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Catabolism of aromatic β-glucosides by bacteria can lead to antibiotics resistance

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Abstract

Antimicrobial resistance is a serious public health threat worldwide today. *Escherichia coli* is known to resist low doses of antibiotics in the presence of sodium salicylate and related compounds by mounting non-heritable transient phenotypic antibiotic resistance (PAR). In the present study, we demonstrate that Bgl⁺ bacterial strains harboring a functional copy of the β-glucoside (*bgl*) operon and are actively hydrolyzing plant-derived aromatic β-glucosides such as salicin show PAR to low doses of antibiotics. The aglycone released during metabolism of aromatic β-glucosides is responsible for conferring this phenotype by de-repressing the multiple antibiotics resistance (*mar*) operon. We also show that prolonged exposure of Bgl⁺ bacteria to aromatic β-glucosides in the presence of sub-lethal doses of antibiotics can lead to a significant increase in the frequency of mutants that show heritable resistance to higher doses of antibiotics. Although heritable drug resistance in many cases is known to reduce the fitness of the carrier strain, we did not see a cost associated with resistance in the mutants, most of which carry clinically relevant mutations. These findings indicate that the presence of the activated form of the *bgl* operon in the genome facilitates the survival of bacteria in environments in which both aromatic β-glucosides and antibiotics are present.

Keywords Phenotypic antibiotic resistance (PAR) · *bgl* operon · Aromatic β-glucosides · Efflux pumps

Abbreviations

PAR   Phenotypic antibiotic resistance
NaSal   Sodium salicylate
SG   Saligenin
MIC   Minimal inhibitory concentration

Introduction

The heavy overuse and misuse of antibiotics due to their increased demand in several sectors have resulted in antimicrobial resistance as predicted by Alexander Fleming

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in 1945 (Levy 1992) Bacteria develop genetic resistance against antibiotics as an evolutionary response involving diverse mechanisms. While most of the studies performed in antimicrobial resistance focus on heritable resistance (that requires mutations), the ability of bacteria to resist antibiotics without any genetic alterations has been largely ignored. The non-heritable and transient ability of bacteria to resist antibiotics is termed as phenotypic antibiotic resistance (PAR) which primarily include drug indifference, persistence, biofilm formation, and reduced permeability of antibiotics or their increased efflux (Corona and Martinez 2013). PAR has been shown to be triggered by salicylate and other related compounds like acetate, acetyl salicylate (aspirin), benzoate, DMSO, and 1-methyl-2-pyrrolidinone in *Escherichia coli* (Rosner 1985). Salicylate has been shown to induce the *marRAB* (multiple antibiotic resistance) operon which results in the expression of MarA, a DNA binding transcription factor. MarA alters the expression of membrane proteins and efflux pumps like AcrAB–TolC (Cohen et al. 1993; Price et al. 2000). In a recent study, the initial observations have been further strengthened by demonstrating that sodium salicylate induces antibiotic resistance and alters growth in *E. coli* (Bhaskarla et al. 2016).

The *bgl* operon involved in the hydrolysis of plant-derived aromatic β-glucosides such as salicin and arbutin is silent and uninducible in most gut isolates (Mukerji and Mahadevan 1997b). A variety of *cis*- and *trans*-acting mutations can transcriptionally activate the operon (Reynolds et al. 1981, 1986; Lopilato and Wright 1990; Schnetz and Rak 1992; Schnetz 1995; Singh et al. 1995; Mukerji and Mahadevan, 1997a). In our laboratory, it has been previously observed that the activated *bgl* operon confers several advantages under selective conditions in addition to enabling the utilization of aromatic β-glucosides as sole carbon source. By enhancing the metabolic capability of the cell, it can confer a growth advantage in stationary phase (Madan et al. 2005). Also, Bgl+ bacteria actively metabolizing the aromatic β-glucosides salicin and arbutin can evade predators such as soil amoebae and nematodes (Sonowal et al. 2013).

Since salicylate is structurally similar to saligenin (SG) and hydroquinone (HQ) (aromatic part of salicin and arbutin respectively) and is known to induce phenotypic antibiotic resistance, we hypothesized that in the case of Bgl+ bacteria that are actively metabolizing salicin or arbutin, the saligenin or hydroquinone released might be involved in conferring phenotypic antibiotic resistance. In such a case, Bgl+ bacteria could exhibit a significant growth advantage in environments where both aromatic β-glucosides and antibiotics are present. To investigate this possibility, laboratory strains of *E. coli* and natural isolates of *Klebsiella* were used to perform assays to detect resistance against the two structurally and mechanistically different antibiotics nalidixic acid and ampicillin. Unlike *E. coli* isolates, *Klebsiella aerogenes* strains exhibit a Bgl+ constitutive phenotype by expressing a homolog of the *bgl* operon (Raghunand and Mahadevan 2003). Several *Klebsiella* strains also carry the *casRAB* genetic system conferring the ability to hydrolyze cellobiose, arbutin and salicin (Lai et al. 1997). The genes of the *casRAB* operon are significantly similar to the permease and phospho β-glucosidase genes of the *bgl* operon of *E. coli*. The active state of β-glucoside utilization in *Klebsiella* could be related to the fact that their primary habitat is soil where aromatic β-glucosides are known to be present.

The results of the experiments described below support the possibility that phenotypic antibiotic resistance is another selective advantage conferred by the activated form of the *bgl* operon. We also report that this low level of non-heritable phenotypic resistance in Bgl+ bacteria can further facilitate the emergence of a genetically resistant population in the presence of aromatic β-glucosides and sub-lethal concentrations of antibiotics.

Materials and methods

Chemicals, media and growth conditions

Bacterial strains (Table 1) were grown in L Broth (HiMedia, India/Difco, USA) or L Agar (1.5%) or M9 minimal salts (1X M9 salts, 1 mM MgSO4, 0.1 mM CaCl2, 0.01% vitamin B1, 0.2% Cas amino acids) supplemented with 0.4% sodium succinate (Sigma Aldrich) at 37 ℃ either static or shaking at 220 rpm, unless specified. Salicin and arbutin utilization phenotypes were checked on MacConkey medium (1% Tryptone, 0.6% Na Taurocholate, 1.5% Agar, 0.015% neutral red) supplemented with either 1% salicin or 1% arbutin. Sodium salicylate (NaSal), saligenin (SG), salicin, and arbutin were obtained from Sigma Aldrich, USA.

DNA manipulations

Plasmid DNA transformations and other DNA manipulations were performed as described previously (Sambrook et al. 1989, 2001). Linear DNA transformation was carried

Table 1 List of bacterial strains and plasmids used in this study

Strain	Genotype and phenotype	Reference or source
E. coli MA200 (Rec+)	F− Δ*lac*X74*thi bglR*11(*bglR*::IS*1*) λ*bglR*7*bglG'lacZ'lacY* φ(*bgl-lac*) (Bgl+ Rec+)	Mahadevan et al. (1987)
MA200Δ*marA*	MA200*marA*::cam, (Bgl+ Camʳ Rec+)	This study
MA200Δ*acrB*	MA200*acrB*::cam, (Bgl+ Camʳ Rec+)	This study
MA200Δ*tolC*	MA200*tolC*::cam, (Bgl+ Camʳ Rec+)	This study
MA200Δ*bgl*	MA200*bgl*::cam, (Bgl− Camʳ Rec+)	This study
MA200-1(Rec+)	MA200 *bglF201*BglG-Constitutive, (Bgl− Rec+)	Mahadevan et al. (1987)
Klebsiella pneumoniae OK8	Natural pathogenic isolate (*bgl* constitutive)	V. Nagaraja
Klebsiella ANK	Natural sewage isolate (*bgl* constitutive)	Kharat (unpublished)
pKD3 (plasmid)	pANTS_ derivative, Camʳ	Datsenko and Wanner (2000)
pKD46 (plasmid)	Ampʳ *beta exo gam* (lambda *red*) *ori*(Ts)	Datsenko and Wanner (2000)

Camʳ Chloramphenicol resistance, *Ampʳ* Ampicillin resistance

out by electroporation using a BIORAD electroporator as recommended by the manufacturer.

One-step chromosomal gene inactivation for strain construction

All chromosomal gene inactivations were generated by following the protocol described previously (Datsenko and Wanner 2000). Hybrid cassettes carrying Chloramphenicol or Kanamycin resistance marker were amplified from plasmid pKD3 or pKD4, respectively, using primers (Table 2) that carried a region of homology to the target gene. The amplified fragments were digested with DpnI to get rid of the vector backbone and the linear DNA of appropriate size was further purified from gel using GeneJET Gel Extraction Kit (Thermo Scientific/EU). The purified DNA was electroporated into the bacterial strain of interest carrying pKD46 plasmid. The pKD46 plasmid has a temperature-sensitive origin of replication and expresses *λredgam* genes under arabinose promoter**.** Electroporated cells were recovered by growing in L Broth for 18 h at 37 ℃, 220 rpm. The knockouts were selected on L Agar plates containing the appropriate antibiotic and confirmed by colony PCR using confirmation primers.

