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The 2019 Garrod Lecture: MDR Efflux in Gram-negative bacteria – how understanding resistance led to a new tool for drug discovery

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Abstract (210 words)

The AcrAB-TolC multidrug (MDR) efflux system confers intrinsic multidrug resistance and overproduction confers clinically relevant resistance to some antibiotics active against Gram-negative bacteria. The system is made up of three components, namely AcrA, AcrB and TolC otherwise known as the AcrAB-TolC tripartite system. Inactivation or deletion of a gene encoding one of the constituent proteins, or substitution of a single amino acid substitution in the efflux pump component, AcrB that results in loss of efflux function, confers increased antibiotic susceptibility. Clinically relevant resistance can be mediated by a mutation in AcrB which changes the way its substrates are transported. However, it is more common that resistant clinical and veterinary isolates overproduce the AcrAB-TolC MDR efflux system. This is due to mutations in genes such as *marR* and *ramR* that encode repressors of transcription factors (MarA and RamA, respectively) that when produced activate expression of the *acrAB* and *tolC* genes thereby increasing efflux. The Lon protease degrades MarA and RamA to return the level of efflux to that of the wild type. Furthermore, the levels of AcrAB-TolC are regulated by the CsrA. Studies with fluorescent reporters that report levels of *acrAB* and regulatory factors allowed the development of a new tool for discovering efflux inhibitors. Screens of the Prestwick library and a large library from a collaborating pharmaceutical company has generated a number of candidate compounds for further research.

Introduction

Multi-drug resistance (MDR) efflux pumps are proteins that are found in all types of cells including mammalian cells. They transport many different molecules (substrates) and some pumps transport antibiotics. The energy source to translocate molecules can be driven by ATP, the proton motive force (PMF) or another electrochemical gradient (e.g. Na+/K+). Irrespective of this, an efflux pump reduces the concentration of a substrate accumulated within the cell by exporting it from the intracellular to the extracellular environment.

There are many different types of MDR efflux pumps and they transport different types of molecules.[1, 2] Some pumps can be very specific, for instance the tetracycline efflux pumps, and others can transport a wide variety of drugs; the latter are termed MDR efflux pumps. For bacteria, about 10% of the bacterial genome encodes transporters, of which a third are estimated to be MDR efflux pumps. It is important to note that not only can the genes coding for efflux pumps be on the bacterial chromosome (and so present in wildtype and resistant bacteria), but some genes are encoded on transmissible elements and so can be shared between bacteria.

There are several MDR efflux pumps that cause resistance to clinically useful drugs (Figure 1). In Gram-positive bacteria, efflux pumps transfer drugs from the inside of the cell to the outside of the cell. In Gram-negative bacteria, the cell envelope is much more complex; it has a double membrane and therefore the efflux pump protein needs to link to two other types of proteins to export its substrates to the outside of the cell. These three component "machines" comprise dimers or trimers of an efflux pump protein, with periplasmic adapter proteins and an outer membrane protein (Figure 2). Different pump proteins can have differing or overlapping substrate profiles. The most clinically important MDR efflux pump in Gram-negative bacteria is the AcrAB-TolC tripartite system and its homologues of the Resistance

Nodulation Division (RND) class in others species e.g. the *Pseudomonas aeruginosa* Mex system, or *Campylobacter* CmeABC system.[3]

The AcrB protein usually captures its substrates from the periplasm and can be described in simple terms as a bacterial 'vacuum cleaner' that removes noxious substances from the bacterial cell. The substrate is then transported via AcrB to TolC and finally expelled to the extracellular environment. AcrA is required for this process.[4] Besides antibiotics, substrates include non-steroidal anti-inflammatory and anticholinergic drugs.[5] Indeed, it is extremely difficult to find a molecule that AcrB does not export. However, if any of the genes encoding the components of the tripartite system are deleted or inactivated, the bacterium becomes more susceptible to many different types of substrates, including antibiotics, dyes, disinfectants and detergents (Table 1; Figure 3). It is not just antibiotics that are exported, substrates also including non-steroidal anti-inflammatory and anticholinergic drugs.[5] Indeed, it is extremely difficult to find a molecule that AcrB does not export.

