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Eliza, M. orcid.org/0009-0009-7304-8455, Hipperson, H. orcid.org/0000-0001-7872-105X and Harrison, E. orcid.org/0000-0002-2050-4631 (2025) Different transcriptional impacts of prophage within the rhizobia-legume symbiosis. Symbiosis, 96. pp. 249-260. ISSN: 0334-5114

https://doi.org/10.1007/s13199-025-01075-w

Symbiosis (2025) 96:249–260
https://doi.org/10.1007/s13199-025-01075-w

ORIGINAL PAPERS

Different transcriptional impacts of prophage within the rhizobia-legume symbiosis

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Received: 7 April 2025 / Accepted: 6 August 2025 / Published online: 29 August 2025

Abstract
Prophages – viruses that infect and integrate into bacteria - have important effects for their bacterial hosts. These include fitness benefits, for example, through carrying adaptive traits or killing competitors, and costs due to regulatory disruption and cell lysis. Such effects can be dramatic, particularly when the bacterial hosts play key ecological roles, for instance, in pathogens where prophages can drive virulence and rapid adaptation. However, their role in non-pathogens, such as symbiotic bacteria where subtle changes may have outsized effects, has been overlooked. Here, we investigate the impact of carrying a prophage on the nitrogen-fixing symbiont, rhizobia. Rhizobia form symbioses with legumes in the form of root nodules. Their nitrogen-fixing ability has an important role in the transition to sustainable agriculture, potentially reducing the use of inorganic fertilisers. In this study, we show that prophage, vTRX32-1, has different effects on gene expression across two *Rhizobium* strains while in symbiosis with clover. While in one strain, the phage has no measurable impact whatsoever, in another strain, prophage carriage is associated with significant downregulation of many rhizobial genes, including those involved in nitrogen fixation. Moreover, phage presence has a positive effect on plant biomass at 4 weeks, which decreased slightly after 8 weeks. However, since these results were non-significant, they suggest that phage presence does not significantly affect the performance of the rhizobia-legume symbiosis. This implies that the rhizobia-legume system can be robust even in the face of prophage-induced disruption to cellular regulation.

Keywords Rhizobia-legume symbiosis · Prophages · RNA sequencing · Transcriptomics · Nitrogen fixation

1 Introduction

Prophages are bacterial viruses that integrate into the bacterial chromosome or on extra-chromosomal elements (Gama et al. 2013; Howard-Varona et al. 2017). The impact of prophages has been best described for host-associating (typically pathogenic) bacteria where they are known to impart benefits to their hosts in many ways (Paul 2008; Pfeifer et al. 2022); their acquisition can alter bacteria-host interactions by encoding novel traits, such as virulence factors, or drive rapid adaptation through mutagenesis (Gazitúa et al. 2021; Wang et al. 2022). They can also benefit their bacterial hosts by providing competitive advantage as 'biological weapons' against non-host related but phage-sensitive competitors (Harrison and Brockhurst 2017; Howard-Varona et al. 2017), particularly beneficial during colonisation (Brown, Inglis, and Taddei 2009). Finally, integration can provide resistance to superinfection of the same and related phages. While temperate phages provide many benefits, they, like other mobile genetic elements (MGEs) – genetic segments that can move between genomes (Frost et al. 2005) – can also incur costs to their bacterial hosts. These costs can be due to alteration of metabolic processes (Zhao et al. 2017), disruption of functional genes due to prophage integration (Coleman et al. 1991) or killing of individual host cells during induction (Harrison and Brockhurst 2017). In other cases, the vulnerability of prophage-infected cells can be exploited by competitors. This is evident by the production

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of Hydrogen peroxide by *Streptococcus pneumoniae*, which can induce prophages in *Staphylococcus aureus*, leading to bacterial lysis and subsequent displacement (Pericone et al. 2003; Selva et al. 2009). Phages have the potential to play important roles in bacterial ecology, with direct and indirect implications for their hosts. However, their impact in non-pathogenic systems is less well understood but of equal importance.

As one of the main and most efficient drivers of biological nitrogen fixation (BNF), rhizobia are essential components of the soil microbiome. Rhizobia interact with legumes to form symbioses in the form of root nodules where they exchange fixed atmospheric nitrogen for carbon compounds (Cangioli et al. 2022). As legume symbionts, rhizobia contribute to the global nitrogen cycle by providing 70 million tons of nitrogen per year globally to the agricultural industry (Brockwell, Bottomley, and Thies 1995; Zahran 1999). Rhizobia interact with many MGEs in their environment, which play an important role in rhizobial ecology and evolution (Heath et al. 2022; Wang et al. 2019). Rhizobial symbiosis plasmids, pSyms, and Integrative Conjugative Elements, ICEsyms, for instance, are a major characteristic of the Rhizobium clade (Wang et al. 2019), as they carry the genes that underlie nitrogen-fixing symbioses. MGEs have also been shown to benefit rhizobia in survival (Mercado-Blanco and Toro 1996), nodule competitiveness (Abdel-Salam et al. 2013; Brom et al. 2000) and stress resistance (Cytryn et al. 2008) in different environments when inside and outside the host. But MGE presence can also be disruptive in the symbiosis. For instance, loss of plasmid RP4 was shown to improve nitrogen fixation by *Rhizobium leguminosarum* bv viciae in peas (O'Connell et al. 1998) and curing of a *Mesorhizobium loti* strain of the plasmid pRlo2037 led to higher nodule competitiveness and nitrogen fixation (Pankhurst et al. 1986), suggesting interference of MGEs in these important processes.

