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Mefloquine and new related compounds target the F_0 complex of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae*

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ABSTRACT

The activity of mefloquine (Mef) and related compounds on previously characterized *Streptococcus pneumoniae* strains carrying defined amino acid substitutions in the *c* subunit of the F_0F_1 H^+-ATPase was studied. In addition, a series of Mef^R strains were isolated and characterized. A good correlation was observed between inhibition of growth and inhibition of the membrane-associated F_0F_1 H^+-ATPase activity. Mef was about 10-fold more active than optochin and about 200-fold more active than quinine in inhibiting both growth and ATPase activities of the laboratory pneumococcal strain R6. Mutant strains showed different degrees of inhibition by the different compounds, depending on their specific mutations at the *c* subunit. The resistant strains studied here had point mutations that change amino acid residues either in the *c* or *a* subunits of the F_0 complex. Changes in the *c* subunit were located in one of the two transmembrane α-helices: residues M13, G14, G20, M23 and N24 of helix-1; residues M44, G47, V48, A49 and V57 of helix-2. Changes at the *a* subunit were also found in either of the transmembrane α-helices 5 or 6: residue L186 of helix 5; residues W206, F209, and S214 of helix 6. These results suggest that the transmembrane helices of the *c* and *a* subunits interact and that the mutated residues are important for the structure of the F_0 complex and proton translocation.

The prevalence of *Streptococcus pneumoniae* strains resistant to common antimicrobial agents (10, 26), and the emergence of resistance to new drugs, as the fluoroquinolones (4, 20), has highlighted the need for novel antimicrobial agents directed at new targets. The membrane-associated F_0F_1 H^+-ATPase could be such a target, given that the unique sensitivity of *S. pneumoniae* to the amino alcohol antimalarial agents optochin (Opt) and quinine (Qin) resides in the characteristics of its F_0 complex (6, 24). The H^+-ATPase could be essential for the viability of the pneumococcus, as demonstrated in *Lactococcus lactis*, a genetically related species (17). The primary role of this enzyme in *S. pneumoniae* is to create a proton gradient with the energy provided by ATP hydrolysis, and to maintain the intracellular pH via proton extrusion (21) as in other related bacteria (7, 14). However, in bacteria with a respiratory chain such as *Escherichia coli*, the primary role of this enzyme is to synthesize ATP from the proton gradient of the respiratory chain. The bacterial F_0F_1 ATPases consist in a cytoplasmic sector F_1 (α, β, δ, γ, and ξ subunits), which contains the catalytic sites for ATP hydrolysis, and a cytoplasmic membrane sector F_0 (*a*, *c*, and *b* subunits), which forms the proton channel. Proton transport through F_0 drives the release of ATP on F_1 by long-range conformational changes. The protonation state of an acidic residue of the *c* subunit produces conformational changes that drive the rotation of an oligomeric ring of these subunits. This rotation, in turn, causes the rotation of the attached γ and ξ subunits, producing conformational changes in the β subunits (28, 33) that result in ATP synthesis (1). Conversely, hydrolysis of ATP in F_1 generates rotation of γ and ξ, resulting in the pumping of protons back across the membrane through F_0. In *S. pneumoniae* (21), as well as in other related bacteria (2, 15-18) an increase in the activity of the F_0F_1 ATPase as the pH of the growth medium decreases has been observed. The regulation of this pH-inducible phenotype occurs in *S. pneumoniae* at the level of initiation of transcription (21).

Although Opt was used at the beginning of the 20th century for the treatment of pneumococcal infections (23), its use is nowadays restricted to diagnostic purposes due to its high toxicity. However, new related, less toxic compounds have been synthesized (19). Given the special characteristics of the pneumococcal F_0F_1 ATPase, and its role in the maintenance of intracellular pH, this enzyme could be considered to be a target for the design of new

antibacterial agents. Moreover, the pneumococcus could be also considered as a model system for the study of the putative antimalarial activity of new compounds (24).