Role of MDR efflux pumps in intrinsic antibiotic resistance

Over the last 20 years, there has been ample evidence to show that lack of a component of a RND efflux pump confers multidrug susceptibility and increased intracellular concentrations of those substrates. [6, 7] These data show that not only does the AcrB pump confer intrinsic MDR, but also provides evidence that this is why many drugs that have good activity against Gram-positive bacteria are ineffective against Gram-negative bacteria. Active efflux also underpins many types of antibiotic resistance and an intact AcrAB-TolC pump is required for clinically relevant levels of resistance due to other chromosomal mechanisms of antimicrobial resistance. For instance, in the absence of any component of the tripartite pump, mutations in topoisomerase genes do not confer clinically relevant levels of resistance to fluoroquinolones [8, 9] and mutations in *fab* genes do not confer triclosan resistance.[10] Likewise,

the same is true for some transferable drug resistances including those to florfenicol and tetracycline resistance encoded by *floR*, *tetA* and *tetG*, respectively [9, 11]. In the absence of AcrB, it is also very hard for bacteria to evolve drug resistance. [12]

For many years it has been accepted that inactivating or deleting AcrB equates with loss of efflux. However, until recently it was unclear as to whether the phenotype of multi-drug susceptibility was due to loss of a large integral membrane protein, or due to loss of efflux function. To explore this, a mutant *Salmonella* Typhimurium was constructed with a single amino acid substitution in AcrB (D408A); the mutant protein could not derive energy from PMF, because it could not translocate protons. The protein is inserted in the membrane, but it can no longer actively efflux substrates.[13] It was also less able to infect tissue culture cells and mice. The mutant's susceptibility to antibiotics, dyes and disinfectants was the same as an isogenic *acrB* deletion mutant (Table 1). Both mutants accumulated much higher levels of fluorescent dyes and antibiotics. However, in the absence of AcrB over half of the genes in the *Salmonella* genome had altered expression, but far fewer genes were altered when there was loss of efflux in the AcrB D408A mutant. Most importantly, when the AcrB protein is lacking, homologous RND efflux pumps such as AcrD and AcrF are produced.[7, 10] It has been proposed that this compensates for the lack of AcrB, not just in terms of providing structural integrity to the bacterial membrane, but also in the ability to maintain efflux, including of some antibiotics. In the loss of AcrB efflux function mutant (D408A), these proteins were not over-produced. In fact, AcrD and AcrF are produced at virtually undetectable levels, which means that they cannot compensate for loss of AcrB efflux function. [13] This is important because strategies to discover efflux pump inhibitors have assumed that inhibition of AcrB, AcrD and AcrF will be required; this may not be necessary.

MDR in clinical isolates can be due to a mutant efflux pump protein

We investigated a series of isolates from a single patient in which multi drug-resistant salmonellae evolved over a period of five months (Table 2). We showed that the post-therapy isolates over-expressed AcrB and accumulated low amounts of ciprofloxacin. Efflux is halted in the presence of carbonyl cyanide 3-chlorophenylhydrazone, an uncoupler that dissipates the proton motive force, as the amount of ciprofloxacin increased to that found in the pre-treatment isolate.[14] This shows that MDR can result from over-production of an efflux pump during treatment. Whole genome sequences of the MDR isolates also revealed a change in the AcrB protein; a single amino acid (glycine) was substituted with aspartate at position 288.[15] All MDR isolates obtained after 2 weeks treatment had this single change in AcrB whereas earlier isolates did not (Table 2). It was hypothesised that the G288D substitution altered the shape of the binding pocket of the AcrB protein, in particular the region that transports substrates. The amount of ciprofloxacin, doxorubicin and minocycline accumulated in laboratory constructed strains of *Escherichia coli* and *Salmonella typhimurium* with mutated AcrB was compared with the amount accumulated by the wildtype strains. Less ciprofloxacin accumulated in bacteria with AcrB G288D unlike doxorubicin and minocycline which accumulated to much higher concentrations. Therefore, the mutated AcrB exported ciprofloxacin better than wildtype protein, but export of some of the other substrates was worse and this was associated with lower MICs of these drugs. These data suggest that minocycline may have been a useful option to treat the disseminated salmonellosis which ultimately proved fatal.

