Typing of *mecD*-islands in genetically diverse methicillin-resistant *Macrococcus caseolyticus* from cattle

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Running title: McRI$_{mecD}$ typing in *Macrococcus caseolyticus*

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ABSTRACT

Macrococcus caseolyticus belongs to the normal bacterial flora of dairy cows and does not usually cause disease. However, methicillin-resistant *M. caseolyticus* strains were isolated from bovine mastitis milk. These bacteria had acquired a chromosomal island (McRI$_{mecD}$-1 or McRI$_{mecD}$-2) encoding the methicillin resistance gene *mecD*. To gain insight into the distribution of McRI$_{mecD}$ types in *M. caseolyticus* from cattle, 33 *mecD*-containing strains from Switzerland were characterized using molecular techniques, including multilocus sequence typing, antibiotic resistance gene identification and PCR-based McRI$_{mecD}$ typing. Additionally, the same genetic features were analyzed in 27 *mecD*-containing *M. caseolyticus* strains isolated from bovine bulk milk in England/Wales using publicly available whole genome sequences. The 60 strains belonged to 24 different sequence types (STs), with strains belonging to ST5, ST6, ST21 and ST26 observed in both Switzerland and England/Wales. McRI$_{mecD}$-1 was found in different STs from Switzerland (n=19) and England/Wales (n=4). McRI$_{mecD}$-2 was only found in 7 strains from Switzerland, all of which belonged to ST6. A novel island, McRI$_{mecD}$-3, which contains a complete *mecD* operon (*mecD-mecR1*$_m$-*mecI*$_m$) combined with the left part of McRI$_{mecD}$-2 and the right part of McRI$_{mecD}$-1, was found in heterogeneous STs from both collections (Switzerland: n=7; England/Wales: n=21). Two strains from England/Wales carried a truncated McRI$_{mecD}$-3. Phylogenetic analyses revealed no clustering of strains according to geographical origin or carriage of McRI$_{mecD}$-1 and McRI$_{mecD}$-3. Circular excisions were also detected for McRI$_{mecD}$-1 and McRI$_{mecD}$-3 by PCR. The analyses indicate that these islands are mobile and may spread by horizontal gene transfer between genetically diverse *M. caseolyticus*.

IMPORTANCE

Since its first description in 2017, the methicillin resistance gene *mecD* has been detected in *M. caseolyticus* from different cattle sources and countries. Our study provides new insights into the molecular diversity of *mecD*-carrying *M. caseolyticus* strains using two approaches to characterize *mecD* elements: (i) multiplex PCR for molecular typing of McRI$_{mecD}$ and (ii) read mapping against reference sequences to identify McRI$_{mecD}$ types in silico. In combination with multilocus sequence typing, this approach can be used for molecular characterization and surveillance of *M. caseolyticus* carrying *mecD*.

INTRODUCTION

Macrococcus caseolyticus is a catalase- and oxidase-positive bacterium related to the genus *Staphylococcus*. *M. caseolyticus* is found as a commensal on the skin of cattle and has been isolated from bovine raw milk and dairy products (1-4). *M. caseolyticus* is considered to have low pathogenic potential; it has only been reported once previously in association with abscesses in lambs (5) and, recently, as causative agents of infections in broiler chicken (6). Furthermore, *M. caseolyticus* strains have been isolated from bovine mastitis milk and from the site of a skin infection on a dog (7). These strains were resistant to all β-lactam antibiotics due to the acquisition of the methicillin resistance gene *mecD* (7). As with other structural *mec* genes, *mecD* encodes an alternative penicillin-binding protein (PBP2a) and is located on a genomic island named the *M. caseolyticus* resistance island *mecD* (McRI$_{mecD}$), which is unrelated to the previously detected *mecA*- and *mecC*-containing staphylococcal cassette chromosome *mec* (SCC*mec*) and *mecB*-carrying elements (8, 9). McRI$_{mecD}$ was found to be site-specifically integrated at the 3' end of the 30S ribosomal protein S9 gene (*rpsI*). McRI$_{mecD}$ carries a *mecD* operon with the complete regulators *mecR1*$_m$ and *mecI*$_m$, a putative virulence gene (*virE*) and an integrase gene (*int*) responsible for element integration and

excision (10). Two island types have been detected to date, McRI$_{mecD}$-1 and McRI$_{mecD}$-2, which differ from each other by their diverse 3' end segments (Fig. 1A). The sequence of this segment contains a restriction modification system (*hsmMI-hsrMI*) and a DNA recombination-mediator protein (*dprA*) in McRI$_{mecD}$-1 and two putative reverse transcriptase genes (*rts*) in McRI$_{mecD}$-2. McRI$_{mecD}$-1, but not McRI$_{mecD}$-2, is delimitated at both ends by direct repeats (DR) and is capable of circularization and excision from the chromosome (7).

Since the first description of *mecD* in 2017, additional methicillin-resistant *M. caseolyticus* strains have been isolated from cattle in Switzerland as well as from bulk tank milk in England and Wales (11), indicating a broader geographical dissemination. In the present study, we characterized *M. caseolyticus* from Switzerland using multilocus sequence typing (MLST), PCR-based McRI$_{mecD}$ typing, and microarray detection of antibiotic resistance genes. Publically deposited whole genome sequences were used to identify the same genetic features in *M. caseolyticus* strains from England and Wales. These analyses provided new insights into the molecular characteristics of methicillin-resistant *M. caseolyticus* strains from cattle from different geographical origins and the spread of different *mecD* islands.

RESULTS

Methicillin-resistant *M. caseolyticus* strains from cattle in Switzerland and England/Wales. A total of 67 methicillin-resistant *M. caseolyticus* strains were analyzed during this study. Thirty-four strains were isolated in Switzerland between 2015 and 2017 from bovine samples, including 13 strains from mastitis milk obtained from 7 different farms at different time points, two strains from milking machines on a farm with a recurrent mastitis problem and 19 strains from the noses of healthy calves all raised on different farms (Table 1). The remaining 33 *M. caseolyticus* strains originated from the study of MacFadyen and

colleagues, who isolated them from bovine bulk milk tanks in England and Wales between 2015 and 2016 (11) (Table 2). Genetic characterization of these strains from England/Wales was performed using publicly available whole genome sequences (NCBI Bioproject PRJNA420921).

The majority of the methicillin-resistant strains contained the *mecD* gene (Switzerland number [n] = 33; England/Wales n = 27) (Table 1 and Table 2). One strain from a Swiss calf and 6 strains from England/Wales carried *mecB*. In addition to *mec* genes, the Swiss strains also contained the tetracycline efflux gene *tet*(L) (n = 16), the ribosome protection genes *tet*(M) (n = 3) or *tet*(S) (n = 1) or both genes *tet*(L) and *tet*(M) (n = 2), the streptomycin nucleotidyltransferase genes *str* (n = 20) and *ant(6)-Ia* (n = 1), the trimethoprim resistance dihydrofolate reductase genes *dfrK* (n = 7) and *dfrD* (n = 1), the macrolide-lincosamide-streptogramin B (MLS_B) 23S rRNA methylase gene *erm*(B) (n = 7), the fusidic acid resistance gene *fusC* (n = 2), and the bifunctional aminoglycoside acetyltransferase and phosphotransferase gene *aac(6')-Ie-aph(2'')-Ia* (n = 3). Twenty strains also carried the kanamycin nucleotidyltransferase gene *ant(4')-Ia*, but *ant(4')-Ia* alone did not confer kanamycin resistance. The kanamycin MIC of these strains ranged from ≤4 to 8 µg/ml, except for one strain (Genton2014), which had an intermediate MIC of 32 µg/ml. One strain, Msa0331, was positive for *erm*(B) according to PCR and microarray analyses but remained susceptible to erythromycin and clindamycin. Otherwise, the presence of resistance genes correlated with increased MICs of β-lactam (n = 34; MIC range of penicillin 1 to >2 µg/ml and MIC of cefoxitin 8 to >16 µg/ml), tetracycline (n = 22; MIC >16 µg/ml), streptomycin (n = 21; MIC 16 to >32 µg/ml), trimethoprim (n = 8; MIC >32 µg/ml), erythromycin (n = 6; MIC >8 µg/ml), clindamycin (n = 6; MIC >4 µg/ml), kanamycin (n = 3; MIC ≥ 64 µg/ml), gentamicin (n = 3; MIC 8 to 16 µg/ml) and fusidic acid (n = 2; MIC 4 µg/ml) (Table 1).

