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AcrS is an Activator of *acr*D Expression in *Escherichia coli* K-12 Following Exposure to Sub-inhibitory Concentration of Kanamycin Pretreatment

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AcrS is known to be a repressor of efflux pump complexes, but its role in the regulation of *acr*D is unknown. AcrD is an aminoglycoside efflux pump, closely related to other drug efflux pumps as part of the resistance-nodulation-division (RND) family in *Escherichia coli*. The RND family is known to be regulated in a co-ordinated manner, so the role AcrS plays in the regulation of *acr*D was investigated. AcrD has been shown to be responsible for inducing adaptive resistance to kanamycin following pretreatment with kanamycin at sub-inhibitory levels. The Δ*acr*S *E. coli* and wild-type *E. coli* BW25113 were pretreated with sub-inhibitory concentrations of kanamycin, and then changes in kanamycin resistance and *acr*D expression were compared. *acr*D amplified cDNA gene transcripts from Δ*acr*S and wild-type showed that only wild-type *E. coli* pretreated with sub-inhibitory kanamycin expressed *acr*D. However, the kanamycin resistance assays showed that Δ*acr*S strain has a higher resistance to kanamycin than the wild-type strain after pretreatment. These results suggest that AcrS is a putative activator of *acr*D, and that the deletion of *acr*S could possibly lead to aminoglycoside export by other overexpressed RND efflux pumps.

The frequent widespread use of antibiotics in clinical settings has lead to the emergence of multi-drug resistant pathogenic strains of bacteria, which is a threat to public health and emphasizes the importance of understanding the mechanisms of how bacteria are able to confer resistance to multiple antibiotics. Sub-inhibitory concentrations of antibiotics can strongly affect mutation rate, horizontal gene transfer and biofilm formation, which all contribute to the emergence of antibiotic resistance. Previously, studies have shown that *Escherichia coli* pretreated with a sub-inhibitory level of kanamycin acquire cross resistance to many antibiotics due to upregulation of multidrug efflux pumps (1-3). Bacterial efflux pumps are membrane transport proteins that are able to transport antibiotics out of the cell, thereby lowering the intracellular concentration of antibiotic which can contribute to antibiotic resistance (5). AcrB is a well-studied major multidrug efflux pump in *E. coli* with a wide substrate range that includes amphiphilic and lipophilic drugs. AcrD is a closely related efflux pump specific for aminoglycoside antibiotics. Both AcrB and AcrD are inner membrane pumps that are part of the resistance nodulation division (RND) family, which form a tripartite complex with the periplasmic membrane fusion protein (MFP), AcrA, and the outer membrane channel protein Tol-C (4,5). While *acr*AB is known to be regulated locally by AcrR and AcrS, the regulation mechanism of *acr*D is unknown (6,7).

Dick *et al*. hypothesized a connected interplay in the regulation of these two efflux pumps by the AcrR repressor in response to aminoglycoside antibiotic stress (7). AcrR is a repressor that must be removed in order for the *acr*AB efflux gene to be expressed. Dick *et al.* showed that AcrR is not involved in *acr*D regulation in response to aminoglycoside stress, and *acr*D expression in their wild-type and Δ*acr*R strains were consistent following pretreatment with sub-inhibitory kanamycin levels (7).

AcrS is also a repressor of *acr*AB and was shown to have a higher affinity for the *acr*AB operon promoter than AcrR. AcrS has been shown to be involved in repression of the *acr*AB multidrug efflux gene in response to antibiotic stress (6). AcrS is a putative repressor of the AcrEF efflux pump, which is also a member of the RND family (5).

