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Global transcriptomic response of the AI-3 isomers 3,5-DPO and 3,6-DPO in *Salmonella* Typhimurium

Authors: Claire Lallement, William P. D. Goldring and Lotte Jelsbak*

Department of Sciences and Environment, Roskilde University, Roskilde, Denmark

*Corresponding author email: ljelsbak@ruc.dk

ORCIDs: Claire Lallement: 0000-0002-3139-4570;

William P.D. Goldring: 0000-0002-8760-7491; Lotte Jelsbak: 0000-0002-6642-7424.

Abstract

Bacterial intercellular signaling mediated by small molecules, also called autoinducers (AIs), enables synchronized behavior in response to environmental conditions, and in many bacterial pathogens, intercellular signaling controls virulence gene expression. However, in the intestinal pathogen *Salmonella enterica subsp. enterica* serovar Typhimurium (*S.* Typhimurium), although three signals, named AI-1, AI-2 and AI-3, have been described, their roles in virulence remain elusive. AI-3 is a 3,6- isomer of a previously described *Vibrio cholerae* signaling molecule; 3,5-dimethylpyrazin-2-ol (3,5-DPO). To elucidate the role of AI-3/DPO in *S.* Typhimurium, we have mapped the global transcriptomic responses to 3,5- and 3,6-DPO isomers in *S.* Typhimurium. Our studies showed that DPO affects expression of almost 8% of all genes. Specifically, expression of several genes involved in gut-colonization respond to DPO. Interestingly, most of the affected genes are similarly regulated by 3,5-DPO and 3,6-DPO, respectively, indicating that the two isomers have overlapping roles in *S.* Typhimurium.

Keywords: Quorum sensing, enteric bacteria, DPO, AI-3, RNA-seq

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Introduction

Intercellular signaling mediated by self-produced small molecules, also called autoinducers (AIs), is a widespread bacterial communication system. Through the synthesis, secretion and sensing of AIs, bacterial populations are able to coordinate gene expression at the population-wide level in response to a specific signal. Similarly, bacteria are able to detect signaling molecules released from other organisms inhabiting the same ecological niche. This is beneficial in many aspects of bacterial life, including the coordinated formation of biofilms, motility and stress survival responses (Bassler 2002). Furthermore, in many bacterial pathogens several types of AIs play a pivotal role in orchestrating virulence (Rutherford and Bassler 2012; Sharma and Casey 2014; Herzog et al. 2019). The intestinal pathogens *E. coli* and *Salmonella enterica subsp. enterica* serovar Typhimurium (henceforth *S*. Typhimurium), employ three main signaling molecules, AI-1, AI-2 and AI-3, that play distinct roles in cell physiology (González Barrios et al. 2006; Soares and Ahmer 2011; Sharma and Casey 2014). For example, AI-3, a molecule that is also produced by the commensal intestinal microflora (Kim et al. 2020), has a prominent role in enteropathogenic and enterohemorrhagic *E. coli* by specifically inducing expression of virulence factors (Hughes et al. 2009). However, in *S*. Typhimurium, the role of AI-3 is less clear.

In both *E. coli* and *S*. Typhimurium the histidine kinase QseC conveys AI-3 signal perception to its cognate response regulators QseB (Bearson and Bearson 2008; Hughes et al. 2009). Still, specific binding of AI-3 to QseC has not been reported. This results in activation of gene expression targets, for example, motility genes *flhDC* and auto-induction of *qseBC* genes (Clarke and Sperandio 2005). QseC has also been reported to respond to

the host-produced signals epinephrine and norepinephrine pointing to a role in interkingdom signaling (Karavolos et al. 2008; Hughes et al. 2009).

A major challenge for studies of AI-3 signaling and the QseBC pathway was that the AI-3 structure was unknown. However, in 2020, AI-3 was assigned the structure 3,6-dimethylpyrazin-2-ol (3,6-DPO), a small molecule derived from threonine catabolism (Fig. 1a) (Kim et al. 2020). Interestingly, a positional isomer of 3,6-DPO, 3,5-dimethylpyrazin-2-ol (3,5-DPO), was described in 2017 as a new signaling molecule in *Vibrio cholerae* (Fig. 1a). In this enteric pathogen, 3,5-DPO downregulates virulence and biofilm genes through specific binding to the receptor VqmA (Papenfort et al. 2017; Wu et al. 2019).