In the methicillin-resistant *M. caseolyticus* strains from England and Wales, tetracycline resistance genes (*tet*(L): n = 13; *tet*(M): n = 2; and *tet*(S): n= 1) and streptomycin resistance genes (*str*: n = 16; and *ant(9)-Ia*: n = 1) were also widespread (Table 2). The strains also carried *ant(4')-Ia* (n = 9), *erm*(B) (n = 6) and *fusC* (n = 5), but neither *dfr* genes nor *aac(6')-Ie-aph(2'')-Ia* were detected; instead, few strains contained the streptothricin acetyltransferase gene *sat4* (n = 4), the kanamycin phosphotransferase gene *aph(3')-III* (n = 4), and the lincosamide nucleotidyltransferase genes *lnuA* (n = 2) or *lnuG* (n = 1). The *mecB*-carrying *M. caseolyticus* strains contained the β-lactamase gene $blaZ_m$ (Table 1 and Table 2). The *sat4* and *aph(3')-III* genes were additionally found in the *mecB*-positive strain from Switzerland. While some *mecD*-positive *M. caseolyticus* strains carried no further resistance genes (Switzerland: n = 6; England/Wales: n = 7), the majority of the strains had acquired three or more additional resistance genes (Switzerland: n = 21; England/Wales: n = 13).

PCR-based McRI$_{mecD}$ typing and characterization of the new McRI$_{mecD}$-3. Three multiplex PCRs (I-III) were developed for typing McRI$_{mecD}$ in the Swiss strains (see Figure 1A for the McRI$_{mecD}$ structures, Table 3 for PCR and Table 4 for the primers). Multiplex PCR I detected site-specific island integration at the *rpsI* locus using primers specific for the integrase gene of McRI$_{mecD}$-1 (*int0819*) and McRI$_{mecD}$-2 (*int0473*). Multiplex reaction II distinguished between the putative virulence genes *virE0819* of McRI$_{mecD}$-1 and *virE0473* of McRI$_{mecD}$-2, which share 75% nt identity. Unique genes present only in McRI$_{mecD}$-1, such as *dprA* and *hsmMI-hsrMI*, or the putative reverse transcriptase gene *rt0473*, which is characteristic of McRI$_{mecD}$-2, were detected by multiplex PCRs II and III. In addition, the specific primers for the putative copper-translocating P-type ATPase gene *cop* were included in multiplex PCR III. The *cop* gene was believed to belong to the core genome of *M. caseolyticus*, and its absence in strain IMD0473 carrying McRI$_{mecD}$-2 indicates a possible chromosomal deletion (Fig. 1A) (7). Multiplex PCRs I-III were tested with the reference

150 strains for McRI$_{mecD}$-1 (IMD0819) and McRI$_{mecD}$-2 (IMD0473) as well as the *M. caseolyticus*
151 strains containing no insert at the *rpsI* locus (KM1352) and a strain containing an alternative
152 insert (JCSC5402) (Fig. 2). Alternative inserts and resistance islands can be integrated at the
153 *rpsI* locus (12) associated with integrases related to Int0473 and Int0819. The *mecD*-negative
154 *M. caseolyticus* strain JCSC5402 contains a unique sequence downstream of the *rpsI* gene
155 that is unrelated to McRI$_{mecD}$, except for an integrase that shares 97% nucleotide (nt) identity
156 with *int0473* and the DRs that delimited the element (Fig. 1A). A specific PCR product was
157 therefore also amplified from JCSC5402 in multiplex PCR I (Fig. 2). The larger size of this
158 fragment (1,823 bp) allowed it to be differentiated from McRI$_{mecD}$-1 (809 bp) and McRI$_{mecD}$-2
159 (1,328 bp).

160 PCR-based McRI$_{mecD}$ typing performed for field strains generated for some strains
161 amplicons specific for *int0473* and *virE0473* of McRI$_{mecD}$-2 as well as amplicons specific for
162 *dprA* and *hsmMI-hsrMI* of McRI$_{mecD}$-1, which is represented by the PCR profile of strain
163 Msa0018 in Figure 2. These results indicated the presence of a third genomic island
164 containing *mecD*. The sequence of the *rpsI* region in strain Msa0018 was determined,
165 revealing a new 17,950-bp island named McRI$_{mecD}$-3. McRI$_{mecD}$-3 was integrated at the 3' end
166 of the *rpsI* gene; it contained the *mecD* operon (*mecD-mecR1*$_m$*-mecI*$_m$) and was flanked by
167 imperfect direct repeats of 123 bp (DR1) and 120 bp (part of DR2) (Fig. 1) (GenBank
168 accession number MH671353). The McRI$_{mecD}$-2-McRI$_{mecD}$-1 hybrid pattern observed by
169 multiplex PCR was confirmed. The left part of McRI$_{mecD}$-3 (4,212 bp; MH671353, positions:
170 1026-5237), including the genes *int0473*, *orf2*, *orf3* and *virE0473*, was 99.98% identical (1
171 single nucleotide polymorphism [SNP]) to that of McRI$_{mecD}$-2 of strain IMD0473 and had
172 overall only 68% nt identity to the corresponding segment of McRI$_{mecD}$-1. The right part of
173 McRI$_{mecD}$-3 (7,150 bp; positions: 11826-18975) downstream of the *mecD* operon was
174 identical to McRI$_{mecD}$-1 of strain IMD0819 apart from 1 SNP. The segment containing *orf5* to

175 *orf9*, the *mecD* operon and *orf13* of McRI$_{mecD}$-3 (6,588 bp, positions: 5238-11825) was
176 identical to McRI$_{mecD}$-1 and differed from McRI$_{mecD}$-2 by 2 SNPs. Strain Msa0018 also
177 carried a 2,774-bp chromosomal island (CI) flanked by extended imperfect direct repeats of
178 405 bp (DR2) and 404 bp (DR3) downstream of McRI$_{mecD}$-3 (Fig. 1). This island shared
179 97.30% nt identity (75 SNPs) with McCI$_{IMD0819}$ of strain IMD0819. The downstream
180 sequence encoded a putative AAA family ATPase, a truncated transposase (*Δtnp*) and part of
181 the *cop* gene and was identical to that of strain IMD0819.

