Characteristics of population structure, antimicrobial resistance, virulence factors, and morphology of methicillin-resistant *Macrococcus caseolyticus* in global clades

Yu Zhang, Shengyi Min, Yuxuan Sun, Jiaquan Ye, Zhemin Zhou* and Heng Li*

**Abstract**

*Macrococcus caseolyticus* is an opportunistic pathogen that is frequently isolated from dairy products and veterinary infections. Recent studies have reported the possibility of methicillin resistance that be transferred among staphylococcal species in foods. The present study examined the population structure, antimicrobial resistance, virulence factors, and morphology of methicillin-resistant *M. caseolyticus* by investigation of 94 genomes derived from both isolates in beef (n = 7) and pork (n = 2) at Shanghai and those deposited in public domain (n = 85). Phylogenetically, *M. caseolyticus* were divided into four clades, which each consisted of genomes isolated from continent of European countries (82.4%, n = 78), Asian countries (11.3%, n = 10), United States (4.1%, n = 4), Australia (1%, n = 1), and Sudan (1%, n = 1). The *M. caseolyticus* isolated from present study formed a genetically distinguished clade, which was characterized by novel alleles in the traditional 7-gene MLST scheme. Furthermore, we identified 24 AMR genes that were associated with 10 classes of antimicrobial agents in *M. caseolyticus*. Most AMR genes were carried by dominant plasmids such as rep7a, rep22 and repUS56. The genomes in the global clades carried significantly less AMR genes (p < 0.05) and more virulence factors (p < 0.001) than present clade. Virulence factors were detected in methicillin resistant *M. caseolyticus* including genes coding hemolysin, adherence, biofilm formation, exotoxin, and capsule that associated to human health and infection. Finally, as the close relative of the genus *Staphylococcus*, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed for morphological comparison that *M. caseolyticus* has a larger diameter and thicker cell wall compared with *S. aureus* ATCC 25,923. Taken together, our study suggested that *M. caseolyticus* mediating divergent antimicrobial resistance and virulence factors could serve as the vector for methicillin resistance habitats in foodborne microorganisms.

**Highlights**

- The global lineage of *M. caseolyticus* strains were divided into four clades from A to D.
- MLST typing revealed novel alleles in *M. caseolyticus* strains isolated in China.
- Global clades carried significantly less AMR genes and more virulence factors than present isolates.
Introduction

Initially described in 1916, *Macrococcus* are catalase-positive cocci that currently consist of 12 species, including *Macrococcus caseolyticus*, *Macrococcus bovis*, *Macrococcus canis*, and many more [9, 25]. As the close relative of the genus *Staphylococcus*, *M. caseolyticus* exhibits a high homology in phenotypical and biological characteristics, and shares similarities with these oxidase-positive and novobiocin-resistant staphylococci [25]. Previously, *M. caseolyticus* was classified as *Staphylococcus caseolyticus* due to the high similarities [5, 7, 29], indicating the necessity to distinguish these two bacteria from a perspective of morphological, phenotypic, and genotypic approaches.

*Macrococcus caseolyticus* is an exotic bacterium that is frequently found in fermented cheeses, sausages, pigs, calves, pork, and beef meat in Europe [17]. It can enhance the flavor of dairy products by producing amino acid and lipid-derived flavor compounds [10, 15, 26]. However, the adaptive acquisition of methicillin resistance genes in *M. caseolyticus* genomes, such as *mecABCD* has been observed over the past decades [11, 28–30]. Comparing to classical *mecA* and *mecC* that are mostly carried by *Staphylococcus aureus*, the methicillin-resistant genes in *M. caseolyticus* are *mecB* and *mecD* respectively. Similar to the mechanism in methicillin-resistant *S. aureus* (MRSA), the mec complex in *M. caseolyticus* JCSC7096 is also associated with a transposon Tn6045, which can be horizontally transferred into other species in the *Macrococcus* genus [12].

