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Influence of Different Transposon Families on Genomic Stability of *Shewanella oneidensis* MR1

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ABSTRACT

Shewanella oneidensis, recognised as an important model organism for exoelectrogenic electron transport, has been extensively studied for its potential applications in bioelectrochemical systems. To date, the activity of transposable elements in this organism has not been conclusively investigated. This study focused on transposases, specifically insertion sequences (IS), which make up approximately 4.7% of the organism's genome, and evaluated their impact on genome stability under stress conditions. Using whole genome sequencing, two IS families, ISSOD1 and ISSOD2, were identified as the most active, both showing similar transposition patterns across all tested stressors. A CRISPR/dCas9 cytosine deaminase system was used to introduce stop codons in the ISSOD2 transposase genes, resulting in a significant reduction of transposition events under stress conditions. Analysis of transposition patterns revealed a high frequency of insertions occurring on the megaplasmid, which predominantly carries non-essential genes. Experiments performed here to delete the megaplasmid resulted in the elimination of approximately 35% of its sequence, including an unexpected complete loss of the ori/repA region. Therefore, it was hypothesised that the megaplasmid either exists in a metastable state, possibly representing a cointegrated intermediate within the ISSOD9 (Tn3 member) transposition mechanism, or consists of two replicons that have been combined in previous assemblies due to long overlapping homologies resulting from the presence of ISSOD9. These findings highlight the dynamics of transposable elements in *S. oneidensis* and suggest strategies to improve strain stability by inactivating these elements and at least reducing megaplasmid sequences. Such approaches could improve the suitability of the organism for industrial applications.

1 | Introduction

Since its discovery in 1988 (Myers and Nealson 1988), *Shewanella oneidensis* has become a model organism for the study of extracellular electron transport. Its ability to use an electrode as an inexhaustible electron acceptor makes it a promising candidate for bioelectrochemical systems (BES) that can be applied in the production of valuable chemicals or in bioremediation efforts (Fredrickson et al. 2008; Bursac et al. 2017). Despite certain limitations in production and biofilm formation associated with its wild-type form, advances in genetic engineering and a deeper exploration of its metabolic pathways have revealed significant potential for industrial applications (Ikeda et al. 2021). However, industrial application will depend on the genetic robustness of the strain. This robustness would be required, for example, to predictably produce heterologous proteins needed for synthetic pathways leading to the production of platform chemicals. Transposable elements (TEs) can pose a challenge to this robustness, and several studies have already reported the significant influence these elements can have on the physiology of *S. oneidensis* (Bordi et al. 2003; Schicklberger et al. 2013; Cheng et al. 2020).

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In prokaryotes, insertion sequences (IS), a condensed form of TEs, predominate. The function and role of TEs, once thought to be simply 'selfish DNA' (Doolittle and Sapienza 1980), is now understood to be more complex. TEs can drive adaptive responses to environmental stresses, including the acquisition of antibiotic resistance (Che et al. 2021; Vandecraen et al. 2017). While many TE activities can be deleterious, others can enhance gene function, rescue gene loss or form beneficial operons (Hall 1999; Fan et al. 2019; Kanai et al. 2022). It has been suggested that newly acquired TEs or TEs residing in stressed cells may undergo a 'transposition burst', characterised by a rapid increase in activity that declines as stability is achieved (Siguier et al. 2014; Wu et al. 2015). Over time, TEs and their hosts often reach an equilibrium in which the effects of TEs remain largely neutral, minimising excessive stress on the host genome that might otherwise lead to reduced TE numbers or lineage extinction (Iranzo et al. 2014; Mira et al. 2001).

As bacterial TE numbers show considerable variation across species and clades—with genome size being one of the few correlating factors (Touchon and Rocha 2007)—categorising TE numbers as particularly high or low is challenging. In *S. oneidensis*, 219 TEs have been detected, representing approximately 4.7% of the genome or 5.6% of all coding sequences (Romine et al. 2008). Plasmids typically have higher insertion frequencies and tend to contain a larger number of TEs (Siguier et al. 2015). For *S. oneidensis*, a megaplasmid (MP) with a size of 161 kbp has been identified in previous studies. Notably, 21% of the total genetic information on the MP consists of IS elements, compared to 5.6% on the chromosome, which may contribute to genomic instability. As the MP appears to lack essential genes (Deutschbauer et al. 2011), its elimination could reduce the number of integration sites, the potential for transposition originating from the MP, and the risk of genetic rearrangements. However, stable propagation of the MP appears to be ensured by 10 type II toxin-antitoxin (TA) pairs. These genetic cassettes encode a stable toxin and a less stable antitoxin. In the event of module loss, the toxin predominates, while the antitoxin is degraded more rapidly. The toxic effect is thus unbalanced, leading to a phenomenon known as post-segregational killing (Cooper and Heinemann 2000).

As high transposon activity can disrupt engineered metabolic pathways or biofilm formation, leading to performance losses and variability in production systems (Cepas et al. 2020; Cheng et al. 2020), and as this activity could jeopardise the stability of traits carried on plasmids used in production processes that are critical for system performance (Endy 2005; Rugbjerg et al. 2018), the aim of this study was to quantify transposon activity in *S. oneidensis* and to develop strains with higher genetic robustness by reducing TE activity. While transcriptomic analysis of TE activity presents several challenges and potential ambiguities (Nagy and Chandler 2004), we designed an experiment to quantify insertion events using whole-genome sequencing (WGS) as a proxy for IS activity and stress induction to stimulate increased IS activity (Capy et al. 2000). By applying repeated stress cycles, we aimed to induce a transposition burst that would help to identify the most active IS elements. We then used a CRISPR-Cas deaminase to introduce stop codons into the most active members of the IS families to reduce their activity. In a second approach, we aimed to remove the MP by expressing antitoxins from an expression plasmid. We achieved a 35% reduction of the MP and obtained evidence that the MP may not exist in the wild-type strain in the configuration previously suggested by (Romine et al. 2008).

2 | Materials and Methods

2.1 | Strains and Strain Cultivation

All strains and plasmids used in this study are listed in Table 1. For oxic experiments, all strains were grown in batch culture with LB medium containing tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L and shaken continuously at 180 rpm. Temperatures were adjusted to 37°C and 30°C for *Escherichia coli* and *S. oneidensis*, respectively. Kanamycin (50 μg/mL), 2,6-diaminopimelic acid (DAP) (60 μM), isopropyl-β-D-thiogalactopyranoside (IPTG) (0.8 mM) and/or arabinose (5 mM) were added as required.

For anoxic experiments, *S. oneidensis* cells were pre-cultured in LB medium under oxic conditions and later transferred to M4 minimal medium as described elsewhere (Arinda et al. 2019). 100 mM lactate and 100 mM ferric citrate were added as electron donor and acceptor, respectively. A total of 100 mL of minimal medium was transferred to 150 mL flasks, which were then sealed with rubber stoppers. To remove oxygen from the flask, the headspace was purged by repeated cycles of nitrogen flushing and vacuum application, each cycle lasting 2 min, for a total of 25 cycles. The flasks were then autoclaved.

