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Revising the Role of the Pneumococcal *vex-vncRS* Locus in Vancomycin Tolerance

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Vancomycin is used increasingly to treat invasive infections caused by multidrug-resistant *Streptococcus pneumoniae*. Although no vancomycin-resistant strains have been isolated to date, tolerant strains that fail to die rapidly and that cause relapsing disease have been described. The *vex123-pep$_{27}$-vncRS* locus, consisting of an ABC transporter, a presumed signaling peptide, and a two-component system, respectively, has been implicated in vancomycin tolerance. Recent findings, however, challenged this model. The data presented here indicate that erythromycin in the growth medium induces a vancomycin-tolerant phenotype and that loss of function of Pep$_{27}$ or VncRS does not alter autolysis. However, a role for the ABC transporter encoded by the *vex123* genes in tolerance was confirmed. A *vex3* mutant was considerably more tolerant to vancomycin treatment than the wild-type strain T4, and the strength of the phenotype depended on the orientation of the resistance cassette used to construct the mutant. Microarray results suggested a number of genes that might be involved in tolerance in the *vex3* mutant. Although the exact function and regulation of the *vex123-pep$_{27}$-vncRS* locus remains to be determined, several factors influence the autolysis behavior of *S. pneumoniae*, including the bacterial capsule, erythromycin, and the *lytA* and *vex3* gene products.

Streptococcus pneumoniae, the causative agent of middle ear infections, pneumonia, bacteremia, and meningitis, has demonstrated increased resistance to previously effective antibiotics (1, 33). Vancomycin has been used more frequently for severe, invasive disease where immediate intervention is required, since no resistant strains have been reported to date. Exposure to vancomycin or β-lactams results in autolysis of sensitive cells (6). Strains which do not undergo autolysis after treatment with these antibiotics show increased survival and are termed tolerant (10, 20, 32). This phenotype is of potential medical significance since tolerance can lead to treatment failures and facilitate the development of antibiotic resistance (20). Recently, reports of vancomycin tolerance have been published, raising anxiety about treatment options, particularly for meningitis (2, 10, 11, 20).

In a study seeking the signaling cascade that activates the autolytic pathway by analysis of loss-of-function mutants, a genetic locus consisting of six genes was identified (Fig. 1) (22, 23). Three of the genes, *vex1*, *vex2*, and *vex3*, have similarity to the transmembrane and ATP-binding proteins of ABC transporters, while the fourth gene, *pep$_{27}$*, was predicted to encode a peptide of 27 amino acids. The last two genes in the locus, *vncR* and *vncS*, have similarity to response regulator and histidine kinase subunits of a two-component system. The proposed function of the proteins was to measure cell density via a quorum-sensing mechanism and to initiate autolysis. The model predicted that Pep$_{27}$ was secreted from the cell by the Vex123 transporter system and that the peptide accumulated outside of the cell. At a threshold concentration in the environment, Pep$_{27}$ was expected to activate the membrane-bound signal sensor VncS. The signal would then be relayed to the DNA-binding protein VncR, which was hypothesized to be a repressor of genes involved in autolysis. The model also predicted that antibiotics, such as penicillin or vancomycin, could short-circuit this activation process and trigger autolysis before stationary growth phase was reached (22, 23).

Recent work has challenged this model. In this second study, Robertson et al. (26) were unable to detect a biological effect of Pep$_{27}$ on a growing culture of *S. pneumoniae*, and deletions in the *vncRS* genes did not alter lysis with respect to vancomycin. As an alternative explanation, it was suggested that erythromycin in the growth medium, which is required to maintain the stability of the mutants created by insertion-duplication mutagenesis, caused a vancomycin-tolerant phenotype regardless of the specific gene that was mutated (26).

This inconsistency between the two studies prompted a detailed reanalysis of the *vex-vnc* locus and the behavior of individual mutants with respect to vancomycin treatment. Our data confirm that the presence of erythromycin increases the tolerance of a strain to vancomycin. Newly constructed mutations in the genes *pep$_{27}$*, *vncR*, or *vncS* had no effect on vancomycin tolerance. The role of the VncRS two-component system remains to be determined, since transcriptional analyses of various mutants also did not demonstrate regulation of *vex123* or other genes. However, the replacement of *vex3* by an *ermB* resistance cassette did yield a strain that was vancomycin tolerant, even in the absence of erythromycin. Transcriptional analyses showed that several genes are differentially regulated in this tolerant *vex3* mutant.

MATERIALS AND METHODS

Bacterial strains and PCR ligation mutagenesis. The strains of *S. pneumoniae* used in this study are listed in Table 1. Null mutants in the genes for *vex3*, *pep$_{27}$*, *vncR*, *vncS*, or *lytA* were generated in *S. pneumoniae* strain T4 by PCR ligation mutagenesis (18). Gene SP2155, coding for a truncated immunoglobulin A1

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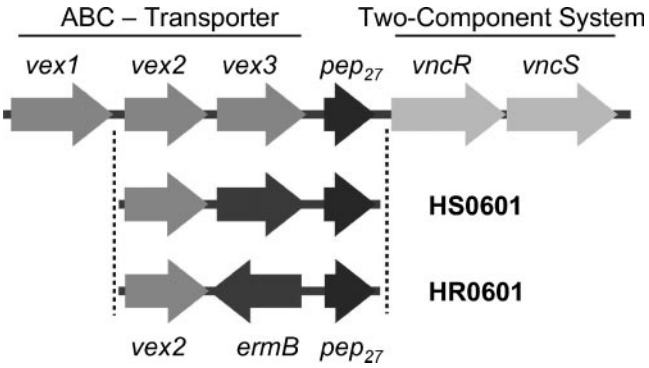

FIG. 1. Organization of the *vex-vnc* locus in *S. pneumoniae*. The Vex1 and Vex3 proteins are predicted to function as transmembrane permeases, while the Vex2 protein contains motifs typical for an ABC cassette protein. The three proteins together represent an ABC transport system. Pep_{27} was reported to be a peptide involved in cell-cell signaling, and VncR and VncS are subunits of a two-component signal transduction system. The *vex-vnc* locus in the mutant strains HS0601 and HR0601 is depicted schematically to indicate the replacement of *vex3* by *ermB*. The arrows indicate the directions of gene transcription.

