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Low-Level Resistance to Rifampin in *Streptococcus pneumoniae*

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Rifampin is recommended for combination therapy of meningitis due to β-lactam-resistant *Streptococcus pneumoniae*. High-level rifampin resistance (MIC, ≥4 mg/liter) has been mapped to point mutations in clusters I and III of *rpoB* of the pneumococcus. The molecular basis of low-level resistance (MICs, ≥0.5 and <4 mg/liter) was analyzed. Spontaneous mutants of clinical pneumococcal isolates were selected on Columbia sheep blood agar plates containing rifampin at 0.5, 4, 10, or 50 mg/liter. Low-level resistance could be assigned to mutations in cluster II ($I_{545}N$, $I_{545}L$). Sensitive (MIC, <0.048 mg/liter) wild-type strains acquired low-level resistance at a rate approximately 10 times higher than that at which they acquired high-level resistance (average mutation frequencies, 2.4×10^{-7} for low-level resistance versus 2.9×10^{-8} for high-level resistance [$P < 0.0001$]). In second-step experiments, the frequencies of mutations from low- to high-level resistance were over 10 times higher than the frequencies of mutations from susceptibility to high-level resistance (average mutation frequencies, 7.2×10^{-7} versus 5.0×10^{-8} [$P < 0.001$]). Mutants with low-level resistance were stable upon passage. Sequencing of a clinical isolate with low-level resistance (MIC, 0.5 mg/liter) revealed a $Q_{150}R$ mutation upstream of cluster I. The frequencies of mutations to high-level resistance for this strain were even higher than the rates observed for the in vitro mutants. Therefore, a resistance-mediating mutation located outside clusters I, II, and III has been described for the first time in the pneumococcus. In vitro low-level rifampin resistance in *S. pneumoniae* could be mapped to cluster II of *rpoB*. Mutants of pneumococcus with low-level resistance may be selected in vivo during therapy in tissue compartments with low antibiotic concentrations and play a role in the development of resistance.

Streptococcus pneumoniae is the most frequent cause of bacterial meningitis, community-acquired bacterial pneumonia, and acute otitis media. Clinical isolates of *S. pneumoniae* with β-lactam resistance have been isolated at increasing frequencies over the past few decades (1). In addition, *S. pneumoniae* isolates not susceptible to penicillin are often multidrug resistant. As a consequence, empirical or targeted monotherapy of severe pneumococcal infections, such as meningitis, with a β-lactam antibiotic is no longer safe. Combination therapy with an expanded-spectrum cephalosporin and rifampin or vancomycin has been recommended (10, 13, 14). The semisynthetic rifamycin rifampin has primarily been used as part of combination therapy for tuberculosis, but it has also been used as a therapeutic agent against (methicillin-resistant) *Staphylococcus aureus* (4) or for chemoprophylaxis for close contacts of patients with invasive infections due to *Neisseria meningitidis* (6) or *Haemophilus influenzae* type b (3).

Rifampin resistance has been described in several bacterial species, such as *Mycobacterium tuberculosis*, *Escherichia coli* (12), *S. aureus* (2), and *N. meningitidis* (21). Resistance has also been reported in *S. pneumoniae* (8, 18, 23). Rifampin resistance is caused by an alteration of the β subunit of RNA polymerase, the target of the antibiotic. Rifampin acts by binding to the β subunit, which leads to the premature termination of DNA transcription. Resistance to rifampin has been linked to amino acid alterations found in three regions of *rpoB*, termed clusters I to III. These alterations arise mainly due to point mutations, but horizontal gene transfer may also play a role in the evolution of rifampin resistance in *S. pneumoniae* (8).

Rifampin resistance in clinical *S. pneumoniae* isolates seems to be rare at present. Reported rates of resistance vary between 0.4 and 1.5% (7, 15, 19). Resistance has been linked to point mutations within cluster I or III of *S. pneumoniae rpoB* (8, 18, 23). This study addresses the molecular basis of the low-level rifampin resistance (MICs, ≥0.5 to <4 mg/liter) and its role in the development of resistance in clinical isolates of *S. pneumoniae*. A large collection of nasopharyngeal *S. pneumoniae* isolates (17) was searched for isolates with low-level resistance. In addition, mutants with low-level resistance were selected in vitro, and the *rpoB* gene was analyzed for mutations. Stepwise increases in the rifampin MIC that resulted in resistance in *S. pneumoniae* were investigated.