S. Typhimurium is a major pathogen causing gastroenteritis in humans that in severe cases may develop into systemic infections. *S*. Typhimurium can inhabit a diverse range of niches including both environmental and numerous host habitats. The first step in human *S*. Typhimurium infections is colonization of the gastro-intestinal tract, home to a complex microbial community. This community offers numerous benefits to the host including niche protection through metabolite inhibition of invading pathogens (Sana et al. 2016; Jacobson et al. 2018; Sibinelli-Sousa et al. 2022). However, *S*. Typhimurium can employ several mechanisms to overcome this barrier. For example, *S*. Typhimurium can scavenge iron with the siderophores enterobactin and salmochelin (Raffatellu et al. 2009; Mey et al. 2021) . Another example is the Type 6 secretion system (T6SS) delivering cellular toxins to target cells thereby promoting *S*. Typhimurium gut establishment (Sana et al. 2016; Jurėnas et al. 2022). Furthermore, *S*. Typhimurium-induced inflammation triggers ecosystem disruption (Winter et al. 2010; Thiennimitr et al. 2012) by promoting a shift to

anaerobic respiration and catabolism of microbiota derived 1,2-propanediol and host-cell derived ethanolamine (Thiennimitr et al. 2011; Faber et al. 2017). Once established in the gut, *S*. Typhimurium causes severe diarrhea and may penetrate deeper into tissues resulting in invasive disease (LaRock et al. 2015). During transition between hosts, *S*. Typhimurium can survive and persist in biofilm communities with an extracellular matrix mainly composed of cellulose and protein (Steenackers et al. 2012).

To elucidate the role of AI-3/DPO in *S*. Typhimurium physiology, we report herein the global transcriptomic response to the 3,5- and 3,6-DPO isomers in *S*. Typhimurium. Specifically, we found that DPO affected almost 8% of all genes at the transcriptional level. The majority of affected genes were similarly regulated by 3,5-DPO and 3,6-DPO, indicating the two isomers are interchangeable. Interestingly, expression of several genes involved in gut-colonization responded to DPO. However, we did not find clear evidence for a role of QseC in response to DPO.

Methods

Bacterial growth and sampling for RNA extraction

Salmonella enterica subsp. *enterica* serovar Typhimurium strain 4/74 (Wallis et al. 1995) was used for this study. The virulence of this strain, originally isolated from the bowel of a calf, is well-defined (Jelsbak et al. 2012; Wallrodt et al. 2014; Søndberg and Jelsbak 2016) and it has been previously used for trancriptomic studies of *S*. Typhimurium (Kröger et al. 2013; Srikumar et al. 2015). An overnight culture of *S*. Typhimurium was diluted 100 fold in M9 minimal media (Miller 1972), pH=6 (48 mM $Na_2HPO_4 \cdot 7H_2O$; 22 mM KH_2PO_4; 8.6 mM NaCl; 18.7 mM NH_4Cl; 0.1mM $CaCl_2$; 2 mM $MgSO_4$) supplemented with 0.4 % glucose

and with either no DPO; 3,5-DPO; or 3,6-DPO (100 μM). DPO isomers were purchased from Sigma-Aldrich/Merck (Catalog numbers ATE413074467 and ENAH93E741A4, respectively). Cultures were grown until stationary phase at 37°C with shaking (150rpm). For RNA extraction, 2x1.5 mL volumes were sampled from each culture. Cells were centrifuged at 4°C, supernatant was removed and pellets were stored at -80°C until extraction of RNA.

RNA extraction.

