ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2007, p. 1515–1519 Vol. 51, No. 4
0066-4804/07/$08.00+0 doi:10.1128/AAC.01201-06


# Effect of Antibiotics on *Staphylococcus aureus* Producing Panton-Valentine Leukocidin▽

Oana Dumitrescu, Sandrine Boisset, Cedric Badiou, Michele Bes, Yvonne Benito, Marie-Elisabeth Reverdy, François Vandenesch, Jerome Etienne, and Gerard Lina*

*INSERM, U851, Lyon F-69008, France, and Université Lyon 1, Centre, National de référence des Staphylocoques, Faculté Laennec, Lyon F-69008, France*

Received 25 September 2006/Returned for modification 19 October 2006/Accepted 10 January 2007

**We examined the capacity of *Staphylococcus aureus* strains to release Panton-Valentine leukocidin (PVL) in the presence of antibiotics. No PVL was detected when *S. aureus* was incubated at inhibitory concentrations, while subinhibitory concentrations of oxacillin enhanced the PVL level; clindamycin, linezolid, and fusidic acid were inhibitory; and vancomycin had roughly no effect.**

*Staphylococcus aureus* is an important human pathogen. It expresses a variety of exoproteins, including Panton-Valentine leukocidin (PVL) (31). While Voyich et al. could not establish clear differences in virulence between isogenic pairs of PVL-positive/negative strains (29), Labandeira-Rey et al. clearly demonstrated the role of PVL as a major determinant of virulence in an acute pneumonia mouse model using other sets of isogenic strains for PVL (13) and thus confirmed the results of the princeps experiments showing that PVL is a virulence factor (15). The apparent discrepancy between these studies basically comes from the choice of the experimental models and the choice of the strains.

PVL is now frequently detected in clinical practice, as it is produced by community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clones currently spreading throughout the world (27). PVL has been linked to specific human *S. aureus* infections such as primary skin and soft tissue disease and severe necrotizing pneumonia, where the mortality rate is about 75% (10, 14). Several lines of evidence incriminate PVL in necrotizing pneumonia pathogenesis, including the strong epidemiological link with PVL-producing *S. aureus* isolates (10) and the immunodetection of PVL in the lung (9) and that solely PVL-producing *S. aureus* isolates are able to reproduce necrotizing pneumonia in experimental models (13). Antibiotics that inhibit PVL production may be more appropriate for the treatment of severe necrotizing pneumonia, by analogy with their use in streptococcal and staphylococcal toxic shock syndrome (8, 25, 26).

We examined the effect of antibiotics on PVL release by methicillin-sensitive *S. aureus* and CA-MRSA strains in vitro. We chose a reference strain lysogenized by phage phiSLT (encoding *luk*-PV) and five isolates representing the main PVL-producing CA-MRSA clones (Table 1) (23, 24, 27, 30). We intended to use experimental procedures as close as possible to Clinical Laboratory Standards Institute (CSLI) recommendations for MIC determinations in terms of the culture medium, bacterial inoculum, and growth conditions in order to be able to extrapolate our results to the clinical setting (20). The PVL concentration was determined in culture supernatants by using a specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Agro-Bio; bioMérieux). Unfortunately, when using Mueller-Hinton (MH) medium and CSLI conditions, the PVL level was close to the detection limit in the absence of antibiotics (data not shown). MH medium was thus replaced by casein hydrolysate and yeast extract (CCY) medium, which increases PVL levels (32). The PVL level was 50 times higher in CCY than in MH medium (data not shown), while the MICs obtained with the two media were of the same order (Table 2), except with gentamicin, which dramatically increased (632-fold). CCY medium was therefore used in the rest of the study, and gentamicin was excluded.