Increased efflux confers resistance to anti-Gram-negative bacterial drugs

The OmpF protein in Enterobacteriales such as *E. coli* and *Salmonella* is a porin that selectively allows passive diffusion of molecules, including many antibiotics, based upon their size, shape and charge, so not all molecules on the outside of the Gram-negative bacterium gain entry.[16] Once the drug has entered the cell, much of it is exported from the

bacterium by the AcrAB-TolC MDR efflux system. [17] Therefore, the intracellular concentration is an equilibrium between drug influx and efflux. This, in turn, influences the amount of drug available to interact with its target. Bacteria have various levels of regulation of important gene products such as OmpF and AcrAB-TolC. There are master regulators and specific regulators that target promoter sequences of genes within their regulon to turn on or off the expression of those genes. Furthermore, once a gene is transcribed into RNA, the amount of RNA can also be controlled by post-transcriptional modification.[18] Finally, the amount of a protein in a bacterium can be adjusted by post-translational modification. [19]

Regulation of the AcrAB-TolC efflux system is complex. The *acrAB* genes are regulated locally by the AcrR protein.[20] Efflux-mediated resistance in clinical isolates is due to overproduction of efflux pumps rather than a mutant AcrB protein. [21] This is due to mutations in genes that regulate how much efflux pump is produced in the cell. [17] Early work in the 1990s on factors that regulate the production of AcrAB-TolC and focused on the *E. coli* MarA protein which is regulated by MarR. The MarA protein is over-produced if there is a mutation that prevents repression of *marA*. This, in turn, activates expression of *acrAB* and *tolC*. MarA also activates expression of an anti-sense RNA, *micF*, that interacts with *ompF* mRNA thereby preventing production of the OmpF protein. In this way, overproduction of MarA reduces influx and increases efflux to give increased MICs of antibiotics which are greater than those attributed to the innate level of MDR in *E.coli*. [21]

Increased efflux can be conferred via several different mechanisms

In *Salmonella, Klebsiella spp* and *Enterobacter spp* inactivation of *acrR* showed AcrR to be a weak regulator. [22, 23] Furthermore, in MDR clinical isolates and veterinary isolates with increased efflux of dyes and antibiotics such as fluoroquinolones, there was no increased expression of *marA*; instead there was overproduction of another gene, *ramA*. RamA is a

homologue of MarA and is from the same family of AraC/XylS regulators; [21] *ramA* is co-located on the genome with *ramR*. However, *E. coli* and *Shigella* spp do not possess *ramRA* genes. RamA is overproduced by mutations that prevent RamR binding and repressing expression of *ramA*. If the *ramR* gene is deleted or inactivated, RamA is over-produced resulting in MDR; [24] this is restored to wildtype by complementation with wildtype *ramR in trans*. The amount of RamA also influences the level of MDR. This was revealed by cloning *ramA* onto a plasmid where expression is inducible. [5] The level of RamA was also mirrored by an increase in AcrB production (Table 3). The amount of *ramA* can also vary in clinical isolates and is one of the reasons why the level of resistance due to overproduction of AcrAB-TolC can vary; different mutations influence the binding of RamR to its target DNA sequence and hence levels of RamA. [25, 26]

Whilst it may be evolutionarily advantageous to constitutively de-repress RamA to allow bacterial survival in the presence of an antibiotic, the RamRA locus is a classic inducible system whereby removal of an inducing condition should allow the amount of RamA to be restored to normal levels. To explore this further, the antipsychotic drug, chlorpromazine, was used as it induces *ramA* in a concentration-dependent manner. Chlorpromazine was removed after inducing the production of *ramA* and the amount of RamA was quickly restored to the pre-induction level with a half-life of 2 minutes. [27] It is assumed that high levels of RamA and consequent overexpression of AcrAB-TolC is harmful to the bacterium and so expression is reset to basal levels as soon as the inducer is removed. Further experimentation showed that, like MarA, [28] the levels of RamA are reset to pre-induction levels by post-translational regulation by the Lon protease that proteolytically degrades RamA. [27] This was shown by inactivating the *lon* gene and observing that *ramA* remained highly expressed.

