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Amino Acid Substitution in the Major Multidrug Efflux Transporter Protein AcrB Contributes to Low Susceptibility to Azithromycin in *Haemophilus influenzae*

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ABSTRACT Clarithromycin-resistant *Haemophilus influenzae* strains with a nonsense mutation in *acrR* generally exhibited susceptibility to azithromycin, although one strain was found to be nonsusceptible; we aimed to clarify the differences. This strain had an amino acid substitution, Arg327Ser, in AcrB. Introduction of this substitution into *H. influenzae* Rd caused an increase in the MIC of azithromycin, suggesting that this substitution contributed to nonsusceptibility. These findings indicate that azithromycin-nonsusceptible isolates could occur through stepwise mutation in the *acr* region.

KEYWORDS BLNAR, azithromycin low susceptibility, AcrR, AcrB

Macrolide-, β-lactam-, and fluoroquinolone-resistant *Haemophilus influenzae* strains have been isolated worldwide (1–4). In particular, clarithromycin (CLR)-resistant strains have been increasing in Japan (1). We reported previously that these strains harbored a nonsense mutation in the negative regulator gene *acrR* that resulted in overexpression of the chromosomal efflux pump AcrAB, which likely contributed to the CLR resistance (MICs of ≥32 μg/ml) (5). Although all of the isolates with a nonsense mutation in *acrR* showed resistance to CLR (MICs of ≥32 μg/ml), almost all of the isolates showed susceptibility to azithromycin (AZM) (MICs of ≤4 μg/ml) and the AZM MICs were even slightly reduced, indicating that this mutation could not be involved in resistance to AZM. However, one of the CLR-resistant isolates with the *acrR* mutation showed nonsusceptibility to AZM (5). Therefore, the aim of the present study was to clarify the difference between AZM-nonsusceptible and -susceptible strains.

For this study, we used the AZM-susceptible strain 2011-70 (CLR MIC of 32 μg/ml and AZM MIC of 4 μg/ml) and the AZM-nonsusceptible strain 2012-42 (CLR MIC of 32 μg/ml and AZM MIC of 8 μg/ml), which were isolated at Hachioji Medical Center of Tokyo Medical University and have mutations at the same *acrR* site (Table 1) (5). These two strains have the same genetic background of sequence type 156 (ST156), as determined by multilocus sequence typing (1). *H. influenzae* ATCC 49247 and *H. influenzae* Rd were used as control strains in the antimicrobial susceptibility tests, and *H. influenzae* Rd was also used as a recipient for transformation.

Antimicrobial susceptibilities were measured in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (6, 7). AZM (LKT Laboratories, St. Paul, MN, USA), CLR (Tokyo Chemical Industry Co., Tokyo, Japan), and ethidium bromide (EB) (Wako Pure Chemical Industries, Osaka, Japan) were used as the antimicrobial agents. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Wako Pure Chemical Industries) was used as the efflux pump inhibitor. The antimicrobial susceptibility breakpoints were

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TABLE 1 Characteristics and MICs of bacterial strains used in this study

Strain	Serotype	ST	Deletion site in *acrR*	MIC (μg/ml)[a]						
					CLR + CCCP			AZM + CCCP		
				CLR	0.75 μg/ml CCCP	1.5 μg/ml CCCP	AZM	0.75 μg/ml CCCP	1.5 μg/ml CCCP	EB
2012-42	NT[b]	156	Nucleotide 141[c]	32	16	4	8	4	1	≥16
2011-70	NT	156	Nucleotide 141[c]	32	16	1	4	1	0.25	8
Rd	NT	47	None[c]	8	4	0.25	1	0.5	≤0.06	1
Rd_{42acr}	NT	47	Nucleotide 141[c]	32	16	2	8	4	0.5	8
Rd_{70acr}	NT	47	Nucleotide 141	32	16	1	4	2	0.13	4

[a]CLR, clarithromycin; AZM, azithromycin; EB, ethidium bromide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.
[b]NT, nontypeable.
[c]Results of a previous study by Seyama et al. (5).

set to ≤4 μg/ml for AZM and ≤8 μg/ml for CLR, according to the CLSI guidelines. In addition, to detect slight changes in susceptibility, we tested a more detailed range of concentrations than recommended by the CLSI, which were established through serial dilutions from 2 μg/ml for CLR and 1 μg/ml for AZM. These experiments were performed four times on independent occasions.

The transcription levels of the chromosomal multidrug efflux pump *acrAB* were determined by semiquantitative reverse transcription (RT)-PCR, as described previously (5). The *acr* operons of isolates 2011-70 and 2012-42 were analyzed according to the sequencing results obtained in the previous study (5).

The efflux pump activity was determined based on the intercalation fluorescence of EB, which is a substrate of AcrAB, according to the method described by Hayashi and colleagues (8, 9), with minor modifications. The isolates were treated with EB, CLR, and AZM at a final concentration of 10 μM. The fluorescence was measured every 5 min with a Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific, Kanagawa, Japan), with excitation at 530 nm and emission at 630 nm. The experiment was performed at least four times on independent occasions.

Comparison of *acr* operon regions, from 100 bases upstream of *acrR* to 30 bases downstream *acrB* (5,021bp), between the strains showed that 2012-42 had a specific amino acid substitution in AcrB, in which arginine was replaced by serine at position 327 (Arg327Ser) (see Fig. S1 in the supplemental material). No other CLR-resistant and AZM-susceptible strains ($n = 6$) tested had this substitution (data not shown) (5).

