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Clarithromycin Resistance Mechanisms of Epidemic β-Lactamase-Nonproducing Ampicillin-Resistant *Haemophilus influenzae* Strains in Japan

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The aim of this study was to clarify the clarithromycin resistance mechanisms of β-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae* strains. In all clarithromycin-resistant strains, the transcript level of *acrB* was significantly elevated, and these strains had a frameshift mutation in *acrR*. Introduction of the *acrR* mutation into *H. influenzae* Rd generated a clarithromycin-resistant transformant with the same MIC as the donor strain. Our results indicate that the *acrR* mutation confers clarithromycin resistance by the increasing the transcription of *acrB*.

Haemophilus influenzae can cause respiratory tract infections, otolaryngology disease, and meningitis (1). β-Lactams have been used as the first-line treatment agents for *H. influenzae* infections. However, β-lactamase-producing ampicillin-resistant (BLPAR) *H. influenzae* and β-lactamase-nonproducing ampicillin-resistant (BLNAR) *H. influenzae* do occur. These β-lactam-resistant strains represent serious clinical problems (2–4).

For the treatment of BLNAR *H. influenzae* infections, macrolides, including azithromycin (AZM) and clarithromycin (CLR), and fluoroquinolones can be used as alternative agents of β-lactams. However, in general, fluoroquinolones cannot be used for children because of the risk of fluoroquinolone-associated cartilage lesions (5). Therefore, macrolides are the most important options for treatment. Furthermore, macrolides are frequently used in the empirical treatment of bronchopulmonary infections without the identification of causative pathogens because of wide antibacterial activity against *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*. However, many pathogens have become macrolide resistant (6–8). We reported previously that clarithromycin susceptibility has decreased among clinically isolated BLNAR *H. influenzae*, suggesting the occurrence of multidrug-resistant BLNAR *H. influenzae* (9).

There are three known macrolide resistance mechanisms in *H. influenzae*. One mechanism is the acquisition of the resistance genes *mefA* and *ermB* that encode a drug efflux pump and 23S rRNA methylase, respectively (10). Another mechanism is the decreased affinity for macrolides achieved by amino acid substitutions on ribosomal proteins L4 and L22 (10). The third mechanism is the overexpression of chromosomal multidrug efflux pumps, such as YieO, YdeA, EmrB, NorM, and AcrB (11–13).

Information regarding the resistance mechanisms might help prevent the spread of clarithromycin-resistant and multidrug-resistant BLNAR *H. influenzae*. In this study, we clarified the macrolide resistance mechanisms among clinically isolated clarithromycin-resistant BLNAR *H. influenzae* strains.

We collected BLNAR *H. influenzae* isolates from patients with respiratory infections at the Tokyo Medical University Hachioji Medical Center between 2010 and 2012 (9). Among them, all clarithromycin-resistant BLNAR *H. influenzae* isolates ($n = 7$; clarithromycin MIC, ≥32 μg/ml) were used in this study. Clarithromycin-susceptible strains (clarithromycin MIC, ≤8 μg/ml) isolated during the same period were extracted at random for comparison with the resistant strains. These isolates were genetically identified as *H. influenzae* in a previous study (9). The definitions of clarithromycin and ampicillin resistance were based on the breakpoints by CLSI (14). *H. influenzae* was cultured on chocolate agar and in supplemented brain heart infusion (sBHI) broth, which contains NAD (15 μg/ml) and hemin (15 μg/ml) added to BHI broth (Oxoid, Hampshire, United Kingdom), at 37°C for 24 h in 5% CO_2 (15).

The detection of *mefA* and *ermB* was performed using the PCR method described by Noguchi et al. (16). *mefA* and *ermB* possessing *S. pneumoniae* clinical isolates were used as a positive control. The detection of mutations in L4 and L22 was performed by PCR and DNA sequencing methods found in previous reports, with minor modifications (17, 18).

