Adhesion and Virulence Properties of Epidemic Canadian Methicillin-Resistant \textit{Staphylococcus aureus} Strain 1: Identification of Novel Adhesion Functions Associated with Plasmin-Sensitive Surface Protein

Mario Huesca,\textsuperscript{1,}\textsuperscript{a} Robert Peralta,\textsuperscript{1} Daniel N. Sauder,\textsuperscript{2,}\textsuperscript{a} Andrew E. Simor,\textsuperscript{1,}\textsuperscript{3,}\textsuperscript{a} and Martin J. McGavin\textsuperscript{1,}\textsuperscript{4}

Epidemic Canadian methicillin-resistant \textit{Staphylococcus aureus} strain 1 (CMRSA-1) comprises related subtypes that differ in phenotype and prevalence, with subtypes 1A, 1B, and 1D representing 1\%, 71\%, and 18\%, respectively, of total CMRSA-1 isolates. The predominant CMRSA-1B subtype possesses a variant of the staphylococcal cassette chromosome mec, harboring \textit{pls}, which encodes plasmin-sensitive surface protein (Pls). CMRSA-1B cells that express Pls exhibited poor adhesion to keratinocyte extracellular matrix. However, CMRSA-1B and purified Pls adhered to cellular lipids and glycolipids, and Pls promoted bacterial cell-cell interactions.

Although exoprotein expression was restricted to a precursor form of lipase in CMRSA-1B, it was not attenuated in virulence relative to CMRSA-1A, which exhibits normal exoprotein expression. In contrast, CMRSA-1D exhibited a pleiotropic defect in exoprotein expression and attenuated virulence relative to CMRSA-1A. These data indicate that the high transmissibility of CMRSA-1B was not achieved at the expense of attenuated virulence and that Pls confers a novel adhesion mechanism.

\textit{Staphylococcus aureus} is a major nosocomial pathogen that causes a broad spectrum of diseases, ranging from superficial skin infections to serious and potentially fatal illnesses, such as endocarditis, pneumonia, and septicemia [1]. The emergence of multiple-drug resistance in methicillin-resistant \textit{S. aureus} (MRSA), including strains with intermediate resistance to vancomycin, the only alternative in the treatment of many MRSA infections [2], has promoted renewed interest in understanding the mechanisms of persistence and virulence of this important pathogen.

MRSA is the most prevalent nosocomial pathogen in the United States [3, 4]; in Canada, a dramatic increase in the incidence of MRSA is associated with the emergence of epidemic strains [5, 6]. Epidemic MRSA exhibit rapid transmission and interhospital spread, long-term persistence, and the ability to cross geographic and continental boundaries [7–10]. Canadian MRSA strain 1 (CMRSA-1) is an example, representing 49\% of MRSA isolates submitted for molecular typing by sentinel hospitals in the Canadian Nosocomial Infection Surveillance program [5, 6]. Although predominant in Ontario hospitals, CMRSA-1 is present in other geographic regions of Canada and also has appeared in Belgium and Switzerland [11].

In a previous study, we established that CMRSA-1 comprises several closely related subtypes that exhibit significant differences in clinical prevalence and phenotypic traits [12]. CMRSA-1A displayed a phenotype that was characteristic of \textit{S. aureus}, including secretion of \textit{α} toxin and proteases and positive results in the coagulase and agglutination assays used for identification of \textit{S. aureus} in clinical microbiology laboratories. However, CMRSA-1A represents <1\% of total CMRSA-1 isolates, whereas subtypes 1B and 1D currently constitute 71\% and 18\%, respectively, and exhibit atypical phenotypes. The phenotype of CMRSA-1D was consistent with a defect in the accessory gene regulator (\textit{agr}) locus, including enhanced fibronectin binding, defective expression of secreted exoproteins, and attenuated transcription of RNAIII [12], which is essential for the induction of exoprotein expression in the postexponential growth phase [13]. Surprisingly, the predominant CMRSA-1B subtype exhibited abundant transcription of RNAIII, but its expression of secreted proteins was restricted to a doublet of 70-kDa proteins of unknown function. These findings were suggestive of a reduced capacity for virulence in the predominant CMRSA-1B and -1D subtypes and supported a view about the relationship between virulence and transmissibility whereby enhanced opportunity for transmission is achieved at the expense of reduced virulence [14].

\begin{thebibliography}{12}

Received 4 September 2001; revised 3 December 2001; electronically published 29 March 2002.

Animal experiments were conducted in accordance with institutional guidelines, and operating protocols were reviewed and approved by the Sunnybrook and Women’s College Health Science Centre Animal Care Committee.

Financial support: Aventis Pasteur (Canadian Universities Research Program grant to M.J.M. and University of Toronto Funded Research no. 72012558); Sunnybrook and Women’s College Health Science Centre Trust fund (Premiers Research Excellence Award to M.J.M. and postdoctoral fellowship to M.H.).

\textsuperscript{*} Present affiliations: Lorus Therapeutics, Sunnybrook and Women’s College Health Science Centre, Toronto, Ontario, Canada (M.H.); Department of Dermatology, Johns Hopkins University, Baltimore, Maryland (D.N.S.).

Reprints or correspondence: Dr. Martin J. McGavin, University of Toronto, Sunnybrook and Women’s College Health Science Centre, S112, Div. of Microbiology, 2075 Bayview Ave., Toronto, Ontario, Canada M4N 3M5 (martin.mcgavin@swchsc.on.ca).

The Journal of Infectious Diseases 2002;185:1285–96
© 2002 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2002/18509-0010$02.00

\textit{Staphylococcus aureus} is a major nosocomial pathogen that causes a broad spectrum of diseases, ranging from superficial skin infections to serious and potentially fatal illnesses, such as endocarditis, pneumonia, and septicemia [1]. The emergence of multiple-drug resistance in methicillin-resistant \textit{S. aureus} (MRSA), including strains with intermediate resistance to vancomycin, the only alternative in the treatment of many MRSA infections [2], has promoted renewed interest in understanding the mechanisms of persistence and virulence of this important pathogen.