To investigate whether this amino acid substitution contributed to AZM nonsusceptibility, the *acr* operon regions, from 134 bases upstream of *acrR* to 258 bases downstream of *acrB* (5,282 bp), of strains 2012-42 and 2011-70 were amplified by PCR and introduced into *H. influenzae* Rd by electroporation, as described previously (5). Transformants (Rd_{42acr} and Rd_{70acr}) were selected by plating on chocolate agar containing 16 μg/ml CLR. The *acr* operon regions, from 100 bases upstream of *acrR* to 30 bases downstream *acrB* (5,021 bp), in the transformants were confirmed by DNA sequencing, which showed that the *acr* operons of the transformants obtained were identical to those of the donor strains (strains 2012-42 and 2011-70). The MICs of CLR and AZM for the transformants increased to the same levels as observed for the donor strains (Table 1). In addition, the *acrB* transcriptional levels of the transformants increased significantly, compared to that of Rd (Table 2).

The antimicrobial susceptibility test recommended by the CLSI was designed as a serial 2-fold dilution method; this method is concise and highly reproducible but would not detect subtler changes. Therefore, the detailed antimicrobial susceptibilities of the transformants were tested (Table 2). The detailed MICs of CLR and AZM in Rd_{42acr} were 1.2 and 2.3 times higher, respectively, than those in Rd_{70acr}.

We hypothesized that this amino acid substitution would alter the ability of the efflux pump AcrAB to recognize the substrate. To test this hypothesis, the macrolide efflux activities were measured with an EB efflux competition assay (Fig. S2), according to the gradient of the curve of the graph indicating the change in fluorescence over

TABLE 2 Detailed MICs of *H. influenzae* strain Rd and strains with *acr* mutations

Strain	Relative *acrB* mRNA level[a]	MIC (μg/ml)		Ratio[b]	
		CLR	AZM	CLR	AZM
Rd	0.82 ± 0.24	8	1	1	1
Rd_{42acr}	1.86 ± 0.35[c]	30	8	3.8	8
Rd_{70acr}	1.45 ± 0.09[c]	26	3.5	3.3	3.5

[a]The transcription levels were calculated in comparison with the *gyrB* transcription level. The measurements of *acrB* transcription levels were performed at least three times on independent occasions.
[b]Ratio of each MIC to that of *H. influenzae* Rd.
[c]$P < 0.05$, Rd versus Rd_{42acr} and Rd versus Rd_{70acr}.

time. After AZM addition, the initial velocity at 5 to 15 min, which might reflect efflux speed, was slightly but significantly larger in Rd_{42acr} than in Rd_{70acr}, by two-tailed Student's *t* test. In contrast, no significant differences between the strains were detected following EB or CLR addition (Fig. S2).

Overall, a nonsense mutation of *acrR* in *H. influenzae* appears to confer resistance to CLR but not to AZM. The present study further demonstrates that a subsequent missense mutation in *acrB* could result in AZM nonsusceptibility. Although the mechanism by which this substitution confers nonsusceptibility to AZM remains unclear, the results of the EB efflux assay showed that AZM addition competed with EB efflux at the early stage, suggesting that this amino acid substitution contributed to alterations in initial recognition and efflux. AcrB is known to form a trimer that acts as a complex with the outer membrane channel TolC and the membrane fusion protein AcrA (10–12). This efflux system discharges the agents from both the inner membrane and the periplasm (10). Several studies with efflux pump inhibitors demonstrated that the sensitivity of the inhibitors was determined by slight steric effects involving the amino acids around the inhibitor-binding pit (13, 14). These findings suggest that the amino acid substitution in the efflux pump influences the activity of the efflux pump. Therefore, we attempted to analyze the precise location of the amino acid 327 substitution in AcrB. The transmembrane protein structure prediction software CCTOP (http://cctop.enzim.ttk.mta.hu), which is a transmembrane region prediction tool (15), predicted that amino acid 327 of AcrB was located outside the outer membrane (data not shown). Amino acid 327 of AcrB in *Escherichia coli* was also reported to lie outside the outer membrane (16). This configuration implies that the amino acid could be involved in substrate recognition.

AZM shows relatively stronger antibacterial activity for *H. influenzae*, with a superior tissue distribution effect, compared to conventional macrolides (17–21). Furthermore, AZM was reported to be effective even when administered over a short period with a small dose, owing to its extended half-life (22, 23). Therefore, the possibility of increases in AZM-nonsusceptible strains could represent a crucial clinical problem, because of the loss of therapeutic options.

Our findings strongly suggest that the amino acid 327 substitution of AcrB contributes to AZM nonsusceptibility. Moreover, it appears that AZM-nonsusceptible isolates have emerged from stepwise mutations in the *acr* region; *H. influenzae* acquired a nonsense mutation in *acrR* as the first step, followed by a missense mutation in *acrB* as the second step. As we reported previously, CLR-resistant strains with a nonsense mutation in *acrR* are already prevalent in Japan. Our present study indicates that these strains could easily become AZM nonsusceptible through a single mutation in AcrB.

Accession number(s). The analyzed nucleotide sequences were submitted to GenBank under accession numbers LC269307 (strain 2012-42) and LC269308 (strain 2011-70).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01337-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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