Antimicrobial susceptibilities were measured using the broth dilution method, according to the CLSI and the Japanese Society of Chemotherapy (19, 20). The tested antimicrobial agents included azithromycin (AZM) (LKT Laboratories, St. Paul, MN), clarithromycin (CLR) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), minocycline (MIN) (Wako, Osaka, Japan), norfloxacin (NOR) (Wako), and ethidium bromide (EB) (Wako). Reserpine (Sigma-Aldrich Japan, Tokyo, Japan), phenylalanine-arginine β-naphthylamide (PAβN) (Sigma-Aldrich Japan), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Wako) were used as efflux pump inhibitors (21). The MICs of clarithromycin and azithromycin were measured with or without efflux pump inhibi-

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TABLE 1 MICs of CAM-resistant BLNAR and CAM-susceptible BLNAR *H. influenzae*

	MIC (μg/ml)[a]												
		CLR + CCCPat:		CLR + PAβNat:			AZM + CCCP at:		AZM + PAβN at:				
Strain	CLR	0.75	1.5	25	50	AZM	0.75	1.5	25	50	MIN	NOR	EB
124-2010	32	16	1	32	16	4	2	0.13	2	2	0.25	0.031	8
54-2011	32	16	4	32	16	4	2	0.5	2	2	0.25	0.031	4
70-2011	32	16	4	16	8	4	1	0.25	2	1	0.25	0.031	8
72-2011	32	16	1	16	8	4	1	≤0.06	2	1	0.25	0.063	8
82-2011	32	16	0.5	16	8	4	2	0.13	2	2	0.5	0.031	8
42-2012	32	16	4	32	8	8	4	1	4	2	0.5	0.031	≥16
46-2012	32	16	4	16	8	4	2	0.5	2	1	0.5	0.016	8
130-2011	1	1	1	1	1	0.13	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.008	2
ATCC 49247	2	2	1	2	2	0.25	0.25	≤0.06	0.25	0.25	0.13	0.016	2
H. influenzae Rd	8	4	0.25	8	8	0.5	0.5	≤0.06	1	1	0.13	0.016	1

[a] CLR, clarithromycin; AZM, azithromycin; MIN, minocycline; NOR, norfloxacin; EB, ethidium bromide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PAβN, phenylalanine-arginine β-naphthylamide. Concentrations are given for the second drug listed in combinations and are measured in micrograms per milliliter.

tors. The concentrations of efflux pump inhibitors were 5 μg/ml reserpine, 25 or 50 μg/ml PAβN, and 0.75 or 1.5 μg/ml CCCP.

The transcription levels of chromosomal multidrug efflux pump genes (*yieO*, *ydeA*, *emrB*, *norM*. and *acrB*) were evaluated by semiquantitative reverse transcription-PCR (RT-PCR) using specific primers (see Table S1 in the supplemental material). The PCR products were electrophoresed, and the gel images were analyzed by ImageJ (http://imagej.nih.gov/ij/). *gyrB* was used as an internal control. These experiments were performed at least three times on independent occasions.

The *acr* operon of clarithromycin-resistant strains was amplified by PCR and sequenced. The primers used in this experiment are listed in Table S1 in the supplemental material. Analyses of the nucleotide sequences were performed using ATGC software (Genetyx, Tokyo, Japan).

A transformation experiment was performed by electroporation. Competent cells were prepared using the method described by Ubukata et al. (1). Transformants were selected by spreading on chocolate agar containing clarithromycin at 16 μg/ml.

To identify clarithromycin resistance mechanisms, the macrolide resistance genes *mefA* and *ermB* and amino acid substitutions in L4 and L22 were examined. *mefA* and *ermB* and the amino acid substitutions in L4 and L22 were not detected (data not shown).

We then investigated whether efflux pumps contributed to macrolide resistance (Table 1). In the presence of CCCP, an efflux pump inhibitor, the MICs of clarithromycin and azithromycin of clarithromycin-resistant strains decreased to the same level as those of clarithromycin-susceptible strains in a dose-dependent manner (Table 1). With the addition of another efflux pump inhibitor, PAβN, the MICs slightly decreased (Table 1). No decrease in the MICs of the clarithromycin-susceptible strains 130-2011 and ATCC 49247 were observed following the addition of CCCP and PAβN. These results suggested that macrolide resistance involves efflux pumps. The other efflux pump inhibitor, reserpine, did not affect resistance.

