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An ABC Transporter of *Streptococcus pneumoniae* Involved in Susceptibility to Vancoresmycin and Bacitracin[▽]

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Vancoresmycin is a novel tetramic acid antibiotic, probably interfering with functions of the cytoplasmic membrane. To investigate its mode of action, mutants of *Streptococcus pneumoniae* exhibiting reduced susceptibility to vancoresmycin were isolated at a low frequency. Four of them were further examined and showed similar pleiotropic phenotypes, including reduced growth rate, early autolysis, and chain formation. In one mutant, the level of transcripts from a single locus encoding the potential ABC transporter Spr0812-Spr0813 was increased sixfold. The corresponding DNA sequence revealed a nonsense mutation (C1744T) in spr0813, leading to the formation of a truncated permease lacking 2 of the 10 predicted transmembrane helices. As demonstrated by deletion and transformation analysis and reconstructing the spr0813$_{C1744T}$ mutation in the wild-type background, this nucleotide exchange was sufficient to cause reduced susceptibility to vancoresmycin and higher amounts of spr0812-spr0813 mRNA. Mapping and reporter assays of the cognate promoter P$_{abc}$ showed that spr0812 and spr0813 are cotranscribed with a preceding small gene and that the spr0813$_{C1744T}$ mutation does not affect the activity of P$_{abc}$. Due to the similarity of Spr0812-Spr0813 to bacitracin transporters of *Streptococcus mutans* and *Bacillus* spp., the bacitracin susceptibility of spr0813 mutants was examined. Both the spr0813$_{C1744T}$ nonsense mutation and the deletion of the transporter genes led to a clearly increased sensitivity to bacitracin. Thus, the intact transporter is required for intrinsic resistance to bacitracin, whereas reduced vancoresmycin susceptibility is mediated by the truncated permease.

Antibiotic-resistant bacterial pathogens are of growing concern worldwide. Thus, extensive and ongoing efforts are required to screen for new drugs active against clinically relevant bacteria such as *Streptococcus pneumoniae*, one of the major human pathogens. Derivatives of tetramic acid (2,4-pyrrolidinedione) represent one group of chemical compounds with a high potential of antibacterial activity (28); however, there is currently no drug of this type in the market. A number of tetramic acid derivatives with antibacterial activity have been detected by testing natural or or chemically synthesized products (see reference 37 [and references therein] and references 24 and 31).

The antimicrobial mechanism of action for most of these compounds is not well characterized. Notable exceptions are reutericyclin (produced by *Lactobacillus reuteri*), which acts as a proton ionophore, dissipating the transmembrane proton potential (4, 5), and several submicromolar inhibitors of undecaprenyl pyrophosphate synthase that were recently designed on the basis of a pharmacophore hypothesis (24). This points to the cytoplasmic membrane as the cellular target of tetramic acid antibiotics and is consistent with the observation that their activity can be improved by introducing more lipophilic side chains at the N-substituted position (37). Partitioning of such compounds into the cytoplasmic membrane is hampered by the asymmetrical gram-negative outer membrane (20), which is the likely reason for their selective activity against gram-positive bacteria (4, 10).

Vancoresmycin (Var) is a natural tetramic acid derivative produced by a strain of the actinomycete *Amycolatopsis* sp. It exhibits potent antibiotic activity against gram-positive bacteria (including *S. pneumoniae* and vancomycin-resistant *Enterococcus* spp.), whereas gram-negative bacteria and fungi are not inhibited. At the 3 position of the tetramic acid core, Var carries a C-45 long partially unsaturated and highly oxygenated alkyl chain replaced by an aminoglycoside (10). Since this structure does not show obvious similarities to the activity-related pharmacophores of reutericyclin or undecaprenyl pyrophosphate synthase inhibitors, there is no reliable clue to the mode of action of Var. We addressed this issue by the isolation and transcription profiling of mutants of *S. pneumoniae* with reduced sensitivity to this antibiotic.

Although the mode of action of Var was not determined, a truncated ABC transporter with protein homology to bacitracin transporters from other species was identified that could confer reduced Var susceptibility and that also appears to be involved in bacitracin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, growth conditions, and transformation. *S. pneumoniae* strains and plasmids used in this work are listed in Table 1. PCR primers were synthesized at Operon Biotechnologies and are listed in Table 2. Primers used for sequencing and confirming the correct integration of DNA sections delivered to the *Streptococcus pneumoniae* genome are not listed. *S. pneumoniae* was grown in C-medium (15) supplemented with 0.2% yeast extract at 37°C without aeration or on D agar supplemented with 3% defibrinated sheep blood (Oxoid). Growth of *S. pneumoniae* in liquid cultures was monitored by nephelometry. MICs of antibiotics were determined by agar dilution using Var concentrations in the range of 0.3 to 0.8 μg/ml (0.05 μg/ml