The *acr*AB, *acr*EF and *acr*D efflux pump genes have been reported to be coordinately regulated in response to antibiotic stress (8,9). The exact roles of AcrS in drug efflux systems are not entirely understood but it has been shown to be involved in the repression of more than one multidrug efflux system in *E. coli* upon exposure to antibiotics (6). AcrS may also be involved in repressing AcrD regulation by acting to coordinate the expression of efflux pumps in response to aminoglycoside antibiotic stress. In this paper, the role of AcrS in AcrD-mediated aminoglycoside antibiotic resistance is investigated by comparing Δ*acr*S strain to wild type *E. coli acr*S$^+$ strain with respect to *acr*D expression and kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains and culturing methods. *E.coli* JW3232-1, the Δ*acr*S strain with kanamycin resistance inserted, was ordered from the Coli Genetic Stock Centre (CGSC) at Yale University (10). JW3232-1 has seven mutations including *Δ(ara*D-*ara*B*)567*, *ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔacrS::kan*, *rph-1*, *Δ(rha*D-*rha*B*)568*, *and hsdR514*. Except *ΔacrS::kan*, the other six mutations are shown to be irrelevant to AcrD-mediated aminoglycoside antibiotic resistance. The wild type control, *E.coli* BW25113 cells, was retrieved from the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia. All strains were grown in Luria Bertani (LB) medium incubated at 30°C on a shaking platform set at 100 rpm.

Confirmation of *acr*S deletion. *acr*S of JW3232-1 was knocked out by Baba *et al.* by targeted replacement with a kanamycin resistant cassette of approximately 1.3 kb in size (10, 14). The Δ*acr*S in *E. coli* JW3232-1 was confirmed using PCR and gel electrophoresis by using a primer pairs that ybridizes

Table 1 NCBI reference sequence, primer sequence, gene size and product size of primers used for PCR. *acr*S-F primers flank the *acr*S gene, and *acr*S-R are primers nested within the *acr*S gene.

Gene	Reference Sequence		Primer Sequence (5' to 3')	Gene Size (bp)	Product Size (bp)
*gap*A	NC_000913.2	Forward	GTCGCATTGTTTTCCGTGCT	996	701
		Reverse	CAGACGAACGGTCAGGTCAA		
*acr*D	NC_000913.2	Forward	TGCTGGCAATCCTGTTGTGT	3114	320
		Reverse	CTGCGGTAACTTTCGCATGG		
*acr*S- F	NC_000913.3	Forward	GCTGGCAACAGAAACAGACA	663	1338
		Reverse	GCTTCACTTTTCGCCAGTTC		
*acr*S- N	NC_000913.3	Forward	CCCGCCATATTCATTAACCA	663	321
		Reverse	CTTGACGGCTGGATTAGAGC		

upstream and downstream of *acrS* and a nested primer pairs that hybridizes within the *acrS* gene. The primer sequences and expected size of flanking and nested fragments are shown in Table 1. Primer3Plus online software was used to design primers. All designed primers were ordered from Integrated DNA Technologies.

Preparation of antibiotic stock solutions. Stock solutions of ampicillin (Sigma-Aldrich #K1377-SG) and kanamycin (Sigma-Aldrich #A-9518) were prepared by dissolving each antibiotic in distilled water to achieve a final concentration of 100 mg/ml. All solutions were passed through 0.2 µm Acrodisc® Syringe Filters for sterilization. Antibiotic stock solutions were stored at -20ºC.

Preparation of competent *E.coli* JW3232-1 cells for transformation. 3 ml of overnight *E.coli* JW3232-1 cell culture was inoculated into 100 ml LB broth and incubated at 30ºC in an air shaker until an OD_{600} of 0.3 was reached. The culture was chilled on ice for 15 minutes and then centrifuged at 4ºC for 10 minutes at 4000 rpm. Cell pellets were resuspended in 0.1 M of cold sterile $CaCl_2$ solution. Cells were kept on ice for 30 minutes and then centrifuged again at 4ºC for 10 minutes. The final pellet was resuspended in 0.1 M $CaCl_2$ with 15% glycerol and stored at -80°C.

