GSC Advanced Research and Reviews

eISSN: 2582-4597 CODEN (USA): GARRC2

Cross Ref DOI: 10.30574/gscarr

Journal homepage: https://gsconlinepress.com/journals/gscarr/

(RESEARCH ARTICLE)

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AcrB-TolC efflux system is essential for macrolide resistance in *Helicobacter pylori*

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GSC Advanced Research and Reviews, 2021, 09(03), 056–062

Publication history: Received on 10 November 2021; revised on 11 December 2021; accepted on 13 December 2021

Article DOI: https://doi.org/10.30574/gscarr.2021.9.3.0292

Abstract

The prevalence of *Helicobacter pylori* strains resistant to macrolide is increasing worldwide. Macrolide molecules can be generally extruded by the AcrB-TolC system in bacteria. The *H. pylori* 26695 genome was assessed for putative translocases and the outer membrane efflux of AcrB (HP607) and TolC (HP605) proteins. We investigated the role of the AcrB-TolC efflux system in macrolide resistant (M-R) *H. pylori*. Both acrB- and tolC-mutant M-R strains were constructed from M-R strains by insertional inactivation of the *acrB* and *tolC* genes. The minimal inhibition concentrations (MICs) of erythromycin (EM) and clarithromycin (CLR) were determined by an agar dilution assay. To investigate the efflux ability of macrolides, intracellular accumulation of radiolabeled EM in the *H. pylori* 26695 strain, M-R strain, and acrB- and tolC-mutant M-R strains was measured by a liquid scintillation counter. For Post antibiotic effect (PAE), EM-treated *H. pylori* was diluted 1000-fold to remove antimicrobial activity. After additional 24 hours incubation, the CFU was measured. The decrease in the levels of resistance to EM and CLR was 32-fold higher for the acrB- and tolC-mutant M-R strains than the M-R strains. The intracellular EM concentration significantly increased in the acrB- and tolC-mutant M-R strains than the *H. pylori* 26695 and M-R strains. Diluted acrB, and tolC M-R mutant *H. pylori* after EM treatment was markedly reduced compared to M-R *H. pylori*. Our result showed that the M-R mechanism of *H. pylori* is significantly associated with AcrB-TolC efflux system.

Keywords: *Helicobacter pylori*; AcrB; TolC; Macrolide; Drug resistant; Post antibiotic effect

1. Introduction

Helicobacter pylori is a pathogenic bacterium that causes gastric ulcer and gastric cancer [1, 2, 3]. In Japan, the present first-line treatment for *H. pylori* eradication is a triple therapy combination with a proton pump inhibitor (PPI), clarithromycin (CLR) and amoxicillin [4]. This combination achieves clinical cure rates of more than 80% [4]. The prevalence of CLR resistance varies with geographical location and is generally estimated to be about 10% in Japan [5]. However, the prevalence of *H. pylori* strains resistant to macrolides is increasing worldwide [6]. Given that CLR has a stronger antibacterial effect on *H. pylori* than other agents, the presence of resistant microbes may result in eradication failure [7]. The mechanism of macrolide resistance in *H. pylori* is attributed to point mutation in 23S rRNA [7]. Since *H. pylori* acquires resistance to CLR and the like due to the gene mutation of 23S ribosome RNA, the therapeutic effect is decreasing. Therefore, the development of new therapeutic agents targeting other than ribosomes is desired.

Generally, in gram-negative bacteria, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamilies, and the resistance-nodulation-cell division (RND) family are in the inner membrane [8]. Therefore, they are also called inner membrane efflux protein (IEP), which is AcrB or an AcrB homologue that acts with two other components, a periplasmic efflux protein (PEP), which facilitates the interaction with the other component, and an outer

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membrane efflux protein (OEP), which is TolC or a TolC homolog [9]. Bacteria may have several different translocases and may act with only a limited number of OEPs. In *Escherichia coli*, four TolC-like proteins may act with an eightfold higher number of translocases [10].

