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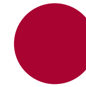

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RESEARCH ARTICLE

Reversible Antibiotic Tolerance Induced in *Staphylococcus aureus* by Concurrent Drug Exposure

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ABSTRACT **Resistance of *Staphylococcus aureus* to beta-lactam antibiotics has led to increasing use of the glycopeptide antibiotic vancomycin as a life-saving treatment for major *S. aureus* infections. Coinfection by an unrelated bacterial species may necessitate concurrent treatment with a second antibiotic that targets the coinfecting pathogen. While investigating factors that affect bacterial antibiotic sensitivity, we discovered that susceptibility of *S. aureus* to vancomycin is reduced by concurrent exposure to colistin, a cationic peptide antimicrobial employed to treat infections by Gram-negative pathogens. We show that colistin-induced vancomycin tolerance persists only as long as the inducer is present and is accompanied by gene expression changes similar to those resulting from mutations that produce stably inherited reduction of vancomycin sensitivity (vancomycin-intermediate *S. aureus* [VISA] strains). As colistin-induced vancomycin tolerance is reversible, it may not be detected by routine sensitivity testing and may be responsible for treatment failure at vancomycin doses expected to be clinically effective based on such routine testing.**

IMPORTANCE **Commonly, antibiotic resistance is associated with permanent genetic changes, such as point mutations or acquisition of resistance genes. We show that phenotypic resistance can arise where changes in gene expression result in tolerance to an antibiotic without any accompanying genetic changes. Specifically, methicillin-resistant *Staphylococcus aureus* (MRSA) behaves like vancomycin-intermediate *S. aureus* (VISA) upon exposure to colistin, which is currently used against infections by Gram-negative bacteria. Vancomycin is a last-resort drug for treatment of serious *S. aureus* infections, and VISA is associated with poor clinical prognosis. Phenotypic and reversible resistance will not be revealed by standard susceptibility testing and may underlie treatment failure.**

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Resistance to antimicrobial agents is now recognized as the principal challenge to efforts to control infectious diseases (1). The bulk of such resistance is attributable to mutations that heritably alter microbial sensitivity to therapeutic agents and which can be transmitted linearly, and in some instances also laterally, among populations of pathogens (2). Additionally, genes that inactivate antibiotics or otherwise affect their actions can be transmitted among microbes and can remain silent until they encounter a signal generated by the cognate antibiotic (for a recent review, see reference 3).

Staphylococcus aureus is a Gram-positive bacterial pathogen that colonizes 30% of healthy individuals and yet gives rise to a wide variety of severe infections (4, 5). The epidemic spread of *S. aureus* strains resistant to penicillins and other beta-lactam antibiotics is a major threat (6) that is made more severe by the increasingly frequent occurrence of heritable decreased susceptibility of these bacteria to the glycopeptide antibiotic vancomycin, commonly considered an antimicrobial agent of "last resort" for treatment of *S. aureus* infections (7). Here, we report the existence of an additional and previously unrecognized threat to vancomycin efficacy: the induction of reversible vancomycin tolerance by an antibiotic that may be administered for treatment of a coexisting infection by an unrelated microbe. When investigating factors that may affect *S. aureus* sensitivity to vancomycin, we discovered that the bactericidal effects of this agent are reduced by concurrent exposure to colistin, a cyclic polypeptide antibiotic used to treat a variety of infections by Gram-negative bacteria. We demonstrate that colistin-induced vancomycin tolerance persists only so long as colistin is present and that induced vancomycin tolerance is accompanied by altered gene expression and other phenotypic properties characteristic of genetically stable VISA (vancomycin-intermediate *S. aureus*) strains, which also show decreased sensitivity to vancomycin. As colistin-induced resistance to vancomycin is transient, it is not detectable by routinely used antimicrobial susceptibility test procedures, and thus may account for treatment failures observed in patients receiving vancomycin doses that are expected on the basis of such testing to be clinically effective.

RESULTS

Screening for inducible antibiotic resistance. During a screen for functional interactions between antibiotic combinations in a community-associated methicillin-resistant *S. aureus* (CA-MRSA)

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TABLE 1 Screening of antibiotics that affect growth in the presence of inhibitory concentrations of other antibiotics

Test compound	Effect on growth after preexposure to inducer compound (target)[a]				
	Colistin (membrane)	Vancomycin (cell wall)	Teicoplanin (cell wall)	Rifampin (DNA/ RNA synthesis)	Gentamicin (protein synthesis)
Colistin	+	+	+	−	−
Vancomycin	−	−	−	−	−
Rifampin	−	+	−	+	−
Ciprofloxacin	−	−	ND	+	−
Tetracycline	ND	−	−	−	−

[a] +, cells grew in the presence of a restrictive concentration of the tester antibiotic after preexposure to the inducer antibiotic; −, no increased tolerance was observed with preexposure to the inducer compound; ND, not determined.

strain, USA300 (Table 1), we observed that if cells were exposed to colistin for 30 min, they were able to form colonies on otherwise-restrictive concentrations of vancomycin (Fig. 1a). When testing the ability of colistin to induce resistance against other cell wall-active agents, we found that resistance was induced against the glycopeptide teicoplanin which, like vancomycin, prevents the synthesis of *N*-acetylmuramic acid and *N*-acetylglucosamine polymers, which form the backbone of the *S. aureus* cell wall (8). In contrast, no resistance was induced against the membrane-active daptomycin (Fig. 1a). Our screen also suggested other possible instances of antibiotic cross-tolerance; however, these were not studied further in the investigation reported here (Fig. 1b; Table 1).