Recent studies have reported high mortality rates in animals infected with *Macrococcus* [4, 5, 20]. For instance, the *M. caseolyticus* SDLY strain isolated from commercial chickens exhibited severe pathogenicity, including hemorrhages and multifocal necrosis, which might be associated with the insertion of capsular polysaccharide synthesis genes in a virulence background [20].

To further characterize antimicrobial resistance, virulence factors, and morphology in global lineage of methicillin-resistant *M. caseolyticus*, a combined analysis of phenotypic, genomic, and morphological approaches was administrated on a set of 94 *M. caseolyticus* isolates. We examined the antimicrobial resistance and virulence factors in a global scale and described morphological comparison between *M. caseolyticus* and its close relative, *Staphylococcus aureus*, which allowed a better understanding of these two bacteria species.

Method

Sampling and collection

Samples from each of 24 outlets, including 11 in wet markets and 13 in the supermarkets, were collected between August and October of 2021 in Shanghai (Supply Table 1). The sampled outlets were distributed across 11 districts from Shanghai city, including Pudong (n = 6), Huangpu (n = 3), Xuhui (n = 2), Jing’an (n = 2), Minhang (n = 2), Yangpu (n = 2), Changning (n = 2), Putuo (n = 2), Songjiang (n = 1), Fengxian (n = 1), and Baoshan (n = 1). Packages of frozen chicken, beef, and pork were purchased from the outlets and transported to the laboratory on ice containers within 4 h of collection. Retail meat products were prepared for analysis as previously described [21]. Briefly, 10 g of samples were homogenized in 0.1% peptone saline in a filter bag (Bkmam, Changde, China) and 100 ul were cultured onto CHROMagar™ MRSA agar (Becton Dickinson, Franklin Lakes, NJ) for selection overnight at 37 °C [17]. The obtained *M. caseolyticus* strains were confirmed by 16 s rRNA sequencing and MALDI-TOF MS (Bio-M’erieux, Craponne, France). Detail isolation and species confirmation were described in Supply Table 1.

Phenotypic antimicrobial susceptibility test

Confirmed *M. caseolyticus* strains were enrolled for antimicrobial susceptibility test by disk diffusion (Oxoid) according to the guideline of Clinical and Laboratory Standards Institute (CLSI 2017). The antimicrobial compounds included ampicillin (10 μg), amikacin (30 μg), cefazolin (30 μg), cefuroxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), cefoperazone (75 μg), doxycycline (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), minocycline (30 μg), penicillin (10 μg), pipercillin (100 μg), streptomycin (10 μg), tetracycline (30 μg), and with cefoxitin as methicillin-resistant drug control. *Escherichia coli* ATCC 25,922 and *S. aureus* ATCC 25,923 were used as the quality control.

Whole genome sequencing

Nine *M. caseolyticus* strains isolated from this study were sent for DNA purification using HiPure Bacterial DNA
Public data retrieval and analysis
A total of 87 SRA genomes of *M. caseolyticus* were evaluated from GenBank (accessed in December 2021). After checking the duplicate samples, 51 SRA genomes were excluded, and addition two strains were removed due to the low identity by species identification. Finally, a total of 85 global strains were downloaded from GenBank (accessed in December 2021). After evaluation with QUAST v2.3 [13, 31]. This resulted to a total of 87 SRA genomes of *M. caseolyticus* were submitted for the China National GenBank under project accession ID CN0002826.

Genomic analysis and SNP tree construction
All 94 *M. caseolyticus* assemblies were submitted for MLST typing (https://pubmlst.org) and screened for antimicrobial-resistant genes and virulence factors using online tools of ResFinder, MobileElementFinder, and VFDB [3, 14, 22]. Alignments with a minimum of ≥ 60% nucleotide identity was kept in all three programs, respectively. The single nucleotide polymorphism (SNP) tree was constructed using CSI Phylogeny v1.4 (http://genomicepidemiology.org/) by aligning other genomes onto a reference genomic sequence from *Macrococcus* sp. (ASM2009413).