Quantitative gene expression (qRT-PCR)

Total RNA was extracted using the acid phenol method (Harwani et al. 2012) from bacterial cells exposed to different growth conditions and treated with DNAse I (Thermo Fisher Scientific). cDNA was prepared from 1 µg of DNAse I-treated RNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, K1621) as per the instruction manual. Transcript levels of target genes were analyzed by performing quantitative real time reverse transcription PCR (Quant Studio 6 Flex System Applied Biosystems) using gene-specific primers (Table 2). The primers were designed using EcoCyc database and NCBI BLAST. The reactions were prepared using Maxima SYBR green qPCR master mix kit. Analysis of gene expression was performed using comparative CT method ($2^{-\Delta\Delta CT}$) (Schmittgen and Livak 2008). Values were normalized to the 16S rRNA transcript. Graphs were generated using GraphPad Prism software.

Analysis of phenotypic antibiotic resistance on solid media

Cells were grown overnight in M9 salts + 0.4% succinate at 37 ℃, 220 rpm. The cultures were diluted to OD_{600} ~ 0.8. Cells were serially diluted and spotted on M9 succinate agar plates containing different compounds (2-mM NaSal, 1.5-mM SG, 5-mM Salicin, or 5-mM Arbutin) in the presence and absence of antibiotics (concentrations indicated in the

respective figures). Plates were kept for overnight incubation at 37 ℃. Imaging was performed using ChemiDoc™ XRS + Imaging System (Bio-Rad). Experiments were repeated thrice.

Quantification of phenotypic antibiotic resistance

Cells were grown overnight in M9 salts + 0.4% succinate at 37 ℃, 220 rpm. The cultures were diluted to OD600 ~ 0.8 and plated on appropriate media as above. Strips containing a concentration gradient of antibiotic (Ezy MIC strip, HiMedia) were placed on the evenly spread bacterial lawns and kept for overnight incubation at 37 ℃. The MIC was read at the point of intersection of bacterial growth and antibiotic strip. Experiments were repeated twice or thrice.

Determination of frequency of genetic mutants

Cells were grown overnight in M9 salts + 0.4% succinate at 37 ℃, 220 rpm. The cultures were diluted to OD_{600} ~ 0.5 and 0.1% was inoculated in three different growth media i.e. (a) M9 salts + 0.4% succinate + 5 mM salicin (control) (b) M9 salts + 0.4% succinate + 1 µg/ml of nalidixic acid (control), and (c) M9 salts + 0.4% succinate + 1 µg/ml of nalidixic acid + 5 mM salicin (test experiment). For the control experiments, 12 independent cultures were inoculated for each strain and in the case of test experiments, 20 independent cultures were inoculated. Cells were grown for 24 h at 37 ℃, 220 rpm. Nalidixic acid resistant colonies were selected on LB agar plates containing 4, 8, 16, and 32 µg/ml of nalidixic acid. Fractions of nalidixic acid-resistant colonies were calculated as Fraction of NAL^R = CFU/ml on NAL plate/CFU/ml on LB agar plate. Plots were generated using MATLAB. A similar set of experiments was performed using 2 µg/ml of NAL (data not shown).

Confirmation of genetic resistance

Colonies from selection plates were randomly isolated and grown overnight in *L* broth at 37 ℃, 220 rpm without addition of nalidixic acid to OD_{600} ~ 1. Cultures were serially diluted in 96-well plates and spotted on LB agar plates containing higher concentrations of nalidixic acid (16, 32, 64, and 128 µg/ml). Plates were kept for overnight incubation at 37 ℃. Imaging was performed using ChemiDoc™ XRS + Imaging System (Bio-Rad). Experiments were repeated twice.

Sequencing of *gyrA* locus in mutants

The *gyrA* locus was amplified from 18 colonies (6 each from 3 nalidixic acid selection plates) using gene specific primers (Table 2) and Phusion high-fidelity DNA polymerase (Thermo

Table 2 List of primers used in this study

marAhyFP	Hybrid forward primer for *marA* deletion
	5′ CTGACGCTATTACCATTCATAGCATTTTGGACTGGTGTAGGCTGGAGCTG CTTCG 3′
marAhyRP	Hybrid reverse primer for *marA* deletion
	5′ CTGTTGTAATGATTTAATGGATGTAAAAAGCGCGCATATGAATATCCTCC TTA 3′
marAcfFP	Forward primer for *marA* deletion confirmation
	5′ TGGCAACACTTGAGTATTTGCTT 3′
marAcfRP	Reverse primer for *marA* deletion confirmation
	5′TGAAAGTGGTTTCATGATTGCCT 3′
acrBhyFP	Hybrid Forward primer for *acrB* deletion
	5′ATGCCTAATTTCTTTATCGATCGCCCGATTTTTGTGTAGGCTGGAGCTGC TTCG 3′
acrBhyRP	Hybrid Reverse primer for *acrB* deletion
	5′TCAATGATGATCGACAGTATGGCTGTGCTCGATACATATGAATATCCTC CTTA 3'
acrBcfFP	Forward primer for *acrB* deletion confirmation
	5′GCACAAGAAGTTACCGCTGA 3′
acrBcfRP	Reverse primer for *acrB* deletion confirmation
	5′CTTACGCGGCCTTAGTGATT 3′
tolChyFP	Hybrid forward primer for *tolC* deletion 5′ATGAAGAAATTGCTCCCCATTCTTATCGGCCTG AGCCTTTCTGGGTTCA GGTGTAGGCTGGAGCTGCTTCG 3′
tolChyRP	Hybrid Reverse primer for *tolC* deletion 5′TCAGTTACGGAAAGGGTTATGACCGTTACTGGT GGTAGTGCGTGCGGA TCATATGAATATCCTCCTTA 3′
tolCcfFP	Forward primer for *tolC* deletion confirmation
	5′CTAAATACTGCTTCACCACAAGGA 3′
tolCcfRP	Reverse primer for *tolC* deletion confirmation
	5′AGATAACCCGTATCTTTACGTTG 3′
RM 1	Hybrid forward primer for *bgl* deletion
	5′ATATAACTTTATAAATTCCTAAAATTACACAAAGTTGTGTAGGCTGGA GCTGCTTCG 3′
RM 2	Hybrid reverse primer for *bgl* deletion
	5′AATGCTTTCGCTGTGGGAAACACACAACCAGTCATGTACATATGAATA TCCTCCTTA 3′
SM1	Forward primer for *bgl* deletion confirmation
	5′GTGGATCCATCTTCTACTACGTGAAG 3′
SM70	Reverse primer for *bgl* deletion confirmation
	5′GAGGATCCTTAAGGTGCTTTAATGG 3′
16SqFP	Forward primer for transcript analysis of 16S rRNA
	5′GAATATTGCACAATGGGCGCAA 3′
16SqRP	Reverse primer for transcript analysis of 16S rRNA
	5′AATTCCGATTAACGCTTGCACC 3′
marAqFP	Forward primer for transcript analysis of *marA*
	5′GTGTCAGAGCGTTCGGGTTA 3′
marAqRP	Reverse primer for transcript analysis of *marA*
	5′GCGATTTCCGTCATCTTACG 3′
acrBqFP	Forward primer for transcript analysis of *acrB*
	5′CGAAGATTGAGCTGGGTGGT 3′
acrBqRP	Reverse primer for transcript analysis of *acrB*
	5′TTTTCAGACCCGACGGGAAG 3′
tolCqFP	Forward primer for transcript analysis of *tolC*
	5′ACGCCTACAAACAAGCCGTA 3′
tolCqRP	Reverse primer for transcript analysis of *tolC*
	5′CATTCGCCAGCTCTTGCTTG 3′
acrDqFP	Forward primer for transcript analysis of *acrD*
	5′CGGTCAGGCATCTGTCACTT 3′

Table 2 (continued)

acrDqRP	Reverse primer for transcript analysis of *acrD*
	5′GTCACGCCCTGATTTTGCAC 3′
acrFqFP	Forward primer for transcript analysis of *acrF*
	5′TCTGAAACCGTGGGAAGAGC 3′
acrFqRP	Reverse primer for transcript analysis of *acrF*
	5′CCAGTTCAACAATGGCTGGC 3′
mdtFqFP	Forward primer for transcript analysis of *mdtF*
	5′GTCCAGTCGGTGTTTACCGT 3′
mdtFqRP	Reverse primer for transcript analysis of *mdtF*
	5′CATTGCCCGCTGAATGATCG 3′
macBqFP	Forward primer for transcript analysis of *macB*
	5′GCGCGTTGGGAATAACACTG 3′
macBqRP	Reverse primer for transcript analysis of *macB*
	5′TGACCGAGCAGAGAAACTCG 3′
gyrAFP	Forward primer for *gyrA* amplification
	5′GGATACAGTAGAGGGATAGCGGTTA 3′
gyrARP	Reverse primer for *gyrA* amplification
	5′AAAATTATTCTTCTTCTGGCTCGTC 3′
gyrAprimer 1	Overlapping primer for *gyrA* sequencing
	5′GCGGCAATCATTAACGGTCGTCG 3′
gyrAprimer 2	Overlapping primer for *gyrA* sequencing
	5′GTTGCCGCGATGCTCGAACGTGC 3′
gyrAprimer 3	Overlapping primer for *gyrA* sequencing
	5′GTCAACCTGCTGCCGCTGGAGCA 3′
gyrAprimer 4	Overlapping primer for *gyrA* sequencing
	5′ATGGGGATGGTATTTACCGATTA 3′

Fisher Scientific) and Sanger sequencing was performed (Eurofins Scientific). The mutations were analyzed using various online tools (NCBI nucleotide BLAST, EXPASy, and ClustalW).