While increasing numbers of prophages are being identified within rhizobial genomes (A Schwinghamer and Reinrardt 1963; Abebe et al. 1992; Dhar et al. 2013; Engelhardt et al. 2013; Ford et al. 2021; Halmillawewa et al. 2016; Joglekar et al. 2023; Sharma et al. 2019; Takahashi and Quadling 1961), their effect on legume-rhizobia symbioses is unclear. To date, no known accessory genes encoded by these prophages have been reported – though many genes remain unannotated. Other potential benefits for the host bacteria, such as competition between strains, are unlikely to be beneficial to the plants themselves as competition would reduce the diversity of potential symbionts. In contrast, prophages may disrupt rhizobial symbiosis by impacting cellular regulation or by resulting in a significant reduction of symbiont cells if prophages are actively entering lysis during nodulation and nitrogen fixation. Studies measuring the impact of prophages on the symbiosis, nodulation and nitrogen-fixing interactions of different rhizobia with their host plants show contradictory effects (Abebe et al. 1992; Dhar et al. 2013; Hatem, El -Sabbagh, and El -Didamony 2017; Uchiumi et al. 1989). For instance, studies on soybean infecting *Bradyrhizobium japonicum* (Abebe et al. 1992; Dhar et al. 2013) and *Rhizobium* infecting *Sesbania aegyptica* (Hatem et al. 2017) found no effects on symbiosis quality. However, Uchiumi et al. 1989 showed that lysogeny negatively affects nodule formation in *Rhizobium leguminosarum* bv trifolii (Uchiumi et al. 1989). Thus, the impact of prophages on the rhizobia symbiosis is unclear, and no study to date has investigated what impacts prophage carriage has on the bacteria itself during nodulation.

Here, we seek to explore the transcriptional impact of prophage carriage in nodulating bacteria. Phage vTRX32-1 is a widely infectious temperate phage isolated from a rhizobial symbiont, *Rhizobium leguminosarum* bv trifolii (*Rlt)* strain TRX32, found in a clover (*Trifolium repens*) nodule in York (Ford et al. 2021). Related prophages have subsequently been found across the world in different *Rhizobium* spp. showing its long evolutionary history with symbiotic *Rhizobium* (Ford et al. 2021). vTRX32-1 has been shown to have a wide host range within the *Rhizobium leguminosarum* species complex, and can form prophages in the vast majority of strains it infects (Ford et al. 2021). As a prophage, it is highly inducible with high rates of spontaneous lysis in broth culture (Ford et al. 2021). To investigate the effect of phage integration on symbiotic strains, we introduced the phage to 2 *Rhizobium* strains isolated from the same community – TRX19 and TRX4 (Kumar et al. 2015) – and measured (1) the transcriptional impact of the prophage in symbiotic nodules, and (2) their symbiotic quality. We find that phage impact on rhizobial gene expression varies widely for each rhizobial strain; in TRX19, the phage results in no measurable transcriptional impacts, while in TRX4, prophage carriage is associated with significant downregulation of rhizobial genes across the genome, including symbiosis genes. Despite this, prophage presence does not affect rhizobia-clover symbiosis in either strain.

2 Materials and Methods

2.1 Bacterial and phage strains

All experiments were conducted on *Rhizobium* strains TRX19 and TRX4, belonging to the *Rhizobium leguminosarum* species complex. These strains were isolated from the root nodules of white clover i.e. *Trifolium repens* in York (Kumar et al. 2015). TRX19 belongs to the species *Rhizobium ruizarguesonis* (Young et al. 2021), previously known as genospecies C (Kumar et al. 2015) and TRX4 belongs to the genospecies D (Kumar et al. 2015). The Average Nucleotide Identity values for both the strains lie between 92.4% and 94.6% (Kumar et al. 2015). Both the strains were isolated from the same soil community (within a 1m^2 area) as the original host of the phage, vTRX32-1 (Ford et al. 2021). They were selected for the absence of other inducible prophages (no response to mitomycin C treatment and no inhibition of growth when supernatant is spotted on a panel of other strains).

The strains were previously labelled with gentamycin-resistant markers and either Green Fluorescent Protein (GFP) or mCherry (MC) fluorescent markers using MiniTn7 (Mendoza-Suárez et al. 2020, 2021). Temperate phage vTRX32-1 (accession number MW023914.1) was introduced into the bacteria by applying 20 µL of 10^7 PFU/mL concentrated single phage cultures in the middle of the bacterial lawn. The plates were incubated for 72 h at 28°C. This allows the phage to naturally integrate into the bacterial genome. Bacteria which have acquired the phage are then able to grow within the zone of clearing despite the presence of the phage, allowing isolation of lysogens by streaking (Bobay, Rocha, and Touchon 2013; Gama et al. 2013). The phage insertion site is highly conserved within a tRNA leucine in the bacterial chromosome and integration was confirmed in lysogenic clones by PCR using phage specific primers targeting the conserved maturase B gene (forward - GTCGAGTGCTTGACCTCCTC, and reverse - AC CTCTTCTTGGTCGCTTCA) and a variable phage region which is specific to vTRX32-1 (forward- CAGTCCTGCC ACCTCAATGT, and reverse - ACGAAGAAATCCGTTG CCCT). Three independently established lysogenic clones were selected for the experiment. Cultures were grown in 6 mL TY (Tryptone −6 g/L, Yeast −3 g/L) liquid medium in 30 mL glass universals at 28°C in an incubator shaken at 180 rpm for 72 h.

2.2 Plant varieties and growth conditions

White clover plants (variety = Avoca, DLF seeds Ltd.) were grown in 1 L tricorn pots containing 900 g autoclaved vermiculite and sand mix (1:4 ratio). Seeds were sterilised by immersing and shaking in 3% bleach for 30 min at room temperature. The seeds were washed 4X with sterile water to remove any traces of bleach, spread on sterile filter paper and left to germinate for 5 days at room temperature. Single seedlings were randomly selected and placed in the tricorn beakers (Simport, United Kingdom). Rhizobia free nitrogen-negative (N-) and nitrogen-positive (N+) controls were included in all experiments. N + seedlings were additionally supplied with 0.01 mL of 0.83 mM/L ammonium sulphate solution every week. This concentration is similar to the volume of fertiliser applied to 1m^3 of topsoil at the rate of 0.5 kg/hectare as it has been shown to increase herbage production (Burchill et al. 2014). The tricorn pots were covered with autoclaved sun bags (Merck) which contain a 0.2 micron filter for gas exchange, and secured tightly to prevent cross contamination. A sterilised silicon watering tube capped with a 0.45 µm sterile filter (Millex™) was used for watering and feeding. Treatment pots were placed in a controlled environment chamber (16/8 hour day/night cycle at 22°C/20°C, 500 µmol/m^2/s or Photosynthetic Photon Flux Density (PPFD). The plants were grown as described and harvested after four and eight weeks with roots processed on the same day.