TABLE 1. Strains, plasmid, and phage

| Strain, plasmid, or phage | Reference or source | Description |
|---|---|---|
| *S. aureus* strains | | |
| RN6390 | 22 | Laboratory strain that maintains its hemolytic activity when propagated on sheep erythrocyte agar (parental strain) |
| LUG855 | This study | RN6390 phiSLT |
| LUG1124 | This study | RN6390 carrying pLUG547 |
| HT20010734 | 18 | ST1; *agr3 mecA*$^+$ *lukS-PV lukF-PV*$^+$ |
| HT20020488 | 27 | ST80; *agr3 mecA*$^+$ *lukS-PV lukF-PV*$^+$ |
| HT20030203 | 24 | ST8; *agr1 mecA*$^+$ *lukS-PV lukF-PV*$^+$ |
| HT20040332 | 30 | ST59; *agr1 mecA*$^+$ *lukS-PV lukF-PV*$^+$ |
| HT20041010 | 23 | ST80; *agr3 mecA*$^+$ *lukS-PV lukF-PV*$^+$ |
| Plasmid | | |
| pLUG547 | This study | Derivative of pTCV-lac containing the *lukS lukF-PV* promoter (nucleotide −480 to the start codon) fused to *lacZ* |
| Phage | | |
| phiSLT | 19 | *lukS lukF-PV*$^+$-containing phage isolated from A980470 |

* Corresponding author. Mailing address: Centre National de Référence des Staphylocoques, INSERM E0230, 7 rue Guillaume Paradin, 69372 Lyon Cedex 08, France. Phone: 33 472 11 07 75. Fax: 33 478 11 07 64. E-mail: gerard.lina@chu-lyon.fr.

▽ Published ahead of print on 22 January 2007.

TABLE 2. MICs of selected antibiotics for MSSA and CA-MRSA isolates in MH and CCY media

| Antibiotic | MIC (μg/ml) in MH medium/MIC in CCY medium[a] | | | | | |
|---|---|---|---|---|---|---|
| | LUG855 (RN6390) | HT20010734 | HT20020488 | HT20030203 | HT20040332 | HT20041010 |
| Oxacillin | 0.12/0.06 | 32/16 | 8/8 | 32/16 | 16/8 | 32/16 |
| Clindamycin | 0.06/0.12 | 0.12/0.25 | >128/>128 | 0.12/0.25 | >128/>128 | >128/>128 |
| Linezolid | 1/2 | 0.5/2 | 0.5/2 | 1/2 | 1/2 | 1/2 |
| Vancomycin | 1/4 | 0.5/4 | 0.5/4 | 1/4 | 1/4 | 2/4 |
| Fusidic acid | <0.03/<0.03 | <0.03/<0.03 | 4/4 | 0.12/0.06 | <0.03/<0.03 | >128/128 |

[a] MICs were determined with a standard microdilution method recommended by the CSLI in MH broth and CCY medium inoculated with $5 \times 10^5$ CFU/ml (20).

To examine the influence of antibiotics on PVL release, PVL was quantified in the culture supernatant of *S. aureus* LUG855 incubated with various concentrations of oxacillin, vancomycin, clindamycin, and linezolid for 24 h. As shown in Fig. 1A, no PVL was detected when bacteria were incubated with inhibitory concentrations of oxacillin, clindamycin, fusidic acid, linezolid, or vancomycin. This could be explained by the fact that PVL production requires bacterial growth (3).

PVL is associated with intense necrosis in vivo, possibly leading to poor antibiotic diffusion and suboptimal concentrations at sites of infection (4). We therefore examined the effect of subinhibitory antibiotic concentrations on PVL. PVL levels released by LUG885 depended on the antibiotic and the concentration used (Fig. 1A). Clindamycin and linezolid induced concentration-dependent decreases in PVL levels from one-eighth the MIC, while it was significantly increased (up to threefold) at one-eighth and one-quarter the MIC with oxacillin and was unmodified using sub-MIC concentrations of vancomycin. As LUG855 is highly sensitive to fusidic acid, the latter antibiotic was not tested for its effect on PVL release.