Johnson et al identified TolC homologs (HP605) in *H. pylori* based on their structural similarities with OEP domains [9][11]. In addition, Bina et al. identified three RND efflux systems, each of which consisted of a translocase, an accessory protein, and a TolC homolog (HP605 to HP607) (*hefABC*) [12]. As the HP605 (*hefA*) are the TolC homolog encoding the outer membrane efflux protein, inactivation of a TolC-like protein may affect the functions of multiple translocases [11]. But Bina et al. could not establish a role for these efflux systems in antibiotic resistance, because knockout mutants for the translocases (HP607) displayed profiles of susceptibility to 19 different antibiotics identical to that of their wild-type strains [12]. As macrolide molecules can be generally extruded by the AcrB-TolC system in bacteria, the contribution of efflux proteins to a macrolide resistant (M-R) strain is not well established in M-R *H. pylori*.

Post antibiotic effect (PAE) is that the antibacterial effect is sustained even when the antibacterial drug is removed and is often seen in Gram-negative bacteria [13]. Since various factors of bacteria are involved in PAE, excretion functional factors can also be candidates for therapeutic targets. However, detailed mode of action has not been investigated.

Therefore, we investigated whether the AcrB and TolC efflux systems of *H. pylori* affects macrolide resistance and PAE.

2. Material and methods

2.1. Bacterial strains, culture condition

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown at 37°C in Luria-Bertani broth or agar supplemented with ampicillin (100 μg/ml), and kanamycin (Km) (25 μg/ml), when appropriate. *H. pylori* strains were grown on Trypticase soy agar (TSA) with 5% sheep blood (Becton Dickinson and Company, MD, USA) or Brucella serum (Becton Dickinson and Company) agar with 10% newborn calf serum (FCS; Gibco BRL, MD, USA) plates at 37°C in 5% CO_2 atmosphere. Antibiotic-resistant *H. pylori* transformants were selected with Km (25 μg/ml), and erythromycin (EM) (1μg / ml). CLR was distributed by Taisho Pharmaceutical Co., Ltd. Other antibiotics were purchased from Fuji film wako pure chemical Co.

2.2. Construction of plasmids and *H. pylori* mutant strains

H. pylori chromosomal DNA was prepared from cells of each strain after 48 h of growth on two agar plates as described previously [14, 15, 16]. Plasmid DNA was prepared from *H. pylori* after 48 h of growth or from *E. coli* after overnight cultures, using a midi-prep protocol (Qiagen N.V., Venlo, CA, USA) according to the manufacturer's instructions.

Table 1 Bacterial strains and plasmids used in this study

Strain (alternate name) or plasmid	Genotype or characteristics	Reference or source
26695	*H. pylori* wild -type	[11]
26695 M-R	*H. pylori* 26695 with 23SrRNA point mutation	[15]
26695 M-R acrB	26695 M-R / HP607: *aphA*	This study
26696 M-R tolC	26695 M-R / HP605: *aphA*	This study
pGEMT Easy	ColE1, Amp^r, PCR cloning vector	Promega
pUC4K	ColE1, Amp^r, *aphA*	Parmacia
pGEMT acrBKm	pGEMT Easy / HP607: *aphA*	This study
pGEMT tolCKm	pGEMT Easy / HP605: *aphA*	This study

Macrolide-resistant (M-R) isogenic *H. pylori* mutants were constructed by natural transformation methods [14, 15]. Domain V of the 23S rRNA gene in *H. pylori*, which is associated with macrolide resistance [15], was amplified by PCR (CAMsense, and CAMantisense). Template DNA was extracted from M-R strains 628 and was found to possess A-to-G point mutations at position 2143 in domain V of the 23S rRNA gene. The PCR products from donor DNA samples were

purified. Briefly, 1 ng of DNA was amplified with 100 pmol of the sense and antisense primers in a 50-µl reaction mixture containing 0.25 µl of Ex Taq polymerase (Takara Bio Inc., Ohtsu, Japan, Ohtsu, Japan).

