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Vancomycin Tolerance Induced by Erythromycin but Not by Loss of *vncRS*, *vex3*, or *pep27* Function in *Streptococcus pneumoniae*

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Vancomycin-tolerant *Streptococcus pneumoniae* is a growing problem among drug-resistant human pathogens. Some vancomycin-tolerant pneumococci have been reported to carry mutations in loci encoding a two-component regulatory system designated VncRS or in a proximal ABC transporter, Vex. A model was advanced proposing that the tolerance phenotype resulted from the inability of a *vncS* mutant to respond to the Vex-transported Pep27 "death peptide" signal and dephosphorylate VncR, thereby preventing relief of repression of autolytic and other cell death functions in response to antibiotics. To explore this hypothesis, we constructed mutations in *vncS*, *vncR*, *vex3*, and *pep27* in *S. pneumoniae* strain R6 and two additional genetic backgrounds. The lytic responses of the isogenic Δ*vncS*, Δ*vex3*, Δ*vncR*, and Δ*pep27* mutants, but not a Δ*lytA* strain, to vancomycin were indistinguishable from that of the parent strain. Δ*vncS* strains also failed to exhibit tolerance to vancomycin at various doses in multiple media and showed wild-type sensitivity to other classes of autolysis-inducing antibiotics. In contrast, addition of subinhibitory levels of the antibiotic erythromycin led to tolerance to vancomycin during late, but not early, exponential-phase growth in a Δ*vncS* strain, in the parent strain R6, and in two other strains bearing erythromycin resistance markers, namely, a Δ*vncR* strain and an unrelated Δ*comD* strain that is defective in competence-quorum sensing. Thus, this tolerance effect resulted from changes in cell growth or other erythromycin-dependent phenomena and not inactivation of *vncS* per se. Consistent with these results, and in contrast to a previous report, we found that a synthetic form of Pep27 did not elicit lytic or nonlytic killing of pneumococci. Finally, microarray transcriptional analysis and β-galactosidase reporter assays revealed VncS-dependent regulation of the *vex123* gene cluster but did not support a role for VncRS in the regulation of autolytic or other putative cell death loci. Based on these findings, we propose that vancomycin tolerance in *S. pneumoniae* does not result from loss of *vncS* function alone.

Antimicrobial tolerance (43) is defined as the capacity of bacteria to survive, but not grow, during antibiotic therapy (22). Tolerant bacteria cease active growth and are not killed by normally lethal doses of a given antibiotic, yet the MICs for tolerant bacteria remain identical to those seen for their nontolerant counterparts. This mechanism for persistence in the presence of antibiotics is distinct from that of antimicrobial resistance, in which bacteria are insensitive to the antibiotic and continue to multiply in the presence of the inhibitor (22). Given the insidious nature of tolerant bacteria, it is thought that replication of these organisms will renew once the drug is removed. In this way, tolerance to antimicrobial agents may promote the failure of antibiotic chemotherapy and may lead to the acquisition of bona fide drug resistance (23, 29, 43).

The gram-positive diplococcus *Streptococcus pneumoniae* is a common cause of bacterial invasive disease and is a leading cause of morbidity and mortality, especially among the elderly and the very young (13). The incidence of antibiotic resistance in pneumococci has increased dramatically in the past decade, and multiple-antibiotic-resistant strains are now being reported worldwide (13, 42). As a result, vancomycin is now recommended for initial treatment of pneumococcal meningitis in children (33). Thus, the recent recovery of clinical isolates of *S. pneumoniae* exhibiting tolerance to vancomycin (1, 10, 20, 22, 44) is of utmost concern, as this may be indicative of selective pressure for the emergence of true vancomycin resistance in this devastating pathogen.

The emergence of drug tolerance in *S. pneumoniae* has been proposed to result from mutations in several distinct genetic loci (4, 26–29, 46), although more recent evidence has called into question the role that certain gene products (i.e., ZmpB, PsaA, and ClpC) play in tolerance to antibiotic challenge (3, 5, 7, 36). Some tolerant laboratory strains (29), as well as a handful of clinical isolates (10), have been reported to carry mutations in a single locus encoding a two-component regulatory system (TCS) designated *vncRS*. TCSs are employed by bacteria to sense and respond to environmental stimuli and typically consist of a membrane-bound histidine protein kinase (e.g., VncS) and a cytoplasmic effector termed a response regulator (e.g., VncR) (reviewed in reference 38). Genomic sequence analysis has revealed the presence of *vncRS*, 12 additional TCSs, and one orphan response regulator in multiple strains of *S. pneumoniae* (12, 41). The functions of most of these TCSs in pneumococci are largely unknown. It was recently reported that SPSJ01, an R6 pneumococcal strain with an insertion of the vector pJDC9 into *vncS*, is tolerant to vancomycin as well as to beta-lactams, cephalosporins, aminoglycosides, and quinolones (29). A later study suggested that a secreted peptide, Pep27, encoded upstream of the *vncRS* locus,

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was the effector molecule sensed by VncS to trigger the cell death pathway (28). Together, these studies led to a model for Pep27-VncRS signal transduction (28). According to the model described previously (28), phosphorylated VncR functions as a repressor of autolytic and other cell death functions under normal growth conditions. As the cells enter stationary phase or following drug treatment, Pep27 is secreted through the Vex ABC transporter and accumulates in the medium. Accumulated Pep27 is then sensed by VncS, converting this histidine protein kinase into a phosphatase that dephosphorylates phosphorylated VncR, allowing derepression of autolytic pathways and resulting in the loss of viability and lysis of pneumococcal cells.

Because TCSs are thought to represent potential targets for broad-spectrum antibiotics (19), we further evaluated the role of *vncS* in antibiotic tolerance in *S. pneumoniae* and tested the proposed Pep27-VncRS signal transduction model. We constructed several defined *vncS* mutations in multiple genetic backgrounds and evaluated antibiotic tolerance in the presence of multiple classes of antibiotics and under different culture conditions. In addition, we constructed defined mutations in genes encoding other reported components of this signal transduction pathway, including the ABC transporter *vex3*, the response regulator *vncR*, the major autolysin *lytA*, and the peptide effector *pep27*. Herein, we report the effects of these mutations on the drug tolerance of *S. pneumoniae* and describe microarray transcriptional profiling and β-galactosidase reporter assays of some of these strains as a first step in understanding the genetic pathways regulated by the VncRS-Pep27 signal transduction system. Our results show that tolerance to vancomycin is induced following treatment of pneumococci with erythromycin but that inactivation of VncRS function is not required for this effect.

MATERIALS AND METHODS

Bacterial strains, growth, and transformation conditions. Bacterial strains employed in this study were derived from *S. pneumoniae* CP1250 (17), D39, and the unencapsulated D39 derivative R6 (12, 37) (Table 1). R6 and D39 were assigned unique strain designations to track isogenic derivatives. Bacteria were cultivated statically in either brain heart infusion broth (BHI; Becton Dickinson BBL), a semisynthetic casein acid hydrolysate medium supplemented with 0.5% (wt/vol) yeast extract (C+Y broth) (14), Todd-Hewitt broth (Difco) supplemented with 0.5% (wt/vol) yeast extract (THB-Y), or chemically defined medium (CDM) (36, 40), as indicated. For enumeration of bacteria and for transformation recovery, bacteria were plated on Trypticase soy agar II blood agar plates (TSAII BA; Becton Dickinson BBL) or on CAT agar (24) at 37°C in an atmosphere of 5% CO_2.

For transformation of *S. pneumoniae*, cells were grown to an optical density at 620 nm (OD_{620}) of ≤0.1, diluted 1:10 in BHI containing 10% heat-inactivated horse serum (Sigma), 10 mM glucose, and 100 ng of synthetic competence stimulatory peptide 1 per ml, and incubated for 15 min at 37°C in an atmosphere of 5% CO_2 (17). Following the addition of 0.2 to 1.0 μg of DNA per ml, the cells were allowed to incubate for an additional 1.5 h to allow phenotypic expression of antibiotic resistance markers. Transformants were recovered on TSAII BA containing appropriate antibiotics in nutrient broth soft agar overlays (0.8% [wt/vol] Bacto Nutrient Broth, 0.4% [wt/vol] Bacto Agar) or by using the double-overlay method (30) at 37°C in an atmosphere of 5% CO_2. For antibiotic selection, media were supplemented with 200 μg of kanamycin per ml, 100 μg of spectinomycin per ml, 2.5 μg of chloramphenicol per ml, or 0.3 or 1.0 μg of erythromycin per ml, as indicated. To test the effect of erythromycin on vancomycin tolerance, media were supplemented with 1.0 μg of erythromycin per ml for strains carrying an *ermAM* (erythromycin resistance) marker and 0.002 and 0.004 μg of erythromycin per ml for R6. In other instances, media were not routinely supplemented with antibiotics.

Construction of insertion-duplication and deletion-replacement mutants of *S. pneumoniae*. Insertion-duplication mutants were constructed by using suicide vector pCZA342 (2), and deletion-replacement mutants were constructed by using synthetic amplicons generated by overlap extension PCR (15) or ligation of *Bam*HI- and *Apa*I-restricted PCR fragments (see below and Table 1). For insertion-duplication mutagenesis, a 495-bp 5′ internal fragment of *vncS*, spanning nucleotides 131 to 626 of the 1,329-bp *vncS* open reading frame (ORF), was inserted into the *Bam*HI site of vector pCZA342 (Fig. 1 and Table 1). For deletion-replacement mutagenesis, each amplicon was designed such that all or most of the target gene was deleted and replaced with the spectinomycin resistance marker *aad9* (16), the erythromycin resistance marker *ermAM* (18), or derivatives of the erythromycin and kanamycin resistance genes under control of a robust synthetic P_c promoter to give P_c::*ermAM* (6) and P_c::*aphIII* (39), respectively (Fig. 1 and Table 1). Methods for amplicon generation by overlap extension PCR (15) were as described previously (36). The *vncS*(Δ78–1224)::P_c::*aphIII* linear-deletion amplicon was generated by incorporating unique heterologous *Bam*HI or *Apa*I restriction sites into the oligonucleotide primers used to generate the flanking DNA fragments (Table 2). Following restriction with *Bam*HI and *Apa*I, these fragments were ligated to a similarly digested PCR-amplified kanamycin resistance cassette (39). The resultant linear-deletion amplicons or the insertion-duplication vector was used to transform *S. pneumoniae* as described above. All mutations were confirmed by PCR analysis of genomic DNA based on diagnostic changes in DNA fragment size following recombination of plasmids or amplicons or by the use of primer sets specific for the mutagenesis cassette and flanking DNA sequences.