2.2 | Stress Induction

Shewanella oneidensis was exposed to different stress factors to induce transposase activity. Hence, *S. oneidensis* MR1 (JG7; Table 1) was precultured on M4 minimal medium containing ferric citrate as an electron acceptor. This preculture, now referred to as MR1.1 (JG1980; Table 1)—because we had already detected transposon activity within this preculture—was divided into three batches, each of which was subjected to different stress conditions (I–III): (I) exposure to UV light for 2 min at 14 W/m^2, resulting in a dose of 1680 J/m^2; (II) a heat shock for 20 min at 42°C; and (III) a sublethal dose of kanamycin (2.5 μg/mL). Each stress condition was tested in triplicate. After treatment, the cells were used for reinoculation into fresh ferric citrate medium. After reaching the exponential phase, the optical density at 655 nm (OD_{655}) was adjusted to 1, and the cells were again exposed to their respective stress conditions. The OD was measured at 655 nm to avoid errors due to scattering of ferric iron at 600 nm. 300 μL of culture was used for subsequent transfers. The process was repeated for 15 cycles. As a control, a single MR1.1 culture was transferred without stress induction to measure transposase activity in the absence of either of the three stress factors.

In an independent second stress experiment, triplicates of the control strain *S. oneidensis* (MR1.1) and the strain with deactivated ISSOD2 transposases (JG1981; Table 1) were grown in LB medium and subjected to UV stress only as described above. After UV treatment, the cells were allowed to grow to log phase.

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TABLE 1 | Strains used in this study.

Strain	Genotype	Source
JG 7	*Shewanella oneidensis* MR1 wild-type strain	Myers and Nealson (1988)
JG22	*Escherichia coli* (DH5α Z1) *laci*^{q} *PN25-tetR* Sp^{R} *deoR supE44* Delta(*lacZYA-argFV169*) Phi80 *lacZ*DeltaM15	Lutz and Bujard (1997)
JG98	*Escherichia coli* (WM3064) *thrB1004 pro thi rpsL hsdS lacZ_* M15RP4–1360_(*araBAD*)*567 _dapA1341*::[*erm pir*(*wt*)]	*W. Metcalf, Universität*
JG574	*Shewanella oneidensis* pBAD_*mtrB*$_{V5}$	This study
JG1459	*Shewanella oneidensis* ΔmtrB + pMK-RQ-Ara_RFP	This study
JG1958	*Escherichia coli* JG98 pMQ150_RFP_ISSOD9/Tn3 of Megaplasmid of SO	This study
JG1959	*Escherichia coli* JG22 pBAD_AT1-10 (SO_RS22245; SO_RS22250; SO_RS22275; SO_RS22300; SO_RS22315; SO_RS22445; SO_ RS22455; SO_RS22490; SO_RS22540; SO_RS22785)	This study
JG1960	*Shewanella oneidensis* JG7 araP_RFP_ISSOD9/Tn3_Megaplasmid (pos. 155,260–15,675)	This study
JG1980	*Shewanella oneidensis* MR1.1—JG7 (MR1) adapted to ferric citrate respiration	This study
JG1981	*Shewanella oneidensis* JG1980 pCYR104_sgRNA_ISSOD2_family deactivated	This study
JG1982	*Escherichia coli* JG98 pCYR104_sgRNA_ISSOD2	This study
JG2034	*Shewanella oneidensis* JG7 pCYR104_sgRNA_ISSOD1_ contains stop_codons in 19 out of 43 copies	This study
JG2038	*Escherichia coli* JG98 pCYR104_sgRNA_ISSOD1	This study
JG2045	*Shewanella oneidensis* JG7 pBAD_AT1-10_after 20 transfers	This study

The optical density was measured at 600 nm and adjusted to an OD_{600} of 1, after which the cells were again exposed to UV light. 50 μL of the cells were then used to inoculate 5 mL of LB medium. This was repeated for 15 cycles.

2.3 | Adaptation Experiment

To determine whether the resistance of the strains to stress conditions increased after completion of the full number of 15 cycles from the above stress experiment, colonies from the triplicate cultures were tested against the precursor (MR1.1). For each of the three cultures developed under antibiotic stress, three individual colonies were exposed to 0, 2.5, 5 or 7.5 μg/mL kanamycin alongside a triplicate of the precursor strain MR1.1. From the triplicate developed under stress, three colonies were exposed to 0, 5, 15 or 20 min of irradiation, again with MR1.1 as a control. To test the effect of elevated temperature on strain development, three colonies from each triplicate were grown at 37°C together with a corresponding triplicate of MR1.1. The experiments were performed in 96-well plates using LB medium. (Growth was measured in a TECAN Infinite 200 PRO plate reader at OD_{600} every 20 min for up to 16 h.)

2.4 | Stop-Codon Integration

Plasmid pCYR104 containing dCAS9-AID and an sgRNA expression cassette was kindly provided by Tianjin University (Chen et al. 2023). The sgRNA target sequence was introduced into the plasmid using 120 bp DNA oligos (forward and reverse) containing the target sequence together with 50 bp overlap. The plasmid was digested with BsaI and purified from an agarose gel using the Promega Wizard SV Gel and PCR Cleanup System. Prior to Gibson assembly, the oligos were annealed to form double-stranded DNA (dsDNA) in duplex buffer (100 mM potassium acetate; 30 mM HEPES, pH 7.5) at a concentration of 1 mM. The mixture was heated to 95°C for 2 min and then gradually cooled to room temperature. The concentration of the resulting dsDNA was measured using the Invitrogen Qubit dsDNA Assay Kit and absorbance was assessed using the Tecan Infinite 200 PRO plate reader at 485/530 nm. The target sequence was integrated using Gibson assembly (Gibson et al. 2009) with a 10-fold excess of oligonucleotide, and the construct was introduced into *E. coli* WM3064 by electroporation. Correct integration was confirmed using primers 4399/4400 (see Table S2) for amplification, followed by Sanger sequencing. The resulting plasmid was then introduced into *S. oneidensis* MR1.1 (JG1980; Table 1) by mating with strain WM3064 on LB plates containing kanamycin and DAP together with IPTG as an inducer. After 48 h incubation, *S. oneidensis* MR1.1 was transferred to plates without DAP. Single colonies were sequenced for stop codon conversion using flanking primers 4420/4421 for ISSOD2 and 4418/4419 for ISSOD1 (Table S2). Nanopore sequencing was then performed to confirm conversions throughout the genome.