182 To evaluate the mobility of McRI$_{mecD}$-3, spontaneous excision and circularization of
183 the McRI$_{mecD}$-3-McCI$_{IMD0819}$ subunits were tested by PCR (Supplementary Table S1). Two
184 PCR products were obtained with divergent primers specific for *mecD* and *int0473* (primers
185 labeled 16 and 19, respectively, in Fig. 1A) and were confirmed by sequencing to be the
186 circularized McRI$_{mecD}$-3 and the composite circular form of McRI$_{mecD}$-3-McCI$_{IMD0819}$. A
187 circular molecule of McCI$_{IMD0819}$ was also detected using the divergent primers araC-F and
188 IMD0819c21-F6 (primers labeled 20 and 21, respectively, in Fig. 1A). Joint chromosomal
189 segments remaining after McRI$_{mecD}$-3 and/or McCI$_{IMD0819}$ subunit excisions were obtained
190 using convergent primers specific for *truA* and *cop* (primers labeled 18 and 15, respectively,
191 in Fig. 1A) and for *orf20* and *cop* (primers labeled 22 and 15, respectively, in Fig. 1A) and
192 short elongation times to avoid amplification of the entire inserts (Supplementary Table S1).
193 The joining sequences of all the circular molecules and chromosomal segments contained the
194 proposed 61-bp core attachment (*att*) site present in the DR regions as well as the 3' end of
195 the *rpsI* gene (7) (Fig. 1B). Mismatches in the imperfect DR sequences allowed identification
196 of the positions of strand exchanges within the first 8 bases of the core *att* sites, which
197 differed between 2 and 4 bases among each other (Fig. 1B). The core *att* sequence present in
198 all the circular DNA molecules (cMcRI$_{mecD}$-3, cMcRI$_{mecD}$-3-McCI$_{IMD0819}$, and cMcCI$_{IMD0819}$)
199 was identical to the left core *att* site used in the recombination reaction. Accordingly, the core

200 *att* sequence that remained on the chromosome after circular DNA excision (ΔMcRI$_{mecD}$-3,
201 ΔMcRI$_{mecD}$-3-McCI$_{IMD0819}$, and ΔMcCI$_{IMD0819}$) contained an identical sequence to that of the
202 right core *att*-site involved in recombination (Supplementary Table S1).

203 **Distribution of the McRI$_{mecD}$ elements in *M. caseolyticus* from Switzerland and**
204 **England/Wales.** To analyze the population of *mecD*-carrying *M. caseolyticus* in cattle, the
205 relatedness of the strains was determined by multilocus sequence typing (MLST) based on
206 seven housekeeping genes, and the distribution of the three different McRI$_{mecD}$ types was
207 investigated by multiplex PCR in the Swiss strains and by read mapping against reference
208 sequences for the strains from England/Wales. A heterogeneous *mecD*-carrying *M.*
209 *caseolyticus* population was observed, including 33 strains belonging to 13 different sequence
210 types (STs) from Switzerland and 27 strains belonging to 15 different STs from
211 England/Wales (Table 1 and Table 2). ST5, ST6, ST21 and ST26 were observed in strains
212 from both geographical regions containing however different McRI$_{mecD}$ elements, except for
213 the ST26 strains, which all contained McRI$_{mecD}$-3. Whereas McRI$_{mecD}$-2 was only found in 7
214 isolates from Switzerland, all of which belonged to ST6, McRI$_{mecD}$-1 and McRI$_{mecD}$-3 were
215 detected in both regions in diverse STs. The most frequently detected *mecD*-islands were
216 McRI$_{mecD}$-1 in strains from Switzerland (n = 19; ST5, ST8, ST9, ST21, ST22, ST23 and
217 ST29) and McRI$_{mecD}$-3 in strains from England/Wales (n = 21; ST5, ST6, ST21, ST26, ST40,
218 ST42, ST43, ST44, ST47 and ST51). McRI$_{mecD}$-1 was found in 4 strains (ST48, ST49 and
219 ST50) from England/Wales, and McRI$_{mecD}$-3 was detected in 7 strains from Switzerland
220 (ST7, ST25, ST26, ST27 and ST28). Mapping assemblies obtained for the McRI$_{mecD}$
221 elements of the strains from England/Wales showed only up to 5 SNPs compared to the
222 reference sequences McRI$_{mecD}$-1 of IMD0819 or McRI$_{mecD}$-3 of Msa0018. The only exception
223 was strain 5804_BC29, which contained a McRI$_{mecD}$-3 that differed by 31 SNPs from the
224 reference strain Msa0018. The 3 ST6 strains from England/Wales carried McRI$_{mecD}$-3, not

McRI$_{mecD}$-2, but they were the only strains that contained upstream of the *s66* gene the two *rt* genes also found in the 3' fragment of McRI$_{mecD}$-2 (Fig. 1A). Two strains from England/Wales, 5459_5_49 and 5782_EF83 (ST46), were identical only for the 5' fragment of McRI$_{mecD}$-3 spanning the first 10.5 kb, including the *mecD* operon. Sequences of the 3' fragment characteristic of McRI$_{mecD}$-1/3 (*dprA*, *hsmMI* and *hsrMI*) or for McRI$_{mecD}$-2 (*rt0473*) were not present. Because the core *att* site of McRI$_{mecD}$ (Fig. 1B) was only found at the 3' end of the *rpsI* gene, the islands were suggested to be truncated and were named McRI$_{mecD}$-3Δ (Table 2).

No amplification of the *cop* gene was observed in Swiss strains carrying McRI$_{mecD}$-2 but amplification was also absent in 4 strains containing McRI$_{mecD}$-1 and in 2 strains containing McRI$_{mecD}$-3 (Table 1). The *cop* gene was absent in the majority of strains from England/Wales (n = 21) (Table 2), indicating that deletion of the chromosomal segment downstream of the *rpsI* gene frequently occurs and is independent of the type of McRI$_{mecD}$ integrated at that position. One *mecB*-carrying strain from England/Wales (5456_3_46) contained an insert at the *rpsI* locus (GenBank NZ_PIWR01000018) that shared 96% nt identity with a 4-kb fragment of McRI$_{mecD}$-2/3 containing *int0473*, *orf2*, *orf3* and *virE0473*. Multiplex PCRs would generate a 2,610-bp fragment from the 5456_3_46 template for the *rpsI*-associated integrase that can be differentiated from those of McRI$_{mecD}$ types (Table 3). Priming of the *virE0473*-like gene might fail because the primers each contain 2 mismatches. The chromosomal island McCI$_{IMD0819}$ was detected in 27 strains from England/Wales either downstream of McRI$_{mecD}$ or directly at the *rpsI* locus in *mecD*-negative strains (Table 2). The mapping assembly showed higher variability for this island, ranging from 97% to 100% nt identity to McCI$_{IMD0819}$ of IMD0819 and Msa0018.

The phylogenetic relationship among the *M. caseolyticus* strains was visualized by generating a maximum parsimony tree based on MLST data (Fig. 3). Strains from dog (ST2:

no *mec*) and chicken (ST31: *mecB*) (Table 1) were included in the analysis and were found on separate branches with an estimated higher evolutionary distance to bovine strains. The clustering of the *M. caseolyticus* strains from cattle did not correlate to their geographical origin except for one branch containing only strains from Switzerland belonging to ST7, ST9, ST22 and ST27. McRI$_{mecD}$-1, McRI$_{mecD}$-3 and *mecB* were carried by distantly related STs. On the other hand, different elements were observed within the same STs: strains belonging to ST21 and ST5 carried either McRI$_{mecD}$-1 or McRI$_{mecD}$-3 and ST48 strains contained either *mecB* elements or McRI$_{mecD}$-1. The clustering pattern suggests that a heterogeneous population of *M. caseolyticus* strains from cattle had acquired methicillin resistance through the acquisition of McRI$_{mecD}$-1, McRI$_{mecD}$-3 or *mecB*-containing elements. McRI$_{mecD}$-2 (ST6) and McRI$_{mecD}$-3Δ (ST46) might represent truncated McRI$_{mecD}$-3 variants that evolved in single clones.

DISCUSSION

Different approaches were used to characterize methicillin-resistant *M. caseolyticus* strains from the cattle environment and determine the distribution of the different McRI$_{mecD}$ elements. Swiss strains were analyzed by conventional molecular techniques, including multiplex PCR for McRI$_{mecD}$ typing. Whole genome sequences were used for in silico analysis of strains from England and Wales. In total, three McRI$_{mecD}$ types were found as well as a truncated McRI$_{mecD}$-3 element and a McRI$_{mecD}$-3 element, followed by a fragment found in the 3' segment of McRI$_{mecD}$-2. All these elements can be identified by multiplex PCR I-III designed for *M. caseolyticus* in this study. This McRI$_{mecD}$ typing method could be easily adapted to other bacteria containing *mecD* using a species-specific primer for the *rpsI* gene.