MRSA is the most prevalent nosocomial pathogen in the United States [3, 4]; in Canada, a dramatic increase in the incidence of MRSA is associated with the emergence of epidemic strains [5, 6]. Epidemic MRSA exhibit rapid transmission and interhospital spread, long-term persistence, and the ability to cross geographic and continental boundaries [7–10]. Canadian MRSA strain 1 (CMRSA-1) is an example, representing 49\% of MRSA isolates submitted for molecular typing by sentinel hospitals in the Canadian Nosocomial Infection Surveillance program [5, 6]. Although predominant in Ontario hospitals, CMRSA-1 is present in other geographic regions of Canada and also has appeared in Belgium and Switzerland [11].

In a previous study, we established that CMRSA-1 comprises several closely related subtypes that exhibit significant differences in clinical prevalence and phenotypic traits [12]. CMRSA-1A displayed a phenotype that was characteristic of \textit{S. aureus}, including secretion of \textit{α} toxin and proteases and positive results in the coagulase and agglutination assays used for identification of \textit{S. aureus} in clinical microbiology laboratories. However, CMRSA-1A represents <1\% of total CMRSA-1 isolates, whereas subtypes 1B and 1D currently constitute 71\% and 18\%, respectively, and exhibit atypical phenotypes. The phenotype of CMRSA-1D was consistent with a defect in the accessory gene regulator (\textit{agr}) locus, including enhanced fibronectin binding, defective expression of secreted exoproteins, and attenuated transcription of RNAIII [12], which is essential for the induction of exoprotein expression in the postexponential growth phase [13]. Surprisingly, the predominant CMRSA-1B subtype exhibited abundant transcription of RNAIII, but its expression of secreted proteins was restricted to a doublet of 70-kDa proteins of unknown function. These findings were suggestive of a reduced capacity for virulence in the predominant CMRSA-1B and -1D subtypes and supported a view about the relationship between virulence and transmissibility whereby enhanced opportunity for transmission is achieved at the expense of reduced virulence [14].
The predominant CMRSA-1B subtype was also unique in possessing a gene, \( pls \), which encodes the plasmin-sensitive cell surface protein (Pls). Expression of Pls previously has been associated with MRSA that exhibit a negative response in the latex agglutination assay [15], and this property is attributed to the ability of Pls to mask the function of other cell surface adhesion proteins [16, 17]. Accordingly, we observed that CMRSA-1B isolates that express Pls exhibited significantly less fibronectin binding than did other CMRSA-1 isolates [12]. However, other functions of Pls and its potential contributions to the predominance of CMRSA-1, have not been determined. The \( pls \) gene also was shown recently to be located in the staphylococcal cassette chromosome (SCC) \( mec \) [18], which contains the \( meca \) gene that confers resistance to methicillin. Although there are at least 3 structural architectures of SCC\( mec, pls \) is exclusive to type I SCC\( mec \), which was present in the first MRSA isolated in England in 1961 and disseminated among MRSA that originated in the 1960s during the early years of chemotherapy [18, 19]. Consequently, type I SCC\( mec \) confers resistance only to methicillin. However, CMRSA-1 is a contemporary MRSA that was not prominent in Canada before 1995, and CMRSA-1B isolates that possess \( pls \) are resistant to multiple antimicrobial agents [12].

Here, we present a detailed examination of the CMRSA-1 strain, with the objectives of defining the relationship between virulence and in vitro expression of secreted exoproteins in different subtypes, confirming the association of \( pls \) with SCC\( mec \) in CMRSA-1B, and identifying possible roles for Pls in contributing to the success of CMRSA-1B.

Materials and Methods

Bacterial strains and growth conditions. \( S.\ aureus \) RN6390 was obtained from R. Novick (Skirball Institute, New York) and has been described elsewhere [13]. \( S.\ aureus \) strain L857 is a clinical isolate cultured from blood of a patient with septic arthritis [20] and expresses high levels of a cell-surface fibronectin-binding protein (FnbP). All CMRSA isolates were provided by the Canadian Nosocomial Infection Surveillance Program. Data from our previous study summarizing the phenotypic traits of CMRSA-1 subtypes are presented in table 1 [12]. Because of a change in nomenclature, isolates defined as CMRSA-1D in the present study are equivalent to those that were previously designated as CMRSA-1E [12] and remain the second most abundant subtype of CMRSA-1. All strains were typed by pulsed-field gel electrophoresis (PFGE) of StmaI-digested genomic DNA [21] and determination of antibiotic resistance profiles, in accordance with National Committee for Clinical Laboratory Standards guidelines [22]. The presence of \( meca \) was confirmed by polymerase chain reaction (PCR) [23]. The American Type Culture Collection strains ATCC-43300 and ATCC-2913 were used as type strains for MRSA and methicillin-susceptible \( S.\ aureus \) (MSSA), respectively, in accordance with National Committee for Clinical Laboratory Standards guidelines. Stock cultures were maintained at \(-70^\circ\mathrm{C}\) in 20% (wt/vol) glycerol and passaged on brain-heart infusion (BHI) agar (Difco) or sheep blood agar (PML Microbiologicals) at \(37^\circ\mathrm{C}\) before selection of colonies for specific assays.