2.5 | Sequencing and Transposition Identification

Library preparation was performed using native barcoding kits from Oxford Nanopore, and genomic DNA was subsequently

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sequenced on a MinION device with an R10.4.1 flowcell (Oxford Nanopore). All procedures were performed according to the manufacturer's instructions. Base-calling was performed using Dorado v.0.7.0 in superaccuracy mode, which included read filtering for quality and trimming of adapters and barcodes.

Subsequent steps were performed using the CLC Genomic Workbench (Qiagen version 20.0.4). Unless otherwise stated, all operations were performed using default parameters. These include the use of blastn (word size 10, match 2, mismatch −3, gapcost open 5, extension 2) with an increased number of hit sequences to 250 k. Long-read mapping was performed in CLC using minimap2 without long-read splice alignment (match score = 2, mismatch-, gap open-, gap extend-, long gap open- and extend-cost of 2, 4, 4, 2, 24, 1 respectively).

Given that the transfers in the stress experiment likely resulted in a community of subpopulations with different insertions of ISs, it was not feasible to assemble the data without losing information about low abundance integration events. Therefore, we used the raw sequencing data to generate a BLAST data set for each experiment. For insertion identification, a BLASTn search was performed for each IS against the corresponding dataset generated from the individual sequencing to extract sequences associated with the target IS. Filters were applied to exclude all reads shorter than 90% of the total IS length and those with less than 90% sequence identity. All remaining reads were then mapped to the *S. oneidensis* pangenome. New insertions were manually identified when reads resulted in gaps in the reference genome (Heidelberg et al. 2002). The threshold for counting a read as an integration event was set to a single read. The identity of the TE was confirmed by BLAST analysis using the ISFinder database (Siguier et al. 2006). For deletions, regions of 500 bp upstream and downstream of each individual IS were fused in silico. All reads were then mapped against these fused sequences. If a mapping yielded reads covering the fused region, it was identified as a deletion at that position.

2.6 | Development of an RFP-Reporter Strain

The gene for red fluorescent protein (RFP) under the arabinose promoter was amplified from the pMK-RQ-Ara_RFP plasmid of strain JG1459 using primers 4372 and 4373 (Table S2), which contain overlapping regions with 500 bp homology regions from ISSOD9 on the MP of *S. oneidensis*. The 500 bp homology regions with overlap to pMQ150 were amplified using primers 4360/4371 and 4374/4365 (Table S2). A PCR was performed to combine the homology regions and the RFP expression cassette into one DNA fragment. Plasmid pMQ150 was digested with BamHI and SalI and purified using Promega Wizard SV Gel and PCR Cleanup System. Gibson cloning was performed to integrate the *rfp* gene with the homology regions into the pMQ150 plasmid, and the cloning mixture was transformed into *E. coli* WM3064. The correct integration of the insert was verified using primers 41 and 42 (Table S2) and subsequently confirmed by Sanger sequencing. Integration into *S. oneidensis* was performed as previously described (Schuetz et al. 2009). The position of integration was determined by test PCRs using primers 4432, 4433, and 4373 (Table S2). Functional expression was confirmed by microscopy.

2.7 | Reduction of MMP Size

A plasmid based on the pBAD plasmid backbone was designed to contain all the antitoxin genes (AT) found on the *S. oneidensis* MP. All ATs were to be cloned into a single expression cassette under arabinose promoter control. ATs were identified using NCBI annotations and cross-checked with the TADB3.0 TA-finder identification tool (Guan et al. 2024) using standard parameters.

To construct the complete AT cassette, individual ATs were amplified with overlapping primers 4325–4334, 4339–4343 and 4356–4359 (Table S2) and later fused by PCR. Purification of the individual and fused products was performed using the Promega Wizard SV Gel and PCR Cleanup System. The full-length PCR product containing all 10 AT genes was ligated to the NcoI and PmeI linearised pBAD plasmid using Gibson assembly (Gibson et al. 2009). Correct integration was tested by PCR and confirmed by Sanger sequencing.

After assembly, pBAD_AT1-10 was transferred by electroporation into *S. oneidensis* cells containing the RFP gene on the MP, and colonies were grown on LB agar containing 50 μg/mL kanamycin and 5 mM arabinose. Colonies were selected when the RFP signal was absent after an appropriate incubation time (< 3 days) and transferred to a new LB plate with antibiotic and inducer. Tests for MP loss were performed by PCR at different positions on the MP using primers: 4284/8283; 4270/4269; 4268/4267; 4488/4489; 4432/4365 (Table S2). After 20 transfers, candidates with the lowest number of positive PCRs were selected for WGS.

2.8 | Data Availability and Genome Assembly

All sequencing data sets generated in this study have been deposited in the NCBI database under the BioProject accession number PRJNA1214554. In particular, the assembly of MR1.1 (JG1980; Table 1) was derived by metadata analysis of the sequencing data from the stress experiment (BioSample SAMN46377464). Similarly, MR1.1 with stop codon-induced ISSOD2 copies (JG1981; Table 1) was assembled from data obtained in the second stress experiment (BioSample SAMN46377465). ISSOD1 inactivation data are available as BioSample SAMN46377466 (JG2034). MP reduction data are included in BioSample SAMN46377467 (JG2045; Table 1). In addition, resequencing data for *S. oneidensis* MR1 (JG7; Table 1) wild type is available as an assembly (BioSample SAMN46377450) and raw sequencing data (BioSample SAMN46377449).

3 | Results

3.1 | Assessment of Transposase Activity

The aim of this study was to assess transposase activity, correlate it with transposase families and develop strategies to reduce this activity. To accurately assess transposase activity, *S. oneidensis* was exposed to three different stress conditions—UV, heat shock and sub-toxic concentrations of antibiotics. These conditions were chosen for their potential to accelerate TE transposition (Eichenbaum and Livneh 1998; Lartigue

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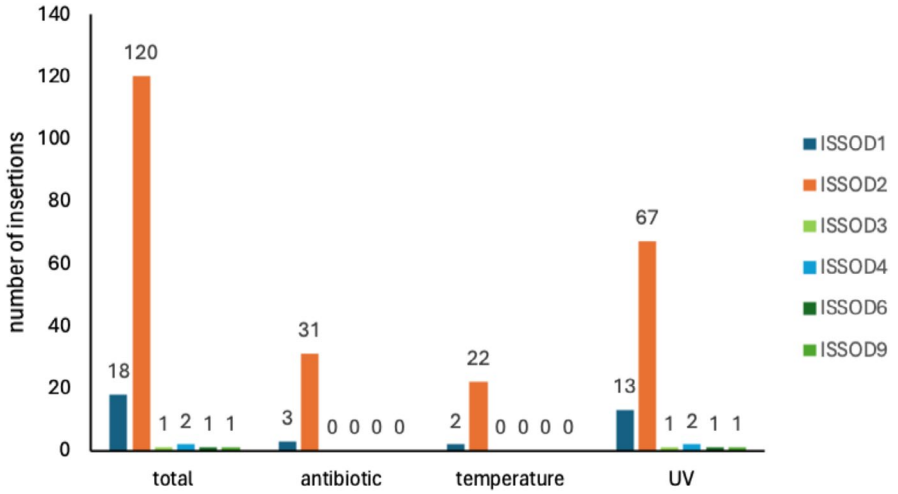

