ARTICLE

Plasmid paradox: Persistence of recombinant plasmid without antibiotic selection elevates tellurite resistance in *Escherichia coli* strain A1 mutant

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ABSTRACT

The Tellurite (TeO_3^{2-}) is a highly toxic by-product of copper refining. Microbiological tellurite reduction into elemental tellurium (Te^0), a photoelectric and semiconductor metalloid used in green technologies such as photovoltaic cells and rechargeable batteries, can alleviate tellurium resource scarcity. *Escherichia coli* Strain A1 transformant harbors a recombinant pHSG398 plasmid (pHSG-A1) expressing a putative tellurite-reducing gene (A1-ORF1) and a chloramphenicol resistance gene (*Cm*^{r}). The instability of tellurite reduction activity due to loss of recombinant plasmid when cultured in media with and without antibiotics is a major concern in the use of *E. coli* Strain A1 in tellurite bioremediation and tellurium recovery. The *E. coli* Strain A1 mutant was isolated and characterized from the parental *E. coli* Strain A1 transformant culture after successive cultivation in Luria Bertani (LB) medium without antibiotic-mediated selection. A1 mutant showed higher tellurite resistance in 2 mM Na_2TeO_3 at pH 7.0. The presence of the A1-ORF1 was validated in the A1 mutant through polymerase chain reaction (PCR) using A1-ORF1-specific primers. The exact mutations that drive the persistence of recombinant pHSG-A1 plasmid in *E. coli* Strain A1 mutant with elevated tellurite resistance without antibiotic-mediated selection pressure merit further investigation. Elucidating the mechanism of plasmid compensatory evolution is important in understanding the persistence of antimicrobial resistance in bacterial populations. This will also help to develop novel strategies to combat the imminent threat of antimicrobial resistance. Moreover, the identification of *E. coli* Strain A1 mutant with elevated tellurite resistance provides an opportunity for the efficient recovery of tellurium in the future.

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INTRODUCTION

Tellurite (TeO_3^{2-}) is a byproduct of copper refining and acts as a strong oxidizing agent, damaging biological molecules and leading to cell death (Trouba 2019). Studies on metal-microbe interactions have improved our understanding of the innate ability of microorganisms to convert toxic mining by-products like tellurite (TeO_3^{2-}) into less toxic elemental tellurium (Te^0) (Avazéri et al. 1997; Trutko et al. 2000; Borsetti et al. 2003). Tellurite reduction using microbial cells has been demonstrated to be a viable and sustainable technology for addressing tellurite pollution consequently allowing for the efficient recovery of tellurium, an extremely valuable photoelectric and semiconductor metalloid used in green technologies such as photovoltaic cells (PVCs) and rechargeable batteries (Narayanan and Sakthivel 2010; Borghese et al. 2014; Grandell and Hook 2015).

KEYWORDS

bioremediation, compensatory evolution, *Escherichia coli*, plasmid, tellurium

Bacterial plasmids may encode traits beneficial for the survival of the host cell, such as antimicrobial resistance, heavy metal tolerance, virulence, and enzymes needed for metabolism (Carroll and Wong 2018). However, plasmids impose competitive disadvantages on host cells. Most studies that reported plasmid-induced burden that reduces bacterial fitness fail to examine the evolutionary relationship of plasmid and host genotypes (Bentley et al. 1990; Lenski et al. 1994; San Millan and MacLean, 2017; Carroll and Wong 2018). The "plasmid paradox" highlights the instability of plasmid maintenance, predicting that plasmids may be lost over time, even when they carry beneficial genes. Consequently, advantageous plasmid-encoded traits may eventually become integrated into the host genome to ensure their long-term persistence (Harrison and Brockhurst 2012). Previous studies have shown that plasmid persistence exists even without positive selection pressure from the antibiotics. Mechanisms on the occurrence of plasmid persistence include host-plasmid co-adaptation, cross-ecotype transfer, and high plasmid transfer rates. However, no mechanism can adequately explain the plasmid paradox (Bergstrom et al. 2000; Koeppel et al. 2013). The lack of knowledge underlying the mechanisms that permit the long-term persistence of plasmids in bacterial populations despite the plasmid-induced burden could shed light on understanding the emergence of antimicrobial resistance (AMR). Understanding the plasmid paradox could, therefore, provide necessary insights to address the imminent threat of AMR.

In a previous study, we reported the discovery of a novel gene, A1-ORF1 (DDBJ Accession No. LC457965) about 214 amino acid residues, conferring tellurite resistance and tellurite reduction to *Escherichia coli* Strain A1 transformant through metagenomic library construction and functional screening approach (Munar et al. 2019). Basic Local Alignment Search Tool using nucleotide sequences (BLASTN) revealed sequence homology (92%, E-value: 0.00) of A1-ORF1 to *Pseudomonas stutzeri* PAP2 family/protein DedA family protein (Altschul et al. 1990). PAP2 type 2 phosphatidylglycerol-phosphate shares catalytic residues with haloperoxidases, bacterial acid phosphatases, and ATP diphosphohydrolase (Zhang et. 2008). Gene Ontology (GO) prediction linked the metagenome fragment to substrate-specific transmembrane transporter (99.5%), and catalytic activity (98.6%) (Ashburner et al. 2000; The Gene Ontology Consortium, 2023). The minimum inhibitory concentration (MIC) of *E. coli* Strain A1 is 1 mM Na_2TeO_3, which is 200 times higher than the wild-type (Aradska et al. 2013). However, the loss of tellurite reduction activity due to the instability of the recombinant plasmid was frequently observed in the transformant cultures. In this study, we report the isolation of *E. coli* Strain A1 mutant, which acquired increased tellurite resistance and reduced robustness of tellurite reduction activity after more than 10 generations of successive cultivation in LB medium without antibiotic-mediated selection.