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TABLE 1. *S. pneumoniae* strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
Strains		
R6	Wild type	22
VarA	R6, Var^r	This work
VarE	R6, Var^r	This work
VarF	R6, Var^r	This work
VarG	R6, $spr0813_{C1744T}$, Var^r	This work
VarGt	R6 transformed with DNA containing $spr0813_{C1744T}$, selected on Var, Var^r	This work
VarGc	R6 transformed with DNA containing $spr0813_{C1744T}$::*aphIII*, selected on Kan, Kan^r Var^r	This work
VarAΔcopY	VarA, *rpsL*, Δ*copY*, Str^r Var^r	This work
VarEΔcopY	VarE, *rpsL*, Δ*copY*, Str^r Var^r	This work
VarFΔcopY	VarF, *rpsL*, Δ*copY*, Str^r Var^r	This work
VarAΔcyl	VarA, Δ*cyl*::*aad9*, Spc^r Var^r	This work
VarEΔcyl	VarE, Δ*cyl*::*aad9*, Spc^r Var^r	This work
VarFΔcyl	VarF, Δ*cyl*::*aad9*, Spc^r Var^r	This work
R6Δabc	R6, Δspr0811a-spr0813::*aphIII*, Kan^r	This work
VarGΔabc	VarG, Δspr0811a-spr0813::*aphIII*, Kan^r	This work
R6-P_{abc}	R6, *bgaA*::*tetM*-P_{abc}*lacZ*, Tet^r	This work
VarG-P_{abc}	VarG, *bgaA*::*tetM*-P_{abc}*lacZ*, Tet^r Var^r	This work
VarGt-P_{abc}	VarGt, *bgaA*::*tetM*-P_{abc}*lacZ*, Tet^r Var^r	This work
VarGc-P_{abc}	VarGc, *bgaA*::*tetM*-P_{abc}*lacZ*, Tet^r Var^r	This work
RP100	R6, *bgaA*::*tetM-lacZ*, Tet^r	7
RP204	R6, *bgaA*::*tetM*-P_{vegM}*lacZ*, Tet^r	7
Plasmids		
pUC19	ColE1, P_{lac}*lacZ′*, *bla*	36
pdel17	pUC19 with Δ*cyl*::*aad9* flanked by fragments of *S. pneumoniae* DNA in SmaI site	This work
pCR2.1spc	ColE1, *bla*, *neo*, *aad9*	18
pPP2	ColE1, integrative promoter probe vector for *S. pneumoniae*, *htrA′-′lacZ* reporter gene, *bla*, *tetM*	7
pPP2Pabc	pPP2, P_{abc}*lacZ*	This work

intervals) and bacitracin concentrations in the range of 0.5 to 6 μg/ml (0.2 μg/ml intervals). Antibiotic resistance genes used for chromosomal integrations in *S. pneumoniae* were selected with 80 μg/ml spectinomycin (Spc [*aad9*]), 200 μg/ml streptomycin (Str [*rpsL*]), 100 or 20 μg/ml kanamycin (Kan [*aphIII*, with or without promoter]), and 3 μg/ml tetracyclin (Tet [*tetM*]), respectively. Various concentrations of Var were prepared from a stock solution (0.5 mg/ml) in methanol and kept at −20°C. Transformation of *S. pneumoniae* was performed using naturally competent cells as previously described (18).

For cloning in the pPP2 vector, *Escherichia coli* DH5α [ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*] (35) was used as an intermediate host. *E. coli* was cultivated in LB media (30) and transformed by using chemically competent cells (8).

RNA extraction. Total RNA was extracted from *S. pneumoniae* by a modified hot phenol procedure as described previously (19). For each strain, cells harvested from two independent 100-ml cultures at a density of 80 nephelometric turbidity units were used. After final precipitation, washing, and drying of the nucleic acids, they were redissolved in 300 μl of diethylpyrocarbonate-treated water. DNA was then digested by the addition of 24 U of RNase-free DNase (NEB) in 33 μl of 10× DNase buffer (NEB) and incubation for 10 min at 37°C. The RNA was further purified using a Qiagen RNeasy minikit according to the manufacturer's instructions.

Microarray-based transcriptome analysis. The microarray used (obtained from MWG Biotech AG) carried 50-mer oligonucleotide probes for all *S. pneumoniae* R6 annotated genes (11), each spotted in duplicate onto Schott Nexterion E slides.

Reverse transcription of RNA into labeled cDNA, prehybridization, hybridization, slide washing, scanning, and analysis of the data were performed exactly as described by McKessar and Hakenbeck (19). Only genes which showed reproducible changes in the transcript amount that were greater than threefold were considered further.

DNA manipulations. Plasmid isolation and routine DNA manipulations were carried out by standard methods (30). Chromosomal DNA was isolated from *S. pneumoniae* as described earlier (12), and PCR products and DNA recovered after restriction endonuclease digestions were purified using the a JETquick spin column technique kit (Genomed) or a NucleoSpin Extract II kit (Macherey-Nagel).

Restriction enzymes and T4 DNA ligase were purchased from Roche Applied Science and used according to the manufacturer's instructions. PCRs were performed using either Goldstar Red *Taq* polymerase (Eurogentec) or iProof high-fidelity DNA polymerase (Bio-Rad) according to the manufacturer's instructions. Nucleotide sequencing was performed using an ABI Prism BigDye Terminator ready-reaction cycle sequencing kit (version 3.1; Perkin Elmer-ABI). Nucleotide sequences were analyzed by using CloneManager and Chromas software.

Construction of delivery cassettes and plasmids. An in-frame deletion (Δ*copY*) in *copY* was constructed via a two-step process in which the central part of the gene was first replaced with the Janus cassette (33), conferring a Kan^r Str^s phenotype in an Str^r background. In the second step, the Janus cassette was deleted, thus restoring the original Str^r phenotype. Two "integration fragments" flanking the central part (346 bp) of *copY* were amplified from chromosomal DNA of *S. pneumoniae* R6 by using primer pair cop_up1 and copYrev_JanusLinker and primer pair copYfwd_JanusLink and copY_down1 to obtain two PCR products (1,253 and 982 bp), each overlapping with one end of the Janus cassette by 29 bp and 28 bp, respectively. These fragments were mixed with the Janus cassette (1,359 bp), and after annealing, the desired product consisting of the Janus cassette flanked by the two "integration fragments" was amplified by using the nested primers cop_up2 and copY_down2. This product was used to transform Str^r derivatives of VarA, VarE, and VarF, which were obtained by transformation of these strains with chromosomal *S. pneumoniae* DNA carrying the AmiA9 resistance marker (29). In the resulting Kan^r Str^s transformants, the correct position of the Janus cassette was confirmed by DNA extraction and PCR with appropriate primers. Two PCR products (1,247 bp and 981 bp) were generated separately using primer pair cop_up1 and Mini-copY_rev and primer pair Mini-copY_fwd and copY_down1 to obtain overlapping fragments flanking the desired *copY* deletion. These products were then mixed and subjected to further PCR amplification with the nested primers cop_up2 and copY_down2 to obtain a product containing the deletion and the flanking DNA regions. This product was used to transform the derivatives of VarA, VarE, and VarF carrying the integrated Janus cassette. DNA from transformants displaying a Kan^s Str^r phenotype was amplified by PCR and sequenced to confirm the deletion in the resulting strains VarAΔcopY, VarEΔcopY, and VarFΔcopY.