2.3 Transcriptional effects of lysogeny within symbiosis

To assess the impact of lysogeny on symbiont activity within the nodule, an RNAseq experiment was conducted.

Pots were set up with either non-lysogen (without the phage) strains, TRX19 or TRX4, or lysogen strains, TRX19v32 (TRX19 + vTRX32-1), or TRX4v32 (TRX4 + vTRX32-1). All strains were GFP labelled to maintain consistency and to reduce any background noise due to different fluorescent labels. Three biological replicates – bacterial clones – were grown per strain. Each was inoculated onto 3 three replicate pots planted with three clover seedlings which were pooled for sequencing. This experiment thus had the following design : 2 strains X 2 phage conditions (presence/absence) X 3 biological replicate clones X 3 replicate pots, for a total of 36 samples. The seedlings were then inoculated with 100 µL of 1×10^7 CFU/mL bacterial culture per plant, 20 mL sterile nitrogen-free Jensen media (Howieson and Dilworth 2016), and 30 mL sterile distilled water. The plants were grown as described above and harvested after 8 weeks. Roots were separated from shoots and washed in distilled water. Nodules from each replicate plant were collected separately to allow nodule counts and transferred to a sterile 2 ml eppendorf. Between 87 and 249 nodules were harvested per plant. Nodules were sterilised in 3% household bleach for 30 min to remove bacteria on the nodule surface, washed 3X in Phosphate Sucrose Magnesium (PSM) buffer (1 M K_2HPO_4, 1 M KH_2PO_4, 300 mM sucrose, 2 mM $MgCl_2$). The nodules from the 3 plants in each replicate pot were pooled and macerated using sterile pestles in 0.6 ml PSM and plant debris removed by centrifugation through 2 layers of sterile muslin for 2 min at 1000 rpm. 0.5 ml of supernatant was added to 1 mL RNA protect and incubated at room temperature for 5 min. Samples were centrifuged, supernatant removed and frozen at -20°C.

RNA was extracted on the following day from the nodule isolate pellets using Qiagen RNeasy mini kit. The pellets were resuspended in 350 µl RLT buffer and lysed in Tissue lyser II using 300 mg 0.1 mm silica and 100 mg 0.1 mm glass beads and centrifuged at 6000 rpm for a minute. RNA from samples was obtained using RNeasy columns following kit instructions. cDNA preparation and sequencing were performed by the Centre for Genomic Research, University of Liverpool. The sequencing library was prepared using ZymoSeq RiboFree Total RNA library preparation kit. The cDNA libraries were prepared and sequenced on the Illumina NovaSeq platform using random primers and S4 chemistry.

Raw sequence data was run through FastQC (Andrews 2010) and multiQC (Ewels et al. 2016) to visualise data quality. The data was further trimmed and filtered using Trimmomatic (Bolger et al. 2014). The trimmed and filtered data was aligned to the relevant reference genome (i.e. self-aligned) using HISAT2 (Kim et al. 2015; Pertea et al. 2016). TRX19v32-1 (with vTRX32-1) (accession number - PRJNA1190931) and TRX4 (accession number - PRJNA1176346) were used as the reference genomes HTSeq 2.0 (Putri et al. 2022) was used for absolute expression, these values were used to generate transcripts per million scores for each gene and assemble all the gene transcripts.

The RNA data was analysed using DESeq2 (version v1.34.0 and v1.46.0)(Love, Huber, and Anders 2014) in R. In addition, packages ggplot2 (version 3.3.5 and 3.5.1) (Wickham 2016), dplyr (version 1.0.8 and 2.5.0)(Wickham et al. 2021) and readr (version 2.1.2 and 2.1.5)(Wickham et al. 2024) were used for visualising data and reading files. Normalisation of data was done using DESeq2, which normalises the data by computing a scaling factor for each sample. This scaling factor is a median of the ratio of the read count of each gene in a sample and its geometric mean across all samples is called a size factor (Anders and Wolfgang 2010; Dillies et al. 2013). The commands used for the analysis are given in Supplementary text T1.

DESeq2 uses negative binomial distribution to calculate the means and variance of the two groups. It further uses the Wald t-test to calculate the adjusted p-values. To compare phage and chromosomal genes in lysogen samples, Trimmed Mean of M-values (M refers to gene-wise log fold changes) (TMM) normalised values from EdgeR (version 3.36.1 and 4.4.2) were used (Robinson, McCarthy, and Smyth 2009). TMM estimates the ratio of RNA production using weighted trimmed mean of the log expression ratios (Chen et al. 2008; Robinson and Oshlack 2010). We used TMM as it is usually recommended for comparisons within the samples (here, we compared lysogen samples) believed to have no differential expression, this is because EdgeR normalises the library sizes rather than the size factors as described above (Chen et al. 2008; Robinson et al. 2009) .

2.4 Measuring symbiotic effectiveness

To assess the impact of lysogeny on the effectiveness of the symbiosis we measured plant growth and nitrogen content in a separate pot experiment. 12 replicate pots were established per treatment with 6 replicates harvested at 8 weeks as above and 6 harvested at 4 weeks to investigate if potential effects are manifested throughout growth or only at later timepoints.