We hypothesized that clarithromycin resistance was conferred by the overexpression of chromosomal multidrug efflux pumps in *H. influenzae*. To test this hypothesis, the transcription levels of chromosomal multidrug efflux pump genes (*yieO*, *ydeA*, *emrB*, *norM*, and *acrB*) in *H. influenzae* were analyzed by RT-PCR. We compared those transcription levels to the level of *gyrB*, and we found that the transcription of *acrB* in the clarithromycin-resis-

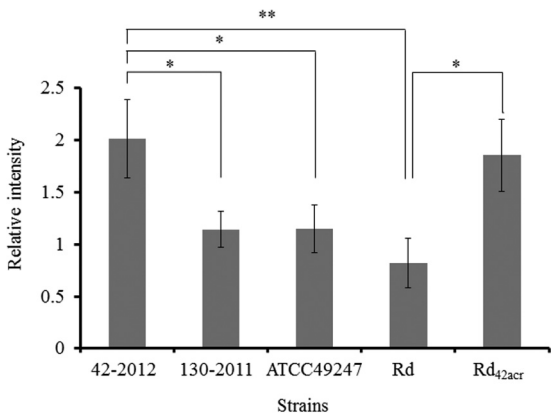

FIG 1 Transcription level of the *acrB* gene. The transcription levels of *acrB*, which encodes a chromosomal multidrug efflux pump in *H. influenzae*, were analyzed by semiquantitative reverse transcription-PCR (RT-PCR). The relative intensity was calculated in comparison with the transcription level of *gyrB*. 42-2012, clarithromycin-resistant strain; 130-2011, clarithromycin-susceptible strain; ATCC 49247, clarithromycin-susceptible control strain; Rd, *H. influenzae* Rd; Rd_{42acr}, Rd strain transformed with the *acrR* gene from strain 42-2012. *, $P < 0.05$; **, $P < 0.01$. Results are shown as the means ± standard deviations.

TABLE 2 Identified amino acid substitutions in the *acrR* gene of *H. influenzae* used in this study

Strain	Mutation sites in *acrR* gene[a]	Protein synthesis (stop site)
124-2010	Deletion of base T in nt 453	Incomplete (152)
54-2011	Substitution of base C to A in nt 400	Incomplete (134)
70-2011	Deletion of base T in nt 141	Incomplete (105)
72-2011	Insertion of 2 bases (GG) behind nt 200	Incomplete (106)
82-2011	Substitution of base C to T in nt 256	Incomplete (86)
42-2012	Deletion of base T in nt 141	Incomplete (105)
46-2012	Substitutions of 2 bases, C to T in nt 246 and G to A in nt 248	Incomplete (83)
130-2011	Multiple substitutions	Complete

[a] Reference with *acrR* genes of *H. influenzae* Rd.

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TABLE 3 MICs of *H. influenzae* Rd strains transformed with the 42-2012 *acr* gene

	MIC (μg/ml)[a]												
		CLR + CCCP at:		CLR + PAβN at:			AZM + CCCP at:		AZM + PAβN at:				
Strain	CLR	0.75	1.5	25	50	AZM	0.75	1.5	25	50	MIN	NOR	EB
H. influenzae Rd	8	4	0.25	8	8	1	0.5	≤0.06	1	1	0.13	0.016	1
H. influenzae Rd_{42acr}	32	16	2	32	16	8	4	0.5	4	4	0.5	0.031	8
42-2012	32	16	4	32	16	8	4	1	4	2	0.13	0.016	≥16

[a] CLR, clarithromycin; AZM, azithromycin; MIN, minocycline; NOR, norfloxacin; EB, ethidium bromide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PAβN, phenylalanine-arginine β-naphthylamide. Concentrations are given for the second drug listed in combinations and are measured in micrograms per milliliter.

tant strain 42-2012 was significantly higher than that in the clarithromycin-susceptible strains 130-2011 and ATCC 49247 ($P < 0.05$) (Fig. 1). No differences in the transcription levels of other efflux pump genes were observed in any strains (data not shown). These results suggested that the overexpression of *acrB* contributes to clarithromycin resistance.