MATERIALS AND METHOD

Bacterial strains, plasmids, and culture conditions

E. coli strains and plasmids used in this study are presented in Table 1. Bacterial strains were grown in a 15-mL Nunc conical tube (Thermo Scientific) with 10 mL LB broth (pH 6.7) with or without 12.5 µg/mL chloramphenicol (CHL). Bacterial cultures were cultivated at 37°C for 24 h to standardize OD_{550} at 0.1-0.2 using SANYO Incubator MIR-162 (SANYO Electric Co., Ltd., Osaka, Japan). Optical density was measured using a WPA CO-7500 Colorimeter (Biochrom Ltd., UK).

Table 1: Plasmids and bacterial strains used in this study

Plasmids/ strains	Description	Reference or source
(A) Plasmids		
pHSG398	Cloning vector; *Cm*^{r}	Takara Bio, Japan
pHSG-A1	pHSG398 plasmid vector harboring a metagenome fragment conferring tellurite resistance through tellurite reduction; *Cm*^{r}	Munar et al. 2019
(B) *E. coli* strains		
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (*Str*^{R}) *end*A1 *nup*G	Thermo Fisher SCIENTIFIC, Waltham, MA, USA
TOP10-pHSG	*E. coli* TOP10 transformed with empty pHSG398 plasmid vector; *Cm*^{r}	Munar et al. 2019
A1	*E. coli* harboring pHSG-A1 plasmid; *Cm*^{r}	Munar et al. 2019
A1 mutant	Chloramphenicol-susceptible *E. coli* Strain A1 with increased tellurite resistance	This study

Plasmid extraction, stabilization, and bacterial transformation

In a previous study, the parental transformant, *E. coli* Strain A1, was isolated from a marine sediment metagenomic library constructed by ligating *Sau*3A1-digested metagenome DNA with *Bam*HI-digested pHSG398 plasmid vector and transformed into One Shot TOP10 *E. coli* competent cells (Munar et al. 2019). FastGene Plasmid Mini Kit was used to extract recombinant pHSG398 plasmid (pHSG-A1) (NIPPON Genetics Europe GnBH Co., Ltd., Japan). Extracted pHSG-A1 was re-transformed into *E. coli* TOP10 competent cells for plasmid stabilization. pHSG-A1 and empty pHSG398 plasmid vector were transformed in LB broth at 37°C for 60 min with intermittent shaking every 10 min using SANYO Incubator MIR-162 (SANYO Electric Co., Ltd., Osaka, Japan). The transformation reaction was plated on LB agar with 1 mM 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Transformants harboring the pHSG-A1 and empty pHSG398 plasmid vectors were grown in LB broth with or without 12.5 µg/mL CHL (Takara Bio). Bio Clean Bench (SANYO, Japan) was used to perform microbiological techniques.

Tellurite reduction activity and minimum inhibitory concentration (MIC) assay

The stock solution of 100 mM Na_2TeO_3 was prepared by dissolving sodium tellurite (Sigma-Aldrich, USA) in ultrapure water (Direct-Q 3 UV Water Purification System, Merck Millipore, USA). The stock solution was filter-sterilized using a 0.20 µm polyethersulfone (PES) syringe filter unit (Advantec, Toyo Roshi Keisha Ltd., Japan). Tellurite reduction activity and MIC were assayed in 96-well clear plates (TrueLine, USA) using 200 µL of LB broth inoculated with 100 µL of bacterial cultures (OD_{550}= 0.2). LB broth with or without 12.5 µg/mL CHL was amended with various concentrations of Na_2TeO_3 (1-6 mM) to determine MIC. Spot plating technique was performed to replicate the tellurite reduction activity of the parental *E. coli* Strain A1 and A1 mutant on LB agar amended with 1 mM Na_2TeO_3. Bacterial cultures were incubated at 37°C for 24-72 h using SANYO Incubator MIR-162 (SANYO Electric Co., Ltd., Osaka, Japan). A SpectraMAX M Series Multi-Mode Microplate Reader was used to measure bacterial growth at OD_{550} (Molecular Devices, USA). Tellurite reduction, exhibited by the production of black tellurium precipitates in the medium, was visually observed (Tucker et al. 1962). The formation of brown coloration in the assay suggests tellurite reduction by non-specific tellurite reductases such as membrane-bound nitrate reductase and cytoplasmic glutathione reductase, which were liberated as a result of cell lysis due to high metal ion concentration (Avazéri et al., 1997; Turner et al., 2001).