Growth of CMRSA-1. For standard growth conditions, overnight cultures were inoculated into Erlemeyer flasks containing either BHI broth (Difco) or protease expression medium [24], to achieve an initial absorbance of 0.1 at 600 nm. The culture volume was maintained at 0.2 of flask capacity to maintain constancy of growth conditions. Cultures were grown with shaking at 250 rpm at \(37^\circ\mathrm{C}\) in an incubator. Cells for adhesion assays were harvested from BHI cultures at midexponential phase (2 h), washed with PBS, heat-killed, and then adjusted to a cell density of \(10^{10}\) cfu/mL and stored in aliquots at \(-70^\circ\mathrm{C}\), as described elsewhere [12]. The heat-killing step was omitted when indicated, to permit biotin-labeling of viable bacteria. For adhesion assays, suspensions of live or heat-killed \( S.\ aureus \) cells \((10^{10}\) cfu/mL\) were labeled with biotinamidocaproate \(N\)-hydroxysuccinimide ester (Sigma), as described elsewhere [20]. For comparison of secreted protein profiles and N-terminal sequence analysis of secreted proteins, cultures were grown for 18 h in pro-

### Table 1. Summary of phenotypic traits of Canadian methicillin-resistant Staphylococcus aureus strain 1 (CMRSA-1) subtypes and specific isolates.

<table>
<thead>
<tr>
<th>CMRSA-1 subtype (%)</th>
<th>( \alpha ) Toxin</th>
<th>Protease, ( A_{214} )</th>
<th>Secreted protein profile</th>
<th>RNAIII transcription</th>
<th>Percentage of fibronectin bound</th>
<th>Pls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMRSA-1A (0.98)</td>
<td>+</td>
<td>0.40</td>
<td>A</td>
<td>+</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>1A318</td>
<td>+</td>
<td>0.39</td>
<td>A</td>
<td>+</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>1A218</td>
<td>+</td>
<td>0.67</td>
<td>A</td>
<td>+</td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>CMRSA-1B (71.1)</td>
<td>–</td>
<td>0.06</td>
<td>B</td>
<td>+</td>
<td>19</td>
<td>+/+</td>
</tr>
<tr>
<td>1B387</td>
<td>–</td>
<td>0.08</td>
<td>B</td>
<td>+</td>
<td>19</td>
<td>+/+</td>
</tr>
<tr>
<td>1B317</td>
<td>–</td>
<td>0.01</td>
<td>C</td>
<td>–</td>
<td>19</td>
<td>+/+</td>
</tr>
<tr>
<td>1B315</td>
<td>–</td>
<td>0.09</td>
<td>C</td>
<td>–</td>
<td>38</td>
<td>+/-</td>
</tr>
<tr>
<td>CMRSA-1D (17.6)</td>
<td>–</td>
<td>0.03</td>
<td>C</td>
<td>–</td>
<td>42</td>
<td>–</td>
</tr>
<tr>
<td>1D375</td>
<td>–</td>
<td>0.01</td>
<td>C</td>
<td>–</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>1D333</td>
<td>–</td>
<td>0.05</td>
<td>C</td>
<td>–</td>
<td>40</td>
<td>–</td>
</tr>
</tbody>
</table>

**NOTE.** \( A_{214} \): absorbance at 574 nm; +, positive; –, negative.

*Percentage of total CMRSA-1 strains, derived from database of 820 CMRSA-1 isolates collected between 1995 and 1998 [12].

A, abundantly expressed secreted proteins; B, restricted expression limited to doublet of 70-kDa proteins; C, pleiotropic defect in expression of secreted proteins.

Pls, plasmin-sensitive surface protein. –, \( pls \) not present; +/+, \( pls \) present and Pls expressed; +/-, \( pls \) present but Pls not expressed.
tease expression medium, and proteins were precipitated from cell-free culture supernatant by mixing with an equal volume of ice-cold 20% (wt/vol) trichloroacetic acid.

**Keratinocyte cell culture and preparation of extracellular matrix (ECM).** Normal human keratinocytes, obtained from neonatal foreskin, were obtained and maintained as described elsewhere [25]. Human squamous carcinoma cells SCC-A431 [26] were cultured in Eagle MEM (Gibco BRL) with 10% fetal calf serum (Gibco BRL). To prepare keratinocyte ECM for adhesion assays, 100-µL aliquots of a trypsinized keratinocyte cell suspension (2 × 10^7 cells/mL) were seeded in individual wells of 96-well flat-bottom cell culture plates (Corning). When cells had achieved confluent growth, ECM was exposed by treating the monolayers with 0.5% Triton X-100 for 30 min at 37°C, followed by treatment with 25 mM ammonium hydroxide for 10 min at 37°C, as described elsewhere [20].

**Purification and biotin labeling of Pls.** CMRSA-1B317 was grown for 8 h in BHI broth, harvested by centrifugation, and converted to protoplasts by digestion of the cell wall with lysostaphin (AMBI), as described elsewhere [20]. After centrifugation, the released cell surface proteins were dialyzed into PBS, followed by affinity chromatography on wheat germ agglutinin coupled to agarose beads (Sigma) [16]. Protein eluted from the wheat germ agglutinin–agarose affinity column with 50 mM N-acetyl glucosamine was dialyzed in 50 mM Tris-HCl (pH 8.0) and applied to a 5-mL Hi-Trap column of Q-Sepharose (Amersham Pharmacia), equilibrated in the same buffer. After washing to remove nonbound protein, a linear gradient of NaCl from 0 to 1.0 M was applied over a volume of 50 mL while collecting 1-mL fractions. Fractions that contained Pls were pooled and desalted on a G-25 column (Amersham Pharmacia) equilibrated in PBS. For biotin-labeling, purified Pls was concentrated to 5 mg/mL by use of a centrifugal filter device (Millipore) and labeled with biotinamidocaproate 5 mg/mL by use of a centrifugal filter device (Millipore) and labeled.

**Isolation and chromatography of keratinocyte lipids and lipid standards.** Total lipids were extracted from normal human keratinocyte cells maintained in serum-free keratinocyte growth medium, standards. Total lipids were extracted from normal human keratinocytes, obtained from neonatal foreskin, and type IV phosphatidylethanolamine from soybean were obtained from Sigma. Human plasma fibronectin was purchased from Gibco BRL. Sphingolipid and ganglioside standards were purchased from Matrey, including lactosyl ceramide, gangliotriosyl ceramide (G3), sialosyl derivatives of Gg3 (monosialoganglioside [GM3] and disialoganglioside [GD3]), gangliotetraosyl ceramide (Gg4), and sialosyl derivatives of Gg4 (monosialoganglioside [GM1], disialoganglioside [GD1a and GD1b], and trisialoganglioside [GT1b]).