(IgA1) protease (29) (http://www.tigr.org), was mutated to serve as the negative control (strain HS2155). *lytA*, coding for the LytA autolysin, was mutated as the positive control (strains HS1937 and HR1937). DNA fragments of ~1 kb representing sequences 5′ and 3′ of the gene to be deleted were amplified by PCR using TaKaRa LA Taq DNA polymerase (Takara, Shiga, Japan) and primers (available on request). PCR products were purified by using Wizard PCR Preps (Promega, Madison, Wis.) and digested with either NgoMIV or EagI (New England BioLabs, Beverly, Mass.). The *ermB* expression cassette from plasmid pTCV-lac (25) was PCR amplified and digested with XmaI and PspOMI. 5′ and 3′ PCR fragments were ligated to the *ermB* cassette to yield ligation products of approximately 3 kb. Alternatively, PCR products were fused together by gene splicing by overlap extension (SOEing) (12, 13). Mutagenic PCR products were transformed into *S. pneumoniae* T4 by using standard methods (3). Erythromycin-resistant clones were selected on tryptic soy agar plates containing 4% sheep blood and 1 μg of erythromycin (Sigma)/ml. MICs were determined by using the E-test. The parental strain T4 was sensitive (MIC, 0.125 μg/ml) to erythromycin, while all mutant strains, containing the *ermB* gene, were resistant (MICs, 48 mg/ml or higher) to erythromycin. Mutant clones were confirmed by DNA sequence analysis using primers that annealed outside the DNA region used for mutagenesis. This analysis was done to ensure the correct location and sequence of the *ermB* gene and surrounding DNA. All sequencing reactions were carried out at the Hartwell Center for Biotechnology at St. Jude Children's Research Hospital.

Bacterial growth and kill curves. Overnight cultures of *S. pneumoniae* were grown from −70°C glycerol stocks. Cultures were routinely grown overnight in 10 ml of C+Y medium in KIMAX culture tubes (18 by 150 mm) at 37°C and 5% CO_2 without agitation. The following day, fresh cultures were started by diluting the overnight cultures 1:100 in prewarmed C+Y medium. The turbidity of the cultures was measured in 30-min intervals with a Turner model 340 spectrophotometer at 620 nm. Vancomycin (5 μg/ml; 10× MIC; Sigma) was added at an optical density at 620 nm (OD_{620}) of 0.25 to 0.30, corresponding to mid-logarithmic growth phase and approximately 3×10^7 CFU/ml. Bacterial viability (CFU/ml) was determined at the time of the addition of vancomycin and 4 h thereafter by plating 10-μl samples on tryptic soy broth-blood agar plates (15) and counting the colonies at 18 to 24 h.

RNA isolation. Bacterial cells were harvested for RNA isolation during logarithmic growth phase at an OD_{620} of 0.45 to 0.5, which corresponds to 7.5×10^7 CFU/ml. RNA isolation was performed by using the QIAGEN RNeasy Mini Kit (QIAGEN, Inc., Valencia, Calif.) with the following modifications. Bacteria were lysed by shaking for 5 min in the presence of 400 mg of 0.1-mm zirconia-silica beads (BioSpec Products) by using a Mini-Beadbeater 3110BX (BioSpec), followed by incubation at 70°C for 10 min. The lysate was centrifuged through a QIAshredder (QIAGEN) and processed according to the manufacturer's instructions, with an on-column DNase digestion step. Quantitation of RNA samples was performed by using a UV spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at OD_{260}.

Northern blot. For Northern blot analysis, 5 μg of total RNA was separated on a 1.2% MOPS (morpholinepropanesulfonic acid)–formaldehyde gel and transferred to nylon membranes (Duralon-UV; Stratagene, La Jolla, Calif.) according to standard procedures (28). Hybridization reactions with *ermB*- or *vncS*-specific probes were carried out by using Church's buffer (5) for 16 h at 66°C. Blots were washed with 1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate at 66°C and exposed at −80°C to BioMax MR film (Eastman Kodak Company, Rochester, N.Y.) with intensifying screens.

Microarray analysis. Whole-genome *S. pneumoniae* cDNA microarrays were received as a grant from the Pathogen Functional Genomics Resource Center (PFGRC; http://pfgrc.tigr.org/; The Institute for Genomic Research, Rockville, Md.). Microarray analyses were performed according to MIAME (minimum information about a microarray experiment) standards by using the protocols provided by PFGRC with minor modifications. cDNA was generated from bacterial RNA with random hexamers and SuperScript II (Invitrogen, Carlsbad, Calif.) in the presence of 5-(3-aminoallyl)-dUTP (Ambion, Inc.). Cy-3 and Cy-5 monoreactive fluorescent dyes were then coupled to aminoallyl group-containing cDNA products according to PFGRC standard operating procedure M007 (8). The fluorolabeled products were then quantified spectrally, and equimolar quantities of Cy-3- and Cy-5-labeled products were combined and used for hybridization according to standard operating procedure M008 (7). All microarray experiments were performed in triplicate with independent biological samples, including one dye flip experiment in which the Cy-3 and Cy-5 labels were reversed to account for dye bias. After hybridization, raw TIFF images from the scanner were loaded into GenePix Pro version 4.1 (Axon, Inc., Union City, Calif.) for analysis. Spot grids (.gal files) were manually fitted to the microarray images. Spots were automatically flagged in instances of high background or stray fluorescent signals. The resulting GenePix result files (.gpr files) were exported into a DecisionSite for Functional Genomics version 7.2 module (Spotfire, Inc., Somerville, Mass.). Further analyses were performed to normalize the data set in an intensity-dependent manner (34) and to perform statistical analysis by analysis

