Transcriptome modulations due to A/C2 plasmid acquisition

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ABSTRACT

Plasmids play an important role in driving the genetic diversity of bacteria. Horizontal gene transfer via plasmids is crucial for the dissemination of antimicrobial resistance genes. Many factors contribute to the persistence of plasmids within bacterial populations, and it has been suggested that epistatic interactions between the host chromosome and plasmid contribute to the fitness of a particular plasmid-host combination. However, such interactions have been shown to differ between bacterial hosts. In this study, RNA-Seq was performed in six different strains, spanning three species, to characterize the influence of host background on the A/C2 plasmid transcriptome. In five of these strains, chromosomal transcriptomes were compared in the presence and absence of A/C2 plasmid pAR060302. Host-specific effects on plasmid gene expression were identified, and acquisition of pAR060302 resulted in changes in the expression of chromosomal genes involved in metabolism and energy production. These results suggest that A/C2 plasmid fitness is, in part, dependent on host chromosome content, as well as environmental factors.

KEYWORDS

A/C2, RNA-Seq, Gene expression, HGT, Crosstalk

1. INTRODUCTION

Antimicrobial resistance among Gram-negative bacterial pathogens is a public health crisis (1). The relative lack of drug discovery and the rapid emergence of multidrug-resistant (MDR) bacterial pathogens is a truly alarming combination. Horizontal gene transfer (HGT) is a major contributor to the spread of genes conferring multidrug resistance. Understanding the factors driving the persistence of the elements facilitating HGT is crucial for the ability to better understand how to curtail the spread of multidrug resistance.

Plasmid family A/C2 is a group of large (~100~200 kb), broad-host-range plasmids that often carry genes conferring resistance to multiple classes of antimicrobials (2–6). They are commonly isolated from Gram-negative Proteobacteria associated with aquatic and terrestrial animals, food products and human infections (7–11). Because A/C2 plasmids commonly confer multidrug resistance, it is easy to understand how this plasmid type persists in environments under selective pressures such as antibiotic therapy. It has been proposed that this plasmid type may even require antimicrobial selection pressure to persist in microbial populations (12), however these experiments focused on only one host strain. The interactions between plasmids and host chromosome have been reported in other plasmid types (13–17). These studies have demonstrated that chromosomal background can be a significant factor in the maintenance of plasmids. Regulatory crosstalk between the broad-host range A/C2 plasmid type and its host chromosome has yet to be explored.

The present study attempts to better understand the A/C2 plasmid-host relationship through the use of total RNA sequencing (RNA-Seq). To determine if there were species-specific changes to

the transcriptome of broad-host range plasmid pAR060302, or if there were common changes to the chromosomal transcriptional program during plasmid carriage, we examined the transcriptome of 6 different strains, spanning three different species of Gammaproteobacteria, carrying pAR060302. We compared the transcriptome of pAR060302 in each host to that in the strain it was originally isolated in, AR060302. Finally, we compared the transcriptomes of the chromosomes of five strains with and without plasmid pAR060302.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

The bacterial strains used were as follows: *Escherichia coli* strain DH10B (DH10), *E. coli* strain AR060302 (AR060302) (4), *Salmonella. enterica* serovar Newport strain SL317 (SL317) (18), *Salmonella. enterica* serovar Heidelberg strain SL486 (SL486) (18), *Salmonella enterica* serovar Enteritidis strain MH16125 (18), and *Shewanella oneidensis* strain MR-1 (MR-1) (19). A nalidixic acid resistant mutant of each strain (except for DH10 (rifampicin resistant) and AR060302) was selected in the following manner. A new 5 mL aliquot of Difco™ Luria-Bertani broth (LB) was inoculated with 50 µL of an overnight culture. These were incubated at 37° C with shaking (200 rpm) and grown to an OD of 1.0. The cells were collected by centrifugation at 8872.5 x g for 10 min. The resulting cell pellet was resuspended in 100 µL of LB and spread onto a LB agar plate supplemented with naldixic acid (30 µg/mL). Resulting isolated colonies were further streak purified on LB agar with naldixic acid (30 µg/mL) and used as a plasmid host. Plasmid pAR060302 was moved by conjugation into each strain, except AR060302, which already harbors the plasmid. Successful transconjugants were selected for using LB agar supplemented with either naldixic acid (30 µg/mL) or rifampicin (100 µg/mL) and florfenicol