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MATERIALS AND METHODS

Bacterial strains. One hundred clinical isolates were randomly selected from a collection of strains (1,211 isolates) obtained from surveillance for nasopharyngeal isolates of *S. pneumoniae* conducted by the Swiss Sentinel Surveillance Network between 1998 and 1999 (17). All isolates were serotyped by use of the Quellung reaction with specific antisera from the Statens Serum Institute (Copenhagen, Denmark). Bacteria were routinely grown on Columbia sheep blood agar (CSBA) plates or in brain heart infusion (BHI) broth and stored at −80°C by using Protect bacterial preservers (Technical Service Consultants, Heywood, United Kingdom).

Susceptibility testing. Rifampin MICs were determined by using E-test strips (AB Biodisk, Solna, Sweden) on CSBA plates.

DNA methods. Chromosomal DNA was obtained from the *S. pneumoniae* isolates as follows. Bacteria from two CSBA plates were resuspended in TE

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TABLE 1. Primers used for amplification and sequencing of *rpoB*

Primer	Sequence[a]	Use	Reference or source
31f	TTTACAACCATATGACGATTAGAA	PCR and sequencing	This study
372f	GGGACGTTCGTNATHAAYGG	PCR and sequencing	16
642r	TGAGAAACCAAGAGCACGAACC	PCR and sequencing	This study
2380r	TCGCGAGTGATYTCYTCMGG	PCR and sequencing	16
3733r	CAACCACTATTTCTTCCCTTTCTA	PCR	This study
399f	GA**T**GAYAT**C**GAYCA**CC**T**C**GGA**A**AA	Sequencing	8
525r	**G**AT**G**TTAGG**T**CC**T**TCA**G**GT**G**T**C**TC	Sequencing	8
1440f	TTGTCACARTTYATGGAYCA	Sequencing	16
1570r	ATACATGCTGTCGCAACGGC	Sequencing	This study

[a] The nucleotides modified for this study are highlighted in boldface.

buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The bacteria were lysed by the addition of the GES reagent (guanidium isothiocyanate, EDTA, sarcosyl). Ammonium acetate was added to the solution, followed by 10 min of incubation on ice. Chloroform-isoamyl alcohol was added to extract the proteins, and then the DNA was precipitated with isopropanol and washed with 70% ethanol. The DNA was resuspended in TE buffer.

PCR amplification and sequencing of *rpoB*. The *rpoB* gene was amplified from bp 405 to 2468 (*S. pneumoniae* strain R6 coordinates) by using primers slightly modified from those published previously (8, 16) (Table 1) and *Taq* DNA polymerase (Roche Molecular Biochemicals, Rotkreuz, Switzerland). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Basel, Switzerland). The *rpoB* genes of different isolates were sequenced by using the sequencing primers described in Table 1. DNA sequences were aligned by using Lasergene (DNASTAR Inc., Madison, Wis.) software. *rpoB* sequences were translated, and the amino acids were aligned.

In vitro generation of rifampin-resistant mutants. BHI was inoculated with a single colony, and the bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6 to 0.7. Aliquots of the cultures were spread in duplicate on CSBA plates containing rifampin at concentrations of 0.5, 1, 4, 10, and 50 mg/liter. The plates were incubated for 48 h prior to counting of the colonies. In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total cell count. Single resistant colonies obtained from the plates with each rifampin concentration were stored at −80°C for further evaluation.

Selection for mutants with high-level rifampin resistance (second-step mutation). Two strains (strains 111.46 and 204.26) were chosen for the selection of mutants with high-level rifampin resistance. For each strain, single colonies each of a rifampin-sensitive (MIC <0.048 mg/liter) wild type and a mutant with low-level resistance (MICs, ≥0.5 and <4 mg/liter) were inoculated in BHI broth, and the bacteria were grown to an OD_{600} of 0.6 to 0.7. Aliquots from each culture were spread in duplicate on CSBA plates containing 4, 10, and 50 mg of rifampin per liter. The plates were incubated for 48 h prior to counting of the colonies. The total cell count was determined as described above. Colonies with high-level resistance (MICs, ≥4 mg/liter) derived from strains with low-level rifampin resistance were stored and further analyzed.