To quantify adhesion of CMRSA, keratinocyte cell culture plates containing exposed ECM were prepared as described above. Alternatively, individual wells of 96-well microtiter plates (Corning) were coated overnight at 4°C with 1 µg/mL purified proteins or glycoproteins. Excess protein-binding sites were blocked by incubation for 60 min with 200 µL of 3% bovine serum albumin (BSA) in PBS. Subsequently, 100-µL aliquots of heat-killed and biotin-labeled CMRSA isolates, diluted to 10^8 cfu/mL in PBS containing 0.1% BSA and 0.05% Tween 20, were added to individual wells, followed by a 60-min incubation with orbital mixing. After extensive washing to remove nonadherent cells, 100 µL of 5000-fold diluted streptavidin–alkaline phosphatase conjugate (Roche) was added to each well, followed by a 60-min incubation. The plates were washed extensively and developed by addition of 100 µL of 1.0 mg/mL Sigma 104 alkaline phosphatase substrate. After 60 min of development, adhesion was quantified by measurement of absorbance at 405 nm with a microplate reader (model 3550; Bio-Rad). To measure the effect of Pls on adhesion to different substrates, the assays were conducted with CMRSA isolates diluted in buffer containing various concentrations of purified Pls.

The assay for binding of CMRSA to lipids was a modification of a protocol described elsewhere [27]. After separation of keratinocyte total cellular lipids or individual lipid and glycolipid standards by thin-layer chromatography, total lipids were visualized by staining with iodine vapor, and glycolipids were detected with orcinol spray. Excess protein-binding sites on duplicate plates were blocked by incubation in 2% gelatin for 2 h at 37°C. Suspensions of live biotin-labeled bacteria (10^8 cfu/mL in 100 mM Tris-buffered saline [pH 7.0]) or 25 µg/mL purified Pls diluted in Tris-buffered saline were overlaid for 2 h at room temperature on a rocking platform mixer. After extensive washing with Tris-buffered saline, adherent bacteria were detected with peroxidase-labeled streptavidin (Roche). Adherent Pls was detected with 2000-fold-diluted anti-Pls rabbit polyclonal antiserum [12], followed by incubation with peroxidase-labeled goat anti-rabbit IgG (Jackson ImmunoResearch). Plates then were developed with 3 mM 4-chloro-1-naphthol (Sigma) in 50 mM Tris-buffered saline (pH 7.0) containing 0.003% H2O2.

**SDS-PAGE, Western blotting, and N-terminal sequence analysis.** Proteins precipitated from cell-free culture supernatants of CMRSA-1 were visualized by SDS-PAGE [28]. Sample loading was adjusted in proportion to the cell density of each culture (absorbance at 600 nm), such that the total protein secreted by an equivalent number of cells was applied to each lane. For detection of Pls, cell suspensions of CMRSA (10^9 cfu/mL) were treated with 25 µg/mL lysostaphin in the absence of osmotic stabilizer to prepare total cell lysates. After centrifugation to remove cellular debris, total protein content was determined by the bicinchoninic acid protein assay (Pierce). Equivalent protein amounts of CMRSA lysates were subjected to SDS-PAGE (7.5% acrylamide), followed by trans-
fer to Immobilon-P membrane (Millipore) by use of the Bio-Rad Mini Trans-Blot apparatus (Bio-Rad) and standard transfer buffer [29]. Thereafter, membranes were processed for detection of PIs with polyclonal antibodies, as described elsewhere [12]. FnBP was detected on duplicate blots by use of biotin-labeled human fibronectin, as described elsewhere [20]. For N-terminal sequence analysis of proteins secreted by CMRSA-1B387, trichloroacetic acid–precipitated proteins were subjected to SDS-PAGE, followed by transfer to Immobilon P membrane in 3-(cyclohexylamino)-1-propanesulfonic acid buffer [30]. After proteins were visualized by brief staining with Coomassie blue in 40% methanol, protein bands were excised with a scalpel and submitted to the University of Toronto Health Science Center Biotechnology Center for N-terminal sequencing.

**Assay of bacterial aggregation.** The ability of soluble PIs to induce bacterial aggregation was investigated by a modification of published methods [31, 32]. Cultures were grown overnight in 10 mL of BHI broth at 37°C and divided into 5-mL aliquots. Bacteria were separated by centrifugation at 2000 g for 20 min at 4°C, suspended in aggregation buffer (10 mM Tris [pH 8.0], 0.1mM CaCl₂, 0.1 mM MgCl₂, 150 mM NaCl, and 0.02% NaN₃), and centrifuged again. Pellets from the 2 aliquots of each culture then were resuspended in either aggregation buffer or aggregation buffer containing 50 μg/mL purified PIs. After vortex-mixing for 10 s, the cell suspensions were incubated at 4°C overnight. After gentle mixing, the cell suspensions were transferred to 16 × 100 mm glass tubes and left to settle at room temperature. At specific intervals, the level of aggregation was estimated by determining the absorbance at 600 nm of a 0.5-mL sample withdrawn from the upper part of each suspension.

**Sequence analysis and PCR.** Homology searches were conducted by use of the BLAST algorithms [33] provided by the National Center for Biotechnology Information (available at http://www.ncbi.nlm.nih.gov). The nucleotide sequences of pls [17] and the complete sequences of types I, II, and III SCCmec have been published elsewhere [18]. Oligonucleotide primers for PCR amplification of probes specific for pls and mecA have been described elsewhere [12, 23]. Primers used for amplification of the transposase tnpA gene segment of transposon Tn554 [34] were 5'-GAGAGGA-GTGGGATAAAGACGTTTG-3' and 5'-GCTTGTCATTTCTGG-3'. PCRs contained 1.0 ng of template DNA in a 25-μL volume, containing 2.0 mM MgCl₂, 12.5 pmol of each forward and reverse primer, 0.2 mM dNTP mix, and 1.875 U of AmpliTaq DNA polymerase (Roche Canada), in buffer provided by the supplier. Amplification consisted of a 4-min denaturation at 94°C, followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 2 min, and extension at 72°C for 1 min. Genomic DNA for use as template in PCRs was purified from CMRSA isolates by use of Qiagen 100G genomic tips (Qiagen).