Phylogenetic clustering based on 7 housekeeping genes showed an overall good correlation with the phylogenetic analysis based on 1550 gene targets performed in the study

of MacFadyen and colleagues (11). The same ST was assigned to strains that were highly related based on whole genome MLST analysis (11). The only exception was strain 5795_EF335, which was assigned to ST51 even though it was on the same terminal branch as the ST21 strains 5193_2_23 and 5818_BC116. The increasing discriminatory power of whole genome MLST analysis was observed for ST6 strains from England and Wales, which were located on a branched clade (11). The reference strain IMD0819 from Switzerland included in their analysis was also located on a separate branch in a clade with ST5 strains from England and Wales. Overall, the phylogenetic analysis revealed a diverse population of methicillin-resistant *M. caseolyticus* strains in cattle. Twenty-seven STs were detected in total, no clustering by geographical origin was observed, and strains belonging to ST5, ST6, ST21 and ST26 were found in both Switzerland and England/Wales.

The data indicate that methicillin-resistant *M. caseolyticus* strains from bovine sources more frequently carry *mecD* than *mecB*, and only *mecD*-containing *M. caseolyticus* strains have to date been associated with cases of bovine mastitis. However, the role of these strains in mastitis is still not clear because bovine mastitis milk samples contained additional bacterial species in approximately 90% of the cases (data not shown). The beta-lactam resistance phenotype of *mecD*-carrying *M. caseolyticus* likely allows them to survive treatment for mastitis in which penicillin or cephalosporins are administered (13). Similar strains carrying *mecD* were also isolated from the nose of healthy calves in Switzerland (this study) and bulk milk tank in England/Wales (11), indicating a broader distribution of these bacteria. All three resistance islands (McRI$_{mecD}$-1, McRI$_{mecD}$-2, and McRI$_{mecD}$-3) were detected in *M. caseolyticus* strains from healthy calves as well as from mastitis milk samples in Switzerland. ST5-McRI$_{mecD}$-1 and ST6-McRI$_{mecD}$-2 were repeatedly obtained from milk samples from the same farms, suggesting the persistence of *mecD*-positive clones over time (Table 1). Methicillin-resistant *M. caseolyticus* have also been isolated in the past, namely

300 strains of unknown mechanisms from bulk milk in the US (14) and *mecB*-carrying strains
301 from chicken sources in Japan, Thailand and China (6, 9). The recent reports of a *mecB*-
302 containing plasmid in *S. aureus* (15) that is highly similar to a plasmid detected in *M. canis*
303 (16) and the demonstrated activity of the integrase of McRI$_{mecD}$-1 in *Staphylococcus* and
304 *Bacillus* species (10) indicate that *mec* genes from *Macrococcus* can spread to other genera.
305 Detection of *mecB* and *mecD* genes should therefore be included in the diagnosis and
306 monitoring of methicillin-resistant staphylococci.

307 McRI$_{mecD}$-3, described here, was the major element carrying *mecD* in strains from
308 England and Wales. McRI$_{mecD}$-3 might represent a precursor of McRI$_{mecD}$-2 that could have
309 been formed through a deletion event. The 3 ST6 strains from England and Wales support
310 this hypothesis because they carry the same rt segment downstream of McRI$_{mecD}$-3.
311 McRI$_{mecD}$-2 may therefore represent a truncated McRI$_{mecD}$-3 element. McRI$_{mecD}$-2 lacks a DR
312 at the right end, and circular excision of the element was not observed (7). However, circular
313 intermediates were detected for McRI$_{mecD}$-3, which encodes an identical Int0473 enzyme but
314 contains DRs at both sites, including the core *att* sites that are supposed to be recognized and
315 recombined by the Int protein of McRI$_{mecD}$ (10). McRI$_{mecD}$-1 and McRI$_{mecD}$-3 were
316 associated with unrelated STs from Switzerland and England/Wales, indicating that these
317 islands are mobile and may be spread by horizontal gene transfer between genetically diverse
318 *M. caseolyticus*. By contrast, McRI$_{mecD}$-2 was only carried by strains belonging to ST6 from
319 Switzerland, indicating that McRI$_{mecD}$-2 is not mobile and spreads with a clone. A second
320 truncated McRI$_{mecD}$-3 element, named McRI$_{mecD}$-3Δ, that probably also lost its potential for
321 mobility, was found in two ST46 strains from England and Wales.

322 Methicillin-resistant *M. caseolyticus* strains seem to be widespread, but their
323 genotypes often remain unknown. In the current study, we suggest possible approaches for
324 characterizing *mecD*-carrying *M. caseolyticus* strains starting from whole genome sequences

or using conventional PCR-based techniques. The data from this study will help to characterize methicillin-resistant *M. caseolyticus* and to surveil the global spread of strains carrying *mecD*.

MATERIALS AND METHODS

Collection and identification of methicillin-resistant *M. caseolyticus* strains from Switzerland. The reference strains IMD0819, IMD0473, KM1352 and JCSC5402 (7, 8), as well as the field strains isolated during this study, are listed in Table 1. Strains from bovine mastitis milk and from milking machines were isolated by routine milk diagnostics using non-selective media as previously described (17). Strains from healthy calves were recovered from nasal swabs collected from different slaughterhouses. These strains were obtained using a two-step enrichment protocol for MRSA and selection on BBL™ CHROMagar™ MRSA II (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (18). After isolation, all strains were routinely cultivated on non-selective trypticase soy agar plates containing 5 % sheep blood (TSA-SB) (Becton, Dickinson and Company) at 37°C. Species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Microflex LT; Bruker Daltonics GmbH, Bremen, Germany). The presence of the *mecD* and *mecB* genes was tested by PCR. All of the relevant primers used in this study are listed in Table 4.

Multilocus sequence typing (MLST) and cluster analysis. Sequencing of seven housekeeping genes (*ack*, *cpn60*, *fdh*, *pta*, *purA*, *sar*, and *tuf*) was performed to determine the allelic profiles and sequence types of the *M. caseolyticus* strains using the definitions available on the pubMLST homepage (http://pubmlst.org/mcaseolyticus/). The maximum parsimony method in BioNumerics v7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to construct a MLST-based phylogenetic tree.

Determination of the antimicrobial resistance profile of *M. caseolyticus* strains from Switzerland. The MIC of antibiotics were measured in Müller-Hinton broth using the microdilution technique and Sensititre™ EUST plates (Thermo Fisher Scientific, Inc., Waltham, USA). The resistance phenotype was determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines (19) and using the breakpoints proposed for *Staphylococcus* sp. in the CLSI supplement M100-S27 (20). Antibiotic resistance genes were detected using a custom-made microarray (AMR+ve-5.1 array tubes, Alere Technologies GmbH, Jena, Germany), allowing the identification of up to 117 resistance genes from Gram-positive bacteria (21). The presence of the *blaZ*$_m$ and *lnuG* genes was tested by PCR (Table 4).

Multiplex PCR for McRI$_{mecD}$ typing. DNA was extracted from *M. caseolyticus* strains using the MOBIO UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Target genes specific for McRI$_{mecD}$ were amplified in three different multiplex PCRs. The locations of the genes and primers and the amplicon sizes are shown in Figure 1 and Table 3. *M. caseolyticus* strains IMD0819, IMD0473, and Msa0018 served as positive controls for McRI$_{mecD}$-1, McRI$_{mecD}$-2 and McRI$_{mecD}$-3, respectively. Strains KM1352 and JCSC5402 were included as negative controls. Multiplex PCRs were performed using HOT FIREPol® DNA polymerase and buffers (Solis BioDyne, Tartu, Estonia). Reactions were performed in 30 µl volumes using 1.5 U polymerase, 200 µM dNTPs and 0.2 µM primers. DNA amplification was performed for 35 cycles with an annealing temperature of 54°C, an extension time of 2 min for Multiplex I, 1 min for Multiplex II, and 1.5 min for Multiplex III.

