## Resistance to Autolysis in Vancomycin-Selected *Staphylococcus aureus* Isolates Precedes Vancomycin-Intermediate Resistance

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Four clinical U.S. glycopeptide intermediate resistant *Staphylococcus aureus* (GISA) isolates were resistant to Triton X-100-induced autolysis. Similar resistance was demonstrated in an isolate obtained after a single passage of a susceptible clinical isolate in low-level vancomycin. Strains with the vancomycin-induced Triton X-100 resistance phenotype produced active murein hydrolases but were resistant to lysis by murein hydrolases.

A common characteristic among glycopeptide intermediate resistant *Staphylococcus aureus* (GISA) isolates is a slightly thickened cell wall (10, 14, 22), although the explanation for this phenomenon is unknown. One hypothesized mechanism is a decrease in activity of murein hydrolases (MH) or autolysins

100 90 80-70-%Absorbance 60-50 40-30 20 10 523 ò Time (Hrs) 523 1x10<sup>9</sup> NJ 1x10<sup>4</sup> MI PC 1x10 – IL-A IL-F 1x10 1x10 1x10 1x10 2 Time (Hrs) â

FIG. 1. Triton X-100 autolysis assay of clinical GISA isolates. GISA isolates IL-F, MI, PC, and NJ are clinical isolates from Illinois, Michigan, Port Chester, N.Y., and New Jersey, respectively (6–9, 21, 23). Cultures were grown to mid-logarithmic growth phase and suspended in buffered 0.05% Triton X-100. At 1-h intervals, an aliquot was collected for absorbance determinations at 600 nm and for determination of viable cell count (CFU/mI). Absorbances are represented as percentages of the absorbance at 600 nm relative to that at time zero for each sample.

\* Corresponding author. Mailing address: Department of Pediatrics, The University of Chicago, 5841 S. Maryland Ave., MC 6054, Chicago, IL 60637. Phone: (773) 702-6401. Fax: (773) 702-1196. E-mail: sboyleva @midway.uchicago.edu. that play physiologic roles in cell separation, penicillin-induced lysis, and ongoing peptidoglycan remodeling (1, 13, 17).

Lysis induced by incubation in Triton X-100 has been used to evaluate autolytic activity (15, 25). We and others observed that resistance to Triton X-100-induced lysis occurs in laboratory-derived GISA mutants obtained by selection of glycopeptide-susceptible clinical isolates in glycopeptide-containing media (3, 19, 24).

This phenomenon has been studied less well among clinical GISA isolates. It was reported that a clinical GISA isolate, IL-F, was resistant to Triton X-100-induced lysis (2), as was IL-A, an earlier blood isolate obtained from the same patient.



FIG. 2. Triton X-100 autolysis assay of vancomycin-susceptible strain 523 and its vancomycin-exposed derivatives, 523a to 523k. Cultures were grown to mid-logarithmic growth phase and suspended in buffered 0.05% Triton X-100. At 1-h intervals, an aliquot was collected for determinations of absorbance at 600 nm and viable cell count (CFU/ml). Absorbances are represented as percentages of the absorbance at 600 nm relative to that at time zero for each sample.