**PFGE and hybridization.** PFGE of SmaI-digested genomic DNA of CMRSA isolates was done as described elsewhere [12, 21]. After electrophoresis, ethidium bromide–stained gels were photographed to visualize the profile of DNA fragments and then blotted by capillary transfer to Hybond N+ membrane (Amersham Pharmacia), by means of the alkaline transfer method [35]. Probes specific for pls, mecA, and tnpA of Tn554 were prepared by PCR, as described above. Labeling of the PCR amplicons with horse-radish peroxidase and processing of the blotted membranes was done with use of the reagents and protocols provided with the ECL Direct nucleic acid labeling and detection system (Amersham-Pharmacia). Kodak Biomax ML autoradiography film was used for visualization of chemiluminescent signals. For Northern hybridization experiments, total RNA was isolated from cells grown for 18 h in protease expression medium, followed by electrophoresis, blotting, and hybridization with a probe specific for RNAIII, as described by us elsewhere [12].

**Results.**

**Identification of secreted proteins and analysis of PIs expression in CMRSA-1B.** As summarized from our previous study [12] (table 1), the predominant CMRSA-1B subtype exhibited a unique profile of exoproteins, which was restricted to a doublet of 70-kDa proteins of unknown function. Because these may represent novel virulence determinants that contribute to the prevalence of CMRSA-1B, N-terminal sequence analyses were done to establish the identity of these proteins, providing the amino acid sequences AEKQVNMGNSQE and EKT(V?)T(M?)(N?)AA, respectively. The former is identical to aa 36–47 of a triacylglycerol lipase precursor of *S. aureus*, after cleavage of a signal peptide at Ala₃₅, whereas the latter is similar to EKTSTAA, spanning aa 39–46 of a glycerol ester hydrolase from *S. aureus*. Lipases of *S. aureus* are secreted as 70-kDa precursors, which undergo proteolytic conversion to mature enzymes of ~42 kDa [37]. Because low protease activity is a characteristic feature of CMRSA-1B (table 1), the secreted lipase appears to remain stable in the precursor form. This unusual profile of secreted exoproteins was a distinguishing feature of CMRSA-1B, as represented by CMRSA-1B387 (table 1). However, isolates CMRSA-1B317 and CMRSA-1B315 did not express the lipase precursor proteins and exhibited a pleiotropic defect in expression of secreted exoproteins (table 1).

The null profile of secreted exoproteins in CMRSA-1B317 and CMRSA-1B315 resembled that of CMRSA-1D. However, PCR analyses revealed that both isolates possessed the pls gene, as did CMRSA-1B387, whereas pls was not detected in CMRSA-
1A or CMRSA-1D isolates (figure 1A). Although plS was present in CMRSA-1B315, it did not express Pls, whereas CMRSA-1B317 expressed a Pls that was slightly larger in size than that of CMRSA-1B387 (figure 1B). Variation in the size of Pls in different strains of MRSA has been noted previously and is attributed to differences in the size of domains that are composed of repetitive amino acid motifs [17]. CMRSA-1B317 and -1B315 were also defective transcription of RNAIII (figure 1C), which is consistent with their pleiotropic defect in expression of secreted exoproteins (table 1). These different combinations of Pls expression and transcription of RNAIII in separate isolates of CMRSA-1 suggest that expression of Pls is not strongly influenced by agr function, which normally serves to repress the expression of S. aureus cell surface proteins in response to increasing cell density [38]. To test this hypothesis, we used Western blots to detect FnBP and Pls in both exponential- and stationary-phase cells of CMRSA-1B387 (figure 1D). FnBP was detected only in exponential-phase cells, whereas Pls was present at both time points, and its expression was not reduced in stationary phase. Therefore, Pls did not display the temporal expression profile characteristic of other cell-surface adhesion proteins in S. aureus.

**Virulence of CMRSA-1 in tissue abscess model.** CMRSA-1B and CMRSA-1D represent 71% and 18%, respectively, of the total CMRSA-1 isolates. Furthermore, CMRSA-1D exhibits agr-null behavior (table 1; figure 1C), whereas most CMRSA-1B isolates secrete only a precursor form of lipase. These traits suggest that the predominant CMRSA-1 subtypes possess a reduced capacity for virulence relative to CMRSA-1A, which exhibits phenotypic traits typical of S. aureus. Accordingly, CMRSA-1D was attenuated in virulence in a tissue abscess infection model relative to CMRSA-1A318 (table 2). However, mice challenged with CMRSA-1B387 developed a tissue abscess that was indistinguishable from that of mice challenged with CMRSA-1A318, with no difference in abscess surface area or colony-forming units per gram of abscess tissue at 24 h and 72 h after challenge, respectively (table 2). In contrast, both variants of CMRSA-1B that failed to transcribe RNAIII, represented by -1B317 and -1B315, exhibited attenuated virulence. Therefore, attenuated virulence was observed among CMRSA-1 isolates that failed to transcribe RNAIII but was not characteristic of the predominant CMRSA-1B.

**pls is associated with the SCCmec of CMRSA-1B.** To evaluate the association of plS with SCCmec in CMRSA-1, Smal-restricted genomic DNA was subjected to PFGE, followed by Southern hybridization with probes specific for plS, mecA, and transposase *tpa* of Tn554 (figure 2). The PFGE profile of CMRSA-1B differed from that of CMRSA-1A or -1D by a single band shift (figure 2A), and both subtypes were closely related to CMRSA-1K, which was responsible for an outbreak of MRSA infection in Nova Scotia in 1998. Although CMRSA-

---

**Table 2.** Virulence of related subtypes of Canadian methicillin-resistant *Staphylococcus aureus* strain 1 (CMRSA-1) in murine tissue abscess infection model.