For 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C in a DNA thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA). The presence of a single 425-bp band was verified on a 1% agarose gel, and the PCR products were purified with a QIAquick PCR Purification kit (Qiagen).

The HP605 and HP607 ORF of strain *H. pylori* 26695 were amplified by PCR using primers (HP605sense, HP605antisense, HP607sense, and HP607antisense), respectively (Table 2) [11]. The product was ligated into pGEM-T Easy (Promega Co, WI, USA) and transformed into *E. coli* DH5α. A unique EcoRI site was created by inverse PCR.

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
HP0605sense	attgctaaaaggggctaccact
HP0605antisense	gtctattttatgcccgctgttg
HP0605inv1	ggaattctagtttctttaagggagcag
HP0605inv2	ggaattcgtaacccttttaatgtccgt
HP0607sense	cggtggtggttactacgact
HP0607antisense	gagcaaaatcaagcctatca
HP0607inv1	ggaattcacttcagcatgtttttcaatcg
HP0607inv2	ggaattcaccaccttgcaagtgggtta
CAMsense	ccacagcgatgtggtctcag
CAMantisense	ctccataagagccaaagccc

HP605inv1, HP605inv2, HP607inv1, HP607inv2), respectively. Plasmid pUC4K was digested with EcoRI (Takara Bio Inc.), after which the Km resistance (Kan^r; *aphA*) cassette was isolated by agarose gel electrophoresis and ligated into the inverse PCR product to disrupt the HP605 ORF and HP607 ORF, creating pGEMT tolC km and pGEMT acrB km. *H. pylori* 26695 M-R was transformed to Kan^r with pGEMT acrB km and pGEMT tolC km, to create 26695 M-R acrB and 26695 M-R tolC, respectively. Chromosomal DNAs isolated from *H. pylori* mutants were confirmed by PCR. PCR oligonucleotide primers specific for HP605 and HP607 or *aphA* were used, and the sizes of the PCR products were evaluated by agarose gel electrophoresis (Table 2).

2.3. Natural transformation

Isogenic M-R strain with a 23S rRNA point mutation (2143 A to G) was constructed from *H. pylori* 26695 and *H. pylori* 628 with a 23S rRNA point mutation by natural transformation. *H. pylori* strain 26695 was used as the transformation recipient. After 48 h of growth, recipient *H. pylori* cells were harvested from one BB agar plate and placed into 1 ml of phosphate-buffered saline (PBS) and then centrifuged at 8,500 × g for 5 min. The pellet was resuspended in 300 µl of PBS. Each transformation mixture, consisting of 25 µl of recipient cells and 1 µg of donor DNA, was spotted onto a BB agar plate (approximately 600 ng of DNA/25 µl of cells is a saturating amount of DNA). The plates were incubated overnight at 37°C in a microaerophilic atmosphere. After 18 h of incubation, the transformation mixture was spread onto BB agar containing 1µg of EM /ml. All plates were incubated for 5 days at 37°C in a microaerophilic atmosphere to select transformants [14, 15]. The presence of point mutations in the transformants was confirmed by PCR and direct sequencing (CEQ2000XL; Beckman Coulter Inc., Fullerton, CA, USA).

2.4. Electroporation

AcrB- and TolC-mutant M-R *H. pylori* strains were constructed from isogenic M-R *H. pylori* strains by insertional inactivation of the *acrB* and *tolC* genes with electroporation. *H. pylori* 26695 was transformed with pGEMT acrB km and pGEMT tolC kmH by electroporation as described elsewhere [14, 15], resulting in strain 26695 M-R acrB and 26695 M-R tolC (Table 1).

