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Insights into macrolide resistance in *Arcobacter butzleri*: potential resistance mechanisms and impact on bacterial fitness and virulence

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Background: Macrolides are recommended for treating the emerging enteropathogen *Arcobacter butzleri*; nonetheless, this bacterium often exhibits highly variable resistance rates, and the mechanisms behind this resistance phenotype remain largely unexplored.

Objectives: To understand the phenotypic and genotypic consequences associated with the acquisition of erythromycin resistance in *A. butzleri*, as well as the effects on the fitness of this species.

Methods: Resistant strains resulting from spontaneous mutations and adaptive laboratory evolution under increasing erythromycin concentrations were examined regarding their cross-resistance and collateral susceptibility profiles. Genetic causes of phenotypic antibiotic resistance were analysed by sequencing and bioinformatics, with functional correlation through ethidium bromide accumulation assays. Growth profiles in the presence and absence of erythromycin, motility and biofilm formation abilities were assessed to detect potential changes in fitness and virulence.

Results: Clones from spontaneous mutation rate evolution demonstrated decreased susceptibility to erythromycin and other classes of antibiotics, associated with mutations in the transcriptional repressor *areR*, causing overexpression of the AreABC efflux pump. In turn, WGS analysis of the evolved strain showed additional mutations in the ribosomal proteins L4 and L22 and in the *areR* gene. Furthermore, the acquisition of macrolide resistance altered *A. butzleri* virulence and entailed a high biological cost.

Conclusions: The findings of this study have proved that efflux activity contributes synergistically with mutations in the ribosomal proteins L4 and L22 to *A. butzleri*'s high-level macrolide resistance. The results further suggest an impact on the bacterial physiology and virulence, with the increased fitness cost justifying the low worldwide prevalence of high-level resistant circulating strains.

Introduction

Arcobacter butzleri is an emergent enteropathogen belonging to the Arcobactereaceae family and the Campylobacterales order.[1] *A. butzleri* is widely distributed and rated as a moderate hazard for human health by the International Commission on Microbiological Specifications for Foods, making it a potential public health concern due to its transmission to humans and animals through the consumption of contaminated water and food.[2] Humans infected with *A. butzleri* may develop gastroenteritis, bacteraemia and septicaemia, with the most commonly reported symptoms being persistent diarrhoeal episodes associated with abdominal pain, nausea, vomiting and, in rare cases, fever.[2,3] Concerning prevalence, this bacterium was found to be the fourth most prevalent *Campylobacter*-like organism found in diarrhoeal samples in humans, as well as the fourth most common pathogen associated with acute enteric disease.[4–7] *A. butzleri* infections are typically self-limiting; however, severe or persistent cases may require antibiotic treatment, with β-lactams, fluoroquinolones and macrolides being the most frequently recommended.[2] Resistance to several classes of antimicrobial agents is reported for this enteropathogen, including multidrug resistance.[2,8] In particular, regarding macrolide resistance, isolates from humans, animals, food, water or

environmental samples have shown highly variable rates, with food isolates being more resistant to erythromycin compared with environmental isolates.[8] The pooled estimated erythromycin resistance rate remains in 9.6% (95% CI 4.8%–18.3%),[8] with some studies reporting even higher rates of resistance.[2,3,8] Despite macrolide-resistant isolates being reported from numerous geographic origins, the mechanisms responsible for this phenotype are largely unexplored. To date, in *A. butzleri*, neither mutations in 23S rRNA genes nor the presence of the methyltransferase gene *erm*(N) have been found in association with resistance to this antimicrobial agent.[9–11] Currently, the only correlation with the erythromycin resistance in *A. butzleri* was an up-regulation of the expression of the AreABC efflux pump, associated with truncation of the transcription repressor AreR;[9,12] or the presence of an *ere*(A) gene.[9–11] However, this does not explain all cases of macrolide resistance described in *A. butzleri*.[12,13] Other genes implicated in macrolide resistance in other bacteria, such as the methyltransferase *erm*(B), *macA*, *macB*, *tolC* and/or the *mexAB-oprM* operon have also been found in *A. butzleri*'s genome.[14,15]

Therefore, aiming to gain insights into the mechanisms of erythromycin resistance and its progression in *A. butzleri*, resistant strains resulting from spontaneous mutation and from adaptive laboratory evolution (ALE) were studied to unravel the associated phenotypic and genotypic changes.

Materials and methods

Bacterial isolates and MIC determination

A representative *A. butzleri* strain Ab_DQ40A1, isolated from the surface of dairy factory equipment,[16] was used for the assays. The strain had undergone WGS and exhibited an erythromycin-susceptible profile.[9]

The MIC was determined by the broth microdilution method[12] for several antibiotics of different classes, such as erythromycin, gentamicin, tetracycline, ampicillin, ciprofloxacin and cefotaxime, adjusting the antibiotic concentrations according to the clones' phenotype. The MIC of the antibiotics was assessed visually, followed by spectrophotometric confirmation at 620 nm, using a value of 0.05 as the cut-off of the OD. All assays were performed in duplicate on at least three independent assays.

Mutation rate and spontaneous emergence of erythromycin resistance

The spontaneous mutation rate was determined by using a fluctuation assay, according to the method previously described by Lea and Coulson,[17] as detailed in the Supplementary Methods (available as Supplementary data at *JAC* Online). From the assay, 16 colonies were randomly selected, and after five successive passages on antibiotic-free tryptic soy agar (TSA), each mutant (designated TM1A to TM1Q) was stored, and the MIC of erythromycin was also determined for each isolate according to the broth microdilution method.