Removal of kanamycin resistance from *E.coli* JW3232-1 cells. The kanamycin resistance gene in the supplied *E. coli* JW3232-1 strain was removed through a method adapted from Dick *et al.*, which uses the pCP20 plasmid (7). The pCP20 plasmid, which contains an ampicillin resistance gene and FLP recombinase, was transformed into the competent *E.coli* JW3232-1 cells by heat shock. Cells were recovered in 1 ml of SOC medium and incubated at 30°C for 1 hour. 100 µl of the recovered cells were spread onto LB + ampicillin (100 µg/ml) plates and incubated at 30°C overnight. Ampicillin resistant colonies were streaked onto LB plates and incubated at 42°C overnight to activate the FLP recombinase and inhibit plasmid replication. Isolated colonies were then grid plated onto LB, LB + ampicillin (100 µg/ml) and LB + kanamycin (50 µg/ml) plates to select for colonies that are both kanamycin and ampicillin sensitive.

Determination of minimal inhibitory concentrations of kanamycin for *E.coli* BW25113 and Δ*acr*S strain. The MIC microdilution assays were carried out in 96-well flat bottom plates using a method adopted from Sidhu *et al.* (2). All cells were grown to an OD_{600} of 0.25 and incubated in triplicate with the following concentrations of kanamycin: 0, 10, 11, 14, 15, 20, 22, 25, 28, 30, 35, 40, 44, 50, 56, 60, 100 µg/ml. The plates were incubated at 30°C for 20 hours and then examined visually for bacterial growth. The MIC was determined as the lowest concentration of kanamycin where no visible growth was observed.

Kanamycin resistance assay for *E.coli* BW25113 and Δ*acr*S strain. *E. coli* BW25113 and Δ*acr*S cells were cultured to an OD_{600} of 0.25 in LB and each divided into pretreatment and no pretreatment conditions. The pretreatment conditions were treated with sub-inhibitory concentrations of kanamycin, determined as half the MIC, and incubated for 2 hours and 20 hours at 30°C. The kanamycin resistance assay was set up in 96-well flat bottom plates in a similar manner to the initial MIC assay. All cells were pated at an OD_{600} of 0.05 and incubated overnight at 30°C with the following concentrations of kanamycin: 0, 10, 11, 14, 15, 18, 20, 22, 25, 28, 30, 35, 36, 40, 44, 50, 56, 60, 72, 100 µg/ml. They were observed visually for bacterial growth to determine MICs.

Total RNA extraction and cDNA conversion. BW25113 and Δ*acr*S cells were grown to an OD_{600} of 0.3 before extraction. Trizol and QIAGEN RNeasy Mini Kit were used to protect and extract total RNA, respectively, followed by DNase treatment to eliminate DNA contaminations. PCR of *acr*D on total RNA before and after DNase treatment confirmed removal of DNA prior to cDNA synthesis. 300 ng of total RNA was then reverse transcribed using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen™) according to manufacturer's protocol. Isolated RNA and cDNA were stored at -80°C.

PCR amplification and gel electrophoresis. Primer sequences, gene sizes and product sized of *gap*A and *acr*D are shown in Table 1. The PCR reaction was carried out with AccuPrime Kit. There were 35 PCR cycles starting with 95°C for 5 min, followed by another 95°C for 30 sec, 51°C to anneal for 30 sec, and extend at 72°C for 45 sec. All PCR products were electrophoresed in 1.0% (w/v) agarose gel at 100 V and then stained with 0.5 µg/ml ethidium bromide for 20 min. The gel was visualized using Alpha Innotech® MultiImage light cabinet.

RESULTS

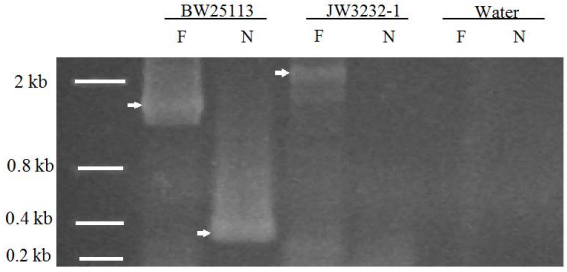

FIG 1 Agarose gel electrophoresis of PCR products from BW25113 and JW3232-1 genomic DNA using *acr*S flanking and nested DNA primers to confirm *acr*S deletion in JW3232-1. "F" refers to PCR using flanking primers, while "N" refers to PCR using nested primers. Water was used as a negative control.