To explore the reason for the increased *acrB* transcription, the *acr* operons of clarithromycin-resistant strains were sequenced. The *acr* operon includes *acrR* (564 bp), *acrA* (1,149 bp), and *acrB* (3,099 bp) (12, 22).

The sequence of *acrR* to *acrB* (4,891 bp) in the clarithromycin-resistant strain 42-2012 was compared with the sequence of *H. influenzae* Rd (GenBank accession no. NC_000907). The clarithromycin-resistant strain 42-2012 had a deletion of 141 nucleotides in *acrR* (accession no. LC126887). This mutation caused a frameshift and generated a stop codon at amino acid 105. These data suggest that the disruption of AcrR by this mutation is related to the overexpression of *acrB*. The *acrR* genes of the other clarithromycin-resistant clinical isolates were also sequenced. All clarithromycin-resistant strains had insertions or deletions in *acrR* that disrupted the function of AcrR (Table 2).

To confirm that a mutation of *acrR* conferred clarithromycin resistance, the *acrR* region of clarithromycin-resistant strain 42-2012 was amplified by PCR and introduced into *H. influenzae* Rd. Consequently, 68 transformants were obtained. The nucleotide sequences of *acrR* in seven transformants were analyzed, and all tested sequences had the same mutation as the donor strain 42-2012. The MICs of clarithromycin and azithromycin for the transformants were tested, and we found that the MICs had increased to the same level as those of the donor strain 42-2012 (Table 3). The transcription of the *acrB* gene of these transformants was also increased significantly compared with that of the parent strain Rd (Fig. 1, $P < 0.05$).

BLNAR *H. influenzae* spreads rapidly, and the incidence of difficult cases with resistance to select antimicrobials has increased. For the treatment of BLNAR *H. influenzae* infections, macrolides are important therapeutic medicine. We previously reported that BLNAR *H. influenzae* strains with reduced susceptibility to clarithromycin were prevalent in a Japanese university hospital (9). In this study, to clarify the macrolide resistance mechanisms of *H. influenzae*, we analyzed the molecular mechanisms of resistance.

mefA and *ermB* and amino acid substitutions of L4 and L22 were not found. These results are similar to those of a recent study by Atkinson and colleagues (23). On the other hand, Roberts et al. (10) reported that macrolide resistance genes in *H. influenzae* were detected. In general, macrolide resistance gene confers high-level resistance to not only clarithromycin but also azithromycin. Our isolates showed resistance to clarithromycin but not to azithromycin, except for one strain, suggesting that these genes were not involved. The MICs of macrolides for clarithromycin-resistant strains decreased in the presence of CCCP and PAβN, and the transcriptional levels of *acrB* in these strains were significantly higher than those in the clarithromycin-susceptible strains.

Several studies have reported that macrolides can be excreted by chromosomal multiple drug efflux pumps; AcrAB belongs to the resistance-nodulation-division (RND) family of transporters (11, 12). AcrAB is encoded by an operon that contains *acrR*, *acrA*, and *acrB*. The transcription of *acrAB* is negatively regulated by the repressor AcrR (24). The *acrRAB* clusters of *H. influenzae* are homologous to those of *Escherichia coli* (12). The *acrAB* gene clusters contributed to reduced susceptibility to macrolides, such as clarithromycin and azithromycin (11, 12, 25).

All clarithromycin-resistant strains had deletions or insertions in *acrR* that disrupted the function of the AcrR protein. When an *acrR* mutation was transformed into *H. influenzae* Rd, the transcription of *acrB* in all transformants was elevated significantly, and the MICs of clarithromycin and azithromycin increased. When the transformation was performed using donor strain 72-2011, which contained a different *acrR* mutation site from that in the clarithromycin-resistant strain 42-2012, the same results were obtained (data not shown).

Our results indicate that the dysfunction of AcrR confers macrolide resistance. However, the mutation sites in *acrR* varied among the strains, suggesting that the *acrR* region in *H. influenzae* might be a hot spot where mutations occur easily.

