Streptococcus pneumoniae plays an important role in causing acute exacerbations in patients with chronic respiratory disease. However, few data are available regarding pneumococcal persistence in adult patients with chronic respiratory diseases. Fifty pneumococci recovered from sputum samples (1995 to 2010) from 13 adult patients with ≥3 episodes of acute exacerbation or pneumonia, with the same serotype and pulsed-field gel electrophoresis (PFGE) pattern, were studied. Multilocus sequence typing (MLST) loci, penicillin-binding protein (PBP) genes (pbp1a, pbp1b, pbp2b), and the quinolone-resistant determining regions (QRDRs) of parC, parE, and gyrA were PCR amplified and sequenced. The average time between the first and last episode was 582 days (standard deviation [SD], ±362). All but two patients received multiple courses of β-lactam treatment, and all persistent strains were resistant to penicillin; however, the PBP sequences were stable over time apart from one variable nucleotide in pbp2x, observed among pneumococci isolated from three patients. In contrast, 7/11 patients treated with fluoroquinolones had fluoroquinolone-resistant pneumococci. In three patients, the initially fluoroquinolone-susceptible strain developed resistance after fluoroquinolone therapy, and in the remaining four patients, the persistent strain was fluoroquinolone resistant from the first episode. QRDR changes involved in fluoroquinolone resistance were frequently observed in persistent strains after fluoroquinolone therapy; however, the PBP sequences and MLST genotypes of these strains were stable over time.

Patients with chronic respiratory disease, such as chronic obstructive pulmonary disease (COPD) and bronchiectasis, are often persistently colonized by respiratory pathogens (27, 32). Airway colonization, mainly by Pseudomonas aeruginosa, Haemophilus influenzae, and Streptococcus pneumoniae, contributes to progressive pulmonary damage, increasing the morbidity and the risk of death of these patients due to frequent and recurrent episodes of acute exacerbations (27).

Most of the acute exacerbations caused by P. aeruginosa are due to a preexisting strain which colonizes the lower airway. Often, these strains are hypermutable (strains with defects in genes involved in DNA repair) and have been related to an increase of antimicrobial resistance due to a stepwise accumulation of point mutations (28). In contrast, when S. pneumoniae has been recovered during an acute exacerbation, this has generally been associated with the acquisition of a new strain, and the high prevalence of multidrug-resistant pneumococci associated with acute exacerbations has been related to the consumption of antimicrobials that these patients receive as empirical treatment (18, 26). The role of pneumococcal hypermutable strains is unclear and could be related to the persistent strains that colonize among 15 to 17% of COPD patients at any time (12, 32).

Antimicrobial treatment for acute exacerbations includes β-lactams, macrolides, and fluoroquinolones, and the high rates of antimicrobial resistance to these classes of antimicrobials in patients with respiratory diseases are a cause of concern (18). Among pneumococci, resistance to β-lactams is the result of alterations in the penicillin-binding proteins (PBPs), most importantly PBP1A, PBP2B, and PBP2X (7). Macrolide resistance is mediated by two main mechanisms, target site modification by methylases encoded by the _erm_ (B) or _erm_ (TR) genes (referred to as the MLSB phenotype) and an efflux pump encoded by the _mef_ (A/E) gene (referred to as the M phenotype) (5). In _S. pneumoniae_, macrolide resistance is frequently associated with tetracycline resistance due to the presence of the Tn916 family of transposons, which can result in the spread of resistance to both antimicrobials (29). Fluoroquinolone resistance is caused mainly by changes in the quinolone-resistant determining regions (QRDRs) of DNA topoisomerase IV subunits (ParC and ParE) and the DNA gyrase (GyrA) subunit (10).