Total RNA was extracted using the QIAgen RNeasy® Mini Kit (QIAGEN, Germany) kit following the manufacturer recommendations, as described previously (Jelsbak et al. 2010, 2012). Briefly, bacterial cells were lysed using the lysis buffer from the kit with addition of β-mercaptoethanol and 3-step sonication (15sec, 30sec and 15sec, with 30sec on ice between pulses) using the in-house instrument, Vibra Cell Sonicator, Sonic and Materials inc. with output 60%. Following spin-column purification, total RNA was eluted with RNAse free water. RNA concentrations were measured using the Nanodrop One™ (Thermo Scientific™) instrument. Integrity was tested using 2100 Bionalyzer (Agilent Technologies) following the Agilent RNA 6000 Nano kit (Agilent Technologies). RNA samples were shipped on dry ice to Novogene, UK (Novogene Co., Ltd.). Novogene conducted library preparation for paired-end 150 nucleotides directional mRNA sequencing reads on NovaSeq 6000, rRNA was removed. Q30>80%. A minimum of 2G raw data per sample.

RNA-Seq analysis

All data were processed in Geneious 2022.0.2 (https://www.geneious.com). Reads were mapped to the reference strain genome (Accession numbers for Chromosome: CP002487; Plasmid 1: CP002488; Plasmid 2: CP002489; Plasmid 3:CP002490).

Calculation of expression was conducted using CDS or manually annotated ncRNA referenced at the SalComMac website (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?db=salcom_mac_HL) (Srikumar et al. 2015). Using DESeq2 (Love et al. 2014), conditions were compared and a log2 of the fold-change (FC) was calculated. For this experiment, cut-offs were defined as a log2(FC) >1 or <-1 and with a *P*-value < 0.01 and confirmed that all Transcripts per Million base pair (TPMs) were above 10. RNA-seq analysis and data visualization were carried out in R v4.1.2. RNA-seq data was deposited in Sequence Read Archive under Bioproject accession number PRJNA923488.

Results and Discussion

Global response to DPO isomers in S. Typhimurium

During gastrointestinal infections, *S*. Typhimurium bacteria interact with members of the microbiome and metabolites produced in the gut, including DPO (Kim et al. 2020), however, the role of DPO/AI-3 in *S*. Typhimurium is still unclear. To investigate the effects of the AI-3 molecule 3,6-DPO, and its isomer 3,5-DPO, on global gene expression, isogenic cultures of *S*. Typhimurium wildtype were cultured in defined M9 minimal media alone or with 100μM of either 3,5-DPO or 3,6-DPO. It has been reported that both *E. coli* and *V. cholerae* can *de-novo* synthesize DPO in the presence of L-Thr (Papenfort et al. 2017; Kim et al. 2020). Therefore, M9 minimal media without amino-acid supplements was chosen as the growth condition to avoid confounding effects from the potential presence of

DPO in the reference samples. Transcriptomic differences between DPO-treated cultures and the reference were assessed by RNA-seq analysis. Treatment with either DPO isomer resulted in significant (P<0.01) Log2(FC)>1 or Log2(FC)<-1 fold regulation of 423 genes in response to 3,5-DPO, and 413 genes in response to 3,6-DPO, signifying a global response to DPO in *S*. Typhimurium (Supplementary Tables S1 and S2). The majority of genes were downregulated (387 CDS and 5ncRNA with 3,5-DPO and 359 CDS and 3 ncRNA with 3,6-DPO) in response to DPO (Fig. 1b;). This pattern of expression was also previously reported for the QseC transcriptome (Hughes et al. 2009). Interestingly, with a few exceptions, the majority of genes (355 genes) were similarly regulated by the two DPO isomers, with only 58 and 50 genes uniquely regulated by 3,5-DPO and 3,6-DPO, respectively (Fig. 1c) . However, for most of these uniquely regulated genes in one condition (for example 3,5-DPO), their regulation in the other condition (for example 3,6-DPO) is just below the thresholds (log2(FC) >1 or Log2(FC) <-1; *P*<0.01), and if these thresholds are slightly adjusted (Log2(FC)>0.6 or Log2(FC) <-0.06 and/or *P*<0.05), the number of uniquely regulated genes are reduced to 10 and 15 by 3,5-DPO and 3,6-DPO, respectively (Supplementary Table S3). Together these results indicate that 3,5-DPO and 3,6-DPO are operating through the same or overlapping signaling pathways. The majority of DPO downregulated genes are involved in metabolism (Fig. 1d and Supplementary tables S4 and S5). These include amino-acid metabolic pathways and carbohydrate metabolism. This pattern could indicate that DPO slows down overall metabolism resulting in a more sessile state. Interestingly, while *qseB* is moderately downregulated by both isomers (1.78 fold and 2.1 fold by 3,5-DPO and 3,6-DPO, respectively), expression of *qseC* is not affected (Fig. 1e). This could indicate that DPO does not act through QseC, as QseC activation by ligand binding upregulates expression of both *qseB* and *qseC* (Clarke

and Sperandio 2005). In concordance with this, the QseBC activated genes *flhDC* are not affected by DPO.

