Correspondence

The Panton-Valentine Leukocidin Is a Virulence Factor in a Murine Model of Necrotizing Pneumonia

To the Editor—We read with great interest the article by Villaruz et al about a point mutation in the *agr* locus of *Staphylococcus aureus* causing phenotypes that we and other investigators had attributed to Panton-Valentine leukocidin (PVL) [1]. In this article, the authors stated that a strain used in one of our studies [2] "contained an unintended mutation in *agr*, which dramatically changed gene expression." The authors also stated that "the virulence phenotype described by Labandeira-Rey et al for the same strain was influenced by the *agr* mutation and not PVL."

The results obtained by Villaruz et al prompted us to check the integrity of the *agr* locus in our own strain stocks. As shown in Figure 1*A*, strains RN6390, LUG855 (Φ*pvl* lysogen of RN6390), LUG776 (LUG855 Δ*pvl*), LUG862 (plasmid *pvl* complementation in LUG776), and LUG1564 (empty vector in LUG776) show normal β-hemolysis when plated in blood-agar medium, whereas RN6911 (the RN6390 Δ*agr* derivative) showed no hemolysis. As described by the authors, β-hemolysis is "a common and simple readout for *agr* functionality" [1]. Therefore, contrary to the results described by Villaruz et al, the LUG855 strain used in the studies of Labandeira-Rey et al displays a normal *agr* phenotype. In addition, we have sequenced the *agr* P2-P3 region (nucleotides 1724–1458) in RN6390, LUG776, and LUG855 and found that the sequences were identical to those reported for the RN6390 parental strain, NCTC8325-4. We performed Northern blot analyses to detect the expression of RNAIII transcripts as a direct readout for *agr* activity (Figure 1*B*). As expected, RNAIII transcripts were generated at similar levels in most strains tested, including LUG855, but they were not present in the Δ*agr* RN6911 or its Φ*pvl* lysogen, LUG856. Expression analyses of protein A by use of immunoblot assays confirmed our previously published results, in which the strain LUG855 showed an increased level of protein A production compared with its isogenic Δ*pvl* derivative (LUG776) and its parental strain (RN6390) (Figure 1*C*). Overexpression of *spa* is also seen in the Δ*pvl* strain complemented by a plasmid encoding PVL (LUG862), but it is not seen in the vector-only control (LUG1564).

Regarding the PVL production, Villaruz et al claim that "the expression of PVL in LUG855 was very low, most likely owing to strong *agr* control of *lukSF-PV*, which was defective in LUG855." Using a specific enzyme-linked immunosorbent assay [3], we have measured the level of *lukS-PV* production in the spent medium from cultures of selected strains (Figure 1*D*). Our results show that LUG855 produced ~500 ng/mL culture medium during early stationary phase, whereas the plasmid-complemented strain LUG862 produced up to 5-fold more, presumably because of the multicopy nature of the expression vector used in this strain. These levels of PVL production are consistent with a functioning *agr* locus. By comparison, the Δ*agr*, Φ*pvl* lysogen strain LUG856 showed a reduced level of PVL production. Therefore, we agree with the authors' statement that *lukSF-PV* may be under *agr* regulatory control.

Finally, we have repeated experiments to compare the ability of the strains used in our studies to cause disease in a nonlethal model of murine pneumonia. As described in our previous reports, the LUG855 strain caused more morbidity (measured as weight loss) compared with its isogenic Δ*pvl* strain (LUG776) and its parental strain (RN6390) (Figure 1*E*). Tissue sections from lungs infected with LUG855 showed a strong recruitment of neutrophils, necrosis, and hemorrhage (Figure 1*F*). Conversely, the lungs infected with the PVL-negative strain (LUG776) showed normal lung structures, despite some leukocyte infiltration (Figure 1*F*)

These results are analogous to those obtained when the LAC strain and the LACΔ*pvl* strain (constructed by M. Otto and donated by F. DeLeo) were used as infecting agents [4]. In our mouse model, the expression of PVL enhances the virulence of *S. aureus*, regardless of whether the infecting agents are laboratory strains or clinical isolates. Furthermore, anti-PVL antibodies effectively block the cytolytic action of PVL [5], and animals actively or passively immunized with the PVL subunits are effectively protected against community-associated methicillin-resistant *S. aureus* pneumonia and skin infections, which confirms the role played by PVL in such infections [4, 6].