<table>
<thead>
<tr>
<th>Strain, trial</th>
<th>No. of mice with abscess</th>
<th>Abscess size, cfu/g (\times 10^6)</th>
<th>Lesion size, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMRSA-1A318</td>
<td>1</td>
<td>6</td>
<td>3.75 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>4.07 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>2.18 ± 0.92</td>
</tr>
<tr>
<td>CMRSA-1B387</td>
<td>1</td>
<td>6</td>
<td>2.70 ± 1.10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>2.68 ± 1.98</td>
</tr>
<tr>
<td>CMRSA-1B317</td>
<td>1</td>
<td>5</td>
<td>0.54 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>0.14 ± 0.16</td>
</tr>
<tr>
<td>CMRSA-1B315</td>
<td>1</td>
<td>1</td>
<td>0.31 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>0.12 ± 0.17</td>
</tr>
<tr>
<td>CMRSA-1D375</td>
<td>1</td>
<td>4</td>
<td>0.98 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>0.28 ± 0.28</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SD. There were a total of 6 mice tested.
transposon 554 (B), reus (MRSA) strain ATCC-43300 or methicillin-susceptible strain 1 (CMRSA-1) subtypes and unrelated methicillin-resistant Staphylococcus aureus Southern hybridization of same strains with probes specific for ATCC-29213, or other CMRSA-1 subtypes (figure 2). Figure 2. A, Pulsed-field gel electrophoresis of Sma I-digested genomic DNA of Canadian methicillin-resistant Staphylococcus aureus strain 1 (CMRSA-1) subtypes and unrelated methicillin-resistant S. aureus (MSSA) strain ATCC-29213 after staining with ethidium bromide. B–D, Southern hybridization of same strains with probes specific for mec (B), transposon 554 (C), or pls (D). M, DNA size markers. Vertical bar in the left margin of panel A represents the region of gel displayed for Southern hybridization in panels B–D.

1B387 and -1B315 differed in expression of Pls, transcription of RNAIII, and expression of lipase precursor, their PFGE profiles were identical, establishing that CMRSA-1B315 was not a separate subtype of CMRSA-1. In Southern hybridization experiments, a probe specific for mecA (figure 2B) hybridized to the same Sma I fragment that was identified by ethidium bromide staining (figure 2A) as a major feature in differentiating subtypes. When the same blot was stripped and hybridized with a probe specific for Tn554 (figure 2C), the hybridization pattern was identical to that of mecA, with the exception of CMRSA-1A168, which did not contain Tn554, whereas CMRSA-1A218 and -1A318 displayed 2 Sma I fragments that hybridized with the Tn554 probe. A probe specific for pls hybridized to the same Sma I fragment that contained mecA and tnpA in CMRSA-1B and -1K but did not hybridize to MRSA ATCC-43300, MSSA ATCC-29213, or other CMRSA-1 subtypes (figure 2D). Therefore, pls is present on the same Sma I fragment that harbors SCCmec in specific subtypes of CMRSA-1, including the major CMRSA-1B subtype.

Pls is not an MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin. Several cell surface proteins of S. aureus are members of the MSCRAMM family of proteins that promote adhesion to tissue ECM, including the FnBP adhesin [39–41]. To determine whether Pls could function as an MSCRAMM, we assayed for adhesion of a panel of MRSA and MSSA to ECM deposited by SCC-A431 keratinocyte cell culture and to fibronectin-coated microtiter plates (figure 3). S. aureus strains RN6390 and MSSA L857, which exhibit low- and high-level binding of soluble fibronectin, respectively [20], also displayed low and high adhesion to microtiter plates coated with either keratinocyte ECM (figure 3A) or fibronectin (figure 3B). The variation in adhesion to ECM and fibronectin by the different subtypes of CMRSA-1 showed the same correlation, which is consistent with a report that fibronectin is the major component of keratinocyte ECM that supports adhesion of S. aureus [41]. CMRSA-1B isolates that express Pls exhibited the lowest level of adhesion to both fibronectin and keratinocyte ECM. This is consistent with a proposed function for Pls in masking the activity of other adhesion proteins [17] and establishes that Pls itself does not function in promoting adhesion to ECM. CMRSA-1B315, which possesses pls but does not express Pls, showed elevated adhesion to both ECM and fibronectin, which is in agreement with a report that inactivation of pls in a clinical isolate resulted in enhanced adhesion to fibronectin [17]. However, because the CMRSA-1B isolates in this study are not isogenic, the enhanced adhesion of CMRSA-1B315 could be due to both an agr-null phenotype, which promotes enhanced expression of adhesion proteins, and failure to express Pls, which would otherwise mask the function of the MSCRAMM adhesins.

Pls promotes binding of CMRSA-1B to keratinocyte lipids and lipid standards. Because cellular lipids are receptors for a number of pathogenic bacteria [42, 43], we tested different CMRSA isolates for binding to lipids extracted from normal human keratinocytes (figure 4A, 4B, 4C, and 4D) and to purified lipid standards (figure 4E, 4F, 4G, and 4H). When detected with iodine vapor after separation by thin-layer chromatography, the predominant lipids extracted from keratinocyte cells (figure 4A, lane 3) exhibited mobilities similar to those of phosphatidylethanolamine and phosphatidylcholine standards (figure 4A, lane 1). The major keratinocyte glycolipid detected with orcinol spray (figure 4B, lane 3) comigrated with the purified glycolipid standard GM3 (figure 4B, lane 2). When identical thin-layer chromatography plates were used in adhesion assays, 3 CMRSA-1B isolates that expressed PIs (1B387, 1B315, and 1B374) exhibited the same specificity of adhesion; each strain was tested twice, with a representative result being shown for CMRSA-1B185 (figure 4C). Each strain bound to the phosphatidylethanolamine and phosphatidylcholine standards (figure 4C, upper bands in lanes 1 and 2, respectively) and also adhered to a keratinocyte lipid that migrated closely with the phosphatidylethanolamine and phosphatidylcholine standards (figure 4C,
CMRSA-1B cells also adhered to a keratinocyte lipid of intermediate mobility that did not correspond to any of the known standards. The mobility of the slowest migrating keratinocyte lipid that supported adhesion of CMRSA-1B cells (figure 4C, lane 3, lower band) was comparable to those of the phosphatidylcholine (figure 4A, lane 1) and GM3 (figure 4B, lane 2) standards. In contrast, CMRSA-1B315, which possesses pls but does not express Pls, did not exhibit adhesion to any keratinocyte lipids or lipid standards (figure 4D).