Point mutations in *acrR* might easily be generated by the selective pressure provided by the frequent use of macrolides.

Nucleotide sequence accession number. Newly reported sequence data related to this article have been deposited (accession no. LC126887).

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REFERENCES

1. **Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, Sunakawa K, Inoue M, Konno M.** 2001. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother **45:**1693–1699. http://dx.doi.org/10.1128/AAC.45.6.1693-1699.2001.
2. **Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR, Appelbaum PC, Sunakawa K, Ubukata K.** 2003. Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the

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United States. Microb Drug Resist **9:**39–46. http://dx.doi.org/10.1089/107662903764736337.
3. **Kishii K, Morozumi M, Hasegawa K, Ubukata K.** 2009. Antibiotic susceptibility and resistance gene analysis of *Haemophilus Influenzae* in clinical tebipenem-pivoxil studies in pediatric patients using PCR method. Jpn J Chemother **57:**67–75.
4. **Medeiros AA, Levesque R, Jacoby GA.** 1986. An animal source for the ROB-1 beta-lactamase of *Haemophilus influenzae* type b. Antimicrob Agents Chemother **29:**212–215. http://dx.doi.org/10.1128/AAC.29.2.212.
5. **Noel GJ, Bradley JS, Kauffman RE, Duffy CM, Gerbino PG, Arguedas A, Bagchi P, Balis DA, Blumer JL.** 2007. Comparative safety profile of levofloxacin in 2523 children with a focus on four specific musculoskeletal disorders. Pediatr Infect Dis J **26:**879–891. http://dx.doi.org/10.1097/INF.0b013e3180cbd382.
6. **Diaz MH, Benitez AJ, Winchell JM.** 2015. Investigations of *Mycoplasma pneumoniae* infections in the United States: trends in molecular typing and macrolide resistance from 2006 to 2013. J Clin Microbiol **53:**124–130. http://dx.doi.org/10.1128/JCM.02597-14.
7. **Shortridge VD, Flamm RK, Ramer N, Beyer J, Tanaka SK.** 1996. Novel mechanism of macrolide resistance in *Streptococcus pneumoniae*. Diagn Microbiol Infect Dis **26:**73–78. http://dx.doi.org/10.1016/S0732-8893(96)00183-6.
8. **Ozawa D, Yano H, Hidaka H, Kakuta R, Komatsu M, Endo S, Kanamori H, Kaku M, Katori Y.** 2014. Twelve-year survey (2001–2012) of the antimicrobial susceptibility of *Streptococcus pneumoniae* isolates from otorhinolaryngology clinics in Miyagi Prefecture, Japan. J Infect Chemother **20:**702–708. http://dx.doi.org/10.1016/j.jiac.2014.07.014.
9. **Wajima T, Seyama S, Nakamura Y, Kashima C, Nakaminami H, Ushio M, Fujii T, Noguchi N.** 2016. Prevalence of macrolide-non-susceptible isolates among β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in a tertiary care hospital in Japan. J Glob Antimicrob Resist. http://dx.doi.org/10.1016/j.jgar.2016.01.014.
10. **Roberts MC, Soge OO, No DB.** 2011. Characterization of macrolide resistance genes in *Haemophilus influenzae* isolated from children with cystic fibrosis. J Antimicrob Chemother **66:**100–104. http://dx.doi.org/10.1093/jac/dkq425.
11. **Peric M, Bozdogan B, Jacobs MR, Appelbaum PC.** 2003. Effects of an efflux mechanism and ribosomal mutations on macrolide susceptibility of *Haemophilus influenzae* clinical isolates. Antimicrob Agents Chemother **47:**1017–1022. http://dx.doi.org/10.1128/AAC.47.3.1017-1022.2003.
12. **Sánchez L, Pan W, Viñas M, Nikaido H.** 1997. The *acrAB* homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. J Bacteriol **179:**6855–6857.