To confirm these results, experiments were reproduced using five different CA-MRSA isolates (Table 1 and Fig. 1B). Linezolid induced a concentration-dependent decrease in the PVL level from one-eighth the MIC (four of five isolates) and from one-quarter the MIC (all isolates) to the MIC. Clindamycin, tested on the two susceptible strains, induced a strong concentration-dependent decrease in PVL levels from one-eighth the MIC to the MIC. Again, PVL release by the CA-MRSA isolates increased in the presence of all subinhibitory oxacillin concentrations, by 2- to 6.5-fold. With vancomycin, PVL levels were unmodified except with HT20020488 at one-quarter and one-eighth the MIC and HT20010734 at one-half the MIC, which decreased and increased PVL release, respectively. Fusidic acid, tested on isolates with MICs higher than

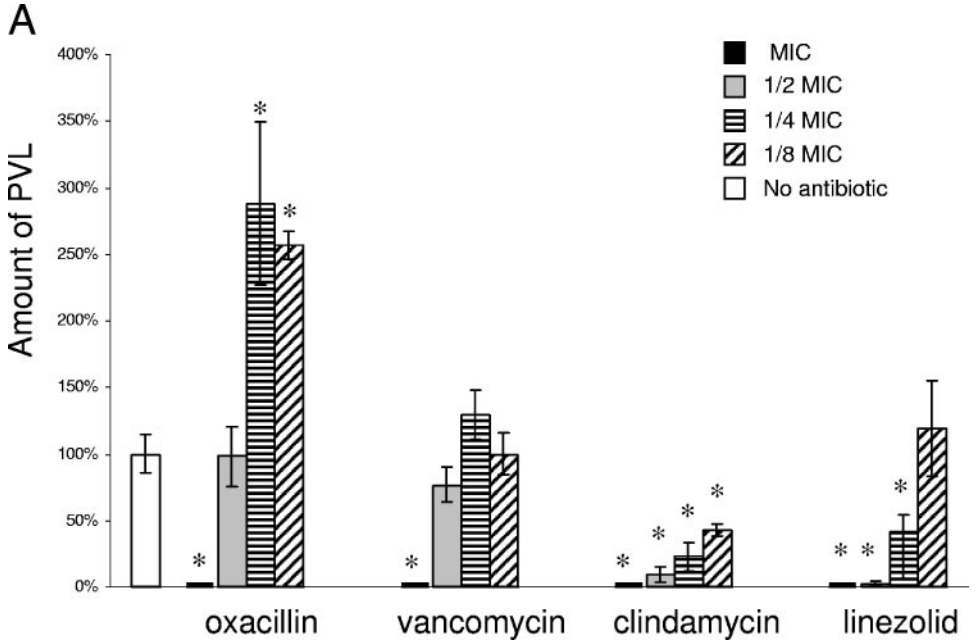


FIG. 1. Effect of antibiotics on PVL. *S. aureus* LUG855 (A) and *S. aureus* strains HT20010734, HT20020488, HT20030203, HT20040332, and HT20041010 (B) were incubated in CCY medium with or without antibiotics (at 1, [1/2], [1/4], and [1/8] MIC), according to standard CSLI procedures, for 25 h at 37°C without shaking. Samples were taken for bacterial counting (plate counting of colonies from diluted broth) and PVL quantification by ELISA. Results are ratios of μg of PVL/$\log_{10}$ CFU of bacteria cultured with the indicated concentrations of antibiotic by means of μg of PVL/$\log_{10}$ CFU of bacteria cultured without antibiotic and expressed as percentage values. Values are means ± standard deviations (five different experiments in panel A and three different experiments in panel B). *, statistically different from the control (corresponding isolate grown without antibiotic), with a $P$ value of <0.05, by one-way analysis of variance followed by a posteriori Dunnett's test. ND, not determined.