8325/4 RN450 Mw-2 IL-A/LL-F N315	1	MARKPNYKLPSNVALTLUGSAVTANQVQAADITQQQTTNKNVLDSNKVKATTEQAXAEVK MARKPNYKJESMVAIT.VGSAVTANQVQAADITQQQTTNKNVLDSNKVKATTEQAXAEVK MARKPNYKJESMVALTLGSAVTANQVQAADITQQQTINKNVLDSNKVKATEQAXAEVK MARKPNYKLPSMVALTLVGSAVTANQVQAADITQQQTINKNVLDSNKVKATTEQAKAEVK MARKPNYKLPSMVALTLVGSAVTANQVQAADITQQQTINKNVLDSNKVKATTEQAKAEVK	60 60 60 60 60	8325/4 RN450 Mw-2 1L-A/IL-F N315	721 721 721 713 713	GTGNQTFXATKQQQTDKSTVLFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT GTGNQTFXATKQQDTDKSTVLFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT GTGNQTFKATKQQDIDKSTULFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT GTGNQTFKATKQQDIDKSTYLFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT GTGNQTFKATKQQDIDKSTYLFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT GTGNQTFKATKQQDIDKSTYLFGTVNGKSGWVSKAYLAVPAAPKKAVAQPKTAVKAYTVT	780 780 780 772 772
8325/4 RN450 Mw-2 11-A/I1-F N315	61 61 61 61 61	NPPQNISGTQYYQDPATVQPXTANNKTGNACVSQKVDTAQVNGDTRANCSATNNTQPVA NPPQNISGTQYYQDPATVQPXTANNKTGNACVSQKVDTAQVNGDTRANCSATNNTQPVA NPPQNISGTQYYQDPATVQXTANNKTGNACVSQKVDTAQVNGDTRANCSATTNNTQVA NPPQNISGTQYYQDPATVQKTANNKTGNACVSQKVDTAQVNGDTRANCSATTNNTQPVA NPPQNISGTQYYQDPATVQPKTANNKTGNACVSQKVDTAQVNGDTRANCSATTNNTQPVA	120 120 120 120 120	8325/4 RN450 Mw-2 IL-A/IL-F N315	781 781 781 773 773	KPQTIQTVSKIAQVKPNNTGIRASYYSKIAKNGAKYADRIFYVIKRAHGNETYVLINNT KPQTIQTVSKIAQVKPNNTGIRASYYSKIAKNGAKYADRIFYVIKRAHGNETYVLINNT KPQTTQTVSKIAQVKPNNTGIRASYYSKIAKNGAKYADRIFYVIKRAHGNETYVLINNI KPQTTQTVSKIAQVKPNNTGIRASYYSKIAKNGAKYADRIFYVIKRAHGNETYVLINNI KPQTTQIVSKIAQVKPNNTGIRASYYSKIAKNGAKYADRIFYVIKRAHGNETYVLINNI	840 840 840 732 732
8325/4 RN450 Mw-2 TL-A/IL-F N315	121 121 121 121 121	KSTŠTTAPKTNENVTNAGYSE VDDEDENSENQIN PRILIKSAAKPAALETQYKTAAPKAAT KSTŠTTAPKTNENVTNAGYSE VDDEDENSENGIN PRILIKSAAKPAALETQYKTAAPKAAT KSTŠTTAPKTNINVTNAGYSE VDDEDDISGEQIN PELIKSAAKPAALETQYKAAP KSTŠTTAPKTNINVTNAGYSE VDDEPINSERGIN PELIKSAAKPAALETQYKAAP KSTŠTTAPKTNINVTNAGYSENDEPINSERGINE ELIKSAAKPAALETQYKAAP	180 180 180 176 176	8325/4 RN450 Mw-2 IL-A/IL-F N315	841 841 733 733	SHNIPLGWENVKJLNVQNLGKEVKTTQKYTVNKSNNG.SMVPWOTKNQVILTCNNLAQGI SHNIPLGWENVKJLNVQNLGKEVKTTQKYTVNKSNNG.SMVPWOTKNQVITCNNLAQGI SHNIPLGWENVKJLNVQNLGKEVKTTQKYTVKSNNG.SMVPWOTKNQVITCNNLAQGI SHNIPLGWENVKJLNVQNLGKEVKTTQKYTVKSNNGLSMVPWOTKNQVITTONLAQGI SHNIPLGWENVKJLNVQNLGKEVKTTQKYTVKSNNGLSMVPWOTKNQVITTONLAQGI	900 900 900 892 892