Adaptive laboratory evolution of A. butzleri under erythromycin stress

The experimental evolution was performed with the WT *A. butzleri* strain Ab_DQ40A1 in the presence of increasing concentrations of erythromycin following the method described by Shuster *et al.*,[18] with slight modifications (Supplementary Methods and Figure S1), and a clone from each ALE was selected for further studies. Throughout this process, the IC_{50} was determined for the populations (Supplementary Methods).

Genetic characterization of the A. butzleri mutants and fitness evaluation

The parental *A. butzleri* strain Ab_DQ40A1, the 16 selected TM mutants and the three ALE *A. butzleri* clones were genotyped by ERIC-PCR, as previously described.[16] The growth profile in the absence and presence of erythromycin with concentrations between 0 and 8 mg/L was assessed (Supplementary Methods).

Amplification and Sanger sequencing of the repressor AreR and the promoter region of the AreABC efflux pump

The *areR* gene of all the TM strains was amplified by PCR and Sanger sequenced, using the primers tetR_F1 and tetR_R1 (Table S1), which allowed the amplification of the full gene and the promoter region of the AreABC efflux pump.[12] Prokaryotic gene promoters were predicted using promoter predictor version 2.2,[19] available at BGDP (https://www.fruitfly.org/index.html). After Sanger sequencing, the nucleotide sequence was aligned using MAFFT with the promoter region of the efflux pump AreABC of the genome Ab_DQ40A1. The alignment was then checked for mutations and insertions/deletions (indels), covering both the gene and intergenic promoter region.

WGS and bioinformatic analysis

DNA samples (prepared as described in Supplementary Methods) were subjected to dual-indexed Nextera XT DNA Library Preparation Kit. Libraries were subjected to cluster generation and paired-end sequencing (2×150 bp) on NextSeq2000 Illumina equipment (Illumina). The FASTX-toolkit (https://github.com/agordon/fastx_toolkit) was utilized for trimming raw reads to improve their quality. The genomes were assembled *de novo* through SPADES v13.3 and subsequently annotated using the Rapid Annotation using Subsystem Technology (RAST) server, employing the RASTtk annotation scheme with default parameters.[20,21] The genome completeness was assessed with BUSCO v5.2.2,[22] using the Campylobacterales dataset. Snippy (https://github.com/tseemann/snippy) was used with default settings to identify SNPs and indels between the reference genome Ab_DQ40A1 (accession number CP000361) and the next-generation sequencing (NGS) sequence reads of the experimental evolution strains, detecting both substitutions (SNPs) and indels.

areB expression by quantitative real-time RT–PCR (RT–qPCR)

After RNA extraction (Supplementary Methods), qPCR was performed using the NZYSpeedy qPCR Green Master Mix (2×) kit (NZY Tech) to determine the expression of the *areB* gene, and the constitutively expressed 16S rRNA gene was used for relative quantification (Table S2), as previously described.[12] All qPCR reactions were performed in a Real-Time CFX Connect™ system (Bio-Rad, Hercules, CA, USA) in duplicate.

Ethidium bromide (EtBr) accumulation

The EtBr accumulation was performed according to Ferreira *et al.*,[12] with slight modifications (Supplementary Methods).

Motility and biofilm formation assessment

The *A. butzleri* strains were tested for motility and biofilm formation ability as previously described;[23] the incubation was done at 30°C in aerobiosis for 48 h and the assay was performed at least three times independently for each strain.

Statistical analysis

Data were presented with mean values ± standard deviation (SD) or error of the mean (SEM) according to the assay. Statistical analysis was done with GraphPad Prism (GraphPad Software version 8, San Diego, CA, USA).

Data availability

Short reads for all sequenced isolates have been submitted to the ENA under the project accession PRJEB68189, under the accession numbers ERR12205045 to ERR12205048.

Results

Evolution of resistance to erythromycin, and selection of cross-resistance and collateral susceptibility to antibiotics

In this work, the macrolide resistance mechanisms of *A. butzleri* were explored using a representative strain selected for its low MIC of erythromycin and other antibiotics, through two different approaches: (i) evaluating genetic alterations resulting from spontaneous mutation; and (ii) evaluating the effect of ALE by stress induction with erythromycin over 25 days and 20 passages with increasing antibiotic concentration.

Using a fluctuation assay, the spontaneous mutation rate in *A. butzleri* was determined as 9.26×10^{-8} (Table S3). Sixteen rising clones (TM) were selected, showing at least a 2-fold increase in erythromycin MIC compared with the parental strain Ab_DQ40A1 (Table S4). Following EUCAST recommendations for *Campylobacter jejuni* (erythromycin MIC > 4 mg/L) due to limited *A. butzleri* criteria, five isogenic mutants (TM1E, TM1F, TM1K, TM1L and TM1P) were chosen for further testing (Figure S2).