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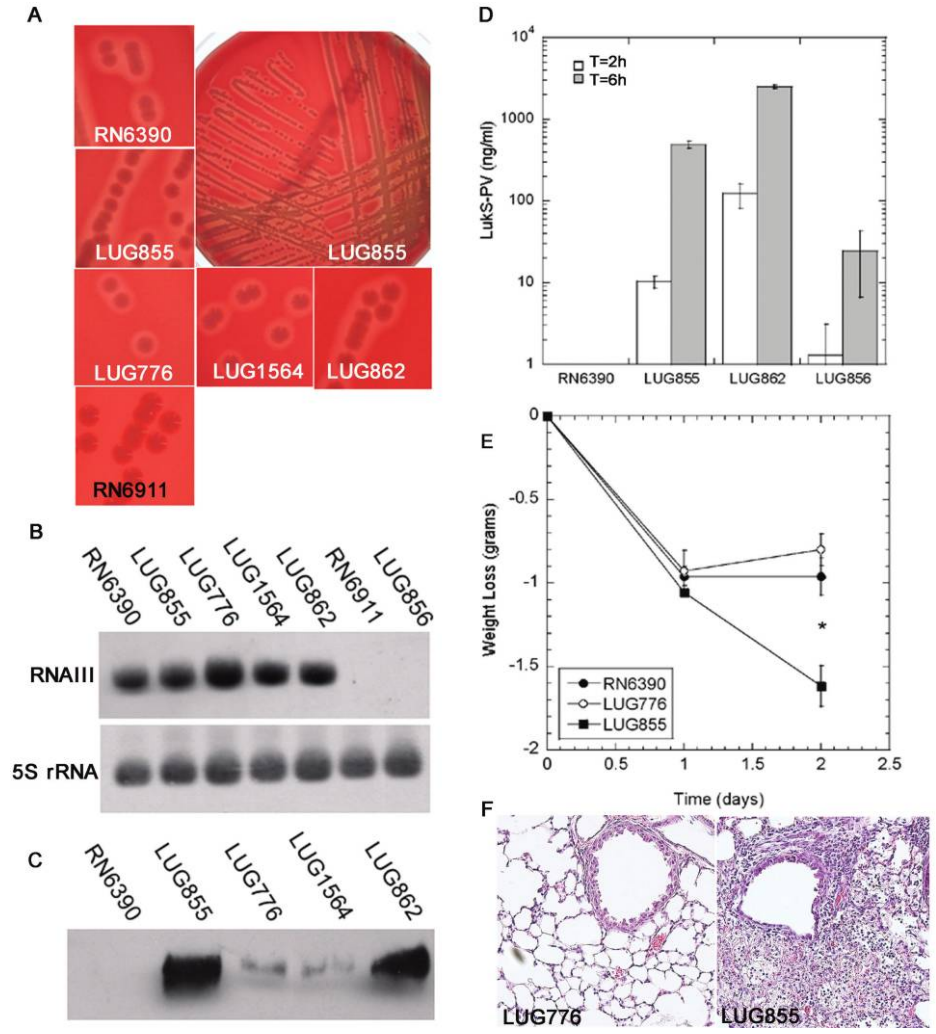

Figure 1. Characterization of *Staphylococcus aureus* strains. *A*, Hemolysis on sheep blood–agar plates of streaked cultures. The LUG855 plate is shown in its entirety to demonstrate a homogeneous phenotype. *B*, RNA detection by use of Northern blot analyses. Total RNA was electrophoresed in a 1% agarose gel containing 2.2 mol/L formaldehyde and vacuum transferred onto a nylon membrane. RNAIII and 5S ribosomal RNA were detected using specific digoxigenin-labeled RNA probes as described elsewhere [7]. *C*, Detection of *spa* in culture supernatants. Strains were grown in casein-casein-yeast medium at 37°C with vigorous shaking for 6 h. Production of *spa* was determined by means of Western blot analysis in standardized samples (optical density at 600 nm, 1). Proteins present in 30 μL of spent culture medium were separated on sodium dodecyl sulfate 12% polyacrylamide gels, blotted to nitrocellulose, and probed with a 1:400 dilution of mouse anti-*spa* monoclonal antibodies (Sigma-Aldrich). Bound antibody was detected with a 1:4000 dilution of goat antimouse immunoglobulin G conjugated to horseradish peroxidase (Sigma) and followed by Western blotting detection (ECL Western blotting systems; GE). *D*, Panton-Valentine leukocidin (PVL) production in selected strains. The *lukS-PV* was quantified in aliquots of standardized supernatants (optical density at 600 nm, 1) by use of a solid-phase sandwich enzyme-linked immunosorbent assay, with a mouse monoclonal antibody and a rabbit peroxidase-conjugated polyclonal F(ab)'2 fragment targeting *lukS-PV*, as recommended by the supplier (R&D Immunoassays, bioMerieux) [3]. *E*, Expression of PVL enhancing the virulence of isogenic *S. aureus* strains. Six-week-old Balb/c female mice ($n = 15$) were intranasally inoculated with suspension of RN6390, LUG776, or LUG855 as described elsewhere [2]. Line graphs indicate weight loss as a measure of morbidity. $^*P < .05$ on day 2 for LUG776 vs LUG855. *F*, Lung tissue sections stained with hematoxilyn-eosin from representative animals infected with LUG776 and LUG855.