13. **Trepod CM, Mott JE.** 2004. Identification of the *Haemophilus influenzae tolC* gene by susceptibility profiles of insertionally inactivated efflux pump mutants. Antimicrob Agents Chemother **48:**1416–1418. http://dx.doi.org/10.1128/AAC.48.4.1416-1418.2004.
14. **CLSI.** 2010. Performance standards for antimicrobial susceptibility testing; 20th informational supplement. CLSI document M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
15. **Nye KJ, Fallon D, Gee B, Messer S, Warren RE, Andrews N.** 1999. A comparison of blood agar supplemented with NAD with plain blood agar and chocolated blood agar in the isolation of *Streptococcus pneumoniae* and *Haemophilus influenzae* from sputum. Bacterial Methods Evaluation Group. J Med Microbiol **48:**1111–1114.
16. **Noguchi N, Tano J, Nasu Y, Koyama M, Narui K, Kamishima H, Saito T, Tsuyuki K, Sasatsu M.** 2007. Antimicrobial susceptibilities and distribution of resistance genes for beta-lactams and macrolides in *Streptococcus pneumoniae* isolated between 2002 and 2004 in Tokyo. Int J Antimicrob Agents **29:**26–33. http://dx.doi.org/10.1016/j.ijantimicag.2006.09.004.
17. **Clark C, Bozdogan B, Peric M, Dewasse B, Jacobs MR, Appelbaum PC.** 2002. *In vitro* selection of resistance in *Haemophilus influenzae* by amoxicillin-clavulanate, cefpodoxime, cefprozil, azithromycin, and clarithromycin. Antimicrob Agents Chemother **46:**2956–2962. http://dx.doi.org/10.1128/AAC.46.9.2956-2962.2002.
18. **Noguchi N, Tamura M, Narui K, Wakasugi K, Sasatsu M.** 2002. Frequency and genetic characterization of multidrug-resistant mutants of *Staphylococcus aureus* after selection with individual antiseptics and fluoroquinolones. Biol Pharm Bull **25:**1129–1132. http://dx.doi.org/10.1248/bpb.25.1129.
19. **CLSI.** 2010. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 8th ed. CLSI document M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
20. **Yamaguchi K, Hirakata Y, Kobayashi I, Ikedo M, Ohno A.** 2012. A report from the committee on microbroth dilution antimicrobial susceptibility testing: issues in antimicrobial susceptibility testing of *Haemophilus influenzae*: a comparison of the Japanese Society of Chemotherapy lysed horse blood and the CLSI HTM broth microdilution methods. J Infect Chemother **18:**134–143. http://dx.doi.org/10.1007/s10156-011-0277-3.
21. **Nakaminami H, Noguchi N, Sasatsu M.** 2010. Fluoroquinolone efflux by the plasmid-mediated multidrug efflux pump QacB variant QacBIII in *Staphylococcus aureus*. Antimicrob Agents Chemother **54:**4107–4111. http://dx.doi.org/10.1128/AAC.01065-09.
22. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, McKenney K, Sutton G, FitzHugh W, Fields C, Gocyne JD, Scott J, Shirley R, Liu L-I, Glodek A, Kelley JM, Weidman JF, Phillips CA, Spriggs T, Hedblom E, Cutton MD, Utterback TR, Hanna MC, Nguyen DT, Saudek DM, Brandon RC, Fine LD, Fritchman JL, Fuhrmann JL, Geoghagen NSM, Gnehm CL, McDonald LA, Small KV, Fraser CM, Smith HO, Venter JC.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512. http://dx.doi.org/10.1126/science.7542800.
23. **Atkinson CT, Kunde DA, Tristram SG.** 2015. Acquired macrolide resistance genes in *Haemophilus influenzae*? J Antimicrob Chemother **70:**2234–2236. http://dx.doi.org/10.1093/jac/dkv093.
24. **Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol Microbiol **19:**101–112. http://dx.doi.org/10.1046/j.1365-2958.1996.357881.x.
25. **Kaczmarek FS, Gootz TD, Dib-Hajj F, Shang W, Hallowell S, Cronan M.** 2004. Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. Antimicrob Agents Chemother **48:**1630–1639. http://dx.doi.org/10.1128/AAC.48.5.1630-1639.2004.

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