Isolation and optimum growth conditions of *E. coli* Strain A1 mutant

The streak plating method was performed to isolate and purify the A1 mutant from the parental culture, showing elevated tellurite resistance on LB agar plates. Optimum temperature and pH for growth and tellurite reduction activity were investigated to determine whether the isolated A1 mutant strain has the same optimum conditions as the A1 parental strain. Optimum growth conditions of A1 mutant in LB broth with and without CHL were determined under varying temperatures (4°C, 32°C, 37°C, 45°C) set in the SANYO Incubator MIR-162 (SANYO Electric Co., Ltd., Osaka, Japan) while Mettler SevenEasy pH meter (Gemini BV) was used to adjust the pH (7.0, 9.0, 10.0) of LB media using 1N NaOH and 1N HCl. The assay includes 100 µL of 24 h bacterial culture (OD_{550}= 0.2) inoculated in 200 µL LB broth using 96-well clear plates (TrueLine, USA). Bacterial growth was measured using a SpectraMAX M Series Multi-Mode Microplate Reader (Molecular Devices, USA) at OD_{550} after 72 h incubation in a shaking incubator (BioShaker BR-43FL, TAITEC, Japan).

Amplification of A1-ORF

A1-ORF1-specific primers (F-5'-ATGGGCTGCGGTATTGCTGTGCCT-3'; R-5'-CTAGGGCTGGACGAGCCTGCGTT-3') were used to validate the presence of A1-ORF1 in the mutant strains (Munar et al. 2019). Polymerase chain reaction (PCR) was performed using a T100 Thermal Cycler (Bio-Rad, CA, USA). PCR reaction includes 1x EmeraldAmp Max PCR Master Mix (Takara BIO), 10 µM each primer, and 556 ng/µL recombinant plasmid. Thermal cycling conditions include: one cycle of pre-denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 75°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were electrophoresed in a 0.7% agarose gel at 100 V for 40 min using the Mupid-exU electrophoresis system (Mupid Co., Ltd., Japan). PCR products were stained with UltraPower DNA Safe Dye (BioTeke), and samples were visualized using Blue Light Transilluminator (LEDB-SBOXH, OptoCode, Tokyo, Japan). Standard 1 Kb DNA Ready-To-Use Ladder was used for amplicon size estimation (Maestrogen, Las Vegas, NV, USA).

Statistical analysis

Growth variations of the A1 mutant at various temperatures and pH in LB media and LB media with or without chloramphenicol supplemented with 1 mM Na_2TeO_3, were evaluated using an unpaired *t*-test at a 95% level of significance to compare the mean between independent variables. Simultaneous effects of temperature and pH were evaluated using two-way analysis of variance (ANOVA) at a 5% level of significance. Statistical analyses were performed using the Microsoft Excel data analysis software.

RESULTS AND DISCUSSION

Isolation of *E. coli* Strain A1 mutant with elevated tellurite resistance

E. coli Strain A1 harboring the recombinant plasmid (pHSG-A1) conferring tellurite resistance through tellurite reduction was successively cultivated in media with and without chloramphenicol. pHSG398 plasmid vector carries the chloramphenicol-resistance gene (Cm^r), which is used in the metagenomic library construction reported in the previous paper (Munar et al. 2019). Tellurite resistance and reduction activity in *E. coli* Strain A1 were frequently lost after one year of successive cultivation, even in chloramphenicol-containing media. To assess the stability of this phenotype, tellurite reduction activity and MIC assays were conducted after at least ten successive generations of cultivation. The recombinant pHSG398 plasmid (pHSG-A1) expressing a putative tellurite-reducing gene (A1-ORF1) was re-transformed into One Shot TOP10 *E. coli* competent cells for plasmid stabilization. Positive transformants identified during the stabilization experiment, which showed tellurite reduction activity, were grown on LB medium with or without CHL. After successive cultivation with at least ten generations, tellurite reduction activity and MIC assay were performed to check the stability of tellurite reduction activity. Unexpectedly, one transformant culture grown in LB medium without CHL exhibited sensitivity to the antibiotic. Upon closer examination, the parental *E. coli* Strain A1 subculture used in the particular assay showed no visible turbidity in wells containing LB media amended with CHL, whereas growth was observed on *E. coli* TOP10 competent cells transformed with an empty pHSG398 plasmid vector (TOP10-pHSG) (Fig. 1). Plasmids can impose metabolic burden on host cells even when expressed at low levels (San Millan and MacLean 2017). Consequently, in the absence of antibiotic selection pressure, cultures may eliminate or degrade recombinant plasmids that do not confer an immediate selective advantage (McLoughlin 1994). Several factors, including thermal degradation of CHL during the incubation period, may limit the potency of the antibiotics used as selective pressure to maintain the recombinant plasmid in the bacterial cell (Lopatkin et al. 2017; Wein et al. 2019).