To determine if anything other than RamR or Lon can regulate *ramA*/RamA an experiment was carried out using a library of *Salmonella typhimurium* in which every gene was inactivated by the random insertion of a transposon. A reporter of *ramA* production linked to that of a green fluorescent protein (GFP) was then introduced. After allowing sufficient growth, the bacterial population was sorted in a fluorescence activated cell sorter (FACS) based on the amount of fluorescence. [29] These populations were sub-cultured and confirmed to have different levels of fluorescence. Whole genome sequencing of representative strains from the populations was used to identify the gene into which the transposon had inserted. The transposon inserted itself into *csrA* gene which codes for a global regulator originally identified as regulating carbon storage. [29] After exhaustive experimentation to determine the mechanism of increased *ramA* expression, it was found that CsrA did not act on *ramA*, but on *acrAB*. In the absence of CsrA, the amount of *acrB* mRNA reduces very quickly indicating that CsrA is required to stabilise the *acrAB* transcripts. Without CsrA, the *acrAB* transcripts quickly degrade and so little AcrA or AcrB proteins are produced. It is hypothesised that the increased expression of *ramA* was in response to the reduced levels of AcrB.

A new tool for discovery of efflux inhibitors

Efflux is a very attractive target for antibacterial drug discovery and small companies have been carrying out such research for over 20 years. An efflux inhibitor will restore clinical effectiveness to currently available antibiotics, prevent many bacteria from infecting their host, and inhibit the evolution of drug resistance. There are various mechanisms by which an efflux inhibitor could work; it could interact directly with an efflux pump protein or another component of a tripartite system, inhibit production of AcrB (and/or its homologues), or prevent increased efflux by repressing the expression of transcription factors such as MarA and RamA. Unfortunately, despite the discovery of numerous efflux inhibitors, none have been licensed for clinical use. [30]

Review of the various inducers and conditions that induced *ramA* showed that exposure to phenothiazines, lack of AcrB, and efflux inhibitors, all induced *ramA* (Figure 4). [31] These data indicate that *ramA* transcription is sensitive to efflux inhibition. A tool for a high throughput screen for efflux inhibitors and counter-screens was constructed based on these findings. The drug discovery screening facility at the University of Birmingham has automated liquid handling equipment including a biochemical and phenotypic platform to screen for activity against intact bacterial cells (https://www.birmingham.ac.uk/facilities/bddf/index.aspx). Chlorpromazine was used as a positive control for the screen. Firstly, the Prestwick Chemical Library comprising a collection of 1,280 molecules containing mostly approved drugs (FDA, EMEA and other agencies) was screened after being selected for their high chemical and pharmacological diversity. Fifty molecules induced production of GFP of which 22 were not antibacterials (manuscript in preparation). Of the remaining 28 molecules, nine were known to have antimicrobial efflux inhibitory activity, five were known to be synergistic with antibacterials, one has been described as an antifungal efflux inhibitor, another is known to bind to RamR, and two have been described as antibacterial efflux inhibitors. The screen was then used with a library of 50,000 novel compounds provided by a collaborating pharmaceutical company. Just over 100 molecules increased GFP production, equating to a hit rate of 0.5% or 1 in 200. This is similar to that of some conventional high throughput antibacterial screens. Subsequent experiments demonstrated that some of the compounds behave as efflux inhibitors and synergise with antimicrobials (Piddock, unpublished data).

Concluding remarks

The AcrAB-TolC efflux system confers intrinsic MDR and the consequent overproduction confers resistance to antibacterials that are active against Gram-negative bacteria. Understanding mechanisms of resistance can inform drug discovery strategies. The evolution