Characterization of McRI$_{mecD}$-3. McRI$_{mecD}$-3 and the flanking regions of strain Msa0018 were obtained by Sanger sequencing of long-range PCR products (Microsynth AG, Balgach, Switzerland). Therefore, an 11-kb fragment encompassing the sequence between

375 *truA* and *mecD* was amplified using the primers truA-F and mecD-R, and a 15-kb fragment
376 encompassing the sequence between *mecD* and *cop* was amplified using the primers mecD- F
377 and cop-R (Table 4). PCRs were performed using the GoTaq® Long PCR Master Mix
378 (Promega, Madison, WI, USA), and the obtained fragments were sequenced using primer
379 walking. Prodigal software for gene finding in prokaryotes was used to define *orfs* (22).
380 Annotation of the *orfs* was manually performed by homology to *orfs* present in McRI$_{mecD}$-1
381 and McRI$_{mecD}$-2. Spontaneous formation of circular DNA molecules and the chromosomal
382 region remaining after excision was analyzed in strain Msa0018 by PCR and sequencing
383 (Supplementary Table S1). PCRs were performed with specific divergent and convergent
384 primer pairs as described (7).

385 **Analysis of *M. caseolyticus* strains from England and Wales.** The reads and contigs
386 used for in silico analysis were from BioProject PRJNA420921 containing 33 methicillin-
387 resistant *M. caseolyticus* strains isolated from bovine bulk tank milk (11). The McRI$_{mecD}$
388 types were identified by paired-end mapping of the MiSeq Illumina reads against reference
389 sequences using the Burrows-Wheeler Alignment tool (bwa v0.7.17) (23) with the –q
390 (trimQuality) option set to 25. The reference sequences were GenBank KY013611.1:5088-
391 35890 (including McCI$_{IMD0819}$) for McRI$_{mecD}$-1, KY013610: 5088-24079 for McRI$_{mecD}$-2 and
392 MH671353 (including McCI$_{IMD0819}$) for McRI$_{mecD}$-3. Alignments were converted to the bam
393 format, sorted and indexed using samtools v1.8 (24). SNP calling was performed using
394 mpileup and bcftools (samtools 0.1.19). Calculations were performed on UBELIX
395 (http://www.id.unibe.ch/hpc), the HPC cluster at the University of Bern. The alignments were
396 then inspected visually using Geneious® 10.2.3. The presence of an element (McRI$_{mecD}$ type
397 and McCI$_{IMD0819}$) was assigned if mapping resulted in an un-gapped alignment. Additionally,
398 the nt sequence identity was determined between the reference sequence and the mapping
399 assembly. The presence or absence of the *cop* gene could also be observed from the

alignment and was additionally confirmed by a BLASTn search against PRJNA420921 contigs. Downloaded assemblies from PRJNA420921 were further analyzed for the presence of antimicrobial resistance genes using ResFinder (25) and for additional resistance genes (*blaZ*$_m$, *fusC* and *sat4*) by a BLASTn search. The assemblies were also used to identify the allelic profiles of the 7 housekeeping genes using the pubMLST scheme for *M. caseolyticus*.

GenBank accession number. The nucleotide sequence of McRI$_{mecD}$-3 and its flanking regions in strain Msa0018 were deposited in GenBank under the accession number MH671353.

SUPPLEMENTAL MATERIALS

Additional supporting information: Table S1.

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510 **TABLES**

511

512 **TABLE 1** Origin, antibiotic resistance and genetic characteristics of the *M. caseolyticus* strains

513 investigated in this study from Switzerland.

Strain	Source	Region/date	Farm	*mecD* element	Resistance phenotype[a]	Resistance genes[b]	ST	*cop* gene	Reference
KM1352	Healthy dog (skin)	Jura/2015	-	-	-	-	2	+	(7)
JCSC5402	Chicken meat	Japan	-	-	FOX, PEN, CLI, ERY, TMP, KAN, GEN, STR	*mecB*, *blaZ*$_m$, *erm*(B), *dfr*, *aac(6')-Ie-aph(2'')-Ia*, *str*	31	+	(8)
Msa0331	Healthy calf (nose)	Basel/2017	27	-	FOX, PEN, TET, KAN, GEN, STR	*mecB*, *erm*(B), *blaZ*$_m$, *tet*(S), *aac(6')-Ie-aph(2'')-Ia*, *ant(6)-Ia*, *aph(3')-III*, *sat4*	11	-	This study
IMD0819	Bovine mastitis milk	Fribourg/2015	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP, STR	*mecD*, *tet*(L), *dfrK*, *str*, *ant(4')-Ia*	5	+	(7)
M1620	Bovine mastitis milk	Fribourg/2015	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP, STR	*mecD*, *tet*(L), *dfrK*, *str*, *ant(4')-Ia*	5	+	This study
M1147	Bovine mastitis milk	Vaud/2016	3	McRI$_{mecD}$-1	FOX, PEN, CLI, ERY, TET, KAN, GEN, TMP, STR	*mecD*, *erm*(B), *tet*(L), *aac(6')-Ie-aph(2'')-Ia*, *dfrK*, *str*, *ant(4')-Ia*	5	+	This study
M1154	Bovine mastitis milk	Fribourg/2016	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP	*mecD*, *tet*(L), *dfrK* , *ant(4')-Ia*	5	+	This study
M1262	Bovine mastitis milk	Fribourg/2017	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP, STR	*mecD*, *tet*(L), *dfrK*, *str*, *ant(4')-Ia*,	5	+	This study
M0615	Bovine mastitis milk	Fribourg/2017	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP, STR	*mecD*, *tet*(L), *dfrK*, *str*, *ant(4')-Ia*	5	+	This study
M1468	Bovine mastitis milk	Fribourg/2017	1	McRI$_{mecD}$-1	FOX, PEN, TET, TMP, STR,	*mecD*, *tet*(L), *dfrK*, *str*, *ant(4')-Ia*,	5	+	This study
M0995	Bovine mastitis milk	Bern/2017	4	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	21	+	This study
Ref0244	Bovine mastitis milk	Bern/2017	5	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(L), *tet*(M), *str*, *ant(4')-Ia*	23	-	This study
Msa0113	Healthy calf (nose)	Zurich/2017	10	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(M), *str*, *ant(4')-Ia*	8	-	This study
Msa0114	Healthy calf (nose)	Zurich/2017	11	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(M), *str*, *ant(4')-Ia*	8	-	This study
Msa0115	Healthy calf (nose)	Zurich/2017	12	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(M), *str*, *ant(4')-Ia*,	8	-	This study
Msa0116	Healthy calf (nose)	Vaud/2017	13	McRI$_{mecD}$-1	FOX, PEN, CLI, ERY, KAN, GEN, TMP	*mecD*, *erm*(B), *aac(6')-Ie-aph(2'')-Ia*, *dfrD*	5	+	This study
Msa0288	Healthy calf (nose)	Grisons/2017	14	McRI$_{mecD}$-1	FOX, PEN	*mecD*	9	+	This study
Z8040	Healthy calf (nose)	Bern/2017	16	McRI$_{mecD}$-1	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	22	+	This study
Genton2014	Healthy calf (nose)	Vaud/2017	15	McRI$_{mecD}$-1	FOX, PEN, TET, FUS, STR	*mecD*, *tet*(L), *tet*(M), *fusC*, *str*, *ant(4')-Ia*	29	+	This study
Msa0856	Healthy calf (nose)	Argau/2017	17	McRI$_{mecD}$-1	FOX, PEN, CLI, ERY	*mecD*, *erm*(B)	9	+	This study
Msa0857	Healthy calf (nose)	Argau/2017	18	McRI$_{mecD}$-1	FOX, PEN	*mecD*	9	+	This study
Msa0858	Healthy calf (nose)	Lucerne/2017	19	McRI$_{mecD}$-1	FOX, PEN, CLI, ERY	*mecD*, *erm*(B)	9	+	This study
IMD0473	Bovine mastitis milk	Bern/2015	2	McRI$_{mecD}$-2	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	6	-	(7)
Ref0166	Bovine mastitis milk	Bern/2016	2	McRI$_{mecD}$-2	FOX, PEN, STR	*mecD*, *str*	6	-	This study
M1867	Bovine mastitis milk	Vaud/2017	6	McRI$_{mecD}$-2	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	6	-	This study
M0926	Milking machine	Bern/2016	8	McRI$_{mecD}$-2	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	6	-	This study
M0927	Milking machine	Bern/2016	8	McRI$_{mecD}$-2	FOX, PEN, TET	*mecD*, *tet*(L), *ant(4')-Ia*	6	-	This study
Msa0705	Healthy calf (nose)	Fribourg/2017	20	McRI$_{mecD}$-2	FOX, PEN, STR	*mecD*, *str*	6	-	This study
Msa0441	Healthy calf (nose)	Bern/2017	21	McRI$_{mecD}$-2	FOX, PEN, TET, STR	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	6	-	This study
Msa0018	Healthy calf (nose)	Argau/2017	9	McRI$_{mecD}$-3	FOX, PEN	*mecD*	7	+	This study
M1659	Bovine mastitis milk	Fribourg/2017	7	McRI$_{mecD}$-3	FOX, PEN, CLI, ERY, TET	*mecD*, *erm*(B), *tet*(L), *ant(4')-Ia*,	25	+	This study
Msa0429	Healthy calf (nose)	Bern/2017	22	McRI$_{mecD}$-3	PEN, FOX	*mecD*	7	+	This study
Msa0852	Healthy calf (nose)	Bern/2017	23	McRI$_{mecD}$-3	FOX, PEN, CLI, ERY, TET, STR	*mecD*, *erm*(B), *tet*(L), *str*	25	+	This study
Msa0706	Healthy calf (nose)	Lucerne/2017	24	McRI$_{mecD}$-3	PEN, FOX	*mecD*	28	-	This study
Msa0913	Healthy calf (nose)	St. Gallen/2017	25	McRI$_{mecD}$-3	FOX, PEN, FUS	*mecD*, *fusC*	26	-	This study
Msa0917	Healthy calf (nose)	Zurich/2017	26	McRI$_{mecD}$-3	FOX, PEN	*mecD*	27	+	This study