TABLE 1. *Streptococcus pneumoniae* strains used in this study

Strain	Characteristics	Reference
T4	Clinical isolate from the blood of a 30-year-old male patient, capsular serotype 4, highly virulent in mouse model	29
T4R	Unencapsulated derivative of strain T4, hyperlytic compared to strain T4	9
R6	Unencapsulated derivative of strain D39, capsular serotype 2, avirulent laboratory strain	14
Tupelo	Clinical isolate from a 10-month-old girl with meningitis, capsular serotype 14, multidrug resistant, naturally vancomycin tolerant	20
HS2155	T4 mutant, truncated immunoglobulin A1 protease gene (SP2155) replaced by *ermB* cassette	This study
HS1937	T4 mutant, autolysin *lytA* gene (SP1937) replaced by *ermB* cassette	This study
HR1937	T4 mutant, autolysin *lytA* gene (SP1937) replaced by *ermB* cassette, *ermB* in reverse orientation relative to *lytA*	This study
HS0601	T4 mutant, *vex3* gene (SP0601) replaced by *ermB* cassette	This study
HR0601	T4 mutant, *vex3* gene (SP0601) replaced by *ermB* cassette, *ermB* in reverse orientation relative to *vex3*	This study
HS0602	T4 mutant, *pep27* gene (SP0602) replaced by *ermB* cassette	This study
HS0603	T4 mutant, *vncR* gene (SP0603) replaced by *ermB* cassette	This study
HS0604	T4 mutant, *vncS* gene (SP0604) replaced by *ermB* cassette	This study
HR0604	T4 mutant, vncS gene (SP0604) replaced by *ermB* cassette, *ermB* in reverse orientation relative to *vncS*	This study

of variance (ANOVA). Genes identified as statistically significant were further subjected to false discovery rate corrections. Genes with at least a twofold change in expression and an ANOVA P value of <0.05 were considered significantly differentially expressed.

Real-time PCR analysis. RNA quantitation by real-time PCR analysis was done on an ABI Prism 7700 sequence detection system (Perkin-Elmer, Foster City, Calif.) by using TaqMan reverse transcriptase and SYBR green reagents according to the protocols provided by the supplier (Applied Biosystems, Foster City, Calif.). PCR primers were designed to yield amplification products of approximately 100 bp and were tested in preliminary experiments to control for primer-dimer formation and amplification efficiency. RNA was isolated as described above, quality tested on agarose gels, and DNase-treated by using a DNA-free kit from Ambion (Austin, Tex.). TaqMan reverse transcriptase was used to transcribe 2.5 ng of total RNA into cDNA; the enzyme was omitted in control reactions. PCRs were performed by adding 5 μl of cDNA to 25 μl of SYBR Green PCR Master Mix in a total reaction volume of 50 μl. Thermocycler conditions were set and data analysis was done according to the manufacturer's recommendations. All real-time PCR data were normalized to the *gyrA* gene as an internal control and are shown relative to the data for the wild-type strain T4 or the control strain HS2155, which were grown in the absence of antibiotics. All data are expressed on a $\log_2$ scale and are the means and standard deviations for three independent biological samples.

Cloning procedures. Plasmid pWHM302 was designed to overexpress the *vncR* gene in *S. pneumoniae*. The −35 and −10 elements of the *fcsK* promoter (4) were PCR amplified, and in the process the sequence for the ribosome binding site changed to GGAGG. This minimal promoter lacks predicted operator sites and is active in the absence of added inducers. The *vncR* gene from strain T4 was amplified and fused to the minimal *fcsK* promoter by overlap-extension PCR. The resulting PCR product possessed SalI and BamHI restriction sites for cloning into the shuttle vector pDL278. The PCR amplifications did not introduce point mutations as determined by DNA sequencing. *S. pneumoniae* clones were selected on tryptic soy broth-blood agar plates containing 500 μg of spectinomycin/ml after transformation with either pDL278 (empty vector) or pWHM302.

Western blot analysis. Cultures were grown in 5 ml of C+Y medium to an OD_{620} of 0.30. Cells were harvested by centrifugation and pellets were resuspended in 100 μl of 20 mM HEPES (pH 8.0) containing protease inhibitors (Complete Mini; Roche). The resuspension was then sonicated on ice in three 20-s bursts. The protein content of the supernatant was determined after centrifugation, and 5 μg of protein was run on a 10% PreCast gel (Bio-Rad). After completion of the run, proteins were blotted onto polyvinylidene difluoride membrane by using standard methods. Immunodetection was performed on the membrane by using anti-VncR antibodies at a 1:2000 dilution overnight at 4°C. Goat anti-rabbit horseradish peroxidase was used as the secondary antibody at a 1:10000 dilution for 30 min at room temperature. Membranes were exposed to film after the membrane was reacted with detection reagent (SuperSignal West Pico Chemiluminescent; Pierce) according to the manufacturer's instructions.

RESULTS

Erythromycin causes a vancomycin-tolerant phenotype. The insertion-duplication mutagenesis method relies on plasmid integration into the chromosome via homologous recombination. Although the requirement for continuous antibiotic selection to prevent reversible loss of the integrated plasmid is a disadvantage of this method (17), it has received longstanding, widespread use, including for analysis of antibiotic action (22, 23). Recent work by Robertson and coworkers (26) suggested that the presence of erythromycin to maintain such mutations results in a tolerant phenotype independent of the gene interrupted by the plasmid. To specifically address this question for the *vex-vnc* locus, genetically stable mutants were generated by using the PCR ligation mutagenesis method described by Lau et al. (18), which uses a PCR product containing a selectable marker that is flanked by DNA fragments that are homologous to regions 5′ and 3′ of the target gene. Homologous recombination completely replaces the target gene with the selectable marker, creating a genetically stable mutant. The *ermB* cassette from plasmid pTCV-lac (25) was chosen as the selectable marker to allow for bacterial growth in the presence or absence of erythromycin. This cassette consists of a strong promoter, a leader peptide, and the *ermB* gene and allows for erythromycin-induced expression of the *ermB* gene. Using this method, we replaced the *vex3*, pep_{27}, *vncR*, *vncS*, and *lytA* genes and the control gene SP2155, encoding a truncated IgA1 protease, with an *ermB* cassette. In contrast to previous experiments that used the unencapsulated laboratory strain R6 (23, 26), we created mutants with the virulent strain T4 to study tolerance in a clinically relevant isolate.