of *acrB* in response to antimicrobial therapy of a patient was very different to that observed *in vitro* and revealed residues that were important for AcrB to transport its substrates. Such information could help drug discovery programmes as molecules that are effluxed less by both wildtype and mutant AcrB will attain high intracellular drug concentrations and exhibit greater antibacterial activity. MDR clinical isolates can have different drug-resistance mutations that have hitherto not been found in laboratory mutants. There are numerous factors that will regulate the AcrAB-TolC efflux system and also regulate production of its regulatory factors. CsrA could become a target for drug discovery, as inhibition will lead to unstable *acrAB* transcripts and, as a consequence, less AcrAB-TolC and reduced efflux. Efflux inhibitors synergise with antibacterials and attenuate virulence. Some currently available drugs such as phenothiazines could form the basis of a discovery programme for efflux inhibitors and research with GFP reporters has led to a new tool to identify new efflux inhibitors. Finally, it should be noted that there has been a tendency for many years for mutants in which the *acrB* or *tolC* genes have been inactivated or deleted to be employed for screening new antibacterial molecules. Research with the loss of function AcrB D408A mutant shows that lack of an efflux protein does not result in the same phenotype as when AcrB loses its efflux function but when the protein is intact. Therefore, drug discoverers are advised to reconsider the use of inactivation or deletion mutants when seeking new molecules with anti-Gram-negative bacterial activity.

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I dedicate this lecture to Dr Vito Ricci who has worked with me on efflux since 1995.

Transparency declaration

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Legends to Figures

Figure 1. Families of MDR efflux pumps and substrates (adapted from reference 1).

Figure 2. Schematic diagram of the assembly of a tripartite MDR efflux pump system.

Figure 3. Disc diffusion assay showing that inactivation or deletion of efflux pump genes confers increased susceptibility to drugs with anti-Gram-negative bacterial activity e.g. ciprofloxacin

Figure 4. Production of GFP by reporter in the presence of CCCP; PAβN; CPZ, chlorpromazine; TDZ, thioridazine. [31]

Table 1. Susceptibility to AcrB substrates.

Strain	MIC (mg/L)													
	ACR	ETBR	CIP	NAL	CHL	TET	NOV	FUS	ATM	CAZ	CTX	ERY	OX	MIN
SL1344	512	>2018	0.03	4	8	2	512	2048	0.5	1	0.5	256	512	2
Δ*acrB*	64	32	0.008	1	2	0.5	8	32	0.12	0.25	<0.008	8	16	0.25
AcrB D408A	32	32	0.008	1	2	0.5	8	16	0.12	0.25	<0.008	8	16	0.25

ACR, acriflavine; ETBR, ethidium bromide; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; NOV, novobiocin; FUS, fusidic acid; ATM, aztreonam; CAZ, ceftazidime; CTX, cefoxitin; ERY, erythromycin; OX, oxacillin; MIN, minocycline. Mode values from four experiments are shown.

Data from Wang Kan *et al.* [13]

368 **Table 2. AcrB G288D and MDR in the clinical isolates of *S*. Typhimurium.**
369

Strain	Weeks post CIP Rx	Minimum Inhibitory Concentration mg/L)						AcrB 288
		NAL	CIP	TET	CHL	CAZ	AZT	
L3[a]	0	2	0.015	1	2	0.12	0.06	G
L10	1	8	0.06	2	8	0.5	0.12	G
L11*	3	16	0.03	2	8	0.25	0.25	G
L12	3	64	0.5	8	32	1	0.5	**D**
L13*	3	64	0.5	8	16	0.5	0.5	**D**
L6	5	64	0.5	8	32	0.5	0.5	**D**
L16	17	64	0.5	8	32	1	0.5	**D**

L18†	19	64	0.5	16	32	2	0.5	**D**

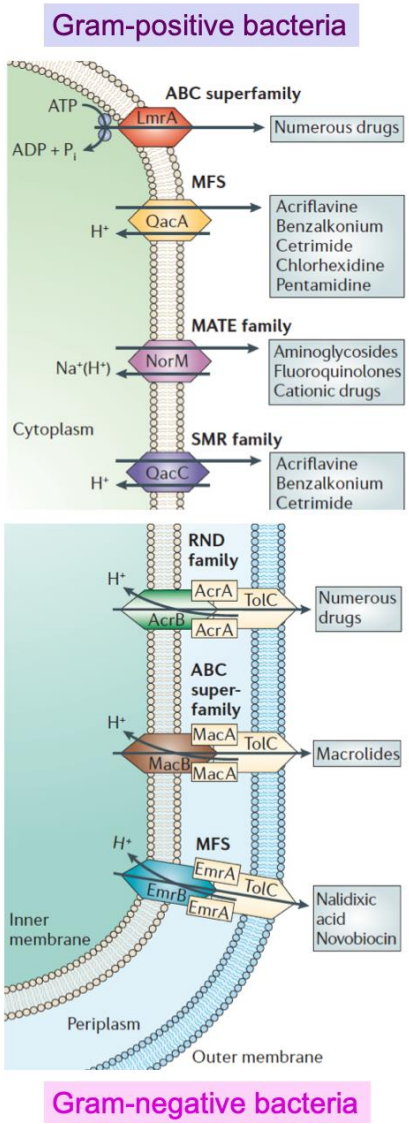
a, pre-ciprofloxacin therapy isolate. * mutation in *gyrA*. †Mutation in *gyrB*. D, aspartate. Data from reference 15.