514 [a] Abbreviation of antimicrobials: CLI, clindamycin; ERY, erythromycin; TET, tetracycline;
515 FUS, fusidic acid; PEN, penicillin; FOX, cefoxitin; KAN, kanamycin; GEN, gentamicin;
516 TMP, trimethoprim; STR, streptomycin.
517 [b] Antibiotic resistance genes and their functions: *mecB*, *mecD*, methicillin-resistance genes
518 encoding PBP2a for resistance to all β-lactam-antibiotics; *blaZ*$_m$, β-lactamase gene; *dfrK*,
519 *drfD*, dihydrofolate reductase gene; *tet*(L), tetracycline efflux gene; *tet*(M), *tet*(S), ribosome
520 protection tetracycline resistance gene; *aac(6')-Ie – aph(2')-Ia*, gentamicin and kanamycin
521 acetyltransferase and phosphotransferase tandem genes; *ant(4')-Ia*, amikacin, kanamycin and
522 tobramycin nucleotidyltransferase gene; *ant(6)-Ia*, streptomycin nucleotidyltransferase gene;
523 *aph(3')-III*, kanamycin phosphotransferase gene; *erm*(B), macrolide, lincosamide and
524 streptogramin B 23S rRNA methylase gene; *fusC*, gene encoding for cytoplasmic protein that
525 protects EF-G from binding fusidic acid; *sat4*, streptothricin acetyltransferase gene.
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528 **TABLE 2** Origin, antibiotic resistance and genetic characteristics of the *M. caseolyticus*

529 strains from England and Wales.

Strain	Region/date	*mecD* element [a]	McCI$_{IMD0819}$ [b]	Resistance phenotype [c]	Resistance genes [d]	ST	*cop* gene [e]	Reference
5194_2_25	Cheshire/2015	-	+	FOX, PEN, (CLI), FUS	*mecB*, *blaZ*$_m$, *fusC*	41	+	(11)
5456_3_46	Shropshire/2015	- (*int0473*)	-	FOX, PEN, CLI, ERY, (TMP)	*mecB*, *blaZ*$_m$, *erm*(B)	45	-	(11)
5812_BC73	Gwent/2016	-	+	FOX, PEN, (CLI), TET	*mecB*, *blaZ*$_m$, *tet*(L), *ant(4')-Ia*	52	-	(11)
5814_BC75	Gwent/2016	-	+	FOX, PEN, (CLI), TET	*mecB*, *blaZ*$_m$, *str*	52	-	(11)
5783_EF107	Gloucestershire/ 2015	-	+	FOX, PEN, (CLI), FUS	*mecB*, *blaZ*$_m$, *fusC*, *str*	48	+	(11)
5816_BC109	Gwent/2016	-	+	FOX, PEN, (CLI), TET, FUS	*mecB*, *blaZ*$_m$, *tet*(L), *fusC*, *str*, *ant(4')-Ia*	48	+	(11)
5813_BC74	Abergavenny/ 2016	McRI$_{mecD}$-1	+	FOX, (CLI), TET, FUS	*mecD*, *tet*(L), *fusC*, *str*, *lnu*(A), *mph*(B)	48	+	(11)
5789_EF199	Devon/2016	McRI$_{mecD}$-1	+	FOX, PEN, CLI, TET	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	49	-	(11)
5784_EF114	Devon/2015	McRI$_{mecD}$-1	+	FOX, PEN, CLI, ERY, TET	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	49	-	(11)
5785_EF123	Wiltshire/2015	McRI$_{mecD}$-1	+	FOX, PEN, CLI, ERY	*mecD*	50	-	(11)
5459_5_49	Cornwall/2015	McRI$_{mecD}$-3Δ	-	FOX, PEN, CLI, ERY,TET	*mecD*, *erm*(B), *tet*(L), *tet*(M), *str*	46	-	(11)
5782_EF83	Dorset/2015	McRI$_{mecD}$-3Δ	-	FOX, PEN, CLI, ERY, TET	*mecD*, *erm*(B), *tet*(L), *tet*(M), *str*	46	-	(11)
5458_5_53	Cornwall/2015	McRI$_{mecD}$-3 (*rts*)	-	FOX, PEN, (CLI)	*mecD*	6	-	(11)
5800_EF393a	Pembrokeshire/ 2016	McRI$_{mecD}$-3 (*rts*)	-	FOX, PEN, (CLI)	*mecD*, *ant(4')-Ia*	6	-	(11)
5799_EF381	Pembrokeshire /2016	McRI$_{mecD}$-3 (*rts*)	-	FOX, PEN, (CLI), TET	*mecD*, *tet*(M), *str*, *ant(4')-Ia*	6	-	(11)
5457_3_80	Cheshire/2015	McRI$_{mecD}$-3	+	FOX, PEN, CLI, ERY, TET, (TMP)	*mecD*, *erm*(B), *tet*(L), *aph(3')-III*, sat4	5	+	(11)
5786_EF153	Cheshire/2016	McRI$_{mecD}$-3	+	FOX, PEN, CLI, ERY, TET, (TMP)	*mecD*, *erm*(B), *tet*(L), *aph(3')-III*, *sat4*	5	+	(11)
5794_EF323	Camarthenshire/ 2016	McRI$_{mecD}$-3	+	FOX, CLI, TET, (TMP)	*mecD*, *tet*(L), *aph(3')-III*, *sat4*	5	+	(11)
5815_BC85	Monmouthshire/ 2016	McRI$_{mecD}$-3	+	FOX, PEN, TET	*mecD*, *tet*(L), *tet*(S), *aph(3')-III*, *sat4*	5	+	(11)
5193_2_23	North Yorkshire/2015	McRI$_{mecD}$-3	+	FOX, (CLI)	*mecD*, *str*	21	-	(11)
5818_BC116	Cheshire/2016	McRI$_{mecD}$-3	+	FOX, PEN, (CLI)	*mecD*, *str*	21	-	(11)
5196_2_38	North Yorkshire/2015	McRI$_{mecD}$-3	+	FOX, PEN, (CLI)	*mecD*	26	+	(11)
5198_3_76	Shropshire/2015	McRI$_{mecD}$-3	+	FOX, PEN	*mecD*	26	-	(11)
5788_EF188	Shropshire/2016	McRI$_{mecD}$-3	+	FOX, PEN	*mecD*	26	-	(11)
5190_42462	Sussex/2015	McRI$_{mecD}$-3	+	FOX, PEN, CLI	*mecD*	40	+	(11)
5787_EF169	Lancashire/2016	McRI$_{mecD}$-3	+	FOX, PEN, CLI, (TMP)	*mecD*	40	+	(11)
5197_42554	Devon/2015	McRI$_{mecD}$-3	+	FOX, PEN, CLI	*mecD*, *str*	42	-	(11)
5450_CC63A	Ceredigion/2016	McRI$_{mecD}$-3	+	FOX, PEN, CLI, TET	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	43	-	(11)
5452_CC83	2016	McRI$_{mecD}$-3	+	FOX, PEN, CLI, TET, (TMP)	*mecD*, *tet*(L), *str*, *ant(4')-Ia*	44	-	(11)
5781_EF64	Wiltshire/2015	McRI$_{mecD}$-3	+	FOX, PEN, CLI, ERY	*mecD*, *str*, *ant(4')-Ia*	47	-	(11)
5798_EF375	Camarthenshire/ 2016	McRI$_{mecD}$-3	+	FOX, PEN, CLI, ERY	*mecD*, *lnu*(A)	47	-	(11)
5795_EF335	Lancashire/2016	McRI$_{mecD}$-3	+	PEN, CLI, ERY	*mecD*, *erm*(B), *ant(9)-Ia*, *str*, *lnuG*	51	-	(11)
5804_BC29	Cheshire/2016	McRI$_{mecD}$-3	-	FOX, PEN, (CLI), FUS	*mecD*, *fusC*	41	+	(11)