Phylogenetic tree with temporal and geographical datasets
EToKi was used to evaluate the 94 *M. caseolyticus* genomes with ASM211982 selected as the reference genome due to the highest N50 value [31]. Then ETok align was applied to generate a matrix file to further build the phylogenetic tree containing the datasets of geography and years [31]. Afterwards, RecHMM and RecFilter were enrolled to filter the reorganization of the phylogenetic tree [31]. Finally, the dated tree was generated by using BactDating v1.1, and was then used to infer geographic distributions using treetime v1.0 [8, 27].

Data visualization and statistical analysis
The genotypic data were visualized in Grapetree and iTOL v4.0 [19, 32]. The violin graph was drawn in GraphPad Prism 7 and statistical significance was calculated using One-way ANOVA with *p* < 0.05, **p** < 0.01, ****p** < 0.001. Description of antimicrobial-resistant genes and MLST were list in Supply Table 3 and Supply Table 4.

Comparative morphological observation
To better understand the morphological differences between *M. caseolyticus* and its closest relative of *S. aureus*, the HB024567 strain from present study and *S. aureus* ATCC 25,923 were employed for SEM and TEM analysis. Prior to scanning and transmission electron microscope (SEM/TEM), the *M. caseolyticus* HB024567 and *S. aureus* ATCC 25,923 cultures were grown in 50 ml of LB at 37 °C with a starting OD600 of 0.02. Cells were harvested at OD600 ~ 0.5 and suspended in fixation solution and incubated overnight at 4 °C. After the treatments, cell pellets were washed twice with cacodylate buffer (0.05 M, pH 7.4) and post-fixed with 2% osmium tetroxide, followed by 0.25% uranyl acetate for contrast enhancement. The pellets were dehydrated with ethanol (30, 50, 70, 80, 90, and 100%), embedded in Epoxy resin, and cut into ultrathin sections for lead citrate staining. The final sections were examined by Philips CM100 BioTWIN transmission electron microscope. For the SEM, the suspended cells after post-fixation and dehydration were placed on stubs and coated with gold–palladium for 2 min. Then samples were observed by JSM-7500F scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Results
Geographic distribution, population structure, and years of isolation
A total of 94 *M. caseolyticus* strains were selected for geographic analysis including nine strains isolated from beef (*n* = 7) and pork (*n* = 2) in Shanghai and additional 85 isolates from GenBank (Fig. 1A/B). All strains were distributed in five continents including European countries (82.4%, *n* = 78), Asian countries (11.3%, *n* = 10), United States (4.1%, *n* = 4), Australia (1%, *n* = 1), and Sudan (1%, *n* = 1) (Fig. 1A/B). The phylogenetic tree showed that global *M. caseolyticus* strains were divided into four clades from A to D. Clade A formed two independent clusters that were separated from the rest strains and this clade was associated with human infection, while clade B from this study was mainly detected in retail meat (beef, *n* = 7; pork, *n* = 2) as a local Chinese cluster. Clade C and D had the dominant proportion isolated from bulk milk (Fig. 1C & 2). MLST typing of clade B showed the presence of novel alleles among the present *M. caseolyticus* strains, e.g., *cpn60, fdh, pta, purA* in HB024539 and *cpn60* and *pta* in HB024569 (Table 1 & Supp. Table 4). Afterwards, the years of isolation were assessed for all global *M. caseolyticus* strains. The present strains formed as a separated clade
that isolated in 2021 (Gray oval, Fig. 1D), whiles the remaining strains showed diverse years of collection between 2003 and 2020 (Fig. 1D).