Data describing the antimicrobial susceptibility, serotype, and pulsed-field gel electrophoresis (PFGE) pattern of a large collection of over 600 pneumococci isolated from COPD patients were recently reported (13). However, little information is available about the evolution of pneumococci associated with multiple acute exacerbation episodes over a long period of time in a patient with chronic respiratory disease. In the present work, we characterized 50 pneumococci isolated from 13 patients with chronic respiratory disease who had 3 or more episodes of acute exacerbations caused by the same pneumococcal strain (as defined by the same serotype and PFGE pattern). Isolates were genotyped by multilocus sequence typing (MLST); _pbp2x, pbp1a, pbp2b, parC, parE_, and _gyrA_ were PCR amplified and sequenced; and _erm_ (B), _erm_ (TR), _mef_ (A/E), and _tet_ (M) were detected by PCR. We hypothesized that the pneumococcal strains that persistently colo-
nize and are associated with multiple episodes of acute exacerbation in these patients would acquire changes in the resistance determinants of β-lactams, macrolides, and fluoroquinolones over time; if so, this has important relevance for the clinical management of these patients.

(This study was presented at the 8th International Symposium on Pneumococci and Pneumococcal Diseases, Foz do Iguaçu, Brazil, 2012 [15a].)

MATERIALS AND METHODS

This study and publication of the results were approved by the “Comité Ético de Investigación Clínica del Hospital Universitario de Bellvitge.”

Study setting, bacterial strains, and antimicrobial susceptibility. Pneumococci isolated from clinical samples (invasive and noninvasive) were prospectively collected in our laboratory. All patients with 3 or more pneumococcal episodes of acute exacerbations detected between 1995 and 2010 were analyzed to identify persistent colonization, defined as the same serotype and PFGE pattern. A new episode was considered when the range of time between episodes was more than 4 weeks, which occurred after a successful outcome. Only sputum samples of good quality (<10 squamous cells and >25 leukocytes per low-power field) in which the diplococcus Gram-positive bacteria were the most frequently detected were cultured (24). Pneumococci were identified by optochin susceptibility and bile solubility. Serotyping was performed by Quellung reaction at the Spanish Reference Laboratory.

An acute exacerbation of COPD or bronchiectasis was defined as any sustained increase in respiratory symptomatology, compared with the baseline situation that required an increase in regular medication and hospital treatment. An episode of pneumonia was considered when fever, leukocytosis, and radiological findings (new infiltrates on chest radiography) were detected. The COPD status was defined according to the international Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (23).

Antimicrobial susceptibility, serotype, and PFGE pattern of six pneumococci isolated from two patients (patients 7 and 10) have been published previously among 611 pneumococci isolated from pneumonia and acute exacerbation episodes of COPD patients (13).

Susceptibility to 22 antimicrobials (MIC) was tested by the microdilution method (STRAHEI; Sensititre, West Sussex, United Kingdom), following the Clinical and Laboratory Standards Institute (CLSI) recommendations (6). The ciprofloxacin MIC of resistant strains (MIC ≥ 4 μg/ml) was confirmed by Etest. S. pneumoniae ATCC 49619 was used as the control strain.

PPB detection and sequence analysis. DNA was extracted from pneumococcal strains using the DNeasy tissue kit (Qiagen). pbp1a, pbp2x, and pbp2b were amplified and sequenced, using primer sets and conditions described previously (4). Sequences were assembled and edited using PegaP4 and Gap4 (Staden Package, http://staden.sourceforge.net/). Once assembled, sequences of PBPs were compared between strains of the same patient.

Gene detection of macrolide and tetracycline resistance. Macrolide resistance genes [erm(B), ermA/E] and the tetracycline resistance determinant tet(M) were studied by PCR as described previously (5).

Characterization of quinolone resistance. The parC, parE, and gyrA genes were amplified as described previously (22). Restriction fragment length polymorphism assay (RFLP) of PCR products was performed to detect point mutations at the main QRDR positions involved in quinolone resistance: S79 and S83 of parC, D435 of parE, and S81 and E85 of gyrA. Briefly, S79 and D83 mutations in the parC gene (using HinII and SfI/SI enzymes, respectively), a D435 mutation in the parE gene (using HinII enzyme), and S81 and E85 mutations in the gyrA gene (using HinII and MboII enzymes, respectively) were detected (2, 22).