TABLE 2. Decrease in OD and CFU after vancomycin treatment for 4 h

Strain	Growth condition[a]	Doubling time (min)	Percent loss of OD_{620}	Log decrease in CFU/ml ± SD[b]
T4		50	70	1.9 ± 0.6
T4R		35	96	6.2 ± 0.5
R6		46	95	2.7 ± 0.4
Tupelo		49	4	0.4 ± 0.1
HS2155		44	86	1.1 ± 0.1
HS2155	+ Erm	74	60	0.6 ± 0.2
HS1937		50	18	0.4 ± 0.1
HS1937	+ Erm	123	−6	−0.1 ± 0.1
HR1937		51	7	0.4 ± 0.1
HS0601		46	67	0.8 ± 0.1
HS0601	+ Erm	62	46	0.9 ± 0.2
HR0601		51	37	0.4 ± 0.1
HS0602		44	89	2.6 ± 0.1
HS0602	+ Erm	69	78	1.2 ± 0.1
HS0603		45	78	2.0 ± 0.4
HS0603	+ Erm	68	77	1.3 ± 0.2
HS0604		44	84	2.6 ± 0.2
HS0604	+ Erm	76	74	1.8 ± 0.1
HR0604		43	79	2.0 ± 0.2

[a] + Erm, grown in the presence of erythromycin.
[b] At 4 h. Mean values for three independent experiments are shown.

For all mutants, cultures grown in the presence of erythromycin had a prolonged doubling time and a decreased loss of OD_{620} and CFU/ml after vancomycin treatment compared to the cultures grown in the absence of erythromycin (Table 2). These results confirm previous observations that erythromycin in the growth medium causes a low level of tolerance to vancomycin (26).

The effect of erythromycin was distinguishable and additive to loss of function of LytA, the major autolysin of *S. pneumoniae*. Mutants that lack a functional *lytA* gene are tolerant and have been used as the gold standard in tolerance experiments. The T4 mutant of *lytA*, strain HS1937, underwent lysis at a much slower rate than the wild-type strain T4, and the rate of lysis was further decreased in the presence of erythromycin (Table 2).

Response of *vex3*, pep_{27}, *vncR*, or *vncS* mutants to vancomycin challenge in the absence of erythromycin selection. Given the increased tolerance invoked by erythromycin, a valid test of vancomycin tolerance would require an assay of the survival of the mutants in the absence of selection by erythromycin. In the absence of erythromycin, interruption of pep_{27} in strain HS0602, *vncR* in strain HS0603, or *vncS* in strain HS0604 did not decrease autolysis. All three strains lysed at least as rapidly as the wild-type strain T4 after the addition of vancomycin (Table 2). These results confirm those of Robertson et al. (26) and indicate that the phenotype observed by Novak et al. (23)

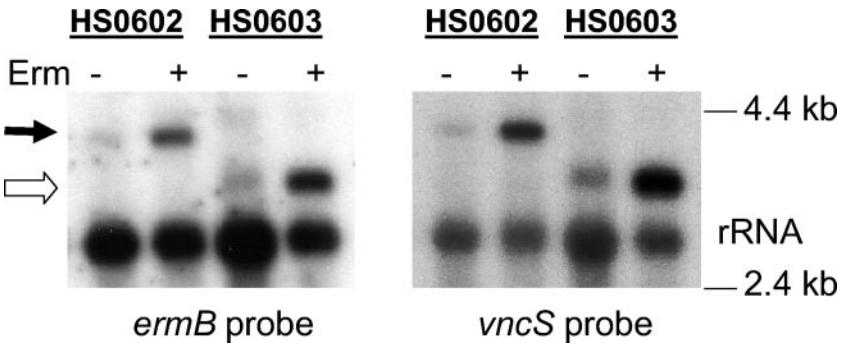

FIG. 2. Northern blot analysis of transcriptional read-through from the *ermB* promoter. RNA from the pep_{27} mutant HS0602 and the *vncR* mutant HS0603 was isolated after the cells were grown in the absence or presence of erythromycin, and samples were subjected to Northern blot analysis with probes specific for *ermB* or *vncS*. High-molecular-weight bands represent *ermB-vncR-vncS* (filled arrow) or *ermB-vncS* (open arrow) polycistronic mRNAs. rRNA is shown as the loading control.

most likely arose due to the presence of erythromycin in the growth medium.

In contrast to the other mutants, the *vex3* mutant HS0601 was the only strain that underwent less vancomycin-induced lysis than the wild-type strain even in the absence of erythromycin (Table 2). Although HS0601 reproducibly underwent less lysis than the parental strain T4, the differences in OD_{620} values were not statistically significant. The viability of the HS0601 mutant decreased by 0.8 log unit, compared to a decrease of 1.9 log units for the wild-type strain T4 and 0.4 log unit for the tolerant *lytA* mutant HS1937. These data show that although pep_{27}, *vncR*, and *vncS* are not directly involved in autolysis as previously suggested (22, 23), *vex3* might play a role in autolysis.

Overexpression of downstream genes due to a polar effect of the *ermB* cassette. Transcripts emanating from the *ermB* promoter might extend into downstream genes, especially when *ermB* expression is induced in the presence of erythromycin. These polar effects may obscure the true phenotype of a gene deletion. To test if the *ermB* cassette had a polar effect, we isolated RNA from strains HS0602 (pep_{27} replaced by *ermB*) and HS0603 (*vncR* replaced by *ermB*) grown in the presence or absence of erythromycin and performed Northern blot analysis (Fig. 2). An *ermB*-specific probe detected a transcript of 4 kb in the HS0602 mutant and a 3-kb transcript in the HS0603 mutant, both of which were also detected with a *vncS*-specific probe. The large transcript in the HS0602 mutant corresponds to a polycistronic transcript that includes *ermB*, *vncR*, and *vncS*, while the transcript seen in HS0603 corresponds to an mRNA that contains the *ermB* and *vncS* genes. The intensities of the bands were relatively weak in RNA samples from cultures grown in the absence of erythromycin and drastically increased when cultures were grown in the presence of erythromycin. These results suggest that insertion of the *ermB* cassette leads to the overexpression of 3′ genes, even in the absence of erythromycin induction of the *ermB* promoter. This hypothesis was supported by real-time PCR analysis (see below). Therefore, further clarification of the tolerance phenotype of the *vex3* mutant was undertaken to determine any possible contribution of overexpression of downstream genes.