FIGURE 1 | The number of observed integrations of the ISSOD families in *Shewanella oneidensis* exposed to different external stress stimuli. Triplicates of cells were exposed to 15 repeating cycles of antibiotic, temperature or UV stress after which they were analysed by WGS to identify new IS insertions. The numbers and family of the respective IS are shown for the individual stressors and given as a total. The numbers represent the insertions as sum of a triplicate of each stressor. ISSOD1 and ISSOD2 are the predominant contributors to overall activity.

et al. 2006; Tanaka et al. 2012). The experimental design aimed to induce transposition events over 15 cycles, allowing the influence of individual stressors on IS family activation to be assessed. All endpoint triplicates and the control strain (with 15 transfers without stressor) were analysed by WGS and checked for transposition events. The resulting insertions caused by each IS family were counted and the total number of transpositions within each triplicate is shown in Figure 1. The number of insertions was used as a proxy for activity. Prior to the experiment, the *S. oneidensis* wild-type strain (MR1—JG7; Table 1) was inoculated in minimal medium containing ferric citrate as electron acceptor to be used as a preculture. Interestingly, we observed that even in the preculture strain, the copy numbers of several ISSOD families had already changed compared to the annotated genome sequence (NCBI RefSeq assembly ASM14616v2), suggesting spontaneous transposition activity prior to experimental stress induction (Table 2). This early activity highlights the dynamic nature of TE integration in *S. oneidensis*, even under non-stress conditions. We therefore refer to the progenitor strain of the stress experiments as MR1.1 (submitted to the NCBI databank as biosample SAMN46377464). Notably, one of the new insertions in MR1.1 corresponds to an integration observed by Cheng et al. (2020), the only difference being that the integration here is from the ISSOD4 family.

Remarkably, our results showed consistent patterns of activation across stressors, with ISSOD2 and ISSOD1 being the most active families regardless of the stress condition applied. Of all observed new insertions, 84.1% were due to ISSOD2 activity, whereas 12.5% were due to ISSOD1 activity. This finding is consistent with the additional IS copies in the precursor strain MR1.1 and previous studies reporting transposition events in *S. oneidensis*, all of which were due to ISSOD1- and ISSOD2-based events (Bordi et al. 2003; Schicklberger et al. 2013; Cheng et al. 2020). In addition, the control strain MR1.1 transferred 15 times without stress stimulus, accumulating 1 additional ISSOD1 and seven additional ISSOD2 copies. This suggests that these families show a stable transposition response, which may represent a general feature of genome dynamics in *S. oneidensis*.

TABLE 2 | Number of transposase copies of the respective families in *S. oneidensis* MR1.1 precursor versus the *S. oneidensis* MR1 wild type genome (NCBI RefSeq assembly ASM14616v2). The numbers correspond to full length TEs. The laboratory strain MR1.1 apparently obtained additional copies of IS before it was used for stress induction experiments.

	Strain	
ISSOD-family	**MR1—JG7**	**MR1.1_SV_ precurser—JG1980**
ISSOD1	42	44
ISSOD2	4	7
ISSOD4	48	49
ISSOD9	2	3

In line with our expectations, the number of integrations was also higher on the MP compared to the chromosome (Table 3). The highest number of new insertions occurred after UV irradiation, accounting for over 50% of the total number of integrations observed. All positions where integration was observed are shown in Figure 2. The positions of integration are distributed throughout the genome. Some hotspots of integration were found. In particular, a 60 kbp region on the MP showed repeated insertions. This could be explained by the abundance of IS and other non-essential genes in this region.

Throughout our analysis, we could only observe one deletion of an ISSOD6 and one deletion of an ISSOD22 IS copy.

3.2 | Adaptation to Stress Induction

To determine whether repeated stress exposure led to adaptation, we compared endpoint strains from individual colonies after stress treatment to the parental strain MR1.1 using triplicate 96-well plate assays in LB medium (Figure S3). We tested

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stress-acclimated strains against the ancestral MR1.1 by progressively increasing stress levels (UV exposure, kanamycin concentration, or temperature). In an antibiotic assay, cells remained viable at 7.5 mg/mL kanamycin—a concentration lethal to MR1.1. At 37°C, one strain entered the lag and exponential phases earlier than MR1.1, while another showed slower growth. Under UV exposure, viability remained comparable for up to 20 min of exposure, after which all UV-acclimated strains appeared more viable than the non-adapted MR1.1 (Figure S1).

3.3 | Stop Codon Conversion and Inactivation of ISSOD1 and ISSOD2

Having identified the most active IS families, we designed targets for the introduction of early nonsense stop codons into the transposases of both ISSOD1 and ISSOD2 using a CRISPR dCas cytosine deaminase. If the stop codons are successfully introduced into the IS reading frame, this should result in a truncated non-functional transposase, thus halting further expansion of an IS family once all copies have been converted. Notably, the high sequence similarity of the transposases in the families allowed the use of a single sgRNA to target all the transposase genes in a family. We used the progenitor MR1.1 (JG1980) to make the following experiments comparable to the insertion frequencies observed in the previous experiment. We observed the planned mutations in all ISSOD2 copies in the genome. To our surprise, whole-genome resequencing of four individual cultures after stop codon integration revealed that we had targeted eight instead of the seven ISSOD2 sequences previously identified in strain MR1.1 (NCBI biosample SAMN46377465). We therefore concluded that a further insertion occurred before the stop codons were introduced in strain MR1.1.

TABLE 3 | Distribution of all insertion events observed in the stress experiment between the chromosome and the megaplasmid. Remarkably, the MP, despite only representing 3.2% of the genetic information, is found to be affected by 20.5% of integration caused by TEs in *Shewanella oneidensis*.