For further clarification of binding specificity, CMRSA-1B cells and purified Pls were assayed for adhesion to a broader range of glycolipid standards (figure 4E, 4F, 4G, and 4H). In figure 4E, glycolipids were detected with orcinol reagent, which revealed in lane 3 the presence of both Gg3 (upper band) and Gg4 (lower band). The same banding pattern was observed when identical plates were incubated with CMRSA-1B cells (figure 4F, lane 3) or purified Pls (figure 4G, lane 3). When Gg4 was mixed with its sialosyl derivatives, the orcinol reagent revealed 4 bands (figure 4E, lane 4), consisting, in descending order, of Gg4 (upper band), GM1, GD1a, and GD1b. However, CMRSA-1B cells and purified Pls adhered only to Gg4 (figure 4F and 4G, lane 4). In figure 4E, lane 5, the orcinol reagent revealed the presence of lactosyl ceramide (upper band) and the siaosyl derivatives of Gg3, including GM3 (middle band) and GD3 (lower band). CMRSA-1B cells adhered well to the lactosyl ceramide standard and also showed some adhesion to GM3 and GD3 (figure 4F, lane 5). Purified Pls showed a similar pattern of adhesion to lactosyl ceramide and GM3/GD3 (figure 4G, lane 5), although less effectively than did intact CMRSA-1B cells. In contrast, CMRSA-1B315, which does not express Pls, did not adhere to any of the glycolipid standards (figure 4H). From these data, it appears that Pls mediates adherence of CMRSA-1B cells to the phospholipid phosphatidylethanolamine and, to a lesser extent, to phosphatidylcholine. Pls also mediates adherence to glycolipids lactosyl ceramide, Gg4, and Gg3 and, to a lesser extent, to the charged sialosyl derivatives of Gg3, including GM3 and GD3. However, it did not promote adhesion to the sialosyl derivatives of Gg4, including GM1, GD1a, and GD1b.

Soluble Pls promotes bacterial cell-cell interaction. Pls is homologous to accumulation-associated protein of Staphylococcus epidermidis [17], which facilitates the formation of multilayer clusters of bacteria in biofilm development [44]. Consistent with a role in promoting cell-cell interactions, purified Pls promoted enhanced adhesion of both CMRSA-1A318 and CMRSA-1B185 to wells of a microtiter plate coated with fibronectin (figure 5A). Because CMRSA-1A does not express Pls, this effect did not appear to depend on Pls promoting a homotypic protein-protein interaction. Soluble Pls also promoted enhanced adhesion of CMRSA-1B185 to other ligands to which S. aureus is known to bind, including fibrinogen, mucin, and IgG (figure 5B), whereas stimulation of adhesion was less evident with substrates that do not normally support adhesion of S. aureus, including gelatin and BSA. Biotin-labeled Pls also bound to wells of microtiter plates coated with different CMRSA isolates but not to wells coated with BSA (figure 5C), fibronectin, or fibrinogen (data not shown). Further evidence of the ability of Pls to promote cell-cell interactions was obtained by assaying for sedimentation of cell suspensions of CMRSA-1A318, -1B185, or -1B315 in the presence or absence of 50 μg/mL soluble Pls (figure 6). All 3 strains exhibited enhanced sedimentation in the presence of soluble Pls, and this was most evident with CMRSA-1A318 and -1B315, which do not express Pls. The reduced rate of sedimentation ob-

**Figure 3.** Binding of heat-killed and biotin-labeled Staphylococcus aureus strains RN6390, L857, and different Canadian methicillin-resistant S. aureus strain 1 isolates to wells of microtiter plates containing exposed extracellular matrix (ECM) produced by human squamous carcinoma cells (A) or coated with purified human fibronectin (B). Each value represents average of triplicate determinations. Each data point represents the mean of triplicate wells from a single experiment. For ECM adhesion assays (A), each experiment was repeated twice with a squamous carcinoma cell line and once with a primary human keratinocyte ECM. For adhesion to fibronectin (B), the experiment was repeated 3 times.
induced in the response to agr, even though it exhibited abundant transcription of RNAIII. Although we cannot exclude the possibility that these traits originated after subculturing in vitro, isolates that exhibit this same phenotype were obtained from different hospitals and geographic locations over a prolonged period of time, supporting the contention that this is a stable strain-dependent phenotype. Recent studies also have noted unusual traits of S. aureus cells studied in vivo [46, 47].

*S. aureus* cells in sputum of patients with cystic fibrosis showed no correlation between cell density and abundance of RNAIII transcript, and there was no correlation between the amount of RNAIII and transcription of *spa* (protein A) or *hla* (α toxin), which are repressed and induced, respectively, in response to RNAIII in vitro [46]. *S. aureus* isolates that were cultured from sputum also exhibited unusual traits, including failure to transcribe RNAIII or lack of *spa* inhibition at high cell density, despite normal transcription of RNAIII. Similar results were obtained with cells recovered from a medical device–related infection model: transcription of *hla* was observed in the absence of RNAIII, and site-directed mutation of *agr* had no consequence for *hla* expression [47]. These findings suggest that *S. aureus* is provided with regulatory circuits in vivo that differ from those characterized in vitro, to ensure expression of secreted virulence factors during infection. Therefore, the in vitro defect of secreted exoprotein expression in CMRSA-1B387 may be alleviated in vivo because of a signaling cue provided by the host environment.