In the present study, we describe the molecular basis of the antipneumococcal behaviour of mefloquine (Mef) and related compounds. The activity of these compounds on the growth and membrane-associated ATPase activity on *S. pneumoniae* strains with defined mutations in the *c* and *a* subunits of the F_0 complex of the F_0F_1 ATPase has been studied. In addition, Mef-resistant (Mef^R) strains have been isolated and characterized.

MATERIALS AND METHODS

Bacterial strains, growth and transformation of bacteria. The strains of *S. pneumoniae* used were: the common laboratory strain R6, the Opt- and Qin-resistant R6 derivatives MJ11, MJ2 (6), MJQ3, and MJQ4 (24), and strains ATCC 6303 and ATCC 49619 that were employed as quality controls for antimalarials (18). Unless otherwise stated, strain R6 was grown in liquid C medium, containing 0.08% yeast extract and transformation was carried out according to Tomasz (34). Cultures containing 9×10^6 CFU per ml were treated with DNA at 0.1 μg/ml for 40 min at 30 ºC, then at 37 ºC for 90 min, before plating on media plates containing 0.3 μg/ml Mef.

Susceptibility tests. MICs were determined by the microdilution method, using cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood as recommended by the National Committee for Clinical Laboratory Standards (25). The inoculum was prepared by suspension of several colonies from an overnight blood agar culture in Mueller-Hinton broth and adjusting the turbidity to the 0.5 McFarland standard (ca. 10^8 CFU/ml). The suspension was further diluted to provide a final bacterial concentration of 10^5 CFU/ml in each well of the microdilution trays. Plates were covered with plastic tapes and incubated in ambient atmosphere at 37ºC for 20-24 h. MIC was defined as the lowest concentration of drug that inhibited visible growth. *S. pneumoniae* ATCC strains 49619 and 6303 were used for the quality control.

PCR amplification and DNA sequence determination. *S. pneumoniae* chromosomal DNA was obtained as described previously (6). PCR amplifications were performed using 1

unit of *Thermus thermophilus* thermostable DNA polymerase (Biotools), 1 μg of chromosomal DNA, 0.4 μM (each) of the synthetic oligonucleotide primers, 0.2 mM of each dNTP, and 2 mM $MgCl_2$ in a final volume of 50 μl. To amplify the *atpC* and *atpA* genes, oligonucleotides atporf1 (5'-ggtcggaaTTCTGGATGGCTAACTTGAACTTG-3'), corresponding to nucleotides –143 to –120 upstream of *atpC*, and atpB56 (5'-GACGGGCTTCTTCAGCTCTGTC-3') (the complementary strand of the primer coding for DRAEEAR of subunit *b*), were used. The 5' end of atporf1 contained a sequence including an *Eco*RI restriction site, which is shown underlined. Amplification was achieved with an initial cycle of 1 min denaturation at 94ºC, and 30 cycles of 30 seconds at 94ºC, 90 seconds at 55ºC and 75 seconds polymerase extension step at 72ºC, with a final 8 min 72ºC extension step and slow cooling at 4ºC. PCR products used in control experiments of transformation were obtained with 2,6 units of a proof-reading enzyme, Expand High Fidelity PCR system (Boehringer Manheim). The *parE* fragment used in these experiments was amplified with oligonucleotides parE105 (5'-GGAGGGAAATTCGGTCAAGGTGGCTA-3') (coding for GGKFGNGGY) and parE483 (9). Gel electrophoresis of PCR products was carried out in agarose gels as described (32). DNA fragments were purified using MicroSpin™ S400 HR columns (Amersham Pharmacia Biotech) and were sequenced on both strands with an Applied Biosystems Prism 377 DNA sequencer, accordingly to protocols provided by the manufacturer with both the primers used for the PCR amplifications and internal primers.

ATPase activity determinations. Membrane-associated ATPase activity was measured as described (24). One unit of activity was defined as the amount of enzyme that resulted in the release of 1 μmol of inorganic phosphate (Pi) per ml per 30 min. IC_{50} values (mean of two independent determinations) were defined as the concentration of the antimalarial agent required for a 50% reduction of enzymatic activity.