Generation of mutants which carry an inverted *ermB* cassette. To distinguish the polar effect of the *ermB* cassette from the phenotype of the deletion mutants, an additional set of mutants was created with the *ermB* cassette cloned in the opposite orientation relative to the gene that was replaced (an example is shown in Fig. 1). Inversion of the *ermB* cassette did not change the slow rate of lysis of the *lytA* mutant HR1937 relative to HS1937 or of the rapidly lytic *vncS* mutant HR0604 relative to HS0604 (Fig. 3A and B, respectively), indicating that mutants bearing this inverse cassette demonstrated their true phenotype. Lysis and killing of the *vex3* mutant HR0601 (*ermB* inverted with respect to *vex-vnc*) indicated that it was even more tolerant than the *vex3* mutant HS0601 (*ermB* not inverted) (Fig. 3C). HR0601 lost only 37% of its initial optical density and showed a decrease of only 0.4 log CFU/ml, thereby fulfilling the standard definition for tolerance (11) (Table 2). The accentuation of tolerance of HR0601 over HS0601 demonstrated that the orientation of the resistance cassette was able to influence the phenotype of the mutant despite the fact that both strains carried a deletion mutation of the *vex3* gene.

***vex12* and *vncRS* are overexpressed in the *vex3* mutant HS0601, but not in HR0601.** Overexpression of genes downstream of the *ermB* cassette might account for the difference in tolerance of HS0601 and HR0601. Expression of the *vex* and *vnc* genes in the control mutant HS2155 and the two *vex3* mutants HS0601 and HR0601 was determined by reverse transcriptase PCR (RT-PCR) (Fig. 4A). Data were first normalized to the *gyrA* gene and then to strain HS2155, which was grown in the absence of antibiotics. While expression of the *vex* and *vnc* genes was not significantly changed in the control strain HS2155 with or without erythromycin, the amount of *ermB* transcript increased 5.6-fold in the presence of the drug, showing that erythromycin induces the *ermB* promoter and that erythromycin by itself does not alter the transcription of *vex123-vncRS*. Transcription of the *lytA* autolysin was indistinguishable for all three strains tested, regardless of the presence of erythromycin. This result demonstrates that the observed effects are specific to the *vex-vnc* locus and the *ermB* gene within it.

Expression of *vncR* and *vncS* was 12- and 8-fold higher in the HS0601 mutant than in the control strain, and these values more than doubled (were 26- and 22-fold higher) in the presence of the antibiotic, indicating that the erythromycin induced transcription of *vncRS* as the result of transcriptional read-through from *ermB*. Consistent with this result, transcription levels of *vncRS* in the HR0601 mutant remained identical to

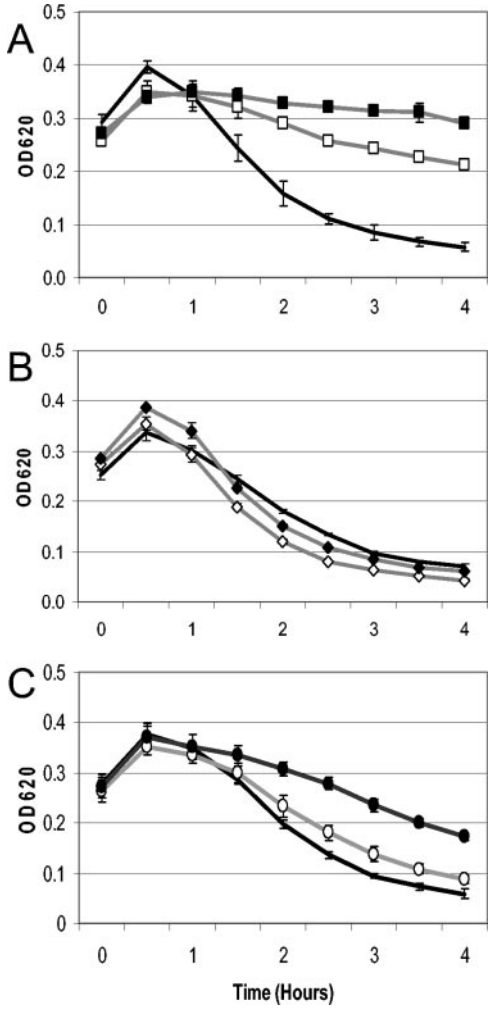

FIG. 3. Effect of the orientation of the *ermB* cassette on autolysis. Wild-type strain T4 (black line) served as the control for all panels. (A) Autolysis of the *lytA* autolysin mutants HS1937 (open squares) and HR1937 (filled squares). (B) Autolysis of the *vncS* histidine kinase mutants HS0604 (open diamonds) and HR0604 (filled diamonds). (C) Autolysis of the *vex3* transmembrane protein mutants HS0601 (open circles) and HR0601 (filled circles). All samples were treated with 10× MIC of vancomycin as the cultures reached an OD_{620} of 0.275 to 0.30. Means and standard deviations for three independent experiments are shown.

those of the control strain. This finding suggests that the deletion of *vex3* per se does not change the expression levels of the *vex-vnc* locus. A Western blot analysis of cellular extracts from strains T4, HS0603, and HS0601, using a VncR-specific antibody, showed a correlation between gene transcription and protein expression levels (Fig. 4B). Thus, the more tolerant mutant HR0601 showed no change in *vncRS* transcription, while the less tolerant HS0601 did show such a change. This result suggests either that tolerance is not a result of change in expression of *vncRS* or that increased expression of *vncRS* counteracts tolerance.

Effect of VncR on the transcription of *vex* or *vnc*. Expression of *vex1* and *vex2* in strain HS0601 in the absence of erythromycin was increased 76- to 96-fold over that in the control strain, and it increased a further 12-fold when erythromycin was present in the culture medium (expression of the deleted *vex3* gene was not detectable).