Although the in vitro expression of secreted exoproteins seemed to be uncoupled from *agr* function in CMRSA-1B387, it exhibited normal expression of FnBP, which was maximal in exponential-phase cells and not detected at stationary phase. This is characteristic of cell-surface adhesion protein expression, which is repressed by both *agr*-dependent and -independent mechanisms in response to increasing cell density. However, the amount of Pls present on CMRSA-1B387 cells was not reduced in response to increasing cell density, distinguishing it from the MSCRAMM family of adhesion proteins. This may be relevant to the in vivo function of Pls, in view of its homology to accumulation-associated protein of *S. epidermidis*, which contributes to biofilm formation by promoting the formation of multilayered cell clusters [44]. In a similar projected role for Pls, it would be detrimental if expression was repressed at high cell density. The ability to grow as a biofilm is an important mediator of medical device infections, because bacteria within biofilms are protected from host defense mechanisms and the lethal action of antibiotics [48, 49]. Recent data also suggest that the ability of *S. aureus* to form biofilms may be restricted to 6% of *agr*-positive strains, whereas 78% of *agr*-negative clinical isolates displayed evidence of biofilm formation [50]. Potentially, the expression of Pls by CMRSA-1B isolates could facilitate biofilm formation without the sacrifice of a functional *agr* locus. An ability to form biofilms could also provide partial compensation for the loss of secreted virulence factor expres-
sion associated with an agr-null phenotype. This may be a factor in the success of the CMRSA-1D subtype of CMRSA-1, which is the second-most-abundant subtype but exhibits an agr-null phenotype and attenuated virulence in the abscess infection model.

In addition to a potential role in promoting cell-cell interactions, another function that we associate with Pls is adherence to cellular lipids, including ganglioside GM3, which constitutes 65% of total gangliosides in keratinocyte cell membranes [51, 52]. This interaction could mediate adhesion of CMRSA-1B to the skin, one of the common sites of CMRSA isolation [5]. Both CMRSA-1B and Pls also bound to phosphatidylethanolamine, which is present in most eukaryotic membranes. A number of pathogens bind to phosphatidylethanolamine in vitro [53–55], including enteropathogenic Escherichia coli; adhesion to epithelial cells by these pathogens can be blocked with phosphati-
dylethanolamine-specific antibodies, and there is a correlation between adherence and the level of phosphatidylethanolamine in the outer leaflet of the host cell membrane [53]. CMRSA-1B isolates also bound the glycolipid Gg4, which is expressed in human lungs and has been proposed as receptor for several pulmonary pathogens, including S. aureus [56]. Although our data suggest that adhesion to Gg4 was unique to CMRSA that express PIs, other S. aureus isolates may possess a gene that confers adhesion to Gg4, which is present or expressed only in strains that cause pulmonary infections.

The binding specificity of PIs also encompassed the recognition of sphingolipids that contain lactosyl ceramide, including the mono- and disialosyl derivatives GM3 and GD3, Gg3 (asialo-GM2), and Gg4 (asialo-GM1). In this respect, sphingolipids and cholesterol-enriched vesicles called caveolae are implicated in internalization of bacteria and viruses, and this mechanism of internalization appears to facilitate intracellular survival [57–60]. Although S. aureus generally is not considered to be an intracellular pathogen, its FnBP adhesins promote internalization by a number of different cell types [61, 62]. The same function is attributed to fibronectin-binding adhesins of Streptococcus pyogenes [63, 64], and internalization of S. pyogenes by epithelial cells has been proposed as a means of evading the lethal effect of antibiotics and maintenance of a carrier state [65, 66]. Therefore, PIs also could confer an alternative mode of internalization, compensating for the reduced functionality of fibronectin-binding proteins that are blocked by PIs expression.

As reported by others [17] and shown in the present study, pIs is associated with the staphylococcal cassette chromosome SCCmec, which confers resistance to methicillin [18]. We have established that subtle variations in the PFGE profiles that distinguish between subtypes of CMRSA-1 are due to variable mobility of the Sma I fragment that carries SCCmec, and this correlates with the presence or absence of pIs. Although SCCmec is present in all MRSA, there are at least 3 different architectures in its structure and organization, and the occurrence of pIs is restricted to type I SCCmec [18, 19]. Therefore, different subtypes of CMRSA-1 may have evolved from a common progenitor strain of MSSA, which acquired different types of SCCmec. However, type I SCCmec is associated with MRSA that originated in the 1960s during the early years of chemotherapy and confers resistance to methicillin only. Type I SCCmec also does not harbor Tn554. In contrast, types II and III SCCmec lack pIs but carry Tn554 and additional resistance genes. Therefore, CMRSA-1B may represent a recent evolution of the MRSA genome, because these strains are resistant to multiple antimicrobial agents, and the genomic Sma I fragment that carries SCCmec also harbors both pIs and Tn554. At present, we cannot determine if other CMRSA-1 subtypes represent strains that acquired a different SCCmec that lacks pIs or if they originally possessed pIs and it was subsequently deleted from SCCmec. However, the functions that we attribute to PIs could facilitate the predominance of CMRSA-1B through several potential mechanisms.

Future studies will focus on identifying functional domains of PIs and the significance of these interactions in contributing to the success of CMRSA-1B. By continuing to identify and determine the functional consequences of adaptations that occur in epidemic MRSA, we will achieve a greater understanding of factors that contribute to the success of these strains in the nosocomial setting.

Acknowledgments

We thank Clifford Lingwood for assistance with the lipid adhesion assays, the Canadian Nosocomial Infection Surveillance Program for providing Canadian methicillin-resistant Staphylococcus aureus isolates, and Christine Watt and Lisa Louie for technical support.

References