2.5. Susceptibility testing

The susceptibilities of the M-R strains were determined by the agar dilution method [16]. The MIC of EM and CLR were defined as the lowest concentration that inhibited the visible growth of isolates completely by the agar dilution method on Mueller-Hinton agar (Becton Dickinson and Company) plates supplemented with 5% sheep blood (Becton Dickinson and Company) (M-H agar). Briefly, all isolates were incubated for 4 days on BB agar. After this incubation, inocula were prepared by suspending growth from the BB agar plates with antimicrobial agents in saline to achieve a suspension equivalent to a 2.0 McFarland standard. Final inocula of 10^6 CFU/spot were applied to M-H agar or BB. All plates were incubated for 3 days at 35°C in a microaerophilic atmosphere, and the number of CFU was counted. In the liquid culture study, aliquots from each culture were applied to BB agar after 3 days of incubation of *H. pylori* at 37°C under microaerophilic conditions. After 4 days of incubation, the number of CFU was counted. The MICs of more than 16 and 1 μg/mL was determined to be EM and CLR resistant.

2.6. Determination of Radiolabeled EM uptake

To investigate the efflux ability of macrolides, intracellular accumulation of radiolabeled EM in the *H. pylori* 26695 strain, M-R strain, and AcrB- and TolC- mutant M-R strains was measured by a liquid scintillation counter. At 20 h before the end of the *H. pylori* culture, N-methyl-^{14}C-EM (NEC777: 50 μmCi/mmol; PerkinElmer, MA, USA) was added to the medium in the wells. To investigate the efflux ability of macrolides, intracellular accumulation of ^{14}C in each strain was measured at every 30 sec by a liquid scintillation counter after incubation with N-methyl-^{14}C-EM. When the culture was finished, the cells were adsorbed on 0.45 μm membrane filters (Advantech Japan, Tokyo, Japan), washed with distilled water, and then dried. The filters were transferred to vials filled with liquid scintillator cocktail, and the radioactivity was measured with a liquid scintillation counter (LSC-6100, Hitachi Aloka Medical, Tokyo, Japan).

2.7. Determination of PAE

The PAE on bacteria was measured by a modification of the previous method [13]. After 4 days of growth of strain 26695 on BB agar, the bacteria were harvested, placed into PBS, and diluted with BB to ~10^8 CFU/ml; and a final inoculum of 10^6 CFU/ml was applied to BB agar. EM was added to BB in a 25-ml tissue culture flask at a concentration of 1.25 μg/ml. After incubation for 8 h, the antibiotics was removed by dilution 1:10^3 into fresh BB and the cultures were incubated for 24 h. Samples were collected for viable counts, plated onto BB agar plates, and incubated for 4 days. The control culture, which was not exposed to any antimicrobial agents, was treated similarly.

2.8. Statistical analysis

All the experiments were repeated at least three times, and the results were expressed as the mean values ± standard deviations and compared using a paired *t* test.

3. Results

3.1. Construction of macrolide-resistant mutants and MIC determination

M-R acrB, and tolC mutants with macrolide resistance were created from strain 26695 M-R. The MICs of CLR were determined by the disk dilution method. M-R *H. pylori* (26695:628) possessed an A-to-G point mutation at position 2143 in domain V of the 23S rRNA gene. The respective MICs of EM and CLR were 0.125 and 0.0625 μg/mL in *H. pylori* 26695 strain, 64 and 32 in 26695 M-R strain, 2 and 1 in 26695 M-R acrB strain, and 2 and 1 μg/mL in 26695 M-R tolC strain, respectively (Table 3). The EM- and CLR-MICs of M-R acrB- and tolC- mutant *H. pylori* were more decreasing than that of M-R *H. pylori*. However, there was no difference in MICs between the M-R acrB-mutant and M-R tolC-mutant strains.