No such increased expression of *vex* genes was detected for strain HR0601, raising the question of whether *vex1* and *vex2* were being upregulated by VncR. To determine if increased levels of VncR and VncS were linked to increased *vex* gene transcription and decreased tolerance of the *vex3* mutant HS0601, a mutant was constructed to overexpress *vncR* from a noninte-

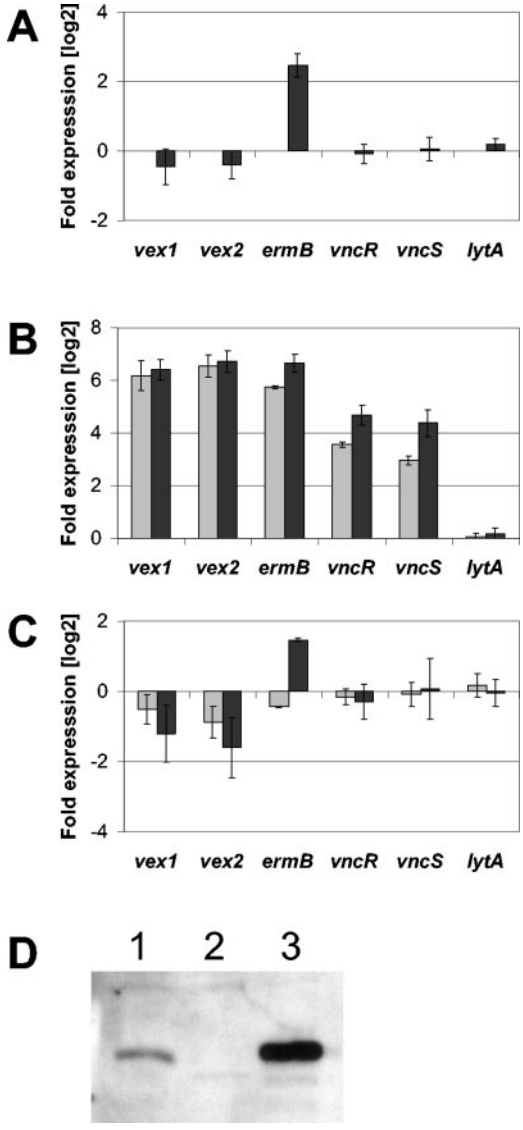

FIG. 4. Expression of *vex12* and *vncRS* in *vex3* mutants. (A) The control mutant HS2155 was grown in the absence (baseline) and presence (dark gray bars) of erythromycin, and total RNA was analyzed by real-time PCR analysis using primers specific for *vex1*, *vex2*, *ermB*, *vncR*, *vncS*, or *lytA*. Gene expression in strains HS0601 (B) and HR0601 (C) is depicted relative to RNA from strain HS2155 grown in the absence of erythromycin (baseline). Cells were grown in the absence (light gray bars) or presence (dark gray bars) of 1 μg of erythromycin/ml prior to RNA isolation. All data were normalized to the *gyrA* transcript and are expression levels on a $\log_2$ scale; the means and standard deviations for three independent experiments are shown. (D) Western blot of cellular extracts from strains T4 (lane 1), *vncR* mutant HS0603 (lane 2), and *vex3* mutant HS0601 (lane 3) probed with VncR-specific antibodies.

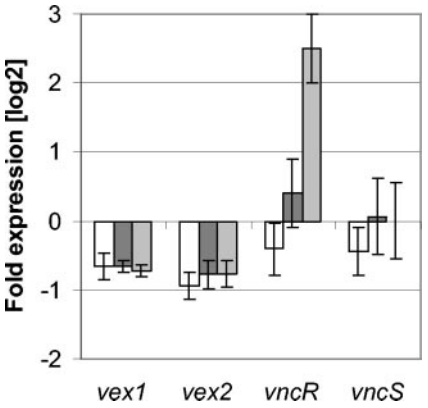

FIG. 5. Effect of *vncR* overexpression on *lytA*, *vex12* and *vncRS* mRNA levels. RNA from strains T4 (baseline), HR0601 (white), HR0601 (pDL278) (dark gray), and HR0601 (pWHM302) (light gray) was analyzed by real-time PCR analysis. Data were normalized to the *gyrA* gene and to RNA from strain T4. The data are expression levels on a $\log_2$ scale; the means and standard deviations for three independent experiments are shown.

grated plasmid. The −35 and −10 elements from the *fcsK* promoter and the *vncR* gene were cloned into the shuttle vector pDL278, yielding plasmid pWHM302. RNA from strains T4, HR0601, HR0601 (pDL278), and HR0601 (pWHM302) were used for real-time PCR analysis to determine if increased expression of *vncR* resulted in increased expression of *vex1* or *vex2* (Fig. 5). The *vncR* gene was 5.7-fold overexpressed in strain HR0601 (pWHM302) compared to strain T4, while *vncR* expression did not change significantly in strain HR0601 (pDL278). Expression of *vex1*, *vex2*, or *vncS* was not significantly altered in any of the three strains tested, demonstrating that the overexpression of *vncR* does not drive increased expression of these genes under the conditions tested. Vancomycin kill curves demonstrated that tolerance to vancomycin was not affected by the overexpression of *vncR* in HR0601 (pWHM302) (data not shown). These results suggest that fivefold overexpression of *vncR* alone is not sufficient to induce expression of the *vex123-vncRS* genes and that the expression level of VncR is independent of tolerance.

Microarray analysis of *vex3*, *vncR*, and *vncS* mutants. To examine genes whose expression is altered in the tolerant mutant HR0601, which might explain tolerance, microarray analysis was used to determine genes controlled by the *vncRS* two-component system (Table 3). Deletion of *vncR* in strain HS0603 upregulated four genes more than twofold: genes encoding ribosomal protein L31 and three cell wall surface anchor family proteins (SP0462 to SP0464) that appear to form one operon. Two genes, *pbuX* and *purK*, were downregulated in the *vncS* mutant HS0604. Although both genes are involved in purine metabolism, their connection to *vncRS* remains to be determined. These scant changes were in agreement with the absence of a phenotype with these deletions.

Seventeen genes were differentially regulated in the *vex3* mutant HS0601, including three hypothetical proteins, cell wall anchor family proteins, and the *vex123-vncRS* locus itself. The expression of *vncRS* was increased 14-fold and that of *vex12* was increased more than 100-fold, values in good agreement with the RT-PCR results. The expression of the gene encoding hypothetical protein SP0598 was also increased (62-fold), although this result may be an artifact since 66 bp of the 162-bp reading frame for SP0598 overlap with the *vex1* gene.