Clover seedlings were grown singly in separate pots and inoculated with 200 µL of 1×10^7 CFU/mL TRX19, TRX4, TRX19v32 or TRX4v32 cultures. Half of the replicates were GFP labelled and half were mCherry labelled to help identify possible cross-contamination, by confirming each pot contains the expected marker, and background noise, by ensuring all isolates are labelled. The negative control and positive nitrogen controls were applied with 200 µl of sterile water. This experiment thus had the following design: 2 strains X 6 biological replicates X 2 phage conditions (presence/absence) X 2 time points X 2 controls for a total of 72 pots. The plants were grown as described above. Roots were separated from shoots and washed in distilled water. Root nodules were carefully removed from the roots, washed and counted. Bulk soil from each replicate was transferred to falcon tubes containing root wash solution i.e. 10 mM $MgSO_4$ and 0.1% Tween and mixed with 4 g of sterile glass bead mix (diameters – 2 mm and 4 mm). Soil was vortexed for a minute, left for 30 min and vortexed again for a minute. The soil wash supernatant was diluted and plated onto TY agar plates supplemented with 3 ng/mL gentamycin and 1 µg/mL cycloheximide (to prevent fungal growth) and kept in incubator at 28°C. Root nodules from each replicate were collected and transferred to a 2 ml eppendorf. The nodules were sterilised in 2 ml of 3% bleach solution and shaken for 30 min at room temperature. The nodules were washed 6X with sterile distilled water and crushed into 0.5 ml of TY media (5 g/L yeast extract, 6 g/L tryptone, 0.5 g/L $CaCl_2$). The supernatant was diluted and plated onto TY agar plates supplemented with 3 ng/mL gentamycin and 1 µg/mL cycloheximide. The plates were incubated at 28°C for 72 h and CFUs counted. The soil wash and nodule wash results have not been presented here as there were no significant differences observed within the treatments. The roots and shoots were kept in drying oven at 60°C for 48 h and weighed.

Natural abundance of ^{15}N method was used to calculate the amount of Nitrogen fixed by rhizobia (He et al. 2009). % ^{15}N of each sample was measured, which is the absolute number of ^{15}N atoms in 100 atoms of total nitrogen. It is calculated as:

$$\% \ 15\text{N} = \frac{\text{N15}}{\text{N14} + \text{N15}}$$

% ^{15}N was converted into milligram of Nitrogen per gram of dry weight plant sample using the following equation:

$$\text{mg N/gm Dry weight} = \frac{\% \text{ N X 10}}{\text{Dry Weight of Plant in grams}}$$

To measure the total Nitrogen and Carbon: Nitrogen ratio, dried shoot material from each treatment was used for Isotope Ratio Mass Spectroscopy (IRMS). Each sample was powdered in Qiagen tissue lyser II using 5 mm tungsten beads. The powdered sample was weighed and packed in a 51 mm X 51 mm tin foil. The tin foil was closed tightly into a pellet and sent for IRMS sampling to Faculty of Science Mass Spectrometry Centre, University of Sheffield. The sampling was done using an ANCA GSL 20–20 Mass Spectrometer made by Sercon PDZ Europa (Cheshire).

2.5 Statistical analyses

The plant dry weight, total shoot nitrogen and carbon to nitrogen ratio data were analysed using linear models i.e. lm (x ~ y, data) and groups were compared using fit.contrast(). Specifically, pairwise comparisons within each strain were done i.e. lysogens and non-lysogens from each strain was compared. The package limma (version 3.50.0 and 3.62.2) (Ritchie et al. 2015) was used to do the analyses. All statistics were performed in R studio - R version 4.1.3 and 4.4.2 (R Core Team 2021).

3 Results

3.1 Transcriptional effects of lysogeny are strain specific

Differential gene expression of TRX19v32 and TRX4v32 relative to their non-lysogenic controls showed very different responses driven by phage carriage between these two strains. In TRX19, out of the 3560 annotated genes, no genes were found to be significantly differentially regulated in response to the phage (Fig. 1 (a)). In TRX4, however, out of the 3494 annotated genes, 410 genes were found to be downregulated and 64 genes were found to be upregulated in response to phage carriage when Log_2FC was set to ≥ 0.5 and adjusted p value was set up to ≤ 0.05 (Fig. 1 (b)).

3.2 Functional analysis on differentially expressed genes

Differentially expressed genes in TRX4 were first investigated using Gene Ontology (GO) functional enrichment analysis to identify significant functional groups affected by lysogeny. In this study, the biological process terms for acetyl-CoA biosynthesis, SOS response/distress signal response and fatty acid biosynthesis were most significant while in the cellular component, cytoplasm associated genes were found to be enriched among differentially expressed genes. In molecular processes, magnesium binding as well as ATP and GTP binding were significantly enriched. In addition to these functional groupings, many individual genes involved in nitrogen fixation, nitrogen regulation as well as key symbiosis genes were altered. Full details of affected genes are given in Supplementary Table 1 with key genes of interest highlighted below. The average distribution