Table 3 MICs of EM and CAM in *H. pylori*

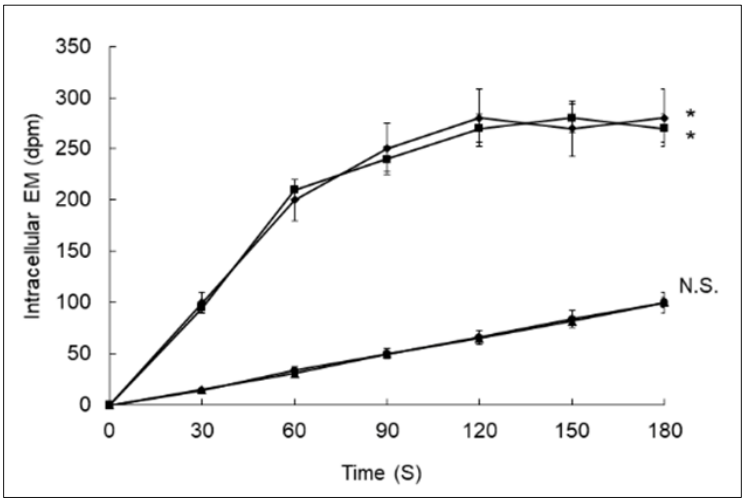
MIC	26695	26695 M-R	26695 M-R	26695 M-R
(μg/mL)			acrB	tolC
EM	0.125	64	2	2
CLR	0.0625	32	1	1

3.2. Ability of AcrB and TolC to efflux macrolides from bacterial cells

Next, we examined the difference in macrolide efflux capacity between the wild strain and the acrB and tolC mutant strains. EM labeled with ^{14}C was added to *H. pylori* and incubated, and ^{14}C in the bacteria was measured by liquid scintillation counter. No difference in ^{14}C levels was observed between the wild-type and macrolide-resistant strains. However, a marked increase in ^{14}C levels was observed in acrB and tolC mutant strains compared to wild-type strains. From these results, we suggested that the intracellular EM concentration significantly increased in the acrB- and tolC-mutant M-R strains than the *H. pylori* 26695 and 26695 M-R strains.

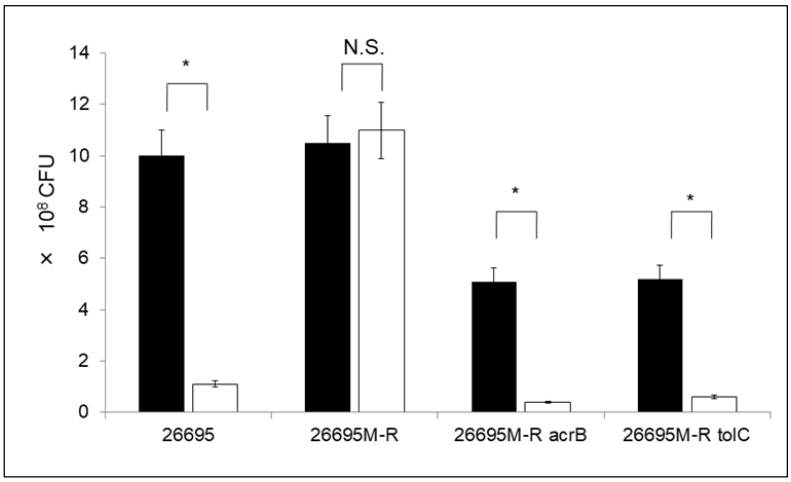
3.3. PAE of AcrB and TolC in M-R *H. pylori*

Finally, we examined the relationship between PAE of macrolides against *H. pylori* and drug efflux factors such as AcrB and TolC. After culturing *H. pylori* with and without EM to steady state, a 1000-fold dilution was used to remove the antibacterial activity of EM. The CFU of the liquid cultured *H. pylori* was measured after 24 hours. The results showed that the CFU of the wild strain of *H. pylori*, strain 26695, significantly decreased with EM addition to about one-tenth of the CFU of the untreated strain. However, for M-R *H. pylori*, there was no difference in CFU with or without erythromycin addition. However, the CFU of the M-R acrB and M-R tolC mutant strains was lower than that of the M-R strain. Furthermore, in the M-R acrB and M-R tolC mutant strains, the CFU of EM addition was significantly reduced to about one-tenth of the CFU of no treatment.