To further explore the potential mechanisms of macrolide resistance in *A. butzleri*, including high-level resistance, the parental *A. butzleri* strain Ab_DQ40A1 was continuously exposed to increasing concentrations of erythromycin by ALE until reaching a concentration of 12× MIC of the parental strain (ALE_DQ40P8). Simultaneously, two controls were used: a growth control without erythromycin (ALE_DQ40P8c); and the solvent control that was subjected to 20 successive passages in tryptic soy broth with ethanol (ALE_DQ40P8solv). The clones from the experimental evolution were also isogenic to the parental strain (Figure S2). Throughout adaptation, a significant increase of the IC_{50}, from 0.31 to 77.42 mg/L erythromycin for the ALE_DQ40P8 evolved population, was observed (Figure 1). In addition, the selected ALE_DQ40P8 clone showed an enhanced resistance profile, with a 256-fold increase in erythromycin MIC compared with the parental strain. The clones from controls, ALE_DQ40P8c and ALE_DQ40P8solv, showed a slight increase in MIC, from 1 to 2 mg/L. Regarding the potential for cross-resistance to other antimicrobial agents,[24] overall, several of the mutants became less susceptible to antibiotics of structurally distinct families (Table 1). Ciprofloxacin exhibited a minimum 2-fold MIC increase in all fluctuation assay mutants, with a >8-fold rise in the MIC for ALE_DQ40P8. Gentamicin susceptibility changed in TM1E, TM1K, TM1P and ALE_DQ40P8c, while tetracycline susceptibility was altered in TM1K and all evolved clones. Conversely, ALE_DQ40P8 displayed increased susceptibility to cefotaxime, indicating collateral susceptibility.

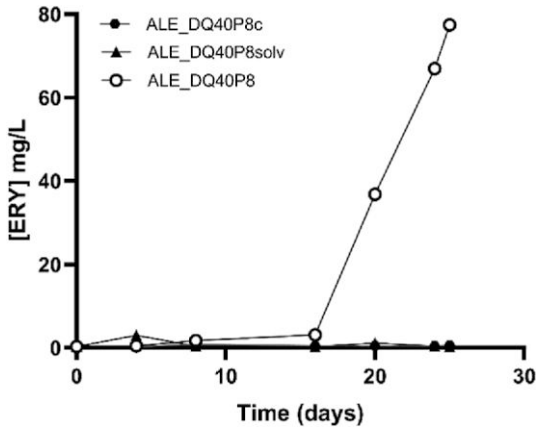

Figure 1. IC_{50} values determined during the ALE to erythromycin (ERY) of the experimentally adapted strain ALE_DQ40P8 and the controls ALE_DQ40P8c and ALE_DQ40P8solv.

Effect of resistance to erythromycin on bacterial fitness

In general, the TM mutant strains showed similar or improved growth compared with the parental strain (Figure 2a), the exceptions being the TM1F and TM1L mutants, which presented significantly lower growth rates compared with the parental strain (Figure S3). When considering the ALE clones, a slight improvement in the growth of the clones from the control populations and an expressive increase in lag phase for the ALE_DQ40P8 mutant were observed (Figure 2b), with a concomitant significant reduction in the growth rate of the ALE_DQ40P8 mutant compared with the parental strain and ALE controls ($P < 0.0001$) (Figure S3).

Assessment of the survivability of the mutants from the fluctuation test in the presence of erythromycin (Figure 3a) showed a more pronounced growth for all mutants compared with the native strain at all erythromycin concentrations tested after 12 h, with the differences being more evident for the TM1K strain, which presented the highest MIC of erythromycin (Table 1). The same scenario was observed for the erythromycin-resistant ALE_DQ40P8 clone (Figure 3b).

Overproduction of efflux pumps contributes to resistance

Efflux pump activity was assessed by examining EtBr accumulation demonstrating that apart from ALE_DQ40P8, all strains showed time-dependent increase of fluorescence, highest in parental strain Ab_DQ40A1, indicating superior EtBr accumulation. In contrast, all mutant strains under study demonstrated enhanced dye extrusion (Figure 4a), with the ALE_DQ40P8 clone exhibiting constant low fluorescence, suggesting heightened efflux activity. In fact, significant differences on EtBr accumulation were observed against the WT *A. butzleri* Ab_DQ40A1 both for TM and ALE strains (data not shown). CCCP, an efflux pump inhibitor, led to an immediate EtBr accumulation increase, supporting efflux pump involvement (Figure S4). The fluorescence emission profile found for ALE_DQ40P8 indicates elevated efflux system expression, preventing the inhibitory effect of CCCP. Results strongly suggest that erythromycin resistance is linked to increased efflux activity.

To confirm the association of macrolide resistance with increased AreABC efflux system expression, the expression of the

Table 1. Susceptibility profile of parental *A. butzleri* Ab_DQ40A1 strain, corresponding mutants TM1E, TM1F, TM1K, TM1L and TM1P, and evolved strains ALE_DQ40P8c, ALE_DQ40P8solv and ALE_DQ40P8

	MIC (mg/L)					
Strain	Erythromycin	Gentamicin	Tetracycline	Ampicillin	Ciprofloxacin	Cefotaxime
Ab_DQ40A1	1	0.5	1	4	≤0.03	8
TM1E	**8**	**1**	1	4	**0.06**	8
TM1F	**8**	0.5	1	4	**0.06**	8
TM1K	**16**	**1**	**2**	4	**0.06**	8
TM1L	**8**	0.5	1	4	**0.06**	8
TM1P	**8**	**1**	1	4	**0.06**	8
ALE_DQ40P8c	**2**	**1**	**2**	4	**0.06**	8
ALE_DQ40P8solv	**2**	0.5	**2**	4	≤ 0.03	8
ALE_DQ40P8	**256**	0.5	**2**	4	**0.25**	**2**

Changes in the MIC value in comparison with the native strain are indicated in bold.