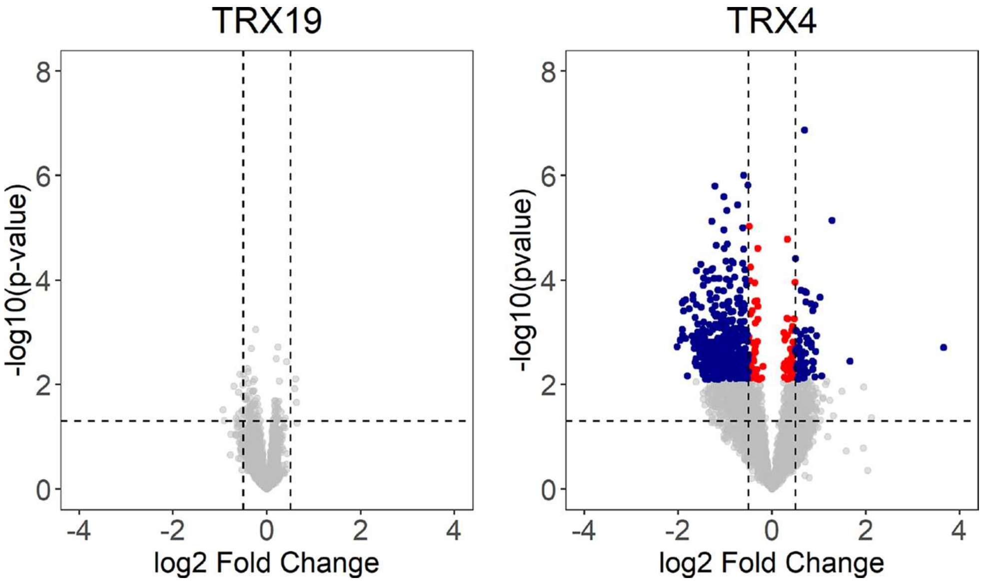

Fig. 1 : **Transcriptional effects of lysogeny are strain specific**. Transcriptional response to prophage carriage in two Rhizobium strains isolated from nodules - **(a)** TRX19, **(b)** TRX4. The x-axis shows Log_2 fold change while the y-axis shows –Log10 of p-value. The points represent individual genes with colour denoting significance: Grey shows non-significant genes; Red shows significant (p adjusted$\leq$0.05); Dark blue shows significant and differentially regulated (p adjusted$\leq$0.05, log_2fold change$\geq$0.5)

of differentially expressed genes and non-expressed genes across the genomes of TRX19 and TRX4 lysogens is shown in Supplementary figure S2.

3.2.1 Nodulation and nitrogen-fixing genes

Many genes involved in nodulation, nitrogen fixation and symbiosis were significantly affected by phage presence in TRX4. Calcineurin-like B protein producing *cbl* genes are Calcium sensors, which perceive NF (nodulating factors) signals and spike calcium ions (Arthikala et al. 2023). The *cbl* gene thus, plays a major role in the initiation of symbiosis and was upregulated, resulting in a Log_2 Fold Change (LFC) of 1.38. In contrast, *nodD2_1* and *nodD2_3*, which are copies of *nodD2* were significantly downregulated (LFC = -1.24 and LFC = -2.48, respectively). The *nodD2* gene plays a critical role in detecting plant-derived flavonoids by acting as transcriptional regulators to trigger the synthesis of other nodulation-related proteins (Ferguson et al. 2020; Schultze and Kondorosi 1998). Both changes in *cbl* and *nodD2_1* and *2* might be expected to increase the response of the bacteria to plant signalling. However, other copies of *nodD2*, *nodD2_3* and *nodD2_4;* and *nod* genes potentially regulated by NodD2 - *nodA*, *nodM* and *nodF* - were not differentially expressed.

Nitrogen fixation genes were also significantly downregulated. These include genes involved in the biosynthesis of nitrogenase and its associated complexes such as *nifB* (LFC = -3.27), involved in adding metal compounds to nitrogenase (Cebolla and Palomares 1994) and *fixP* (LFC = -2.69), part of larger nitrogenase-associated complexes. The *fixP* gene is specifically part of the membrane-bound cytochrome oxidase (Cebolla and Palomares 1994) that is essential for nitrogenase functioning. The gene *mopA (*LFC = -2.00) was also downregulated. *mopA* belongs to the *mopA-modABC* operon, part of the large nitrogen fixation gene cluster containing the structural genes of Mo-nitrogenase, *nifHDK* (Demtröder et al. 2019). In addition, key regulators were also found to be downregulated such as *nifA* (LFC = -3.31), a positive regulator of *fix* and *nif* genes and; *regA* (LFC = -2.95), part of *regB/regA* transduction synthesis, which may have an indirect role in the regulation of *nifHDK*. Finally, genes involved in the regulation of cellular nitrogen - *glnK*, *glnB* and *ntrC* (LFC = -1.98, LFC = -3.09 and LFC = -1.17, respectively), were also downregulated. In total, ~7% of the TRX4 genome was downregulated.

3.2.2 Additional Upregulated genes and their involvement in symbiosis

About ~1% (annotated genes) of the TRX4 genome was upregulated. Upregulated genes were involved in processes such as general stress response, nucleotide synthesis, protein synthesis, glutathione synthesis, amino acid synthesis, RNA synthesis, carbohydrate metabolism, chemotaxis and motility; ribose, potassium and C4-dicarboxylate transport. *pspA*, a phage shock protein had the highest upregulated value, LFC = 7.31. *pspA* is involved in maintaining membrane stability against stresses such as high temperature and phage infection (DeAngelis et al. 2019). However, not all significant genes associated with phage infection were upregulated; a phage-resistant gene, *wcaJ* (de Melo, Morency, and Moineau 2024; Tan et al. 2020), was significantly downregulated with LFC = -2.35.

Some of the upregulated genes found have been reported to be important for symbiosis - *iolB_2*, (LFC = 1.67) and *iolG_15* (LFC = 1.02), are involved in myoinositol catabolism and nodulation competitiveness of rhizobia (Fry et al. 2001). Similarly, adenine utilizing gene, *ade_2*, (LFC = 1.26) has been found as important for the nodulation of roots (George and Robert 1991).

3.2.3 SOS response/distress signal

Apart from some general stress proteins - *gspA* (LFC = -1.65), *msrA* (LFC = -2.59), *oxyR_4* (LFC = -1.99) and; heat shock proteins - *hrp1* (LFC = -2.72), *hspQ* (LFC = -3.77), *hrcA* (LFC = -1.27), many SOS genes were also downregulated.

The genes, *recA* (LFC= -3.35), *lexA_1* (LFC = -2.61) and *lexA_2* (LFC = -3.26) are central components of the SOS response and responsible for DNA damage repair. RecA is an inducer of LexA, which represses the expression of SOS response genes but has also been shown to regulate MGEs, including phage expression. Both, RecA and LexA are known to play a major role in maintaining lysogeny in pathogenic bacteria (Burrus and Waldor 2003; Waldor and Friedman 2005). Down-regulation of *lexA* would be expected to increase the expression of SOS and phage genes resulting in lysis. However, *uvrB* a central gene involved in DNA repair as part of the SOS response was also downregulated (LFC = -1.98), in contrast to the expected response to elevated LexA levels.