Microarray analysis of RNA from the mutant strain HR0601 showed that 58 genes were differentially regulated greater than twofold. Only the gene encoding hypothetical protein SP2233 was downregulated greater than twofold in both *vex3* mutants HS0601 and HR0601. SP1394, annotated as the gene encoding amino acid transporter-amino acid binding protein, is the only gene that was downregulated in HS0601 and upregulated in HR0601 at statistically significant levels. Differential regulation of these 57 genes may represent part of the cellular response to the loss of *vex3* gene expression that results in tolerance.

DISCUSSION

The main objective of this study was to reexamine the role of the *vex-vnc* locus in vancomycin tolerance in *S. pneumoniae*. Early reports had indicated that the *vex123*, pep_{27}, and *vncRS* genes played a major role in autolysis and vancomycin tolerance (22, 23). Later studies by Robertson and coworkers (26) challenged this model, suggesting that erythromycin in the culture medium engendered the vancomycin-tolerant phenotype. Our results confirmed elements of both studies. In agreement with the work of Robertson et al., deletions in pep_{27}, *vncR*, and *vncS* did not confer vancomycin tolerance. Consistent with the work of Novak et al., deletion of *vex3* resulted in mutants that showed various levels of vancomycin tolerance. The basis for this reduced rate of vancomycin-induced lysis remains to be determined.

In studies of the 13 complete pneumococcal histidine kinase response regulator pairs (16, 31), deletion of the *vncRS* two-component system did not change viability, growth rate, competence (16), or virulence in the lung (31). The *vncRS* genes were deemed not essential (30) and not important for virulence in a mouse respiratory tract infection model (31). Our microarray analyses failed to suggest a function for VncRS, stressing that the role of this two-component system in pneumococcal biology remains to be determined. A point mutation at position 440 in VncS was thought to cause the vancomycin tolerance of three type 9V clinical isolates on the basis of the similar phenotype of the analogous laboratory mutant SPSJ01 (23). However, since the mutant was generated by insertion duplication mutagenesis by using plasmid pJDC9, which contains an *erm* resistance gene, this interpretation appears to be no longer valid and the underlying defect of these clinical isolates remains to be determined. The tolerance of the laboratory-constructed *vncS* deletion mutant (22) can be attributed to either of two artifacts introduced by the antibiotic selection. Ng and coworkers determined that a number of genes are differentially regulated in response to erythromycin (21). One or more of these genes could be involved in vancomycin tolerance. Alternatively, the reduced rate of autolysis could be the result of the decreased growth rate of cultures exposed to erythromycin. In the case of the *vex3* mutants HS0601 and HR0601, however, tolerance is not the result of a decrease in growth rate since the strains' growth rates are comparable to those of the wild-type strain T4.

A recent publication by Ortega et al. (24) reported that the

TABLE 3. Microarray results showing genes that are differentially regulated in the *vncR* mutant HS0603, the *vncS* mutant HS0604, and the *vex3* mutants HS0601 and HR0601 in the absence of erythromycin[a]

Microarray ID[b]	Annotation	Fold change for:			
		HS0603	HS0604	HS0601	HR0601
vex-vncRS locus					
SP0598	Hypothetical protein			62.0	
SP0599	Transmembrane protein, *vex1*			114.7	
SP0600	ABC transporter, ATP-binding protein, *vex2*			121.6	
SP0601	Transmembrane protein, *vex3*			−33.0	−10.4
SP0603	DNA-binding response regulator, *vncR*	−150.6		14.8	
SP0604	Sensor histidine kinase, *vncS*		−45.5	14.5	
Transport functions					
SP0729	Cation-transporting ATPase, E1–E2 family				−2.5
SP1062	ABC transporter, ATP-binding protein				2.1
SP1215	Transporter, FNT family, putative				−2.7
SP1241	Amino acid ABC transporter, permease protein				2.9
SP1242	Amino acid ABC transporter, ATP-binding protein				2.4
SP1394	Amino acid ABC transporter			−1.7	2.0
SP1527	ABC transporter, oligopeptide-binding protein, *aliB*			−2.0	
SP1572	Nonheme iron-containing ferritin				2.5
SP1848	Xanthine permease, *pbuX*		−2.0		
SP1871	Iron-compound ABC transporter				−3.0
SP2108	Maltodextrin ABC transporter, *malX*				−1.7
SP2109	Maltodextrin ABC transporter, *malC*				−2.0
SP2110	Maltodextrin ABC transporter, *malD*				−1.8
Ribosomal proteins					
SP0441	Ribosomal protein L28, *rpmB*			−2.4	
SP1107	Ribosomal protein L27, *rpmA*				2.3
SP1299	Ribosomal protein L31, *rpmE*	2.0			
Biosynthesis and degradation of polysaccharides					
SP0107	LysM domain protein				2.8
SP0268	Alkaline amylopullulanase, putative				−2.4
SP2106	Glycogen phosphorylase family protein				−2.2
SP0240	Phosphoglycerate mutase family protein				2.1
SP0285	Alcohol dehydrogenase, zinc containing				−2.2
SP0447	Ketol acid reductoisomerase, *ilvC*				2.1
SP0502	Glutamine synthetase, *glnA*				3.4
SP0730	Pyruvate oxidase, *spxB*				−2.1
SP0837	DNA topology modulation protein FlaR, putative				2.3
SP0965	Endo-beta-*N*-acetylglucosaminidase, *lytB*				2.0
SP1052	Phosphoesterase, putative				2.1
SP1113	DNA-binding protein HU, *hup*				2.1
SP1470	Thiamine biosynthesis protein ApbE, putative				−2.6
SP1471	Oxidoreductase, putative				−3.1
SP1472	Oxidoreductase, putative				−2.8
SP1963	CBS domain protein				2.2
SP2026	Alcohol dehydrogenase, iron containing				−4.0
SP2056	*N*-acetylglucosamine-6-P deacetylase, *nagA*				−2.1
SP2157	Alcohol dehydrogenase, iron containing				−2.6
SP2216	Secreted 45-kDa protein, *usp45*				2.4
Hypothetical proteins					
SP0088	Hypothetical protein				2.0
SP0097	Conserved domain protein				−3.7
SP2107	4-Alpha-glucanotransferase, *malQ*				−2.0
Regulatory functions					
SP0501	Transcriptional regulator, MerR family				3.3
SP0837	DNA topology modulation protein FlaR, putative				2.3
SP1061	Protein kinase, putative				2.7
SP1362	Putative transcriptional regulator, *mecA*				2.0
SP1809	Transcriptional regulator				2.0
SP1821	Sugar-binding transcriptional regulator, LacI family				2.1
SP2234	Transcriptional regulator, TetR family			−2.2	
SP2235	Response regulator, *comE*				2.9
SP2236	Putative sensor histidine kinase, *comD*				2.5