Point mutations were confirmed by sequencing. The oligonucleotide pairs parE98/parE483, parC50/parC152, gyrA44/gyrA170, and gyrB376/gyrB512 were used to amplify and sequence parE, parC, gyrA, and gyrB QRDRs, respectively (11).

Molecular typing. Genotyping was performed by MLST, as described previously (15). Allele numbers and sequence types (ST) were assigned using the pneumococcal MLST website (http://spneumoniae.mlst.net/).

RESULTS

Patients, pneumococcal strains, and antimicrobial resistance. During the study period (1995 to 2010), 231 adult patients were identified who had 3 or more episodes of acute exacerbation, and S. pneumoniae was isolated. A total of 218 of these 231 patients had S. pneumoniae strains that differed by serotype and/or genotype. Thirteen (6.1%) patients had at least 3 different episodes during which the same strain was isolated (i.e., with the same serotype and PFGE pattern) and were selected for this study. Eleven of the thirteen patients had chronic respiratory diseases: 8 had COPD (1 patient with GOLD II status, 2 patients with GOLD III status, and 3 patients with GOLD IV status; the GOLD status of two patients was not available), and 3 had bronchiectasis. The remaining two patients had an endotracheal prosthesis implanted due to a posttracheostomy stenosis.

A total of 50 pneumococci isolated from the 13 patients were analyzed. The average time between episodes was 210 days (range, 30 to 531 days), and the average time between first and last episodes was 582 days (standard deviation [SD], ±362). All 50 pneumococci analyzed were nonsusceptible to penicillin using oral breakpoints and also showed resistance to at least one other antimicrobial class. Strains from 11 patients were multidrug resistant (≥3 antimicrobial classes; Table 1). All pneumococci examined from the same patient had the identical ST as defined by MLST genotyping, with the exception of patient 7, who had 8 pneumococcal isolates, 5 of which expressed serotype 15A (ST63) and 3 of which expressed serotype 35B (ST558). Moreover, antimicrobial MICs for all strains were conserved over time apart from those of the fluoroquinolones. All macrolide-resistant strains possessed erm(B) and tet(M) genes, and no acquisition or loss of macrolide or tetracycline resistance determinants was observed. Additionally, we analyzed six transient pneumococcal strains collected from patients 5 (Sp338A and Sp338T), 6 (Sp337), 7 (Sp338), and 10 (Sp338), whose serotypes were different from the persistent one (Fig. 1). These transient isolates also were fully susceptible to all antibiotics tested and had a different PFGE type from that of the persistent strain.

Finally, the same pbp1a and pbp2b DNA sequences were maintained in each persistent strain over time (Table 1). The sequence of pbp2x was maintained in all but 3 patients (3, 6, and 11) whose strains had a single nucleotide polymorphism (SNP) that was not involved in an increase of the β-lactam MICs. These results strengthen the suggestion that these were persistent strains in all 13 patients (Fig. 1).

All but two patients (8 and 9) received multiple courses of β-lactam therapy (amoxicillin-clavulanic acid and ceftriaxone). All persistent strains from these 11 patients were susceptible to ceftriaxone and cefotaxime, whereas 5 persistent strains (patients 3, 4, 5, 6, and 13) were amoxicillin-clavulanic acid resistant. Seven patients had macrolide-resistant strains, but previous macrolide consumption could be documented in only one of them (patient 7). Furthermore, all but 3 patients (2, 6, and 13) received at least one course of fluoroquinolone treatment between acute exacerbation episodes. In three cases (patients 3, 10, and 12), the persistent
### TABLE 1 Antimicrobial MICs, characteristics of the 13 patients, and molecular characterization of the persistent pneumococcal strains