Vancomycin-induced autolysis. The methods and materials for measuring the sensitivity of *S. pneumoniae* to the cell wall synthesis inhibitor vancomycin were those described previously (29), unless otherwise stated in the text. Briefly, cells were inoculated directly from frozen glycerol stocks into 5 ml of fresh C+Y broth and serially diluted in C+Y broth for static overnight growth at 37°C in an atmosphere of 5% CO_2. Cultures with equivalent OD_{620}s from the dilution series were then diluted in fresh C+Y broth to an OD_{620} of 0.02 (~5×10^6 CFU per ml) and incubated at 37°C in an atmosphere of 5% CO_2 until the OD_{620} reached 0.15 to 0.20 by direct measurement in 1.4-cm-path-length tubes. This absorbance corresponds to 0.25 to 0.30 when converted to the standard 1-cm path length, as was reported previously (29). Vancomycin was added at 5 μg per ml (10 times the MIC), and incubation of cultures was continued. At various times after the addition of vancomycin, cell death and autolysis were measured by viable cell counts and direct measurement of the change in the OD_{620}, respectively. For viable cell counts, samples of cultures were aseptically removed and serially diluted in C+Y broth. Portions of the diluted cultures were immediately spread onto TSAII BA, which were incubated overnight at 37°C in an atmosphere of 5% CO_2. Bacterial colonies were enumerated the following day. The OD_{620} was determined with a Spectronic 20 Genesys spectrometer (Spectronic Instruments, Rochester, N.Y.) fitted for direct measurement of 1.4-cm capped tubes.

Nonionic detergent-induced autolysis. Cells grown in BHI as described above were diluted in 8 ml of fresh BHI to an OD_{620} of ~0.02 (~4×10^6 CFU per ml). These cells were incubated at 37°C in an atmosphere of 5% CO_2 until the OD_{620} reached ~0.13 (~2×10^7 CFU per ml). Each culture was divided into prewarmed tubes and mixed with 0.4% (wt/vol) deoxycholate to a final concentration of 0.05% or with an equal volume of water. Cultures were returned to 37°C, and growth or autolysis was measured as changes in OD_{620} over time. An R6 *lytA* deletion mutant (EL556) (Table 1), which lacks the major autolysin of *S. pneumoniae*, was employed as a control for deoxycholate-induced autolysis.

In vivo assay for vancomycin tolerance. Experiments with animals were conducted strictly according to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (25). Vancomycin tolerance was monitored in immunosuppressed outbred ICR mice in a thigh model of infection (45). Briefly, neutropenia was induced in female ICR mice (Harlan Sprague Dawley), each weighing ~20 g, by intraperitoneal injection with 150 and 100 mg cyclophosphamide per kg of body weight at 4 days and 1 day prior to infection, respectively. *S. pneumoniae* D39 and derivatives were harvested from TSAII BA after overnight incubation, diluted in sterile 0.9% (wt/vol) NaCl (saline) to yield an OD_{580} of ~0.3, and then further diluted 1:100 in BHI to yield ~10^6 CFU per ml. Following brief isoflurane anesthetization, mice were infected by intramuscular inoculation of 0.1 ml of bacterial suspension into each thigh. One mouse from each group was immediately sacrificed by CO_2 asphyxiation, and the mean infecting dose per thigh was determined (see below). Of the remaining animals, half were treated by subcutaneous injection with 10 mg of vancomycin per kg of body weight at 1 and 5 h postinfection. At 8 and 24 h postinfection, animals were sacrificed by CO_2 asphyxiation and the thighs were removed and homogenized in 9 ml of saline. Homogenized tissues were serially diluted in saline and immediately plated onto TSAII BA.

TABLE 1. Bacterial strains, DNA amplicons, and plasmids used in strain construction

Bacterial strain, amplicon, or plasmid	Relevant characteristics[a]	Genotype and/or phenotype[b]	Source or reference
Bacterial strains			
CP1250	*hex malM511 str-1 bgl-1*	Derivative of strain Rx	17
CP1257	CP1250 Δ*vncS*41::P_c::*ermAM* Er^r	CP1250 transformed with linear *vncS*(Δ41–729)::P_c::*ermAM* amplicon	This study
CP1260	Avirulent, unencapsulated	R6 parent[c], derivative of D39	J.-P. Claverys
CP1261	CP1260 Δ*vncS*41::P_c::*ermAM* Er^r	CP1260 transformed with linear *vncS* (Δ41–729)::P_c::*ermAM* amplicon	This study
CP1292	CP1250 Δ*vncS*78::P_c::*aphIII* Km^r	CP1250 transformed with linear *vncS* (Δ78–1224)::P_c::*aphIII* amplicon	This study
EL23	EL59 *vncS*::(pCZA342)::*vncS* Er^r	EL59 transformed with *vncS*::(pCZA342)::*vncS*	This study
EL59	Avirulent, unencapsulated	R6 parent,[d] derivative of D39	A. Tomasz; 12
EL117	EL59 Δ*comD*::*ermAM* Er^r	EL59 transformed with linear *comD*(Δ325–1014)::*ermAM* amplicon	This study
EL121	EL59 Δ*vncS*273::*ermAM* Er^r	EL59 transformed with linear *vncS* (Δ273–1021)::*ermAM* amplicon	This study
EL161	Virulent, encapsulated type 2	D39 parent, subclone of clinical isolate	J. Yother; 37
EL381	EL161 Δ*vncS*273::*aad9* Sp^r	EL161 transformed with linear *vncS* (Δ273–1021)::*aad9* amplicon	This study
EL395	EL161 Δ*vncR*::*aad9* Sp^r	EL161 transformed with linear *vncR*(Δ147–511)::*aad9* amplicon	This study
EL555	EL59 Δ*bgaA*::*ermAM* Er^r	EL59 transformed with linear Δ*bgaA*::*ermAM*	36
EL556	EL59 Δ*lytA*::*aad9* Sp^r	EL59 transformed with linear Δ*lytA*::*aad9*	36
EL562	CP1250 Δ*vncS*273::*ermAM* Er^r	CP1250 transformed with linear *vncS* (Δ273–1021)::*ermAM* amplicon	This study
EL822	EL59 Δ*vex3*::*ermAM* Er^r	EL59 transformed with linear *vex3*(Δ1–1377)::*ermAM* amplicon	This study
EL842	EL59 Δ*pep27*::*ermAM* Er^r	EL59 transformed with linear *pep27* (Δ1–81)::*ermAM* amplicon	This study
EL898	EL59 Δ*vnc*R::*ermAM* Er^r	EL161 transformed with linear *vncR*(Δ147–511)::*ermAM* amplicon	This study
EL1066	EL555 *vex2*::(pEVP3)::*vex2* Er^r Cm^r	EL555 transformed with pEL48	
EL1069	EL1066 Δ*vncS*273::*aad9* Er^r Cm^r Sp^r	EL1066 transformed with linear *vncS*(Δ273–1021)::*aad9* amplicon	This study
Amplicons			
vncS(131–626)		EL59 genomic DNA, (PS198, PS199), partial construct internal bp 131 to 626 of 1,329-bp *vncS*, for insertion into the *Bam*HI site of pCZA342	This study
vncS273e-a		EL59 genomic DNA, (PS481-a1, PS483-a2E), partial construct	This study
S-ermAM-b		pVA838, (PS485-b1E, PS487-b2E), partial construct	This study
VncS273e-c		EL59 genomic DNA, (PS489-c1E, PS490-c2), partial construct	This study
vncS(Δ273–1021)::*ermAM*		vncS273e-a, S-ermAM-b, vncS273e-c, (PS481-a1, PS490-c2), completed construct, *ermAM* replacing bp 273 to 1021 of 1,329-bp *vncS*	This study
vncS273s-a		EL59 genomic DNA, (PS481-a1, PS482-a2SP), partial construct	This study
S-aad9-b		pDL278, (PS484-b1SP, PS486-b2SP), partial construct	This study
vncS273s-c		EL59 genomic DNA, (PS488-c1SP, vncSc2), partial construct	This study
vncS(Δ273–1021)::*aad9*		vncS273s-a, S-aad9-b, vncS273s-c, (PS481-a1, PS490-c2), completed construct, *aad9* replacing bp 273 to 1021 of 1,329-bp *vncS*	This study
vncS41-a		CP1250 genomic DNA, (UpL41, UpR41), partial construct	This study
41-Pc::ermAM-b		M13EryAD, (DAM212, DAM213), partial construct	This study
vncS41-c		CP1250 genomic DNA, (DnL41, DnR41), partial construct	This study
vncS(Δ41–729)::P_c::*ermAM*		vncS41-a, 41-Pc::ermAM-b, vncS41-c, (UpL41, DnR41) completed construct, Pc::*ermAM* replacing bp 41 to 729 of the 1,329-bp *vncS*	This study
vncS78-a		CP1250 genomic DNA, (UpL78, UpR78), partial construct	This study
78-Pc::*aphIII*-b		pR410, (DAM301, DAM302), partial construct	This study
vncS78-c		CP1250 genomic DNA, (DnL78, DnR78), partial construct	This study
vncS(Δ78–1224)::P_c ::*aphIII*		Ligation of vncS78-a (*Bam*HI), 78-Pc::*aphIII*-b(*Bam*HI, *Apa*l) vncS78-c (*Apa*I), completed construct, Pc::*aphIII* replacing bp 78 to 1224 of the 1,329-bp *vncS* by ligation of restricted DNA fragments	This study
vncRs-a		EL59 genomic DNA, (PS291-a1, PS292a-a2SP), partial construct	This study
R-aad9-b		pDL278, (PS293a-b1SP, PS294a-b2SP), partial construct	This study
vncRs-c		EL59 genomic DNA, (PS295a-c1SP, PS296-c2), partial construct	This study
vncR(Δ147–511)::*aad9*		vncRs-a, R-aad9-b, vncRs-c, (PS291-a1, PS296-c2), completed construct, *aad9* replacing bp 147 to 511 of the 657-bp *vncR*	This study
vncRe-a		EL59 genomic DNA, (PS291-a1, PS292b-a2E), partial construct	This study
R-ermAM-b		pVA838, (PS293b-b1E, PS294b-b2E), partial construct	This study