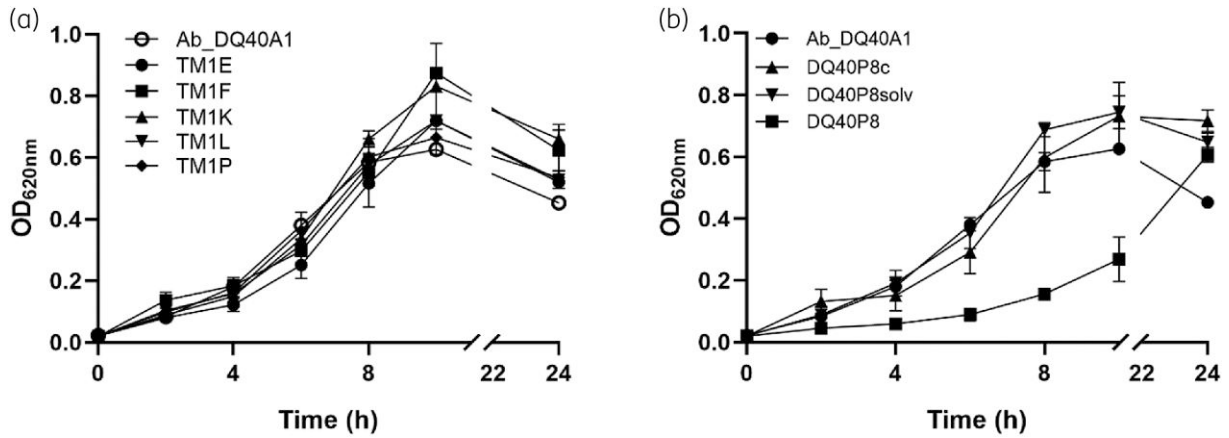

Figure 2. Growth profile of parental *A. butzleri* strain Ab_DQ40A1 and the selected strains from (a) the fluctuation and (b) the ALE assays, in the absence of erythromycin. Data represent the mean ± SD from at least three independent experiments.

areB gene (coding for the inner membrane protein) was assessed (Figure 5). The *areB* transcript levels significantly increased in all TM clones compared with the parental strain (Figure 5a; $P < 0.05$), with ALE clones, especially ALE_DQ40P8, exhibiting increased transcript levels (Figure 5b).

Genetic basis of resistance to erythromycin

To identify mutations responsible for the observed erythromycin resistance in TM mutants, the genetic sequence of the *areR* gene was determined by Sanger sequencing and compared with the parental strain (Table 2). SNP analysis of parental and TM strains revealed multiple *areR* gene changes in four mutants, which displayed missense alterations, or an insertion at position 186, which rendered a premature codon stop in the TM1F mutant. TM1E had no *areR* gene nucleotide changes; nonetheless, an SNP in the intergenic *areR_areA* region was predicted as the AreABC promoter location (Table 2).

In turn, parental strain and ALE clones were subjected to WGS. The parental strain DQ40A1 was *de novo* assembled, achieving a BUSCO score of 97.8%, which indicates good genome completeness. WGS analysis revealed 21 mutations among the three clones selected from the ALE, including SNPs, insertions and deletions spanning 14 genes and three intergenic regions (Table 3). Five variants were common to all three clones, four unique to ALE_DQ40P8c, and eight to ALE_DQ40P8, while ALE_DQ40P8solv shared variants with others. Unique mutations in the erythromycin-evolving clone involved *areR*, L4 and L22 ribosomal proteins genes, previously linked to erythromycin resistance in other bacteria. Additionally, the clone showed an insertion in the *rpoA* gene encoding a DNA-dependent RNA polymerase and accumulated mutations in a LysR family transcriptional regulator gene.

Selection by erythromycin alters the virulence potential of A. butzleri

Acquiring antibiotic resistance can alter bacterial virulence and pathogenicity. The impact of spontaneous and induced erythromycin resistance on key features for bacterial resistance, survival and spread in the environment, such as motility and biofilm formation were evaluated.

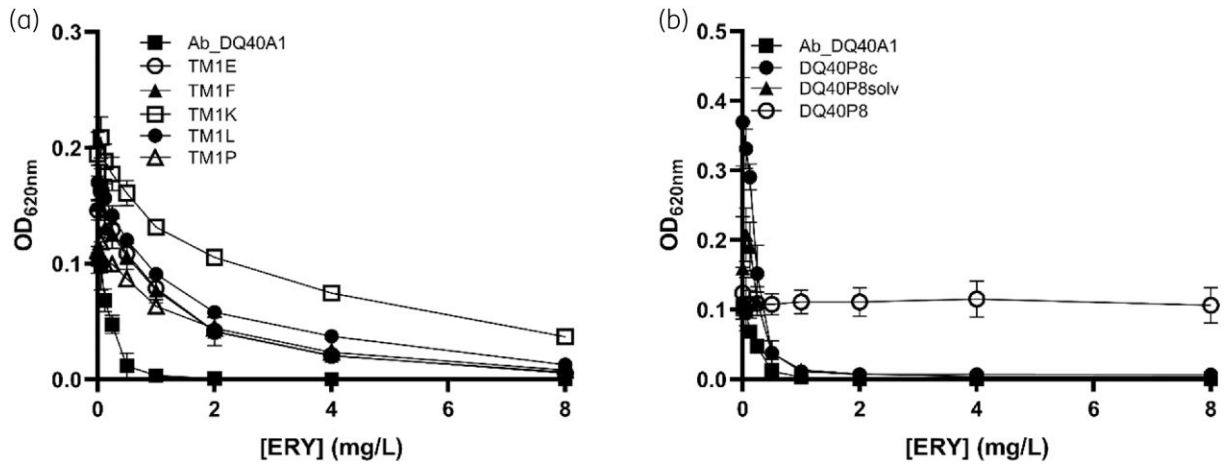

Figure 3. Growth profile, in the presence of increasing concentrations of erythromycin (ERY) between 0 and 8 mg/L at 12 h of exposure, of the parental *A. butzleri* strain Ab_DQ40A1 and the selected strains from (a) the fluctuation and (b) the ALE assays. Data represent the mean ± SEM from at least three independent experiments.