Co-culture experiments

Strains were individually grown overnight in M9 salts + 0.4% succinate at 37 ℃, 220 rpm. The cultures were diluted to $OD_{600} \sim 0.5$. The bacterial strains to be competed were mixed in M9 salts + 0.4% succinate medium in 1:1 ratio and grown at 37 ℃, 220 rpm. At every 4 h of interval, 1% of the previous culture was inoculated in fresh medium and grown to a total period of 12 h. At every sub-culture, aliquots were plated to determine CFU/ml. CFU/ml for the parent strain was calculated by subtracting the CFU on LB agar plates containing 16 µg/ml nalidixic acid (selection concentration) from CFU on LB agar plate (no antibiotic).

Results

Aromatic β-glucosides induce MarA-dependent PAR in Bgl⁺ strains

In an attempt to assess the induction of the *marRAB* operon upon hydrolysis of the aromatic β-glucosides salicin and arbutin, we initially measured the transcript levels of *marA* and its downstream target genes *acrB* and *tolC* in the Bgl⁺ *E. coli* strain MA200. Total RNA was isolated from exponential phase cultures of MA200 exposed to specific growth conditions. The mRNA levels of *marA*, *acrB*, and *tolC* were quantified by qRT-PCR using gene specific primers. Figure 1a represents the fold change of specific mRNA normalized to the 16S rRNA gene *rrnC* (internal control). The mRNA levels of *marA*, *acrB*, and *tolC* were two–threefold higher when cells were grown in

Fig. 1 Aromatic β glucosides induce PAR in Bgl[+] bacteria. **a** qRT-PCR analysis of *marA, acrB,* and *tolC* in MA200 exposed to different growth conditions indicated. Mean ± SEM is plotted. ($n^* = 3$, ANOVA, $p < 0.001 = ***$ $p < 0.01 = **$, $p < 0.05 = *$ $p > 0.05 = ns$). **b** Growth assay of MA200 and MA200Δ*marA* on M9 succinate agar plates containing NaSal, SG, salicin and arbutin in the presence and absence of nalidixic acid or ampicillin. Representative image of $n^* = 3$ biological replicates
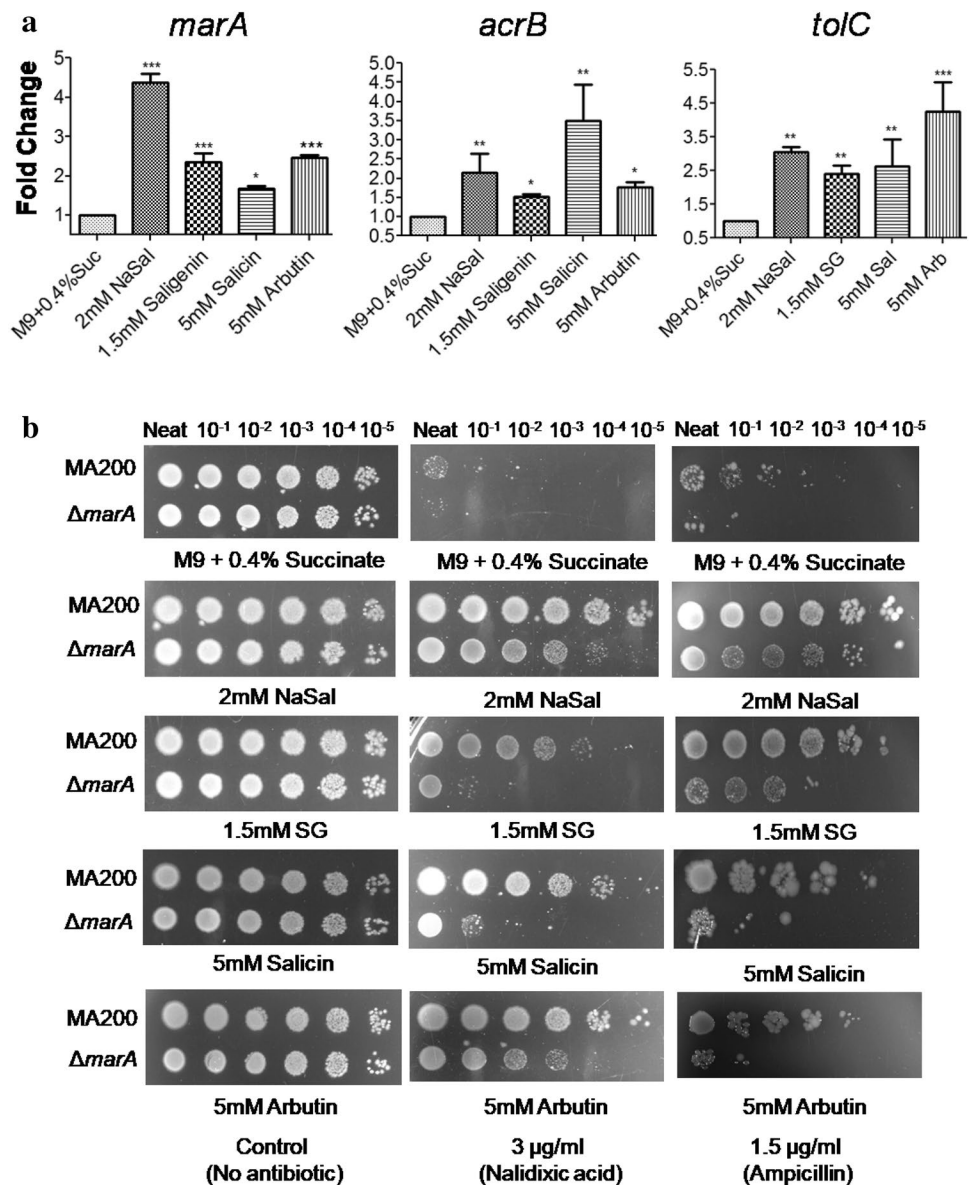
the presence of saligenin (SG), salicin and arbutin compared to M9 succinate control, indicating that saligenin, salicin and arbutin can induce the expression of the *marRAB* operon. To test whether this can lead to phenotypic antibiotics resistance, we examined the ability of the laboratory *E. coli* strain MA200 and pathogenic isolates of *Klebsiella pneumoniae* to grow in the presence of different concentrations of antibiotics. Overnight cultures of the strains were spotted on M9 succinate agar plates containing NaSal, SG, salicin, or arbutin along with nalidixic acid or ampicillin. The strain MA200 was able to tolerate 3 µg/ml of nalidixic acid and 1.5 µg/ml of ampicillin in the presence of aromatic compounds (Fig. 1b) compared to M9 succinate control. The *Klebsiella pneumoniae* strain was able to resist 3 µg/ml of nalidixic acid upon exposure

to aromatic compounds (Fig. S1) as opposed to the M9 succinate control.

To investigate the role of *marA* in the PAR phenotype observed, the strain MA200Δ*marA* was constructed and growth assays were performed as before. The deletion strain MA200Δ*marA* showed loss of the resistant phenotype upon exposure to antibiotics in the presence of saligenin, salicin (SG) and arbutin (Fig. 1b). Thus, Bgl[+] bacteria exhibit *marA*-dependent PAR in the presence of aromatic β-glucosides and low drug pressure. Owing to technical limitations and toxicity issues, hydroquinone (aromatic byproduct of arbutin) was not included in the assays. In these studies, we have also used lower concentrations of sodium salicylate (positive control) and saligenin compared to the concentrations used in a previous study (Cohen et al. 1993).

### Involvement of the *bgl* operon in conferring PAR

To address the question whether the *bgl* operon is involved in the phenotypic antibiotics resistance, the *bgl* operon was deleted in the strain MA200. The deletion was further confirmed by the loss of the Bgl[+] phenotype on MacConkey salicin plates. Growth assays were carried out as described earlier using the strain MA200Δ*bgl* and the permease defective strain MA200-1. Both strains showed loss of the resistant phenotype at 3 µg/ml of nalidixic acid and 1.5 µg/ml of ampicillin when grown in the presence of salicin (Fig. 2d). However, the strains were able to grow on the antibiotic plate in the presence of 1.5-mM SG (Fig. 2c) and 2-mM

NaSal (Fig. 2b) indicating that the functional *bgl* operon is necessary to generate saligenin by the hydrolysis of salicin and can be bypassed if the hydrolyzed product is provided directly in the medium.