Transformation. Rifampin-susceptible (MIC, 0.023 mg/liter) pneumococcal isolate 106.44 was used as a recipient for transformation with *rpoB* from strains with low-level resistance. Competent cells were prepared by inoculating BHI broth with a single colony and growth overnight. A fresh culture was started in the morning by diluting the overnight culture 1:100 in fresh broth and was grown to an OD_{600} of 0.15. An aliquot of the culture was diluted 1:20 in BHI broth prewarmed at 30°C and incubated for 15 min. Competence-stimulating peptide (Neosystems, Strasbourg, France) was added to a final concentration of 200 ng/ml, and the culture was allowed to incubate for 30 min at 30°C. A total of 1 μg of DNA consisting of the *rpoB* gene (PCR fragment bp 372 to 2380) was added to the culture, which was allowed to incubate for 40 min at 30°C and then for 90 min at 37°C. Aliquots of the cultures were then spread onto CSBA plates containing rifampin at concentrations of 0.5, 1, 4, 10, and 50 mg/liter. The plates were incubated for 48 h prior to counting of the colonies. In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total cell count. Single colonies from the plates with each rifampin concentration were stored at −80°C for further evaluation.

RESULTS

Susceptibilities of clinical isolates to rifampin. Among the 100 nasopharyngeal isolates, 99 strains were sensitive to rifampin, with an MIC at which 50% of strains are inhibited (MIC_{50}) of 0.012 mg/liter, an MIC_{90} of 0.023 mg/liter, and an MIC range of 0.008 to 0.032 mg/liter. The MIC was 0.5 mg/liter for only one isolate (strain 205.09). Since the MIC for this isolate was more than 10 times greater than the highest MIC

TABLE 2. Frequency of single- and second-step mutations to low-level and high-level rifampin resistance in clinical isolates of *S. pneumoniae*

Mutation and strain[b]	MIC (mg/liter)	Mutation frequency (log10) with rifampin at concn (mg/liter) of[a]:			
		0.5	4	10	50
First-step mutation					
104.72-I	0.032	−6.49 ± 0.14	−7.30 ± 0.14	−7.49 ± 0.22	−7.51 ± 0.09
111.46-I	0.016	−7.09 ± 0.20	−7.43 ± 0.52	−7.59 ± 0.42	−7.79 ± 0.37
111.81-I	0.032	−6.52 ± 0.01	−7.30 ± 0.12	−7.65 ± 0.03	−8.12 ± 0.03
204.26-I	0.016	−7.06 ± 0.24	−7.74 ± 0.67	−7.96 ± 0.44	−7.97 ± 0.55
777-I	0.032	−6.33 ± 0.39	−7.43 ± 0.20	−7.24 ± 0.13	−7.58 ± 0.35
Second-step mutation					
111.46-II	0.5		−6.15 ± 0.36	−6.50 ± 0.39	−6.63 ± 0.48
204.26-II	0.75		−6.12 ± 0.24	−6.60 ± 0.64	−7.25 ± 0.50

[a] The values represent the means and standard deviations of four independent experiments for each strain except strain 111.81, for which the values represent the means and standard deviations of two experiments. The results of statistical analyses (unpaired Student's *t* test for all comparisons) were as follows: (i) for first-step mutations, frequencies of mutations for resistance to rifampin at 0.5 mg/liter compared to the frequencies of resistance to higher rifampin concentrations, $P < 0.001$; (ii) for comparison of mutation frequencies between mutants with first- and second-step mutations, for rifampin at 4 mg/liter, $P < 0.001$; for rifampin at 10 mg/liter, $P < 0.001$; for rifampin at 50 mg/liter, $P = 0.007$.

[b] Wild-type strains are labeled with the suffix I; mutants with low-level resistance used for second-step experiments are labeled with the suffix II.