Chemicals. Opt and Qin were purchased from Sigma Chemical., St. Louis, Mo. Mef (Ro 21-5998-000) was provided by Roche Laboratories, Basel, Switzerland. Other antimalarial drugs are identified by The Ohio State University (OSU) code numbers (18). New OSU compounds used were, OSU1, OSU207 and OSU255 with molecular weights of 387.3, 427.4, and 444.35, respectively.

Nucleotide sequence accession numbers. The sequences reported here have been deposited in GenBank under accession numbers AY061860 to AY061873.

RESULTS

Antimicrobial activity of Mef and OSU compounds. To test if Mef and OSU compounds specifically target the F_0F_1 ATPase of *S. pneumoniae*, their MICs against Opt^R and Qin^R strains were determined (Table 1). The strains used were R6 mutants with defined point mutations in *atpC*, selected either *via* Opt resistance (strains MJ11 and MJ2) (6), or Qin resistance (strains MJQ3 and MJQ4) (24). Susceptible strains used as controls were R6, and the ATCC strains 6303 and 49619. When these strains are considered, Mef and OSU compounds were 2.4 to 10-fold more active than Opt and 40 to >300-fold more active than Qin in inhibiting the growth of *S. pneumoniae*. When the MICs against R6 were compared to those against their MJ2, MJ11, MJQ3, and MJQ4 mutants, significant increases (variations ≥ 2-fold the MIC) in resistance to Mef, OSU 1, and OSU 8 were observed for strains MJQ3 and MJQ4, and MJQ3 also showed an increase in resistance higher than 15-fold to OSU 199 and OSU 207. However, strain MJ2 only showed increased resistance to Mef, and OSU207, whereas strain MJ11 did not showed any differences for the compounds assayed. None of the mutant strains had increased resistance to OSU compounds 95, 99, 255, and 259 (Table 1). Taking together, these results suggest that the *c* subunit of the F_0 complex of the F_0F_1 ATPase is the target for Mef and OSU compounds 1, 8, 199 and 207, and that each mutant displays a characteristic pattern of inhibition with the various compounds.

Characterization of Mef^R *S. pneumoniae* strains. Mef^R strains were obtained by plating 2×10^{10} R6 cells in 0.3 μg/ml Mef (2-fold the MIC). One isolate, designated as MJM1, was obtained. The *atpC* and *atpA* genes of this strain were PCR amplified with oligonucleotides atporf1 and atpB56, and sequenced. A single mutation was observed in *atpA*, i.e., an TTT codon in strain R6 was changed to TCT that would produce a F209S change in the predicted amino acid sequence of the *a* subunit of the MJM1 F_0F_1 ATPase (not shown). To provide genetic evidence that this change was indeed involved in Mef resistance, the PCR product obtained from strain MJM1 DNA carrying *atpCA* was used to transform competent R6 cells,

and the same PCR product, but from R6 DNA, was used as a control. As expected, the PCR product from MJM1 transformed R6 with high efficiency (higher than 10^5 transformants/ml) to Mef resistance. However, the R6 PCR product was also able to transform, although with much lower efficiency (10^3 transformants/ml). Two colonies, selected from the transformation with the MJM1 PCR product, showed to carry the F209S amino acid change in the *a* subunit, indicating that this mutation was indeed involved in Mef resistance. One of these transformants was named MJM11 (Table 2) and used for further studies. From the transformation experiment with the R6 PCR product, several colonies were selected and the sequences of their *aptCA* genes determined. These strains carried point mutations either in the *atpC* gene (MJM5, 7, 8, 9, 10, 13, 14, and 15) or in *atpA* (MJM3, 6, 11, 21, and 22) (Table 2). Genetic evidence showing that the mutations carried by these strains were indeed involved in resistance was obtained by genetic transformation. PCR products carrying those mutations were able to transform the Mef^S strain R6 with high efficiency (higher that 10^5 transformants/ml) to Mef^R. The susceptibilities of these strains to Mef and OSU compounds was analyzed, with exception of MJM10 and 14 that had amino acid substitutions identical to MJQ3 and Q4, respectively. All mutant strains selected for resistance to Mef showed significant increases in their CMIs to this compound (from 4 to 64-fold) and to OSU199 (4 to 32-fold). All but MJM7 had increased resistance to optochin (4 to 32-fold) and OSU1 (4 to 64-fold), and all but MJM6, MJM8 and MJM11 to quinine (4 to 8-fold) (Table 1).