Involvement of multidrug efflux transporters in the acquisition of PAR in Bgl[+] bacteria

The results reported so far imply that the release of the aromatic moiety saligenin/ hydroquinone upon hydrolysis of salicin/arbutin is responsible for the phenotypic antibiotic resistance in a *marA*-dependent fashion. Consistent with this observation and the enhanced transcript levels of *acrB* and

Fig. 2 Involvement of the *bgl* operon and AcrAB–TolC efflux pump in conferring the PAR phenotype in Bgl[+] strains. Growth assays of the *bgl* deletion strain MA200Δ*bgl*, permease-deficient strain MA200-1, and *acrB* deletion strain MA200Δ*acrB* in the presence and absence of antibiotics (nalidixic acid or ampicillin). **a** Growth pattern of these strains in M9 succinate medium. **b**, **c**, **d** Growth of these strains in M9 succinate medium containing NaSal, SG, and salicin, respectively. Representative image of $n^* = 3$ biological replicates
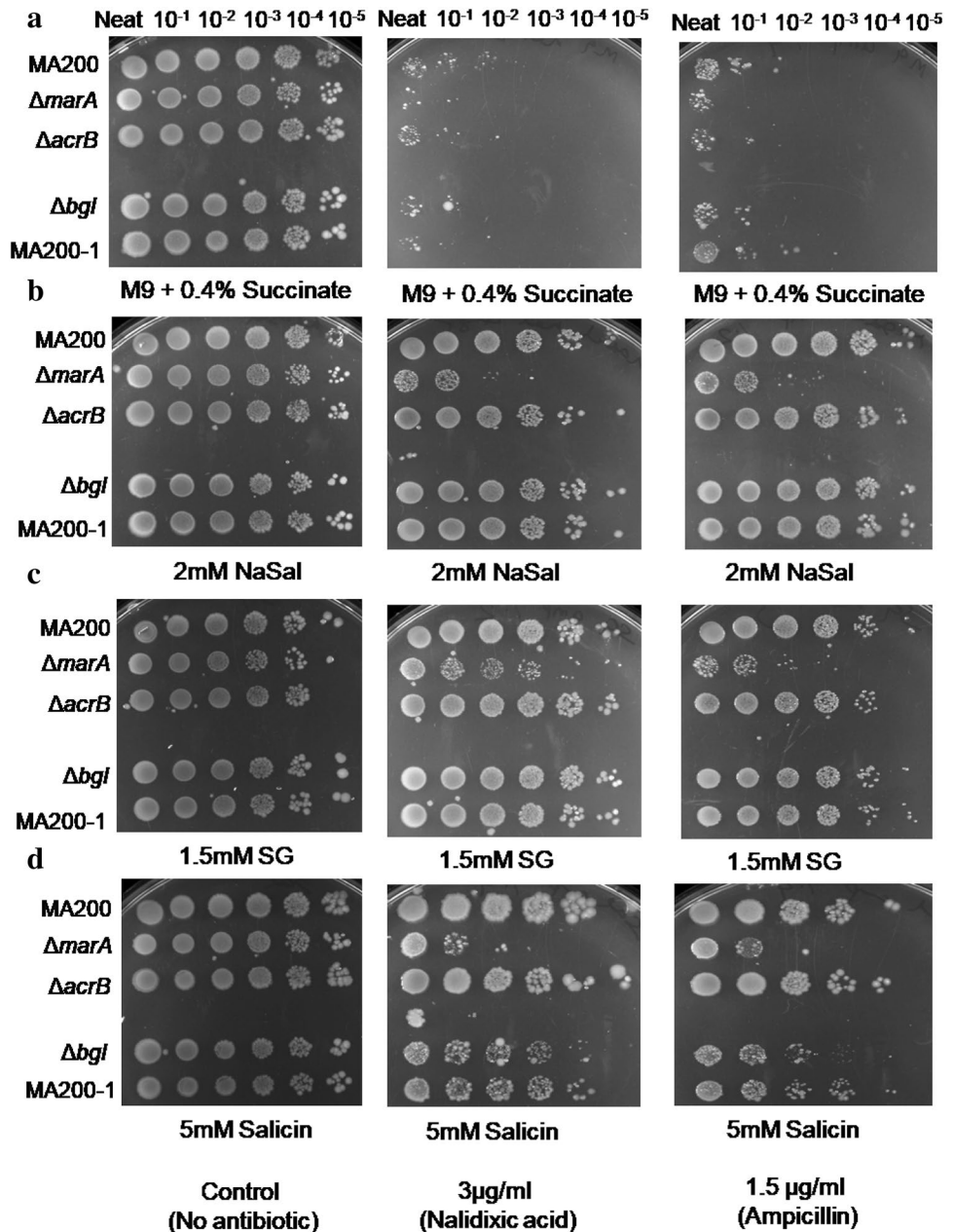
tolC genes (Fig. 1a), we presumed that the AcrAB–TolC efflux pump could be playing an important role in antibiotics resistance. AcrAB–TolC multidrug efflux pump, a member of RND superfamily (Resistance–Nodulation–Division) (Saier et al. 1994; Tseng et al. 1999), has been shown to be involved in antimicrobial resistance in bacterial pathogens (Poole 2004; Piddock 2006). It has also been observed earlier that AcrAB–TolC efflux pump is over-expressed in clinical isolates of *E. coli* in spite of the presence of other Acr pumps (Mazzariol et al. 2000). To test the possible role of AcrAB–TolC efflux pump in the resistance phenotype, we constructed an *acrB* deletion strain in MA200 background. Surprisingly, the patterns of growth in the presence of NaSal and SG were similar (Fig. 2b, c) in the case of MA200 and MA200Δ*acrB* indicating that AcrAB–TolC efflux pump is not solely responsible for the MarA-dependent phenotypic antibiotic resistance in Bgl⁺ bacteria. This was consistent with a previous report by Shuster et al. where they have demonstrated that in the absence of AcrB, other TolC-dependent

MDTs (Multidrug Transporters) provide necessary support in the acquisition of high-level antibiotic resistance (Shuster et al. 2016).

To determine the involvement of other multidrug transporters, we estimated the transcript levels of *acrD, acrF, mdtF*, and *macB* by qRT-PCR using gene-specific primers. *acrD* codes for a multidrug efflux pump RND permease AcrD, a homolog of AcrB (Ma et al. 1994). AcrF, a key component of AcrEF–TolC efflux pump, is not highly expressed in wild-type *E. coli* strains (Ma et al. 1994). MdtF, an inner membrane subunit of MdtEF–TolC efflux pump, belongs to the RND superfamily. *macB* codes for the ATPase subunit of an ABC-type tripartite MacAB–TolC efflux pump. All these TolC-dependent multidrug efflux pumps have been shown to be involved in antibiotic resistance. Results of quantitative RT-PCR using RNA isolated from exponential phase cultures of MA200Δ*acrB* confirmed 1.5–2-fold higher expression levels of *marA, acrD, acrF*, and *mtdF* in the presence of the aromatic compounds (Fig. 3). However, the transcript

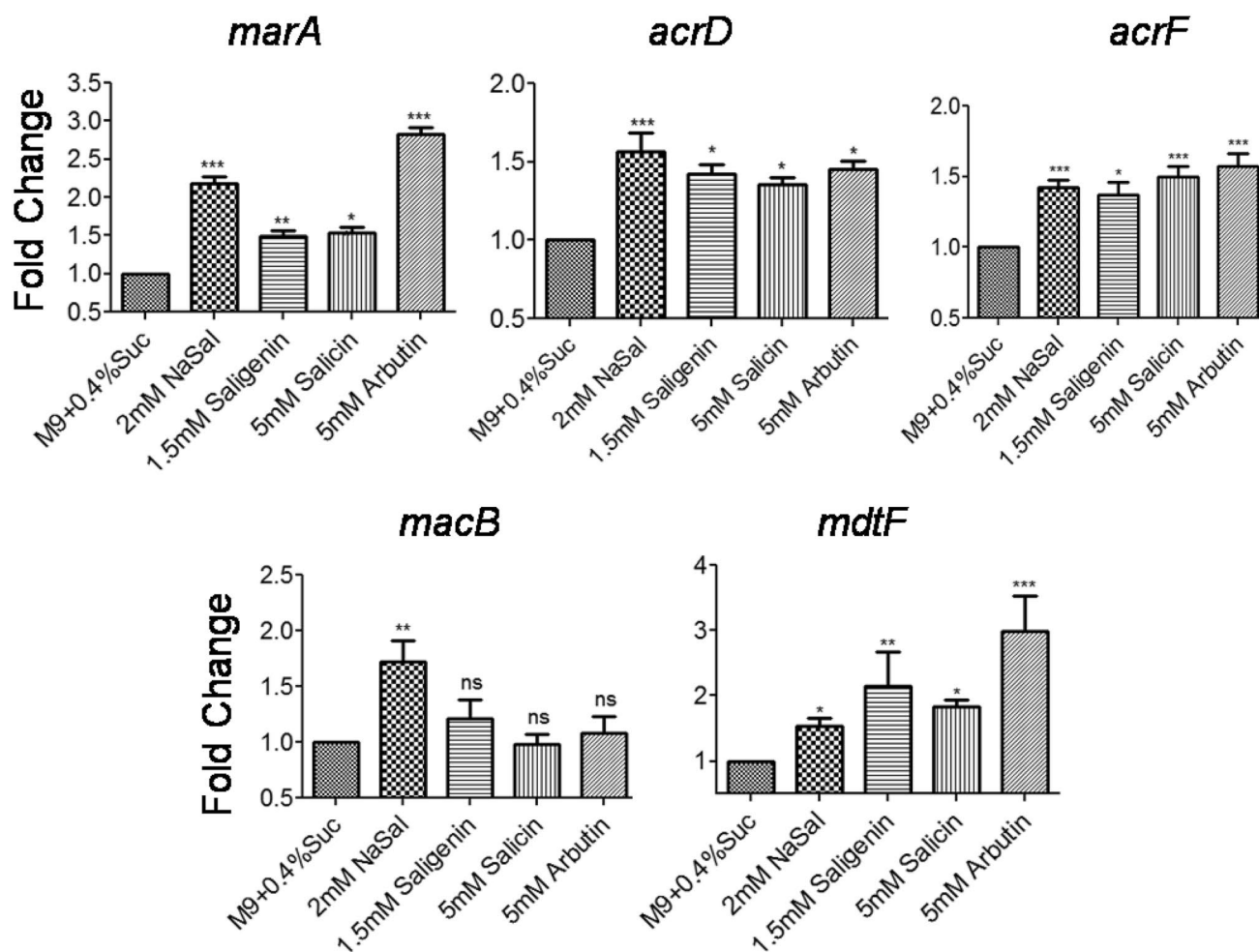**Fig. 3** Involvement of multi-drug transporters in PAR. qRT-PCR analysis of *marA, acrD, acrF, macB*, and *mdtF* in MA200Δ*acrB* upon exposure to growth conditions indicated. Mean ± SEM is plotted. ($n^* = 3$, ANOVA, $p < 0.001 = ***$, $p < 0.01 = **$, $p < 0.05 = *$ $p > 0.05 = ns$)

levels of *macB* were unaffected in the presence of SG, salicin and arbutin indicating that *macB* is not involved in the phenomenon. These results suggest the involvement of other multidrug transporters apart from AcrAB–TolC in phenotypic antibiotic resistance in Bgl[+] bacteria.