Continued on following page

TABLE 1—*Continued*

Bacterial strain, amplicon, or plasmid	Relevant characteristics[a]	Genotype and/or phenotype[b]	Source or reference
vncRe-c		EL59 genomic DNA, (PS295b-c1E, PS296-c2), partial construct	This study
vncR(Δ147–511)::*ermAM*		vncRc-a, R-ermAM-b, vncRe-c, (PS291-a1, PS296-c2), completed construct, *ermAM* replacing bp 147 to 511 of the 657-bp *vncR*	This study
vex3-a		EL59 genomic DNA, (JZ109-a1, JZ 110-a2), partial construct	This study
X-ermAM-b		pVA838, (JZ111-b1er, JZ112-b2er), partial construct	This study
vex3-c		EL59 genomic DNA, (JZ113-c1, JZ114-c2), partial construct	This study
vex3(Δ1–1377)::*ermAM*		vex3-a, X-ermAM-b, vex3-c, (JZ109-a1, JZ114-c2), completed construct, *ermAM* replacing bp 1 to 1377 of the 1,377-bp *vex3*	This study
pep27-a		EL59 genomic DNA, (JZ115-a1, JZ116-a2), partial construct	This study
27-ermAM-b		pVA838, (JZ117-b1er, JZ118-b2er), partial construct	This study
pep27-c		EL59 genomic DNA, (JZ119-c1, JZ120-c2), partial construct	This study
pep27(Δ1–81)::*ermAM*		pep27-a, 27-ermAM-b, pep27-c, (JZ115-a1, JZ120-c2), completed construct, *ermAM* transcribed in the opposite orientation and replacing bp 1 to 81 of the 81-bp *pep27*	This study
comD-a		EL59 genomic DNA, (PS431-a1, PS433-a2E), partial construct	This study
D-ermAM-b		pVA838, (PS435-b1eE, PS437-b2E), partial construct	This study
comD-c		EL59 genomic DNA, (PS439-c1E, PS440-c2), partial construct	This study
comD(Δ325-1014)::*ermAM*		comD-a, D-ermAM-b, comD-c, (PS431-a1, PS440-c2), completed construct, *ermAM* replacing bp 325 to 1014 of the 1,323-bp *comD*	This study
Plasmids			
pEVP3		Insertion duplication *lacZ* reporter plasmid, Cm^r	6
pCZA342		Cloning vector for insertion duplication, Em^r Am^r	2
pGD024		*Bam*HI restricted vncS(131–626) (above), ligated to *Bam*HI pCZA342, Er^r	This study
pEL48		PCR blunt, *Bam*HI restricted vex3-a (above), ligated to *Sma*I, *Bam*HI pEVP3, Cm^r	This study

[a] Er^r, resistant to erythromycin; Km^r, resistant to kanamycin; Sp^r, resistant to spectinomycin; Cm^r, resistant to chloramphenicol; and Am^r, resistant to apramycin. Strain construction was carried out by transformation of the indicated recipient with a linear double-stranded synthetic PCR amplicon or circular plasmid DNA, as indicated.

[b] For amplicons, the source of template DNA is indicated followed in parentheses by the primer pair sets used to generate the given amplicon.

[c] R6 isolate R800 from J. Claverys.

[d] R6 isolate ATCC BAA 255, laboratory stock.

Bacterial colonies were enumerated following overnight incubation at 37°C in an atmosphere of 5% CO_2.

Synthesis of Pep27. Synthetic peptides corresponding to either the predicted (MRKEFHNVLSSDQLLTDKRPARDYNRK; Lilly-Pep27) (12) or published (MRKEFHNVLSSGQLLADKRPARDYNRK; Pub-Pep27) (28) sequences of Pep27 were prepared on an Applied Biosystems ABI433A peptide synthesizer with 9-fluorenylmethoxy-carbonyl amino acids. Each synthesis was started from 0.1 mmol of 9-fluorenylmethoxy-carbonyl Lys(Boc) WANG resin (Midwest Biotech) by using dicyclohexyl carbodiimide/hydroxybenzotriazole-activated single couplings (10-fold excess). The completed peptidyl resins were cleaved with 5 ml of reagent K (0.75 g of phenol, 0.5 ml of thioanisole, 0.25 ml of ethanedithiol, and 0.5 ml of water, each per 10 ml of trifluoroacetic acid [TFA]). The TFA filtrates were added to 40 ml of ethyl ether, and the precipitates were centrifuged. The pellets were washed with ether and dried in vacuo. Each crude product was dissolved in 10 ml of aqueous acetic acid and loaded onto a 2.2- by 25-cm Vydac C_{18} column. Peptide was eluted with a gradient of 15 to 35% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA on a fast protein liquid chromatograph (Pharmacia) at 4 ml per min while the UV absorption at 214 nm was monitored and 5-min fractions were collected. The appropriate fractions were combined, frozen, and lyophilized to give 38 mg of Lilly-Pep27 and 28 mg of Pub-Pep27. The peptide purities were ~90% according to high-performance liquid chromatography and were verified by mass spectrometry by using the electrospray source of a PESciex API III mass spectrometer in the positive-ion-detection mode.

Pep27 challenge. Cells grown overnight in CDM were diluted in fresh CDM containing either 50 μM Lilly-Pep27 or 50 μM Pub-Pep27 or a volume of sterile water equivalent to that added with each peptide to yield an OD_{620} of ~0.004 (~2×10^5 CFU per ml) in a 1.4-cm path length. Cultures were placed at 37°C in an atmosphere of 5% CO_2 and monitored for changes in OD_{620} over time. To exclude the possibility that Pep27 was active only at a high cell density, a late-exponential-phase CDM culture (OD_{620}, ~0.45 in a 1.4-cm path length; ~2×10^8 CFU per ml) was split into 12 equal 0.5-ml aliquots and challenged with a 0, 0.1, 1.0, or 200 μM concentration of either Lilly-Pep27 or Pub-Pep27. Cultures were placed at 37°C in 5% CO_2, and viability was determined at 0, 60, and 240 min after peptide addition by serial dilution in saline and plating on TSAII BA.

RNA isolation and microarray analysis. Microarray transcription profiles were determined for *S. pneumoniae* EL59 (R6 parent), EL121 (R6 Δ*vncS273*::*ermAM*), EL822 (Δ*vex3*::*ermAM*), and EL898 (Δ*vncR*::*ermAM*) grown in CDM to an OD_{620} of 0.3 (mid-exponential phase; ~1×10^8 CFU per ml) and 0.7 (late-exponential phase; ~3×10^8 CFU per ml). Total RNA was extracted, purified, and labeled as described previously (36). Relative transcript amounts were estimated by hybridizing labeled RNA to low-density custom Affymetrix *S. pneumoniae* R6 microarrays covering >95% of predicted *S. pneumoniae* ORFs (36). Data were analyzed by using Affymetrix Microarray Suite version 5.0 and Data Mining Tool version 3.0. The signal log ratio algorithm (details available at the Affymetrix website) was used to estimate the total number of transcriptional changes observed between a control and an experimental microarray. For these studies, changes in the expression of an experimental array probe relative to the baseline that were greater or equal to a signal log ratio of 1 ($\log_2$ of 1 equals an ~2-fold change) and that were observed in two or more independent experiments were considered significant. For the select gene changes shown herein (i.e., *vex123*, *vncRS*, *lytA*, *lytB*, *lytC*, and *murMN*), a ratio of the average difference call, which represents the relative hybridization signal above that of the mismatch controls, was used to estimate the change in target transcript amounts between RNA from the experimental strains (i.e., *vncR*, *vncS*, or *vex3* strains) and the control strains (R6 parent), where a ratio of 1 indicated no change.

β-Galactosidase reporter assays. A transcriptional fusion between *vex2* and the *Escherichia coli lacZ* gene was generated by ligation of PCR-amplified vex3-a (Table 1) following digestion with *Bam*HI to create a 1.3-kb fragment bearing a 3′ overhang for directional cloning into the *Sma*I and *Bam*HI sites preceding *lacZ* in the pneumococcal reporter vector pEVP3 (6). The resultant pneumococcal insertion-duplication plasmid pEL48 (Table 1) was propagated in *E. coli* HB101 and introduced by transformation into *S. pneumoniae* EL555 (R6 Δ*bgaA*::*ermAM*) to yield strain EL1066 (Table 1). The Δ*vncS*273::*aad9* mutation

was introduced into this background to yield an isogenic reporter for *vex* gene expression in the absence of *vncS* (EL1069). For assay of *vex2*::*lacZ* reporter activity, bacterial cultures with an OD_{620} of ~0.6 (~5×10^8 CFU per ml) after overnight incubation in BHI at 37°C were diluted in 11 ml of BHI to yield an OD_{620} ~0.03 (~5×10^6 CFU per ml). In all cases, media were supplemented with 2.5 μg of chloramphenicol per ml to ensure maintenance of the *vex2*::(pEVP3)::*vex2* insertion-duplication. Cultures were placed at 37°C in an atmosphere of 5% CO_2, and growth was monitored by direct measurement of changes in OD_{620} in a 1.5-cm path length. At 60, 180, 270, and 400 min postinoculation, 1 ml of cell suspension was removed, lysed with 0.1% (vol/vol) Triton X-100 at 37°C for 2 min, and assayed for β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Results of β-galactosidase assays are expressed in Miller units normalized to the OD_{620} of the culture at each time point (21).