The spr1764-spr1773 region (*cylM* gene cluster) of *S. pneumoniae* R6 was

TABLE 2. Primers

Primer	Sequence[a]
17xxspec_for	GTTACATTTGCATTGCTAGGATCGATTTTCGTTCGTGAATAC
17xxspec_rev	GAGCCTTCAACACAGATTACCATATGCAAGGGTTTATTG
274-lacZ	GGGAAGGGCGATCGGTGCGGG
abcB-down_r	ACGGCAGATAATGGAACGGAATTCAATCGC
abcB-down_rr	TTCTGAGGAGCACATCTATTATGCGCACCC
abcB-downSal_f	tagtgggtcgaCCAAATGTAAAAAAAGATACCTCGACTTCAAAATCGAGG SalI
abcB-up_f	CCTTCTTCATAGCTGTTTTACTGGTTATCTTTGGG
abcB-up_ff	TGTTTAATGCAGGGATTACCGTTTTCCTCC
abcB-upBam_r	cgcggatccTTTA*ATCTAAACCGACTTTCTGCAAGATAATAAAGCGC BamHI
aph3Bam_f	cgcggatccAAAGAGGAAGGAAATAATAAATGGCTAAAATGAGAATATCACC BamHI
aph3Sal_r	tagtgggtcgaCGAACCAATTGGTTAATTGTAGGCATCTACATTCTCC SalI
aphIII_fwd	ATGGCTAAAATGAGAATATCACCGG
aphIII_rev	CTAAAACAATTCATCCAGTAAAATATAATA
bgaA_rev	GCTAAACCTGCTGCTACTGCTGC
cop_up1	CTCTAATTCCCTTAAAGTGGGAACTAGAG
cop_up2	TTGACTGGACCAGAACCATGACCGAGTTGAG
copY_down1	GAGTTCCCAGCGCAACCAAGGTATCC
copY_down2	CCTGCAACTAACATAATAGGCGTTGTTGC
copY_fwd+JanusLin	ACGTCCAAAAGCATAAGGAAAGGGGCCCAAGTAAGATGTAATTGTATGTAAAGGAGACG
copY_rev+JanusLin	CGGATCCGATCCATTTCCTCTGGAATAGGCCTGCCATTCTGCATCTGAAATCTGCAT
ko-TR_aphIII_fwd	TATTATATTTTACTGGATGAATTGTTTTAGAAAAAGATACCTCGACTTCAAAATCGAGG
L17xx_for	TCCAGCATTTCCTACAAGAATAAGTAGAGG
L17xx_rev	ATTCACGAACGAAAATCGATCCTAGCAATGCAAATGTAAC
Mini-copY_fwd	ATGCAGATTTCAGATGCAGAATGGCAGGAAGTAAGATGTAATTGTATGTAA
Mini-copY_rev	TTACATACAATTACATCTTACTTCCTGCCATTCTGCATCTGAAATCTGCAT
Proorf1_Kanlink_rev	CCGGTGATATTCTCATTTTAGCCATGATGTTACTCCTTTGTTCTTTATGAGTC
Pspr0812_fwd1	gacgcatgcCTTATATTTTACAACTTTAAAAATAGG SphI
Pspr0812_rev1	cgcggatccCTTTATGAGTCTAGTTTACATCAAAAAAAG BamHI
R17xx_for	ACAATAAACCCTTGCATATGGTAATCTGTGTTGAAGGCTC
R17xx_rev	CAGAAGAGTTGATTGACCAGCACTATCAGG
RACE-PCR_5	GATATGCGCGAATTCCTG
RTgyrAf	TATCACAGCAGTACGTGATGAG
RTgyrAr	GGATAGCGAGCATATTGAAACC
RTspr0812_fwd5	AAGTCGTGGTCAGGTTTAC
RTspr0812_rev7	GCGGAAGCAAGATATTGTC
RTspr0813_fwd1	AAACCGCAAACTCTACTATCC
RTspr0813_rev1	GACGACAAACATACCAAATCC
spr0812_down3	TGCTTGTCTTGACGGATCGAAAGAGTAG
spr0812_down4	TTCTTTCCATCACGGCAGATAATGGAAC
spr0812_rev5	GCGGTGTCAGTTCCATTCAAGTAAACC
spr0812_rev6	GATTTACCAGAACCAGACTCACCC
spr0812_up1	ACAGGCTGTAATTTAGTCGGCAATGTGAAG
spr0812_up2	ATTAATAATATTTCGCCAGCTTCATCC

[a] The nucleotides printed in lowercase letters were added to introduce the underlined cleavage sites for the restriction enzymes indicated next to the sequences. The position of the $C_{1744}T$ exchange introduced into spr0813 by the use of the mutagenic primer abcB-upBam_r is indicated by an asterisk.

exchanged for the Spc^r marker *aad9* (Δ*cyl*::*aad9*) with the help of plasmid pdel17, which contains the *aad9* gene between two "integration fragments" corresponding to the flanking regions of the desired deletion. The "integration fragments" were amplified from chromosomal strain R6 DNA by using primer pair L17xx_for and L17xx_rev and primer pair R17xx_for and R17xx_rev to obtain two PCR products (996 bp and 820 bp), each overlapping by 20 bp with one end of the *aad9* cassette. These fragments were joined with the *aad9* cassette (1,198 bp; obtained by amplification from the pCR2.1spc plasmid with the primer pair 17xxspec_for and 17xxspec_rev) in two consecutive rounds of overlapped PCRs. The resulting product was ligated with SmaI-linearized pUC19 vector, and the desired plasmid, pdel17, was isolated after transformation of *E. coli* DH5α. pdel17 was used to transform *S. pneumoniae* VarA, VarE, and VarF. DNA from Spc^r transformants was amplified by PCR and sequenced to confirm replacement of spr1764-spr1773 with *aad9* in the resulting strains VarAΔcyl, VarEΔcyl, and VarFΔcyl.