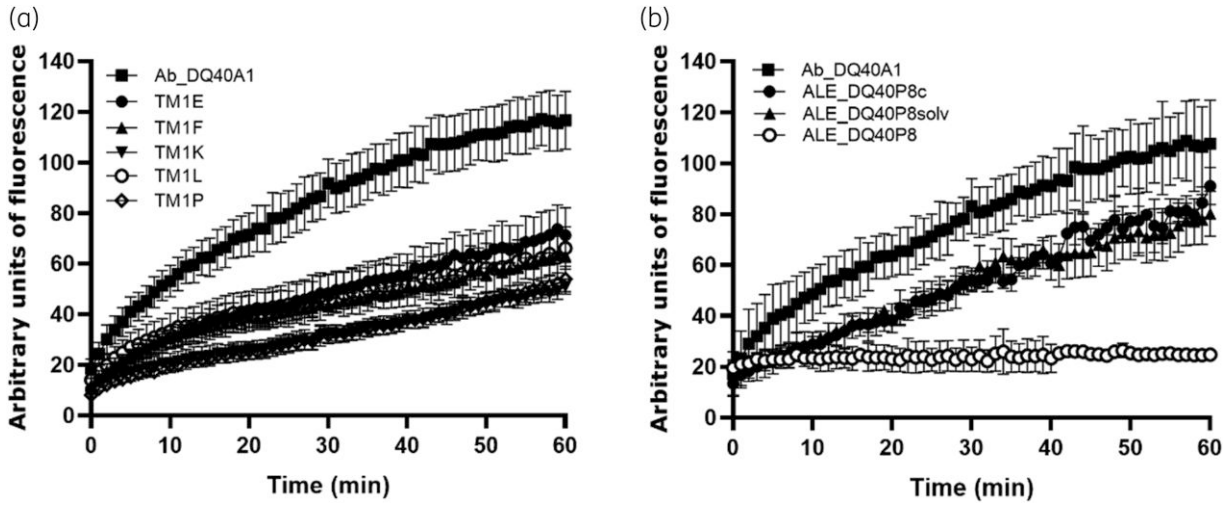

Figure 4. Accumulation of EtBr throughout 60 min of incubation for the parental strain and the selected strains from (a) the fluctuation and (b) ALE assays. Data represent the mean ± SEM of at least three independent experiments.

All the TM mutants showed a reduction in motility of at least 20% ($P < 0.05$) and a decrease in biofilm formation of >45% ($P < 0.01$), with the exception of TM1P. Regarding the ALE clones (Figure 6a), a significant decrease of motility was observed for both ALE_DQ40P8c and ALE_DQ40P8, with the ALE_DQ40P8 clone displaying a reduction of almost 70% ($P < 0.001$), but with no associated change in biofilm formation (Figure 6b).

Discussion

General macrolide resistance mechanisms described so far include: (i) decreased intracellular antibiotic concentration through efflux pump activity; (ii) ribosome modification by target mutations in the rRNA or in ribosomal proteins, or by ribosomal methylation by the members of the Erm family of methyltransferases; or (iii) drug modification by enzymatically catalysed modification of these antibiotics.[25] However, unlike macrolide resistance in other bacteria, these mechanisms in *A. butzleri* remains largely unexplored. Point mutations in domain V of the 23S rRNA, and mutations in the ribosomal proteins L4 and L22 or methylases usually associated with macrolide resistance in *Campylobacter* spp. have not been described for *A. butzleri*.[9,10,26] We previously suggested that the resistance of *A. butzleri* to macrolides may be associated with mutations in the transcription repressor gene *areR*, which affects the expression of the AreABC efflux pump.[9,12,27] In addition, in 2022, Ma *et al.* [11] reported an association between the carriage of the macrolide resistance gene *ere*(A) with an erythromycin MIC value of ≥64 mg/L. Here, we analysed phenotypic and adaptive outcomes of spontaneous mutations and *in vitro* laboratory evolution increasing erythromycin concentration. Our goal was to assess the resistance mechanisms and to explore the impact of acquiring macrolide resistance