8325/4 RN450 Mw-2 TL-A/IL-F N315	181 181 181 177 177	TSAPKAKTEATPKVITFSASAQPRSVAATPKTSLEPKYKPQVNSSINDYTCKNNLKAPKIE ISAPKAKIAATPKVITFSASAQPRSVAATPKTSLEPKYRPQVNSSINDYIKNNLKAPKIE TSAPKAKIEATPKVITFSASAQPRSVAATPKTSLEPKYRPQVNSSINDYIKNNLKAPKIE KAKIEATPKVITFSASAQPRSVAATPKTSLEPKYRPQVNSSINDYIRKNNLKAPKIE KAKIEATPKVITFSASAQPRSVAATPKTSLEPKYRPQVNSSINDYIRKNNLKAPKIE	240 240 240 232 232	8325/4 RN450 Mw~2 IL-A/IL-F N315	901 901 901 893 893	FNATKQVSVCKDVYLYGIINNRIGNVNAKDITAPTAVKPTTSAARDYNYTYVIKNCNGYY ENATKQVSVCKDVYLYGTINNRIGNVNAKDITAPTAVKPTTSAARDYNYTYVIKNCNGYY FNATKQVSVCKDVYLYGTINNRIGNVNAKDITAPTAVKPTTSAARDYNYTYVIKNCNGYY FNATKQVSVCKDVYLYGTINNRIGNVNAKDITAPTAVKPTTSAARDYNYTYVIKNCNGYY FNATKQVSVCKDVYLYGTINNRIGNVNAKDITAPTAVKPTISAARDYNYTYVIKNCNGYY	960 960 960 952 952
8325/4 RN450 Mw-2 1L-A/11-F N315	241 241 241 233 233	EDYTSYFPKYAYRNGVGRPEGIVVHDTANDRSTINGEISYMKNNYQNAFVHAFVGGDRII EDYTSYFPKYAYRNGVGRPFGIVVHDTANDRSTINGEISYMKNNYQNAFVHAFVGGDRI EDYTSYFPKYAYRNGVGRPFGIVVHDTANDRSTINGEISYMKNNYQNFHHAFVGGDRI EDYTSYFPKYAYRNGVGRPECIVVHDTANDRSTINGEISYMKNNYQNAFVHAFVGDRII EDYTSYFPKYAYRNGVGRPEGIVVHDTANDRSTINGEISYMKNNYQNAFVHAFVGDRII	300 300 300 292 292	8325/4 RN450 Mw-2 IL-A/IL-F N315	961 96: 961 953 953	YVTPNSDTARYSLKAFNRQPPAVVXEQVINCQTWYYCKLSNGKLAWIKSTDLAXELIKYN YVTPNSDTARYSLKAFNEQPFAVVXEQVINCQTWYYCKLSNGKLAWIKSTDLAXELIKYN YVTPNSDTARYSLKAFNEQPFAVVXEQVINCQTWYYCKLSGKLAWIKSTDLAXELIKYN YVTPNSDTARYSLKAFNEQPFAVVKEQVINCQTWYYCKLSNGKLAWIKSTDLAKELIKYN YVTPNSDTARYSLKAFNEQPFAVVKEQVINCQTWYYCKLSNGKLAWIKSTDLAKELIKYN	1020 1020 1020 1012 1012 1012