RESULTS

Addition of erythromycin, but not inactivation of *vncS*, promotes tolerance to vancomycin in *S. pneumoniae* R6. To address whether the VncS histidine protein kinase is required for tolerance to vancomycin, we constructed a defined deletion-replacement mutation in *vncS* (EL121) of *S. pneumoniae* R6 (Table 1). The *vncRS* locus from *S. pneumoniae* R6 and the site of DNA insertion or replacement in the mutants constructed in this study are schematically represented in Fig. 1. The Δ*vncS* strain (EL121) and its isogenic *vncS*$^+$ R6 parent (EL59) were tested for tolerance to 10 times the MIC of vancomycin (5 μg of vancomycin per ml) at an OD_{620} equivalent to that reported previously (29). Based on numerous repetitions of the tolerance assay, we failed to demonstrate reproducible tolerance to vancomycin for our *vncS* deletion strain (EL121) either by measuring the change in absorbance at 620 nm (Fig. 2A) or by direct measurement of cellular viability following 4 h of treatment with this cell wall biosynthesis inhibitor (Fig. 2A, inset). We also failed to demonstrate tolerance when cells were challenged with vancomycin at a significantly lower cell density (OD_{620}, 0.05 to 0.06) to reflect the actual number of viable CFU per ml (~0.5×10^7 to 1.0×10^7 CFU per ml) reported previously (29) (Fig. 2B; also data not shown). Similar results were observed for a *vncS* insertion-duplication mutant (EL23) of *S. pneumoniae* R6 (Table 1 and Fig. 1) challenged with various concentrations of vancomycin in BHI medium (data not shown). This indicates that the loss of *vncS* function alone does not confer a vancomycin tolerance phenotype to *S. pneumoniae*. In contrast, reproducible tolerance to vancomycin was observed for the Δ*vncS* strain (EL121) when erythromycin was added to the C+Y medium at ~3.3 times our normal selection dose for the erythromycin marker derived from pVA838 (1.0 versus 0.3 μg of erythromycin per ml [18]) to reflect the methods reported previously (29) (Fig. 2A). This was not due to selection for Δ*vncS* isolates that have retained their *ermAM* marker, as serial dilution and duplicate plating revealed equivalent numbers of bacteria on both selective and nonselective media and PCR analysis revealed no loss of the *ermAM* antibiotic resistance marker in EL121 (data not shown). The ability of erythromycin to promote tolerance of the Δ*vncS* strain (EL121) to vancomycin was growth stage dependent, as challenge of cells during early exponential growth (OD_{620}, 0.05 to 0.06) failed to produce a similar result (Fig. 2B). This concentration of erythromycin did, however, reduce the overall yield of the Δ*vncS* strain (EL121) and increased the doubling time by 78% (~46 min versus ~82 min) in C+Y medium, extending the time required to reach an equivalent OD_{620} by as much as 2 h (Fig. 2C).

To test whether erythromycin alone at the cell density reported previously (29) can promote a drug tolerance phenotype, the effect of high levels of erythromycin on growth and tolerance of the Δ*vncS* (EL121), Δ*vncR* (EL898), and the unrelated *vncS*$^+$ Δ*comD* (EL117) strains was evaluated. These bacteria were grown overnight in C+Y medium, diluted to an OD_{620} of 0.03 (~5×10^6 CFU per ml) in fresh C+Y or in C+Y supplemented with 1.0 μg of erythromycin per ml of medium, and compared to the untreated R6 parental control (EL59). At an OD_{620} of ~0.2 (~5×10^7 CFU per ml), cultures were challenged with 5 μg of vancomycin per ml of medium and monitored for changes in OD and viable counts through 4 h of vancomycin treatment (Fig. 3). Comparison to control cultures to which vancomycin was not added confirmed that the addition of 1.0 μg of erythromycin per ml of medium reduced the growth rate of erythromycin-resistant bacteria in C+Y medium by a factor of ~1.8 to 2.3 (data not shown). This change in growth rate coincided with a dramatic increase in tolerance to vancomycin in all strains tested, including the unrelated *vncS*$^+$ Δ*comD* strain (EL117) (Fig. 3). This indicates that the addition of high levels of erythromycin to *S. pneumoniae* expressing erythromycin resistance from the *ermAM* marker (18) can in and of itself promote a vancomycin tolerance phenotype. To determine if this effect could be reproduced in erythromycin-susceptible bacteria,

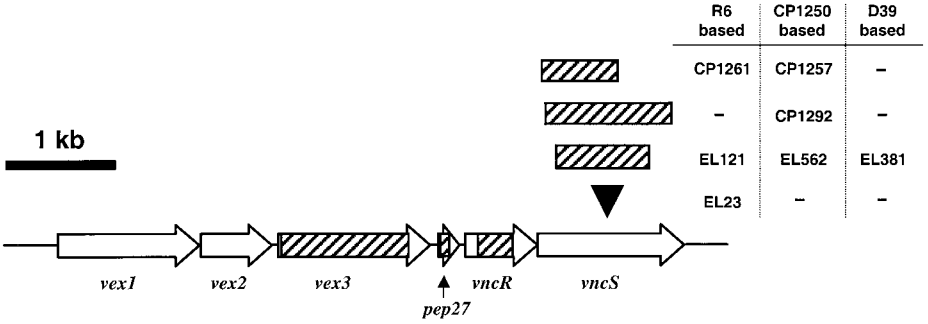


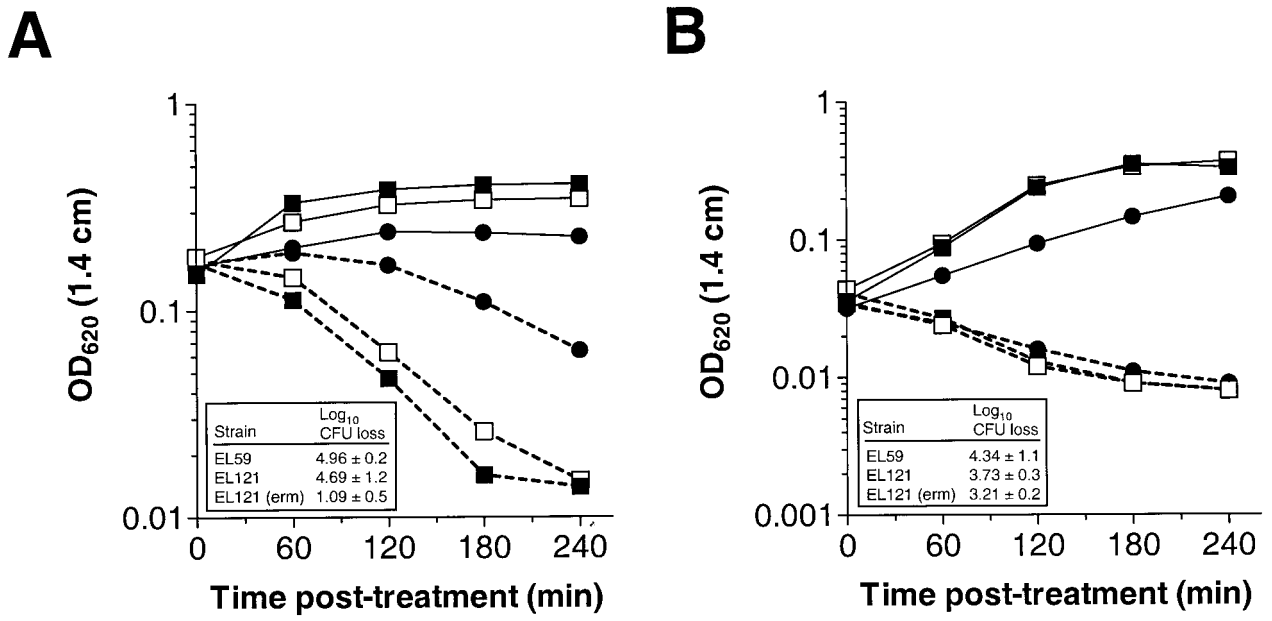
FIG. 1. Organization of the *vncRS* gene cluster in *S. pneumoniae* R6. The genes encoding the Vex1, Vex2, and Vex3 ABC transporter, the putative peptide Pep27, the VncR response regulator, and the VncS histidine protein kinase are indicated with arrows (drawn to scale). The black arrowhead indicates the site of pCZA342 insertion in EL23; the hatched areas indicate the regions of DNA that were replaced in individual deletion-replacement mutants. For *vncS* strains, the name of the corresponding mutant is adjacent to the region deleted.