To delete the spr0811a-spr0813 genes (encoding a putative ABC transporter), they were replaced with the promoterless Kan^r *aphIII* gene (Δspr0811a-spr0813::*aphIII*). Two "integration fragments" flanking the desired deletion were amplified from chromosomal R6 DNA by using primer pair spr0812_up1 and Proorf1_Kanlink_rev and primer pair ko-TR_aphIII_fwd and spr0812_down3 to obtain PCR products (752 bp and 1,161 bp), each overlapping with one end of the *aphIII* gene by 22 bp and 30 bp, respectively. These fragments were mixed with the *aphIII* DNA (795 bp; obtained by amplification from the Janus cassette with the primer pair aphIII_fwd and aphIII_rev), and the desired product containing the *aphIII* gene flanked by the two "integration fragments" was amplified by using the nested primers spr0812_up2 and spr0812_down4. This product was used to transform *S. pneumoniae* VarG and R6. DNA from Kan^r transformants was amplified by PCR and sequenced to confirm replacement of spr0811a-spr0813 with *aphIII* in the resulting strains VarGΔabc and R6Δabc.

A derivative of R6 carrying the C1744T nonsense mutation in spr0813 was constructed by introducing this nucleotide exchange together with the Kan^r marker *aphIII* (spr0813$_{C1744T}$::*aphIII*). Two "integration fragments" (1,071 bp and 1,030 bp) flanking a 232-bp 3′-terminal part of spr0813 were amplified from chromosomal R6 DNA by using primer pair abcB-up_f and abcB-upBam_r (introducing the C1744T exchange) and primer pair abcB-downSal_f and abcB-down_r, and the promoterless *aphIII* gene (904 bp) was amplified from the Janus cassette by using the primer pair aph3Bam_f and aph3Sal_r. The three resulting DNA fragments were restricted with BamHI and SalI as appropriate and ligated, and the desired product (2,886 bp) was amplified from the ligation mixture by using the nested primers abcB-up_ff and abcB-down_rr. This product was used to transform *S. pneumoniae* R6. DNA from Kan^r transformants was amplified by PCR and sequenced to confirm the presence of the spr0813$_{C1744T}$ mutation in the resulting VarGc strain.

To assay the promoter of the spr0811a-spr0813 genes (P_{abc}), a P_{abc}*lacZ* re-

porter fusion was constructed. A 176-bp fragment carrying P_{abc} was amplified from chromosomal DNA of *S. pneumoniae* R6 by using the primer pair Pspr0812_fwd1 and Pspr0812_rev1. The PCR products were cleaved with SphI and BamHI and ligated with the SphI- and BamHI-digested promoter probe pPP2 vector, and the desired plasmid, $pPP2P_{abc}$, was isolated after transformation of *E. coli* DH5α. $pPP2P_{abc}$ was used to transform *S. pneumoniae* R6, VarG, VarGt, and VarGc. DNA from Tet^r transformants was amplified by PCR and sequenced to confirm the presence of the P_{abc}*lacZ* fusion in the resulting R6-P_{abc}, VarG-P_{abc}, VarGt-P_{abc}, and VarGc-P_{abc} strains.

Realtime RT-PCR. Quantification of RNA levels by real-time reverse transcriptase PCR (RT-PCR) was carried out as previously described (19). For each strain, RNA prepared from two independent cultures was used, and each sample was measured in duplicate. Primer pairs were designed for the amplification of products of 149 to 154 bp in length. As an unregulated control, the gyrase *gyrA* gene was probed with the primer pair RtgyrAf and RTgyrAr. For detection of mRNA transcribed from spr0812 and spr0813, primer pair Rtspr0812_fwd5 and RTspr0812_rev7 and primer pair Rtspr0813_fwd1 and RTspr0813_rev1 were used.

Determination of transcriptional start site. The start point of spr0811a-spr0813 transcription was determined by 5′ rapid amplification of cDNA ends (RACE) as described previously (13). The primer spr0812_rev5 was used for reverse transcription of RNA ligated to the RNA adapter, and the nested primer spr0812_rev6 was used for amplification of cDNA. The PCR products were analyzed using a 2% agarose gel, and the nucleotide sequence of the resulting fragment was determined.

Determination of β-galactosidase activity. Preparation of cell extracts from cultures of *S. pneumoniae*, grown to a density of 80 to 90 nephelometric turbidity units in C-medium, and determination of specific β-galactosidase activities were performed as described by Halfmann et al. (7).

TABLE 3. Properties of strains exhibiting reduced Var susceptibility

Strain	Growth			MIC [μg/ml][d]	
	Rate (min^{-1})[a]	Time of autolysis (h)[b]	Chain formation[c]	Var	Bacitracin
R6	0.022	6.0	−	0.4	4.8
VarA	0.022	3.9	++	0.7	5.2
VarE	0.021	3.0	+	0.7	5.2
VarF	0.020	3.4	++	0.65	5.2
VarG	0.018	4.0	++	0.5	1.0
VarGt	0.019	ND[e]	ND	0.5	1.0
VarGc	ND	ND	ND	0.5	1.0
R6Δabc	0.022	ND	ND	0.4	1.0
VarGΔabc	0.019	ND	ND	0.4	1.0

[a] Bacterial growth was monitored by nephelometry and is expressed in nephelometry units (NU). Growth rates were calculated as $\ln NU_{t2} - \ln NU_{t1}/t2 - t1$, where $t1$ and $t2$ are limits of a time interval during exponential growth.

