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Mutations in 23S rRNA Confer Resistance against Azithromycin in *Pseudomonas aeruginosa*

Rasmus Lykke Marvig,[a] Mette S. R. Søndergaard,[a] Søren Damkiær,[a] Niels Høiby,[b] Helle Krogh Johansen,[b] Søren Molin,[a] and Lars Jelsbak[a]

Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark,[a] and Department of Clinical Microbiology, Rigshospitalet and ISIM, University of Copenhagen, Copenhagen, Denmark[b]

The emergence of antibiotic-resistant *Pseudomonas aeruginosa* is an important concern in the treatment of long-term airway infections in cystic fibrosis patients. In this study, we report the occurrence of azithromycin resistance among clinical *P. aeruginosa* DK2 isolates. We demonstrate that resistance is associated with specific mutations (A2058G, A2059G, and C2611T in *Escherichia coli* numbering) in domain V of 23S rRNA and that introduction of A2058G and C2611T into strain PAO1 results in azithromycin resistance.

Systematic long-term, low-dose azithromycin treatment has been used for cystic fibrosis (CF) patients chronically infected with *Pseudomonas aeruginosa* in the Copenhagen CF center in Denmark since 2001 (3). Even though the use of azithromycin on CF lung infections has been found to have beneficial clinical effects (1, 3), it remains unclear how azithromycin works on *P. aeruginosa* and if macrolide resistance can emerge.

Using whole-genome sequencing, we have recently described the dissemination of the DK2 lineage of *P. aeruginosa* among a cohort of CF patients in Copenhagen, Denmark (15). The DK2 lineage has persisted for decades in the CF lung environment and has evolved as independent sublineages in concurrently infected patients. This makes it possible to search for convergent mutational events that occurred independently in isolates from different patients. Isolates CF333-2007 and CF66-2008 sampled from two different patients were found to have independently accumulated the same 23S rRNA A2045G mutation, while their most recent common ancestors did not (15). Mutations in the proximity of A2045 (position 2058 in *Escherichia coli* numbering) in the secondary structure of domain V of 23S rRNA have previously been shown to confer resistance toward macrolide antibiotics in other bacterial species (13). This led us to investigate if the observed mutations could be associated with azithromycin resistance and if such putative resistance mutations were found in strains of the DK2 lineage type present in other CF patients from the Copenhagen CF clinic.

We sequenced and analyzed the 23S rRNA genes of nine clinical *P. aeruginosa* isolates sampled from nine CF patients attending the Copenhagen CF center at the University Hospital (Table 1). Sequence data for three of the nine isolates were available from our previous study (15) (Table 1). Bacterial isolation from sputum and whole-genome sequencing were done as described previously (6, 15). Sequence reads were mapped against the 23S rRNA alleles of the *P. aeruginosa* DK2 reference genome (CF333-2007; GenBank accession no. CP003149), and single nucleotide polymorphisms in the 23S rRNA gene were called by SAMtools release 0.1.7 (10). The number of reads that supported a detected polymorphism relative to the total number of reads covering the polymorphic site was used to estimate the fraction of 23S rRNA genes in an individual clone which contained the polymorphism. Accordingly, polymorphisms present in one, two, three, or four of the four copies of 23S rRNA would be supported by approximately 25%, 50%, 75%, and 100% of the reads, respectively.

Three of the strains (CF206-2002, CF223-2002, and CF311-2002) had mutations in close proximity to the A2045 position in the secondary structure of domain V of 23S rRNA (13) at which isolates CF333-2007 and CF66-2008 were found to be mutated (Table 1). These mutations were A2046G (position 2059 in *E. coli* numbering) and C2598T (position 2611 in *E. coli* numbering), and both mutations have been reported to confer macrolide resistance in other bacterial species (13). The three remaining strains (CF180-2002, CF240-2002, and CF173-2005) did not contain any polymorphisms relative to the ancestral strain CF30-1979 in domain V of 23S rRNA.