372 **Table 3. The level of RamA influences MDR.**
373

Strain	**MIC (mg/L)**						**Fold change in gene expression[a]**	
	Cip	**Nal**	**Chl**	**Tet**	**Tri**	**Cyc**	***acrB***	***ramA***
SL1344	0.03	2	4	2	0.12	S	1	1
SL1344 *ramR::aph* (L1007)	**0.06**	**16**	**16**	**8**	**0.25**	**T**	**1.5**	**1.4**
[1]SL1344 *ramA*::*aph* (L133)	0.03	2	2	2	0.12	S	0.2	0
[1]L133 pTRC*ramA*	**0.03**	**16**	**8**	**4**	**0.25**	**S**	**2.3**	**1.7**
[1]L133 pTRC*ramA*[b]	**0.03**	**64**	**32**	**16**	**1**	**T**	**14.9**	**37.6**

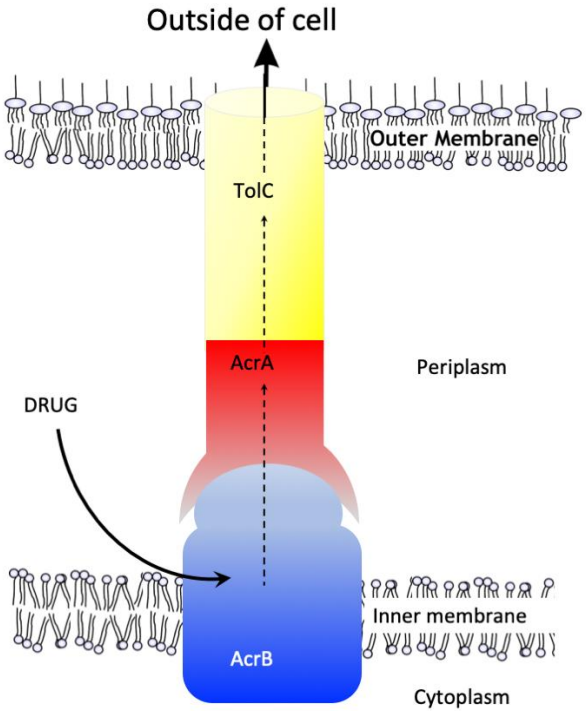
374
375 [a]Compared to SL1344. [b]*hisA::ramA* under the control of IPTG inducible promoter
376
377 Data from references 5 and 12.
378