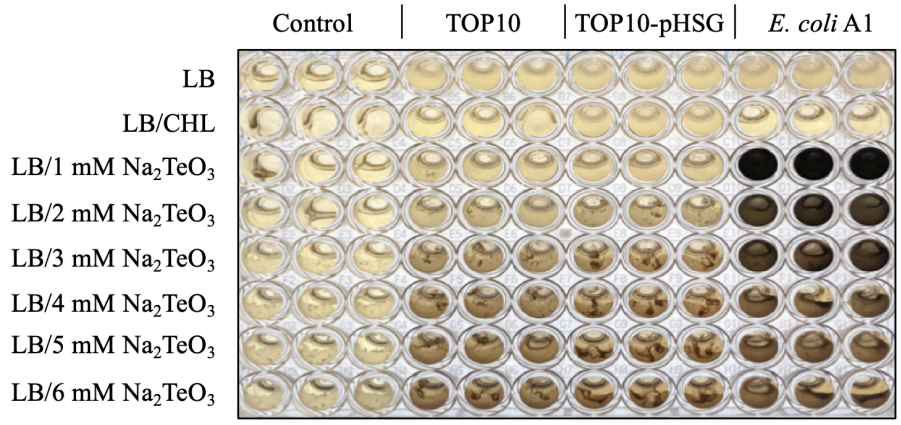

Figure 1: Tellurite reduction activity and minimum inhibitory concentration (MIC) assay revealed chloramphenicol (CHL) susceptibility and higher MIC at 3 mM Na_2TeO_3 of parental *E. coli* Strain A1 transformant culture after successive cultivation in LB broth (pH 7.0) without CHL. The original MIC of the parental *E. coli* Strain A1 transformant was 2 mM Na_2TeO_3. There was no visible growth of *E. coli* Strain A1 in LB amended with CHL, which suggests a loss of chloramphenicol resistance. *E. coli* competent cells (TOP10) and competent cells transformed with an empty plasmid vector (TOP10-pHSG) showed tellurite reduction (brown coloration) by non-specific tellurite reductases such as membrane-bound nitrate reductase and cytoplasmic glutathione reductase liberated as a result of cell lysis due to high metal ion concentration (Avazéri et al., 1997; Turner et al., 2001). The assay was incubated at 37°C for 72 h.

Furthermore, higher tellurite resistance and reduction activity at 2 mM Na_2TeO_3 were recorded in the subculture showing CHL susceptibility. The observed MIC in the particular assay has increased by 200-fold as compared to the original MIC recorded in the parental culture. Taken together, these results have led to the isolation of *E. coli* Strain A1 mutant with chloramphenicol-susceptibility and elevated tellurite resistance (Fig. 2, 3). The brown coloration observed during the tellurite reduction assay in TOP10 competent cells and the empty TOP10-pHSG plasmid vector is likely attributable to non-specific tellurite reductases previously reported in *E. coli*, such as membrane-bound nitrate reductase or cytosolic glutathione reductase. These enzymes may have been released as a consequence of cell lysis induced by high tellurite concentrations, although no direct evidence for their upregulation was obtained in the strains tested (Avazéri et al. 1997; Turner et al. 2001).

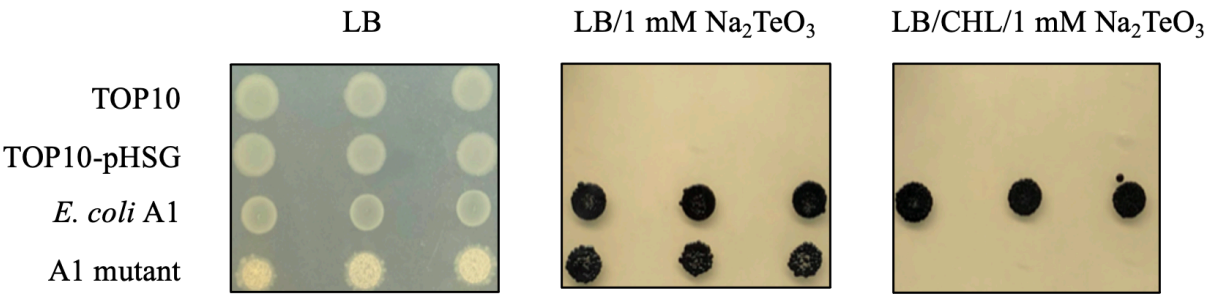

Figure 2: Parental *E. coli* Strain A1 showed tellurite reduction activity on LB agar (pH 7.0) with or without CHL, whereas the A1 mutant strain showed tellurite reduction activity on LB agar without CHL only. Tellurite reduction activity was not observed in *E. coli* competent cells (TOP10) and competent cells transformed with an empty plasmid vector (TOP10-pHSG) after incubation at 37°C for 72 h.