530 [a] *mecD* elements were determined by mapping illumina reads to McRI$_{mecD}$-1, McRI$_{mecD}$-2 and

531 McRI$_{mecD}$-3 references.

[b] Presence (+) and absence (-) of McCI$_{IMD0819}$ was determined by read mapping.

[c] Resistance phenotypes were from MacFadyen et al, 2018 measured by using Vitek2 AST-P634 card and by Etest (BioMérieux) for cefoxitin MIC values, parentheses indicate intermediate resistance.

[d] Resistance genes were identified using ResFinder (25) and NCBI BLASTn for identification of *blaZ$_m$*, *fusC* and *sat4*.

[e] Presence (+) and absence (-) of *cop* gene was determined by BLASTn.

[c, d] Abbreviations for antibiotics and resistance genes are explained in legend of Table 1. Lincosamide resistance genes *lnuA* and *lnuG* encode lincosamide nucleotidyltransferases.

TABLE 3 Features of multiplex PCR for typing the *Macrococcus caseolyticus* resistance island *mecD* (McRI*mecD*).

PCR	Target	Present in McRI$_{mecD}$ 1	2	3	Primer 1 (label Fig. 1)	Primer 2 (label Fig. 1)	Poitive control	Amplicon size/bp
Multiplex I	*rpsI*-associated integrase:							
	int0819	+	-	-	rpsI-F (1)	int0819-F (2)	IMD0819	809
	int0473	-	+	+	rpsI-F (1)	int0473-F2 (3)	IMD0473, Msa0018	1,328
					rpsI-F (1)	int0473-F2 (3)	JCSC5402	1,823
Multiplex II	Putative virulence and recombination-mediator genes:							
	virE0819	+	-	-	virE0819-F (4)	virE0819-R (5)	IMD0819	468
	virE0473	-	+	+	virE0473-F (6)	virE0473-R (7)	IMD0473, Msa0018	250
	dprA	+	-	+	dprA-F (8)	dprA-R (9)	IMD0819, Msa0018	671
Multiplex III	Restriction-modification system, reverse transciptase and copper-translocating P-type ATPase genes:							
	hsmMI-hsrMI	+	-	+	hsmMI-F (10)	hsrMI-R (11)	IMD0819, Msa0018	1,327
	rt0473	-	+	-	rt0473-F (12)	rt0473-R (13)	IMD0473	1,059
	cop	-	-	-	cop-F (14)	cop-R (15)	IMD0819, Msa0018	286
					cop-F (14)	cop-R (15)	JCSC5402	286
					cop-F (14)	cop-R (15)	KM1352	262

546 **TABLE 4** Oligonucleotide primers.

Primer label	Primer name	Sequence (5'-3')	Target	Reference
16	mecD-F	TCCTTTAGCGATAGATGGTGAA	*mecD*	(7)
17	mecD-R	CTCCCATCTTTTCTCCATCCT		
1	rpsI-F	TGGTCAAGCACAAGCTATC	*rpsI*	This study
2	int0819-F	TGGCTAAGGACAAAGATCAG	*int0819* of McRI$_{mecD}$-1	(7)
3	int0473-F2	TGAACTGCGTAAATTACAACTTC	*int0473* of McRI$_{mecD}$-2/McRI$_{mecD}$-3	This study
4	virE0819-F	GTCATCCGCATGATACAACG	*virE0819* of McRI$_{mecD}$-1	This study
5	virE0819-R	GATGATTCGTTTCACCGTCC		
6	virE0473-F	ATTGTTCGGAAAGGATGCAC	*virE0473* of McRI$_{mecD}$-2/McRI$_{mecD}$-3	This study
7	virE0473-R	TATCCCGTCCCATTCCAAAC		
8	dprA-F	AAAGCTAGCGATACACAACTA	*dprA* of McRI$_{mecD}$-1/McRI$_{mecD}$-3	This study
9	dprA-R	GCTGTATGCATAGTACCACTT		
10	hsmMI-F	GATGAAAACTGTGTTCCGTT	*hsmMI* of McRI$_{mecD}$-1/McRI$_{mecD}$-3	This study
11	hsrMI-R	TCTATCGGGAAAAGCAGTCA	*hsrMI* of McRI$_{mecD}$-1/McRI$_{mecD}$-3	
12	rt0473-F	TAAAGACCTGCCCCTTATGT	*rt0473* of McRI$_{mecD}$-2	This study
13	rt0473-R	TTCCAATCACTTCGAGTTCC		
14	cop-F	TATACTCACATTATCTTATTACTATCTC	*cop*	This study
15	cop-R	GCAAGAATTAATACAATCCAATCTG		(7)
18	truA-F	GACAGTATCCCTGCAATCATTC	*truA*	(7)
19	int0473-F	TCATGGCTTCAGGCATACAC	*int0473* of McRI$_{mecD}$-2/McRI$_{mecD}$-3	(7)
20	araC-F	TACCGTCATTCTGGCAAAC	*araC* of McCI$_{IMD0819}$	(7)
21	IMD0819c21-F6	GTACAGAAATTATAGGAAGGAAG	Left side of McCI$_{IMD0819}$	This study
22	orf20-F	GTATTCCCAACTTCGTCTGGA	*orf20* of McRI$_{mecD}$-1 and *orf19* of McRI$_{mecD}$-3	(7)
-	lnuG-F	AGGAGAGGGAGATCAATACT	*lnuG*	This study
-	lnuG-R	CATTTAATCGGGCAGTAGTC		
-	blaZm-fw	AAGTACAATATTCAAGCGGGTGT	*blaZ$_m$*	(26)
-	blaZm-rv	AATTAGCTCCCTGCCCACTT		