Since TolC serves as a common outer membrane channel for all the tested efflux pumps, its involvement was tested. To investigate its role, *tolC* was deleted in MA200 strain background and growth of the mutant strain was monitored as before. Growth of this strain was significantly reduced upon exposure to 3 µg/ml of nalidixic acid or 1.5 µg/ml of ampicillin even in the presence of the aromatic compounds (Fig. 4). Therefore, the PAR observed in the Bgl[+] strain is through over-expression of TolC-dependent efflux pumps.

Quantification of PAR using EzyMIC strip assay

To estimate the level of PAR in Bgl[+] bacteria, we performed EzyMIC strip-based assays using the *E. coli* strain MA200 and two natural isolates of *Klebsiella* (strains OK8 and ANK). The objective was to determine the alterations in the MIC for antibiotics when Bgl[+] bacteria were exposed to aromatic compounds. MA200 grown in the presence of salicin or arbutin showed an increase in MIC of antimicrobial agents (Table 3). MIC for the tested antimicrobial compounds were two–threefold higher when MA200 was exposed to NaSal (positive control) and SG (aromatic component of salicin) compared to control. Both the *Klebsiella* strains OK8 and ANK were found to be resistant to

Fig. 4 Role of *tolC* in PAR Growth assays of the *tolC* deletion strain MA200Δ*tolC* in the presence/absence of antibiotics. **a** M9 succinate agar (control). **b**, **c**, **d**, **e** M9 succinate agar containing NaSal, SG, salicin, and arbutin, respectively. Representative image of $n* = 3$ biological replicates
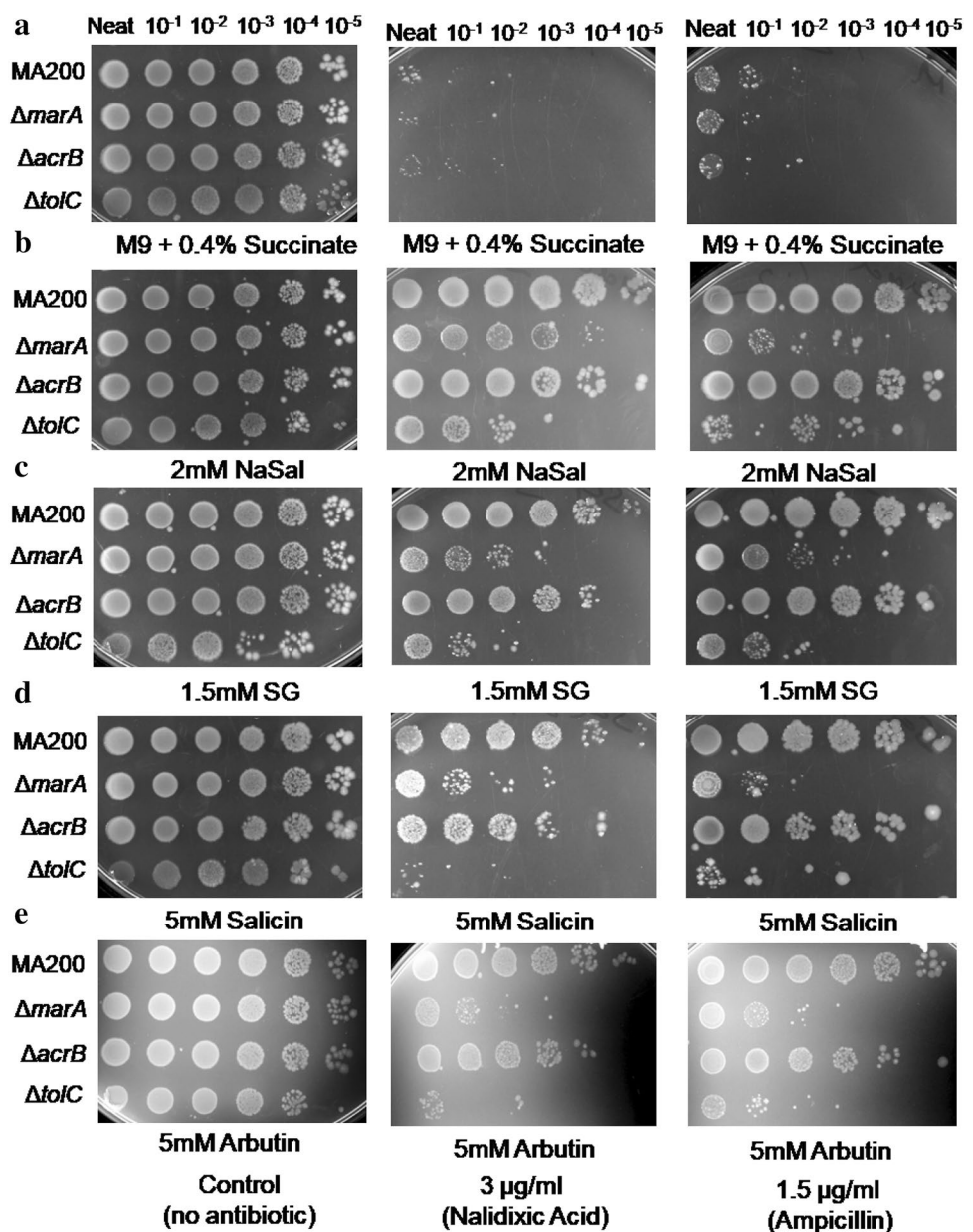
Table 3 Ezy MIC strip-based assay ($n* = 3$, mean ± SEM). Both the *Klebsiella* strains were found to be intrinsically resistant to ampicillin, oxacillin, and trimethoprim

Bacterial strains	M9 succinate (μg/ml)	2 mM Nasal (μg/ml)	1.5 mM SG (μg/ml)	5 mM salicin (μg/ml)	5 mM arbutin (μg/ml)	Antibiotics
E. coli. MA200	2.28 ± 0.1	5.14 ± 0.4	5.14 ± 0.34	3.71 ± 0.18	3.92 ± 0.07	Nalidixic acid
K. pneumoniae OK8	2.35 ± 0.09	4.28 ± 0.28	4	4 ± 0.1	7.71 ± 0.18	Nalidixic acid
Klebsiella ANK	5 ± 1	8	7.5 ± 0.5	7.5 ± 0.5	4.5 ± 0.5	Nalidixic acid
K. pneumoniae OK8	Sensitive	0.0266	0.016	0.042	0.084	Ciprofloxacin
Klebsiella ANK	0.0206	0.029	0.026	0.029	0.042	Ciprofloxacin
E. coli. MA200	1	2.33 ± 0.33	2.33 ± 0.33	2	1.9 ± 0.83	Ampicillin
E. coli. MA200	0.19	1.125 ± 0.21	0.7825 ± 0.14	0.9375 ± 0.06	0.375 ± 0.07	Trimethoprim

ampicillin and trimethoprim (MIC < 256 μg/ml and 32 μg/ml, respectively). OK8 showed a twofold increase in MIC for nalidixic acid when growth medium contained NaSal, SG, or salicin as compared to minimal medium (Table 3). A fourfold increase in MIC for nalidixic acid was observed in the presence of arbutin. Upon exposure to ciprofloxacin, OK8 was completely sensitive when grown in control medium (M9 succinate), but the strain was able to resist the antibiotic when supplemented with any of the compounds NaSal, SG, arbutin, or salicin (Table 3). Similar results were observed in the natural isolate *Klebsiella* ANK. Approximately, 1.5–1.6-fold increase in MIC of nalidixic acid was observed in the presence of NaSal, SG, and salicin. For unknown reasons, MIC was found to be slightly lower (4.5 ± 0.5 μg/ml) in the case of the ANK strain when exposed to arbutin as compared to control (5 ± 1 μg/ml). However, in the presence of ciprofloxacin, we observed an increase of ≈ 1.5- and 2-fold for the ANK strain upon exposure to NaSal, SG, salicin, and arbutin.