(16 µg/mL). All strains were grown in 10 mL LB broth aliquots at 37° C with shaking (200 RPMS), except MR-1, which was grown at 30° C, until an OD_{600} of 0.5 was achieved. Cells were pelleted and RNA was purified using a commercially available RNA extraction kit (RNEasy mini kit, Qiagen). Treatments were included to remove DNA contamination (Qiagen) and ribosomal RNA (MicrobExpress, Ambion). Two biological replicates for each strain were pooled for paired-end library sequencing (either 50 or 100 bp reads) via Illumina Genome Analyzer II at the Biomedical Genomics Center at the University of Minnesota. The details of each sequencing run are reported in Table S1. All of the sequencing data are publically available under the NCBI BioProject ID PRJNA273283.

2.3 RNA-Seq Analysis

All Perl scripts and other computational biology resources used in this study can be found at https://github.com/kevinslang. cDNA reads were first trimmed so that the quality at each base position was above 30 and then mapped to the appropriate genome or plasmid sequence (for pAR060302, GenBank accession no. NC_012692, for DH10, the *E. coli* K-12 MG1655 published sequence was used GenBank accession no. NC_000913, for all *S. enterica* strains the LT2 published sequence was used, GenBank accession no. NC_003197, for *S. oneidensis*, GenBank accession no. NC_004347 and its megaplasmid, Genbank accession no. NC_004349). Read mapping was done using BOWTIE (20). For each host, transcriptome maps of pAR060302 were constructed using Circos (21). To achieve this, a table was generated containing the average number of reads mapped per 250 bp of plasmid sequence. Each average was then normalized per 1 million total reads in the cognate sequence library. These averages were then log transformed and plotted as a line plot.

For statistical testing of differentially expressed genes, the total number of reads mapped to each coding sequence (CDS) was calculated using Perl. These values were then analyzed using the R package EdgeR (22, 23). We conservatively estimated the dispersion at 0.001. A fold-change cutoff of > 1 or < -1 and an adjusted p-value of < 0.05 were used to define significantly differentially expressed genes. Clusters of orthologous groups (COGs) analysis was done using the online tool DAVID (24, 25).

3. RESULTS

3.1 Analysis of the transcriptome of pAR060302 carried by different bacterial hosts

To understand how chromosomal background influences the pAR060302 transcriptome, a total of six strains, spanning three different species, carrying the plasmid were subjected to RNA-Seq. The sequencing reads were mapped to the pAR060302 reference sequence. Figure 1 shows the transcriptome map of plasmid pAR060302 while being carried by different bacterial hosts. Similar to what we have previously reported in *E. coli* strain DH5α (26), the genes most highly transcribed amongst all host species were resistance genes $bla_{\text{CMY-2}}$, *floR*, *strA*, *strB*, hybrid gene cassette *aadA2/1* and *aac*(3)-*VIa*, as well as the putative toxin-antitoxin module (locus tags pAR060302_0025 and 0026). Interestingly only 2 genes, *qacEΔ1* and *sul1*, were differentially expressed across all host bacteria (Table S2). Both genes were significantly down-regulated in all strains compared to their expression in AR060302. The genome sequence of strain AR060302 is not known and it can't be ruled out that these genes might be expressed from somewhere on the chromosome, as well as pAR060302. The plasmid pAR060302 carries 4 copies of IS*26*, which, given their similarity, could cause read mapping issues in our analyses. The expression of many