TABLE 3. DNA mutations and amino acid changes leading to rifampin nonsusceptibility in *S. pneumoniae* in vitro

Strain[a]	Parent strain[a]	Rifampin concn used for selection (mg/liter)[b]	MIC (mg/liter)	DNA sequence change	Amino acid change[c]	Alteration in cluster
104.72-I	None, wild type	None	0.032	None	None	None
104.72-II	104.72-I	0.5	1	ATC to AAC	$I_{545}N$	II
104.72-III	104.72-I	10	>256	CAC to GAC	$H_{499}D$	I
104.72-II4	104.72-II	4	64	AAC to AAA	$N_{545}K$	II
104.72-II10	104.72-II	10	96	AAC to AAA	$N_{545}K$	II
104.72-II50	104.72-II	50	>256	ATC to AAC	$I_{545}N$	I and II
104.72-II50	104.72-II			CAC to TAC	$H_{499}Y$	
111.46-I	None, wild type	None	0.016	None	none	None
111.46-II	111.46-I	0.5	0.5	ATC to CTC	$I_{545}L$	II
111.46-III	111.46-I	10	>256	TCT to TTT	$S_{495}F$	I
111.46-II4	111.46-II	4	>256	ATC to CTC	$I_{545}L$	I and II
111.46-II4	111.46-II			CAC to CTC	$H_{499}L$	
111.46-II10	111.46-II	10	>256	ATC to CTC	$I_{545}L$	I and II
111.46-II10	111.46-II			GAC to TAC	$D_{489}Y$	
111.46-II50	111.46-II	50	>256	ATC to CTC	$I_{545}L$	I and II
111.46-II50	111.46-II			CGT to CAT	$R_{502}H$	
111.81-I	None, wild type	None	0.032	None	None	None
111.81-II	111.81-I	0.5	1.5	ATC to AAC	$I_{545}N$	II
111.81-III	111.81-I	10	>256	CAC to GAC	$H_{499}D$	I
204.26-I	None, wild type	None	0.016	None	None	None
204.26-II	204.26-I	0.5	0.75	ATC to AAC	$I_{545}N$	II
204.26-III	204.26-I	10	>256	TCT to TTT	$S_{495}F$	I
204.26-II4	204.26-II	4	12	ATC to AAC	$I_{545}N$	II
204.26-II	204.26-II			TTG to GTG	$L_{544}V$	
204.26-II10	204.26-II	10	24	ATC to AAC	$I_{545}N$	I and II
204.26-II10	204.26-II			GAC to AAC	$D_{489}N$	
204.26-II50	204.26-II	50	>256	ATC to AAC	$I_{545}N$	I and II
204.26-II50	204.26-II			CAG to AAG	$Q_{486}K$	
777-I	None, wild type	None	0.032	None	None	None
777-II	777-I	0.5	2	ATC to AAC	$I_{545}N$	II
777-III	777-I	10	12	TCT to TTT	$S_{495}F$	I
777-II4	777-II	4	>256	ATC to AAC	$I_{545}N$	I and II
777-II4	777-II			CAG to CAC	$Q_{483}H$	
777-II10	777-II	10	>256	ATC to AAC	$I_{545}N$	I and II
777-II10	777-II			CAC to TAC	$H_{499}Y$	
777-II50	777-II	50	>256	ATC to AAC	$I_{545}N$	I and II
777-II50	777-II			CGT to TGT	$R_{502}C$	

[a] Rifampin-susceptible *S. pneumoniae* strains are designated with the suffix I; their mutants with low-level and high-level resistance are designated with the suffixes II and III, respectively.
[b] Concentration used for selection of mutants with spontaneous rifampin resistance.
[c] The numbering is according to that for *S. pneumoniae* strain R6.

detected for the remaining 99 isolates, for this study low-level rifampin resistance was defined as an MIC ≥0.5 and <4 mg/liter. The low-level resistance in isolate 205.09 was stable in vitro upon multiple passages.