Effect of PAR on the emergence of genetic resistance in Bgl⁺ bacteria

Earlier studies have shown an enrichment of genetically resistant bacteria upon exposure to sub-MIC concentrations of antibiotics (Gullberg et al. 2011; Liu et al. 2011; Andersson and Hughes 2012, 2014; Sandegren 2014). Our observation of PAR in Bgl⁺ bacteria growing in the presence of aromatic β-glucosides and low doses of antibiotics raised the possibility of facilitation of the emergence of genetic resistance. To test this possibility, we designed an experimental set up (Fig. S2) under low drug pressure settings (0.3 X MIC) wherein bacterial growth is not attenuated. Multiple independent cultures of MA200 and MA200Δ*bgl* strains were pre-exposed to sub-inhibitory concentration of nalidixic acid (NAL, 1 μg/ml) and salicin (SAL, 5 mM) individually or in combination in M9 succinate medium. Nalidixic acid-resistant variants were selected on LB agar

plates containing higher concentrations of nalidixic acid (4, 8, 16, 32 μg/ml) and the fraction of resistant mutants was calculated. Both the strains showed similar frequency of mutants upon selection on nalidixic acid plates when grown in the presence of either NAL or SAL (Figs. S3 and S4). However, a significant increase in the frequency of mutants was observed in the case of the Bgl⁺ strain MA200 (Fig. 5a) grown in the presence of NAL and SAL compared to the isogenic Bgl⁻ strain MA200Δ*bgl* (Fig. 5b). For example, in the case of MA200, 17/20 and 15/20 cultures showed resistance to 8 and 16 μg/ml of nalidixic acid, respectively (Fig. 5c). A significant reduction in frequency of mutants was detected in the case of the Bgl⁻ strain MA200Δ*bgl* (Fig. 5c). For instance, only 7/20 and 2/20 cultures showed resistance to 8 and 16 μg/ml of nalidixic acid, respectively. Similar results were obtained when strains were pre-exposed to 2 μg/ml nalidixic acid (data not shown). These findings indicate that the presence of the activated form of the *bgl* operon in the genome facilitates the survival of bacteria in environments in which both aromatic β-glucosides and antibiotics are present by inducing PAR and also by enhancing the emergence of antibiotic resistant mutants.

Confirmation of genetic resistance in Bgl⁺ mutant isolates

Detection of nalidixic acid resistant colonies on selection media at higher antibiotic concentrations (16 and 32 μg/ml) seen in the previous section can be explained only by invoking genetic resistance. To verify this, randomly isolated colonies from selection plates used in previous experiment were grown in L broth in the absence of antibiotic and plated on medium containing increasing concentrations of nalidixic acid. For instance, colonies isolated from selection plate containing 8 μg/ml of nalidixic acid were exposed to 16, 32, 64 and 128 μg/ml of nalidixic acid along with the parent strain MA200. Growth assays (Fig. 6a) showed that randomly picked isolates from selection plates containing
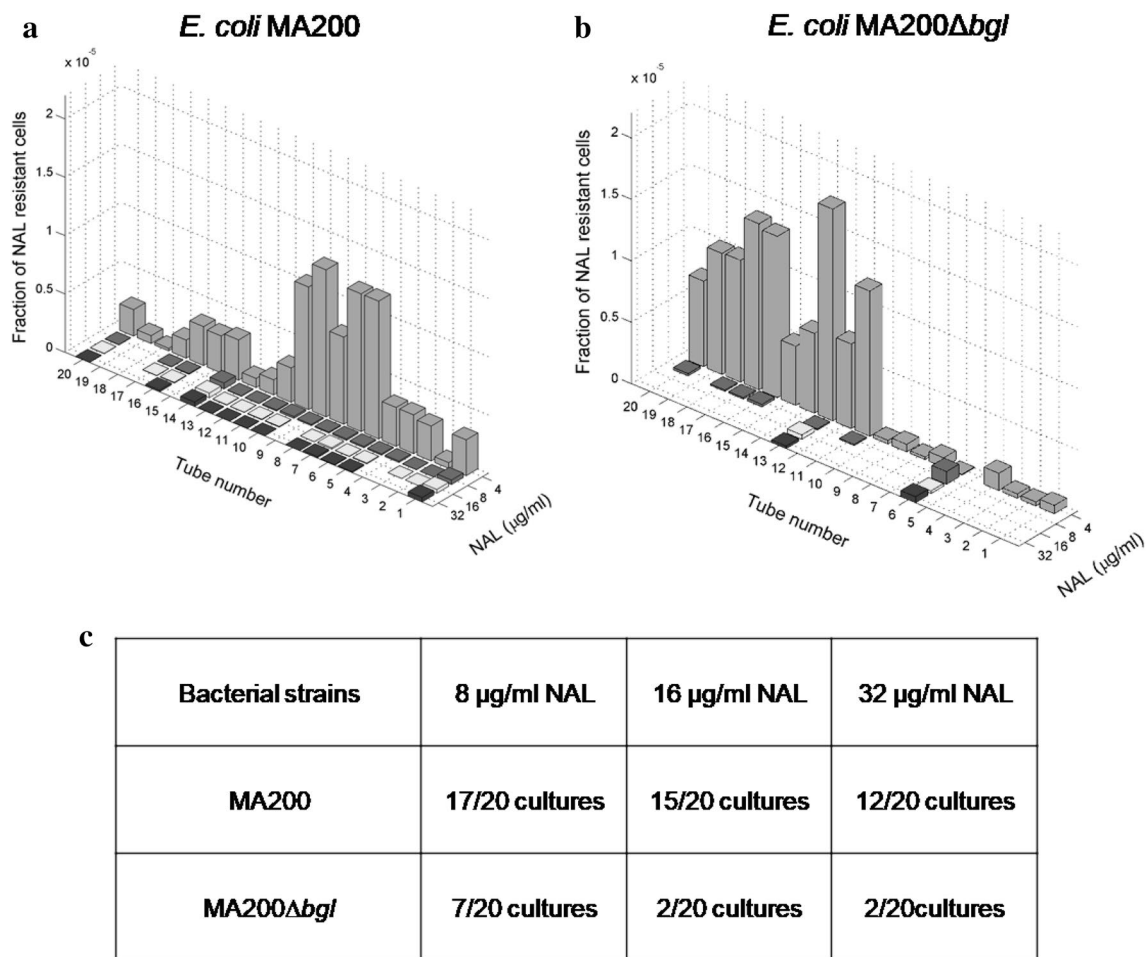
Fig. 5 Emergence of genetic mutants in a Bgl+ strain in the presence of salicin and low drug concentrations (**a**) Frequency of nalidixic acid resistant mutants obtained when strain MA200 was exposed to M9 succinate + 5 mM SAL + 1 µg/ml NAL. **b** Frequency of mutants when strain MA200Δ*bgl* was exposed to similar culture condition. **c** Tabular summary of frequency of mutants at each concentration. X axis denotes individual culture tube number, Y axis denotes fraction of nalidixic acid resistant mutants normalized to LB control for individual culture tube (height of the bars), Z axis denotes nalidixic acid concentration used for selection. Shades of the bars represent individual concentration used for selection viz. 4, 8, 16, and 32 µg/ml. Empty spaces without bar represent no growth
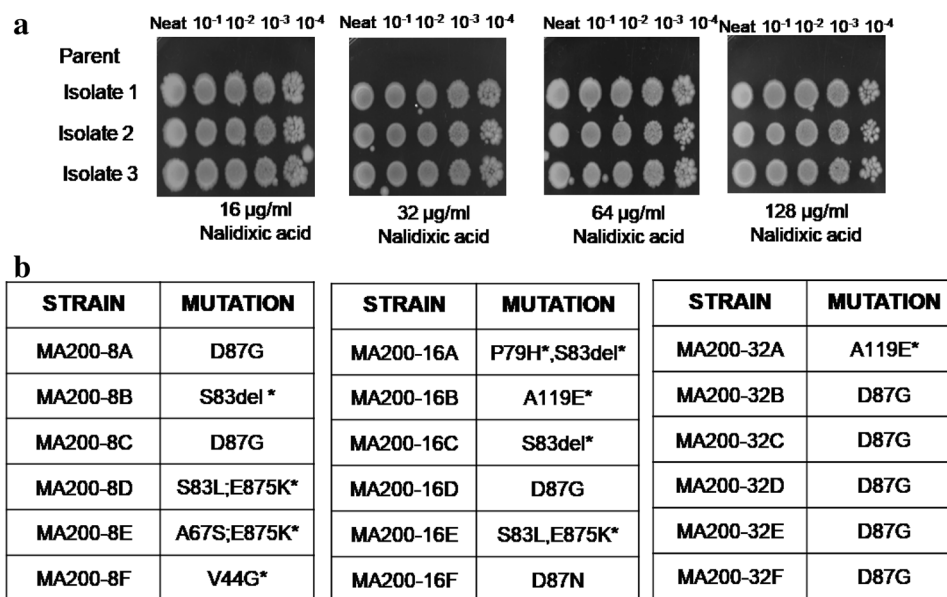
8 µg/ml of nalidixic acid can resist up to 128 µg/ml of nalidixic acid, while the parent showed sensitivity to even lower concentrations. Similar growth patterns were seen with colonies from the selection plates containing 16 and 32 µg/ml nalidixic acid (data not shown). Therefore, growth conditions promoting PAR can lead to the acquisition of mutations that can confer resistance higher concentration of antibiotics.