of the copies of IS*26* are significantly down-regulated in all strains except DH10B. Still, the overall number of reads originating from IS*26* transcripts are lower in abundance in these strains compared to AR060302. It is possible that strain AR060302 has copies of IS*26* somewhere on its chromosome that may contribute to the total number of reads aligning to copies of IS*26* on the plasmid. In either scenario, IS*26* may be an active player in gene transfer events in the original host AR060302. Conversely, in *S. oneidensis,* the putative transposase gene of IS*CR16,* and the transposase genes of IS*CR2* and IS*Ecp1* are all significantly up-regulated, suggesting that genetic rearrangements are possible in this strain via mechanisms apart from IS*26*. Also of note, an entire region of genes from locus tag pAR060302_0098-0133 is significantly up-regulated in strain SL486. Many of these genes have unknown functions and are dissimilar to any coding sequences in Genbank with known functions. A few of the coding sequences in this region do have predicted functions, most notably a phage-like site specific recombination operon, *ssb-bet-exo*.

3.2 Summary of chromosomal transcriptome changes due to carriage of pAR060302

A/C2 plasmids are large, broad host-range plasmids (6, 27, 28). To better understand how acquisition of such a plasmid would influence chromosomal gene expression we compared chromosomal transcriptomes in five strains with and without pAR060302. In order to characterize broad shifts in chromosomal gene expression, lists of differentially expressed genes were subjected to COG ontology analysis using DAVID (24, 25). Across all hosts, carriage of pAR060302 resulted in changes in expression of COGs related to oxidation/reduction reactions, cellular metabolism and/or metal cofactor binding (Table S3). This suggests that plasmid carriage alters the metabolic demands of cells, which has been reported as a general phenomenon

of plasmid carrying bacteria (29). Aside from central metabolism, the carriage of pAR060302 appears to change the expression of genes involved in localization, motility and adhesion in *Salmonella* strains, which may indicate a conserved interaction in that genus.

3.2.1 Specific pathways up-regulated in cells bearing pAR060302

The two main functional classes of genes upregulated in DH10 in response to carriage of pAR060302 were those involved in two-carbon and fatty acid metabolism and amino acid degradation (Figure S1). All genes involved in glycolate metabolism (*glcDEFGBA*) and glyoxylate cycle (*aceBAK*) were up-regulated. These two operons can be induced by either acetate or glycolate and are used to replenish pools of TCA cycle intermediates lost due to synthesis of amino acids (30). Similarly, in the case of MR-1, which lacks the *glc* operon, genes in the glyoxylate cycle are up-regulated (*aceBAK*). Other genes involved with acetate production were also up-regulated (*pta* and *ackA*). MH16125 exhibited increased expression of genes involved in transportation across the cell membrane and those involved with pathogenicity. Some of the transport genes are involved with movement of carbon sources (*wza*, *glpF*, *glpT* and *proV*). The other genes are all located on pathogenicity islands and are involved in type three secretion (*ssaJ, ssaK*, *ssaL*, *invH* and *sicP*), intermacrophage survival (*mgtB* and *mgtC*), and iron uptake (*iroN*). SL486 and MR-1 also up-regulated iron transport genes, although these are not associated with pathogenicity. SL317 exhibited increased expression of several amino acid biosynthesis genes as well as genes involved in metabolism of alternative carbon sources such as propionate (*prpR* and *prpE*).

3.2.2 Specific pathways down-regulated in cells bearing pAR060302

Several genes involved in the biosynthesis of various amino acids were down-regulated in DH10. Also, the *citCDEFXG* operon, which converts malate into acetate and oxaloacetate, was down-regulated. In MH16125, two operons involved in anaerobic respiration were down-regulated in response to plasmid carriage (*fdnI, fdnH, fdnG, narH, narJ, narG*). Several homologs of these anaerobic respiration genes were also down-regulated in MR-1. Whereas in strain MH16125 a few genes involved in pathogenesis were up-regulated, in SL317 and SL486 several genes spanning multiple pathogenicity islands were down-regulated. The functions of these genes include both invasion and type III secretion. In strain SL317 many genes involved in chemotaxis and motility were also down-regulated.