Colistin induces reversible vancomycin tolerance. We found that exposure of USA300 to colistin for as little as 7 min resulted in a 50% increase in the vancomycin MIC (from 1.1 μg/ml to 1.6 μg/ml) (Fig. 2a). The colistin-induced elevation of the vancomycin MIC resulted in a 5-fold increase in bacterial CFU at a vancomycin concentration (i.e., 1.5 μg/ml) that normally inhibits colony formation (Fig. 2b); colistin itself had no effect on reproduction of USA300 at this concentration (Fig. 2c). In both liquid and solid media, colistin-induced tolerance of *S. aureus* to a normally inhibitory concentration of vancomycin required exposure to the inducer prior to vancomycin treatment (Fig. 2a and d). Importantly, vancomycin tolerance was dependent on the continued presence of colistin and was reversed by culturing bacteria in colistin-free medium for just 30 min prior to exposing them to vancomycin (Fig. 2e).

Colistin-induced gene expression resembles gene expression in genetically stable VISA strains. To further investigate the mechanism underlying the colistin-induced reversible tolerance of *S. aureus* to vancomycin, we first used DNA microarray analysis to profile the effects of colistin on individual mRNAs. As phenotypic effects on vancomycin sensitivity were evident after only 10 min of prior exposure to colistin (Fig. 2), alterations in mRNA abundance were investigated at this time point. Using DNA probes for 2,572 *S. aureus* genes, we identified 27 genes that were significantly ($P < 0.05$) upregulated by colistin exposure (Table 2). Remarkably, among these genes were 11 (shown in boldface type in Table 2) that have been reported to be upregulated in clinically isolated VISA strains (representation factor for overlap, 21.8; $P < 1.995e{-14}$), showing stably inherited decreased susceptibility to vancomycin (MIC, 4 to 8 μg/ml) (9, 10). Twenty-two of the 27 genes are known to be components of either the VraSR (11) or the GraXRS (12) operons, both of which have been implicated in genetically inherited multidrug resistance (13, 14). Among the loci regulated by GraXRS is the *dltABCD* operon, which encodes proteins that decrease the negative charge at the bacterial cell surface and mediate the production and export of D-alanyl-D-alanine residues on staphylococcal peptidoglycans targeted by vancomycin (15).

Among the genes showing elevated expression on microarrays as a result of colistin exposure were two known to mediate alterations in *S. aureus* cell walls, *mprF* and *dltB* (16, 17). The rapidity of the onset of vancomycin tolerance to colistin exposure led us to investigate the temporal relationship between expression of these genes and such tolerance. As seen in Fig. 3, both events were tightly correlated temporally; elevation of mRNAs encoded by the *mprF* and *dtB* genes were elevated after 10 min of colistin exposure ($P = 0.00012$) and returned to preinduction levels by 30 min after colistin was removed, consistent with our finding that continuous exposure to colistin is needed for the maintenance of vancomycin tolerance (Fig. 2d). These findings suggest that expression of these two genes is useful as a rapidly assessed biomarker for colistin-induced vancomycin tolerance.

The effects of colistin on gene exposure in *S. aureus* were bidirectional. Sixty-nine of the 2,572 genes probed in our microarray assays were significantly downregulated ($P < 0.05$) after 10 min of colistin exposure (see Table S1 in the supplemental material). Among these was the *saePQRS* operon as well as the *agrB* and *agrD* genes from the *S. aureus* quorum-sensing operon. Both operons encode two-component systems that control the expression of multiple virulence factors (18–20) and, accordingly, we observed that 38% of the downregulated genes were indeed virulence genes. Downregulation of virulence genes is commonly observed in VISA strains (10, 21) and, possibly as a consequence, the virulence of these strains can be attenuated (22).

Collectively, our gene expression data indicated that exposure of USA300 to colistin induces a rapid and reversible transcriptional response that bears striking similarities to the stably inherited transcriptional changes observed in genetically mutated VISA strains (10, 23–25). These alterations in gene expression were highly specific and correlated temporally with elevated vancomycin MICs.

VISA-like phenotypes of bacteria exposed to colistin. Earlier work indicated that autolysis, which occurs in some *S. aureus* populations during normal growth in culture, is reduced in VISA strains (26), which also show both a decrease in the negative charge at the cell surface (27) and an increase in cell wall thickness (28). Exposure of non-VISA USA300 to colistin produced a decrease ($P < 0.007$) (Fig. 4a) in autolysis comparable to that seen in clinically isolated bacteria with the genetically inherited VISA phenotype (26). Additionally, colistin-treated cells showed dramatically reduced binding to cytochrome *c* (10% ± 13% versus