Identification of AMR genes and mobile elements
Given the importance of antimicrobial resistance (AMR) in opportunistic pathogens, we compared the distributions of AMR genes in 94 M. caseolyticus strains from different clades (Fig. 2). A total of 24 AMR genes associated with 10 classes of antimicrobial agents were present in the isolates from four clades, among which 33% (1/3, clade A), 90% (9/10, clade B), 50% (7/14, clade C) and 46.3% (31/67, clade D) of isolates from the corresponding clades were identified as multi-drug resistance (Fig. 2). Notably, all the nine Chinese isolates contained mecB gene whiles HB024566, HB024567, and HB024568 carried multi-antimicrobial resistance genes including ant(6)-Ia, dfrG, isaE, inuB, qacZ, and tetL. In addition, tetL was detected in all present isolates from Shanghai city, China, which may be associated with the horizontal plasmid transfer of rep22. The mobile genetic elements analysis showed that rep7a, rep22, and repUS56 were the top dominant plasmids in M. caseolyticus with the prevalence of 35%, 35%, and 34% in global clades, respectively (Fig. 2). Further statics indicated that the number of AMR genes from clade B (Chinese clade) was significantly higher than those from clade A, C and D (* p < 0.05, ** p < 0.01, Fig. 3).

Phenotypical antimicrobial resistance of present strains
All nine M. caseolyticus isolates from present study were screened for phenotypic antimicrobial-resistant profiles. Specifically, all nine isolates were resistant to ampicillin, cefazolin, ceftazidime, lincomycin, piperacillin, penicillin, streptomycin, and tetracycline, whereas a majority were sensitive to amikacin, cefturoxime, and gentamicin, indicating a broad but complex multi-drug resistance in these M. caseolyticus strains isolated from the current retail meat in Shanghai, China (Table 2).

Distributions of virulence factors in global clades
Virulence factors were compared to determine the pathogenicity within the four clades (Fig. 4). Totally 69 virulence factors were assessed among 94 M. caseolyticus strains including the functional factors of adherence, biofilm formation, exotoxin, capsule, and others. Remarkably, the majority of Chinese clade B lacked the factor of CTC01574 (hemolysin) but contained CD1208 which were associated with hemolysin. Meanwhile, most clade B strains contained cytolyisin-related genes, e.g., hpt, manA, however, lacked genes regulating capsule synthesis such as capE, capF, capO, and capM (capsular polysaccharide synthesis enzyme), indicating the weak ability
Fig. 2 Distributions of MGEs and AMR genes in global *M. caseolyticus* clades. All *M. caseolyticus* strains were divided into four clades including A, B, C, and D based on phylogenetic tree. The squares colored by trait category represented the presence of MGE or AMR genes.

**Table 1** Multi-locus sequence typing (MLST) of nine *M. caseolyticus* strains isolated from beef and pork meat in Shanghai, China

| Strains   | MLSTa | ack | cpn60 | fdh  | pta | purA | sar | tuf |
|-----------|-------|-----|-------|------|-----|------|-----|-----|
| HB024538  | 18/53 | 6   | 2     | 9    | 5b  | 3    | 7   | 4   |
| HB024539  | 18/53 | 6   | 13b   | 9b   | 10b | 3b   | 7   | 5   |
| HB024564  | 18/53 | 6   | 13b   | 9b   | 5   | 20b  | 7   | 5   |
| HB024565  | 62    | 6   | 2     | 9b   | 10b | 20b  | 17b | 5   |
| HB024566  | 18/53/62 | 6 | 2/12b | 9b | 5 | 20b | 1 | 5 |
| HB024567  | 18/53/62 | 6 | 2/12b | 9b | 5 | 20b | 1b | 5 |
| HB024568  | 18/53 | 6   | 2b    | 9b   | 5   | 20b  | 2b  | 5   |
| HB024569  | 3/31/32/33/62 | 6 | 3b | 3 | 1b | 3 | 1 | 4 |
| HB024570  | 18/53 | 6   | 3b    | 9b   | 5   | 2    | 1   | 5   |