8325/4 RN450 Mw-2 IL-A/IL-F N315	301 301 301 293 293	RTAPTDYLSWGVGAVGNPRFINVELVHITHDYASFARSMNNYADYAADQLQYYGLKPDSAR RTAPTDYLSWGVGAVGNPRFINVELVHTHDYASFARSMNYADYAADUQYYGLKPDSAR RTAPTDYLSWGVGAVGNPRFINVELVHITHDYASFARSMNYADYAADQLQYYGLKPDSAR ETAPTDYLSWGVGAVGNPRFINVELVHITHDYASFARSMNYYADYAADQLQYYGLKPDSAR RTAPTDYLSWGVGAVGNPRFINVELVHITHDYASFARSMNYYADYAADQLQYYGLKPDSAR	360 360 352 352	8325/4 RN450 Mw-2 IL-A/IL-F N315	1021 1021 1021 1013 1013	QTCMTLNQVAQIQACLQYXPQVQBVPCKWTDAXFNDVXHAMDTXRLAQDPALKYQFLRLD QTCMTLNQVAQIQACLQYXPQVQBVPCKWTDAXFNDVXHAMDTXRLAQDPALKYQFLRLD QTCMTLNQVAQIQACLQYXPQVQBVCKWTDANFNDVXHAMDTXRLAQDPALKYQFLRLD QTCMTLNQVAQIQACLQYXPQVQRVPCKWTDANFNDVXHAMDTXRLAQDPALKYQFLRLD QTCMTLNQVAQIQACLQYXPQVQRVPCKWTDANFNDVKHAMDTXRLAQDPALKYQFLRLD	1080 1080 1080 1072 1072
8325/4 RN450 Mw-2 IL-A/1L-F N315	361 361 361 353 353	YDGNGTWWTHYAVSKYLGGYDHADPHGYLRSHNYSYDQIYDLINEKYLLKWCKVAPWGTQ YDGNGTWWTHYAVSKYLGGTDHADPHGYLRSHNYSYDQIYDLINEKYLLKMCKVAPWGTQ YDGNGTWWTHYAVSKYLGGTDHADPHGYLRSHNYSYDQIYDLINEKYLLKMCKVAPWGTQ YDCNGTWWTHYAVSKYLGGTDHADPHGYLRSHNYSYDQLYDLINEKYLLKMCKVAPWGTQ YDCNGTWWTHYAVSKYLGGTDHADPHGYLRSHNYSYDQLYDLINEKYLLKMCKVAPWGTQ	420 420 420 412 412	8325/4 RN450 Mw-2 IL-A/II-F N315	1081 1081 1091 1073 1073	OPQNISIDKINQFLKGKGVLENQGAAFYKAAQMYGINEVYLISHALLEGNGISQLAKGA OPQNISIDKINQFLKGKGVLENQGAAFYKAAQMYGINEVYLISHALLEGNGISQLAKGA OPQNISIDKINQFLKGKGVLENQGAAFYKAAQMYGINEVYLISHALLEGNGISQLAKGA OPQNISIDKINQFLKGKGVLENQGAAFYKAAQMYGINEVYLISHALLEGNGISQLAKGA	1140 1140 1132 1132
8325/4 RN450 Mw-2 II-A/II-F N315	421 421 421 413 413	SITT PTT PSKPT I PSKPSTCKLI VAANNOVAQI KPTNSGI YTTYYD XTOKATNEVOKT HA SITT PTT PTT PSKPI T PSK VSTCKLI VVAANNOVAQI KPTNSGI YTTYYD KYGKATNEVOKT FA SITT PTT PSKPI I PSKPSTCKLI VVAANNOVAQI KPTNSGI YTTYYD KYGKATNEVOKT FA PITT PTI PSKPI I PSKPSTGKLI VVAANNOVAQI KPTNSGI YTTYYD KYGKATNEVOKT FA PITT PTI PSKPI I PSKPSTGKLI VVAANNOVAQI KPTNSGI YTTYYD KYGKATNEVOKT FA	480 480 480 472 472	8325/4 RN450 Mw-2 IL-A/IL-F N315	1141 1141 1141 1133 1133	DVVNNKVVTNSNTKYHNVEG LAAVDN DELREGI KYAXQAGWDTVSKATVGGAKFI GNSYV DVVNNKVVTNSNTKYHNVEG LAAVDN DELREGI KYAXQAGWDTVSKATVGGAKFI GNSYV DVVNNKVVTNSNTKYHNVEG LAAVDN DELREGI KYAXQAGWDTVSKATVGGAKFI GNSYV DVVNNKVVTNSNTKYHVEG LAAVDN DELREGI KYAXQAGWDTVSKATVGGAKFI GNSYV DVVNNKVVTNSNTKYHVEG LAAVDN DELREGI KYAXQAGWDTVSKATVGGAKFI GNSYV	1200 1200 1200 1192 1192