We tested the resistance of the isolates toward azithromycin as described by Köhler et al. (9). Stationary-phase cultures of *P. aeruginosa* were incubated with and without 50 μg/ml azithromycin (16 to 62 μg/ml have been measured in CF sputum [14]) for 2 h, and after incubation, CFU were determined by plating onto LB agar plates with and without 20 μg/ml azithromycin, respectively. Inhibition of cell growth by azithromycin was calculated as the number of CFU from cultures grown with azithromycin relative to cultures grown without azithromycin.

As shown in Table 1, only minor or moderate effects of azithromycin treatment were observed for strains CF333-2007, CF66-2008, CF206-2002, CF223-2002, and CF311-2002 harboring mutations in domain V of 23S rRNA (15%, 4%, 25%, 12%, and 15% reduction in CFU, respectively). In contrast, strain CF30-1979, which closely resembles the ancestor of all DK2 clones isolated after 1979 (15) and harbors only wild-type 23S rRNA alleles, was found to be more inhibited by azithromycin (one-tailed Student's *t* test; $P < 0.05$) and exhibited a 64% reduction in CFU (Table 1).

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Address correspondence to Lars Jelsbak, lj@bio.dtu.dk.
R.L.M. and M.S.R.S. contributed equally to this article.

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TABLE 1 Resistance against azithromycin of clinical and laboratory strains used in this study

Strain	Mutations in 23S rRNA[b]	Fraction of reads supporting the mutations (%)	Start of systematic treatment with azithromycin (yr)	Resistance against azithromycin (%)[c]
Clinical isolates[a]				
CF30-1979[d]	None		2001	36 (±39)
CF333-2007[d]	A2045G (A2058G)	100	2006	85 (±14)
CF66-2008[d]	C1433T, A2045G (C1446T, A2058G)	21, 100	2001	96 (±39)
CF206-2002	C2598T (C2611T)	53	2006	75 (±32)
CF223-2002	C2598T (C2611T)	73	2001	88 (±35)
CF311-2002	A93G, A2046G (A106G, A2059G)	26, 70	2004	85 (±23)
CF180-2002	C1433T (C1446T)	25	2001	14 (±27)
CF240-2002	None		2006	35 (±32)
CF173-2005	None		2006	0
Laboratory strains				
PAO1	None	N/A[f]	N/A	0
PAO1-pMES-23S	None	N/A	N/A	1 (±1)
PAO1-pMES-23S(A2045G)	A2045G[e]	N/A	N/A	96 (±22)
PAO1-pMES-23S(C2598T)	C2598T[e]	N/A	N/A	92 (±14)

[a] Names of clinical isolates are given according to their patient origin and sampling year (i.e., isolate CF30-1979 was sampled from patient CF30 in 1979). All clinical isolates belong to the *P. aeruginosa* DK2 clone type.
[b] The corresponding *E. coli* 23S rRNA position is given in parentheses.
[c] Inhibition of cell growth by azithromycin was calculated as the number of CFU from cultures grown with azithromycin relative to cultures grown without azithromycin. The assay was replicated at least three times for each strain, and 95% confidence intervals are given in parentheses. No CFU appeared in cultures grown with azithromycin for strains PAO1 and CF173-2005.
[d] Whole-genome sequence of the isolate has been described previously (15).
[e] The strain carries a mutated 23S rRNA allele on the low-copy-number plasmid vector pME6031.
[f] N/A, not applicable.

The three strains (CF180-2002, CF240-2002, and CF173-2005) that did not contain any polymorphisms in domain V of 23S rRNA were all inhibited by azithromycin (one-tailed Student's *t* test; $P < 0.05$) (Table 1). Interestingly, the results also indicated that CF30-1979, CF180-2002, and CF240-2002 are less susceptible than PAO1. It is possible that this difference is due to other antibiotic-related mutations, e.g., in drug efflux pumps or ribosomal proteins L4 and L22 (8, 11, 12), present in the clinical strains.