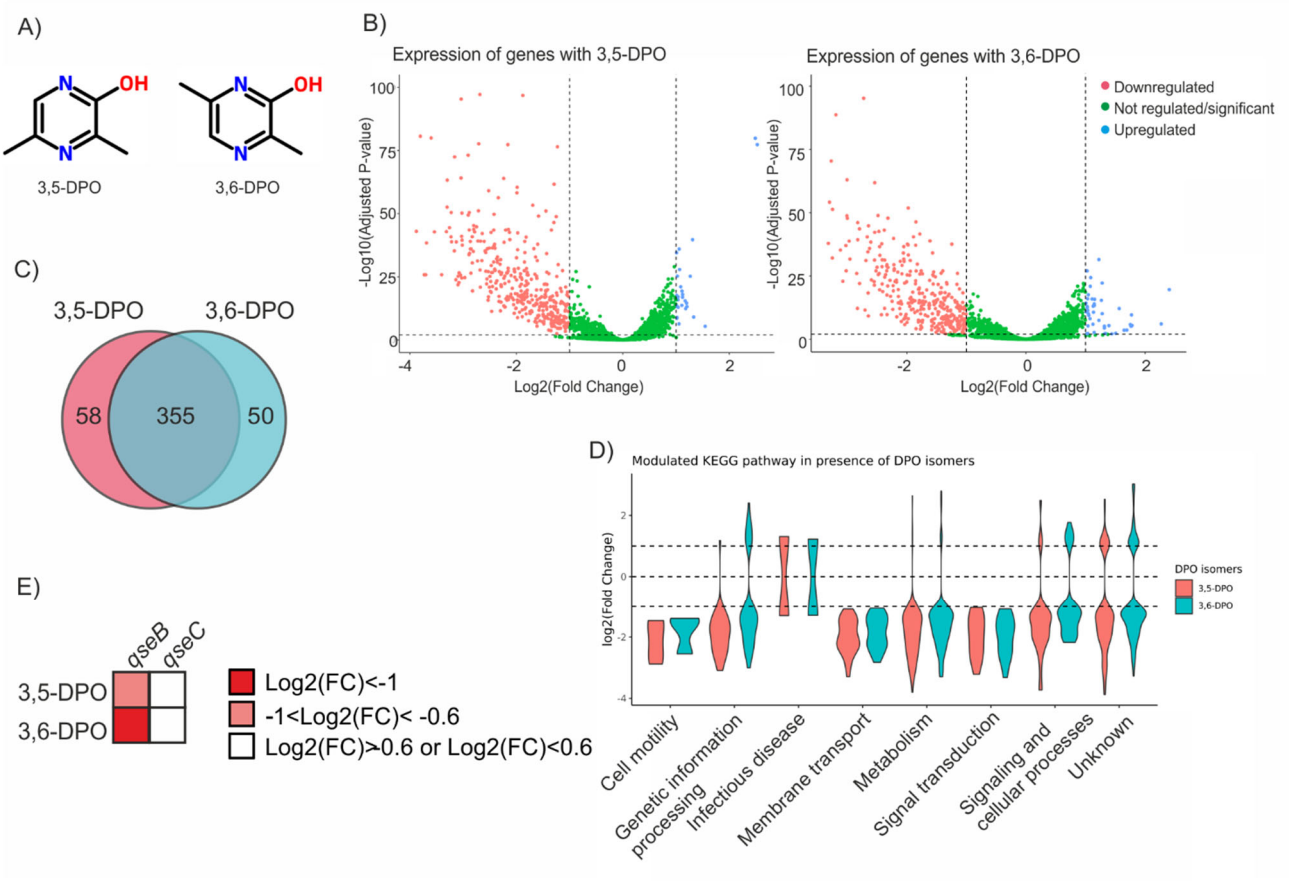

Fig. 1 DPO treatment differentially regulates gene expression in *Salmonella* Typhimurium. (**A**) Schematic of DPO-isomers 3,5-DPO and 3,6-DPO, respectively. Hydroxyl groups are marked in red, Nitrogen is marked in blue. (**B**) Volcano plots indicating differentially regulated genes when comparing wildtype (WT) + 3,5- or 3,6-DPO to WT without treatment. Horizontal dashed lines indicate *P*-value cut-off set to *P*<0.01 for significance. Vertical dashed lines indicate $\log_2$(FC) cut-offs set to $\log_2$(FC)< -1 or $\log_2$(FC)>1 for regulated genes. Red: Downregulated; Green: Not regulated; Blue: Upregulated. (**C**) Venn diagram comparing gene overlap between 3,5- and 3,6-DPO treatment, respectively. Red: 3,5-DPO; Green: 3,6-DPO. (**D**) Violin-plots showing KEGG pathways categories of DPO affected genes. Red: 3,5-DPO; Green: 3,6-DPO. (**E**) Regulation of *qseB* and *qseC* in response to 3,5- and 3,6-DPO, respectively. Dark red signifies downregulation with $\log_2$(FC)<-1. Light red signifies downregulation but with $\log_2$(FC) between -1 and -0.6. No fill signifies no regulation with $\log_2$(FC)>-0.6 or $\log_2$(FC)<0.6. *P*-values<0.01. Log2(FC) indicates log2(Fold Change)

DPO-isomers downregulate genes involved in gut-colonization and biofilm formation

Several of the DPO-regulated genes play important roles during the initial steps of gut-colonization and gut expansion (Raffatellu et al. 2009; Thiennimitr et al. 2011; Sana et al. 2016; Faber et al. 2017). These include genes involved in iron-uptake, competitive interactions (SPI6), and utilization of ethanolamine (*eut*-genes) and 1,2-propanediol (*pdu*-genes) (Fig. 2). Specifically, in