8325/4 RN450 Mw-2 IL-A/IL-F N315	481 481 481 473 473	VSKTATLGNQXFYLVQDYNSGNXFGWVXEGDVVYNTAXSPVNVNQSYSIXPGTX.YTVPW VSKTATLGNQXFYLVQDYNSGNXFGWVEGDVVYNTAXSPVNVNQSYSIXPGTX.YTVPW VSKTATLGNQKFYLVQDYNSGNXFGWVEGDVVYNTAXSPVNVQSYSIXPGTX.YTVPW VSKTATLGNQKFYLVQDYNSGNXFGWVKFGDVVYNTAKSPVNVNQSYSIXGGTX.YTVPW VSKTATLGNQKFYLVQDYNSGNXFGWVKFGDVVYNTAKSPVNVNQSYSIXGGTKI.YTVPW	540 540 532 532	8325/4 RN450 Mw-2 IL-A/IL-F N315	1201 1201 1201 1193 1193	KAGQUNTLYKMRWNPAHPGTHQYATDYDWANINAKIIKGYYDKIGEYGKYFDIPQYK         1256           KAGQUNINKMRWNPAHPGTHQYATDYDWANINAKIIKGYYDKIGEYGKYFDIPQYK         1256           KAGQUNINKMRWNPAHPGTHQYATDYDWANINAKIIKGYYDKIGEYGKYFDIPQYK         1256           KAGQUNINKMRWNPAHPGTHQYATDYDWANINAKIIKGYYDKIGEYGKYFDIPQYK         1248           KAGQUNINKMRWNPAHPGTHQYATDYDWANINAKIIKGYYDKIGEYGKYFDIPQYK         1248	
8325/4 RN450 Mw-2 II-A/IL-F N315	541 541 533 533	GISKQVAGSVSGSQNQTFKASXQQQIDKSIYLYQSVNGKSGWVSKAYLVDTAXPTPTPT GISKQVAGSVSGSQNQTFKASXQQQIDKSIYLYGSVNGKSGWVSKAYLVDTAXPTPTPTP GISKQVAGSVSGSQNQTFKASXQQQIDKSIYLYGSVNGKSGWVSKAYLVDTAKPTPTPTP GTSKQVAGSVSGSQNQTFKASKQQQDKSIYLYGSVNGKSGWVSKAYLVDTAKPTPTPTP GTSKQVAGSVSGSQNQTFKASKQQQDKSIYLYGSVNGKSGWVSKAYLVDTAKPTPTPTP GTSKQVAGSVSGSQNQTFKASKQQDLSKIYLYGSVNGKSGWVSKAYLVDTAKPTPTPTP	600 600 592 592	FIG strains ID: BA ID: A	. 3. IL- AB4 P004	Clustal W alignment of the Atl amino acid sequer A and IL-F (accession number AF537210), N315 (g 2150.1), RN450 (protein ID: BAA04185.1), MW2 (g 825.1) and 8325/4 (protein ID: AAA99982.1) As	ices of protein protein terisks
8325/4 RN450 Mw-2 IL-A/IL-F N315	601 601 593 593	XPSTPCTNKKLTVSSINGVAQINAKNGLFTIVYDKTGKPTKEVQKTFAVTKEASLGGNK KPSTPCTNNKLTVSSINGVAQINAKNGLTTVYDKTGKPTKFVQKTFAVTKEASLGGNK KPSTPTTNNKLTVSSINGVAQINAKNGLTTTVYDKTGKPTKEVQKTFAVTKEASLGCNK KPSTPTTNNKLTVSSINGVAQINAKNGLTTVYDXSCKPTKEVQKTFAVTKEASLGCNK	660 660 652 652	indica positic groups MILF	te po ons v s full , HY	ositions with a single, fully conserved residue; colons ir with polymorphism but with one of the following y conserved: STA, NEQK, NHQK, NDEQ, QHRK, I , FYW; periods indicate positions with one of the fol	dicate strong MILV, lowing