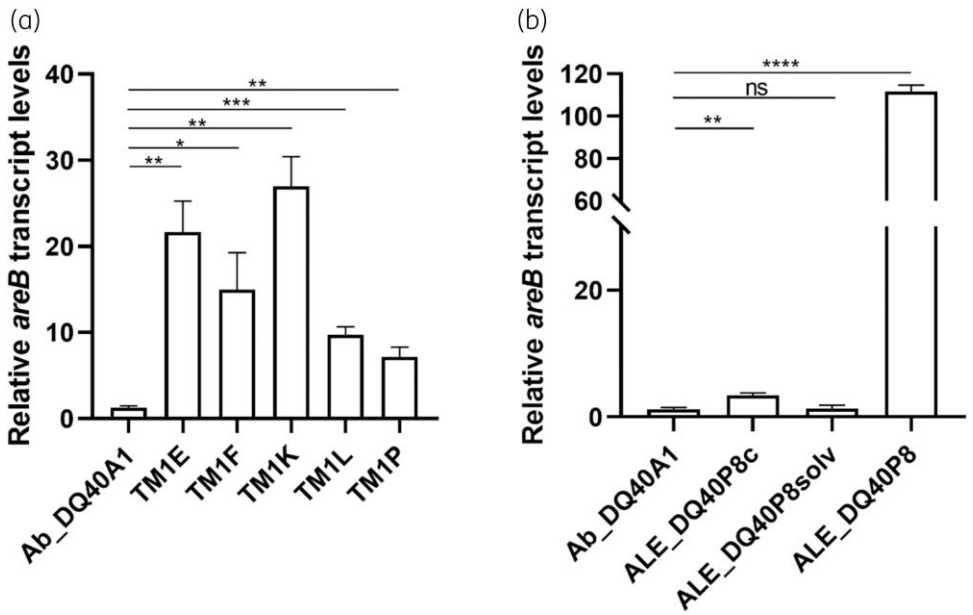

Figure 5. Relative *areB* transcript levels determined by RT–qPCR for selected clones from (a) the fluctuation and (b) ALE assays. Transcript levels were normalized to the housekeeping 16S rRNA gene, and all values are relative to transcript levels in the parental strain Ab_DQ40A1. Error bars indicate SEMs of two replicates from at least three biological replicate assays. Statistically significant differences (unpaired Student's *t*-test): ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Table 2. Mutations found in the *areR* gene for erythromycin-resistant *A. butzleri* mutants

	areR	
Strain	Genetic event	Amino acid change
TM1E[a]	—	—
TM1F	Insertion of G (186)	Premature stop codon
TM1K	G80A	Gly27Asp
TM1L	G128A	Gly43Glu
TM1P	T186G	Asn62Lys

[a]Presents an SNP in the intergenic region *areR_areA* probably corresponding to the AreABC promoter region.

on the robustness of *A. butzleri*. From the spontaneous mutation rate assessment, a stable decrease in susceptibility to erythromycin occurred for *A. butzleri* clones (TM clones), along with a slight decrease in susceptibility to other antibiotic classes. The acquisition of erythromycin resistance was not linked to an important fitness cost but favoured the growth of TM clones in the presence of erythromycin. Despite different SNPs in the *areR* gene or in the *areR_areA* intergenic region being found among the five studied TM clones, a common aspect was that all mutations appeared to cause an overexpression of the AreABC efflux pump, which resulted in increased efflux activity and a consequent decrease in EtBr accumulation. Mutations in the *areR* regulator, namely causing a premature stop codon, have been previously reported by us in human, animal and environmental *A. butzleri* strains to be linked to erythromycin resistance,[9,12] and afterwards supported by Concha-Toloza *et al.*[28] in 2023. The contribution of the AreABC efflux system in decreasing erythromycin susceptibility was further endorsed by the *A. butzleri* ALE_DQ40P8 clone, for which the overexpression of the AreABC efflux pump was associated with a frameshift in the *areR* gene. The role of the AreABC efflux system in decreased susceptibility to other antibiotics classes was supported by the cross-resistance observed, with the erythromycin-resistant mutants displaying an increased MIC of ciprofloxacin, a putative substrate of the AreABC efflux system.[12,27] In addition, the ALE_DQ40P8 clone became more susceptible to cefotaxime, suggesting that the induction of erythromycin resistance may have caused non-mutational collateral susceptibility. Similarly, cefotaxime-treated *Salmonella enterica* serovar Typhimurium populations previously showed lower resistance to erythromycin, supporting the collateral susceptibility between these two antibiotics.[29,30] Other examples of collateral susceptibility resulting from acquisition of antibiotic resistance have been reported, such as for *Pseudomonas aeruginosa*, which developed hypersusceptibility to amikacin, aztreonam, ceftazidime, imipenem and fosfomycin after evolution in the presence of subMICs of ciprofloxacin.[24]

In addition to the alteration in the *areR* regulatory gene, the WGS analysis of the ALE clones also revealed the presence of point mutations in the genes encoding the ribosomal proteins L4 and L22 in the ALE_DQ40AP8 clone, whose presence can be correlated with the high level of erythromycin resistance displayed by this clone. This aligns with findings in *C. jejuni* and *Campylobacter coli*, where resistance to macrolides is also associated with mutations in the ribosomal proteins L4 and L22, which are responsible for the decreased affinity of macrolides to the binding site, acting synergistically with

Table 3. Variants identified in each ALE *A. butzleri* clones, compared with the parental strain