*a* MLST was presented with the closest types

*b* Novel alleles were indicated with the closest alleles numbers
of invasiveness. In clade D, the replacement and loss of functional genes were also observed that KPHS_39850 (protein disaggregation chaperone) was only present in ASM767322 while A225_4443 (clpB factor) were widely distributed in all global isolates. Notably, the aur panC, and panD genes were absent in Chinese isolates in clade B. To summarize, the number of virulence factors in clade B was significantly lower than that of clade C and D (** **p < 0.001, Fig. 5), which may be due to the deletion of corresponding proteins related to capsule synthesis, zinc metalloproteinase aureolysin, and pantothenic acid synthesis.

**Comparative morphological observation of M. caseolyticus and S. aureus**

To better understand the morphological differences between M. caseolyticus and its closest relative of S. aureus, the HB024567 strain from present study and S. aureus ATCC 25,923 were employed for SEM and TEM analysis. SEM results showed that M. caseolyticus was 1.1 ± 0.05 μm with a round shape and smooth surface, while S. aureus was 0.46 ± 0.01 μm which was smaller than the M. caseolyticus strain (Fig. 6). Then the TEM results demonstrated that M. caseolyticus had a broad and thick cell wall with a diameter of around 65 ± 5 nm, whereas the cell wall was much narrow and thin in S. aureus (21 ± 1 nm) (Fig. 7).

**Discussion**

In China, *Macrococcus caseolyticus* has been isolated from many fermented foods, such as Chinese sausages and the specialty food "ChouGuiYu", a type of fermented mandarin fish [6]. Furthermore, *M. caseolyticus* can be isolated from human, animal, and raw meat during food processing and transportation [1, 16, 17], allowing it to be transmitted internationally via commercial shipments of agricultural products.
China is the largest import market and the 6th largest export market of agricultural products. With these commercial shipments it was expected that *M. caseolyticus* in China would be greatly sharpened. To evaluate the hypothesis, *M. caseolyticus* were isolated from both beef (*n* = 7) and pork (*n* = 2) from Shanghai. Nine isolates presented a novel sequence type. In particular, six isolates were found to each carry >3 novel virulence factors.
alleles, indicating that they were genetically distinct from all existing isolates in pubMLST. Phylogenetic analysis also confirmed that nine Chinese *M. caseolyticus* isolates formed a distinct cluster of Clade B in the tree, which did not intermingle with isolates from other countries, with the exception of one isolate from the US (ASM435972).

Most *M. caseolyticus* isolates carried at least one of *mecB* and *mecD* elements (Fig. 2), both homologs of *meca*, which induced resistance to methicillin [28]. A recent study also reported the presence of *mecB* in *S. aureus* strains, which could be horizontally transferred from *M. caseolyticus* [2]. All nine Chinese isolates carried the *mecB* gene. Furthermore, six Chinese isolates were multidrug-resistant, probably due to the acquisitions of MDR mobile genetic elements. For example, it was identified that *str* gene was carried by rep7a, *aadD* (aminoglycoside nucleotidyltransferase gene) and *tetL* (tetracycline efflux protein gene) genes were carried by rep22, among others. Intriguingly, the Chinese isolates in Clade B had the more AMR genes then other isolates. This may be associated with the higher usage of antimicrobial agents in Chinese veterinary [20].

Antimicrobial resistance was tested phenotypically and associated encoding genes identified in genome-based analysis of all nine *M. caseolyticus* strains. In silico detection showed that *M. caseolyticus* isolates harbored antimicrobial resistance genes supporting the results of the phenotypic resistance to methicillin, tetracycline, and streptomycin including the *mecB* gene (n = 9), *tetL* genes (n = 9), *str* genes (n = 9), and other genes (Fig. 2 and Table 2).