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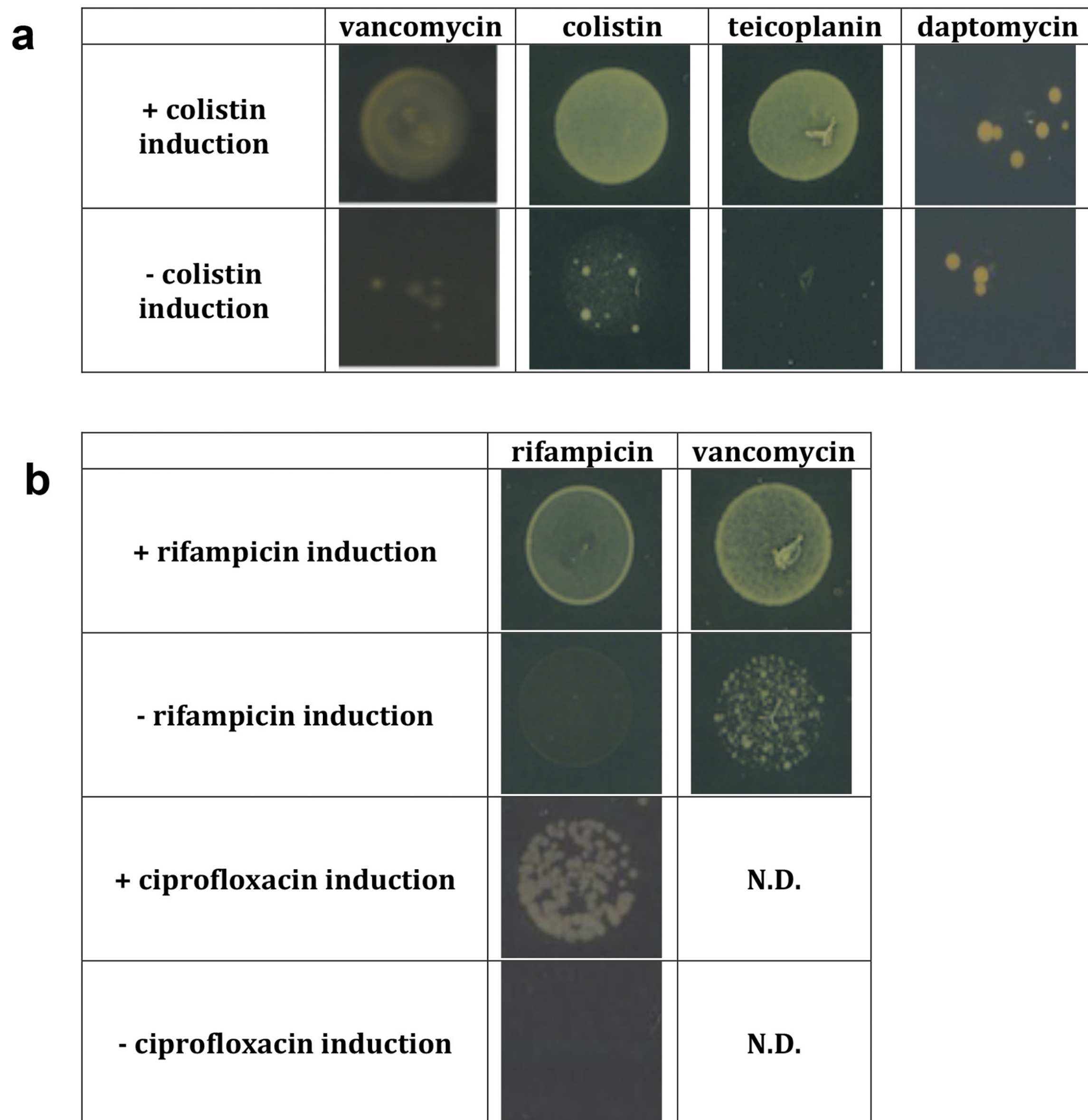

FIG 1 Mitigation of antibiotic lethality by subinhibitory antibiotic concentrations in CA-MRSA USA300. (a) The ability of USA300 to form colonies at inhibitory concentrations of the cell wall-active antibiotics vancomycin and teicoplanin as well as the cell membrane-active antibiotics colistin and daptomycin was assayed after preexposure to sublethal doses of colistin. (b) The same experiment was carried out with preexposure to the ansamycin rifampin or the fluoroquinolone ciprofloxacin, followed by plating on inhibitory concentrations of rifampin or vancomycin.

70% ± 6% in untreated cells [means ± standard deviations]; $P = 0.001$) (Fig. 4b), which has been used to assay the decrease in negative cell surface charge characteristic of VISA (27). However, cell wall thickness, which commonly is increased in VISA strains, was not altered in cells treated with colistin (22.81 nm ± 1.55 nm versus 22.45 nm ± 1.43 nm in nontreated cells) (Fig. 4c).

Vancomycin tolerance regulated by cell wall stress operons. The adjacent *graXRS* and *vraFG* operons together encode a five-component system that along with the *vraRS* two-component regulatory system is central to *S. aureus* defense against vancomycin and other agents that damage the bacterial cell wall (13, 14). The VraFG transporter senses antimicrobial peptides (13) that activate GraXRS-mediated transcription of the *dlt* operon and *mprF*, which reduce the negative cell wall charge by increasing D-alanylation of teichoic acids and increasing incorporation of cationic phospholipid lysyl-phosphatidyl glycerol into the cytoplasmic membrane, respectively (16, 17). VraRS separately impedes the actions of vancomycin by inducing genes that mediate cell wall biosynthesis and the cell's response to the stress of cell wall damage (11, 14, 29). We found that deletion of *vraR* did not significantly affect colistin-induced vancomycin tolerance. However, deletion of *graR* completely abolished such tolerance (Fig. 5), directly demonstrating that a function of this gene is necessary for colistin-mediated reduction of susceptibility to vancomycin in USA300.