Clone	Type	Gene	Product	Variant type	Genetic event	Amino acid change
ALE_DQ40P8c	CDS	*rpoB*	DNA-directed RNA polymerase β unit	SNP: missense	**T2893C**	**Ser965Pro**
	CDS	*cheD*	Chemotaxis protein CheD	SNP: missense	**G415A**	**Ala139Thr**
	CDS		Two-component system histidine kinase RacS	SNP: missense	G449T	Cys150Phe
	Intergenic			SNP	G>C	
	CDS	*actP*	Acetate permease ActP (cation/acetate symporter)	SNP: missense	A1081G	Thr361Ala
	CDS		GGDEF	Ins: fs	**842dupG**	**Ile283fs**
	Intergenic			Ins	C>CT	
	CDS		Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	SNP: missense	A944T	Tyr315Phe
	CDS	*ybhF*	ABC-type efflux pump, duplicated ATPase component YbhF	SNP: missense	A654T	Lys218Asn
	CDS		Hypothetical protein	SNP: missense	T1459A	Phe487Ile
	CDS		Hypothetical protein	SNP: missense	**G292T**	**Asp98Tyr**
ALE_DQ40P8solv	CDS		Two-component system histidine kinase RacS	SNP: missense	G449T	Cys150Phe
	CDS	*actP*	Acetate permease ActP (cation/acetate symporter)	SNP: missense	A1081G	Thr361Ala
	Intergenic			Ins	C>CT	
	CDS		Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	SNP: missense	A944T	Tyr315Phe
	CDS	*ybhF*	ABC-type efflux pump, duplicated ATPase component YbhF	SNP: missense	A654T	Lys218Asn
	CDS		Hypothetical protein	SNP: missense	T1459A	Phe487Ile
	CDS		Transcriptional regulator, LysR family	SNP: missense	C802G	Gln268Glu
ALE_DQ40P8	CDS	*cheD*	Chemotaxis protein CheD	SNP: missense	**C347A**	**Ala116Glu**
	CDS		Two-component system histidine kinase RacS	SNP: missense	G449T	Cys150Phe
	Intergenic			SNP	G>C	
	CDS	*actP*	Acetate permease ActP (cation/acetate symporter)	SNP: missense	A1081G	Thr361Ala
	Intergenic			Ins	CATCT	
	Intergenic			Ins	A>ATTATC	
	CDS		Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	SNP: missense	A944T	Tyr315Phe
	CDS	*ybhF*	ABC-type efflux pump, duplicated ATPase component YbhF	SNP: missense	A654T	Lys218Asn
	CDS	*areR*	Transcriptional regulator, Tet(R) family	Del: fs	**119_129delCAATAGGTGGA**	**Ser40fs**
	CDS		Hypothetical protein	SNP: missense	T1459A	Phe487Ile
	CDS	*rplD*	Ribosomal protein L4 (L1e)	Del	**169_177delGGTGGTGGT**	**Gly57_Gly59del**
	CDS	*rplV*	Ribosomal protein L22 (L17e)	SNP: missense	**C251T**	**Ala84Val**
	CDS	*rpoA*	DNA-directed RNA polymerase alpha subunit	Ins	**659_661dupATT**	**Tyr220dup**
	CDS		Transcriptional regulator, LysR family	SNP: missense	C802G	Gln268Glu
	CDS		Transcriptional regulator, LysR family	SNP: missense	**A797T**	**Lys266Ile**
	CDS		Transcriptional regulator, LysR family	SNP: missense	**787_790delATTAinsCTTC**	**IleThr263LeuPro**

CDS, coding sequence; ins, insertion; del, deletion; complex, combination of ins/del; fs, frameshift; reference genome Ab_DQ40A1. Unique variants are marked in bold.

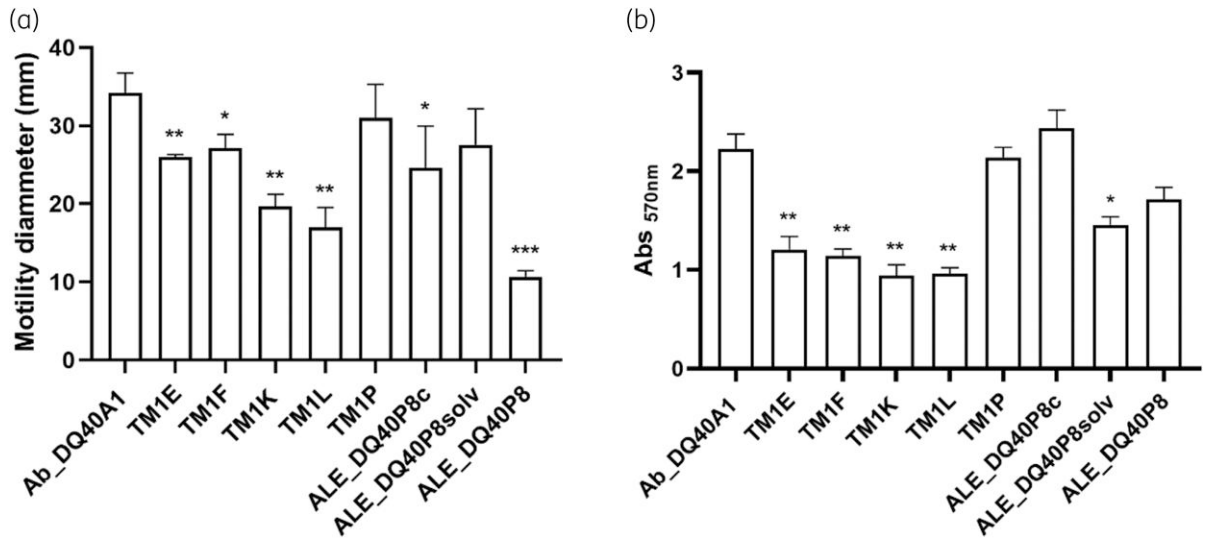

Figure 6. Virulence phenotype of *A. butzleri* strains considering (a) motility and (b) biofilm formation, measured for the parental, fluctuation test and ALE assay clones. Data represent the mean ± SD of at least three independent experiments. Statistically significant differences were analysed against the WT *A. butzleri* Ab_DQ40A1 using Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

other mechanisms, also including increased efflux pump expression.[31]