[b] Values represent the time between the start of the stationary phase and the onset of autolysis. The time point of entering the stationary phase was determined from semilogarithmically blotted growth curves as the intersection of two lines obtained by elongating the linear sections of the blots representing exponential and stationary growth.

[c] −, mainly diplococci; +, mainly short chains of 4 to 8 cells; ++, mainly long chains of more than 10 cells.

[d] Mean values calculated from the results of three independent experiments.

[e] ND, not determined.

RESULTS

Spontaneous mutants of *S. pneumoniae* show reduced susceptibility to Var. The MIC of Var for the *S. pneumoniae* R6 strain was 0.4 μg/ml. This concentration of the antibiotic reduced the number of colony-forming cells about 2,000-fold. In the presence of 0.5 μg of Var/ml, colonies were obtained with a frequency of 2×10^{-7}; at 0.6 μg/ml, no colonies at all were recovered.

Four mutants (VarA, VarE, VarF, and VarG), isolated from agar plates containing 0.5 μg of Var/ml after 24 h of incubation, were examined in more detail. They had distinct phenotypes with respect to MIC and growth properties (Table 3), indicating that reduced susceptibility to Var may be acquired by different mutational pathways. All mutants showed a tendency for chain formation during growth in liquid medium. The growth rates of the mutants were not severely affected; however, after the stationary phase was entered, autolysis occurred earlier than in cultures of the parent R6 strain.

Transcription profiles of the VarA, VarE, and VarF mutants did not reveal primary resistance determinants. To identify genetic determinants involved in susceptibility of *S. pneumoniae* to Var, global transcription patterns of the mutants were compared to those of the parent R6 strain by using oligonucleotide microarrays of the *S. pneumoniae* R6 genome (19). For each strain, data sets from at least four hybridizations were used for normalization and statistical analysis. Only data which showed P values below 10^{-4} in a paired t test (1) and relative changes greater than threefold are listed in Table 4.

Strains VarA, VarE, and VarF showed partly overlapping profiles of differentially expressed genes, sharing some of the most prominent increases in mRNA levels observed. These changes concerned *copY*, encoding a transcriptional repressor involved in the control of the intracellular copper concentration (26), and a major part of the spr1764-spr1773 locus, which contains homologs of the enterococcal *cylM* gene cluster, specifying the production, processing, and secretion of and immunity against cytolysin (32). Mutant VarA in addition showed increased amounts of transcripts from the *blpBA* genes (spr0466-spr0468), which encode an ABC transporter involved in bacteriocin production of *S. pneumoniae* (16). Simultaneous affection of transcription of the *cylM* cluster and the *blpAB* genes has previously been observed in a variety of other *S. pneumoniae* mutants with altered cell wall biochemistry and might be related to some kind of general stress response (18).

To test the relevance of increased expression of the loci retrieved by transcriptome analysis for the susceptibility to Var, they were individually deleted in the respective mutants. In the case of *copY*, it appeared that this gene may be transcriptionally coupled with the downstream genes *ctpA* and *spxB*. To minimize polar effects on the expression of these genes, *copY* was deleted by constructing an in-frame minigene (Δ*copY*). In the case of the spr1764-spr1773 locus, a deletion mutant (Δ*cyl*) was constructed by replacing the entire DNA region with an Spc resistance cassette. The two deletions were individually introduced into each of the VarA, VarE, and VarF mutants, and the MICs of Var were established. Neither Δ*copY* nor Δ*cyl* had any effect on Var susceptibility, suggesting that enhanced expression of the respective genes in the VarA, VarE, and VarF mutants was not the primary cause of their reduced susceptibility but was rather an indirect effect of yet-unidentified determinants.

Reduced Var susceptibility is associated with increased expression of an ABC transporter in the VarG mutant. The transcription profile of VarG was unique in that it showed only one single signal beyond the threefold threshold (Table 4). This signal was not observed with the other mutants and revealed an 8.9-fold-increased level of mRNA transcribed from

TABLE 4. Differentially expressed genes in strains exhibiting reduced Var susceptibility

Locus	Gene	Predicted function(s) and description	Change (fold) in transcript amt in mutant			
			VarA	VarE	VarF	VarG
spr0030	NA[a]	Unknown		0.32		
spr0446	*hsdS*	Type I restriction modification enzyme specificity protein		3.76		
spr0448	*hsdS*	Type I restriction modification enzyme specificity protein	0.29			
spr0466	*blpB*	Transport protein, C-terminal fragment	3.18			
spr0467	*blpB*	Transport protein, N-terminal fragment	3.61			
spr0468	*blpA*	Bacteriocin transport-processing ATP-binding protein	3.70			
spr0639	*copY*	Copper transport transcriptional repressor	5.24	3.15	3.99	
spr0812	NA	ABC transporter ATP-binding protein				8.90
spr0881	*coiA*	Competence protein, possible transcription factor		0.32		
spr1548	NA	Unknown	0.30			
spr1549	NA	Unknown	0.31			
spr1628	*pilD*	Type IV prepilin peptidase		0.26		
spr1758	*cinA*	Competence damage-inducible protein		0.29		
spr1764	NA	Unknown	6.67			
spr1765	NA	Unknown	4.15		4.08	
spr1766	NA	Unknown	7.11	7.50	8.40	
spr1767	*cylM*	Cytolysin subunit modifying protein	3.47	4.49	5.87	
spr1768	NA	Unknown	3.94	6.32	3.38	
spr1769	NA	Unknown	3.50	5.24		
spr1770	*cylB*	Toxin secretion ABC transporter ATP-binding-permease protein	4.73	7.17	5.46	
spr1773	NA	ABC transporter ATP-binding protein	3.22	3.94	3.30	
spr1859	NA	Competence protein		0.28		
spr1863	*cglB*	Competence protein, type II secretion system protein		0.24		
spr1864	*cglA*	Competence protein, type II secretion system ATPase		0.24		

[a] NA, not available.

spr0812, encoding the ATP-binding component of a putative ABC transporter.