Confirmation of *acrS* deletion in *E. coli* JW3232-1. In order to confirm that JW3232-1 ordered from CGSC is indeed Δ*acr*S strain, PCR and gel electrophoresis was performed. Figure 1 shows that the PCR using flanking and nested primers in the BW25113 strain produced fragments at approximately 1.3 kb and 0.3 kb respectively. For strain JW3232-1, neither the flanking or nested primer pairs result in bands in the 1.3 kb and 0.3 kb range. The lane with flanking primers for JW3232-1 strain showed a band at 2 kb, which was also been expected since the inserted kanamycin cassette (1.3kb) and designed primer flanking region (675bp) added up to around 2 kb (10, 14). Negative controls show that there are no signs of contamination. Taken together these results confirm the *acr*S mutation in strain JW3232-1.

Table 2 Minimum inhibitory concentrations of kanamycin for *E. coli* BW25113 and Δ*acr*S following no pretreatment (-) or kanamycin pretreatment with 20 houts (+). Data shows mean values.

	Kanamycin MIC (µg/ml)			
	WT		Δ*acr*S	
Pretreatment	-	+	-	+
2 hours	23.5	25	23.5	25
20 hours	25	38	26.5	50

Pretreatment of BW253113 and Δ*acr*S strains with sub-inhibitory kanamycin for 20 hours differentially affected the MICs. The MICs for the 20 hour untreated BW25113 and *E. coli* Δ*acr*S strain was 25 µg/ml and 26.5 µg/ml respectively, which shows both strains have similar resistance to kanamycin (Table 2). After pre-treatment with sub-inhibitory kanamycin for 20 hours, the MICs for BW25113 strain and Δ*acr*S strain increased to 38 µg/ml and 50 µg/ml, respectively. The results suggested that the Δ*acr*S strain has an increased resistance to kanamycin than the wild type BW25113 strain after the 20 hour exposure period to sub-inhibitory kanamycin. In comparison, with a 2-hour exposure period to sub-inhibitory kanamycin, differences in resistance between strains or treatment types were not apparent (Table 2), suggesting that a 2-hour pretreatment is not sufficient to induce adaptive resistance to kanamycin.

***acrD* is only expressed in BW25113 strain following pre-treatment.** Following the pretreatment, RNA was extracted and reverse transcribed to cDNA in order to semi-quantitatively analyze the expression of *acr*D in both the Δ*acr*S and wild type strains. DNase treatment was performed after RNA extraction, followed by PCR and gel electrophoresis to confirm that the DNase treatment removed genomic DNA contaminants from the extracted RNA. As displayed in all four lanes for the total extracted RNA before DNase treatment (Fig 2), there were strong bands of amplified *acr*D resulting from genomic DNA contaminants. Following DNase treatment, none of the lanes showed the band (Fig 2). Lack of bands in negative control confirmed no genomic DNA contamination in the extracted RNA**.** Then, PCR amplification of cDNA,

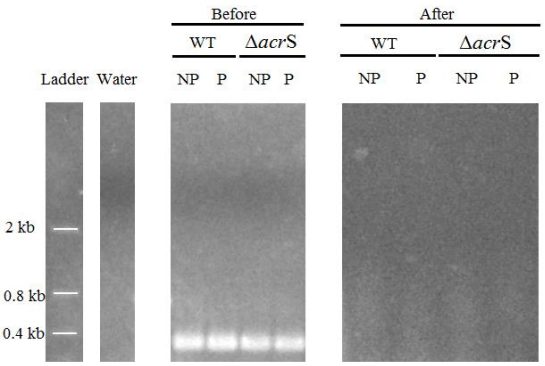

FIG 2 Agarose gel electrophoresis of PCR products from RNA extract with *acr*D amplified before and after DNase treatment. Water is the negative control, "Before" represents the extracted RNA before DNase treatment, while "After" means the RNA after DNase treatment. "WT" refers to BW25113 strain, "NP" and "P" refers to no pre-treatment and pre-treatment respectively.