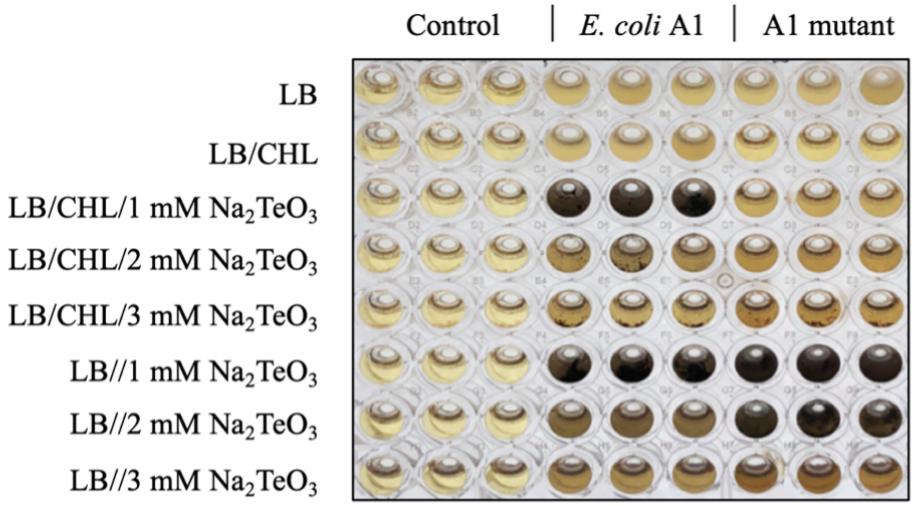

Figure 3: Minimum inhibitory concentration (MIC) assay using pure cultures of parental *E. coli* Strain A1 and A1 mutant in LB broth (pH 7.0) supplemented with increasing concentrations of Na_2TeO_3 (1-3 mM) with or without chloramphenicol (CHL), incubated at 37°C for 72 h. *E. coli* Strain A1 and A1 mutant showed tellurite resistance and reduction activity at 1 mM and 2 mM Na_2TeO_3, respectively.

Tellurite reduction pathways in *E. coli*

Previous studies on tellurite reduction in *E. coli* reported enzymatic and non-enzymatic mechanisms that facilitate the detoxification of tellurite and mitigate its oxidative stress effects. Several enzymes which include nitrate reductase, formate dehydrogenase, and catalases have been reported to catalyze the reduction of tellurite into less toxic elemental tellurium (Avazéri et al. 2002; Tremaroli et al. 2007). Furthermore, proteins involved in oxidative stress response, such as glutathione and superoxide dismutase, were implicated in tellurite resistance by mitigating reactive oxygen species (ROS) generated during tellurite exposure (Taylor et al. 2012). Some bacteria have developed tellurite resistance genes, such as *ter* genes, which encode proteins that facilitate efflux or detoxification of tellurite (Turner et al. 1994).

***E. coli* A1 mutant shows reduced robustness and wider pH for tellurite reduction activity**

Table 2 shows the optical density (OD) of the A1 mutant at various pH and temperatures. The growth of A1 mutant was significantly higher at 32°C and 37°C in pH 7.0 and pH 9.0 than at 4°C and 45°C in pH 10.0 based on an unpaired *t*-test with 95% confidence level (*p*-value < .05) (Fig. 4-A). Tellurite reduction activity of A1 mutant was recorded at 32°C and 37°C and pH 7.0, pH 9.0, and pH 10.0 (Fig. 4-B). The parental *E. coli* Strain A1 showed no apparent tellurite reduction activity at pH 10.0 (Fig. 4-C). The optimum temperature for tellurite reduction activity is the same in *E. coli* Strain A1 and the A1 mutant. However, the A1 mutant showed wider pH tolerance up to pH 10.0 for tellurite reduction (Fig. 4-D). The tellurite reduction activity of the parental *E. coli* Strain A1 coincided with the stationary growth phase in LB media observed after 15 h, whereas the A1 mutant showed delayed tellurite reduction activity observed only after 72 h (Fig. 5). Based on two-way ANOVA, the *p*-value for pH (0.09) is greater than 0.05, suggesting that pH does not have a statistically significant effect on the tellurite reduction activity at 5% significance level. Furthermore, the *p*-value for temperature (0.06) is also greater than 0.05, suggesting that temperature does not have a significant effect at the 5% level. Simultaneous effect of pH and temperature analyzed using two-way ANOVA at 5% level of significance, suggests no significant effect in the tellurite reduction activity in A1 mutant. However, it is noteworthy that increasing the dataset to determine the simultaneous effect of pH and temperature will make the *p*-values more conclusive. The difference in robustness in tellurite reduction activity observed could be attributed to the reduced fitness of the A1 mutant strain associated with the loss or mutation in the recombinant plasmid (Bentley et al. 1990; Carroll and Wong 2018). However, other factors might also contribute to the reduced fitness observed in the A1 mutant. The expression of resistance genes, even at low levels, uses up energy and resources that would otherwise support growth (San Millan and MacLean 2017). Mutations that improve tellurite resistance could also affect nutrient uptake and disrupt the cell membrane (Ferenci 2005; Poole 2012). Moreover, detoxification of tellurite may promote the accumulation of toxic by-products, which may further contribute to growth inhibition. (Kurland and Dong 1996; Imlay 2013). Together, these issues suggest a trade-off between resistance and fitness, which could be better understood through genomic or transcriptomic studies.

Table 2: Mean values of optical density (OD) at 550 nm of A1 mutant in LB media and LB media amended with 1 mM Na_2TeO_3.

		Temperature*			
	pH*	4°C	32°C	37°C	45°C
A1 mutant/LB	7.0	0.27	0.52	0.66	0.31
	9.0	0.30	0.58	0.66	0.26
	10.0	0.27	0.31	0.25	0.24
A1 mutant/LB/1 mM Na_2TeO_3	7.0	0.24	0.38	0.39	0.14
	9.0	0.22	0.38	0.37	0.11
	10.0	0.13	0.36	0.31	0.04

*No significant effect at 5% level, temperature (*p*-value= 0.06); pH (*p*-value=0.09) based on two-way ANOVA.