Reduced expression of the quorum-sensing and virulence reg-

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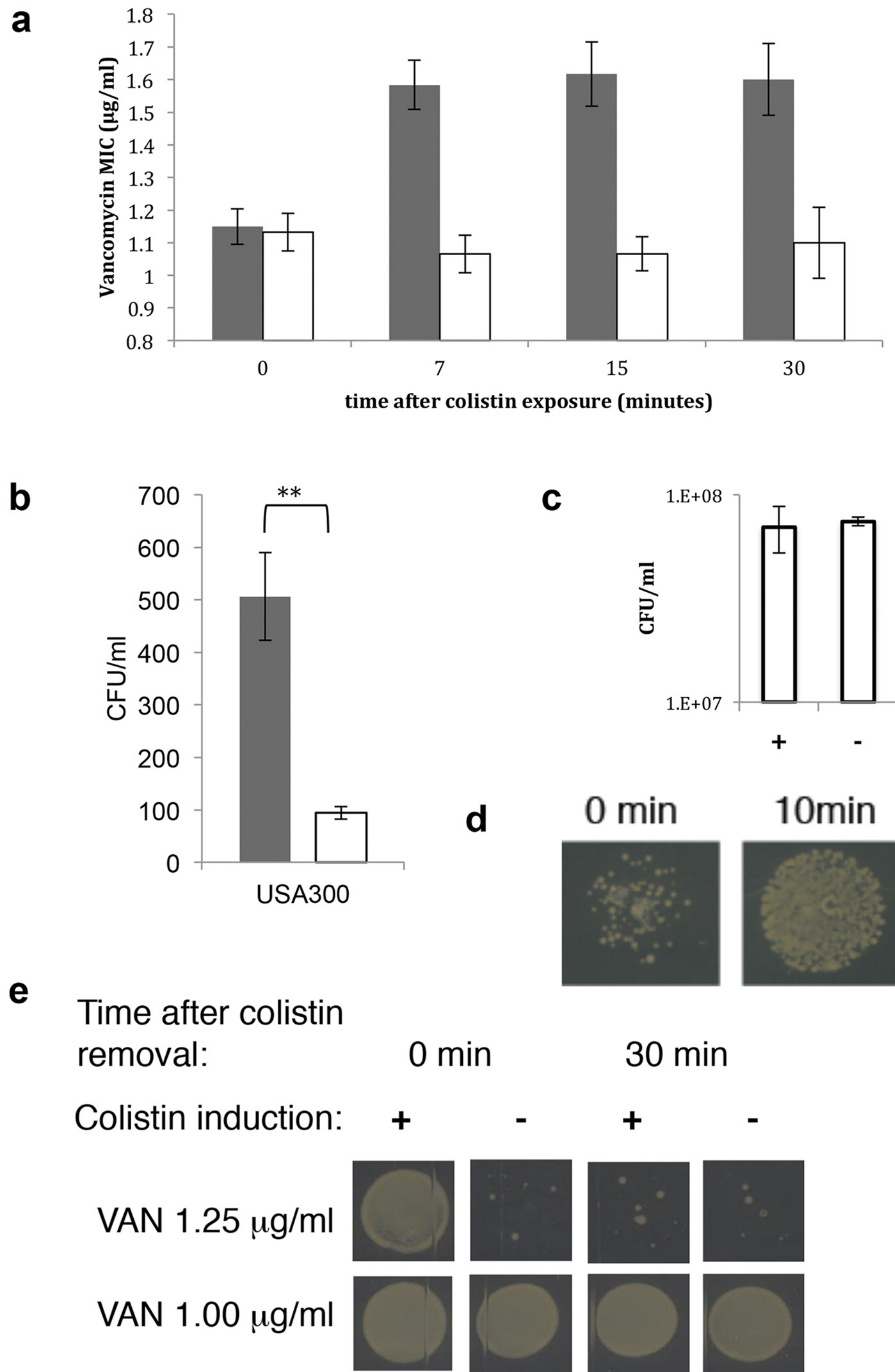

FIG 2 Colistin induces transient vancomycin tolerance in CA-MRSA USA300. (a) The MIC of vancomycin was determined in liquid medium for USA300 after treatment with colistin (shaded bars) for the indicated times and compared to results with the untreated control (no fill). (b) The colony-forming ability of USA300 on plates containing inhibitory concentrations of vancomycin was assayed for cells that had been preexposed to colistin for 30 min (shaded bars) and compared to results with untreated cells (no fill). **, $P < 0.01$, *t* test. (c) The colony-forming ability of USA300 on agar plates without antibiotics following exposure (+) or no exposure (−) to colistin for 30 min. (d) Ten-minute preexposure of USA300 to colistin in liquid culture was compared to a nonexposed control for the ability to grow on plates containing inhibitory concentrations of vancomycin. (e) USA300 was incubated with or without colistin for 30 min in liquid culture. After removal of colistin from the medium and an additional 0 min or 30 min of incubation, the cultures were spotted on agar plates containing vancomycin (VAN). All error bars represent standard deviations ($n = 3$).

ulator *agr* operon as well as that of the downstream regulon has previously been observed in VISA strains (21, 30), but no functional link has been made between *agr* and the VISA phenotype. The ability of colistin to induce vancomycin tolerance in an *agrC* deletion mutant indicated that the *agr* system is not required for the phenotype (Fig. 5).