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Reply to Vandenesch et al

To the Editor—We are grateful for this opportunity to reply to the comments of Vandenesch et al [1] regarding our article [2].

The Panton-Valentine leukocidin (PVL) is a staphylococcal toxin with a well-established cytolytic mechanism that specifically targets phagocytic leukocytes [3]. It has recently been the focus of attention because of an epidemiological association with strains that cause community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections [4]. It is important to note that the present dispute is not about the role of PVL in CA-MRSA disease, which remains incompletely determined. Rather, our study simply addressed the validity of findings in the study by Labandeira-Rey et al [5], in which laboratory strains were used and, thus, the role of PVL in CA-MRSA was not directly explored.

In the study by Labandeira-Rey et al, the authors reported that PVL contributes to virulence in a mouse model of *S. aureus* pneumonia. Importantly, the results of this and other studies that showed an impact of PVL in mouse infection models are at odds with the relative insensitivity of mouse neutrophils to PVL toxicity in vitro [6]. Thus, to be plausible, the observation by Labandeira-Rey et al would require a mechanism of PVL that is independent of leukocyte plasma membrane pore formation or lysis. The pronounced gene regulatory effect of PVL on the expression of major *S. aureus* virulence determinants described by Labandeira-Rey et al would have constituted such a mechanism, thus representing a crucial part of their investigation. However, neither the impact on virulence in the mouse infection model nor the gene regulatory effect could be confirmed using CA-MRSA strains [7–9]. Thus, independent of the technical problem we discovered, the effects of PVL described in the study by Labandeira-Rey et al represent laboratory strain peculiarities, underscoring the notion that laboratory strains are often problematic for investigating the contribution of virulence determinants to disease.

In addition, it is difficult to understand how the regulatory effect of PVL would be limited to laboratory strains when it is completely absent from CA-MRSA strains [9]. The clear resemblance of the reported PVL-regulatory effect to that exerted by *agr*, findings of frequent spontaneous mutations in that locus [10, 11], and the lack of appropriate controls to rule out an effect of second-site mutations suggested an unintended mutation in *agr*. Indeed, we determined that in one of the key strains of the Labandeira-Rey et al study (LUG855) *agr* functionality was dramatically impaired because of a mutation in the *agr* P2 promoter [2]. Notably, because this mutation could have arisen after the strain was used for the experiments in the study by Labandeira-Rey et al, we did not base our conclusions solely on the finding that the strain was mutated. Rather, we repaired the mutation to obtain a strain that is *agr* positive. None of the phenotypes described by Labandeira-Rey et al could be reproduced with that correct strain, demonstrating that they were incorrectly attributed to PVL. Thus, the discrepancies with the studies that used CA-MRSA strains [7–9] were due to technical reasons rather than due to differences between the laboratory strains and the clinical strains.

In their correspondence, Vandenesch et al present a copy of their strain LUG855, which was ascertained to be *agr* positive, similar to the strain in which we had repaired the *agr* mutation. Yet reporting that such a correct strain exists in the authors' collection is of no significance to the dispute, because our results clearly indicate that such a documented *agr*-positive strain was not used in the original study. In addition, the authors repeated a limited set of experiments using that correct strain. We certainly appreciate the adequate comparisons and control strains that were used. However, the results of these experiments contrast with those we achieved with a correct strain and even contradict the authors' previous observations, as in the case of the PVL gene regulatory effect. With regard to the latter phenomenon, data shown in their Figure 1*B* clearly contradict those used to indicate regulation of *agr* by PVL in the authors' original article [5], in which the authors stated that "The expression of the *luk-PV* genes results in an attenuated *agr* system." Specifically, similar RNAIII levels in the current data set (Figure 1*B*) suggest that there is virtually no effect of PVL on *agr*, given that RNAIII expression is directly correlated with *agr* functionality. This lack of consistency is in accordance with the results of our analysis, indicating that key experiments of Labandeira-Rey et al were performed with an *agr*-mutated strain. Thus, it is vital to routinely ascertain and reascertain the *agr* status of *S. aureus* strains used for key experiments.

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