	Insertions from total	Insertions total (%)	% of bp of pangenome
Megaplasmid	29	20.3	3.2
Chromosome	114	79.7	96.8

After confirming that all copies of ISSOD2 did indeed contain stop codons, we tested whether this modification was able to stop further transposition. We performed a stress experiment similar to Section 3.1 using oxic LB media instead of anoxic minimal media to reduce experimental run time as the focus was on ISSOD2 activity instead of mimicking BES conditions. A control without stop codons (MR1.1) was compared to a triplicate of ISSOD2 stop codon containing (ΔISSOD2) MR1.1 strains. These were subjected to 15 cycles of UV irradiation and reinoculation. At the end of these cycles, the strains were analysed by WGS and manual IS integration identification. It was found that the stop codon modification halted transposition, as indicated by the absence of ISSOD2 insertions compared to 14 insertions

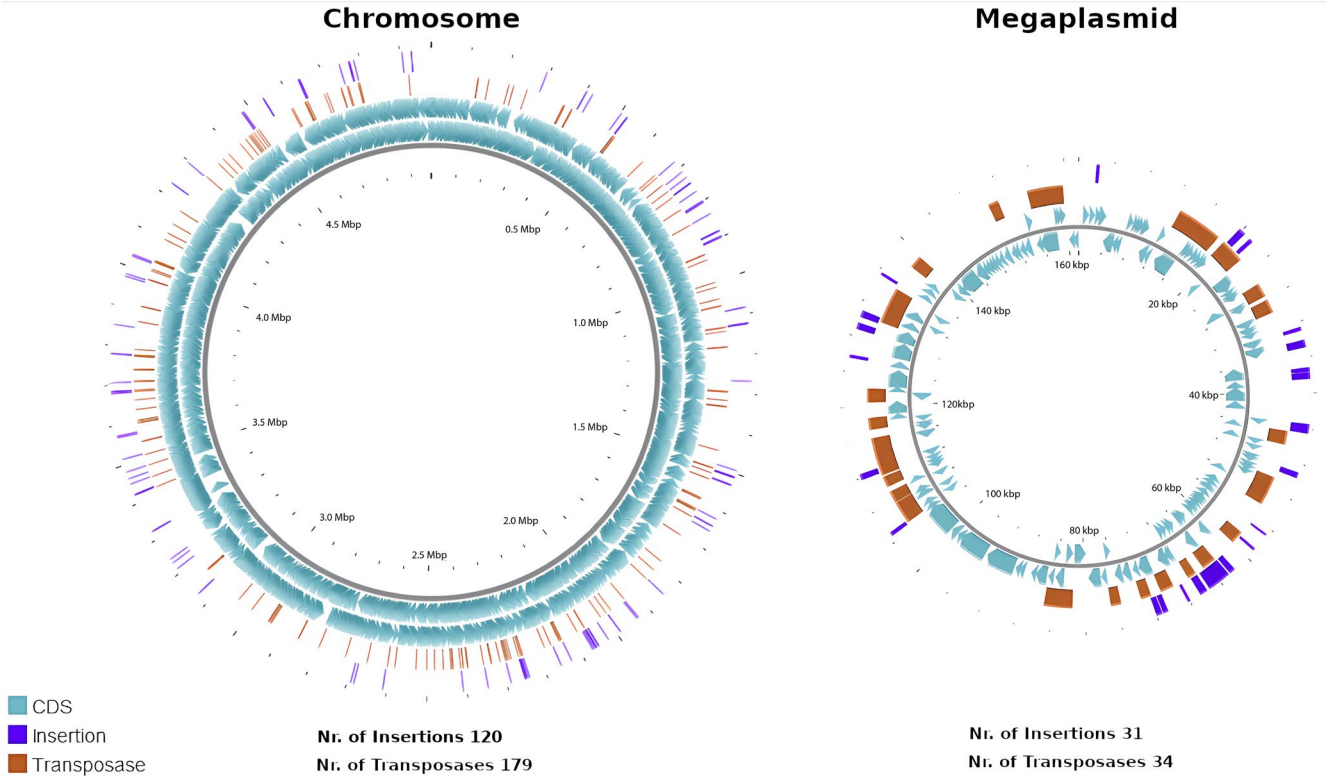

FIGURE 2 | Representation of the chromosome and megaplasmid of *Shewanella oneidensis* with all transposases and positions of integrations resulting from stress induction. The number of identified transposase copies in the precursor is given below and the positions are marked. Notably, the MP appears to be more prone to integrations compared to the chromosome when the respective sizes are considered. Some areas appear to accumulate more insertions, as can be seen for a region around 60 kbp on the MP termed hotspot area in this study (Table 4).

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TABLE 4 | Regions that were deleted during MP reduction with the respective size: A total of 55,716 bp could be deleted from the original MP size of 161,613 bp. The deleted areas were named after the feature that is most representative of the region. Below, the positions on the original MP are given.

Area	*Ori/repA*	Hotspot	RelA/ISSOD1	Tandem-95-repeat	Total	Megaplasmid SO MR1
Size (bp)	19,252	16,509	1933	18,022	55,716	161,613
Position on the original MP	[159947..17585]	[53121..16508]	[19054..20986]	[76764..94785]	—	—

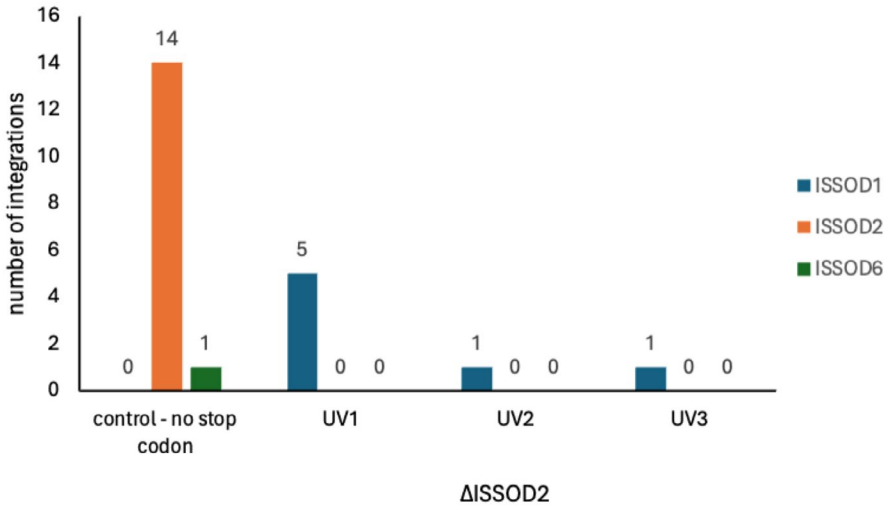

FIGURE 3 | Number of observed integrations after introduction of stop codons in the ISSOD2 IS-amily in a second stress experiment. Control and triplicate of ISSOD2 stop codon-containing strains were exposed to UV radiation (15 cycles) to aim at maximum transposition rate. As seen in the comparison to the control, ISSOD2 activity could be halted by introducing stop codons.

observed in the MR1.1 control under the same stress conditions (Figure 3). Here, the preference for integration into the MP was even more pronounced at 41%. These results clearly indicate that stop codon conversion effectively silences ISSOD2 activity.

For ISSOD1, we tried the same CAS deaminase approach in four individual experiments. Between 6 and 19 out of 43 transposase gene targets were mutated (NCBI biosample SAMN46377466). In all four experiments, a total of 75% of all ISSOD1 copies were converted. It was later found that in all four cases, ISSOD9 integration into the dCAS9 deaminase disrupted further conversion.