The spr0812 gene appeared to constitute an operon together with the preceding (not annotated) short open reading frame spr0811a (108 bp) and the downstream gene spr0813, which has the potential to encode a membrane-spanning permease of unknown specificity (Fig. 1). In order to investigate whether the products of this putative operon are involved in reduced Var susceptibility, a deletion of all three genes (Δspr0811a-spr0813) was introduced into strain VarG by replacing them with the Kan resistance gene *aphIII*. In the resulting mutant, VarGΔabc, Var susceptibility was increased to the level seen with the wild-type R6 strain (Table 3). Introducing the same deletion into the R6 strain (R6Δabc), in contrast, did not affect its Var MIC. Moreover, transformation of R6 with a PCR product amplified from the spr0811a-spr0813 region of the VarG mutant yielded transformants resistant to 0.5 μg of Var/ml (VarGt) at a high frequency.

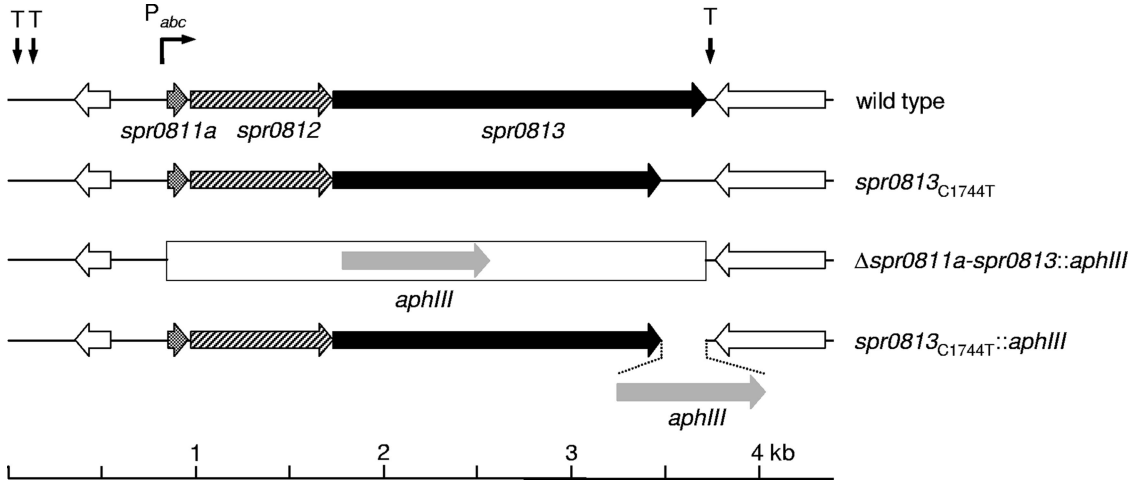

FIG. 1. Genetic organization of the *S. pneumoniae* R6 spr0811a-spr0813 region and derivatives. spr0811a has not been annotated (11), but its expression has been experimentally demonstrated (not shown). Wide horizontal arrows indicate the directions and lengths of spr0811a (dotted), spr0812 (hatched), spr0813 (black), the Kan^r marker *aphIII* (gray), and the flanking genes spr0811 and spr0814 (empty). The positions of the P_{abc} promoter and of putative ρ-independent terminators (T [$\Delta G > 12$ kcal/mol]) are given by an angled arrow and vertical arrows, respectively. Replacement of spr0811a-spr0813 with *aphIII* is shown as a wide box (giving the extend of the deletion) containing a gray arrow. Insertion of *aphIII* is indicated with dotted lines. Relevant genotypic features are given at the right side of the figure.

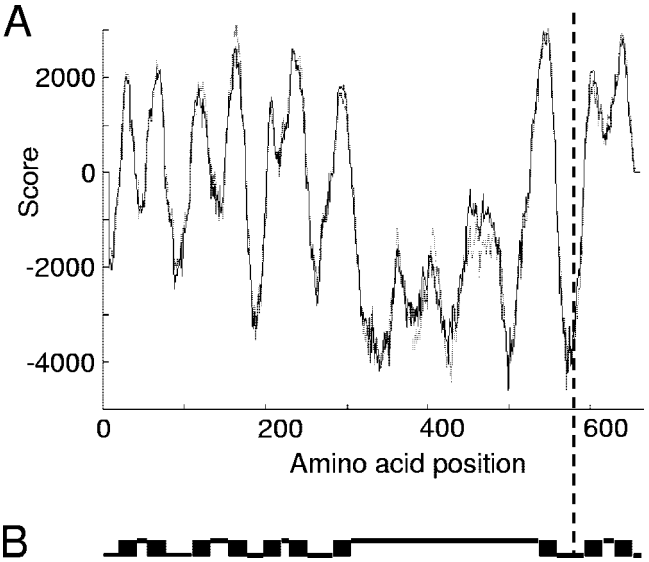

FIG. 2. Transmembrane topology prediction for Spr0813. The dashed vertical line indicates the C terminus of the truncated permease $Spr0813_{Q582*}$. (A) Probabilities for transmembrane helices were calculated with the Tmpred program (9). The solid curve shows the strongly preferred prediction (N terminus inside); the dotted curve shows the less likely prediction (N terminus outside). (B) Transmembrane helices (black boxes) were predicted with the [TMHMM] program (14). Lower lines represent protein sections located inside the membrane; upper lines represent sections located outside.