response to either DPO-isomer we observed between 1.3- and 7-fold down regulation of the *fepABDGC* genes encoding proteins involved in the synthesis of the siderophore enterobactin and uptake of enterobactin complexed with ferric iron, the *iroBCDE* genes involved in uptake of salmochelin and the *fhuACDB* genes encoding the ferrichrome transport system (Fig. 2a, Tables S1 and S2). Iron uptake genes are induced by iron starvation and repressed by the Fur regulator if iron is not limited (Mey et al. 2021). Furthermore, several genes encoded on SPI6 involved in microbial competition by secretion of microbial and eukaryotic toxins were also affected by DPO (Fig. 2b). Specifically, the chaperone gene *clpV* (*sciG*), the toxin gene *rhs1* and the structural genes *sciA* (*tssA*), *sciC* (*tssF*), *sciS* (*tssM*), *sciT* (*tagF*), *vgrG* were 2.1- to 7.1-fold downregulated indicating a reduced activity of T6SS in response to DPO. The ability to utilize 1,2-propanediol and ethanolamine depends on two large horizontally acquired gene clusters, *pdu* and *eut*, respectively, encoding enzymes and structural proteins involved in the respective catabolic processes (Stewart et al. 2020). The *eut*- and *pdu*-operons are between 1.5 and 12-fold downregulated in response to either isomer of DPO (Fig. 2c and 2d). Together, these data suggest that DPO could be a signal for *S*. Typhimurium to downregulate gut-colonization genes and may thus be a signal for dispersion to a new host. Interestingly, DPO (and AI-3 activity) has been reported to be present in the gut

environment of healthy mice, thus DPO could be a signal for *S*. Typhimurium to downregulate gut-colonization genes (Kim et al. 2020).