Analysis of isolate 205.09 with low-level resistance. The *rpoB* gene of strain 205.09 was amplified in two PCRs with primer pair 31f and 2380r and primer pair 399f and 3733r, respectively. Sequencing revealed no mutations within cluster I, II, or III. However, a $Q_{150}R$ (*S. pneumoniae* strain R6 numbering) amino acid change was identified upstream of cluster I. Transformation of strain 106.44 with this mutation led to low-level resistance (MIC, 0.5 mg/liter) and confirmed its resistance-mediating nature.

In vitro generation of *S. pneumoniae* strains with low-level rifampin resistance. Spontaneous mutants with low-level resistance were selected by plating *S. pneumoniae* cultures grown to the exponential phase on agar plates containing 0.5 mg of rifampin per liter. The cultures were also exposed to higher levels of rifampin to detect and compare high-level resistance to rifampin. The frequencies at which such spontaneous mutants arose are shown in Table 2. Sensitive (MICs, <0.048 mg/liter) wild-type strains acquired low-level resistance at a rate approximately 10 times higher, on average, than that at which they acquired high-level resistance (average mutation rates, 2.4×10^{-7} for low-level resistance versus 2.9×10^{-8} for high-level resistance [$P < 0.0001$]). However, there was some variability between different strains. Mutants with low-level resistance were stable upon multiple passages and storage at −80°C.

Mapping of low-level resistance to rifampin. Among the mutants generated from five single strains, one mutant with low-level resistance and one mutant with high-level resistance were chosen from each test strain for further evaluation. The *rpoB* gene from each mutant and wild-type strain was amplified by PCR with primers 372f and 2380r and sequenced. Clusters I to III were analyzed in detail. Amino acid changes were found in cluster II in all five strains with low-level resistance (Table 3). All the amino acid changes in cluster II were targeted to amino acid 545 (*S. pneumoniae* strain R6 numbering). Four mutant strains (strains 104.72-II, 111.81-II, 204.26-II, and

777-II) demonstrated an $I_{545}N$ alteration (ATC/AAC), and one mutant strain (strain 111.46-II) demonstrated an $I_{545}L$ alteration (ATC/CTC). These mutations have not yet been described in *S. pneumoniae*. Substitutions at this position have been demonstrated in *E. coli*; however, either threonine or phenylalanine replaced the isoleucine (12). In all mutants with low-level resistance tested, no amino acid changes could be detected in cluster III or in the DNA regions of *rpoB* outside clusters I to III.

Mapping of high-level resistance to rifampin. Table 3 depicts the mutations detected in mutants isolated from plates containing rifampin at concentrations equal to or above the National Committee for Clinical Laboratory Standards breakpoint of resistance of 4 mg/liter. For each test strain, one mutant growing on CSBA plates containing 10 mg of rifampin per liter was chosen for subsequent sequencing and analysis of clusters I to III. Again, cluster III was not altered in these mutants. In contrast to the alterations detected in mutants with low-level resistance, no amino acid changes could be detected in cluster II; all changes were localized within cluster I. Three mutants contained an $S_{495}F$ amino acid change (TCT/TTT), which has already been described in *E. coli* (12). An $H_{499}D$ mutation (CAC/GAC) was found in two strains; this mutation has previously been reported in *S. pneumoniae* and *E. coli* (8, 12).

Transformation for low-level rifampin resistance. To investigate whether the point mutations found in mutants with low-level resistance and mutations in cluster II mediated rifampin resistance, *rpoB* from two different strains, strains 777 and 111.46, were used for the transformation of rifampin-susceptible strain 106.44. The MICs for the transformants selected on agar plates containing rifampin at 0.5 mg/liter were the same as those for the donor strains. Transformation frequencies ranged between 0.3 and 0.5%. These experiments indicate that both mutations within cluster II do confer low-level rifampin resistance.

Selection for high-level rifampin resistance in mutants with low-level resistance (second-step mutation). The question of whether low-level resistance to rifampin can promote a further increase in resistance was addressed in two mutants with low-level resistance in vitro (strains 111.46-II and 204.26-II) and in the strain with natural low-level resistance (isolate 205.09). Spontaneous mutants were selected in the presence of rifampin at concentrations of 4, 10, and 50 mg/liter. The frequencies at which mutants with high-level resistance could be observed are summarized in Table 2.