These observations clearly showed (i) that the sequence of the spr0811a-spr0813 region of the VarG mutant was different from that of the wild type and (ii) that this difference was necessary to confer the reduced-susceptibility phenotype.

Reduced Var susceptibility depends on C-terminal truncation of the permease Spr0813. The nucleotide sequence of the spr0811a-spr0813 region of the VarG mutant, including the distance to the preceding, divergently oriented gene spr0811 (Fig. 1), was established. The sequence was identical to that of the parent R6 strain, except for one single nucleotide exchange (C to T) at the first position of codon 582 in spr0813, generating the nonsense triplet TAA. The truncated reading frame ($spr0813_{C1744T}$) encodes a fragment of the putative permease Spr0813 in which 81 C-terminal amino acids are missing. From topology predictions, it appeared that this fragment ($Spr0813_{Q582*}$) has only 8 of the 10 transmembrane helices calculated for the wild-type protein (Fig. 2).

In order to exclude the possibility that secondary mutations at unknown sites which may contribute to the reduced susceptibility phenotype had been acquired during the selection of VarG and VarGt, the nonsense mutation in spr0813 was introduced into the wild type without Var selection. This was achieved by transforming R6 with a synthetic cassette in which the truncated gene $spr0813_{C1744T}$ was linked to the *aphIII* resistance marker and by selection of the resulting strain construct, VarGc, in the presence of Kan. VarGc showed the same Var MIC as the original VarG mutant and the VarGt transformant. Thus, the C-terminally truncated protein $Spr0813_{Q582*}$ was sufficient to confer reduced Var susceptibility to *S. pneumoniae*.

Spr0813 truncation does not affect the activity of the P_{abc} promoter. The spr0811a-spr0813 locus had initially been noticed due to the increased level of spr0812 mRNA in the transcriptome of VarG (Table 4). To confirm this observation, transcript amounts of spr0812 and spr0813 were quantified by real-time RT-PCR. In the VarG mutant as well as in the VarGt transformant and the VarGc construct, the mRNA levels of the spr0812 and spr0813 genes were about sixfold higher than in the R6 strain (Table 5). To decide whether this was the consequence of enhanced transcription of the spr0811a-spr0813 region, the 5′ end of the corresponding mRNA was mapped by RACE and located to a position 23 bp upstream of the spr0811a locus. The inferred promoter, P_{abc} (Fig. 1), was used to drive the expression of the *lacZ* reporter gene after single-copy integration at the *bgaA* locus of R6, VarG, and VarGt (7). As shown in Table 5, the activity of the P_{abc} promoter was rather weak compared with that of the P_{vegM} reference promoter. Surprisingly, no significant activity changes of P_{abc} were detected in VarG and VarGt as a consequence of the $spr0813_{C1744T}$ mutation. Therefore, the higher amounts of spr0812-spr0813 transcripts observed in these mutants may have been due to increased mRNA stability and/or increased activity of one or more additional promoters which have not yet been identified.

Spr0812 and Spr0813 are also involved in susceptibility to bacitracin. The protein products predicted for spr0812 and spr0813 showed remarkable degrees of sequence identity with the ABC transporters MbrA (56%) and MbrB (30%) of *S. mutans*, BceA (48%) and BceB (24%) of *Bacillus subtilis*, and BcrA (47%) and BcrB (24%) of *B. licheniformis*. For each of these transporters, it has been demonstrated that they are directly involved in resistance to the nonribosomal peptide antibiotic bacitracin (21, 25, 34). Therefore, the bacitracin susceptibility of the *S. pneumoniae* $spr0813_{C1744T}$ nonsense mutants was tested. All of them (VarG, VarGt, and VarGc) showed a strongly reduced bacitracin MIC (1 μg/ml) compared with the value of 4.8 μg/ml for the parent R6 strain (Table 3). Deletion of the complete spr0811a-spr0813 locus (Δspr0811a-spr0813) in VarG did not lead to a further decrease of baci-

TABLE 5. Transcription of spr0811a-spr0813 in strains exhibiting reduced Var susceptibility

Strain	Amt of transcript[a]		Strain[b]	Activity of P_{abc} (U of β-galactosidase)[c]
	spr0812	spr0813		
R6	1	1	R6-P_{abc}	7
VarG	6.4	6.6	VarG-P_{abc}	6
VarGt	6.3	6.5	VarGt-P_{abc}	6
VarGc	5.8	5.9	R6Δabc-P_{abc}	6
			VarGΔabc-P_{abc}	5
			RP100[d]	0
			RP204[e]	1,140

[a] Values were determined by quantitative RT-PCR. Relative transcript amounts with respect to the wild-type R6 strain results are shown. Transcripts were detected with primer pairs specific for spr0812 and spr0813, respectively.

[b] All strains were deficient in endogenous β-galactosidase due to disruption of the *bgaA* gene.

[c] U, units expressed as nanomoles of nitrophenol produced per minute per milligram of protein. Mean values obtained from the results of experiments using two independent cultures are given.

[d] Negative control, containing promoterless *lacZ*.

[e] Positive control, containing P_{vegM}*lacZ*.

tracin MICs for those strains, whereas the same deletion reduced the bacitracin resistance of the wild-type R6 strain to the level seen with the spr0813$_{C1744T}$ nonsense mutants. This indicated that truncation of Spr0813 was sufficient to completely abolish the contribution of the Spr0812-Spr0813 transporter to bacitracin resistance of *S. pneumoniae*. It thus appeared that the intact transporter was required for resistance to bacitracin, whereas the truncated Spr0983$_{Q582*}$ permease mediated reduced susceptibility to Var.