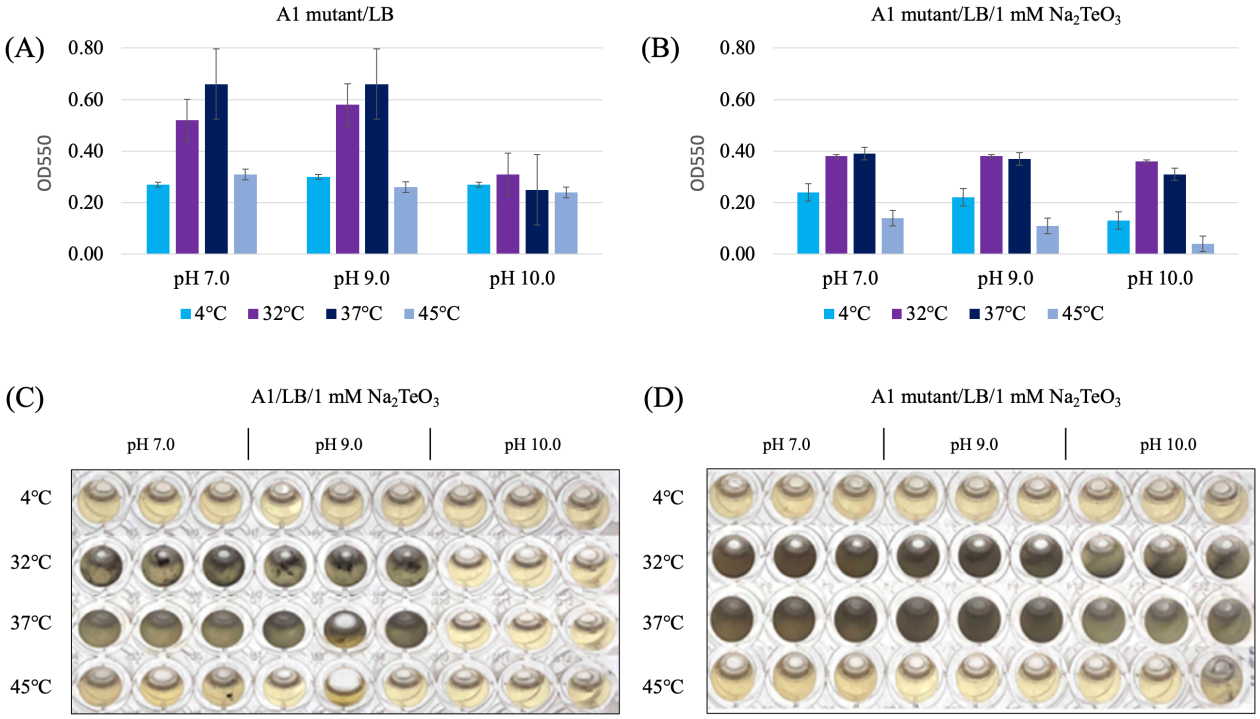

Figure 4: Optimum growth and tellurite reduction activity of A1 mutant at different temperatures and pH, grown in LB broth (A) and LB broth amended with 1 mM Na_2TeO_3 (B) after 72 h incubation. A1 mutant showed significant higher growth at 32°C and 37°C in pH 7.0 and pH 9.0 than at 4°C and 45°C in pH 10.0, based on unpaired t-test with 95% confidence level (*p*-value < .05). Tellurite reduction activity of parental *E. coli* Strain A1 (C) and A1 mutant (D) was observed at pH 7.0 and pH 9.0 and 32°C and 37°C. However, the A1 mutant showed wider pH tolerance for tellurite reduction activity, observed even at pH 10.0.

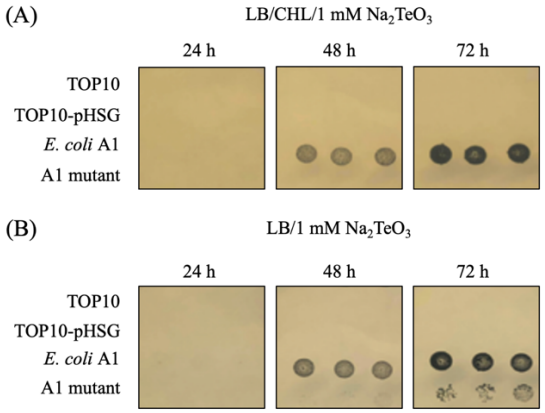

Figure 5: *E. coli* Strain A1 mutant showed reduced robustness, showing tellurite reduction activity after 72 h, whereas *E. coli* Strain A1 showed tellurite reduction activity after 48 h. TOP10 competent cells and TOP10-pHSG showed no visible tellurite reduction activity on LB media (pH 7.0) with CHL (A) and without CHL (B) amended with 1 mM Na_2TeO_3 and incubated at 37°C.