Based on our experiments, we propose that low-level resistance to erythromycin is associated with an increase of the AreABC efflux activity (due to *areR* mutations). The high level of resistance is likely caused by the combination of mutations underlying different resistance mechanisms: (i) increased efflux activity; and (ii) reduced affinity of the antibiotic to its target (due to L4 and L22 ribosomal protein mutations). Supporting the first mechanism, an increase in AreABC expression has been previously associated with low-level erythromycin resistance.[12] The role of the resistance-nodulation-division (RND) efflux system family in macrolide resistance has been previously described for other bacterial species.[26,31,32] Overexpression of these systems confers clinically relevant levels of multidrug resistance by extruding diverse substrates. Such overexpression is often associated with mutations in the regulatory network controlling these pumps, whether affecting a local repressor, a global transcription factor or intergenic regions,[33] such as the ones reported here for *A. butzleri.* Mutations in the *cmeABC* regulatory region have been shown to affect macrolide susceptibility in *C. jejuni* and *C. coli*.[26,31] In particular, a *C. jejuni* variant harbouring an A-to-G substitution in the CmeR-binding site of CmeABC has been identified, resulting in CmeABC system overexpression and a phenotype of macrolide resistance.[34]

Concerning the second suggested resistance mechanism, it is described that alterations at amino acids 55 to 77 of the large loop of the L4 protein and in the amino acids 78 to 98 of the highly conserved large loop of the L22 protein are associated with macrolide resistance in diverse bacterial species.[35] Here, a deletion of three glycines from positions 57 to 59 in ribosomal proteins L4 and a transition from an alanine to a valine at position 84 in the ribosomal protein L22 were observed in the macrolide-resistant mutant ALE_DQ40P8, suggesting that these mutations may have an active role in the adaptation to macrolide selection in *A. butzleri*. In *Campylobacter*, when mutations are absent in the 23S rRNA gene, variations in ribosomal *rplD* and *rplV* are linked to a lower level of resistance.[26] However, in *in vitro*-selected mutants of *C. jejuni* and *C. coli*, these mutations may synergistically interact with the CmeABC efflux pump, conferring an intermediate level of resistance.[36] Similarly to *C. jejuni*, *A. butzleri* may be experiencing a comparable phenomenon, where increased efflux gene expression facilitates the development of highly resistant mutants, while some L4 mutations, like the one at position 57, may restrict the emergence of mutations in the 23S rRNA gene.[35]

The development of macrolide resistance usually imposes a fitness cost when compared with susceptible strains.[26,37–39] Here, the acquisition of erythromycin resistance clearly entailed a fitness advantage in the presence of the antibiotic in comparison with parental and control strains, but in the absence of antibiotic a high biological cost was observed, indicating that the resistant strains can be outcompeted by the susceptible ones.[40,41] This may explain the low prevalence of high-level erythromycin-resistant strains found in the environment. This fitness disadvantage was absent in TM strains, exhibiting a low-level resistance phenotype. This aligns with the assumption that mutations conferring higher MICs are more costly,[26,40] a concept also proposed for macrolide-resistant *Campylobacter*, linking the low prevalence of this phenotype to lower strain fitness.[37]

Resistance is, in general, associated with a reduction in fitness, with resistant mutants growing more slowly and showing reduced virulence and survival in comparison with their susceptible siblings.[38,42] Thus, several features associated with colonization and persistence of *A. butzleri* in different hosts and environments, such as motility and biofilm formation ability,[2,3] were assessed in the parental strain, and TM and ALE clones. Changes in the virulence phenotype were noticed, with a general reduction of motility and biofilm formation ability for TM clones, except for TM1P,

and of motility for the ALE_DQ40P8 clone. These results are consistent with previous studies in *C. jejuni*, where a motility decrease was observed for resistant strains, possibly associated with motility gene down-regulation in macrolide-resistant mutants and a loss of flagellar filaments.[35,38] Despite no clear relationship between resistance and biofilm formation modification existing, the role of motility in biofilm formation[43] may support the observed phenotype, where reduced motility correlates with decreased biofilm formation in TM strains. The development of macrolide resistance in *C. jejuni* influences physiological and metabolic pathways, explaining the reduced fitness and virulence of macrolide-resistant strains.[35,38] However, further antibiotic adaptation may support reversion to the WT-state transcriptome in highly resistant mutants,[35] suggesting that the ALE_DQ40P8 biofilm phenotype may be associated with some biofilm-associated genes returning to their WT level. Biofilm formation modification may in fact be associated with a non-mutational compensation reducing the effects of the resistance-associated mutations.

The findings of this study have proved that efflux systems can play a decisive role in the acquisition of erythromycin resistance in *A. butzleri*, with the accumulation of mutations in ribosomal proteins L4 and L22 acting synergistically with efflux and being associated with high-level resistance. The results further suggest that the development of macrolide resistance in *A. butzleri* impacts the bacterial physiology and virulence. Finally, this study highlights that the increased fitness cost associated with high-level erythromycin resistance may explain the low worldwide prevalence of these strains.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Figures S1 to S4 and Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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