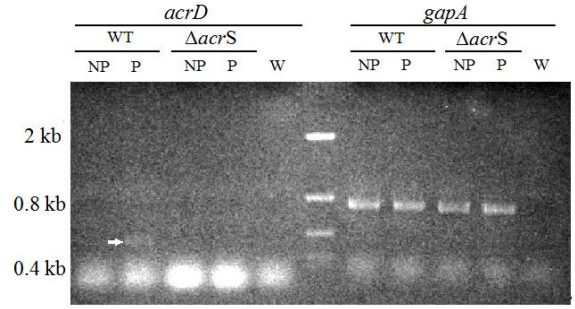

FIG 3 Agarose gel electrophoresis of PCR products from cDNA BW25113 and Δ*acr*S strains with *acr*D and *gap*A amplified. "WT" refers to BW25113, "W" refers to the water negative control. "NP" refers cells that unpretreated, while "P" refers to cells that pretreated for 20 hours. The white arrow refers to a band at around 0.3 kb.

reverse transcribed from the RNA, with *acr*D primers was performed to test the expression of *acr*D in each sample. The gel image (Fig 3) shows that only the pre-treated BW25113 strain lane had a band at around 0.3 kb, which is indicated by the arrow. The size of the fragment is consistent with the predicted size (320 bp) of the *acr*D PCR product (Table 1). Fig 3 also shows that the kanamycin unpretreated samples for both wild type and Δ*acr*S strains did not display bands, as expected, because *acr*D is not expressed if there is no aminoglyoside antibiotic stress (1-3). Semi-quantitative spot densitometry was not needed since there is only one band present across all four samples. PCR of cDNA transcripts were verified for each sample by the amplification of *gap*A, which is indicated by the presence of bands at 701 bp in both pretreated and unpretreated Δ*acr*S strain and BW25113 strain (Fig 3). Water controls show no contamination of the reaction mixture.

DISCUSSION

The hypothesis that AcrS is not a repressor, but possibly an activator, in AcrD-mediated aminoglycoside

antibiotic resistance is proved by experimentally comparing kanamycin resistance and *acr*D expression of Δ*acr*S strain to a wild type strain. If *acr*S was a repressor of *acr*D, we would expect overexpression of *acr*D in the Δ*acr*S strain pretreated with sub-inhibitory levels of kanamycin in comparison to the BW25113 strain. Fig 3 does display a relationship between the deletion of *acr*S and the expression levels of *acrD*, as Δ*acr*S strain were unable to express *acr*D after pretreatment, whereas the pretreated BW25113 strain expressed *acr*D. This observation is counter to our hypothesis and suggests *acr*S is not a repressor of acrD (Fig 3).

Based on our data, we propose that acrS may act as an activator of *acr*D. If *acr*S was required for activating the expression of *acr*D, then a deletion of *acr*S would lead to the downregulation of *acr*D in the Δ*acr*S strain compared to the BW25113 strain , which is consistent with the results of our *acr*D expression analysis (Fig 3). Expression of *acr*D was not observed in the unpretreated controls for both strains (Fig 3), which was expected because previous studies showed that *acr*D was only upregulated when the cells were exposed to aminoglycoside antibiotic stress (1, 2).