The appearance of Mef^R colonies in the transformation with the 1,277-bp PCR product carrying the R6 *atpCA* genes might be attributed to the error rate of the polymerase. This rate is of 1 error/ 10 kbp, and then an error would be expected for 10 molecules of 1-kb. From our results, about 1 % (10^{-2}) of the putative nt changes would result in a residue change conferring Mef^R. Since 9 x 10^6 cells were used in the transformation experiments, the expected number of clones with Mef^R mutations would be of 9 x 10^3, a figure consistent with the results obtained. Furthermore, when a 1,158-bp fragment of an unrelated gene (*parE*, coding for the ParE subunit of DNA topoisomerasa IV), was amplified and used in transformation experiments, the frequency of Mef^R colonies was equivalent to that obtained in the control with no DNA (no colonies appeared in 0.1 ml of the transformation mixture, that gives a

frequency lower that 10 transformants/ml). In addition, similar number (5 x 10^2 transformants/ml) of Mef^R colonies were observed when a proofreading enzyme was used in the amplification of the *atpCA* genes, and no Mef^R colonies appeared when the *parE* was amplified with this enzyme.

Characteristics of the mutations affecting the *c* and *a* subunits of the F_0F_1 ATPase of *S. pneumoniae*. Predictions of the secondary structure of subunits *c* and *a* subunits were made by the PHD method (29-31) using an electronic mail server (http://www.embl-heidelberg.de). Those calculations predicted 2 transmembrane α-helices for the *c* subunits and 6 for the *a* subunits of *S. pneumoniae* and *E.coli* (Fig. 2). Among the 13 Mef^R strains obtained by selection with Mef (named MJMX, M for Mef, Table 2), 8 carried mutations in *atpC* and 5 in *atpA*. All amino acid residues that changed in the Mef^R stains were located in a predicted transmembrane α-helix (Table 2, Fig. 2). In the *c* subunit, 5 strains showed changes in helix-1 and 3 in helix 2, while the changes in the *a* subunit were at residues located in helix 5 (one strain) and in helix 4 (4 strains).

Inhibition of the F_0F_1 ATPase by Mef and OSU compounds. Biochemical evidence for the targeting of the F_0F_1 ATPase by Mef and related compounds was obtained by testing the inhibition of the ATPase activity of membranes obtained from R6 and the mutant strains. All compounds inhibited the R6 ATPase activity, including those compounds (OSU95, 99, 255, and 259) that inhibited the growth of both sensitive and resistant strains (Table 1). The inhibition of the strains by Mef, OSU1, OSU8, OSU 199, and OSU207 of the ATPase activity (IC_{50} values) showed a good correlation with their MICs (Fig. 1), indicating that the primary target for these compounds in the cell is the membrane ATPase. A good correlation was also obtained when the IC_{50} values of the different compounds were plotted against MICs for strain MJQ4, a strain that showed an intermediate sensitivity to most of the compounds (Fig. 1), indicating that all compounds share the same target in the cell.

DISCUSSION

Combined genetic and biochemical studies support a hairpin-like structure for the *c*