3.3. Summary of changes to genes carried on S. oneidensis megaplasmid due to carriage of pAR060302

S. oneidensis strain MR-1 stably maintains a 161,613 bp plasmid termed a "megaplasmid" (19). This offered the opportunity to explore potential interactions between genes carried on the megaplasmid and genes carried on pAR060302. We mapped the transcriptome of MR-1's megaplasmid in strains with and without plasmid pAR060302 (Figure 2). Surprisingly, the MR-1 strain used suffered a ~12 kb deletion on its megaplasmid which includes several ORFs including that of *repA*. This could mean either 1) the plasmid has integrated into the genome of MR-1 2) or the megaplasmid still exists as an extrachromosomal element that replicates via some alternative mechanism. Remarkably, few differentially expressed genes were observed. Most interestingly were those genesinvolved in toxin secretion (Table S4).

4. DISCUSSION

The purpose of this work was to characterize how the acquisition of broad-host-range A/C2 plasmid pAR060302 might change the transcriptional network within the host cell. In addition, we were interested in how the host chromosome might influence the transcription of genes on the plasmid. Analysis of the transcriptome map of pAR060302 revealed that there are chromosome-plasmid interactions that are specific for individual genomes. The differential expression in genes ORF0099-0133 on pAR060302 observed in strain SL486 and, in part, MH16125 and DH10, is curious as the genes in this region have received relatively little attention concerning their biological role. Once thought to be completely conserved, as more A/C2 plasmids have been sequenced this has proven not to be true (31). More work focused on the molecular function of genes in this region is needed to further elucidate the role they play in the biology of this plasmid type.

In order to analyze changes in chromosomal gene expression conferred by acquisition of plasmid pAR060302 we compared the transcriptomes of *E. coli* strain DH10B, *S. enterica* servar Newport, *S. enterica* servar Heidelberg, *S. enterica* servar Enteritidis and *S. oneidensis*, with and without the plasmid. We used gene ontology analysis to distill meaning from large lists of differentially expressed genes. It is clear that plasmid acquisition is altering the energy flux of the cell. This has been reported elsewhere, primarily in the biotechnology literature (29, 32). These studies suggest that plasmid containing cells require more oxygen and increase the rate of glucose uptake in order to generate the additional ATP needed to replicate the plasmid, and to express any genes that may be present on the plasmid. In the present study, all strains were grown in LB broth, which is nutritionally rich, but does not contain glucose. The vast array of

carbon-containing compounds available to cells may explain why expression of genes involved in metabolism was altered, but with no clear pattern when comparing all of the strains. Strains DH10 and MR-1 shared up-regulation of genes involved in glycolate metabolism and the glyoxolate bypass. These pathways are used to generate precursors for the TCA cycle (30). Conversely, the majority of the genes is these pathways were unchanged in the *Salmonella* strains tested in this study. Both MR-1 and MH16125 showed down-regulation of anaerobic metabolism genes, which suggests that A/C2 plasmid acquisition might also influence oxygen flux, but that the rate might be dependent on the host chromosome. Strain-specific metabolic effects has been reported for plasmid containing strains (29). These previous studies examining the effects of plasmid carriage on host metabolism have primarily been conducted using small cloning vectors. Many of the differentially regulated metabolic pathways contrast the results that we observed, indicating that there are likely plasmid-specific influences on the host chromosome. There is a clear need for understanding how large plasmids, such as A/C2, with many genes of unknown function, might be affecting host metabolism. Future experiments should focus on strictly defined media to better understand utilization of specific carbon sources. Work in this area might help elucidate new barriers to host range or explain fitness landscapes in natural environments.