3.3 Phage and Chromosomal gene expression vary in the two strains

To assess the level of activity of phages in these two genomes, the normalised expression of phage genes within each lysogen genome was compared to the expression of non-phage genes using EdgeR. As TRX4 and TRX19 differ somewhat in gene content the genomes were aligned to both references, TRX19v32-1 and TRX4v32-1, separately to ensure the outcome was not dependent on the chosen reference. The expression of different phage genes within the genomes of the lysogens in the two strains was quite similar. Most of the genes expressed were hypothetical but others were structural. Some of these genes were highly expressed such as the gene producing DNA directed RNA polymerase while others were lowly expressed such as the gene producing the major capsid protein. There were also genes that lay in the middle such as genes producing an endolysin and an endonuclease. Moreover, only 24 of the phage genes were expressed in TRX19 as compared to 40 in TRX4. Some of the structural genes (such as the tyrosine recombinase and DNA directed DNA polymerase) present in TRX4 were thus absent in TRX19. Phage gene expression in TRX19 was significantly lower than the rest of the genome ($p \leq 0.05$) regardless of which reference genome was used. In comparison, in TRX4, phage gene expression was the same as – or, where the TRX4v32-1 genome was the reference, slightly higher than the chromosomal gene expression ($p \leq 0.05$) (Fig. 2).

3.4 Plant dry weight and total nitrogen fixed

3.4.1 Plant dry weight is not affected by phage presence

The biomass of four week and eight week old clover plants growing in N-free substrate was compared for the following treatments – phage free TRX19 and TRX4, phage carrying TRX19v32 and TRX4v32, rhizobia-free controls both with (N+) and without (N-) nitrogen. The total biomass i.e. shoot and root biomass, varied significantly among treatments after 4 weeks (Fig. 3 (a) : $p \leq 0.001$, $F_{5,27} = 6.78$) driven primarily by the N- control. In pairwise comparisons, all rhizobial treatments grew significantly larger compared to the negative control ($p \leq 0.05$) as well as the N+control ($p \leq 0.05$), except TRX4 ($p > 0.05$). Surprisingly, plants inoculated with lysogens grew, if anything, larger than those inoculated with non-lysogens.

However, pairwise comparisons using fit.contrasts() between rhizobial treatments found no effect of lysogeny for TRX19 (t = -0.54, $p = 0.59$) and a marginal but non-significant effect for TRX4 (t = -2.01, $p = 0.054$).

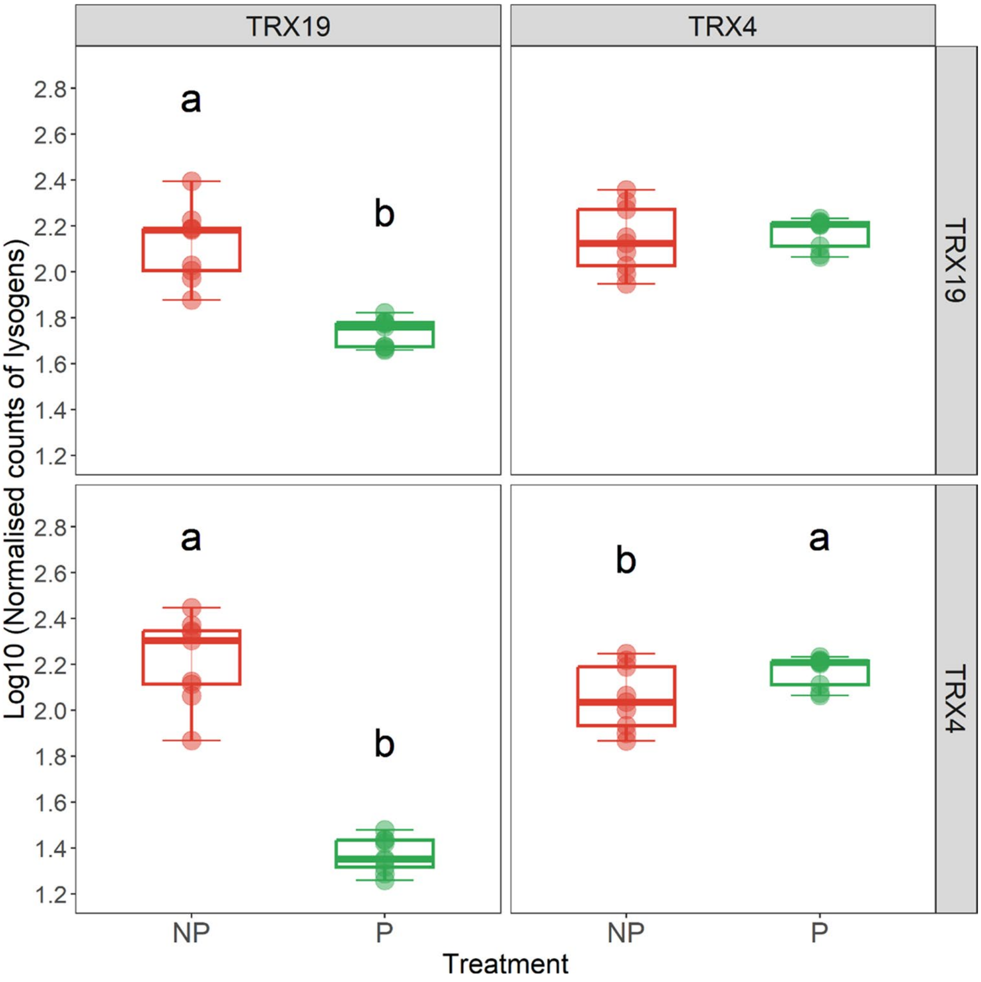

Fig. 2 : Expression of phage and bacterial genes significantly vary in the two strains. Average expression of phage and bacterial genes within TRX19 lysogens and TRX4 lysogens. The x-axis shows the different gene groups (phage and non-phage) while the y-axis shows the log 10 of normalised counts where normalised counts refer to Trimmed means of M-values (TMM). The top grey column titles show the strains whose genomes were compared while the right grey row titles show the reference genomes. Different alphabets denote significant difference