subunit with two α-helices that transverse the membrane in an antiparallel fashion, separated by a conserved polar loop region that forms the F_1 binding region (14). The Mef^R pneumococcal strains had point mutations that change amino acid residues located in one of the two transmembrane α-helices of the *c* subunit at positions close to the two acidic residues E19 and E52 (Table 2, Fig. 2). Although previously characterized Opt^R strains, selected *via* optochin resistance (strains MJ2 and MJ11) had mutations in helix-1 (6) and strains selected *via* quinine resistance (MJQ3 and MJQ4) had mutations in helix-2 (24), the strains selected *via* Mef resistance had mutations in either helix-1 or -2 (Table 2, Fig. 2). However, independently of the compound used for mutant selection, cross-resistance to those three antimalarial agents was observed (Table 1, Figs 1-2), suggesting that these chemically similar drugs interact with the *c* subunit in the same region of the protein, the ion-binding pocket. In the *E. coli* pocket, the D61 side chain is positioned within a four-helix bundle formed by the front and back faces of two adjacent monomers, D61 of helix-2 of one monomer packing in close proximity to residues A24 and I28 of helix-1, and A62 of helix-2 of the neighbouring monomer (5, 13) in a cavity created by the absence of side-chains at G23, G27, and G58 (8). Interestingly, two of the *S. pneumoniae* resistant strains (MJ11, MJM5) had amino acid changes at positions equivalent to G23 and G58 of *E. coli* (Table 2, Fig. 2), and changes G to A are found in strains MJQ4 and MJM15. All these results suggest a structure for the oligomeric subunit *c* ring of *S. pneumoniae* compatible with that of *E. coli*. However, whereas the *E. coli c* subunit has only one carboxyl group (D61 of α helix-2), the *S. pneumoniae c* subunit has two groups, located in a centered position in one of each alpha helices (E19 and E52, equivalent to I28 and D61 of *E. coli*), suggesting that both carboxylic groups would be involved in proton translocation. In agreement with this hypothesis, functional ATPases from *E. coli* mutants in which the essential carboxyl group of the *c* subunit has been switched to position 24 of helix-1 (equivalent to V15 of *S. pneumoniae*) (22) or in which a second carboxyl group has been introduced at position 28 (equivalent to E19 of *S. pneumoniae*) (12) have been described.

Additionally to mutations in the *c* subunit, five of the Mef^R strains carried mutations in two α-helices of the *a* subunit: position L186 of the penultimate helix, and W206, F209 and

S214 of the ultimate helix (Fig. 2). These two helices are also present in the *E. coli a* subunit (Fig. 2). Studies of cysteine cross-linking (11) and nuclear magnetic resonance (28) have established that helix-2 of subunit *c* makes contact with the penultimate helix of subunit *a* (Fig. 2), residues S207, N214, A217, I221, I223, L224 and I225 lying in one face of the transmembrane helix, which then interact with helix-2 of subunit *c*. This proximity of the helices supports the possibility of an interaction between R210 of the *a* subunit and D61 of the *c* subunit during proton translocation. The location of the L186P change in the MJM21 *S. pneumoniae* strain is in accordance to this model of the F_0 complex of *E. coli*.

Four residue changes present in the *S. pneumoniae* resistant strains are located in the last helix of subunit *a* (Fig. 2), suggesting an interaction between this helix of the *a* subunit and any of the helices of the *c* subunit. Although no cross-linking formation has been observed between helix-1 of the *E. coli* subunit c and the last helix of subunit a (11), a mutant strain with a H245D substitution shows a 45% reduction of normal proton translocation (3) and structural studies have defined one hydrophilic pathway between R210 of the *E. coli a* subunit and the periplasmic surface including residues N238, H245, and Q252 of the last helix of this protein (11).

The location and nature of the amino acid changes found in the *c* subunit of the R6 descendants reported here are in accordance with those observed in pneumococcal optochin-resistant clinical isolates, which showed G20S, M23I, A49T, V48F, and F50L changes (6, 27). In addition, a change in the *a* subunit (W206S), that corresponds to that found in strain MJM3, has also been recently reported in a clinical isolate of *S. pneumoniae* (27).