<table>
<thead>
<tr>
<th>Patient no. (age, gender)</th>
<th>MLST genotype</th>
<th>Episode no. (day/mo/yr)</th>
<th>β-lactam MIC (µg/ml)</th>
<th>PBP1A/2B/2X allele</th>
<th>Fluoroquinolone MIC (µg/ml)</th>
<th>Aa substitutions in PorC/GyrA</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (58, male)</td>
<td>ST156&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>1st (19/10/1995)</td>
<td>A/A.1/A.1</td>
<td>8</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>2 (46, male)</td>
<td>ST156&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>1st (24/07/1995)</td>
<td>A/A.1/A.1</td>
<td>8</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>3 (76, male)</td>
<td>ST838&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>1st (06/11/2002)</td>
<td>2</td>
<td>A/B.1/A.2</td>
<td>&gt;32</td>
<td>4</td>
<td>S79F/S81F</td>
</tr>
<tr>
<td></td>
<td>4 (77, female)</td>
<td>ST838&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>1st (11/08/2007)</td>
<td>A/B.1/A.2</td>
<td>&gt;32</td>
<td>4</td>
<td>S79F/S81F</td>
</tr>
<tr>
<td>5 (62, male)</td>
<td>ST838&lt;sup&gt;IV&lt;/sup&gt;</td>
<td>1st (29/10/1997)</td>
<td>2</td>
<td>A/B.1/A.2</td>
<td>&gt;32</td>
<td>4</td>
<td>S79F/S81F</td>
</tr>
<tr>
<td></td>
<td>6 (60, male)</td>
<td>ST6521&lt;sup&gt;1A&lt;/sup&gt;</td>
<td>1st (21/05/2009)</td>
<td>A/B.2/A.2</td>
<td>2</td>
<td>1</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>7 (75, male)</td>
<td>ST63&lt;sup&gt;1A&lt;/sup&gt;</td>
<td>1st (09/11/2008)</td>
<td>0.25</td>
<td>B/C/B</td>
<td>2</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>8 (59, female)</td>
<td>ST63&lt;sup&gt;1A&lt;/sup&gt;</td>
<td>1st (12/06/2000)</td>
<td>B/C/B</td>
<td>2</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>9 (78, male)</td>
<td>ST88&lt;sup&gt;1F&lt;/sup&gt;</td>
<td>1st (24/12/1999)</td>
<td>0.25</td>
<td>B/C/B</td>
<td>2</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>10 (64, male)</td>
<td>ST87&lt;sup&gt;1F&lt;/sup&gt;</td>
<td>1st (26/10/2007)</td>
<td>0.25</td>
<td>B/C/B</td>
<td>2</td>
<td>2</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>11 (73, male)</td>
<td>ST2100&lt;sup&gt;1F&lt;/sup&gt;</td>
<td>1st (08/01/2008)</td>
<td>E/A.2/D.2</td>
<td>&gt;32</td>
<td>4</td>
<td>S79F/S81Y</td>
</tr>
<tr>
<td></td>
<td>12 (38, male)</td>
<td>ST276&lt;sup&gt;1A&lt;/sup&gt;</td>
<td>1st (12/12/2007)</td>
<td>E/A.2/D.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;32</td>
<td>4</td>
<td>S79F/S81Y</td>
</tr>
<tr>
<td>13 (65, male)</td>
<td>ST1624&lt;sup&gt;1B&lt;/sup&gt;</td>
<td>1st (19/06/2006)</td>
<td>4</td>
<td>G/B/E</td>
<td>1</td>
<td>0.5</td>
<td>&lt;0.25</td>
</tr>
</tbody>
</table>

a PEN, penicillin (susceptible: MIC ≤ 0.12 µg/ml); CTX-CRO, ceftoxime-cefoxime (susceptible: MIC ≤ 1 µg/ml); A/C, amoxicillin-clavulanic acid (susceptible: MIC ≤ 4/2 µg/ml); CIP, ciprofloxacin (susceptible: MIC ≤ 4 µg/ml); LEV, levofloxacin (susceptible: ≤ 2 µg/ml); MOX, moxifloxacin (susceptible: ≤ 1 µg/ml); ERY, erythromycin (susceptible: ≤ 0.25 µg/ml); and TET, tetracycline (susceptible: ≤ 2 µg/ml). Boldface font indicates resistant isolates.