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TABLE 2 Upregulated genes in USA300 following 10-min exposure to colistin[a]

Functional group and gene ID no.	Gene	Assignment	Fold change
Defense/stress response			
SAUSA300_0835	*dltA*	D-Alanine–poly(phosphoribitol) ligase subunit 1	3.13
SAUSA300_0836	*dltB*	DltB protein	4.12
SAUSA300_0837	*dltC*	D-Alanine–poly(phosphoribitol) ligase subunit 2	3.62
SAUSA300_0838	*dltD*	D-Alanine-activating enzyme/D-alanine-D-alanyl, DltD protein	3.86
SAUSA300_1255	***mprF***	**Oxacillin resistance-related FmtC protein**	**3.33**
SAUSA300_2573	*isaB*	Immunodominant antigen B	3.78
Gene regulation			
SAUSA300_0647	***vraF***	**ABC transporter ATP-binding protein**	**5.25**
SAUSA300_0648	***vraG***	**ABC transporter permease**	**4.71**
SAUSA300_1866	***vraS***	**Two-component sensor histidine kinase**	**1.63**
SAUSA300_1865	***vraR***	**Two-component response regulator**	**1.54**
SAUSA300_2599	*tetR*	Intercellular adhesion operon transcription regulator (IcaR)	1.67
Metabolism			
SAUSA300_0684	*fruB*	Fructose 1-phosphate kinase	3.01
SAUSA300_1640	*icd* (*citC*)	Isocitrate dehydrogenase	2.97
SAUSA300_1641	*gltA*	Citrate synthetase	3.75
SAUSA300_2319		Pyridine nucelotide-disulfide oxidoreductase	6.26
SAUSA300_2377		Glycerate kinase	3.64
Transport			
SAUSA300_1790	***prsA***	**Foldase protein PrsA**	**2.52**
SAUSA300_2630	*nixA*	High-affinity nickel transporter	1.81
SAUSA300_2409	*nikC*	Oligopeptide ABC transporter permease	1.66
SAUSA300_2411	*nikA*	Oligopeptide permease, peptide-binding protein	1.80
Miscellaneous			
SAUSA300_0964	***iraE***	**Chitinase-related protein**	**2.35**
SAUSA300_1298		Putative XpaC protein	1.64
SAUSA300_1674	*htrA*	Putative serine protease HtrA	1.78
Hypothetical genes			
SAUSA300_1606		**Hypothetical protein**	**3.50**
SAUSA300_2269		**Hypothetical protein**	**2.18**
SAUSA300_2378		**Hypothetical membrane protein**	**1.73**
SAUSA300_2493		**Hypothetical exported protein**	**7.03**

[a] $P < 0.05$ for all genes listed. Boldface indicates genes that are also upregulated in clinical VISA strains.

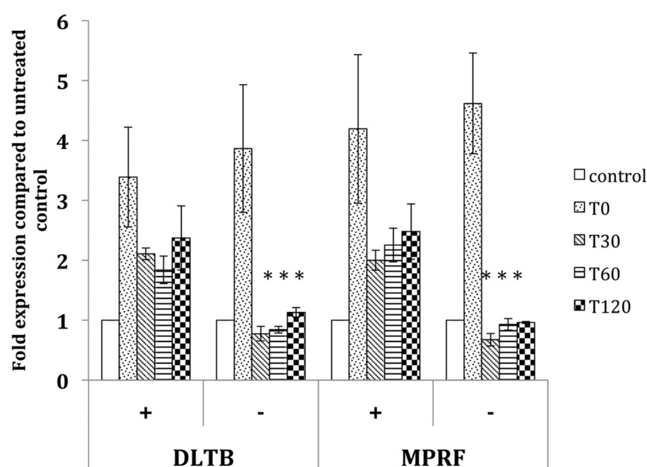

FIG 3 Continued colistin exposure is required for cell wall gene expression. After a 10-min colistin exposure (T0), USA300 cells were passaged in medium with (+) or without (−) colistin. Gene expression of *dltB* and *mprF* was investigated by Northern blotting at 0, 30, 60, and 120 min after removal of colistin from the medium. Asterisks indicate significantly different expression levels ($P < 0.01$, *t* test) between (+) and (−) cultures. Error bars represent the standard deviations ($n = 3$).

DISCUSSION

The experiments reported here show that concurrent exposure to antimicrobials used for treatment of an unrelated infection may reversibly reduce susceptibility to antimicrobial drugs in the major human pathogen *S. aureus*. In particular, exposure of *S. aureus* to colistin reduces susceptibility to vancomycin and is associated with altered gene expression and phenotypic changes, including reduced autolysis and altered cell wall surface charge, that in VISA strains lead to stably inherited decreases in susceptibility to vancomycin resulting from mutation of one or more chromosomal genes. Our results demonstrated that the colistin-induced phenotypic and temporal changes are mediated by induction of the GraRS regulon, which previously was implicated also in the VISA phenotype. In contrast to the previously studied acquired and inducible *vanA*-mediated resistance observed in enterococci, the inducible resistance we report here for *S. aureus* does not require the acquisition of genes from other bacteria.