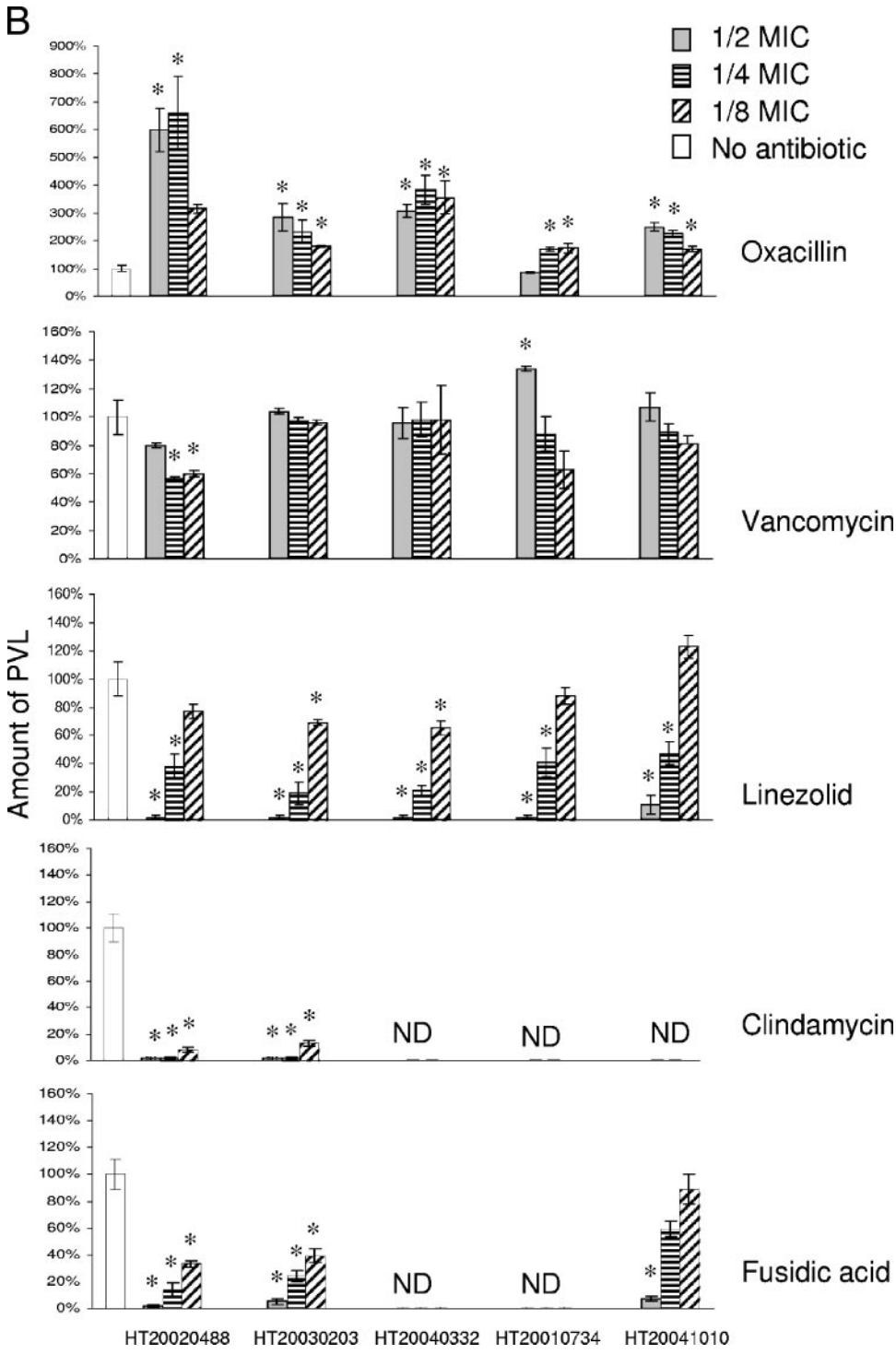


FIG. 1—*Continued*.

0.03 μg/ml, induced a concentration-dependent decrease in the PVL level.

To examine the effect of antibiotics on PVL gene transcription, *S. aureus* LUG1124 (containing the plasmid-borne *luk-PV* promoter fused to the *lacZ* gene described in Table 1) was cultured with or without oxacillin, vancomycin, or linezolid at one-eighth, one-quarter, and one-half the MIC and assayed for β-galactosidase activity. Samples were adjusted to an optical density at 600 nm of 1 before cell lysis with the FastPrep instrument (QBiogen). Protein concentrations and β-galactosidase activity were determined in the lysates by using the Bradford method (1) and the Beta-Glo system (Promega), respectively. As shown in Fig. 2, β-galactosidase activity was significantly enhanced, by 3- to 20-fold, by oxacillin at sub-MIC