b Capital letters were used to define different alleles of each PBP, and numbers were used to differentiate small differences among sequences with identical capital letter (for details of the amino acid substitutions, see Fig. 3).

c Acquisition of an SNP in the PBP2X with respect to the first episode of the same patient.

d Gene erm(B) was detected only in ERY-resistant strains: gene tet(M) was detected only in TET-resistant strains.
strains remained fluoroquinolone susceptible over time, whereas strains of 4 patients (1, 4, 5, and 11) were ciprofloxacin resistant from the first episode, and pneumococci isolated from another 3 patients (7, 8, and 9) became ciprofloxacin resistant after fluoroquinolone therapy.

**Sequence type, serotype, and mechanisms of resistance.** The pbp2x, pbp1a, and pbp2b sequences of the persistent strains were compared to those of the susceptible pneumococcal R6 strain (Fig. 2). The pbp2b gene was the most conserved; this gene has been described to be involved in amoxicillin-clavulanic acid resistance. The pbp1a and pbp2x genes possessed mosaic blocks that conferred amino acid substitutions in their transpeptidase domains, which have been associated with a reduction in the affinity for penicillin and cefotaxime (16). Those amino acid substitutions in PBP1A, PBP2B, and PBP2X that were related to β-lactam resistance are shown in Fig. 3.

The most frequent serotype was 9V, expressed by pneumococci isolated from 5 patients, and all 16 pneumococci were either ST156 (Spain9V-ST156 Pneumococcal Molecular Epidemiology Network [PMEN] clone) or ST388, a single locus variant (SLV) of ST156 (Table 1). These 16 pneumococci had the same pbp1a allele, one of two pbp2b alleles, and the same pbp2x allele, albeit with some minor nucleotide changes in a few isolates. Pneumococci of patient 6 expressed serotype 11A and were ST6521, which is an SLV of ST838, and shared the pbp1a, pbp2b, and pbp2x sequences with ST8389V isolates, with the exception of an amino acid substitution in the PBP2X (P504L) in the isolates recovered from the 2nd and 3rd episodes.

The persistent strain of patient 1 (ST1569V) was ciprofloxacin resistant (mutation S79F) from the first episode and after a previous ciprofloxacin course (Fig. 1). All three isolates of patient 4 (ST8389V) were ciprofloxacin resistant due to changes in ParC(S79F) and GyrA(S81F). All four isolates (ST8389V) from patient 3 remained fluoroquinolone susceptible throughout the period studied, in spite of one course of ciprofloxacin in the third episode. Finally, an analysis of the QRDRs of isolates collected from patient 5 was previously published, describing the acquisition of low-level and subsequent high-level fluoroquinolone resistance (9).

**FIG 1** Number of acute exacerbation and pneumonia episodes of each patient, bacterial pathogen isolated, and courses of antimicrobial treatment. Horizontal lines are proportional time lines between the first and the last episode analyzed. Vertical lines mean infection episodes (acute exacerbation, continuous lines; pneumonia, dotted lines). Pathogens isolated were Sp, *Streptococcus pneumoniae*; Hi, *Haemophilus influenzae*; Mc, *Moraxella catarrhalis*; Pa, *Pseudomonas aeruginosa*; Sa, *Staphylococcus aureus*. Pneumococcal serotypes are superscripted (NS means nonserotyped isolate, and NT means nontypeable isolate). Those pneumococci examined in detail are in boldface font. Colored dots indicate the course of antimicrobial therapy: fluoroquinolone, black; amoxicillin-clavulanic acid, green; ceftriaxone, yellow; and macrolide, white. Abbreviations of the QRDR substitutions are showed in red color (no abbreviation means no changes in the QRDRs).
Strains of patients 7 and 8 expressed serotype 15A and were ST63, PMEN clone Sweden15A-ST63. Both persistent strains developed mutations at QRDRs over time after levofloxacin and ciprofloxacin courses, respectively. The persistent strain of patient 8 showed a new ParC change (D78N) in the 2nd episode, and in the case of patient 7, the persistent strain acquired and maintained high-level ciprofloxacin resistance at the third episode, due to changes in ParC (S79F) and GyrA (S81F). Patient 7 also had 3 episodes caused by a serotype 35B, ST558 (SLV of Utah35B-ST377 clone) persistent strain. The first strain of ST55835B was detected between the fourth and the fifth episode of the former ST6315A persistent strain; thereafter, two new episodes were caused by the ST55835B strain. Since these two last episodes occurred in 2010 and the sputum sample was available, the DNA was extracted and an attempt was made to detect serotype 15A by PCR, but the PCR was negative (data not shown).