Closed triangle: 26695, Closed circle: 26695 M-R, Closed diamond: 26695 M-R acrB, Closed box: 26695 M-R tolC. * : $p < 0.05$ compared with 26695

Figure 1 Comparison of EM efflux among *H. pylori*



Closed box: post antibiotic effect (-), Open box: post antibiotic effect (+). * : $p < 0.05$ compared with 26695, N.S. : not significant compared with 26695

Figure 2 Comparison of post antibiotic effect among *H. pylori*

4. Discussion

Our experimental results showed that AcrB and TolC were involved in the macrolide resistance of *H. pylori*. AcrB and TolC were also found to be closely related to the efflux of macrolides from the bacterial cell and PAE.

Our result demonstrated that inactivation of TolC increased the drug susceptibility of macrolides. Previous report showed that inactivation of TolC resulted in *H. pylori* being more susceptible to novobiocin and deoxycholate or to ethidium bromide, respectively [12]. This result is consistent with the findings obtained with a *Haemophilus influenzae* TolC-knockout mutant, in which the levels of susceptibility to β-lactams, chloramphenicol, tetracycline, and fluoroquinolones were also not increased [17, 18]. TolC is also involved in the efflux of a large variety of small molecules, including antimicrobials. As mentioned above, TolC is shared by different translocases [19]. For example, in *E. coli* the TolC-AcrAB export system is responsible for the efflux of many antibiotics, dyes, detergents, fatty acids, bile salts, and organic solvents, whereas the TolC-EmrAB efflux system exports hydrophobic uncouplers of oxidative phosphorylation, organomercurials, and antibacterial drugs like nalidixic acid and thiolactomycin [19]. *E. coli* TolC-knockout mutants are hypersensitive to a variety of compounds, including detergents, bile salts, and hydrophobic antibiotics [20].

PAE is defined as the period of sustained inhibition of bacterial growth after a brief exposure to an antimicrobial agent. [21]. It depends on the type of antibiotic used, the concentration of the drug, and the type of bacteria. [21]. For example, bacteria in the post-antibiotic (PA) stage are less susceptible to the bactericidal effects of antibiotics, but the degree of inhibition depends on both the bacteria and the antibiotic used to kill the PA stage [21]. Many methods have been used to demonstrate and quantify PAE using batch cultures. These techniques include rapid removal of antimicrobial agents by repeated washing of bacteria, dilution into antibiotic-free medium, filtration through membrane filters, or measurement of microbial growth kinetics after drug inactivation. They are equally useful, and all give reproducible results [13]. However, the problem with the currently used definition of PAE stems from the fact that the cell population is homogeneous and the recovery of individual cells follows the same time course [21]. Even though the number of viable bacteria in the incubator is increasing at a normal rate, the cell size distribution of the bacterial population is significantly reduced. [21]. Bacterial cell physiology (cell size, adherence, and ability to kill host defenses) is greatly altered when exposed to antibiotics [21]. These physiologically altered populations may differ in their susceptibility to food action and their ability to produce pathogenicity, such as toxins [21]. Also, these parameters take longer to recover than the ability to form visible biomass on agar plates [21].

5. Conclusion

AcrB and TolC appear to directly confer efflux-mediated resistance to macrolides in *H. pylori*. The M-R mechanism of *H. pylori* is significantly associated with both the point mutation in 23S rRNA and the AcrAB-TolC efflux system. Further studies on the AcrB and TolC of *H. pylori* may provide more detailed information on the mechanism of the bacterial efflux systems.

Our result demonstrated that macrolide is less likely to be excreted extracellularly due to a decrease in AcrB and TolC function and accumulates in the cells, which may enhance the antibacterial effect including PAE. It is desired to develop a new therapeutic drug targeting AcrB and TolC system to enhance the antibacterial effect.

Compliance with ethical standards

Acknowledgments

We would like to thank Mr. Masashi Ishihara, Ms. Miwako Fujimura, Ms. Teruko Ohkura, Dr. Keizo Torii, and Prof. Tadao Hasegawa for excellent support through this investigation.

Disclosure of conflict of interest

All authors declare no conflict of interest of regarding the publication of this paper.

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