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REFERENCES

1. **Abrahams, J. P., A. G. W. Leslie, and J. E. Walker.** 1994. Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature **370:**621-628.
2. **Bender, G., S. Sutton, and R. Marquis.** 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect. Immun. **53:**331-338.
3. **Cain, B. D., and R. D. Simoni.** 1988. Interaction between Glu-219 and His-245 within the *a* subunit of F_1F_0-ATPase in *Escherichia coli*. J. Biol. Chem. **263:**6606-6612.
4. **Chen, D. K., A. McGeer, J. C. de Azavedo, and D. E. Low.** 1999. Decreased susceptibility of *Streptococcus pneumoniae* to fluoroquinolones in Canada. New Engl. J. Med. **341:**233-239.
5. **Dmitrev, O. Y., P. C. Jones, and Fillingame R. H.** 1999. Structure of the subunit *c* oligomer in the F_1F_0 ATP synthase: model derived from solution structure of the monomer and cross-linking in the native enzyme. Proc. Natl. Acad. Sci. USA **96:**7785-7790.
6. **Fenoll, A., R. Muñoz, E. García, and A. G. de la Campa.** 1994. Molecular basis of the optochin-sensitive phenotype of pneumococcus: characterization of the genes encoding the F_0 complex of the *Streptococcus pneumoniae* and *Streptococcus oralis* H^+-ATPases. Mol. Microbiol. **12:**587-598.
7. **Futai, M., T. Noumi, and M. Maeda.** 1989. ATP synthase (H^+-ATPase): results by combined biochemical and molecular biological approaches. Annu. Rev. Biochem. **58:**111-136.
8. **Girvin, M. E., V. K. Rastogi, F. Abrildgaard, J. L. Markley, and R. H. Fillingame.** 1998. Solution structure of the transmembrane H^+-translocating subunit c of the F_1F_0 ATP synthase. Biochemistry **37:**8817-8824.
9. **González, I., M. Georgiou, F. Alcaide, D. Balas, J. Liñares, and A. G. de la Campa.** 1998. Fluoroquinolone resistance mutations in the *parC*, *parE*, and *gyrA* genes of clinical isolates of viridans group streptococci. Antimicrob. Agents Chemother. **42:**2792-2798.
10. **Hoffman, J., M. S. Cetron, M. M. Farley, W. S. Baughman, R. R. Facklam, J. A. Elliot, K. A. Deaver, and R. F. Breiman.** 1995. The prevalence of drug-resistant *Streptococcus pneumoniae* in Atlanta. N. Engl. J. Med. **333:**481-486.

11. **Jiang, W., and R. H. Fillingame.** 1998. Interacting helical faces of subunits *a* and *c* in the F_1F_0 ATP synthase of *Escherichia coli* defined by disulfide cross-linking. Proc. Natl. Acad. Sci. USA **95:**6607-6612.

12. **Jones, P. C.** 2001. Introduction of a carboxyl group in the first transmembrane helix of *Escherichia coli* F_1F_0 ATPase subunit *c* and cytoplasmic pH regulation. J. Bacteriol. **183:**1524-1530.

13. **Jones, P. C., W. Jiang, and R. H. Fillingame.** 1998. Arrangement of the multicopy H^+-translocating subunit *c* in the membrane sector of the *Escherichia coli* F_1F_0 ATP synthase. J. Biol. Chem. **273:**1718-1785.

14. **Kakinuma, Y.** 1998. Inorganic cation transport and energy transduction in *Enterococcus hirae* and other streptococci. Microbiol. Mol.Biol. Rev. **62:**1021-1045.

15. **Kobayashi, H., T. Suzuki, N. Kinoshita, and T. Unemoto.** 1984. Amplification of the *Streptococcus faecalis* proton-translocating ATPase by a decrease in cytoplasmic pH. J. Bacteriol. **158:**1157-1160.

16. **Kobayashi, H., T. Suzuki, and T. Unemoto.** 1986. Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. J. Biol. Chem. **261:**627-630.

17. **Koebmann, B. J., D. Nilsson, O. P. Kuipers, and P. R. Jensen.** 2000. The membrane-bound H^+-ATPase complex is essential for growth of *Lactococcus lactis*. J Bacteriol. **182:**4738-4743.

18. **Kullen, M. J., and T. R. Klaenhammer.** 1999. Identification of the pH-inducible, proton-translocating F_1F_0-ATPase (*atpBEFHAGDC*) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. Mol. Microbiol. **33:**1152-1161.