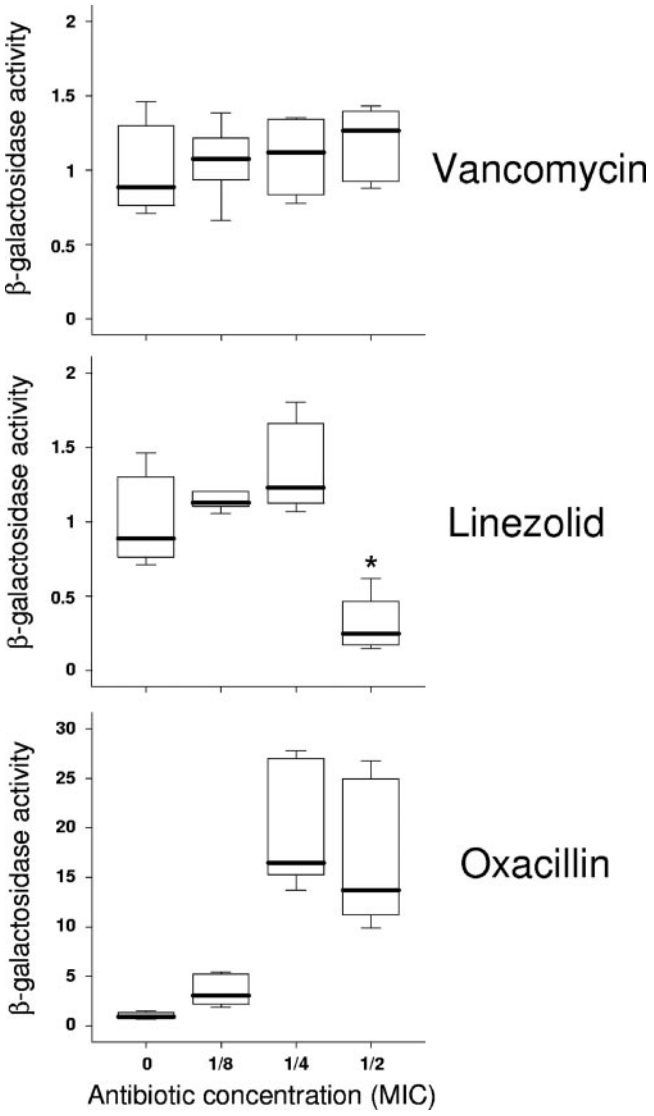


FIG. 2. Variation of *lukS-PV lukF-PV* gene transcription induced by subinhibitory concentrations of antibiotics. *S. aureus* LUG1124 containing a plasmid-carried *luk-PV* promoter-*lacZ* fusion was grown during 24 h at 37°C with or without one-eighth, one-quarter, and one-half the MIC of oxacillin, vancomycin, and linezolid and assayed for β-galactosidase activity. β-Galactosidase activity is expressed as a ratio of arbitrary units per milligram of bacterial protein cultured with the indicated concentration of antibiotic by arbitrary units per milligram of bacterial protein cultured without antibiotic. Values are means ± standard deviations (three different experiments). *, statistically different from control (LUG1124 grown without antibiotics), with a $P$ value of $<0.05$, by one-way analysis of variance followed by a posteriori Dunnett's test.

concentrations, reflecting *luk-PV* promoter activation. It was higher than expected with ELISA quantification, but we used a nonlinear luminometric method to quantify β-galactosidase activity. By contrast, LacZ expression was strongly reduced by linezolid at one-half the MIC, indicating repression of the *luk-PV* promoter, and was not modified by lower concentrations of linezolid or vancomycin. Clindamycin and fusidic acid were not examined upon *luk-PV* transcription, because the plasmid carrying the PVL::*lacZ* fusion also harbored a macrolide resistance gene, and the strain was too sensitive to fusidic acid.