379 Figure 1.



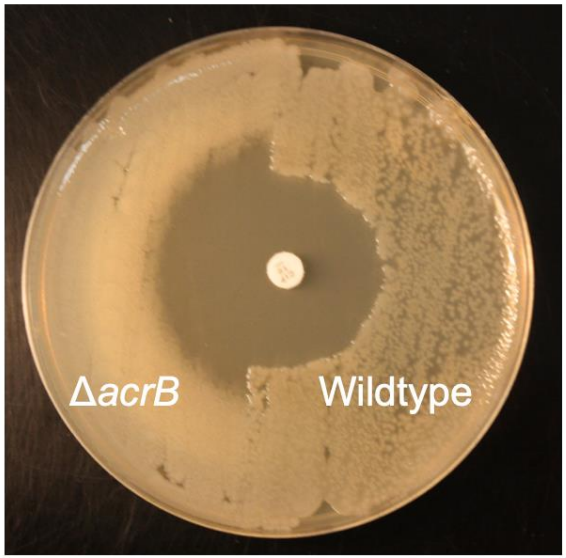
380

381 Figure 2.



382

383 Figure 3.



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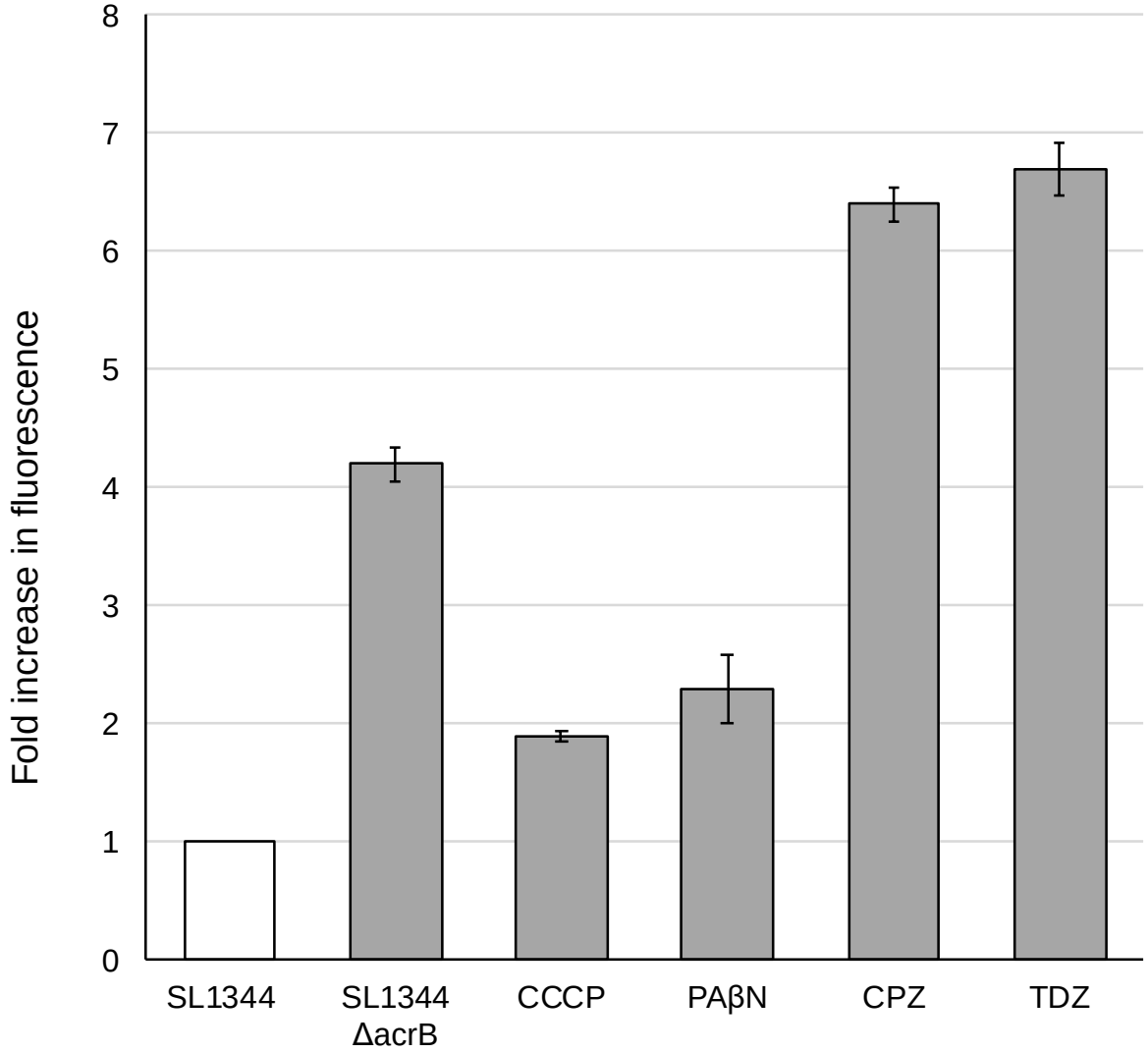
386 Figure 4.



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