In the second-step mutation experiments, the frequencies of mutations from low- to high-level resistance were over 10 times higher than the frequencies of mutations from susceptibility to high-level resistance (average mutation frequencies, 7.2×10^{-7} versus 5.0×10^{-8}; $P < 0.001$ by the unpaired Student t test). The frequencies of mutations to high-level resistance for strain 205.09 (for rifampin at ≥4 mg/liter, 1.8×10^{-6}; for rifampin at ≥10 mg/liter, 9.1×10^{-7}; for rifampin at ≥50 mg/liter, 1.3×10^{-7}) were even higher than the frequencies observed for the in vitro mutants.

Mapping of rifampin resistance in mutants obtained from second-step mutation experiments. Clusters I to III were sequenced and analyzed for mutants with high-level resistance obtained from the four strains with which the second-step mutation experiments were performed. The amino acid alterations (*S. pneumoniae* strain R6 numbering) found in these mutants are shown in Table 3. In strains 777 and 111.46, cluster II was unaltered and second-step mutations were localized in cluster I. The $H_{499}Y$ amino acid change has been described in *S. pneumoniae*; but the $Q_{483}H$, $H_{499}L$, and $R_{502}C$ alterations have not been reported in *S. pneumoniae*. However, the $R_{502}C$, $D_{489}Y$, and $R_{502}H$ alterations have been described in *E. coli* (12). Different amino acid changes could be observed in strain 104.72. The amino acid change $N_{545}K$ (AAC/AAA) in cluster II could be detected in the second-step mutants of this strain selected on plates containing 4 and 10 mg of rifampin per liter. No additional point mutations occurred in cluster I in either mutant. Therefore, in these mutants the same DNA region which was already mutated during selection for low-level rifampin resistance was targeted again upon selection for high-level resistance. This was also the case in mutant 204.26-II4, in which amino acid 544, which is just adjacent to the cluster II mutation found in amino acid 545, was altered ($L_{544}V$). The additional mutants of these two strains (mutants 104.72 and 204.26) kept the cluster II mutations and obtained additional amino acid changes in cluster I. The $H_{499}Y$ alteration has been described above, and the $D_{489}N$ and $Q_{486}K$ alterations have not yet been reported in *S. pneumoniae* but have been reported in *E. coli* (12). The resistance-mediating nature of the observed mutations and the not yet described mutations was confirmed by transformation experiments with PCR fragments containing the corresponding mutation (data not shown). No transformants could be obtained for the $Q_{483}H$ mutation (strain 777-II4). We do not believe that this indicates that the $Q_{483}H$ mutation has no influence on the rifampin MIC. Rather, transformation of cluster I with a PCR fragment obtained with primers 372f and 525dn (Table 1) is technically difficult, since the mutation to be transformed is close to one end of the DNA fragment. Transformation with second-step mutations yielded MICs ≥4 mg/liter.

DISCUSSION

This study investigated the role of low-level resistance to rifampin in *S. pneumoniae*, which has not been studied before. Low-level resistance was analyzed both in clinical isolates and in in vitro-generated rifampin-resistant mutants.

Only 1 of 100 clinical isolates screened was not susceptible to rifampin, according to the National Committee for Clinical Laboratory Standards breakpoints, but the MIC for the isolate was elevated (0.5 mg/liter). This is in accordance with the results of previous studies, in which the reported rifampin resistance rates were also low. One study performed in Spain (15) observed that 0.4% of isolates were rifampin resistant, another study conducted in the United States (7) observed that 0.5% of isolates were rifampin resistant, and a third one conducted in Brazil (19) observed that 1.5% of isolates were rifampin resistant. In the multicenter study performed in the United States, 7 of 1,527 *S. pneumoniae* isolates were resistant to rifampin. The MIC range detected was <0.015 to 32 mg/liter, the MIC_{50} was 0.03 mg/liter, and the MIC_{90} was 0.06 mg/liter.