 8325/4
 661
 FYLVKDYNSFILIGWVKQGDVIYNNAKSPVNVMCTYTVKPGTKLYSVPMGTYKQSAGAVS
 720

 RX450
 661
 FYLVKDYNSFILIGWVKQGDVIYNNAKSPVNVMCTYTVKPGTKLYSVPMGTYKQSAGAVS
 720

 Mw-2
 661
 FYLVKDYSFILIGWVKQGDVIYNNAKSPVNVMCTYTVKPGTKLYSVPMGTYKQSAGAVS
 720

 1L-A/IL-F
 653
 FYLVKDYNSFILIGWVKQGDVIYNNAKSPVNVMCTYTVKPGTKLYSVPMGTYKQSAGAVS
 712

 N315
 653
 FYLVKDYNSFILIGWVKQGDVIYNNAKSPVNVMCTYTVKPGTKLYSVPMGTYKQSAGAVS
 712

weak groups are defined as a strong score of >0.5 and a weak score of  $\le 0.5$ , respectively, according to the Gonnet Pam250 matrix.

weaker groups fully conserved: CSA, ATV, SAG, STNK, STPA,

SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Strong and

IL-A and IL-F were both obtained after vancomycin therapy had been initiated. IL-A was susceptible to vancomycin but contained a bacterial subpopulation that could grow on media containing 4  $\mu$ g of that antimicrobial per ml (2). Subsequently, clinical GISA isolates from Michigan, Port Chester, N.Y., and New Jersey were studied (6–9, 21, 23), and all were found to be relatively resistant to Triton X-100-mediated lysis compared with a vancomycin-susceptible and -naïve control *S. aureus* strain, 523 (10) (Fig. 1); another clinical GISA isolate, Mu50, from Japan did not have this phenotype.

Previously characterized isolates (523a to 523k) (10), obtained by step-wise incubation of isolate 523 in medium containing vancomycin, provided an opportunity to study the time course of acquisition of Triton X-100 resistance and compare it with the acquisition of vancomycin resistance. This model is relevant, since laboratory-derived GISA isolate 523k has many properties accorded to clinical GISA isolates (2, 10). We found that the first isolate, 523a, was already resistant to Triton X-100-induced autolysis compared with the parent strain 523 (Fig. 2), although the MIC of vancomycin was 3 to 4  $\mu$ g/ml (susceptible). The subsequent isolates (523b to 523k) were also resistant to Triton X-100-induced autolysis.

The *atl* gene, encoding the major bifunctional autolysin, was sequenced from strains IL-A and IL-F to assess whether the Triton X-100 resistance phenotype in these vancomycin-exposed strains was due to a change in the function of the *atl* gene product. For sequencing, a 5,890-bp PCR product containing the *atl* gene, two contiguous upstream open reading frames, and a portion of open reading frame 1 (18) was obtained with primers ATL-F (5'-GGTACCAAAAATTAAAT GGTGATG-3') and ATL-R (5'-GCACGTTGCGAATTGAT TGAAGC-3') and the Advantage2 PCR kit (Clontech



FIG. 4. Zymograms containing overlays of heat-killed cells from strains IL-A and IL-F showing resistance to murein hydrolases extracted from the extracellular (EC), cell wall (CW), and intracellular (IC) fractions from strains IL-A and IL-F. Shown are murein hydrolases extracted from strains IL-A (lanes 1) and IL-F (lanes 2).

Laboratories, Inc., Palo Alto, Calif.). Sequencing reactions were performed with fluorescent dye-labeled terminator chemistry with the use of primers designed against the sequence of strain N315 (16).

Sequence alignment (Fig. 3) and mutation detection were performed using public-domain software, namely, Blast (on the NCBI web site at www.ncbi.nlm.nih.gov/blast/Blast.cgi) and ClustalW version 1.8 (http://clustalw.genome.ad.jp). The sequence of the 5,890-bp fragment was identical between strains IL-A, IL-F (accession number AF537210), and vancomycinsusceptible methicillin-resistant *S. aureus* control isolate N315 (16). The amino acid sequence alignment of ATL from five strains of *S. aureus* is shown in Fig. 3. The identity of the *atl* gene sequence with that of N315 suggests that ATL is not responsible for vancomycin resistance in strains IL-A and IL-F.