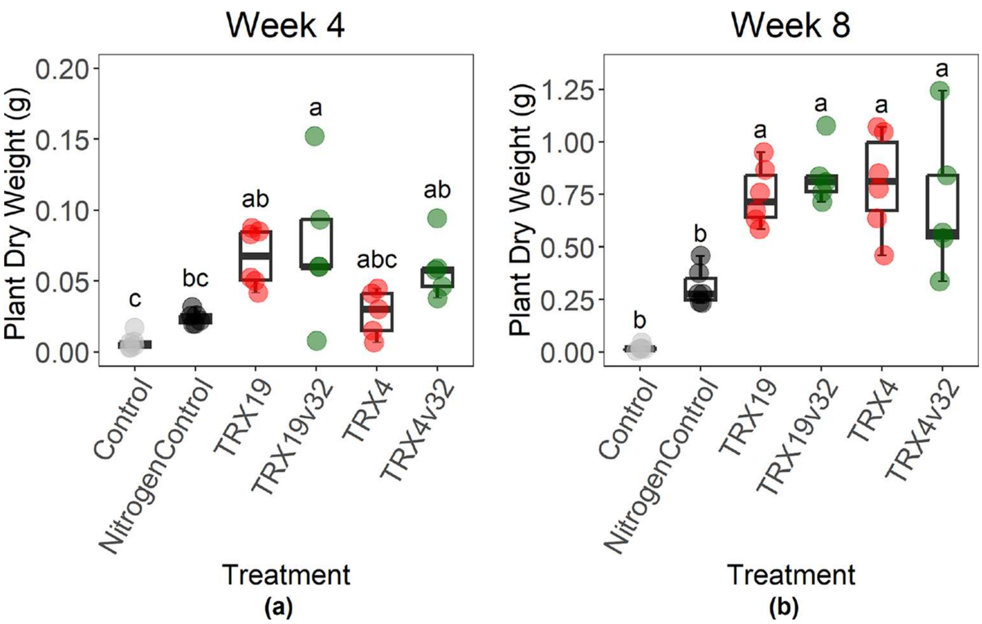

Fig. 3 : Presence of phage vTRX32-1 in the two rhizobium strains does not affect the plant biomass as compared to their non-lysogenic counterparts. The plant dry weight of control, nitrogen control, TRX19, TRX19v32, TRX4 and TRX4v32 after **(a)** 4 weeks and **(b)** 8 weeks. Different colours represent the different replicates within the treatments, grey shows negative control (N-), black shows rhizobia free nitrogen positive control (N+), red show phage free treatments while green show phage treatments. Different alphabets denote significant difference

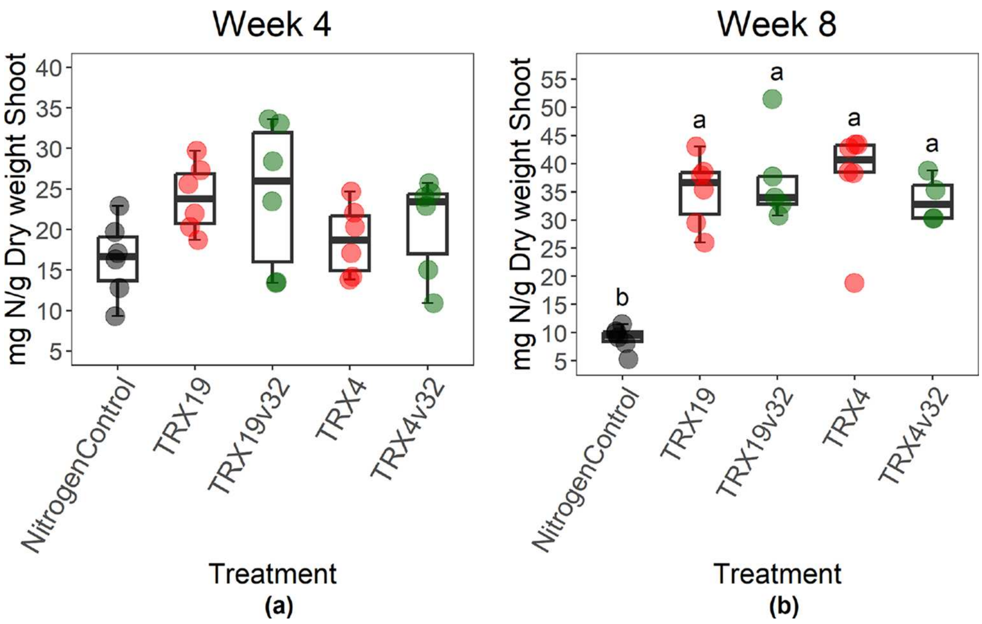

Fig. 4 : Nitrogen fixed by rhizobia is significantly higher than fertiliser treatments after week 8. Total nitrogen measured in nitrogen control, TRX19, TRX19v32, TRX4 and TRX4v32 as mg N/g dry weight after **(a)** 4 weeks and **(b)** 8 weeks. Different colours represent the different replicates within the treatments, black shows rhizobia-free nitrogen positive (N+) control, red shows phage-free treatments and green shows phage treatments. Different alphabets denote significant difference

These results were reversed in the 8 week grown plants, where lysogen-inoculated plants were on average smaller than non-lysogen inoculated plants – but again these effects were non-significant (Fig. 3 (b); $p > 0.05$). All the rhizobial treatments, including TRX4, were significantly larger than the N- and the N+controls ($F_{5,28} = 18.11$, $p \leq 0.05$).