In our experiments, many genes located on pathogenicity islands in *Salmonella* were differentially expressed upon acquisition of pAR060302. These effects were not consistent across all strains of *Salmonella*. Two studies have demonstrated the ability of A/C2 plasmids to mobilize genomic islands located on the *Salmonella* genome (33, 34). Burrus *et al*. confirmed that this is due to the positive regulators of A/C2 conjugative transfer, AcaDC. It remains to seen

whether or not A/C2 plasmids might also encode regulators that could alter pathogenic phenotypes, such as invasion or secretion. The activation or repression of virulence genes could be tied to other plasmid functions, such as replication. The scenario where a plasmid that confers resistance to multiple antibiotics and also alters expression of virulence genes is truly an alarming one. Future work is needed to further characterize the myriad of putative transcriptional regulators encoded on A/C2 plasmids.

Changes to host metabolic pathways observed in the present study beg the question: are these changes merely due to new energy requirements associated with plasmid acquisition or are they due to specific interactions of genes encoded on the plasmid directed at altering host gene expression? Plasmid pAR060302 carries several interesting hypothetical proteins that could directly mediate such interactions with chromosomal metabolic pathways: ORF0053 shares similarity to MhpR, an activator of phenylpropionate degradation; ORF0108 shares similarity with BglG (BglC), an activator of the sugar-transporting phosphotransferase *bgl* operon; and ORF0112 shares similarity to iron-sulfur cluster proteins involved in electron transport. Other plasmid types have been shown to encode novel catabolic proteins (35). It is tantalizing to consider that A/C2 plasmids may carry genes that might finely tune host metabolic pathways to reduce their fitness burden. Future work is needed to clearly define the function of these putative protein and substantiate these ideas.

5. CONCLUSIONS

(1) The results obtained from this study indicate that the expression of broad host-range plasmid genes is influenced by host chromosomal background.

(2) Plasmid acquisition alters host metabolic pathways and these alterations appear to be strain dependent. This is in response to the assumed increase in ATP required for DNA replication and gene expression.

(3) In *Salmonella* strains tested in this study, plasmid acquisition affects expression of genes located on pathogenicity islands.