3.4 | MP Deletion Attempts

We tried to delete the MP to further stabilise *S. oneidensis*. A pBAD-based plasmid was developed containing all the antitoxin genes from the 10 TA pairs on the MP under an inducible promoter. The hypothesis was that the introduction of a plasmid containing the antitoxins would lead to the loss of the MP over time, as post-segregational killing should be prevented (Harms et al. 2018). An RFP reporter strain was developed in which the *rfp* gene was introduced into a copy of ISSOD9. This was used to track either loss or rearrangement at this position and thus changes in MP composition or mutation. We then introduced pBAD_AT1-10 and started a series of transfers to detect cells with deleted MP segments previously stabilised by antitoxin-toxin pairs. The AT cassette was continuously expressed throughout the experiment. Once the RFP signal was lost, further transfers were combined with test PCRs on the MP backbone until colonies were selected for WGS. Although we were unable to induce complete MP loss, we were able to reduce the size of the MP by almost 56 kbp to approximately 65% of its total size. The DNA segments that were deleted are shown in Figure 4. The sizes of the segments corresponding to the total reduction are listed in Table 4. In total, six TA gene pairs were removed from the MP (Seq data SAMN46377467).

Interestingly, we observed a complete loss of the ori/repA region, suggesting a separate, previously undefined ori on the MP. Although the loss of this region was surprising, we observed the situation where the ori/repA region was located on a separate DNA segment from the rest of the MP backbone in an independently available sequencing dataset (NCBI ref. ASM3384304v1). In addition, a resequencing of *S. oneidensis* MR1 wild-type (JG7; Table 1) performed for this study showed only a few reads corresponding to the ori/repA region and placed the rest of the MP on a separate contig in the assembly (assembly SAMN46377450). All other *S. oneidensis* WGS results yielded 3 contigs when assembled. One contig for the chromosome and two for the MP. Both of the resulting MP fragments contained a copy of ISSOD9. The current situation therefore suggests two possibilities for the *S. oneidensis* MP. Either this plasmid never existed, but was in fact two separate plasmids, and/or the two separate plasmids are the result of cointegrate resolution (see Figure 5). The Tn3 transposase uses a replicative

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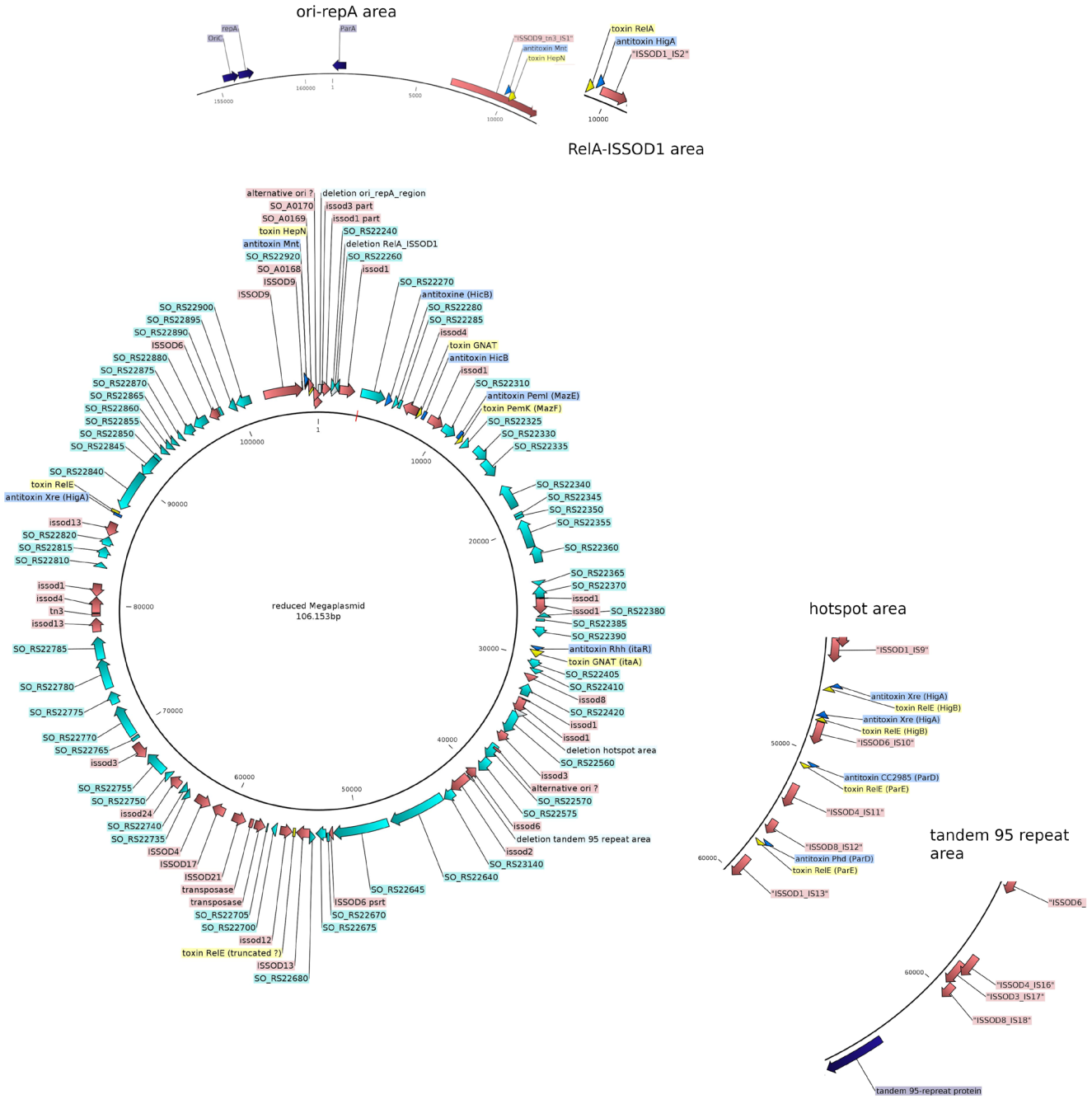

FIGURE 4 | The reduced MP with the regions that were successfully deleted. Parts were named according to their features. Positions of the fragments are placed approximately where they were residing on the MP before deletion. IS are highlighted in red, coding sequences (CDS) in turquois, toxins in yellow and antitoxins in blue. The tandem repeat being homologous to SO_RS22640 and SO_RS22645.

transposition mechanism ('copy and paste') in which the 3′ ends of the transposon and target DNA are first cleaved. These ends are ligated by the transposase activity and the resulting single-stranded DNA is converted to double-stranded DNA (dsDNA) by the host replication system. During this process, the donor DNA (e.g., the plasmid carrying the transposase) fuses with the target DNA, forming an intermediate product known as a cointegrate. In the final transposition step, the cointegrate is resolved by Tn3 resolvase through site-specific recombination between the original and newly created transposon copies. This resolution results in two separate replicons: the donor remains intact and the recipient acquires a new Tn3 copy.