19. **Kunin, C. M., and W. Y. Ellis.** 2000. Antimicrobial activities of mefloquine and a series of related compounds. Antimicrob. Agents Chemother. **44:**848-852.

20. **Liñares, J., A. G. de la Campa, and R. Pallarés.** 1999. Fluoroquinolone resistance in *Streptococcus pneumoniae*. N. Engl. J. Med. **341:**1546-1548.

21. **Martín-Galiano, A. J., M. J. Ferrándiz, and A. G. de la Campa.** 2001. The promoter of the operon encoding the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae* is inducible by pH. Mol. Microbiol. **6**:1327-1338.

22. **Miller, M. J., M. Oldenburg, and R. H. Fillingame.** 1990. The essential carboxyl group in subunit *c* of the F_1F_0 ATP synthase can be moved and H^+-translocation retained. Proc. Natl. Acad. Sci. USA **87:**4900-4904.

23. **Moore, H. F., and A. M. Chesney.** 1917. A study of ethylhydrocuprein (optochin) in the treatment of acute lobar pneumonia. Arch. Intern. Med. **19:**611-682.

24. **Muñoz, R., E. García, and A. G. de la Campa.** 1996. Quinine specifically inhibits the proteolipid subunit of the F_0F_1 H^+-ATPase of *Streptococcus pneumoniae*. J. Bacteriol. **178:**2455-2458.

25. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa.

26. **Pallarés, R., J. Liñares, M. Vadillo, C. Cabellos, F. Manresa, P. F. Viladrich, R. Martín, and F. Gudiol.** 1995. Resistance to penicillin and cephalosporin and mortality from severe pneumococcal pneumonia in Barcelona, Spain. N. Engl. J. Med. **333:**474-480.

27. **Pikis, A., J. M. Campos, W. J. Rodríguez, and J. M. Keith.** 2001. Optochin resistance in *Streptococcus pneumoniae*: mechanism, significance, and clinical implications. J. Infect. Dis. **184:**582-590.

28. **Rastogi, V. K., and M. E. Girvin.** 1999. Structural changes linked to proton translocation by subunit *c* of the ATP synthase. Nature. **402:**263-268.

29. **Rost, B.** 1996. PHD: predicting one-dimensional protein structure by profile-based neural networks. Methods Enzymol. **266:**525-539.

30. **Rost, B., P. Fariselli, and R. Casadio.** 1996. Topology prediction for helical transmembrane proteins at 86% accuracy. Protein. Sci. **5:**1704-1718.

31. **Rost, B., and C. Sander.** 1993. Prediction of protein secondary structure at better than 70% accuracy. J. Mol. Biol. **232:**584-599.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

33. **Stock, D., A. G. W. Leslie, and J. E. Walker.** 1999. Molecular architecture of the rotary motor in ATP synthase. Science. **286:**1700-1705.

34. **Tomasz, A.** 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. **101:**860-871.

35. **Valiyaveetil, F. I., and R. H. Fillingame.** 1998. Transmembrane topography of subunit *a* in the *Escherichia coli* F_1F_0 ATP synthase. J. Biol. Chem. **273:**16241-16247.

FlGURE LEGENDS

Figure 1. Correlations between inhibition of the ATPase activity and inhibition of growth by Mef and OSU compounds of membranes the strains indicated (A) and of membranes of MJQ4 with the different compounds.

Figure 2. Comparisons of the amino acid sequences and secondary structures of the *c* and *a* subunits of *S. pneumoniae* (*SPN*) and *E. coli* (*ECO*). Predicted α-helices are showed above the *SPN* sequence and below the *ECO* sequence: gray and solid bars indicate, respectively, predictions made by the PHD method and according to Miller *et al.* (for the *c* subunit) (22) or to Valiyaveetil and Fillingame (35) (for the *a* subunit). Residues mutated in the pneumococcal resistant strains are showed in boldface and underlined. Residues of the ECO *a* subunit involved in interactions with the *c* subunit (11) are shown boldfaced and doubly underlined. Residues involved in proton translocation in *E. coli* and the equivalent *S. pneumoniae* residues are boxed.

