Cloning and Occurrence of \textit{czrC}, a Gene Conferring Cadmium and Zinc Resistance in Methicillin-Resistant \textit{Staphylococcus aureus} CC398 Isolates\textsuperscript{v}

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We recently reported a phenotypic association between reduced susceptibility to zinc and methicillin resistance in \textit{Staphylococcus aureus} CC398 isolates from Danish swine (F. M. Aarestrup, L. M. Cavaco, and H. Hasman, Vet. Microbiol. 142:455–457, 2009). The aim of this study was to identify the genetic determinant causing zinc resistance in CC398 and examine its prevalence in isolates of animal and human origin. Based on the sequence of the staphylococcal cassette chromosome mec (SCC\textit{mec}) element from methicillin-resistant \textit{S. aureus} (MRSA) CC398 strain SO385, a putative metal resistance gene was identified in strain 171 and cloned in \textit{S. aureus} RN4220. Furthermore, 81 MRSA and 48 methicillin-susceptible \textit{S. aureus} (MSSA) strains, isolated from pigs (31 and 28) and from humans (50 and 20) in Denmark, were tested for susceptibility to zinc chloride and for the presence of a putative resistance determinant, \textit{czrC}, by PCR. The cloning of \textit{czrC} confirmed that the zinc chloride and cadmium acetate MICs for isogenic constructs carrying this gene were increased compared to those for \textit{S. aureus} RN4220. No difference in susceptibility to sodium arsenate, copper sulfate, or silver nitrate was observed. Seventy-four percent \((n=23)\) of the animal isolates and 48\% \((n=24)\) of the human MRSA isolates of CC398 were resistant to zinc chloride and positive for \textit{czrC}. All 48 MSSA strains from both human and pig origins were found to be susceptible to zinc chloride and negative for \textit{czrC}. Our findings showed that \textit{czrC} is encoding zinc and cadmium resistance in CC398 MRSA isolates, and that it is widespread both in humans and animals. Thus, resistance to heavy metals such as zinc and cadmium may play a role in the coselection of methicillin resistance in \textit{S. aureus}.

Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) isolates mainly belonging to CC (clonal complex) 398 have emerged recently in livestock production around the world (7, 10, 20, 22). This clonal lineage is able to persist in the farm environment and therefore constitutes a large reservoir for transmission to humans working in such environments, which raises occupational health concerns (14, 22, 27). Resistance to antimicrobials, as well as other factors, may have contributed to the success of MRSA CC398 and its emergence in the animal reservoirs (1).

Metal-containing compounds are widely used as a feed supplement or for the prevention of gastrointestinal diseases in food animals. Resistance to metals is widely disseminated among bacterial species, and we have shown previously that the use of copper in feed can coselect for resistance to macrolides and glycopeptides in \textit{Enterococcus faecium} (5). Heavy-metal resistance, i.e., resistance to arsenic, mercury, and cadmium, has been described among \textit{S. aureus} isolates, including MRSA (2, 17–19). We recently described a phenotypic association between reduced susceptibility to zinc and MRSA CC398 from pigs in Denmark (1). It was suspected that the genetic background for the metal resistance was genetically linked to the methicillin resistance, and it was hypothesized to be present in the staphylococcal cassette chromosome mec (SCC\textit{mec}) element carried by these MRSA isolates, as metal resistance genes had been described previously in the SCC\textit{mec} element type III of MRSA isolate ST239 (9).

The purpose of this study was to identify the genetic determinant(s) involved in the observed zinc resistance phenotype among the CC398 MRSA isolates from animals in Denmark and to investigate its effects on susceptibility to several metal compounds. Furthermore, the prevalence of resistance to zinc and the putative resistance gene were determined among a larger collection of swine and human \textit{S. aureus} CC398 isolates from Denmark.

\textbf{MATERIALS AND METHODS}

\textbf{Strains.} A total of 31 MRSA and 28 methicillin-susceptible \textit{S. aureus} (MSSA) CC398 strains isolated from 2000 to 2008 from pigs in Denmark (1), as well as 50 MRSA and 20 MSSA strains of CC398 isolated from humans in Denmark, were included in the screening for the putative zinc resistance gene.

The 20 human MSSA isolates were isolated from bacteremia cases. The 50 human MRSA isolates were randomly chosen among 115 CC398 MRSA isolates referred to the Statens Serum Institut. The reporting of MRSA outbreaks has been required in Denmark since November 2006.

The strain collection from animals that had been tested previously for zinc susceptibility contained 74\% of the zinc chloride-resistant isolates among the
TABLE 1. Results of susceptibility testing against heavy-metal compounds of *Staphylococcus aureus* recipient strain and clones

<table>
<thead>
<tr>
<th>Strain</th>
<th>czrC status</th>
<th>MIC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ZnCl₂</td>
</tr>
<tr>
<td>RN4220</td>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>RN4220 + pAT18</td>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>RN4220 + pAT18 with czrC 1-1</td>
<td>Positive</td>
<td>8</td>
</tr>
</tbody>
</table>

* All MIC determinations were performed in agar dilution assays as described before (2), and a tentative breakpoint for resistance was defined as MIC > 2 mM.