The persistent strains of patients 9, 10, and 11 all expressed serotype 19F, although the genotypes were different (ST88, ST87, and ST2100). The strain of the 2nd episode of patient 9 (ST8819F) developed high-level ciprofloxacin resistance (ParC-S79F and GyrA-S81Y mutations) after a levofloxacin course, and the strain was also recovered during the 3rd episode. Persistent strains of patient 10 belonged to ST87 (SLV of ST88) and remained stable over time. Finally, the isolates from patient 11 were ST210019F (SLV of ST63). The strain from the 2nd episode acquired an amino acid substitution in the PBP2X (L600S). After multiple courses of moxifloxacin, the pneumococci isolated from the first acute exacerbation episode was high-level ciprofloxacin resistant (mutations S79F in ParC and S81Y in GyrA) and persisted over time. Finally, all isolates from patient 12 were of the same serotype, ST, and susceptibility pattern over time; the same was true of all isolates from patient 13.

**DISCUSSION**

Once *S. pneumoniae* causes an acute exacerbation episode in patients with chronic respiratory disease, the isolate is usually replaced by another *S. pneumoniae* strain with a different serotype/genotype or by a different bacterial species, such as *P. aeruginosa*, *H. influenzae*, or *M. catarrhalis* (26, 27, 32). However, in the present study, we showed, based on stability in capsular type, ST, PBPs, and other resistance determinants, that pneumococci can persist over a long period of time, colonizing and causing acute

**FIG 2** Schematic of the mosaic genes encoding penicillin-binding proteins (PBPs) 1A, 2B, and 2X of the persistent strains. Alleles of each PBP are shown as bars. Mutations associated with resistance to β-lactams are indicated at the top of each PBP. The PBP sequences of the susceptible pneumococcal R6-strain were used as the reference. Blocks showing the percent sequence divergence from the corresponding regions of R6 are indicated. White boxes, regions highly conserved (<1.5% divergence); striped boxes, regions that differed by 1.6 to 9.0%; black boxes, regions that differed by >9.0%. Percentage value indicates rate of divergence of each allele with respect to the R6-susceptible sequence.
exacerbation or pneumonia episodes in patients with chronic respiratory disease.

Persistent bacterial colonization in patients with bronchiectasis and/or severe COPD is frequently associated with *P. aeruginosa* but rarely with pneumococci. In fact, in the present study, the same strain was recovered from only 13 of 213 (6.1%) patients with multiple pneumococcal episodes.

Persistence of *P. aeruginosa* in COPD is often associated with hypermutable strains that dramatically speed up resistance development due to the acquisition of point mutations during exposure to antimicrobial agents (20, 28). In contrast, our study shows that among this collection of isolates, most pneumococcal genes involved in antimicrobial resistance were stable over time, with the exception of *parC* and *gyrA*, which were associated with the development of fluoroquinolone resistance after treatment.

Several factors could explain the persistence of pneumococcal strains in patients with COPD or bronchiectasis. First, it is well known that these patients have several impairments in innate lung defenses, facilitating the permanent colonization by microorganisms (27). Second, the persistence of the strains could be related to their serotype and/or genotype. The capsule is the main virulence factor of *S. pneumoniae*, since it prevents the opsonization by macrophages (19). An association between capsular type polysaccharide, susceptibility to neutrophil-mediated killing, and carriage prevalence has been demonstrated, and the serotypes expressed by several persistent strains of the present study (19F, 6B, 11A, 19A, and 9V) are able to avoid neutrophil-mediated killing (30).