TABLE 2. Synthetic oligonucleotides used in these studies

Name and description[a]	Sequence (5′→3′)[b]
Primer set used for *vncS* insertion-duplication cassette construction	
vncS::(pCZA342)::*vncS* (Em^r)	
PS198	GTCATCGTCAggatccCATTGGTCAAAAGGC
PS199	AACAGTCAAGgatccTGGTAGAGGCTATTG
Primer sets used for deletion replacement cassette construction	
vncS(Δ273–1021)::*ermAM* (Er^r) and *vncS* (Δ273–1021)::*aad9* (Sp^r)	
PS481-a1	GGCTCAAGCCTCTAACCTTGGTGGT
PS482-a2SP	CGAACGAAAATCGATGAGGAAGACTGTCCTTGAC
PS483-a2E	TTTCCATAACTTCTTGAGGAAGACTGTCCTTGAC
PS484-b1SP	GTCAAGGACAGTCTTCCTCATCGATTTTCGTTCGTGAAT
PS485-b1E	GTCAAGGACAGTCTTCCTCAAGAAGTTATGGAAA
PS486-b2SP	GGTTTAGATAAGCCTGCTGATTTAGAATGAATATTTCC
PS487-b2E	GGTTTAGATAAGCCTGCTGATTTAGCTCCTTGGAAGC
PS488-c1SP	GGAAATATTCATTCTAAATCAGCAGGCTTATCTAAACC
PS489-c1E	GCTTCCAAGGAGCTAAATCAGCAGGCTTATCTAAACC
PS490-c2	GCAGTTATGTTGTCCTGTTC
vncS(Δ41–729)::P$_c$::*ermAM* (Er^r) (CP1261)	
UpL41	AGATGAGTGCCTTTGCCTCTTTGG
UpR41	ATCAAACAAATTTTGGGCCCGGTAAACCTGTTCGTTTCATTTTCGC
DnL41	AATTCTATGAGTCGCTGCCGACTGAAAACACCGCTGGCTAGTTTGA
DnR41	TTCTCCTTCTCTTTCTCCAATTCG
DAM212	CCGGGCCCAAAATTTGTTTGAT
DAM213	AGTCGGCAGCGACTCATAGAAT
vncS(Δ78–1224)::P$_c$::*aphIII* (Km^r) (CP1292)	
UpL78	CTCTAACCTTGGTGGTGGTGCAGAAGTA
UpR78	ATggatccTGAAGGCAGATAACCAGAACACTAA
DnL78	ATgggcccTTTGTGGTTAAGAGTCTATTAGAA
DnR78	ATGTTACCGATACCAGCTGCCAAGAAGT
DAM301	CGCGCAAGCTGGggatccG
DAM302	ACGTgggcccTAGGTACTAAAACAATTCATCCAGTAA
vncR(Δ147–511)::*aad9* (Sp^r) and *vnc*R (Δ147–511)::*ermAM* (Er^r)	
PS291-a1	GAAACGCCAGAAACCAAGAAGAATC
PS292a-a2SP	CGAACGAAAATCGATGCATCTGGATATCCAG
PS292b-a2E	TTTCCATAACTTCTTGCATCTGGATATCCAG
PS293a-b1SP	CTGGATATCCAGATGCATCGATTTTCGTTCGTGAAT
PS293b-b1E	CTGGATATCCAGATGCAAGAAGTTATGGAAA
PS294a-b2SP	GAGACCGAGTCAAGGCCTTAGAATGAATATTTCC
PS294b-b2E	GAGACCGAGTCAAGGCCTTAGCTCCTTGGAAGC
PS295a-c1SP	GGAAATATTCATTCTAAGGCCTTGACTCGGTCTC
PS295b-c1E	GCTTCCAAGGAGCTAAGGCCTTGACTCGGTCTC
PS296-c2	TCCCATTTACATTGTTCATTTTATCA
vex3(Δ1–1377)::*ermAM* (Er^r)	
JZ109-a1	AATCCTTATCCACGAAGAATTGGCTA
JZ110-a2	TTTCCATAACTTCTTAGATTCCCTTTCTAGATTTTGTTTTAATC
JZ111-b1E	CTAGAAAGGGAATCTAAGAAGTTATGGAAATAAGACTTAGAAGCAAAC
JZ112-b2E	CATTTTTTCAAATTTATTAGCTCCTTGGAAGCTGTCAGTAGTATACCT
JZ113-c1	GCTTCCAAGGAGCTAATAAATTTGAAAAAATGAGTCTGGAATAAAG
JZ114-c2	TGGTTTTACGAATCTCAGCTAGAACTTC
pep27(Δ1–81)::*ermAM* (Er^r)	
JZ115-a1	ACGATGCAGATAATGAAAAAGGAGCC
JZ116-a2	CTTCCAAGGAGCTAAAAATTCCATAGCTTTAGTATATTATATTTA
JZ117-b1er	TAAAGCTATGGAATTTTTAGCTCCTTGGAAGCTGTCAGTAGTATAC
JZ118-b2er	TAAATACCTACTCTAAAGAAGTTATGGAAATAAGACTTAGAAGCA
JZ119-c1	TTTCCATAACTTCTTTAGAGTAGGTATTTATTCTAAGAAAAAT
JZ120-c2	GGCGGTCTGTGTCCAGAGGAAGACTG
comD(Δ325–1014)::*ermAM* (Er^r)	
PS431-a1	GTTGTAACAGTTGGGAAACTG
PS433-a2E	TTTCCATAACTTCTTGAGGAGAAAGTTACGG
PS435-b1eE	CCGTAACTTTCTCCTCAAGAAGTTATGGAAA
PS437-b2E	CGCTCATGATACGAACCTTAGCTCCTTGGAAGC
PS439-c1E	GCTTCCAAGGAGCTAAGGTTCGTATCATGAGCG
PS440-c2	GCAACTGAATCTAAATACCACCG

[a] Er^r, resistant to erythromycin; Sp^r, resistant to spectinomycin; and Km^r, resistant to kanamycin.

[b] Lowercase letters indicate heterologous restriction sites engineered into the oligonucleotide to facilitate ligation. Underlined portions of oligonucleotides represent short heterologous extensions that are complementary to the fragments of DNA to be linked by overlap extension PCR.



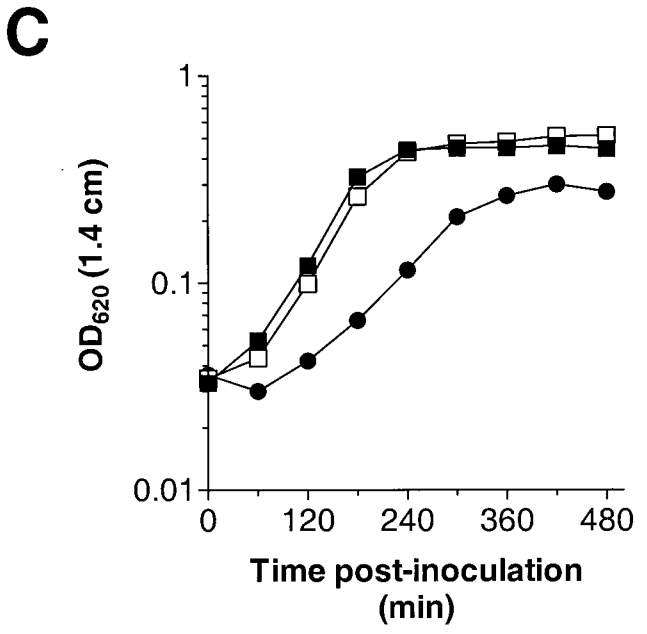


FIG. 2. (A and B) Lack of vancomycin tolerance in a defined deletion-replacement mutant of *vncS* during mid-exponential to late exponential (OD_{620}, ~0.16 to 0.2) (A) or early exponential (OD_{620}, ~0.05 to 0.06) (B) growth. Duplicate samples grown in C+Y medium without supplementation or C+Y medium supplemented with 1 μg of erythromycin per ml were left untreated (growth control) or were treated with 5 μg of vancomycin per ml (10 times the MIC). Growth or autolysis was measured as a change in OD_{620} in a 1.4-cm-path-length tube. Data are representative of at least two independent experiments. Solid lines, untreated growth control; dashed lines, treated with 5 μg of vancomycin per ml. Insets show log_{10} CFU loss (mean ± standard deviation of results from two independent experiments) following 4-h challenge with vancomycin as determined by serial dilution in C+Y medium and plating on TSAII BA. (C) Growth kinetics of the *S. pneumoniae vncS* strain altered in the presence of high levels of erythromycin. Cells were inoculated into C+Y medium with no supplementation or C+Y medium supplemented with 1 μg of erythromycin per ml, and growth was measured as changes in the OD_{620} over time. Symbols: closed squares, R6 parent strain (EL59); open squares, R6 Δ*vncS*273::*ermAM* (EL121); closed circles, R6 Δ*vncS*273::*ermAM* (EL121) supplemented with 1 μg of erythromycin per ml.

our erythromycin-sensitive *vncS*$^+$ R6 parent strain (EL59) was treated with 0.002 to 0.004 μg of erythromycin per ml of medium and assayed for phenotypic tolerance to vancomycin in C+Y medium. At the highest concentration of erythromycin, the R6 parent strain continued to grow but had a doubling time of ~1.7 times that of the untreated control (data not shown) and showed greatly reduced susceptibility to lysis in the presence of vancomycin (Fig. 4). These findings are consistent with a reduction in the sensitivity of *S. pneumoniae* to the action of the cell wall synthesis inhibitor vancomycin following changes in cell growth or other erythromycin-associated effects, but not by the loss of *vncS*. It is not yet known whether this is an erythromycin-specific effect or if other inhibitors of cell growth or protein synthesis would confer a similar result. Clearly, additional experiments are necessary to more fully address this question.

Inactivation of *vncS* in different genetic backgrounds does not result in tolerance to vancomycin or other antibiotics in vitro or in vivo. It is possible that subtle unknown genetic differences might exist between our R6 parent strain and the strain employed in a previous study (29). Thus, our inability to demonstrate vancomycin tolerance in the absence of erythromycin could be due to unknown differences between these separate clones of the common laboratory strain R6. To eliminate this possibility, we compared the effects of Δ*vncS* deletion-replacement mutations on tolerance to vancomycin challenge in C+Y medium in three common genetic backgrounds of *S. pneumoniae*, namely, R6, D39, and CP1250 (Table 1). As shown in Table 3, none of the Δ*vncS* strains derived from the R6, D39, or CP1250 parental strains exhibited any degree of tolerance to 10 times the MIC of vancomycin in C+Y medium (Table 3). By comparison, a deletion-replacement mutation in *lytA* (EL556), which encodes the major pneumococcal autoly-