The failure of vancomycin treatment in certain *S. aureus* infections which, based on standard susceptibility testing, should have been susceptible to the bactericidal effects of this antimicrobial (i.e., VSSA strains) has been well documented (31–40). MIC elevations comparable to those induced by colistin exposure have

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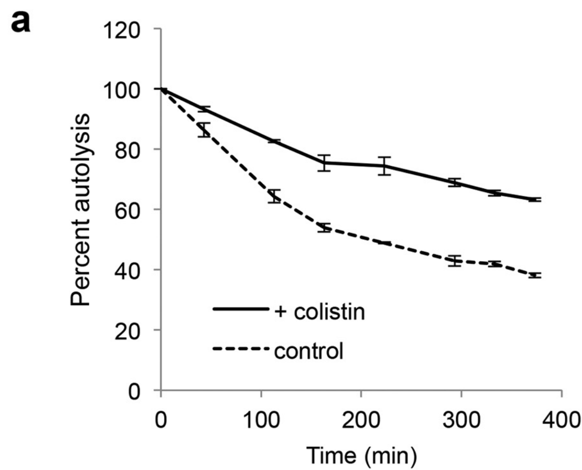

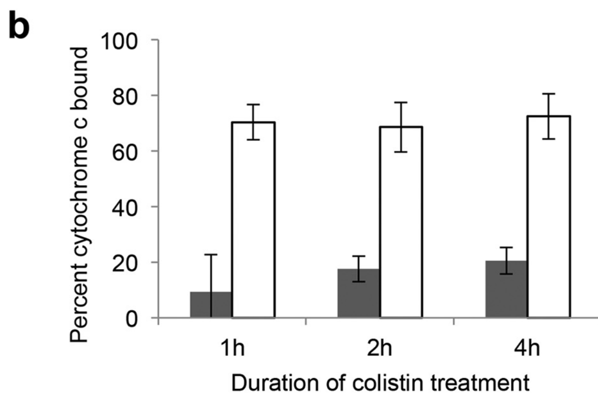

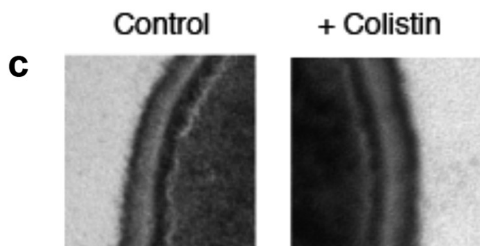

FIG 4 Colistin induces VISA phenotypes. (a) Assay for decreased autolysis. Cells were grown with or without colistin for 1 h before being washed and resuspended in buffer containing detergent. Autolysis was determined based on the decrease in optical density over time compared to that of a control culture with no added detergent ($n = 3$). (b) Assay for diminished negative cell surface charge. After growth with (shaded bars) or without (no fill) colistin exposure for various periods of time, cells were washed and resuspended in buffer containing the positively charged molecule cytochrome *c*, which binds to the negatively charged cell surface. Differences between cultures with or without colistin in terms of the amount of unbound cytochrome *c* in the supernatant after removal of cells were measured spectrophotometrically and used as an indicator for cell surface charge. More cytochrome *c* bound indicates a more negatively charged cell surface ($n = 3$). (c) Cell wall thickness. Cells were grown with or without colistin for 1 h before being harvested and prepared for TEM analysis. Inspection of 50 cells under each condition revealed no significant difference in cell wall thickness. In all cases, error bars represent the standard deviations.

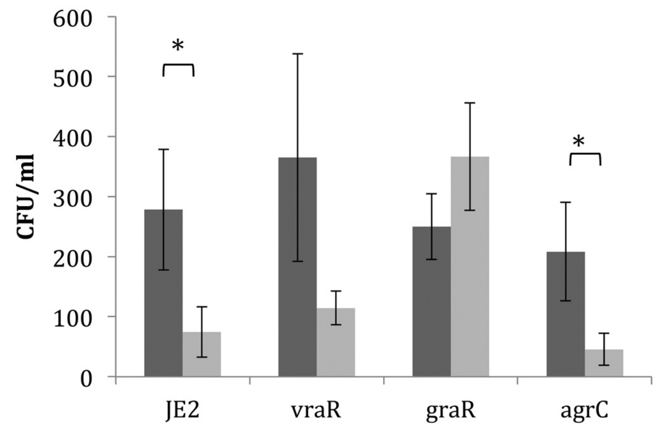

FIG 5 Regulatory two-component systems that mediate colistin-induced vancomycin tolerance. USA300 JE2 and derived mutants were grown in liquid medium with (shaded bars) and without (no fill) colistin for 30 min. Subsequently, the colony-forming ability on plates containing inhibitory concentrations of vancomycin was determined in standard plating assays. Error bars represent the standard deviations ($n = 3$). *, $P < 0.05$.

been associated with increased mortality (32, 35, 37, 41). As removal of colistin restored vancomycin sensitivity to the precolistin level, our results raise the prospect that an infecting *S. aureus* strain isolated from a patient receiving colistin may be reported as being highly sensitive (42), whereas the actual susceptibility of bacteria present in that patient may be reduced. However, the gene expression alterations we have identified provide a potential biomarker for the occurrence of reversible vancomycin resistance in uncultured bacteria isolated from humans receiving concurrently administered therapeutic agents.