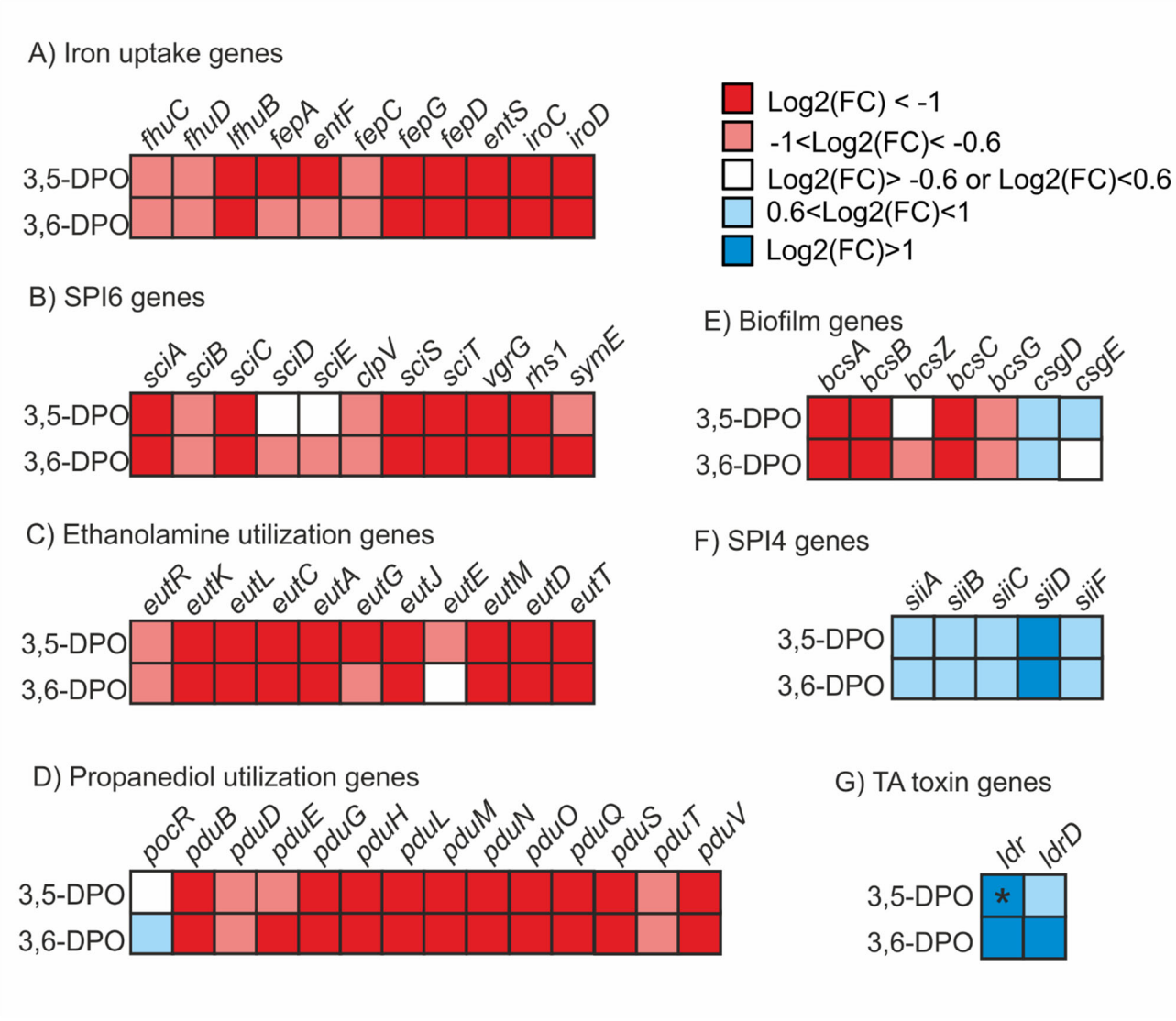

Fig. 2 Differentially regulated virulence genes in response to DPO. Significantly regulated genes in response to 3,5- and 3,6-DPO, respectively. Dark red signifies down regulation with log_2(FC)<-1. Light red signifies down regulation but with log_2(FC) between -1 and -0.6. No fill signifies no regulation with log_2(FC)>-0.6 or log_2(FC)<0.6. Dark blue signifies up regulation with log_2(FC)>1. Light blue signifies up regulation but with log_2(FC) between 0.6 and 1. (**A**) Iron-uptake genes. (**B**) SPI6 genes. (**C**) Ethanolamine utilization genes. (**D**) Propanediol utilization genes. (**E**) Biofilm genes. (**F**) SPI4 genes. (**G**) TA toxin genes. Asterix indicates that the *P*-value is between 0.05 and 0.01. Log2(FC) indicates log2(Fold Change). *P*-values<0.01 unless otherwise indicated