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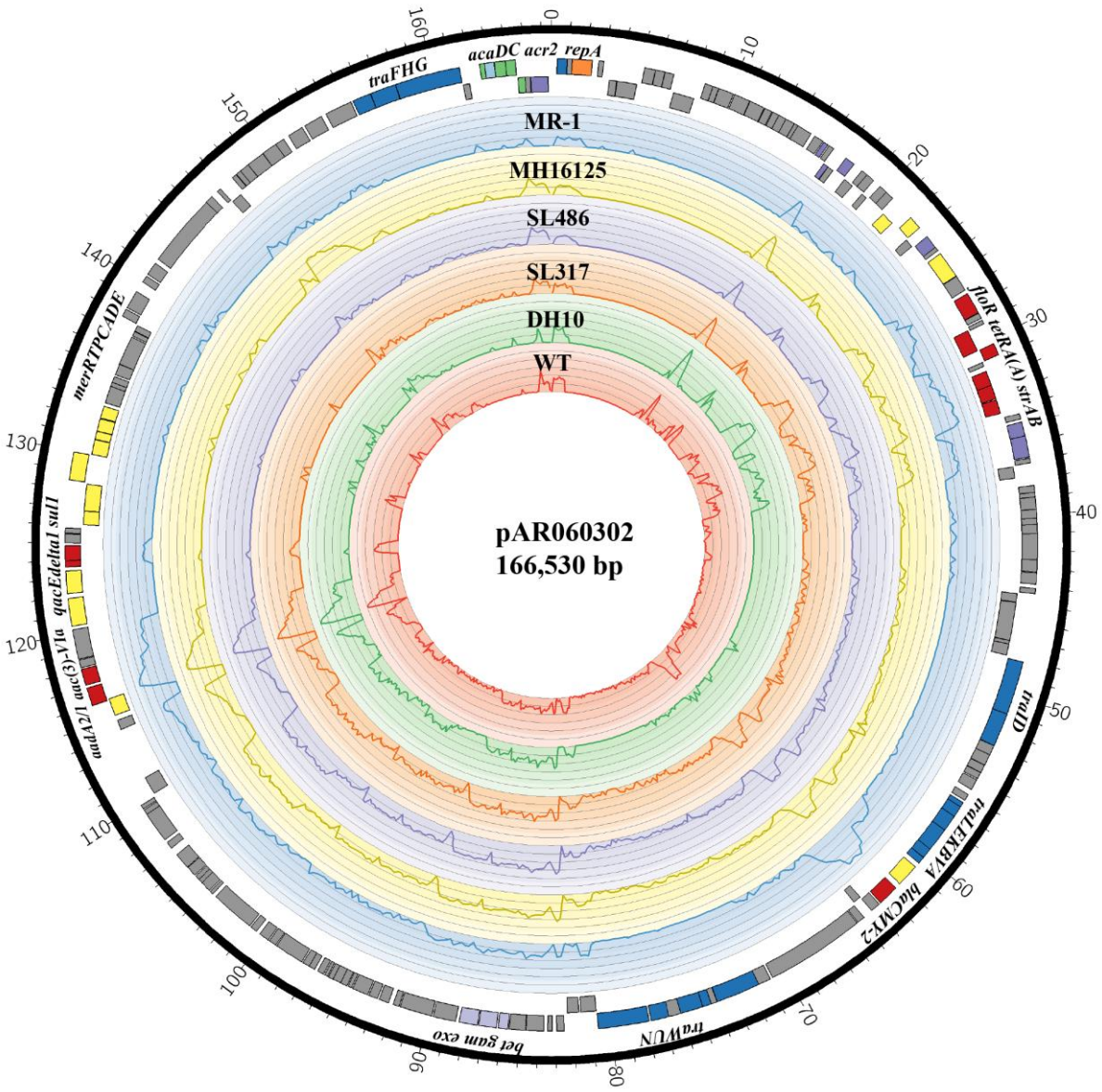
Figure 1. Transcriptome map of pAR060302 carried by different hosts. The rings from outside-in: 1) plasmid base pair coordinates; 2) gene map of pAR060302; 3-8) transcriptome maps of pAR060302 carried by *S. oneidensis* strain MR-1, *S. enterica* serovar Enteritidis strain MH16125, *S. enterica* serovar Heidelberg strain SL486, *S. enterica* serovar Newport strain SL317, *E. coli* strain DH10B, and *E. coli* strain AR060302. Genes on the map are colored by function: gray, hypothetical protein; red, antibiotic resistance; yellow, mobile genetic elements;

green, transcriptional regulation; light purple, recombination; purple, DNA binding; blue, conjugal transfer; light blue, transglycosylase; orange, replication. Line plots represent average reads aligned to 250 bp windows along pAR060302, normalized for sequencing library size and log-transformed. Each line on the transcriptome maps represents 0.5 log.