Our data indicate that the inducible and reversible antibiotic tolerance may not be restricted to the colistin-vancomycin combination, as several antibiotics unrelated to colistin by class and mode of action induced reversible antibiotic tolerance toward a variety of antibiotics. While the mechanistic details of these drug interactions await further investigation, the inducible drug resistance phenotype points to a potential need to take induced and noninherited resistance into account when testing for antimicrobial susceptibility.

MATERIALS AND METHODS

Strains and growth conditions. *S. aureus* USA300 FPR3757 was obtained from the American Type Culture Collection (ATCC) and routinely grown in Mueller-Hinton (MH) medium (Sigma) at 37°C in Erlenmeyer flasks with aeration. A plasmid-cured version of USA300 Lac (JE2), and mutants in this background, Δ*graR* and Δ*vraR*, were retrieved from The Nebraska Transposon Mutant Library (http://www.narsa.net). JE2 was routinely grown in MH medium at 37°C in Erlenmeyer flasks with aeration, and the mutants were grown in the presence of 10 μg/ml erythromycin except when performing the induction experiments, in which case they were cultured as the wild type was. For colistin induction, exposure to 150 μg/ml colistin for 30 min was used unless noted otherwise.

Determination of vancomycin MICs. USA300 was grown exponentially for 3 h to mid-log phase in MH before being diluted to an optical density at 600 nm (OD_{600}) of 0.25 in MH or MH containing 150 μg/ml colistin sodium sulfate (Sigma). Immediately after mixing (at 7, 15, and 30 min), subsamples of 5e−5 CFU per ml were aliquoted into 96-well microtiter plates containing MH and vancomycin (ranging from 0.8 μg/ml to 1.8 μg/ml; Sigma) as well as 150 μg/ml colistin for the preinduced cultures. The plates were incubated at 37°C for 24 h, and growth

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was determined by using a Tecan Infinite 200 apparatus. The MIC was determined from the optical density measurements of 8 technical and 3 biological replicates.

Susceptibility screen. Exponential-phase cultures were adjusted to an OD_{600} of 0.02 in warm MH broth, and inducer antibiotics were added at sublethal concentrations (60 μg/ml colistin sodium sulfate [Sigma], 0.001 μg/ml rifampin [Sigma], or 0.2 μg/ml ciprofloxacin [Sigma]). After 90 min at 37°C and 180 rpm, 10-μl aliquots of the cultures were spotted on freshly prepared MH agar plates containing inhibitory concentrations of the tester antibiotic (1.5 μg/ml vancomycin, 360 μg/ml colistin sodium sulfate, 1.125 μg/ml teicoplanin, 2 μg/ml daptomycin, 0.009 μg/ml rifampin). The plates were incubated overnight at 37°C.

Vancomycin susceptibility testing by plating. Exponential-phase cultures were adjusted to an OD_{600} of 0.25 in warm MH broth, and 150 μg/ml colistin was added to one culture. After 30 min, serial 10-fold dilutions were prepared, and 100-μl aliquots of the appropriate dilutions were plated on freshly prepared MH agar plates containing inhibitory concentrations of vancomycin. The plates were incubated overnight at 37°C, and CFU were determined. In one experiment, the cultures were also spotted at 0 and 30 min after removing colistin from the broth by spinning (5,000 $\times$ *g*, 5 min, room temperature) and resuspending in warm MH broth.

Determination of Triton X-100-induced autolysis. The autolysis assay was performed as described previously (27). Briefly, strains were grown in MH broth at 37°C, 180 rpm, to mid-exponential phase. The cultures were diluted to an OD_{600} of 0.15 in warm MH, grown to an OD_{600} of 0.25, and 150 μg/ml colistin was added to one subset while another subset served as the nonexposed control. After 1 h, the cultured cells were washed twice in ice-cold sterile distilled water and resuspended in the same volume of 0.05 M Tris-HCl (pH 7.2) containing 0.05% Triton X-100. Cells were incubated at 30°C, and the OD_{600} was measured every 30 min. Data are expressed as the percent loss of OD_{600} at the indicated times compared to findings at time zero. Each data point represents the mean and standard deviation from three independent experiments.

TEM. For transmission electron microscopy (TEM), strains were grown in MH broth at 37°C, 180 rpm, to mid-exponential phase. The cultures were diluted to an OD_{600} of 0.15 in warm MH, grown to an OD_{600} of 0.25, and 150 μg/ml colistin was added. After 1 h the cultures were harvested by low-speed centrifugation for 5 min at 0°C. The pellets were resuspended in 10 ml of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and incubated at 5°C overnight. Again, cells were harvested by low-speed centrifugation. The pellets were resuspended in 5 ml glutaraldehyde and stored at 5°C. The samples where rinsed in 0.15 M sodium cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 0.12 M sodium cacodylate buffer for 2 h at room temperature. Then, they were dehydrated in a graded series of ethanol solutions according to standard procedures, transferred to propylene oxide, and embedded in Epon. On one-hole copper grids, ultrathin sections of 80 nm where stained with uranyl acetate and lead citrate. The samples were studied using a Philips CM 100 transmission electron microscope. The mean thickness and standard deviation of at least 50 cells from each treatment condition were determined at magnifications of 60$\times$ to 120,000$\times$ using iTEM (Olympus). The cells examined were located in different fields. All cells were intact and were visualized in full cross-section without a section artifact. Cell wall thickness was measured from the outer aspect of the cell membrane to the outer aspect of the cell wall.