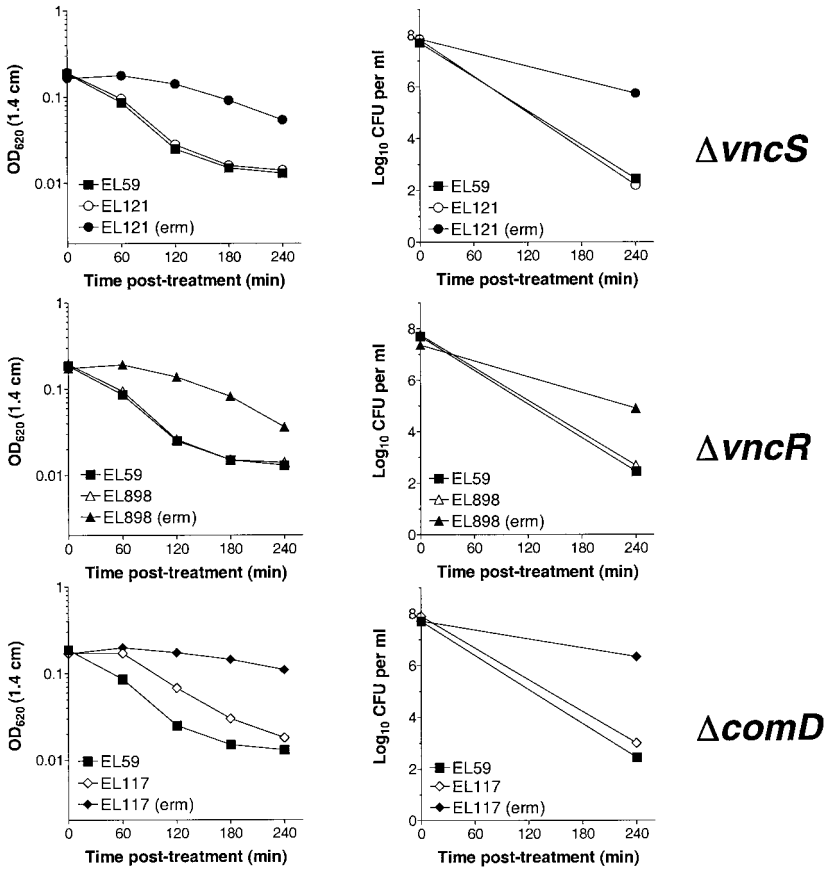


FIG. 3. Addition of erythromycin to *S. pneumoniae vncS*, *vncR*, and *comD* strains promotes a vancomycin tolerance phenotype. Cells grown in C+Y medium with no supplementation or C+Y medium supplemented with 1 μg of erythromycin per ml [indicated by (erm)] were treated with 5 μg of vancomycin per ml (10 times the MIC). Growth or autolysis was measured as the change in OD_{620} (left panels), and loss of viability (right panels) was monitored by serial dilution and plating on TSAII BA. Viability data are presented as $\log_{10}$ CFU per milliliter of medium.

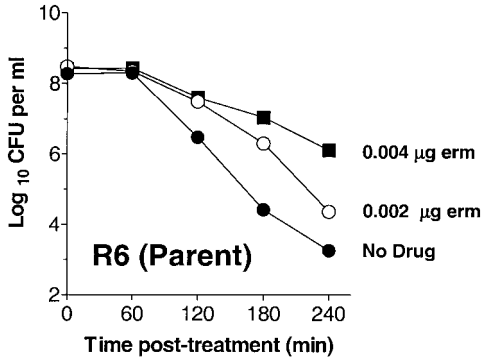


FIG. 4. Addition of sublethal concentrations of erythromycin promotes a vancomycin tolerance phenotype in the erythromycin-sensitive R6 parent strain (EL59). R6 grown in C+Y medium with no supplementation or in C+Y medium supplemented with 0.002 or 0.004 μg of erythromycin (erm) per ml was challenged with 5 μg of vancomycin per ml (10 times the MIC). Data are presented as $\log_{10}$ CFU per ml of medium. Symbols: closed circles, EL59 (R6 parent) without erythromycin supplementation; open circles, EL59 (R6 parent) supplemented with 0.002 μg of erythromycin per ml; closed squares, EL59 (R6 parent) supplemented with 0.004 μg of erythromycin per ml.

sin, was highly tolerant to vancomycin and, as expected, did not undergo autolysis in the presence of this antibiotic (Table 3). These findings strongly argue that LytA activity is essential for lysis in the presence of 10 times the MIC of vancomycin but that the VncS histidine protein kinase is not required for this process.

In a previous study (29), a *vncS* insertion-duplication mutant derived from D39 was reported to exhibit little to no killing during vancomycin therapy in a rabbit meningitis model and was found to have a selective advantage over an otherwise isogenic *vncS*$^+$ parental stain. This finding led to the proposal that the loss of VncS function may lead to the failure of vancomycin therapy in vivo (29). To test the generality of this proposal, we employed an established murine neutropenic thigh model of infection to evaluate the resistance of *vncS*-deficient pneumococci to vancomycin therapy in vivo (see Materials and Methods) (45). In control mice that were not treated with vancomycin, the *vncS* strain (EL381), the *vncR* strain (EL395), and the D39 parent strain (EL161) were found to replicate extensively and to nearly equivalent levels (data not shown). However, in mice that received subcutaneous vancomycin therapy at 1 and 5 h postinfection, significant clearance (>4 log reduction in CFU) of all strains was observed at 8 and 24 h postinfection, and the kinetics of clearance of the

TABLE 3. Vancomycin-induced autolysis is not altered in *vncS* mutants constructed in different genetic backgrounds and is not dependent on *vex3* or *pep27* function[a]

Strain	Description	Log_{10} CFU per ml at:		Log_{10}CFU loss[b]
		0 h	4 h	
EL59	R6 parent	7.86 ± 0.16	3.20 ± 0.45	4.66 ± 0.39
EL121	R6 *vncS*::*ermAM*	8.07 ± 0.07	3.15 ± 0.45	4.92 ± 0.52
EL822	R6 *vex3*::*ermAM*	8.00 ± 0.08	4.40 ± 0.33	3.60 ± 0.26
EL842	R6 *pep27*::*ermAM*	7.76	4.40	3.36
EL556	R6 *lytA*::*aad9*	8.48	7.77	0.71
EL161	D39 parent	8.15	4.11	4.03
EL381	D39 *vncS*::*aad9*	7.95	4.45	3.51
CP1250	Rx-based parent	8.26	2.90	5.35
EL562	CP1250 *vncS*::*ermAM*	8.26	2.04	6.21

[a] Values with standard errors represent means of results of three independent experiments. Values without standard errors are the results of a single set of experiments.
[b] Log_{10} CFU loss of ≥ 2.0 is not considered tolerant (29).

vncS strain (EL381) was found to be nearly identical to that of either the *vncR* strain (EL395) or the D39 parent (EL161) (data not shown). Thus, inactivation of *vncS* appears to afford no obvious protection from vancomycin therapy in vivo in this established murine infection model system.

Our failure to demonstrate tolerance of *S. pneumoniae vncS* mutants to vancomycin prompted an investigation of the role of this gene product in resistance to other antibiotics to which the inactivation of *vncS* was reported to confer tolerance (29). Again, to ensure that the lack of observed vancomycin tolerance was not due to the nature of our previous mutations, an additional *vncS* deletion-replacement mutation was generated and introduced into a derivative of strain Rx (CP1250) and an independent R6 isolate (CP1260) obtained from another laboratory (Table 1). In these strains, the front half of *vncS*, including the active histidine, was removed and replaced with a region encoding an erythromycin resistance gene expressed from a robust synthetic promoter (P_c::*ermAM*) (6) transcribed in the same orientation as the original gene (CP1257 and CP1261) (Fig. 1 and Table 1). Using these strains, we found that while the kinetics of vancomycin killing for the R6-based *vncS* mutant (CP1261) and its R6 parent (CP1260) were somewhat more rapid than that observed for the Rx-based pair (CP1250 and CP1257), again there was no significant difference between the *vncS* mutant strains and their $vncS^+$ parents (data not shown). The R6-based *vncS* strain (CP1261) and its isogenic parent (CP1260) were further tested for sensitivity to an aminoglycoside (streptomycin), which inhibits protein synthesis at the level of the ribosome, a fluoroquinolone (ciprofloxacin), which targets toposiomerase IV and DNA gyrase A, and two cell wall inhibitory agents, a beta-lactam (ampicillin) and cephalosporin (11). In each of the four cases, the bactericidal effect on the *vncS* strain (CP1261) was the same as that on its isogenic $vncS^+$ parent (CP1260) (data not shown). Thus, in contrast to what was reported previously for the SPSJ01 *vncS* insertion-duplication mutant (29), these data indicate that the loss of VncS function affords pneumococci no protection from killing by these diverse autolysis-inducing antibiotics.

Given the surprising sensitivity of multiple *vncS* strains constructed in distinct genetic backgrounds to killing by vancomycin and four additional antibiotics, we evaluated whether the inactivation of *vncS* might cause a subtle degree of tolerance, observable in specific media at inhibitor levels close to the MIC of vancomycin. To eliminate any possibility of unknown polar effects of our previous mutations on downstream genes, an additional larger deletion of *vncS* was constructed; in this deletion, the majority of the coding sequence was removed and replaced with a kanamycin resistance gene expressed from the P_c synthetic promoter (P_c::*aphIII*) (39) transcribed in an orientation opposite to that of the original gene (strain CP1292) (Fig. 1 and Table 1). For these experiments, early-exponential-phase cultures (OD_{550}, 0.1) of the Δ*vncS*::P_c::*aphIII* strain (CP1292) and its parent strain (CP1250) were diluted 1:10 into fresh media (i.e., CDM, THB-Y, and CAT agar) containing various concentrations of vancomycin and monitored for changes in OD_{550} over the course of 3 h of incubation at 37°C. Interestingly, while the sensitivity of *S. pneumoniae* to vancomycin differed slightly in various media, the responses of the *vncS* strain (CP1292) and its $vncS^+$ parent strain (CP1250) to this inhibitor at various doses of drug were indistinguishable (Fig. 5). Susceptibility to vancomycin was also unaffected by growth of these strains in CDM or THB-Y in an atmosphere of CO_2 (data not shown), and in no case was the delay in vancomycin-induced autolysis at ≥2 times the MIC extended for the *vncS* mutant (CP1292) relative to that of its parent strain (CP1250). Thus, mutation of *vncS* does not appear to afford any obvious protection against vancomycin, even at graded doses of inhibitor in rich or defined media.