Ten out of 14 strains belonged to three clonal complexes (CC156, CC88, and CC63), suggesting a major role of the genetic background on persistence. In agreement with this finding, a major genotype (related to CC177) was recovered from children attending day care centers, and prolonged colonization was observed in 22% of children (25). Genetic characteristics could favor the colonization over time of these clonal complexes, such as the presence of biofilm or adhesins such as PspC or PspA and/or pilus, which can facilitate the adhesion to the epithelial cells (14, 17). The presence of pilus has been shown to be a clonal property, and when the type I pilus was analyzed, it was detected only in persistent strains belonging to both genotypes CC156 and CC90 (data not shown), as was documented previously (21).

Third, possible biofilm formation (by *S. pneumoniae* or another pathogen) in the respiratory tract of these patients may prevent the appropriate diffusion of antibiotics and therefore may result in a decrease in the bacterial load but not bacterial eradication. This might explain why the persistent, amoxicillin-clavulanic acid-susceptible (MIC ≤ 2 mg/liter) strains of 6 patients could persist over time in spite of multiple courses of this antibiotic.

On the other hand, it is well known that pneumococci can acquire β-lactam resistance by acquisition of exogenous DNA at their PBPs from either β-lactam-resistant pneumococci or commensal streptococci, such as *Streptococcus oralis* or *Streptococcus mitis* (31). Surprisingly, in spite of the amoxicillin-clavulanic acid pressure on the six persistent pneumococci resistant to amoxicillin-clavulanic acid, neither new recombination events nor point mutations in the *pbp2b* of the resistant strains were observed. These results suggest that an optimal combination of *pbp* genes is maintained to compensate for the fitness cost imposed by additional changes in these genes, either by point mutation or recombination, as has previously been shown (1).

In contrast, the development of fluoroquinolone resistance was observed among the persistent strains isolated from 3 of 6 patients after receiving single or multiple courses of fluoroquinolone treatment. The frequency of mutation to ciprofloxacin resistance in *S. pneumoniae* has been shown to be in the range of $10^{-9}$ to $10^{-8}$; hence, it is possible that the fluoroquinolone pressure on the high pneumococcal inoculum ($\geq 10^6$ CFU/ml) observed in patients with COPD or bronchiectasis could select for spontaneous mutants at the QRDRs (8). The persistence of fluoroquinolone-resistant isolates could be related both to an inadequate treatment and to the fitness cost of the mutations. Some patients with chronic respiratory disease are colonized by *P. aeruginosa* and *H. influenzae* and sometimes receive multiple courses of fluoroquinolones, usually empirically. Furthermore, the mutations found in these persistent strains are not related to a decrease in bacterial fitness [fitness has been associated only with the amino acid change GyrA(E85K), which is not present in any of the persistent resistant isolates] (3). Overall, our study demonstrates the risk of the development of fluoroquinolone resistance among persistent pneumococci after fluoroquinolone therapy. This fact should be considered before starting a new empirical fluoroquinolone treatment in order to avoid therapeutic failures.

The analyses of the sequences determined in this study (7

FIG 3 Amino acid substitutions in PBP1A, PBP2B, and PBP2X, compared to those PBPs of the susceptible pneumococcal R6 strain. Highlighted positions indicate changes related to β-lactam resistance. Dots are placed when amino acids are identical to R6. Allele differences between sequence types, i.e., alleles A and B of PBP2B and alleles A and D of PBP2X, are divided into two alleles (e.g., A1 and A2), and differences are shaded. See also Table 1.
housekeeping genes, QRDRs of parC, parE, and gyrA; pbp1a, pbp2b, pbp2x), together with the analyses of macrolide and tetracycline determinants, suggests that a pneumococcal strain can colonize the respiratory airways for an extended period of time. Moreover, the low clonal diversity observed among these persistent strains also suggests that some pneumococci are successfully adapted to persist over a long period of time in patients with chronic respiratory disease and thus potentially cause multiple acute exacerbation episodes.

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