DISCUSSION

In this study, mutants of *S. pneumoniae* that exhibit reduced susceptibility to the tetramic acid antibiotic Var were characterized. Mutants could be isolated at a rather low frequency, and their drug MICs (0.5 to 0.7 μg/ml) were only moderately increased compared to that seen with the parental R6 strain (0.4 μg/ml). This indicated that Var might not interact with one specific cellular target at which single point mutations would be expected to lead to a more distinct resistance phenotype. Due to its suggested partitioning into the cytoplasmic membrane (37), the antibiotic probably interferes with a variety of functions that depend on the integrity of the cell envelope. In agreement with this view, transcription profiling revealed that genes differentially expressed in the mutants investigated here mostly encode products which are associated with or depend on the cytoplasmic membrane for activity: the *cylM* gene cluster encoding the production of a homologue of the potent membrane-active cytolysin of *Enterococcus* spp., a bacteriocin transporter, a regulator of copper transport, and proteins involved in genetic competence (see Table 4). It is therefore not surprising that all four mutants were resistant at only low levels and showed pleiotropic phenotypes such as a reduced growth rate, a shorter stationary phase followed by early lysis, and a tendency for chain formation.

From these properties of the mutants, it appeared that there are different routes by which mechanisms protective against Var may emerge. One of these routes relies on modification of the ABC transporter Spr0812-Spr0813, which is also involved in the susceptibility of *S. pneumoniae* to the structurally unrelated antibiotic bacitracin. Since Var, like bacitracin, possibly displays its antimicrobial activity by interference with essential functions of the cytoplasmic membrane, both drugs may be accessible to the Spr0813 permease, the substrate-specific component of the transporter. C-terminal truncation of the permease (in strain VarG) and deletion of the complete transporter gene locus (in strain VarGΔabc), however, had opposing effects on the susceptibility of the bacteria to Var, whereas resistance to bacitracin was abolished by both mutations.

In *B. licheniformis* and *B. subtilis*, it seems that BcrAB and BceAB act by transporting the bacitracin molecule itself, thus directly removing the antibiotic from its membrane target (25, 27). The issue of whether these transport systems mediate bacitracin efflux or influx, however, is still a matter of debate (3, 27). Based on very similar transmembrane topology predictions for BceB and Spr0983 (9 or 10 transmembrane helices, with a large hydrophilic domain between helices 7 and 8), the Spr0812-Spr0813 transporter of *S. pneumoniae* may also use bacitracin itself as a substrate, and the C-terminal pair of predicted transmembrane domains in Spr0813 (Fig. 2) may be indispensable to this function. The absence of these transmembrane domains, in contrast, was the primary cause of reduced susceptibility to Var. As one plausible explanation of this finding, truncation of the Spr0813 permease may lead to altered substrate specificity so that Var itself can be transported. Alternatively, the mutated transporter could export an unknown substance inactivating the antibiotic outside the cell. Apart from that, the possibility remains that the (sixfold-) elevated level of spr0812-spr0813 transcripts in spr0813$_{C1744T}$ mutants leads to overexpression of the transporter, which in turn may either exclusively or partly (together with the spr0813$_{C1744T}$ mutation) account for the reduced-susceptibility phenotype. Consistent with this possibility, it has been reported that mutations affecting the specificity of an enzyme can be compensated for by overexpression, which in turn permits a broader range of substrates to be used (2).

The spr0813$_{C1744T}$ mutation had no detectable effect on the activity of the P_{abc} promoter, indicating that higher amounts of spr0812-spr0813 mRNA may be due to increased stability of the transcripts or increased activity of unidentified promoters. The interrelation between spr0813 truncation and these effects, however, remains unclear.

It is also not known whether the 36-amino-acid peptide encoded by spr0811a is functionally associated with the Spr0812 and Spr0813 transporters. As verified by assaying a translational spr0811a::*lacZ* fusion, this peptide is in fact expressed in *S. pneumoniae* (not shown). BLAST searches, however did not reveal any hints to possible functions of Spr0811a, and no open reading frames with the potential to encode similar products are present upstream of the genes of the related bacitracin transporters BcrAB and BceAB of *Bacillus* spp. or MbrAB of *S. mutans*.

In the case of the BceAB system of *B. subtilis*, it was recently shown that it is not only active as a bacteriocin detoxification pump but is also crucial for bacitracin perception by the histidine kinase BceS (27). BceS and the cognate response regulator BceR are encoded immediately upstream of *bceAB* and mediate induction of these genes in the presence of bacitracin (21). Similarly, the genes for the homologous ABC transporter MbrAB of *S. mutans* are clustered with genes for the two-component system MbrCD. In contrast, no two-component system is encoded in the vicinity of spr0812-spr0813 in *S. pneumoniae*. Of all proteins predicted for *S. pneumoniae*, the Rr01 response regulator and the Hk01 sensor kinase of the so-far-uncharacterized 01 two-component system (23) show the highest identities with BceR (43%) and BceS (28%) of *B. subtilis* and MbrC (41%) and MbrD (32%) of *S. mutans*, respectively. As with BceS and MbrD, the N-terminal input domain of *S. pneumoniae* Hk01 indeed shows the typical architecture (two deduced transmembrane helices, with no extracytoplasmic linker in between) of intramembrane-sensing histidine kinases (17) belonging to the phylogenetically conserved HPK3i subgroup (6). In contrast to the members of this subgroup, which is characterized by the location of the respective genes adjacent to those encoding functionally linked ABC transporters, the 01 two-component system of *S. pneumoniae*, however, is encoded 0.64 Mb away from the spr0812-spr0813 transporter genes. Further studies will be necessary to establish whether there is a regulatory link between these two loci.

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