The hypothesis that *acr*S acts as an activator is in conflict with the phenotypic results as Table 2 shows increased MICs for the Δ*acr*S strain (89%) in comparison to the BW25113 strain (52%) following a 20 hour pretreatment. Since *acr*D is an aminoglycoside efflux pump (2), it was expected that the lack of its expression resulting from deletion of the putative activator, *acr*S, would result in decreased kanamycin resistance in Δ*acr*S strain compared to the BW25112 strain. However, it is important to note that *acr*S is a known repressor of *acr*AB (6), and that the *acr*AB homolog in other gammaproteobacteria, such as *Salmonella enterica* and *Yersinia pestis*, were shown to be able to extrude kanamycin (11,12). Therefore, the overexpression of *acr*AB caused by the lack of *acr*S might be responsible to the increased efflux of kanamycin and higher MICs in the Δ*acr*S strain after 20 hours pretreatment. Another potential explanation for this increase in Δ*acr*S strain resistance is that *acr*EF, another multidrug efflux pump putatively repressed by AcrS, is overexpressed in the absence of *acr*S (6). Similar to *acr*AB overexpression, *acr*EF overexpression might also result in increased kanamycin efflux and resistance, as both pumps are known to have similar substrate ranges (6). Furthermore, *acr*S was proposed to act as a switch between the alternative expression of *acr*AB and *acr*EF, so that only one efflux pump was expressed in response to their substrates in order to prevent excess efflux pump protein production (6). Therefore, the deletion of *acr*S, can lead to the overexpression of both *acr*AB and *acr*EF, which may explain the higher kanamycin resistance seen in the 20 hour pretreated Δ*acr*S strain in comparison to the BW25113 strain. The expression levels of *acr*AB and *acr*EF in Δ*acr*S strain would need to be analyzed in order to confirm this explanation.

BW25113 strain had 52% increased resistance compare with the unpretreated strain (Table 2). Since *acr*D expression was observed after 20 hours pretreatment (Fig 3), the increase in resistance could be attributed to the expression of *acr*D, as other studies had shown that pretreatment with kanamycin confers adaptive resistance to subsequent treatment with aminoglycosides via upregulation of *acr*D (1, 2, 4).

Both BW25113 and Δ*acr*S strains displayed an increased kanamycin resistance following a 20 hour pretreatment in comparison to a 2-hour pretreatment, which was consistent with what has been previously reported in the literature, where an increased pretreatment time was observed to have a higher adaptive kanamycin resistance (3,7).

Transcriptional analysis suggests that acrS is not a repressor of *acr*D. On the contrary, our data indicate a positive relationship between between *acr*S and the expression of *acr*D, suggesting that *acr*S may actually be an activator of *acr*D expression. Paradoxically, resistance of both Δ*acr*S and BW25113 strain increased after pretreatment compared to unpretreated cultures, in which the increased resistance in Δ*acr*S strain was greater than in BW25113 strain, possibly due to compensatory expression of alternate aminoglycosides efflux pumps such as *acr*AB or *acr*EF in the absence of *acr*D.

FUTURE DIRECTIONS

Further experiments should be done to investigate the expression of *acr*AB and *acr*EF in relation with *acr*S. The upregulation of *acr*AB and *acr*EF was proposed to be responsible for the increased kanamycin resistance observed in the Δ*acr*S strains. A similar experiment should be done with a deletion in *acr*B and *acr*F in addition to *acr*S to provide evidence of the potential involvement of these efflux pumps in kanamycin-induced aminoglycoside resistance. If *acr*S is an activator of *acr*D, and *acr*B and *acr*F are involved in the effux of kanamycin, then it would be expected that the kanamycin MICs after pretreatment would decrease in this multiple knockout strain in comparison to the BW25113 strain. Another experiment can be done to overexpress *acr*S to test how this would affect kanamycin MICs and *acr*D, *acr*B, and *acr*F expression levels after kanamycin pretreatment. If *acr*B and *acr*F are repressed by *acr*S and involved in kanamycin efflux, then we would expect to see decreased MICs and decreased expression levels of these transcripts. If *acr*D expression is activated by acrS, then we would expect to see increased MICs and overexpression of *acr*D.

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