Continued on following page

TABLE 3—*Continued*

Microarray ID[b]	Annotation	Fold change for:			
		HS0603	HS0604	HS0601	HR0601
Cell surface proteins					
SP0462	Cell wall surface anchor family protein	2.7		4.6	
SP0463	Cell wall surface anchor family protein	3.1		4.2	
SP0464	Cell wall surface anchor family protein	2.6		4.3	
SP2190	Choline binding protein A, *cbpA*			3.3	
Various functions					
SP0054	Phosphoribosylaminoimidazole carboxylase, *purK*		−2.5		
SP0098	Hypothetical protein				−4.4
SP0099	Hypothetical protein				−4.2
SP0100	Conserved hypothetical protein				−3.2
SP0430	Hypothetical protein				−2.2
SP0448	Hypothetical protein				2.4
SP0728	Hypothetical protein				−2.0
SP0742	Conserved hypothetical protein				2.8
SP0861	Hypothetical protein				2.2
SP0958	Hypothetical protein				−2.2
SP1053	Conserved domain protein				2.1
SP1059	Hypothetical protein				3.3
SP1140	Hypothetical protein				2.0
SP1141	Hypothetical protein				2.1
SP1145	Hypothetical protein				2.0
SP1793	Hypothetical protein				2.0
SP1810	Hypothetical protein				2.0
SP2191	Conserved hypothetical protein			2.3	
SP2232	Conserved hypothetical protein, authentic frameshift			−4.3	
SP2233	Hypothetical protein			−3.4	−3.7

[a] Mean changes (*n*-fold) from three independent biological samples are shown. Genes that are differentially regulated more than twofold are shown. Statistical significance was verified by ANOVA.
[b] Microarray ID, identification number from microarray analysis.

cell wall-inhibiting antibiotics penicillin or cefotaxime resulted in a great decrease in CFU when the antibiotics were given individually compared to results with the same antibiotics given jointly with erythromycin. This finding agrees with the present and published findings (19, 26) and highlights the need to better understand the effects that antibiotics have on bacteria.

Interpretation of the tolerant phenotype of the HS0601 mutant generated by gene replacement must consider the polar effects generated by the antibiotic resistance cassette. Overexpression of *vncS*, located 3′ to the resistance cassette, was demonstrated with the pep_{27} and *vncR* mutants HS0602 and HS0603, respectively. The *ermB* promoter used to generate the mutants in this study was shown to be constitutively active at low levels and inducible by erythromycin. Read-through transcription from this promoter may explain the high level of *vncRS* transcripts evident by RT-PCR and microarray analyses of the *vex3* mutant HS0601. However, fivefold overexpression of *vncR* in strain HR0601 (pWHM302) did not increase the rate of transcription of the *vex* or *vnc* genes, suggesting that *vncS* needs to be overexpressed as well or that other factors are involved in the high level of expression of the *vex12* genes in strain HS0601. It also remains possible that higher expression of *vncR* or *vncRS* may be required to change *vex12* expression.

Robertson et al. (26) reported that *vex123* expression is derepressed in the absence of *vncS*, a result that we were unable to confirm by microarray analysis. This inconsistency may be due to several factors, such as differences in pneumococcal strains (R6 versus T4) or growth media (CDM versus C+Y) or the method of generating mutants (partial versus complete gene replacement). Alternatively, the same factors that lead to overexpression of *vex12* and *vncRS* in the *vex3* mutant HS0601 might have played a role in Robertson's work as well.

The *vex3* mutant HS0601 produced large amounts of *vex12* mRNA, but was tolerant to vancomycin only to a small degree. In contrast, the *vex3* mutant HR0601 produced less *vex12* mRNA than the control or wild-type strain, but was more tolerant to vancomycin. One possible explanation for these results is that the Vex123 ABC transporter is involved in tolerance, a phenotype that is evident in strains lacking *vex3*. This phenotype is strongest in HR0601 and might be partially attenuated in HS0601 because overexpression of Vex1 might be able to compensate for the loss of Vex3. The two proteins have a similar domain organization, with an FtsX-like permease domain at the carboxy terminus, and share 28% sequence identity at the amino acid level (http://smart.embl-heidelberg.de/). Verification of this model and determination of the function of Vex123 in pneumococcal cell biology will require further investigation.

Further evidence for the role of the *vex123* genes in tolerance comes from a study by Rodriguez et al. (27), in which the *vex123-*pep_{27} genes from tolerant and nontolerant strains were sequenced. The authors found two alleles each for the *vex2* and pep_{27} genes, one allele combination exemplified by strain T4 and the other by strain R6. The mixed pairing of R6 pep_{27} and T4 *vex2* alleles was frequently found with tolerant isolates, whereas nontolerant isolates mostly possessed pairs of alleles restricted to either T4 or R6 only. The T4 pep_{27} and R6 *vex2*

alleles were never found together in any strain, indicating a strong negative selection. These findings suggest that the sequence of the *vex* locus is an epidemiological marker for tolerance. The present study indicates that this marker may be related to the biological function of Vex123, the absence of which leads to tolerance.

The results presented in the present study indicate that the original model of a death signaling pathway consisting of the *vex123-pep*$_{27}$*-vncRS* gene products must be corrected. Tolerance can arise by loss of function of Vex3. It is possible that high expression levels of Vex1 can functionally substitute for the lack of Vex3, thereby attenuating the tolerant phenotype. This phenotype is not related to increased expression of VncR, since the VncRS two-component system does not appear to regulate transcription of *vex123*. Vancomycin tolerance is a multifactorial phenomenon arising from the effects of the bacterial capsule (6), erythromycin treatment (23), the autolysin LytA (32), and the ABC transport system Vex123. The connection between these factors and stationary phase autolysis remains to be determined.

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