To determine whether decreased autolysis in vancomycinexposed S. aureus isolates IL-A, IL-F, 523a to -k, and clinical GISA isolates was due to a changed physical property of vancomycin-exposed cell walls, heat-killed cells from strains 523, 523a, 523k, IL-A, and IL-F were incorporated as overlays into zymograms. Zymography was performed as described previously (20) with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis to resolve MHs. Proteins were obtained from the intracellular, cell wall, and extracellular fractions, as described previously (5). Overlays of strain 523 could be hydrolyzed by MHs from any fraction of any of the S. aureus test strains (data not shown). In contrast, overlays of the Triton X-100-resistant S. aureus strains 523a, 523k (data not shown), IL-A, and IL-F were resistant to MHs from all fractions from strains IL-A and IL-F (Fig. 4) and all other sources we tested (data not shown). The poor activity was not due to inactive enzymes, since the MHs from the Triton X-100-resistant S. aureus isolates were highly active (IL-F more so than IL-A) when evaluated on heat-killed cells of Micrococcus luteus or S. aureus strain 523 (Fig. 5). These data demonstrate that vanco-



FIG. 5. Intracellular murein hydrolases evaluated on zymograms containing overlays of heat-killed cells from *M. luteus* (A) or *S. aureus* strain 523 (B). Lanes: 1, MH from strain IL-A; 2, MH from strain IL-F; m1, Bio-Rad Kaleidoscope prestained standards; m2, Bio-Rad High Range prestained sodium dodecyl sulfate-polyacrylamide gel electro-phoresis standard.

mycin-selected resistance to Triton X-100 in strains IL-A, IL-F, 523a, and 523k is correlated with resistance to MHs, presumably due to change(s) in the cell wall.

The data from isolates 523 to 523k prove that exposure of a vancomycin-naïve strain to low-level vancomycin in vitro was sufficient for selecting Triton X-100 resistance concurrently with decreasing susceptibility to vancomycin and that Triton resistance preceded the vancomycin-intermediate resistance phenotype. Moreover, we have now shown that four clinical GISA isolates and one vancomycin heteroresistant isolate (IL-A) all have decreased lytic capability compared with vancomycin-susceptible strains; therefore, the phenotype of Mu50 is an exception among the clinical GISA isolates we studied.

These data suggest that autolysis plays a role in vancomycinmediated killing in *S. aureus*, and that a strain with decreased autolytic capacity could evade the lysis-inducing effect of vancomycin at an early stage. Thus, we propose a model whereby acquisition of intermediate vancomycin resistance requires at least two steps. The first involves acquisition of a decrease in autolytic activity mediated by resistance of cell walls to MHs. An isolate that could avoid lysis would have a prolonged survival time and an enhanced opportunity for a second-step mutation to lead to intermediate vancomycin resistance.

Resistance to autolysis was not likely due to a defect in the enzymatic activity of ATL in clinical isolates IL-A and IL-F. Although it might be hypothesized that decreased autolysis might explain the thickened cell walls described in GISA isolates, the cell wall of strain IL-F is 1.5-fold thicker than that of strain IL-A (2) without a further increase in resistance to autolytic activity. Understanding the increase in resistance to an exogenous MH, lysostaphin, as documented in GISA strain IL-F (2), may provide insight into the mechanism by which this occurs.

Several regulators of autolysin activity have been identified in *S. aureus* (4, 11–13). Although strains IL-A and IL-F were resistant to lysis, the MH activity was higher in strain IL-F than in strain IL-A. Since this increase was not explained by a change in the sequence of ATL, it will be interesting to learn if any of the regulators of autolysin activity were involved in that change.

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