Normal autolytic functions are unperturbed in *vex3*, *pep27*, and *vncR* mutants. Our inability to reproduce the reported tolerance phenotype of *vncS*-deficient pneumococci led us to examine whether other components of the proposed VncRS-Pep27 signal transduction pathway were required for drug tolerance. To test this proposal, defined deletion-replacement mutants of *vncR*, *vex3*, and *pep27*, the latter two having been previously implicated in the reported tolerance phenomenon (28), were constructed and assayed for tolerance to vancomycin at 10 times the MIC in C+Y medium. None of the individual mutations tested afforded any protection to vancomycin challenge (Table 3). Again, all strains evaluated exhibited similar kinetics of autolysis following challenge with vancomycin (Table 3) or with another well-characterized autolysin trigger for pneumococci, namely, 0.05% deoxycholate (data not shown) (3). In contrast, the *lytA* control strain (EL556) stopped growing but failed to undergo autolysis under either condition (Table 3 and data not shown). In total, these results indicate that the normal autolysis-signaling pathway is unperturbed in the absence of any one component of the proposed VncRS-Pep27 signal transduction pathway.

Synthetic Pep27 does not induce cell autolytic or other death functions in pneumococci. A small peptide, Pep27, encoded upstream of the *vncRS* locus (Fig. 1), was reported to function as a "death peptide," signaling both autolytic and nonautolytic cell death functions following its accumulation in the surrounding growth medium (28). As our studies indicate that mutation of the gene responsible for synthesis of Pep27 (*pep27*) or of those encoding the putative transporter (*vex*) or sensor (*vncS*) did not have any discernible effect on tolerance to vancomycin, we tested whether Pep27 exhibited any inhibitory activity against *S. pneumoniae*. Comparison of the *pep27* sequence obtained from the R6 genomic sequencing project (12) and

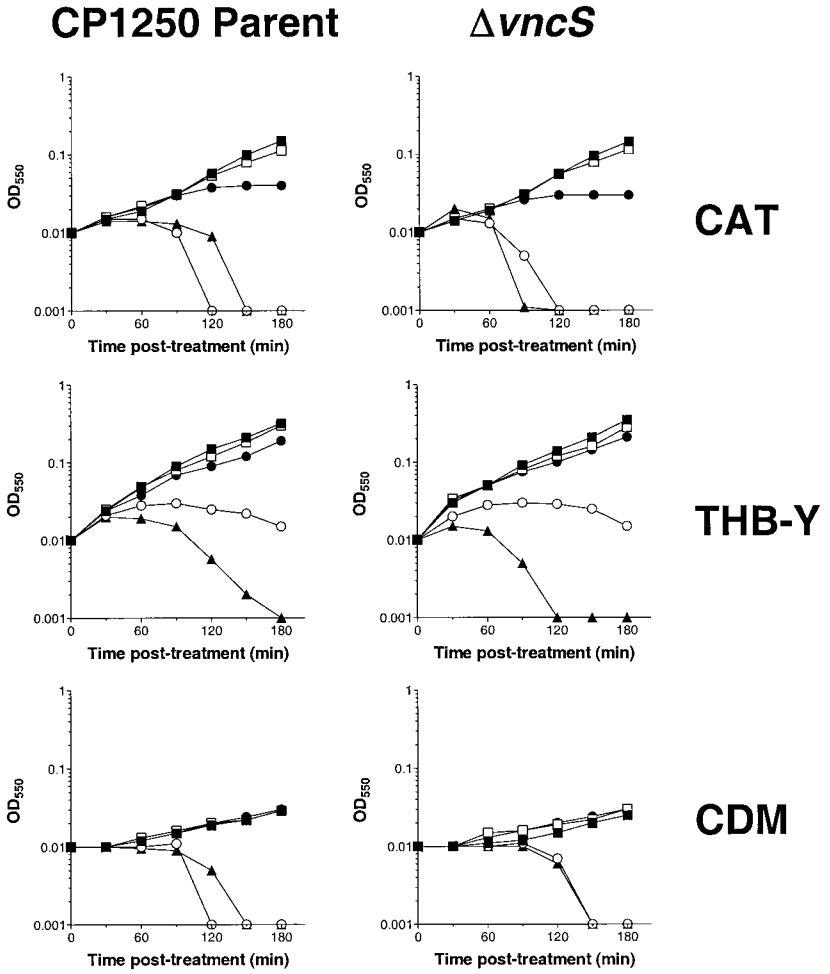
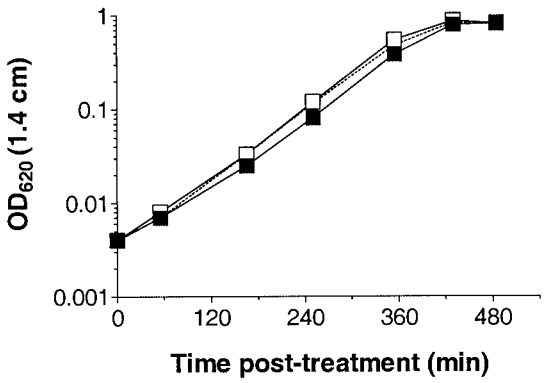


FIG. 5. Response of *S. pneumoniae* to challenge with vancomycin in three different growth media. CP1250 parent cells (left panels) and Δ*vncS* cells (right panels) grown in media specified on the right were challenged with various doses of vancomycin. Cell growth or autolysis was measured by the change in OD_{550}. Symbols: closed squares, no vancomycin; open squares, 0.25 μg of vancomycin per ml; closed circles, 0.5 μg of vancomycin per ml; open circles, 1.0 μg of vancomycin per ml; closed triangles, 5.0 μg of vancomycin per ml.

that previously reported for R6 (28) predicted two nonconserved amino acid substitutions within Pep27 (see Materials and Methods). This finding is consistent with the reported heterogeneity of peptide quorum-sensing systems in *S. pneumoniae* (32, 34). Therefore, to be consistent with the previous study (28), two versions of synthetic Pep27, reflecting both the published (Pub-Pep [28]) and predicted (Lilly-Pep [12]) Pep27 sequences, were synthesized and tested for their killing effects on *S. pneumoniae* R6 (EL59). Using peptide that was predicted to be intact and more than 90% pure (see Materials and Methods), we challenged *S. pneumoniae* with 50 μM peptide at a low cell density (Fig. 6A) or with graded doses of peptide at a high cell density (Fig. 6B). In both cases, we observed no negative effect on bacterial cell growth, lysis, or viability following Pep27 treatment. Indeed, a previously described disk sensitivity assay (36) using concentrations of synthetic Pep27 ranging from 3 to 300 μM failed to reveal any detectable zone of inhibition for our R6 (EL59) or D39 (EL161) parent strains on TSAII BA (data not shown). These results are consistent with the lack of tolerance observed for our *pep27* deletion-replacement mutant (EL842) (Table 3) and indicate that it is unlikely that Pep27 functions as a general effector of cell death in pneumococci.

Expression of *vex123* is derepressed in the absence of VncS. As a first step in determining the physiologic function of the VncRS signal transduction pathway, we employed microarray global transcriptional analysis to map changes in gene expression in the absence of *vncS*, *vncR*, or *vex3* during mid- or late-exponential-phase growth in CDM (see Materials and Methods). By this approach, we found that very few (19 to 35) genes showed expression changes of ≥2-fold (data not shown). This is surprisingly low compared to results of similar studies evaluating the competence TCS pathways of *S. pneumoniae* (31, 35) and may indicate that the true physiologic inducing condition for this TCS is not accurately achieved under these experimental conditions. However, in no case was the transcription of known autolysis-related genes or genes that may regulate tolerance and cell death, such as the recently described *murMN* operon (9), altered in the absence of individual components of the VncRS signal transduction system (Fig. 7A and B). However, under both experimental growth conditions, we did observe induction of the *vex123* gene cluster in the *S.*

A



B

Log_{10} CFU per ml of culture following challenge of *S. pneumoniae* R6 with synthetic Pep27 at late-log phase growth [a].

	μM Lilly-Pep27				μM Pub-Pep27			
Time (min)	0	200	1	0.1	0	200	1	0.1
0	8.32	8.08	8.30	8.43	8.36	8.27	8.53	8.36
60	8.49	8.68	8.51	8.60	8.72	8.60	8.69	8.72
240	8.91	9.20	9.04	8.90	9.20	9.34	9.08	8.78

[a] See Materials and Methods

FIG. 6. Treatment of *S. pneumoniae* EL59 (R6 parent strain) with synthetic Pep27 does not inhibit growth or induce autolysis. (A) *S. pneumoniae* grown in CDM medium was diluted to an OD_{620} of ~0.004 in CDM containing 50 μM synthetic Pep27. Growth was monitored by the change in the OD_{620} over time. Symbols: closed squares, no peptide control; open squares, 50 μM Pub-Pep27; open squares with dashed line, 50 μM Lilly-Pep27. (B) Cells grown in CDM to an OD_{620} of ~0.45 (late exponential phase) were challenged with various doses of the two synthetic forms of Pep27. Viability was monitored by serial dilution and plating on TSAII BA. Data are presented as log_{10} CFU per milliliter of medium.

pneumoniae vncS strain (EL121) but not in other strains evaluated (Fig. 7A and B). Derepression of *vex123* in the absence of VncS was highly reproducible, being observed in four additional microarray experiments, and was found to occur irrespective of the growth stage of the bacteria evaluated (Fig. 7A and B; also data not shown). To further investigate this relationship and to examine the kinetics of *vex* gene expression, we constructed a *vex2* transcriptional fusion with the *E. coli lacZ* gene by insertion-duplication of the pneumococcal suicide plasmid pEVP3 in the native *S. pneumoniae vex* locus (see Materials and Methods) (6). In contrast with the parent strain (EL555), which contains a deletion of the *bgaA* gene to eliminate endogenous β-galactosidase activity (47), strain EL1066 showed β-galactosidase activity resulting from the strain's *vex2*::*lacZ* fusion that was highest during early-exponential-phase growth in BHI and that declined steadily as the cultures reached mid-exponential to late exponential growth and stationary phases relative to the corresponding increase in OD_{620} (Fig. 7C). Introduction of a *vncS*(Δ273–1021)::*aad9* mutation into the EL1066 background (EL1069) did not alter the overall pattern of *vex* gene expression but did result in a four- to fivefold increase in β-galactosidase activity at all time points (Fig. 7C). This result is consistent with that of our global transcriptional analysis (Fig. 7A and B) and strongly argues that expression of the *vex* gene cluster is induced in the absence of VncS.