Genes encoding for the biosynthesis of cellulose for biofilm formation, *bcsABZCG*, were downregulated in response to DPO (Fig. 2e). This is a similar response as reported for *V. cholerae* where 3,5-DPO inhibited biofilm formation (Papenfort et al. 2017). In contrast, the SPI4 genes *siiABCD* and *siiF* were up-regulated in response to either DPO-isomer (Fig. 2f). These genes are involved in adhesion to the host-cell and is an essential first step in invasion of epithelial cells (Wille et al. 2014).

Two toxin genes (*ldr* and *ldrD*) were upregulated in response to DPO (Fig. 2g). These genes are part of bacterial Toxin-Antitoxin (TA) modules in which the toxin induces growth arrest that can be alleviated by expression of the antitoxin (Kawano et al. 2002; Harms et al. 2018). TA modules are linked to cellular dormancy and phenotypic antibiotic persistence in many species including *S*. Typhimurium (Cheverton et al. 2016).

DPO-isomers affect expression of ncRNAs

Non-coding RNAs (ncRNAs) play a central role in the swift adaptation to changes in environmental conditions by affecting translation or mRNA stability. In *S*. Typhimurium several ncRNAs have been linked to the regulation of virulence, biofilm formation, starvation response and more (Vogel 2009). Furthermore, the 3,5-DPO signaling response in *V. cholerae* occurs via regulation of ncRNAs expression (Papenfort et al. 2017). Our RNA-seq data reveal that expression of 27 ncRNAs in *S*. Typhimurium were affected by the presence of either or both DPO isomers (Fig. 3). Specifically, *ssrS*, *ryeF*, *spf* (spot 42) and *sdsR* (*ryeB*) were upregulated in response to DPO (Fig. 3a). *ssrS* and *ryeF* have roles in acid-resistance (Hobbs et al. 2010; Ren et al. 2017), whereas *spf* and *sdsR* promote mRNA stability of the virulence regulator *hilD* (El Mouali et al. 2018; Abdulla et al. 2022).

Of note, the expression of *hilD* itself is not modulated above the threshold (Log2(FC)=0.47, *P*<0.05) and neither are the known targets of HilD; *hilA*, *hilC* and *rtsA*. Downregulated ncRNAs included *isrG* and *sroA* (Fig. 3b). These have been implicated in *S*. Typhimurium intracellular survival and systemic infections, respectively (Santiviago et al. 2009; Ortega et al. 2012).