Comparing the findings regarding the prevalence of antimicrobial resistant *M. caseolyticus* in this study with other studies. A regional-wide investigation on *M. caseolyticus* in England and Wales shows that all strains (n = 33) were determined as methicillin-resistant with both *mecB* and *mecD* detected in these diverse isolates [23]. A Chinese study describes the isolation of *M. caseolyticus* from broiler chickens containing genes that confer resistance to amikacin, penicillin G, piperacillin, kanamycin, chloramphenicol, and ceftriaxone, which is similar to our findings in the present study [20].

At the same time, all *M. caseolyticus* genomes were screened for virulence factors. The Chinese *M. caseolyticus* isolates were clustered together with ASM435972. Comparative genomic analysis revealed
several functional gene substitutions and losses between ASM435972 and the other clade B isolates. Notably, genes related to capsule synthesis were absent in Chinese isolates, which have previously been reported as the dominant virulence factors in broiler disease [20]. The panC and panD genes with pantothenic acid synthesis functions were also lost in the present Chinese isolates. Such reduction in virulence factors in Chinese M. caseolyticus isolates indicated a weaker pathogenicity, which might be due to the isolated environment of retail raw meat, rather than human skin or nasal samples.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) results showed that M. caseolyticus HB024567 possessed a diameter of 1.1 ± 0.05 μm with a cell wall of 65 ± 5 nm (Fig. 6 A & B), which showed larger values of diameter and cell wall compared to S. aureus ATCC 25,923. The genus Macrococcus was measured in previous studies with the diameters of M. caseolyticus ATCC13548 (1.1–2 μm), M. equiperadicus ATCC51831 (1.3–2.3 μm), M. bovicus ATCC51825 (1.2–2.1 μm), M. carouselicus ATCC5128 (1.4–2.5 μm), M. brunensis CCM4811 (0.89 -1.2 μm), M. hajekii CCM4809 (0.89 μm), M. lamae CCM4815 (0.74 -0.92 μm), and M. canis KM45013 (0.8 μm) [4, 9, 18, 24]. SEM results showed that M. caseolyticus had a smooth surface, while it was rough in M. bovicus, M. equiperadicus and M. carouselicus, with spiny protrusions observed in M. equiperadicus stains [18].

In conclusion, comparative genomic analyses were enrolled for the construction of population structure as well as for the prediction of antimicrobial resistance and virulence factors in 94 M. caseolyticus strains collected from humans, animals, meat, and dairy products. Four clades were identified globally that Chinese isolates clustered together as clade B, carrying significantly more AMR genes ($p<0.05$) and less virulence factors ($p<0.001$). MLST typing and phylogenetic trees indicated a potential local evolution of M. caseolyticus in China. The comparative electron microscope demonstrated the morphological variation between M. caseolyticus and S. aureus, showing that M. caseolyticus has a larger diameter and thicker cell walls. Together, the present study provided a phylogenetic and genotypic comparison of the global M. caseolyticus stains. Our results suggested that both human and animal reservoirs could contribute to contamination in food products and such products might serve as a vector for M. caseolyticus habitats in foodborne microorganisms.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-022-02679-8.

Additional file 1: Supply Table 1. Isolation and identification of nine M. caseolyticus isolates from this study by rIDBAC (http://fbac.dmicrobe.cn).

Supply Table 2. Description of the genomes used in this study. A total of 85 global strains were downloaded from GenBank including 34 sets of filtered SRA genomes and 51 already assemblies. Supply Table 3. Description of the 24 antimicrobial resistance genes used in this study. Supply Table 4. Multi-locus sequence typing (MLST) identified in additional 85 global M. caseolyticus isolates.

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Authors’ contributions

YZ, HL, ZZ wrote the main manuscript text and SM, YS, JY prepared the figures. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The raw reads from this study are submitted to the China National GenBank under project accession ID CNP0002826.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The Author hereby consents to publication of the Work in any and all BMC publications.

Competing interests

The authors declare no conflicts of interest.

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