Overcoming plasmid costs may include a change in plasmid gene expression, conjugation rates, or loss of plasmid genes (San Millan and MacLean, 2017). Compensatory evolution may reduce plasmid costs by either chromosomal or plasmid mutation (Zwanzig et al. 2019). The loss of chloramphenicol resistance in the A1 mutant may be attributed to the mutation of the Cm^r gene in the pHSG-A1 plasmid. Reduced robustness of tellurite reduction activity of A1 mutant and higher resistance to tellurite as compared to the parental *E. coli* Strain A1 suggests compensatory evolution to ameliorate plasmid-induced metabolic burden.

Mechanisms of compensatory evolution in *E. coli* and other bacterial systems

Compensatory evolution occurs when secondary mutations offset the fitness costs imposed by a primary mutation, such as antibiotic resistance. A study by Barrick et al. (2009) demonstrated that compensatory evolution in *E. coli* can involve mutations in essential genes related to transcriptional regulation, translation, membrane transport, and oxidative stress response pathways. In *E. coli*, specific compensatory mutations have been identified in efflux pump regulators (*baeS, crp, hns, and rpoB*), which enhance bacterial fitness after initial deleterious mutations (Cho and Misra 2021). Mechanisms of compensatory evolution have been observed in other bacterial species, including *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. In *P. aeruginosa*, mutations in efflux pumps conferring antibiotic resistance can be compensated by secondary mutations in regulatory genes that regulate the expression of efflux systems to reduce fitness costs (Pacheco et al. 2017). Moreover, in *M. tuberculosis*, mutations in *rpoB* (RNA polymerase β-subunit) that confer rifampicin resistance can be compensated through mutations in *rpoC* (RNA polymerase β'-subunit), which may restore bacterial growth and virulence (Comas et al. 2012).

Interestingly, the loss of chloramphenicol (CHL) resistance in the A1 mutant strain coincided with the emergence of enhanced tellurite reduction activity. Rather than being independent outcomes, we interpret this phenotypic shift as a case of compensatory evolution. Previous studies revealed condition-dependent compensatory mechanisms of antibiotic resistance, such as the shift from respiratory to fermentative metabolism upon overexpression of efflux pumps (Zampieri et al. 2017). Investigating metabolic constraints may provide a possible explanation for why improving one trait, such as tellurite resistance, can lead to a reduction in another, like antibiotic resistance or growth. Bacteria have limited resources, so producing more resistance proteins, such as efflux pumps or reductases, can divert energy away from cell reproduction and other cellular functions (San Millan and MacLean 2017). These trade-offs could often lead to compensatory mutations, genetic changes that help restore balance. Previous studies have also demonstrated that loss-of-function mutations can trigger genome-wide adaptive changes aimed at restoring fitness (Pál et al. 2014). In our case, the inactivation of CHL resistance, potentially due to mutations in the plasmid-encoded CHL resistance gene, may have imposed a fitness cost, such as impaired growth under antibiotic-free conditions or a metabolic burden. This cost likely exerted selective pressure for secondary mutations or regulatory rewiring that alleviate the stress, resulting in the observed gain-of-function in tellurite reduction. While tellurite metabolism is not directly linked to CHL resistance, the enhancement of this pathway may reflect a broader shift in cellular resource allocation or redox balance that improves overall viability. This is consistent with reports that compensatory evolution often involves pleiotropic changes and does not necessarily restore the original function, but instead redirects the population toward alternative adaptive strategies (Alonso-Del Valle et al. 2021). Thus, we propose that the increased tellurite reduction capacity observed in the A1 mutant strain represents a possible compensatory adaptation following the loss of antibiotic resistance.

It is important to note, however, that the proposed mechanism has not yet been experimentally validated. To test the hypothesis of compensatory evolution, future studies should focus on identifying the genetic mutations responsible for CHL resistance loss and enhanced tellurite reduction. Whole-genome sequencing of parental and mutant strains can elucidate candidate mutations. Targeted gene knockouts of suspected compensatory genes or redox regulators using Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes, followed by phenotypic assays for antibiotic resistance and tellurite reduction, would help establish the proposed correlation. Complementation studies, reintroducing deleted genes via plasmids, could further confirm the roles of these genes in fitness restoration and metabolic rewiring (Cui and Bikard 2016). These approaches will provide a mechanistic understanding of the compensatory processes possibly at play in the *E. coli* strain A1 mutant.

Validation of A1-ORF1 in A1 mutant

Recombinant plasmid (pHSG-A1) (Fig. 6-A) was extracted from the parental *E. coli* Strain A1 and the A1 mutant to confirm the presence of A1-ORF1. PCR revealed the presence of A1-ORF1 in the parental *E. coli* Strain A1(Fig. 6-B) and A1 mutant (Fig. 6-C). *E. coli* TOP10 competent cell used in the transformation of recombinant plasmid has the *rec*A1 gene, which limits unwanted recombination of the cloned gene. At the same time, it is also an F- strain which limits the transfer of the recombinant plasmid through conjugation. An unknown mutation in the pHSG-A1 plasmid could have driven compensatory evolution, which led to the emergence of *E. coli* Strain A1 mutant with reduced robustness in tellurite reduction activity and elevated tellurite resistance. Determination of compensatory mutations often requires genome-wide approaches and functional validation through genetic knockouts and complementation studies, as previously described (Barrick and Lenski 2013). Exact mutations in the pHSG-A1 plasmid have not been elucidated due to the lack of sequencing. Therefore, further exploration of the exact mutation leading to plasmid compensatory evolution, albeit very difficult, necessitates additional investigation.