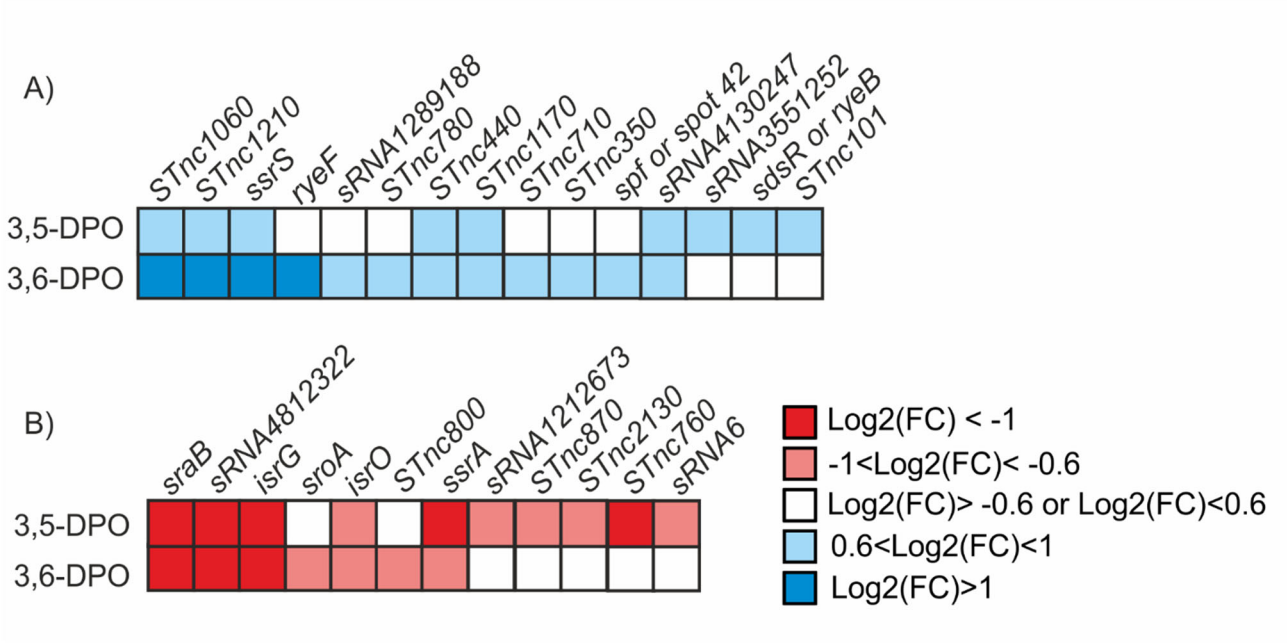

Fig. 3 Differentially regulated ncRNAs in response to DPO. Significantly regulated ncRNAs in response to 3,5- and 3,6-DPO, respectively. Dark red signifies down regulation with $\log_2$(FC)<-1. Light red signifies down regulation but with $\log_2$(FC) between -1 and -0.6. No fill signifies no regulation with $\log_2$(FC)>-0.6 or $\log_2$(FC)<0.6. Dark blue signifies up regulation with $\log_2$(FC). Light blue signifies up regulation but with $\log_2$(FC) between 0.6 and 1. (**A**) Up regulated ncRNAs. (**B**) Down regulated ncRNAs. Log2(FC) indicates log2(Fold Change). *P*-values<0.01

Conclusions

In the present study, we have mapped the global response in *S*. Typhimurium to the DPO-isomers of which 3,6-DPO was previously reported to be the elusive AI-3 in *S*. Typhimurium and *E. coli* (Kim et al. 2020), whereas 3,5-DPO was shown to function as a signaling molecule in *V. cholerae* (Papenfort et al. 2017).

Our results reveal that DPO has global effects on gene expression and show a large degree of overlap in response to the two isomers indicating that they operate through the same or overlapping pathway(s). The receptor for AI-3 in *E. coli* and *S*. Typhimurium was reported to be the cell-wall anchored histidine kinase QseC (Bearson and Bearson 2008; Moreira et al. 2010; Moreira and Sperandio 2012), whereas another study did not support this conclusion (Hamed et al. 2022). In *V. cholerae* the 3,5-DPO-receptor is the cytosolic regulator, VqmA (Papenfort et al. 2017).

Both DPO-isomers are expected to be freely diffusible across the cell-wall suggesting that the DPO-receptor is possibly located in the cytosol, as is the case for AI-1 receptors. Our data are inconclusive with respect to the role of QseC in the 3,6-DPO signaling cascade in *S*. Typhimurium.

It is interesting that many of the affected genes play a role in establishing *S*. Typhimurium infections in the gastrointestinal tract. For example, genes involved in metabolizing gut-associated metabolites, like 1,2-Propanediol and Ethanolamine are downregulated in response to DPO. Furthermore, genes involved in T6SS mediated intermicrobial competition are also downregulated, including several of the T6SS secreted toxins. Together these results indicate that DPO does not serve to increase virulence in *S*. Typhimurium by promoting gut-colonization, but rather functions as a microbiota produced signaling molecule that inhibits *S*. Typhimurium gut-infections or promotes the release of *S*. Typhimurium to the environment. This is similar to the role of 3,5-DPO in *V. cholerae*, where both virulence genes and biofilm genes are downregulated thus promoting release of *V. cholerae* to the environment (Papenfort et al. 2017).

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Data availability: The datasets generated during and/or analysed during the current study are available in Sequence Read Archive under Bioproject accession number PRJNA923488.