indicates that it confers some survival advantage, and that we should continue to monitor it's presence in the national dairy herd. The work that has been performed provides valuable baseline phylogenetic data against which future resistant isolates can be compared. It is of some concern that the serendipitous discovery of a human isolate possessing the divergent *mecA* gene shares the sequence type of a bovine divergent *mecA* isolate from the same geographical region. Although the human isolate may well have represented carriage rather than infection, it is possible that this human isolate was acquired from a dairy farm. Any risk assessment of the significance of divergent *mecA* will require data on the prevalence and distribution of *S. aureus* possessing divergent *mecA* in human and animal populations.

Reasonable attempts were made to show that possession of the divergent *mecA* gene was associated with phenotypic high MIC β-lactam resistance but technical limitations prevented our being able to confirm the association of the high MIC β-lactam resistant phenotype with the divergent mecA gene. A number of alternative, albeit more elaborate or expensive, approaches could be attempted to demonstrate the effect of deleting the divergent *mecA* gene.

If the divergent mecA is not conferring β-lactam resistance it seems incredible, given that we have an annotated copy of the entire genome of one of the resistant isolates, that we are not able to find an alternative explanation for the high level of β-lactam resistance. Clearly it would also be of interest to attempt the deletion of the penicllllinase gene (*blaZ*) to be absolutely certain that this gene is not conferring the resistance. We should also attempt to discover if the divergent *mecA* gene is being transcribed by resistant strains in the presence of β -lactams. In any event, it is clear that strains of *S. aureus* are present in the dairy population that have levels of β-lactam resistance that would be described as MRSA according to BSAC guidelines (and would be identified as such using the techniques employed by the majority of human laboratory testing in the UK). We may not have definitive evidence that the divergent *mecA* gene is responsible for this phenotype but the antibiotic resistance phenomenon has been confirmed by every laboratory that has encountered strains of *S. aureus* that possess divergent *mecA*.

Now that genome sequencing has become economically viable, considerable information could be obtained from genome analysis of isolates obtained from epidemiological investigations of infected farms. Not only would this enable a fine resolution phylogenetic analysis to be performed but it would help enable us to identify any other genes that might be contributing to this puzzling antibiotic resistance phenomenon.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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