3.4.2 Total shoot nitrogen in plants is significantly higher in rhizobium treatments after 8 weeks

Total shoot nitrogen in the plants was measured after 4 weeks and 8 weeks. Nitrogen positive controls were used as the controls in the models. There was no significant difference in the treatments after 4 weeks (Fig. 4 (a): $F_{4,25} = 1.905$, $p > 0.05$). After 8 weeks, however, there was a significant difference between all the rhizobial treatments and the N+control (Fig. 4 (b): $F_{4,22} = 19.08$, $p \leq 0.001$). The nitrogen added plants had 25% less nitrogen as compared to the rhizobial treatments. There was no significant difference ($p > 0.05$) observed in strain specific comparisons amongst lysogens and phage free treatments (Fig. 4). A similar pattern was observed for Carbon: Nitrogen ratio, which was higher for nitrogen control plants than those inoculated at 8 weeks ($F_{4,22} = 177.52$, $p \leq 0.0001$; Supplementary figure S1).

4 Discussion

The interactions of rhizobia with MGEs such as ICEs and plasmids (Wardell et al. 2021; Zahran 2017) are well studied but other MGEs still need further exploration. Here, we show that prophage vTRX32-1 can have very different impacts on their hosts depending on genotype. While in one strain, TRX4, major transcriptional effects on the host rhizobia were observed, the same phage produced no effects in another host, TRX19.

One possible explanation for this difference in outcome is in the level of phage gene expression in these two hosts. The majority of phage genes are tightly repressed during lysogeny and expressed only on entering lysis (Owen et al. 2020), thus expression of phage genes implies that phages are in active lysis. Under expression of phage genes relative to chromosomal genes in TRX19 implies the phage was more tightly repressed in this strain, while in TRX4 higher expression of phage genes indicates greater lytic activity in this strain.

Greater lytic activity would be consistent with changes in regulation in SOS gene, as observed here. The interaction between lysogeny and the RecA-LexA controlled SOS response has been well described in other phage systems and can both trigger (Sedgwick, Yarranton, and Heath 1981) and be triggered by phage lysis (Campoy et al. 2006). However, down-regulation of both *lexA* as well as its antagonist *recA*, makes it challenging to infer the outcome of these changes. Reduced LexA production is expected to increase expression of the SOS response, however, reduced RecA levels would be expected to buffer this effect by reducing the rate of LexA cleavage. High *recA* expression, through initiation of the SOS response or direct interaction with phage repressors has been shown to increase phage induction (Quinones et al. 2005; Sedgwick et al. 1981), Interestingly *uvrB* – a key SOS response gene – was also downregulated. This implies that overall, the SOS response was, if anything, dampened in TRX4.

The significant downregulation of many key symbiosis genes was also unexpected. Again, the direction of regulatory change makes the impact of these changes unclear. Downregulation of *nodD2*, for example would be expected to increase bacteria-plant signalling and nodulation rates (Ferguson et al. 2020; Honma et al. 1990; Schultze and Kondorosi 1998). Reduced lipid A biosynthesis meanwhile has been shown to delay nodule formation with smaller white, irregularly shaped nodules due to unstable cell membranes (Bourassa et al. 2017; Brown et al. 2011). And of course, downregulation of genes involved in nitrogenase functioning and nitrogen uptake suggests reduced symbiotic functions once in the nodule.

However, despite these changes in TRX4 – or lack thereof in TRX19 – we saw no significant impact of lysogeny on the outcome of the symbiosis between strains. It is possible that the high level of natural variance in plant growth experiments may have hindered evidence of marginal effects – the slight increase at 4 weeks and subsequent decrease in biomass of TRX4 lysogens relative to non-lysogens is intriguing. However, these subtle effects would remain in contrast to the extent of the changes in symbiosis-related gene expression. One possibility is that the transcriptional response seen in TRX4 represents a recent shift in the phage population prior to the sampling point rather than a persistent state throughout symbiosis. At 8 weeks post-inoculation (which is the typical timespan over which to measure symbiotic performance (Mendoza-Suárez et al. 2020; Westhoek et al. 2017)), plants are entering flowering and fruiting stage – representing a point of peak nitrogen fixation and it is possible that the effects of lysogeny in TRX4 would have been detectable later in the growth season.

This finding that the phage vTRX32-1 does not disrupt rhizobia-legume symbiosis has important implications in rhizobia research for expanding our understanding of the role of phage in rhizobial populations generally, and the potential of phages to enhance rhizobial inoculants. However, prophages could provide other advantages, for instance, in competition with other bacteria, that would make them useful agents for improving the success of inoculant strains. The absence of negative effects, in a range of bacterial hosts with very different interactions with the phage is key to ensuring that there are no negative impacts on the symbiosis. However, an understanding of the transcriptional responses, and their impacts on symbiosis over time is clearly needed to understand these effects comprehensively.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-025-01075-w.

Acknowledgements We thank Dr Heather Walker and Professor Duncan Cameron for helping with the Isotope Ratio Mass Spectroscopy.

Author contributions Conceptualization: Ellie Harrison and Mary Eliza; Methodology: Ellie Harrison and Mary Eliza; Formal Analysis and Investigation: Mary Eliza and Helen Hipperson; Writing: Mary Eliza; Writing-review and editing: Ellie Harrison and Helen Hipperson; Funding acquisition: Ellie Harrison; Resources: Ellie Harrison and Helen Hipperson; Supervision: Ellie Harrison.

Funding This work was supported by funding from the Institute for Sustainable Food, The University of Sheffield.

Data availability The reference genomes used in RNA sequencing, TRX19 (with vTRX321) and TRX4 were deposited in the NCBI BioProject database under the accession numbers PRJNA1190931 and PRJNA1176346, respectively. The phage vTRX321 data is available under the accession number MW023914.1. The raw data from

RNA sequencing, total Nitrogen, Carbon and dry weight is available on the European Nucleotide Archive under the accession number PRJEB82617.

Declarations

Competing interests The authors declare no competing interests to declare that are relevant to the content of this article.

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