547

548

549 **FIGURE LEGENDS**

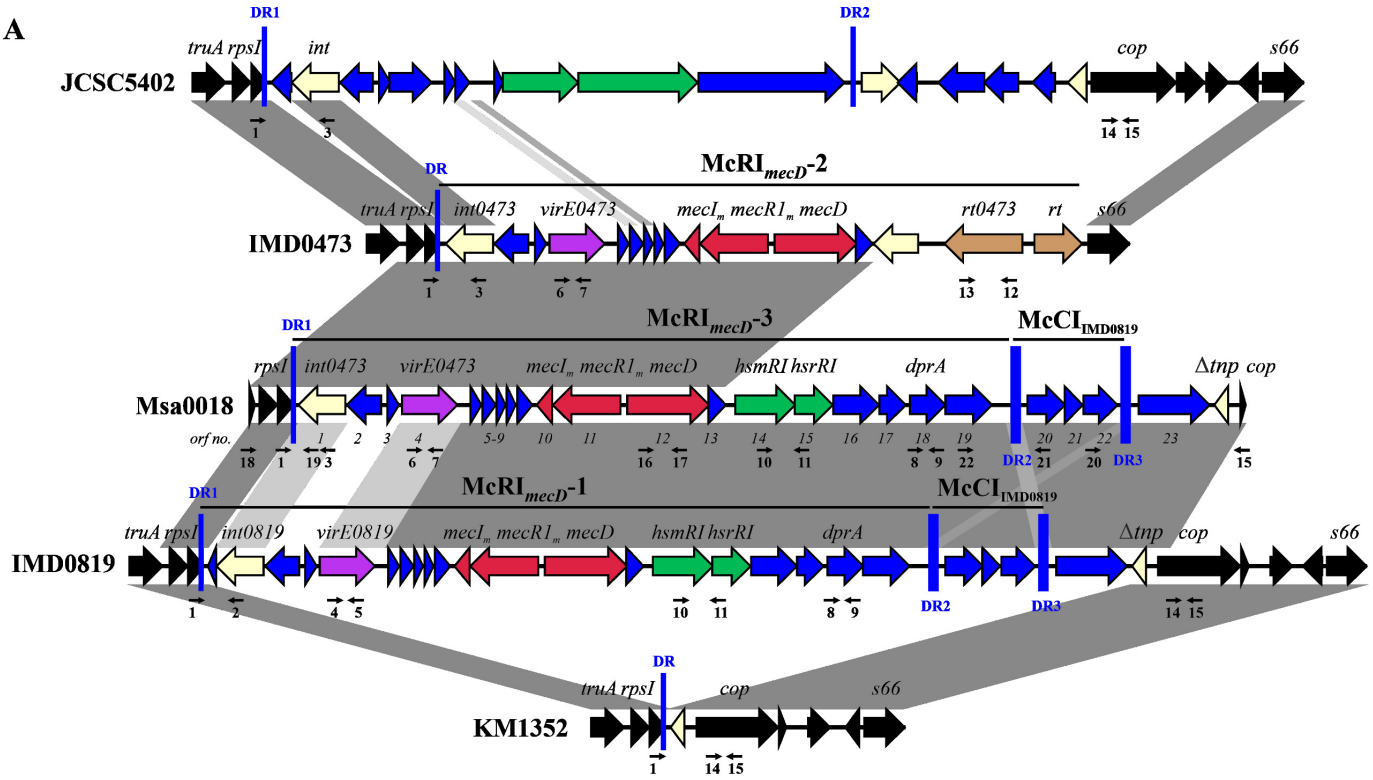
550 **FIG 1** Genomic islands and core attachment sites at the 30S ribosomal protein S9
551 gene locus *rpsI* in *M. caseolyticus*. (A) Comparison of strains containing the *M. caseolyticus*
552 resistance islands McRI$_{mecD}$-1 (strain IMD0819), McRI$_{mecD}$-2 (IMD0473), McRI$_{mecD}$-3
553 (Msa0018); an alternative accessory island (JCSC5402); or no insert (KM1352). The
554 chromosomal island McCI$_{IMD0819}$ found in some strains is indicated, and the imperfect direct
555 repeats (DRs) delimiting genomic elements are represented as vertical lines. All putative
556 open reading frames are shown by arrows: *mec* operon genes are in red, restriction
557 modification systems are in green, recombinases are in pale yellow, *virE* genes are in purple,
558 reverse transcriptases are in beige, other unique genes of *rpsI*-associated islands are in blue
559 and core genome genes are in black. The primers used for PCRs are represented as small
560 black arrows labelled by numbers below them (see Table 4 for the primer names and
561 sequences). Gray areas indicate regions with between 68 % and 100 % nucleotide sequence
562 identity. The figure was generated using Easyfig software (27) and the sequences of the *M.*
563 *caseolyticus* strains JCSC5402 (GenBank acc. no: region, AP009484: 220254-247942)
564 IMD0473 (KY013610: 5075-24092), Msa0018 (MH671353), IMD0819 (KY013611: 5075-
565 35902) and KM1352 (KY013613: 5075-12916). (B) Putative core attachment (*att*) sites
566 found in the extended DRs delimiting McRI$_{mecD}$-3 and the chromosomal island McCI$_{IMD0819}$
567 in strain Msa0018. The numbers indicate additional bases belonging to DRs upstream and
568 downstream of the core *att* sites. The positions that include variant bases within the core *att*
569 sites are unshaded.

570

571 **FIG 2** Characterization of McRI$_{mecD}$ in *M. caseolyticus* by multipex PCR I, II and III.
572 PCR products are shown for the reference strains containing McRI$_{mecD}$-1 (IMD0819),
573 McRI$_{mecD}$-2 (IMD0473), or McRI$_{mecD}$-3 (Msa0018) as well as for the two negative control

574 strains (JCSC5402 and KM1352). Specific amplicons (obtained by multiplex PCR I, II, or
575 III) are indicated on the right side of the agarose gel. For the amplicon sizes, see Table 3. The
576 DNA markers used were from Solis Biodyne (M1, 1-kb DNA ladder; M2, 100-bp DNA
577 ladder).
578
579 **FIG 3** Phylogenetic relationship and carriage of the *mec* element in *M. caseolyticus*
580 strains from Switzerland and England/Wales. The maximum parsimony tree was constructed
581 based on 7 gene multilocus sequence typing (MLST) data. The sequence types (STs) are
582 specified by the numbers next to the nodes, and the origins of the strains and McRI$_{mecD}$ types
583 are visualized by color code. STs that differ in 4 or more variants are linked by dashed and
584 dotted lines, respectively.
585
586

A



B

core attachment site

```
                1                                                  61
Msa0018 DR1 [  0+ GAACGTAAAAAACCAGGTCTTAAAGGCGCTCGTCGTTCACCACAGTTCTCAAAACGTTAAT + 62 b]
Msa0018 DR2 [146+ GAACGTAAAAAACCAGGTCTTAAAGGTGCTCGTCGTTCACCACAATTCTCAAAACGTTAAT +198 b]
Msa0018 DR3 [146+ GAACGTAAGAAGCCAGGTCTTAAAGGCGCTCGTCGTTCACCACAGTTCTCAAAACGTTAAT +197 b]
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