Figure 2. Transcriptome map of *S. oneidensis* megaplasmid in MR-1 with and without pAR060302. The rings from outside-in: 1) plasmid base pair coordinates (red indicating possible deletion); 2) gene map of the megaplasmid; 3-4) transcriptome maps of the megaplasmid in strain MR-1(pAR) (green) and MR-1 (red). Genes on the map are colored by function: gray, hypothetical protein; yellow, mobile genetic elements; light purple, recombination; orange, replication; red, plasmid partitioning; light blue, DNA restriction/modification; purple, toxin/antitoxin; blue, bacterocin related; green, lipoprotein; dark red, metal efflux; dark yellow, transcriptional regulator. Line plots represent average reads aligned to 250 bp windows along the megaplasmid, normalized for sequencing library size and log-transformed. Each line on the transcriptome maps represents 1 log.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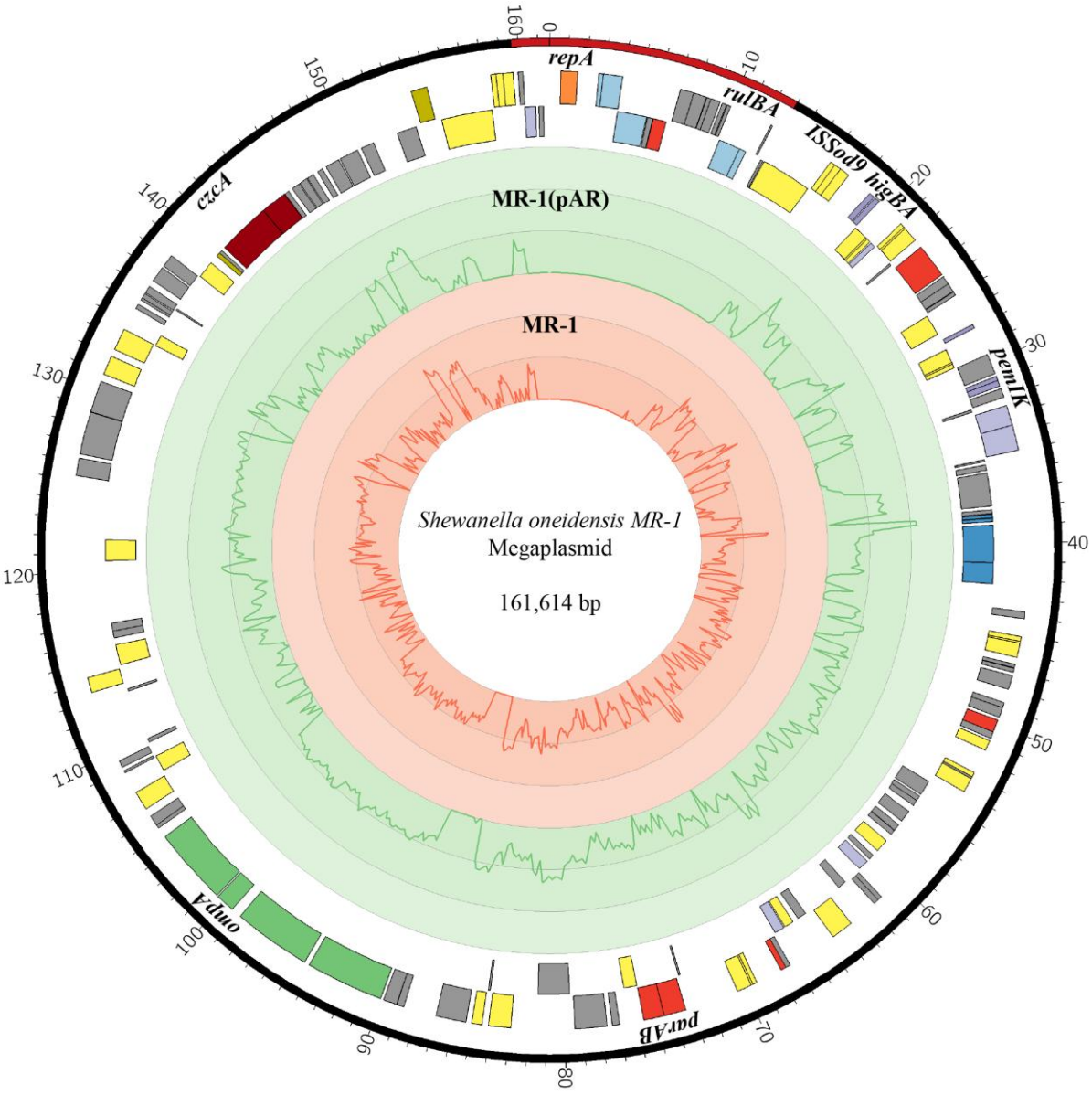
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