In conclusion, subinhibitory concentrations of oxacillin enhanced PVL levels by all the isolates through PVL promoter activation as previously observed for *S. aureus* alpha-hemolysin (21). How oxacillin enhances *luk-PV* transcription remains to be determined. We could hypothesize the involvement of SOS pathway stimulation by β-lactams (16) and those of response regulatory pathways engaged in peptidoglycan synthesis (7, 12). By contrast, subinhibitory concentrations of clindamycin, linezolid, and fusidic acid significantly reduced PVL release. This was not explained by the impact of these antibiotics on bacterial growth because PVL was detectable at the cell density achieved (data not shown). These antibiotics have previously been shown to reduce the production of several other toxins (2, 5, 6, 8, 25, 26, 28), possibly through their impact on bacterial protein synthesis and transcription (11, 17).

These data showing that subinhibitory antibiotic concentrations can either up-regulate or down-regulate PVL release by *S. aureus* may have therapeutic implications. It provides a logical basis for future studies to examine whether linezolid, clindamycin, or fusidic acid administration could improve the outcome of severe infections due to PVL-producing *S. aureus* strains.

We thank Christine Courtier, Christine Cardon, Céline Spinelli, Caroline Bouveyron, Martine Rougier, Annie Martra, and Florence Couzon for their technical advice and David Young for editorial guidance.

The laboratory received a research grant from Pfizer.

## REFERENCES

1. **Ausubel, F., R. Brent, R. Kingston, B. Moore, J. Seidman, J. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Wiley Interscience, New York, NY.
2. **Bernardo, K., N. Pakulat, S. Fleer, A. Schnaith, O. Utermohlen, O. Krut, S. Muller, and M. Kronke.** 2004. Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. Antimicrob. Agents Chemother. **48:**546–555.
3. **Bronner, S., P. Stoessel, A. Gravet, H. Monteil, and G. Prevost.** 2000. Variable expressions of *Staphylococcus aureus* bicomponent leucotoxins semiquantified by competitive reverse transcription-PCR. Appl. Environ. Microbiol. **66:**3931–3938.
4. **Cars, O.** 1990. Pharmacokinetics of antibiotics in tissues and tissue fluids: a review. Scand. J. Infect. Dis. Suppl. **74:**23–33.
5. **Coyle, E. A., R. Cha, and M. J. Rybak.** 2003. Influences of linezolid, penicillin, and clindamycin, alone and in combination, on streptococcal pyrogenic exotoxin A release. Antimicrob. Agents Chemother. **47:**1752–1755.
6. **Dickgiesser, N., and U. Wallach.** 1987. Toxic shock syndrome toxin-1 (TSST-1): influence of its production by subinhibitory antibiotic concentrations. Infection **15:**351–353.
7. **Gardete, S., S. W. Wu, S. Gill, and A. Tomasz.** 2006. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **50:**3424–3434.
8. **Gemmell, C. G., and C. W. Ford.** 2002. Virulence factor expression by gram-positive cocci exposed to subinhibitory concentrations of linezolid. J. Antimicrob. Chemother. **50:**665–672.
9. **Genestier, A. L., M. C. Michallet, G. Prevost, G. Bellot, L. Chalabreysse, S. Peyrol, F. Thivolet, J. Etienne, G. Lina, F. M. Vallette, F. Vandenesch, and L. Genestier.** 2005. *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. J. Clin. Investig. **115:**3117–3127.
10. **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne.** 2002. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet **359:**753–759.
11. **Herbert, S., P. Barry, and R. P. Novick.** 2001. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. Infect. Immun. **69:**2996–3003.
12. **Kuroda, M., K. Kuwahara-Arai, and K. Hiramatsu.** 2000. Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus*

*aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. Biochem. Biophys. Res. Commun. **269:**485–490.
13. **Labandeira-Rey, M., F. Couzon, S. Boisset, E. L. Brown, M. Bes, Y. Benito, E. M. Barbu, V. Vazquez, M. Höök, J. Etienne, F. Vandenesch, and M. G. Bowden.** 2007. *Staphylococcus aureus* Panton Valentine leukocidin causes necrotizing pneumonia. Science, in press.
14. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne.** 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin. Infect. Dis. **29:**1128–1132.
15. **Lina, G., F. Vandenesch, and J. Etienne.** 2006. A brief history of *Staphylococcus aureus* Panton Valentine leucocidin. *In* V. L. Yu (ed.), Antimicrobial therapy and vaccines: microbes, 2nd ed., vol. I. ESun Technologies, LLC, Pittsburgh, PA.
16. **Miller, C., L. E. Thomsen, C. Gaggero, R. Mosseri, H. Ingmer, and S. N. Cohen.** 2004. SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science **305:**1629–1631.
17. **Mukhtar, T. A., and G. D. Wright.** 2005. Streptogramins, oxazolidinones, and other inhibitors of bacterial protein synthesis. Chem. Rev. **105:**529–542.
18. **Naimi, T. S., K. H. LeDell, K. Como-Sabetti, S. M. Borchardt, D. J. Boxrud, J. Etienne, S. K. Johnson, F. Vandenesch, S. Fridkin, C. O'Boyle, R. N. Danila, and R. Lynfield.** 2003. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA **290:** 2976–2984.
19. **Narita, S., J. Kaneko, J. Chiba, Y. Piemont, S. Jarraud, J. Etienne, and Y. Kamio.** 2001. Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. Gene **268:**195–206.
20. **National Committee for Clinical Laboratory Standards.** 2004. Performance standards for antimicrobial susceptibility testing. Approved standard M100-S14. National Committee for Clinical Laboratory Standards, Wayne, PA.
21. **Ohlsen, K., W. Ziebuhr, K. P. Koller, W. Hell, T. A. Wichelhaus, and J. Hacker.** 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **42:**2817–2823.
22. **Peng, H. L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J. Bacteriol. **170:**4365–4372.
23. **Ramdani-Bouguessa, N., M. Bes, H. Meugnier, F. Forey, M. E. Reverdy, G. Lina, F. Vandenesch, M. Tazir, and J. Etienne.** 2006. Detection of methicillin-resistant *Staphylococcus aureus* strains resistant to multiple antibiotics and carrying the Panton-Valentine leukocidin genes in an Algiers hospital. Antimicrob. Agents Chemother. **50:**1083–1085.
24. **Said-Salim, B., B. Mathema, K. Braughton, S. Davis, D. Sinsimer, W. Eisner, Y. Likhoshvay, F. R. Deleo, and B. N. Kreiswirth.** 2005. Differential distribution and expression of Panton-Valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* strains. J. Clin. Microbiol. **43:**3373–3379.
25. **Schlievert, P. M., and J. A. Kelly.** 1984. Clindamycin-induced suppression of toxic-shock syndrome-associated exotoxin production. J. Infect. Dis. **149:**471.
26. **Sriskandan, S., A. McKee, L. Hall, and J. Cohen.** 1997. Comparative effects of clindamycin and ampicillin on superantigenic activity of *Streptococcus pyogenes*. J. Antimicrob. Chemother. **40:**275–277.
27. **Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg. Infect. Dis. **9:**978–984.
28. **van Langevelde, P., J. T. van Dissel, C. J. Meurs, J. Renz, and P. H. Groeneveld.** 1997. Combination of flucloxacillin and gentamicin inhibits toxic shock syndrome toxin 1 production by *Staphylococcus aureus* in both logarithmic and stationary phases of growth. Antimicrob. Agents Chemother. **41:**1682–1685.
29. **Voyich, J. M., M. Otto, B. Mathema, K. R. Braughton, A. R. Whitney, D. Welty, R. D. Long, D. W. Dorward, D. J. Gardner, G. Lina, B. N. Kreiswirth, and F. R. DeLeo.** 2006. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J. Infect. Dis. **194:**1761–1770.
30. **Wannet, W. J., M. E. Heck, G. N. Pluister, E. Spalburg, M. G. van Santen, X. W. Huijsdans, E. Tiemersma, and A. J. de Neeling.** 2004. Panton-Valentine leukocidin positive MRSA in 2003: the Dutch situation. Eur. Surveill. **9:**28–29.
31. **Ward, P. D., and W. H. Turner.** 1980. Identification of staphylococcal Panton-Valentine leukocidin as a potent dermonecrotic toxin. Infect. Immun. **28:**393–397.
32. **Woodin, A. M.** 1959. Fractionation of a leucocidin from *Staphylococcus aureus*. Biochem. J. **73:**225–237.