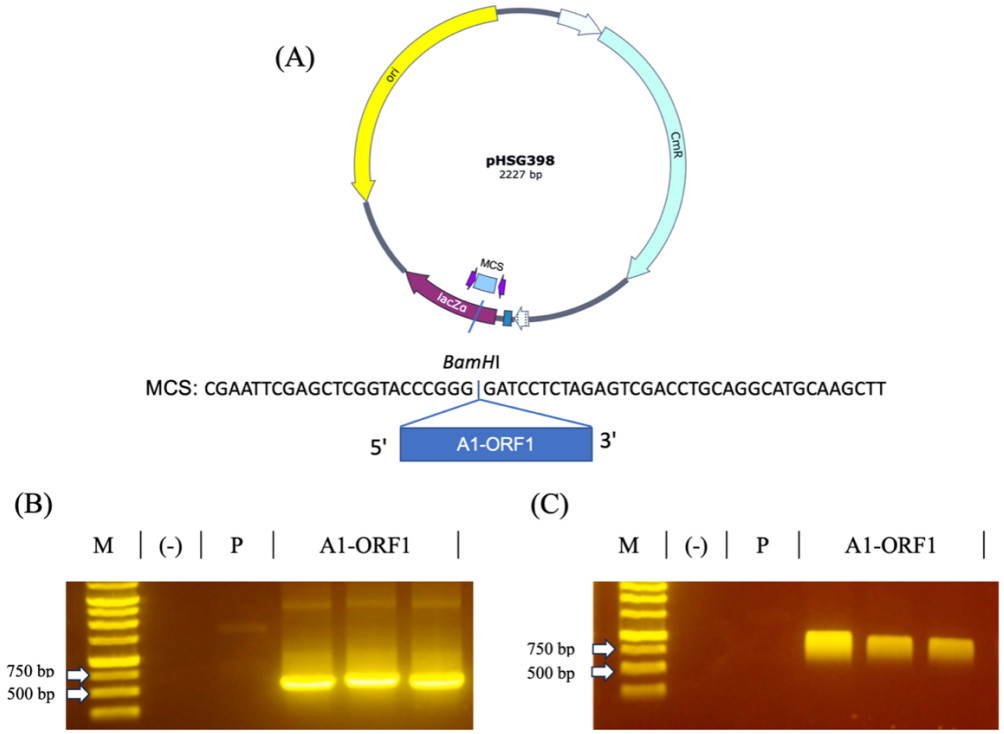

Figure 6: pHSG398 plasmid vector showing *BamH*I ligation site of the metagenome fragment harboring A1-ORF1 (A). Plasmids from three independent subcultures of parental *E. coli* Strain A1 (B) and A1 mutant (C) show amplicons of A1-ORF1 between 500-750 bp, corresponding to the expected amplicon size of 644 bp. There were no visible amplicons in the negative controls, UltraPure H_2O (-), and the empty pHSG398 plasmid vector (P).

Recombinant plasmid persistence in the absence of antibiotic-mediated selection in the *E. coli* strain A1 mutant

The persistence of recombinant plasmids in the *E. coli* strain A1 mutant was inferred from the continued tellurite reduction activity observed after at least ten generations of subcultivation without antibiotic selection. Plasmids are unstable and are usually expelled from the host cell in the absence of selective pressure. Daughter cells without a plasmid-induced metabolic burden tend to outcompete plasmid-bearing cells (Mei et al. 2019). Plasmid stabilization timing is critical to prevent the loss of recombinant plasmid in the host cell. Metabolic burden, induced by plasmid carriage such as plasmid replication, expression, and repair, has significant fitness costs on the host cell reproduction. Compensatory mutations on plasmids or bacterial chromosomes can reduce plasmid-induced fitness costs, which allow for plasmid-encoded genes to persist in the bacterial host population (Liu et al. 2024). Compensatory evolution on chromosomes occurs from mutations in transcriptional regulatory factors, whereas for plasmids, compensatory evolution occurs through plasmid copy regulation, conjugation transfer efficiency, and expression of antimicrobial resistance genes (Liu et al. 2024). In the absence of positive selection for plasmid-encoded traits, compensatory evolution increases plasmid persistence, thus increasing the risks of spreading plasmid-encoded antibiotic resistance in the environment (Zwanzig et al. 2019).

CONCLUSION

Understanding compensatory evolution in *E. coli* and other bacteria is essential for unraveling the dynamics of plasmid-host interactions and their consequences for antimicrobial resistance. The interplay between oxidative stress responses, efflux systems, and metabolic adaptations highlights the complexity of bacterial evolution under selective pressure. Future research should focus on genome-wide approaches to elucidate compensatory mutations and their effects on fitness, as well as investigating potential applications in biotechnology and antimicrobial resistance management.

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CONFLICT OF INTEREST

The author declared no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

MP Munar performed the experiments and wrote the manuscript.

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