Determination of whole-cell surface charges. The assay for whole-cell surface charges was performed as described previously (16, 43). Briefly, strains were grown in MH broth at 37°C, 180 rpm, to mid-exponential phase with and without exposure to colistin for 1 to 4 h. To ensure continued exponential growth during treatment, cultures were diluted in prewarmed medium with or without colistin, thereby keeping them in the log-phase state. After treatment, cells were harvested by centrifugation (800 rpm, 2 min) and washed twice with morpholinepropanesulfonic acid buffer (20 mM, pH 7). The cells were resuspended in the buffer containing 0.125 mg/ml cytochrome *c* (Sigma) to an approximate OD_{600} of 7 and incubated at room temperature. After 10 min, the suspensions were centrifuged at 8,000 rpm for 2 min. The amount of cytochrome *c* remaining in the supernatant was quantified based on the OD_{530}; this measure is negatively correlated to the negative charge of the bacterial cell surface. The data shown are the means and standard deviations from three independent experiments.

RNA isolation for DNA microarray gene expression studies. USA300 was grown exponentially for 3 h in MH before 62.5 μg/ml colistin was added at an OD_{600} of 0.25. After 10 min of exposure, 50 ml culture was harvested onto Whatman nitrocellulose filters and snap-frozen in liquid nitrogen. RNA was isolated by pulverizing the filters containing bacteria with a mortar and pestle in liquid nitrogen followed by purification using the TRIzol (Invitrogen) procedure according to the manufacturer's recommendations. Samples were DNase treated and cleaned using the RNeasy (Qiagen) protocol according to the manufacturer's recommendations. RNA quantity and quality were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

DNA microarray analysis. Approximately 10 μg of total RNA was used for the Cy3/Cy5 labeling procedure. Labeling, cDNA purification, hybridization, and washing were performed as described previously (44), except that USA300-specific DNA microarray slides were used (purchased from MYcroarray). The slides were scanned in a GenePix 4000B reader and processed using GenePix Pro software.

The slides were analyzed in R with the linear models for microarray data (LIMMA) package. Background correction was conducted using the normexp method (45), and normalization was conducted using the "normalizeWithinArrays" method (46). Log ratios (M) and log intensities (A) were calculated, and differential expression analysis was conducted using the lmFit and eBayes functions (47). Genes were ranked by their adjusted *P* values by using the topTable function. R scripts and specific commands used for the analysis are available upon request. Clustering analysis was performed by using Cluster 3.0 (48) and the hierarchical clustering of genes method, and the clusters were visualized using Java TreeView (49).

Significance levels for overlapping genes to VISA strains were determined by calculation of the representation factor using the software at http://nemates.org/MA/progs/representation.stats.html. A representation factor of $>$1 indicated more overlap than expected between two independent groups.

RNA isolation and quantification of transcript levels by Northern blotting. Exponential-phase cultures were adjusted to an OD_{600} of 0.25 in warm MH broth, and 150 μg/ml colistin was added. At different time points, cells were harvested by centrifugation at 8,000 rpm for 3 min. The cells were lysed mechanically using the FastPrep system (Bio101; Qbiogene), and total RNA was extracted using a Qiagen RNeasy minikit according to the manufacturer's instructions. RNA quantity and quality were measured on a NanoDrop ND-1000 spectrophotometer based on the absorbance (the A_{260} and the A_{260}/A_{280} and A_{260}/A_{230} ratios, respectively). Four micrograms of RNA from each preparation was loaded onto a 1% agarose gel and transferred to a positively charged nylon membrane (GE Healthcare). The hybridization was performed using gene-specific probes labeled with [^{32}P]dCTP (PerkinElmer) and Ready-to-Go DNA-labeling beads from GE Healthcare. Internal fragments of the genes were amplified using the following primers: graR-forward, TGCTGGTATTGA AGATTTCG; graR-reverse, CCTACTTTTGTTTCGATTGC; vraR-forward, GTGGATGATCATGAAATGGT; vraR-reverse, TGGAATGCA TAGATAACAGC; mprF-forward, CTGTGGTGTAATTGTTGACG; mprF-reverse, TAATTACCGCCGTACTGATT; dltB-forward, ACCAAC AGGCAATGAATATC; dltB-reverse, TAAGTGCTGTTGTGAAACCA. The PCR products were used as the templates in the labeling reactions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02268-14/-/DCSupplemental.

Table S1, PDF file, 0.3 MB.

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Mutant strains were supplied by the NARSA Strain Repository (http://www.narsa.net).

J.H., C.F., S.N.C., and H.I. conceived of the experiments. J.H. and C.F. carried out the experiments. All authors analyzed data. J.H., S.N.C., and H.I. wrote the manuscript.

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