Analysis of the wild-type isolate for which the rifampin MIC was increased (0.5 mg/liter) revealed a $Q_{150}R$ amino acid al-

teration upstream of cluster I that mediated low-level rifampin resistance; no mutations could be found within clusters I to III of this isolate. Interestingly, low- and high-level resistance-mediating mutations were described in the analogous region upstream of *rpoB* in *E. coli* (20). A mutation for rifampin resistance outside classical clusters I to III has not yet been described in *S. pneumoniae*. In fact, earlier studies concentrated on the classical clusters and did not investigate for (further) mutation sites outside clusters I to III. There is only one description in the literature of a clinical isolate of *S. pneumoniae* with low-level resistance (MIC, 2 mg/liter), and the isolate has been sequenced (18). Also, reports of studies with other bacterial species have proposed alternative mechanisms for rifampin resistance, including modification of the antibiotic (5, 22) and the presence of mutations in other subunits of the polymerase (11).

The in vitro-generated mutants with low-level resistance carried point mutations in cluster II of *rpoB*. These mutations have not been reported in *S. pneumoniae*. Exposure of *S. pneumoniae* to higher concentrations of rifampin therefore yielded mutations different from those obtained after exposure to low concentrations. This stands in contrast to a study performed with *E. coli*, in which no difference between the mutations that occurred after exposure to low or high concentrations of rifampin was found (12). As in this study, mutants with low-level resistance were selected about 10 times more frequently than mutants with high-level resistance. However, in contrast to the mutants of *S. pneumoniae* described here, mutants of *E. coli* with low-level resistance were not always stable upon multiple passages (12). Experiments with *S. aureus* showed that rifampin-resistant mutants were selected at similar frequencies on low and high concentrations of rifampin (2). On the basis of the results, it was suggested that resistance to high levels of rifampin arises in a single-step fashion and not by sequential independent events. We observed both mechanisms in our experiments. Mutants with high-level resistance could be selected directly upon exposure to high concentrations of rifampin but could also be selected by exposing mutants with low-level resistance to higher concentrations of the antibiotic. These mutants acquired new mutations within cluster I, which can explain the observed increase in the MIC, but more importantly, the mutants did preserve the mutations within cluster II. In addition, one of the mutants acquired a second point mutation in the same codon of cluster II.

In our experiments the frequency at which mutants with mutations for high-level resistance could be selected was significantly higher for bacteria with low-level resistance than for their rifampin-susceptible wild-type strain. This was also the case for the natural clinical isolate with low-level resistance. Therefore, low-level rifampin resistance may predispose *S. pneumoniae* and possibly other bacterial species to the acquisition of high-level resistance more rapidly. In a recent report, the emergence of a rifampin-resistant pneumococcus was described in three patients in The Netherlands receiving rifampin therapy (23). Interestingly, in two of the three patients, who were sampled repeatedly, isolates with low-level rifampin resistance (MICs, 1.5 and 3 mg/liter, respectively) were recovered by culture; this was followed over time by the recovery of isolates for which the MICs were higher (8 and 4 mg/liter, respectively). This observation provides further strength to our hypothesis that the stepwise development of rifampin resistance may be clinically important.

Rifampin is not one of the most routinely used antibiotics. However, it has an important role in the treatment of mycobacterial infections, osteomyelitis, and foreign-body infections, such as prosthetic valve endocarditis and infections of prosthetic joints due to staphylococci. Also, the drug is often used for prophylaxis for the contacts of patients with invasive meningococcal infection. More recently, rifampin has been recommended for the treatment of meningitis due to β-lactam-resistant pneumococci in combination with an expanded-spectrum cephalosporin (10, 13, 14). Exposure of pneumococci to low concentrations of rifampin may especially occur in the nasopharynx during colonization (9), but it may also take place in the cerebrospinal fluid during the treatment of meningitis. The data presented here demonstrate the possibility for the stepwise selection of rifampin-resistant *S. pneumoniae* isolates.

In conclusion, this study shows that *S. pneumoniae* isolates with low-level resistance can easily be selected in vitro and that the point mutations responsible are concentrated in cluster II of *rpoB*. The results obtained in this study indicate the risk that *S. pneumoniae* isolates with low-level resistance may more rapidly gain high-level resistance. Furthermore, the study describes for the first time a (low-level) resistance-mediating mutation in *rpoB* outside clusters I, II, and III in *S. pneumoniae*.

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