To corroborate the hypothesis for two instead of one MP, we generated in silico replicons (as shown in Figure 5), potentially resulting in the 3 contigs observed in the sequence alignment. We then examined the reads spanning the flanking regions of ISSOD9 and identified uninterrupted reads crossing these boundaries. These reads do not align to an MP configuration in which ori/repA remains flanked by two ISSOD9 copies, supporting the possibility that the putative MP is in fact absent or an unstable replicon that may be subject to recombination into two separate replicons (see Figures S4 and S5). Interestingly, when we compare the coverage of the MP backbone to that of the ori/repA region in the combined metadata from all stress

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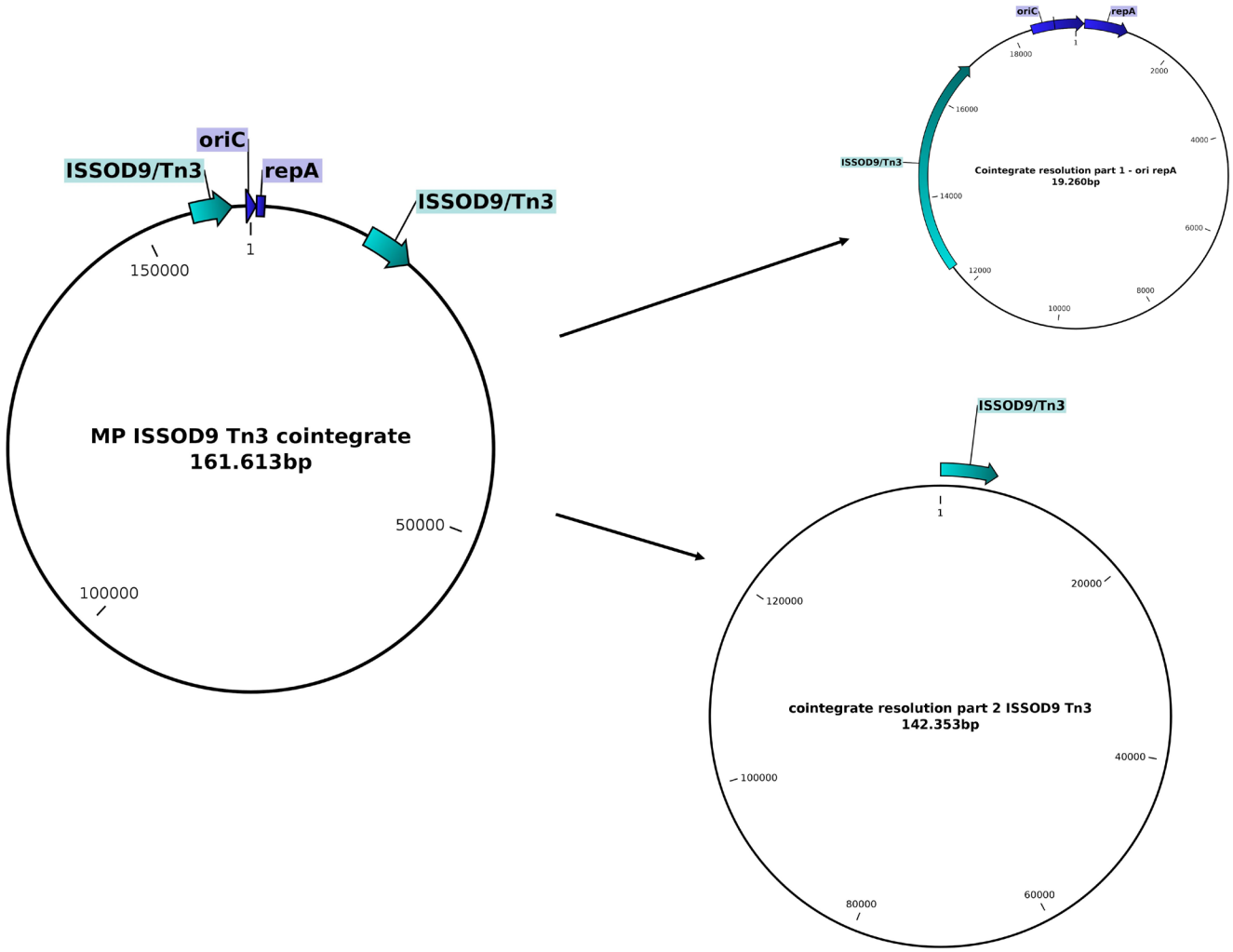

FIGURE 5 | Hypothetical configuration of a meta-stable MP precursor that might be resolved into 2 replicons. On the left side is the cointegrate that could have been formed by ISSOD9 transposition mechanism (copy in) of a horizontally transferred replicon. The resolution would fit multiple resequencing datasets and represent the final step in Tn3 transposition. The corresponding part 1 was lost in the MP reduction experiment. Independent of a cointegrate precursor, these two replicons could exist as separate entities.

experiment sequencing, we observe a significant difference: the ori region has a coverage of 0.45 ± 0.11, whereas the backbone has a higher coverage of 2.55 ± 0.17. This data corroborates again the presence of two plasmids which seem to be present in different copy numbers.

The loss of regions containing IS and AT pairs indicates that the expression cassette was at least partially functional. Analysis of the pBAD_AT1-10 plasmid after 20 transfers shows multiple insertions of ISSOD2 and ISSOD9. Therefore, at this point we cannot assess whether the complete loss of the MP was due to essential genes, incomplete expression of all ATs, or disruption of AT expression caused by integration of ISSOD2 or ISSOD9.

4 | Discussion

4.1 | Identification of Active ISSOD Families in *S. oneidensis*

In biotechnology, genetic changes can lead to non-productive strains, which pose a challenge to maintaining stable productivity—especially in continuously operating systems (Andrianantoandro et al. 2006; Czajka et al. 2020). In these cases, genetic changes within the bacterial chassis are often subtle and difficult to detect, resulting in productivity losses that may be inaccurately attributed to system parameters rather than genetic changes (Csörgo et al. 2012; Rugbjerg and Sommer 2019; Wehrs et al. 2019). An observation that underscores this issue in *S. oneidensis* is the disruption of the *flrA* gene by transposases identified here (ISSOD4 integration in the ancestral strain) and by Cheng et al. (2020). Deactivation of this flagellar cluster was found to affect biofilm formation in two independent studies (Thormann et al. 2004; Cheng et al. 2020). Without understanding the dynamics of transposition, it is difficult to predict or anticipate the potential impact on experimental results. As *S. oneidensis* is a promising candidate for applications in BES (Zhou et al. 2022), we focused on understanding the role of TEs on genetic stability in this model organism.