To more directly assess the effects of the A2045G and C2598T mutations, we separately cloned both mutated and wild-type rRNA operons into the low-copy-number plasmid vector pME6031 (5 to 7 copies per cell) (4). The entire rRNA operon flanked by the genes PA4690 and PA4691 was PCR amplified from CF30-1979, CF333-2007, and CF223-2002 template DNA and ligated into plasmid pME6031, resulting in the complementation vectors pMES-23S, pMES-23S(A2045G), and pMES-23S(C2598T), respectively, which were introduced into the common laboratory strain *P. aeruginosa* PAO1 (Table 2).

Strain PAO1-pMES-23S was susceptible to azithromycin like its parent strain PAO1, whereas no inhibitory effect of azithromycin was observed for either PAO1-pMES-23S(A2045G) or PAO1-pMES-23S(C2598T) containing mutated 23S rRNA alleles, and both strains were significantly more resistant than PAO1-pMES-23S (one-tailed Student's *t* test; $P < 0.001$) (Table 1). This result confirmed the hypothesis that the A2045G and C2598T mutations confer resistance to azithromycin in *P. aeruginosa*.

We note that the resistance phenotype is observed both in strains PAO1-pMES-23S(A2045G) and PAO1-pMES-23S(C2598T) despite their capacity to express chromosomally encoded wild-type 23S rRNA and plasmid-encoded mutated 23S rRNA and in isolates CF206-2002, CF223-2002, and CF311-2002 in which none of the polymorphisms were fixed in all four copies of the 23S rRNA gene (Table 1). This indicates that not all four 23S rRNA alleles have to contain the mutations in order to confer the resistance phenotype (12).

With the exception of CF180, none of the other patients infected with susceptible isolates (CF30, CF240, and CF173) had been subjected to systematic long-term, low-dose treatment with azithromycin against *P. aeruginosa* at the time of sampling (Table 1). Moreover, resistant isolates CF333-2007, CF66-2008, and CF223-2002 were sampled from their respective host patients after initiation of systematic treatment with azithromycin. In con-

TABLE 2 List of plasmids and strains used for cloning purposes

Strain or plasmid	Relevant characteristics	Reference
E. coli CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recAl*	5
P. aeruginosa PAO1		7
pME6031	PVS1-p15A shuttle vector; Tc^r	4
pMES-23S	A 5.95-kb SacI-PstI fragment containing the rRNA operon from CF30-1979 inserted into pME6031	This study
pMES-23S(A2045G)	A 5.95-kb SacI-PstI fragment containing the rRNA operon from CF333-2007 inserted into pME6031	This study
pMES-23S(C2598T)	A 5.95-kb SacI-PstI fragment containing the rRNA operon from CF223-2002 inserted into pME6031	This study

trast, the resistant isolates CF206-2002 and CF311-2002, both carrying mutations in domain V of 23S rRNA, were sampled from patients who had not been subjected to systematic treatment with azithromycin. It is possible that the presence of these resistant *P. aeruginosa* isolates is either the outcome of earlier treatments with macrolide antibiotics directed against infections caused by other CF pathogens (e.g., *Staphylococcus aureus*) in these patients or the result of transmission from patients treated with azithromycin.

In this study, we report the occurrence of azithromycin resistance among clinical *P. aeruginosa* isolates of the DK2 lineage. Sequencing of 23S rRNA genes revealed the resistance to be associated with mutations at specific loci (A2045, A2046, and C2598), and the effect of two of the mutations (A2045G and C2598T) were validated by *trans* complementation into the laboratory strain PAO1. Even though the use of azithromycin on *P. aeruginosa* CF lung infections has been found to have beneficial effects (1, 3), the exact mechanism by which azithromycin exerts this effect is not well understood. *In vitro*, the effect has been shown to be dependent on interaction with the ribosomes (2, 9), and our results suggest that the mode of action of azithromycin *in vivo* also involves binding to the 50S ribosomal subunit and that the action is blocked by mutations in 23S rRNA.

Further studies are required to investigate the diversity of resistance mutations, how different antibiotic treatment regimens influence the resistance development, and how the effect of azithromycin is dependent on the growth physiology of *P. aeruginosa*.

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