Characterization of Bgl+ nalidixic acid-resistant mutants

Since nalidixic acid belongs to the class of quinolones and is a known DNA gyrase inhibitor, we sequenced the *gyrA* locus of resistant strains using gene-specific primers. The *gyrA* subunit was amplified from 18 nalidixic acid resistant colonies which were randomly picked from selection

plates (6 colonies each that appeared on 8, 16, and 32 µg/ml of nalidixic acid, respectively). The DNA sequences of mutants were compared with *gyrA* sequence of the sensitive parent (MA200 Bgl+). Mutations in the *mar* locus and efflux pumps were ruled out as cross resistance to ampicillin in the isolated mutants was not observed (data not shown). Importantly, DNA sequencing results revealed that all the 18 nalidixic acid-resistant colonies carried mutations in their *gyrA* locus (Fig. 6b). Most of the mutations were found in the QRD region (quinolone resistance determining region; amino acids 67–106) of GyrA subunit. Upon quantifying the resistance against nalidixic acid, it was observed that the mutants show MIC > 16 µg/ml of nalidixic acid (Ezy MIC strip assay). Some of the mutants were completely resistant to the highest concentration used (> 256 µg/ml of nalidixic acid). All the mutants were also able to resist ciprofloxacin

Fig. 6 Characterisation of the Nal^R mutants **a**, Confirmation of heritable resistance by streaking randomly chosen colonies that appeared on selection plates containing 8 µg/ml of nalidixic acid on plates with higher concentrations of nalidixic acid (16, 32, 64, and 128 µg/ml). **b** List of mutations found in *gyrA* locus of 18 randomly isolated colonies from selection plates

STRAIN	MUTATION
MA200-8A	D87G
MA200-8B	S83del *
MA200-8C	D87G
MA200-8D	S83L;E875K*
MA200-8E	A67S;E875K*
MA200-8F	V44G*

STRAIN	MUTATION
MA200-16A	P79H*,S83del*
MA200-16B	A119E*
MA200-16C	S83del*
MA200-16D	D87G
MA200-16E	S83L,E875K*
MA200-16F	D87N

STRAIN	MUTATION
MA200-32A	A119E*
MA200-32B	D87G
MA200-32C	D87G
MA200-32D	D87G
MA200-32E	D87G
MA200-32F	D87G

(* Indicates novel mutations)

as opposed to the parent which was completely sensitive (Table S1). A variety of mutations which include transitions, transversions and deletions were found (Table S1). Interestingly, several mutations viz. S83del, E875K, V54G, P79H, and A119E were novel, and not reported in literature earlier. We also identified various combinations of mutations like S83L and E875K, A67S and E875K, P79H and S83del in the isolates.

Absence of fitness cost in the isolated mutants

Acquisition of genetic antibiotic resistance is often associated with compromised fitness due to the impact of the mutations on the function of the target genes (Björkman and Andersson 2000; Hughes and Andersson 2015; Melnyk et al. 2015). To observe whether there is a fitness cost associated with antibiotic resistance in the mutants described above, exponential phase competition experiments were performed. Three sets of competitions were performed wherein MA200-8A (D87G), MA200-16F (D87N), and MA200-8D (S83L, E875K) were competed against MA200 (parent) in M9 succinate medium. Results indicate the absence of attenuated growth in the case of MA200-8D (S83L, E875K) when competed with the parent MA200 in exponential phase (Fig. 7a) in the absence of drug. The ratios of final versus initial viable cell count of both the strains were similar (Fig. 7b) confirming that Bgl^+ nalidixic acid-resistant mutant MA200-8D does not suffer a fitness cost. None of the tested mutants viz. MA200-8A (D87G) and MA200-16F (D87N) showed reduction in growth during co-culture experiments (Fig. S5).
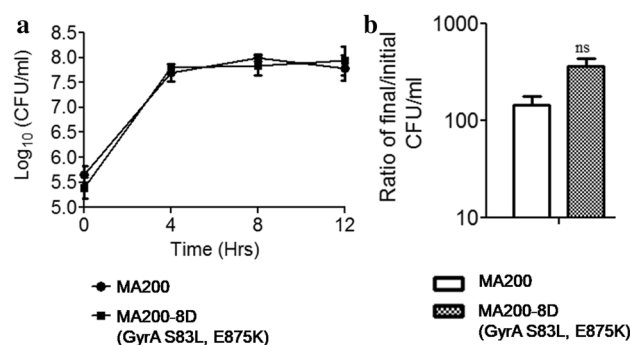

Fig. 7 Absence of cost of resistance in the mutants **a**, Competition in the exponential phase between the parent MA200 and the mutant MA200-8D mixed in 1:1 ratio. **b** The respective ratio of final verse initial CFU/ml. ($n* = 3$)

Discussion

The existence of cryptic or silent genetic elements such as the *bgl* operon of *Escherichia coli* is an evolutionary enigma because the *bgl* operon is maintained as an integral part of the genome over the course of evolution despite the fact that it is silent in most wild isolates. Specific growth conditions such as the presence of plant-derived aromatic β-glucosides can lead to the selection of the active state brought about by a single mutational event. The activated operon has been shown to provide additional fitness advantages under environmental conditions such as stationary phase and the presence of predators (Madan et al. 2005; Sonowal et al. 2013) indicating its highly dynamic role in

survival. In the light of these observations it was important to explore other physiologically relevant conditions where the presence of an activated copy of the allele is beneficial to the bacterium.

The present study directed at the role of the *bgl* operon in conferring PAR was initiated based on the possibility that the release of the aglycones saligenin and hydroquinone upon the breakdown of salicin and arbutin by Bgl+ bacteria can derepress the *marRAB* operon. In support of this, growth assays revealed that laboratory strains of *E. coli* and natural isolates of *Klebsiella* carrying inducible and constitutive forms of the *bgl* operon, respectively, were able to resist sub-lethal doses of nalidixic acid and ampicillin upon exposure to NaSal, SG, salicin and arbutin. This finding also confirmed the role of MarA as loss of the phenotype was observed in the Δ*marA* strain. *E. coli* strains deleted for the *bgl* operon or deficient for transport and hydrolysis functions failed to show the phenotype in the presence of salicin or arbutin (data not shown) confirming the role of the activated *bgl* operon in the phenotype. The underlying mechanism for conferring PAR was suspected to be the increase in efflux due to enhanced transcript levels of the transcription factor MarA and its downstream targets *acrB* and *tolC* (AcrAB–TolC efflux pump) in the Bgl+ strain MA200 grown in the presence of aromatic compounds. Although earlier reports showed the enhanced expression of AcrAB–TolC efflux pump in *E. coli* clinical isolates (Mazzariol et al. 2000; Oethinger et al. 2000; Webber and Piddock 2001), interestingly, deletion of *acrB* in MA200 background did not affect the ability to resist the antibiotics. This suggested the involvement of additional Multi Drug Transporters (MDTs) of *E. coli* in conferring the phenotype in Bgl+ bacteria as a recent report (Shuster et al. 2016) also demonstrated the presence of a multidrug transporter interactome in *E. coli*. Consistent with the hypothesis transcript levels of other TolC-dependent efflux pumps, i.e., *acrF*, *acrD*, and *mdtF* were approximately 1.5–2-fold higher in the Δ*acrB* strain upon exposure to aromatic compounds in the growth medium. Since the identity of the efflux pump involved was unclear, deletion of the common transporter *tolC* was created in the Bgl+ background. The Δ*tolC* strains showed highly attenuated growth in the presence of nalidixic acid and ampicillin. These findings indicate that the function of AcrB is dispensable in specific environmental conditions; in the absence of AcrB, additional MDTs present can take over and provide necessary support for conferring the phenotype.

Upon quantification of PAR, an increase of two–three-fold MIC for nalidixic acid, ampicillin and trimethoprim was observed in the case of *E. coli* MA200. The pathogenic isolate of *Klebsiella pneumoniae* OK8 showed four-fold increase in MIC when exposed to arbutin. This strain was completely sensitive to ciprofloxacin when grown in minimal medium; however in the presence of NaSal, SG,

salicin and arbutin, low level of resistance was observed further strengthening the initial observations. In the case of another natural sewage isolate of *Klebsiella* ANK, a similar phenomenon was observed upon exposure to both antibiotics and inducers (NaSal, SG, salicin and arbutin). Both *Klebsiella* strains were found to be intrinsically resistant to ampicillin, trimethoprim, and oxacillin.

Globally, extensive usage of several antibiotics in various sectors (medical, animal husbandry, agriculture etc.) in an unregulated manner has led to a widespread distribution of several antibiotics in concentrations ranging from nanogram to microgram (Baquero et al. 2008; Fram and Belitz 2011; Jiang et al. 2013; Khan et al. 2013; Andersson and Hughes 2014). Furthermore, exposure to sublethal doses has been shown to facilitate the rise/enrichment of genetically resistant populations under laboratory settings (Gullberg et al. 2011; Liu et al. 2011). As the presence of phenotypic antibiotic resistance and the possible mechanism involved could be demonstrated in Bgl+ strains which are actively metabolizing salicin or arbutin, it was important to explore the possibility of emergence of genetic resistance triggered by phenotypic resistance at sub-lethal concentrations of the antibiotics. Towards this end, Bgl+ (MA200) and Bgl− (MA200Δ*bgl*) strains were cultured under three different growth conditions (combinations of M9 succinate, salicin and sublethal dose of nalidixic acid). A remarkable increase in the frequency of mutants that show nalidixic acid resistance was observed when Bgl+ bacteria (MA200) were exposed to salicin and sub-lethal concentration of nalidixic acid as compared to the Bgl− strain (MA200Δ*bgl*). However, the precise mechanism behind this observation is unknown. Interestingly, the frequency of mutants remained unaffected when cells were pre-exposed either to salicin or sub-lethal dose of nalidixic acid individually. In conclusion, these results suggest that in environments where aromatic -glucosides and low amount of antibiotics are present, bacteria harboring a functional *bgl* operon stand a better chance of survival as opposed to bacteria having the silent counterpart.