TABLE 1. Susceptibilities of *S. pneumoniae* strains to amino alcohol antimalarials

Strains	MIC (μg/ml) [a] of										
	Opt	Qin	Mef	OSU1	OSU8	OSU95	OSU99	OSU199	OSU207	OSU255	OSU259
ATCC 6303	0.75	25	0.15	0.15	0.15	0.15	0.15	0.31	0.15	0.07	0.15
ATCC 49619	1.5	50	0.15	0.15	0.31	0.62	0.62	0.62	0.15	0.15	0.15
R6	1.5	50	0.15	0.15	0.31	0.15	0.62	0.31	0.15	0.62	1.25
MJ11	24	72	0.15	0.15	0.31	0.15	0.62	0.15	0.07	0.62	1.25
MJ2	24	230	0.62	0.31	0.62	0.31	0.31	0.15	0.62	1.25	1.25
MJQ3	3	144	5	2.5	5	0.31	0.31	5	5	1.25	1.25
MJQ4	18	180	2.5	1.25	2.5	0.31	0.31	0.31	0.62	1.25	1.25
MJM3	6	200	2.5	5	—	—	—	5	—	—	—
MJM5	48	200	5	5	—	—	—	5	—	—	—
MJM6	12	100	2.5	0.62	—	—	—	1.25	—	—	—
MJM7	3	200	1.25	0.31	—	—	—	0.62	—	—	—
MJM8	6	100	5	2.5	—	—	—	2.5	—	—	—
MJM9	12	200	0.62	1.25	—	—	—	1.25	—	—	—
MJM11	12	100	2.5	1.25	—	—	—	2.5	—	—	—
MJM13	24	400	10	10	—	—	—	10	—	—	—
MJM15	12	200	5	2.5	—	—	—	2.5	—	—	—
MJM21	6	200	2.5	2.5	—	—	—	2.5	—	—	—
MJM22	24	200	2.5	2.5	—	—	—	5	—	—	—

[a] Opt, optochin; Qin, quinine, Mef, mefloquine; —, not determined.

TABLE 2. Mutational substitutions of the *c* and *a* subunits of *S. pneumoniae* strains resistant to amino alcohol antimalarial agents

Strain	Amino acid change (codon change)[a]			
	c subunit		*a* subunit	
	α-helix 1	α-helix 2	α-helix 5	α-helix 6
R6	^{13}**MG**VSVG<u>E</u>**G**LL**MN**^{24}	^{44}**M**FL**GVA**FI<u>E</u>GTFF**V**^{57}	^{178}GEVMTSLL**L**L^{187}	^{205}A**W**TA**F**SVFI**S**^{214}
MJ11	—	A49T (GCC→ACC)	—	—
MJ2	—	V48L (GTT→CTT)	—	—
MJQ3	M23I (ATG→ATA)	—	—	—
MJQ4	G20A (GGT→GCT)	—	—	—
MJM3	—	—	—	W206G (TGG→GGG)
MJM5	G14S (GGC→AGC)	—	—	—
MJM6	—	—	—	S214P (TCC→CCC)
MJM7	—	V57L (GTA→CTA)	—	—
MJM8	M13T (ATG→ACG)	—	—	—
MJM9	—	M44V (ATG→GTG)	—	—
MJM10	M23I (ATG→ATT)	—	—	—
MJM11	—	—	—	F209S (TTT→TCT)
MJM13	N24Y (AAT→TAT)	—	—	—
MJM14	G20A (GGT→GCT)	—	—	—
MJM15	—	G47A (GGT→GCT)	—	—
MJM21	—	—	L186P (CTT→CCT)	—
MJM22	—	—	—	W206C (TGG→TGT)

[a] The amino acid positions indicated refer to the *S. pneumoniae* R6 coordinates (accession number Z26851, see ref. 6). Residues that changed in the mutant strains indicated are showed in boldface. The E residues possibly involved in proton translocation are showed underlined in the α-helices of the *c* subunit.