DISCUSSION

The VncRS signal transduction system of *S. pneumoniae* has been suggested to play a key role in the regulation of cell death and autolysis in response to vancomycin challenge (10, 29). By using four distinct mutations in *vncS*, in some cases in multiple genetic backgrounds of *S. pneumoniae*, we have demonstrated that the loss of VncS function alone does not result in tolerance to vancomycin challenge or to other autolysis-inducing antibiotics to which the inactivation of *vncS* was reported to confer a selective advantage (28, 29). We did observe a reproducible tolerance of *S. pneumoniae* to vancomycin challenge during mid-exponential-phase to late exponential-phase growth (OD_{620}, >0.15 in a 1.4-cm path length) following exposure of erythromycin-resistant or -susceptible bacteria to sublethal concentrations of erythromycin (Fig. 2A, 3, and 4). This was not dependent on mutation of *vncS*, as it could be

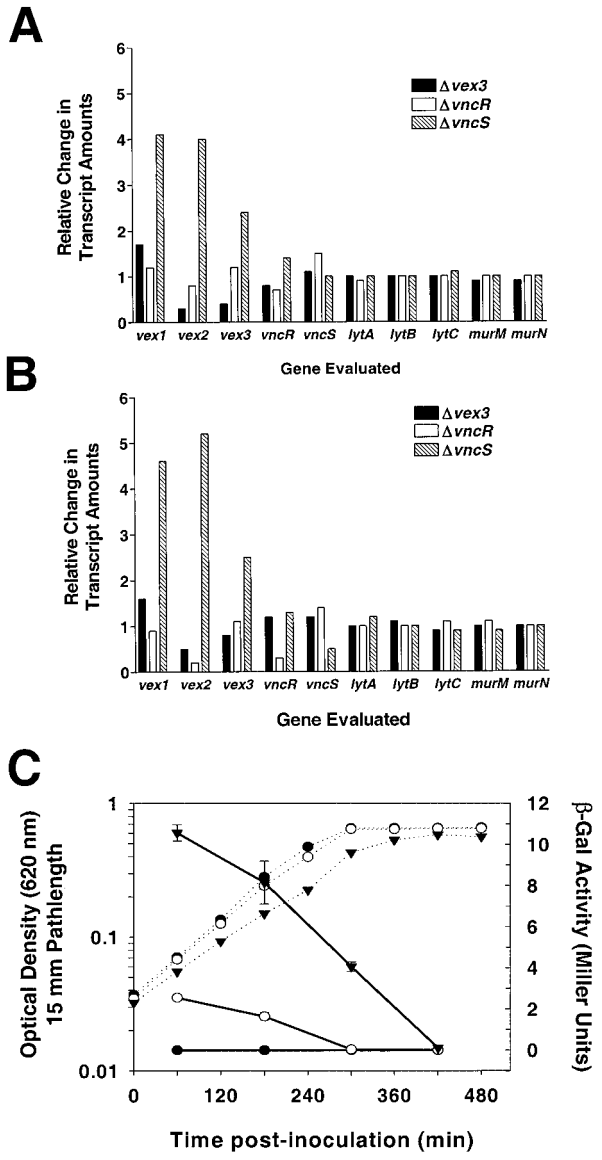


FIG. 7. Transcriptional expression of *vex123* is induced in the absence of VncS. (A and B) Relative changes in amounts of mRNA from select genes from microarray gene expression profiling experiments using *vex3* (EL822) (closed bars), *vncR* (EL898) (open bars), and *vncS* (EL121) (hatched bars) mutants during mid-exponential-phase growth (OD_{620}, ~0.3) (A) or late-exponential-phase growth (OD_{620}, ~0.7) (B) are shown in comparison to that of the R6 parent strain (EL59) grown under the same conditions. Data are presented as ratios of the hybridization signals (see Materials and Methods) from the experimental strains (i.e., *vex3*, *vncR*, or *vncS* mutant) to those of the control (i.e., R6 parent). (C) Expression of a *vex2*::*lacZ* fusion in the presence or absence of VncS. Symbols: dashed lines, OD_{620}; solid lines, β-galactosidase activity; closed circles, Δ*bgaA* parent strain (EL555); open circles, *vex2*::*lacZ* reporter strain (EL1066); closed triangles, Δ*vncS vex2*::*lacZ* reporter strain (EL1069); vertical bars, standard errors of the means. The experiment was conducted in triplicate and was performed twice.

reproduced in a Δ*vncR* strain (EL898), a Δ*comD* strain (EL117) (Fig. 3), or in the *vncS*$^+$ erythromycin-sensitive parent strain R6 (EL59) (Fig. 4). This could partly explain the discrepancy between our results and those reported previously (29), because in the prior study, vancomycin tolerance was routinely assayed in C+Y medium containing a concentration of erythromycin that was found to promote tolerance to vancomycin challenge in our studies (Fig. 2A and 3). Interestingly, we also found that this concentration of erythromycin impairs growth of pneumococci in C+Y medium (Fig. 2B), a property not similarly reported in the earlier study (29). Thus, whether the erythromycin-induced tolerance to vancomycin is due solely to changes in cell growth and division or to other unknown erythromycin-related effects is not yet clear. The proposition that erythromycin increases tolerance to vancomycin by altering cell growth is a testable hypothesis; however, it could also be the case that specific, as yet unidentified physiologic changes resulting from erythromycin treatment are responsible for this effect. One serious but as yet untested implication of this observation is that sublethal concentrations of erythromycin, or possibly of other static antibiotics, may promote slowed growth and phenotypic tolerance of the pneumococcus to secondary autolysis-inducing antimicrobials (i.e., vancomycin) in the clinical setting.

Growth in the presence of sublethal concentrations of erythromycin clearly induces tolerance of pneumococci to vancomycin, yet this phenomenon does not reconcile why Novak and associates (29) did not similarly observe tolerance for other erythromycin-resistant mutants [i.e., *vncR*::(pJDC9)] in vitro (Fig. 3) nor does it explain the discrepancies between their in vivo assays in a rabbit meningitis model and ours in neutropenic mice (see Materials and Methods). Given the number of diverse mutations constructed for this study, we find it highly unlikely that the *vncS* mutants evaluated here are not functionally equivalent to those reported elsewhere (10, 29). Thus, in addition to the effect of erythromycin on the tolerance of *S. pneumoniae*, additional factors may contribute to the phenotypes observed in previous studies (29), such as polarity or other effects of the insertion mutation or some special features of the strain pool from which SPSJ01 was isolated.

In addition to our inability to reproduce antibiotic tolerance in *vncS*-deficient *S. pneumoniae*, we were unable to demonstrate vancomycin tolerance in strains deficient in the proposed peptide signal for this response (e.g., Δ*pep27* [EL842]) or deficient in a component of the proposed Pep27 ABC transporter (e.g., Δ*vex3* [EL822]) (Table 3). Moreover, a synthetic form of the Pep27 death peptide failed to elicit a negative physiologic response in our R6 parent strain (EL59) at concentrations reported to promote both lytic and nonlytic killing of this bacterium (28). Both results argue against the model for vancomycin tolerance in *S. pneumoniae* that was advanced previously (28), and taken together, they support the lack of tolerance observed for our Δ*vncS* mutants. Microarray global transcription analysis of *S. pneumoniae* Δ*vncR*, Δ*vncS*, and Δ*vex3* mutants failed to identify altered expression of genes known to contribute to autolysis (i.e., *lytA*, *lytB*, and *lytC*) or tolerance to vancomycin (i.e., *murMN*). These studies did reveal a strong correlation between the loss of *vncS* function and the induction of the upstream *vex123* gene cluster (Fig. 7A and B). These microarray data were supported by direct measurement of *vex2* gene expression with β-galactosidase reporter assays in *vncS*$^+$ and Δ*vncS S. pneumoniae* strains (Fig. 7C) and are consistent with a role for the VncRS signal transduction system in the transcriptional regulation of *vex* gene expression. This is not uncommon for TCSs whose targets are often prox-

imal to the genes encoding the corresponding histidine protein kinase and response regulator (8).

As transcriptional changes of the *vex* gene cluster were not similarly altered in the absence of *vncR* or *vex3* itself, the effect of the *vncS* deletion on *vex123* may be either indirect and dependent on VncR being locked in a nonphosphorylated state or, alternatively, dependent on other properties resulting from the loss of VncS activity. We propose either that VncR in its nonphosphorylated state functions as an activator of *vex123* gene expression or, alternatively, that phosphorylated VncR is required for repression of these genes. Both models can adequately explain the induction of *vex123* transcription in the absence of *vncS* function. However, given the lack of *vex* induction for the *vncR* mutant (EL898), we favor the former model. Additional experiments are needed to confirm such a relationship. It also remains to be determined what represents the true physiologic signal(s) for VncRS signal transduction and what roles this poorly characterized TCS plays in pneumococcal biology. If, as was previously reported (28), Pep27 promotes dephosphorylation of VncR through stimulation of VncS phosphatase activity, we would anticipate seeing the induction of *vex* gene expression following challenge with synthetic forms of Pep27. However, preliminary experiments using microarrays and *vex2*::*lacZ* reporter assays have failed to establish such a correlation (data not shown). It is noteworthy that while inactivation of *vncR* or *vncS* does not alter vancomycin-mediated clearance of pneumococci from a neutropenic murine thigh model (see Results), this TCS has been implicated in the colonization of the mouse lung (41). Thus, while inactivation of the *vncRS* signal transduction system does not confer a general vancomycin tolerance phenotype, this TCS may contribute to the capacity of pneumococci to produce disease in a host. Given the small number of transcriptional changes observed in our microarray studies (Fig. 7A and B and data not shown), it is not possible to speculate on the basis of this attenuation. Indeed, this may suggest that the activation signals for this TCS are observed only during colonization of a susceptible host. Regardless, given that antibiotic tolerance is not a general property of strains lacking VncS activity and the requirement for this and other TCSs in pathogenesis and viability of *S. pneumoniae* (41; G.T. Robertson, unpublished data), TCSs may still represent potential targets for broad-spectrum antibiotics.

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