To characterize the nalidixic acid-resistant mutants further, the nucleotide sequence of the *gyrA* locus of 18 randomly isolated mutants was analysed as mutations in *gyrA* are more common in clinical isolates of quinolone resistant *E. coli* (Nakamura et al. 1989). The sequencing data revealed that indeed all the 18 mutants carried mutations in the *gyrA* locus and most of them were located in the QRDR—quinolones resistance determining region—spanning the amino acids 67–106 (Yoshida et al. 1990). Both single and combinations of clinically relevant mutations (Lindgren et al. 2003) such as S83L, D87N, and D87G were observed. Some of the identified mutations like S83del, E875K, V54G, P79H, and A119E were novel, not reported in literature earlier. A significant increase in the MIC of both nalidixic acid

and ciprofloxacin was observed in the case of the characterized mutants.

Since antimicrobial resistance is often associated with a fitness cost in the absence of the drug because of the impact of the mutation on the functioning of the target gene or protein (Björkman and Andersson 2000; Hughes and Andersson 2015; Melnyk et al. 2015), it was possible that these mutants might carry a fitness cost of drug resistance. Exponential phase competition experiments between the sensitive parent and genetically resistant mutants indicated that the mutants do not carry a fitness cost imposed by resistance at least under the conditions tested. This observation is consistent with an earlier report which suggests that quinolone resistant mutations in *gyrA* along with other mutations in the genome do not exhibit a cost of resistance in *Streptococcus pneumoniae* (Gillespie et al. 2002). This can be a possible explanation for the prevalence of quinolone resistant pathogens in clinical settings (Redgrave et al. 2014). In the light of these observations, it is apparent that the presence of the *bgl* operon in its active form facilitates the enhanced survival of the bacteria in environments in which both aromatic β-glucosides and antibiotics are present, such as soil, thereby providing a strong selective force for its retention in the genome.

References

Andersson DI, Hughes D (2012) Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist Updat 15:162–172

Andersson DI, Hughes D (2014) Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12:465

Baquero F, Martínez J-L, Cantón R (2008) Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 19:260–265

Bhaskarla C, Das M, Verma T, Kumar A, Mahadevan S, Nandi D (2016) Roles of Lon protease and its substrate MarA during sodium salicylate-mediated growth reduction and antibiotic resistance in *Escherichia coli*. Microbiology 162:764–776

Björkman J, Andersson DI (2000) The cost of antibiotic resistance from a bacterial perspective. Drug Resist Updat 3:237–245

Cohen SP, Levy SB, Foulds J, Rosner JL (1993) Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. J Bacteriol 175:7856–7862

Corona F, Martinez J (2013) Phenotypic resistance to antibiotics. Antibiotics 2:237–255

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97:6640–6645

Fram MS, Belitz K (2011) Occurrence and concentrations of pharmaceutical compounds in groundwater used for public drinking-water supply in California. Sci Total Environ 409:3409–3417

Gillespie SH, Voelker LL, Dickens A (2002) Evolutionary barriers to quinolone resistance in *Streptococcus pneumoniae*. MDR 8:79–84

Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI (2011) Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 7:e1002158

Harwani D, Zangoui P, Mahadevan S (2012) The β-glucoside (*bgl*) operon of *Escherichia coli* is involved in the regulation of *oppA* encoding an oligo-peptide transporter. J Bacteriol 194:90–99

Hughes D, Andersson DI (2015) Evolutionary consequences of drug resistance: shared principles across diverse targets and organisms. Nat Rev Genet 16:459

Jiang L, Hu X, Xu T, Zhang H, Sheng D, Yin D (2013) Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. Sci Total Environ 458:267–272

Khan GA, Berglund B, Khan KM, Lindgren P-E, Fick J (2013) Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities—a study in Pakistan. PLoS One 8:e62712

Lai X, Davis F, Hespell R, Ingram L (1997) Cloning of cellobiose phosphoenolpyruvate-dependent phosphotransferase genes: functional expression in recombinant *Escherichia coli* and identification of a putative binding region for disaccharides. Appl Environ Microbiol 63:355–363

Levy SB (1992) The antibiotic paradox: from tragedy the antibiotic age is born. Springer, Berlin.

Lindgren PK, Karlsson Å, Hughes D (2003) Mutation rate and evolution of fluoroquinolone resistance in *Escherichia coli* isolates from patients with urinary tract infections. Antimicrob Agents Chemother 47:3222–3232

Liu A, Fong A, Becket E, Yuan J, Tamae C, Medrano L, Maiz M, Wahba C, Lee C, Lee K (2011) Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. Antimicrob Agents Chemother 55:1204–1210

Lopilato J, and Wright A (1990) Mechanisms of activation of the cryptic *bgl* operon of *Escherichia coli* K-12. In: Drlica K, Riley M (eds) The bacterial chromosome. American society for microbiology, Washington, DC, pp 435–444

Ma D, Cook DN, Hearst JE, Nikaido H (1994) Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol 2:489–493

Madan R, Kolter R, Mahadevan S (2005) Mutations that activate the silent *bgl* operon of *Escherichia coli* confer a growth advantage in stationary phase. J Bacteriol 187:7912–7917

Mahadevan S, Reynolds A, Wright A (1987) Positive and negative regulation of the *bgl* operon in *Escherichia coli*. J Bacteriol 169:2570–2578

Mazzariol A, Tokue Y, Kanegawa TM, Cornaglia G, Nikaido H (2000) High-level fluoroquinolone-resistant clinical isolates of *Escherichia coli* overproduce multidrug efflux protein AcrA. Antimicrob Agents Chemother 44:3441–3443

Melnyk AH, Wong A, Kassen R (2015) The fitness costs of antibiotic resistance mutations. Evol Appl 8:273–283

Mukerji M, Mahadevan S (1997a) Characterization of the negative elements involved in silencing the *bgl* operon of *Escherichia coli*: possible roles for DNA gyrase, H-NS, and CRP–cAMP in regulation. Mol Microbiol 24:617–627

Mukerji M, Mahadevan S (1997b) Cryptic genes: evolutionary puzzles. J Genet 76:147–159

Nakamura S, Nakamura M, Kojima T, Yoshida H (1989) *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob Agents Chemother 33:254–255

Oethinger M, Kern WV, Jellen-Ritter AS, McMurry LM, Levy SB (2000) Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. Antimicrob Agents Chemother 44:10–13

Piddock LJ (2006) Multidrug-resistance efflux pumps? Not just for resistance. Nat Rev Microbiol 4:629

Poole K (2004) Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect 10:12–26

Price CT, Lee IR, Gustafson JE (2000) The effects of salicylate on bacteria. Int J Biochem Cell Biol 32:1029–1043

Raghunand TR, Mahadevan S (2003) The β-glucoside genes of *Klebsiella aerogenes*: conservation and divergence in relation to the cryptic *bgl* genes of *Escherichia coli*. FEMS Microbiol Lett 223:267–274

Redgrave LS, Sutton SB, Webber MA, Piddock LJ (2014) Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. Trends Microbiol 22:438–445

Reynolds AE, Felton J, Wright A (1981) Insertion of DNA activates the cryptic *bgl* operon in *E. coli* K12. Nature 293:625–629

Reynolds AE, Mahadevan S, LeGrice SF, Wright A (1986) Enhancement of bacterial gene expression by insertion elements or by mutation in a CAP-cAMP binding site. J Mol Biol 191:85–95

Rosner JL (1985) Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in *Escherichia coli* K-12. Proc Natl Acad Sci USA 82:8771–8774

Saier M Jr, Tam R, Reizer A, Reizer J (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 11:841–847

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold spring harbor press, Cold spring harbor, New york

Sambrook J, Russell DW, and Maniatis T (2001). Molecular cloning, vol 1–3. Cold Spring habour laboratory press, New York.

Sandegren L (2014) Selection of antibiotic resistance at very low antibiotic concentrations. Ups J Med Sci 119:103–107

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C_T method. Nat Protoc 3:1101

Schnetz K (1995) Silencing of *Escherichia coli bgl* promoter by flanking sequence elements. EMBO J 14:2545–2550

Schnetz K, Rak B (1992) IS5: a mobile enhancer of transcription in *Escherichia coli*. Proc Natl Acad Sci USA 89:1244–1248

Shuster Y, Steiner-Mordoch S, Cudkowicz NA, Schuldiner S (2016) A transporter interactome is essential for the acquisition of antimicrobial resistance to antibiotics. PLoS One 11:e0152917

Singh J, Mukerji M, Mahadevan S (1995) Transcriptional activation of the *Escherichia coli bgl* operon: negative regulation by DNA structural elements near the promoter. Mol Microbiol 17:1085–1092

Sonowal R, Nandimath K, Kulkarni SS, Koushika SP, Nanjundiah V, Mahadevan S (2013) Hydrolysis of aromatic β-glucosides by nonpathogenic bacteria confers a chemical weapon against predators. Proc R Soc B 280:20130721

Tseng T-T, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier MH Jr (1999) The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol 1:107–125

Webber MA, Piddock LJ (2001) Absence of mutations in *marRAB* or *soxRS* in *acrB*-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of *Escherichia coli*. Antimicrob Agents Chemother 45:1550–1552

Yoshida H, Bogaki M, Nakamura M, Nakamura S (1990) Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob Agents Chemother 34:1271–1272