RESULTS

Cloning and metal susceptibility testing of the isogenic strains. Isogenic strains of *S. aureus* RN4220, one containing the vector pAT18 alone and one with the cloned fragment containing the czrC gene, were compared. The presence of czrC conferred a 4-fold increase in the MIC of zinc chloride, from 2 mM in RN4220 with pAT18 to 8 mM in the isogenic strain containing pAT18 with czrC. Additionally, the resistance level of cadmium also was affected, and the strains carrying czrC showed an 8-fold increase in the MIC of cadmium acetate. On the contrary, the MICS of copper sulfate, silver nitrate, and sodium arsenate did not show any observable changes (Table 1).

Metal susceptibility testing. A total of 74% (*n* = 23) of the MRSA from swine were resistant to zinc chloride (MIC > 2 mM), whereas all 28 MSSA strains were susceptible. All of the zinc-resistant MRSA strains harbored czrC, whereas all of the MSSA isolates and the eight MRSA isolates that displayed a zinc-susceptible phenotype tested negative for this gene. Among the human isolates, 48% (*n* = 24) of the MRSA strains tested were resistant to zinc chloride and harbored czrC, whereas 52% (*n* = 26) were susceptible. All 20 human MSSA isolates tested were susceptible to zinc chloride. All of the MSSA isolates and zinc-susceptible MRSA isolates were negative for the presence of czrC. These results show 100% correlation between phenotypic zinc chloride resistance and the presence of czrC. Additionally, the SCCmec typing data revealed that all of the MRSA strains positive for the czrC gene harbored a SCCmec cassette classified as type V according to the Kondo multiplex PCR method (11), whereas the negative strains harbored either a type IVa or V cassette in both strain collections.

DISCUSSION

MRSA CC398 isolates have emerged as a livestock-associated zoonotic pathogen in many countries, mostly in relation to occupational contact with animals (10, 14, 22, 25). Factors related to this emergence need to be understood before effi-
cient counteractive measures and interventions can be designed.

MSSA CC398 isolates belong to one of three major clonal complexes that have been found to be associated with pigs (6). Among the MRSA population, mostly strains of CC398 have been isolated from pigs, although other CCs have been found in China (CC9) and some countries in Europe (CC1, CC8, CC9, CC30, and CC97) (3, 4, 16, 23, 26).

In our previous study, it was observed that most MRSA CC398 strains isolated from pigs in Denmark had a high prevalence of zinc resistance (74%), whereas the corresponding MSSA isolates were susceptible, and it was hypothesized that zinc resistance was a factor involved in the selection of MRSA of this clonal complex (1).

In the present study, a gene was cloned and expressed in *S. aureus* RN4220 and found to encode reduced susceptibility to zinc and cadmium but not to arsenic, copper, or silver. We therefore suggest renaming this gene *czrC* (for cadmium and zinc resistance gene C). The screenings performed in animal and human isolates confirmed that *czrC* is strongly associated with the resistance phenotype observed.

The MRSA CC398 strains from animals had a higher prevalence of zinc resistance than the strains from humans. This difference might be explained by differences between the SCCmec cassettes. It was observed that *czrC* was associated with SCCmec type V (5C2&5) in the two sequenced SCCmec cassettes (19a and Li et al., unpublished) and also with SCCmec type VIII (28). Although classified as type V SCCmec elements based on the identification of the *crrC* and the class 1 *mec* element using the method described by Kondo and colleagues, there are variations within the cassette types that might not be detectable using the multiplex PCR strategy for typing (8). This also may explain the observation of both zinc-resistant isolates from pigs (n = 23) and humans (n = 24) but also zinc-suscetible MSSA isolates from pigs (n = 7) and humans (n = 15) carrying SCCmec elements classified as type V. Further information about the SCCmec elements circulating can be retrieved only by full sequencing, which may reveal more diversity of SCCmec elements (8).

The finding of a gene causing resistance to zinc that is linked to the SCCmec element is of concern, as compounds such as copper sulfate and zinc oxide are widely used in food-producing animals for the prevention of diarrheal diseases. Thus, the potential selective effect of heavy metals might affect the flora by providing a competitive advantage to resistant strains. The use of metallic compounds might be an important factor to consider in the future when investigating the selection dynamics of MRSA.

In conclusion, we cloned a resistance determinant, *czrC*, that is involved in zinc resistance in *S. aureus*. *czrC* was present in 74% of MRSA CC398 isolates from swine and 48% of MRSA CC398 isolates from humans in Denmark and was strongly related to zinc resistance. Further studies are needed to determine the occurrence of this gene among other populations and the importance for the coselection of MRSA.

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