To assess transposition activity, we designed a stress-induced experiment to create a 'time-lapse' of transposition events occurring under 'non selective' growth conditions. The results were consistent across different stress conditions, highlighting

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ISSOD2 activity. Notably, resequencing of the strain after MP size reduction revealed a similar pattern with 70% of the transposition activity coming from ISSOD2 (data not shown). This suggests that the activity pattern is a general characteristic of *S. oneidensis*. Consequently, any long-term studies or applications should take into account the significant role of this family in the IS-driven genetic dynamics of this organism. The genetic manifestation of new IS copies was used as a proxy for transpositional activity, since their presence can only result from transposition or, much less likely, from a rare genetic duplication event. As transcriptomic and proteomic data are subject to numerous regulatory influences and do not necessarily reflect actual transposition activity, we considered genomic analysis to be the most reliable method for assessing IS activity.

To accurately capture low-frequency transposition events without the risk of information loss from traditional genome assembly or errors from transcriptional data of homologous elements (Lanciano and Cristofari 2020), we combined WGS with manual identification of new insertions. Although this method is labour-intensive, it allows the detection of single-read transposition events using only raw reads from nanopore sequencing. This approach, combined with manual filtering and mapping, should also reduce error rates. However, any transposition detection method may underestimate activity, as deleterious insertions may be lost in subpopulations due to reduced growth or lethal genetic alterations (Bourque et al. 2018).

4.2 | Transposition Activity and Inactivation of ISSOD Families

We investigated transposition activity by growing cells on ferric citrate to mimic conditions in BES. In a pre-culture without stressors, we detected five additional TE copies, highlighting the plasticity of the *S. oneidensis* genome influenced by TEs. By applying stress conditions, we increased the transposition event rates. Among all elements, the ISSOD2 and ISSOD1 families emerged as the most active under all tested conditions. Different stressors did not activate different IS families, but only affected the frequency of activation. Using a dCas9 deaminase system to inactivate the ISSOD2 transposase resulted in complete silencing of this family. Strains with stop codons in ISSOD2 showed no new insertions, in contrast to the MR1.1 control, which accumulated 14 insertions of ISSOD2 after UV irradiation. Considering the ratio of ISSOD2 to total IS activity, this should reduce total IS activity by 70%–84%. Our attempts to silence the entire ISSOD1 family met with limited success, although we were able to modify up to 19 loci; ISSOD9-based deactivation of the deaminase stopped further stop codon integration. We hypothesise that deleting or inactivating ISSOD9 would increase the success rate in this endeavour.

4.3 | ISSOD9 Interaction With Mobile DNA and Hypothesised Co-Integration

During stress experiments, ISSOD9 showed low activity, with only a single insertion observed. However, we found that ISSOD9 integrated into plasmids designed for ISSOD1 transposase inactivation and antitoxin production, suggesting that foreign DNA, particularly plasmids, can stimulate Tn3 activity. This finding is consistent with Tn3's dependence on the host replication machinery, particularly the beta-slide clamp interaction, for transposition (Tang et al. 2024). This suggests that ISSOD9 has a tendency to integrate into plasmids that would facilitate horizontal gene transfer.

This property has important implications for engineered strains, as plasmid instability due to TEs such as Tn3 can reduce the duration of continuous production of plasmid-encoded genes. In addition, the presence of ISSOD9 flanking the origin of replication region on the MP may actually represent a resolved cointegrated structure with two separate replicons, resulting from the transposition mechanism of the element (replicative transposition). The observation that Tn3 invasion on plasmids prevents further integration via a process termed transposition immunity further supports this hypothesis (Lee et al. 1983). However, at this stage it is also likely that there was an initial mis-annotation in ASM14616v2, where these two separate replicons were incorrectly combined due to the large overlapping homology of the ISSOD9 copies. Furthermore, the differences in coverage for the ori/repA region and the MP backbone in the sequencing metadata highlight the inconsistencies coming with the assumption of a single MP.

4.4 | MP Reduction as a Strategy to Increase Genetic Stability

Our results support the hypothesis that removal of the MP could genetically stabilise *S. oneidensis* with respect to IS activity. Although the MP comprises only 3.2% of the genome, it accounts for 20.5% of all integrations, a figure that increased to 41% in the second stress experiment. MPs are often acquired by horizontal gene transfer and typically lack essential genes, making them prone to high integration rates. Removing the MP could therefore also remove an infectious genomic region from which insertional elements could spread. Since the MP contains at least 34 TEs, its removal is expected to reduce genetic plasticity. In this study, we successfully deleted portions of the MP, including a significant 16-kbp region rich in ISs, toxin genes and other non-essential functions. This region also showed the highest integration frequency. Its removal coincided precisely with a region flanked by ISSOD1 elements, suggesting that it was excised by homologous recombination. Given its apparent role as a hotspot for IS integration and transposition activity, deletion of this segment is likely to increase genome stability by reducing transpositional mobility and recombination potential. Another deleted region also contained an AT pair linked to an ISSOD1 copy.

The MP appears to be stably propagated using TA systems, as no essential function has been ascribed to it. Therefore, we constructed a plasmid containing all identified antitoxin genes to reduce selection pressure. In addition, we introduced an RFP reporter system to monitor recombination events at ISSOD9, allowing the detection of recombination events by observing the RFP signal. We expected that bypassing post-segregation killing by antitoxin expression would allow the elimination of the MP backbone. Instead, we observed an unexpected loss of the ori/

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repA region. This finding, combined with the complete absence of ori/repA reads, suggests a secondary ori on the MP. Using OriFinder, we predicted two possible alternative replication origins near the parA gene at positions [30.067–30.252] and [105.1818 to 325] (positions on the reduced MP). If these alternative origins can be confirmed in subsequent studies, they may serve as targets for complete MP deletion.

Further analysis of the pBAD_AT1-10 plasmid revealed the integration of ISSOD2 and ISSOD9, rendering the plasmid physiologically inactive with respect to AT production – highlighting the importance of silencing these elements in any applied strain. It remains speculative whether complete loss was prevented by disruption of the AT cassette, resulting in loss of AT expression for the remaining four TA pairs. However, it cannot be excluded that the remaining modules—relE, hepN, GNAT, and pemK—are retained due to their integration into and function within the host cell regulatory network. These systems are known to interact with key cellular processes, particularly during stress responses or in the context of host defence (Bleriot et al. 2022; Griffin et al. 2013).

Overall, our study demonstrated the potential to stabilise the *S. oneidensis* genome by silencing the ISSOD1 and ISSOD2 transposases by continuous expression of a single sgRNA per ISSOD family. The study also showed that when using plasmid-based expression systems, special care must be taken with ISSOD9-catalysed events, which may require routine checking of plasmid stability even for short-term laboratory studies.

Author Contributions

Benjamin Fritz: investigation, writing – original draft, writing – review and editing, conceptualization, data curation, formal analysis, methodology, visualization. **Christian Jonas Lapp:** data curation, validation, writing – review and editing, formal analysis, methodology. **Johannes Gescher:** funding acquisition, project administration, resources, supervision, writing – review and editing, conceptualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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