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Genome-wide assessment of antimicrobial tolerance in *Yersinia pseudotuberculosis* under ciprofloxacin stress

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Abstract

Yersinia pseudotuberculosis is a Gram-negative bacterium capable of causing gastrointestinal infection and is closely related to the highly virulent plague bacillus *Yersinia pestis*. Infections by both species are currently treatable with antibiotics such as ciprofloxacin, a quinolone-class drug of major clinical importance in the treatment of many other infections. Our current understanding of the mechanism of action of ciprofloxacin is that it inhibits DNA replication by targeting DNA gyrase, and that resistance is primarily due to mutation of this target site, along with generic efflux and detoxification strategies. We utilized transposon-directed insertion site sequencing (TraDIS or TnSeq) to identify the non-essential chromosomal genes in *Y. pseudotuberculosis* that are required to tolerate sub-lethal concentrations of ciprofloxacin *in vitro*. As well as highlighting recognized antibiotic resistance genes, we provide evidence that multiple genes involved in regulating DNA replication and repair are central in enabling *Y. pseudotuberculosis* to tolerate the antibiotic, including DksA (yptb0734), a regulator of RNA polymerase, and Hda (yptb2792), an inhibitor of DNA replication initiation. We furthermore demonstrate that even at sub-lethal concentrations, ciprofloxacin causes severe cell-wall stress, requiring lipopolysaccharide lipid A, O-antigen and core biosynthesis genes to resist the sub-lethal effects of the antibiotic. It is evident that coping with the consequence(s) of antibiotic-induced stress requires the contribution of scores of genes that are not exclusively engaged in drug resistance.

DATA SUMMARY

The authors confirm all supporting data and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

Yersinia pseudotuberculosis is a facultatively anaerobic, Gram-negative pathogen of humans and animals, capable of causing fever and acute gastrointestinal infection, yersiniosis, when transmitted by the faecal–oral route [1]. In rare instances, infection can result in septicaemia, which carries an associated risk of mortality [1]. Most cases of enteric disease caused by *Y. pseudotuberculosis* are self-limiting and do not require treatment, although there is some evidence that antibiotics can reduce faecal shedding, potentially reducing the risk of transmission [2, 3]. Some *Y. pseudotuberculosis* strains may express a superantigenic toxin causing Far Eastern Scarlett-like fever, which is a childhood disease with rash, arthralgia and polyarthritis most commonly reported in eastern Russia, Korea and Japan [4]. There is also epidemiological evidence linking *Y. pseudotuberculosis* with Kawasaki disease in children [1]. There are other rare immunological complications associated with enteric yersiniosis [4], which is why there is a requirement for diagnosis of what is usually a self-limiting gastric infection. In cases with more serious clinical presentation, such as scarlet-like fever or sepsis, antibiotic therapy is required, usually with cefotaxime, ceftriaxone or ciprofloxacin.

In the *Y. pseudotuberculosis* genome, 75 % of the predicted genes are 97 % identical at the DNA level to their orthologous counterparts in *Yersinia pestis*, the causative agent of

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Abbreviations: HU, hydroxyurea; LPS, lipopolysaccharide; MDR, multi-drug resistance; MIC, minimum inhibitory concentration; OM, outer membrane; PGN, peptidoglycan; ROS, reactive oxygen species; TraDIS, transposon-directed insertion site sequencing.
Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary figure and five supplementary tables are available with the online version of this article.
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bubonic and pneumonic plague [5]. If plague is suspected, antibiotic treatment is started immediately without waiting for laboratory confirmation and patients must be placed in isolation to reduce the risk of spread in the event of pneumonic plague developing. In the past, streptomycin has been the drug of choice for the treatment of plague, but the toxicity of streptomycin makes this less than ideal and ciprofloxacin has now been included in Centers for Disease Control and Prevention guidelines for therapy. The fluoroquinolone antibiotic levofloxacin was approved recently by the US Food and Drug Administration for the therapy or prophylaxis of plague infections, based on efficacy studies in African green monkeys [6]. Untreated, the case fatality rate is 40–60 %, but where therapy is used this can be reduced to around 14 %. Due to the high degree of genetic conservation and the fact that *Y. pseudotuberculosis* can be worked with at a lower biosafety containment level than *Y. pestis*, it is a useful surrogate for its more virulent relative.

While most *Y. pseudotuberculosis* clinical isolates are susceptible to antibiotics that are active against Gram-negative bacteria [7, 8], multidrug-resistant (MDR) strains of *Y. pseudotuberculosis* have occasionally been reported [9]. MDR strains of *Y. pestis* have also been reported, including mutations that confer resistance to ciprofloxacin [10]. While there is good evidence that mutation of DNA gyrase and topoisomerase – the key targets of fluoroquinolone activity – mediate resistance to this class of antibiotics in many bacteria, including *Y. pestis* [11], modification of the bacterial envelope by decreasing porin production or increasing the expression of efflux pump systems also contributes to the MDR phenotype. For example, AraC family regulators, such as MarA, can respond to antibiotic stress and confer MDR by increasing expression of the AcrAB-TolC multidrug efflux pump and MicF, a small regulatory RNA that down-regulates the ompF porin [12]. Mutation of MarA homologues in *Y. pestis* [13] resulted in increased susceptibility to a range of antibiotics, including ciprofloxacin. The combined action of these mechanisms confers a significant decrease in bacterial sensitivity to therapy and favours the acquisition of additional mechanisms of resistance.

Thus, the bacterial response to antibiotic stress is more complex than merely the interaction of a single compound with a single target, and there is still much to be learned in the field of drug–pathogen interactions. Identifying and targeting the antibiotic stress regulon may be a valid strategy in improving the activity of currently used antibiotics. Transposon-directed insertion site sequencing (TraDIS or TnSeq) is an approach that can be used to elucidate genome-wide essentiality under selective pressure. It has been applied to a range of pathogens to explore how bacteria cope with antibiotic stress, including *Pseudomonas aeruginosa* [14], *Mycobacterium tuberculosis* [15], *Acinetobacter baumannii* [16] and *Klebsiella pneumoniae* [17]. We recently constructed and validated a TraDIS library in *Y. pseudotuberculosis* to identify the genes required for growth in optimized *in vitro* conditions [18]. In the present work, we utilize this resource to establish, for the first time, the genes involved in resistance to ciprofloxacin stress in enterobacteria.

Impact Statement

Our understanding of the mechanism of action of many antibiotics tends to be restricted to a specific, singular target site that interacts directly with the compound. Beyond this, we can use transcriptomics to describe the response of microbes to treatment, which may capture some of the wider stresses exerted by the antibiotic. However it is unclear from such techniques which genes are essential for surviving the antibiotic stress, and which are just associated with the treatment. By sequencing a transposon library in *Yersinia pseudotuberculosis* subjected to sub-lethal concentrations of ciprofloxacin, we have identified all the genes required by the pathogen to tolerate this stress, as without functional versions of the gene(s), the mutants cannot survive. This approach has generated insightful data that show that dozens of genes normally involved in housekeeping functions are also critical in tolerating ciprofloxacin, including, surprisingly, cell-wall lipopolysaccharide synthesis genes. Not only does this expand our understanding of how ciprofloxacin causes toxicity in microbes, it also highlights mechanisms by which the bacteria can resist the antibiotic. This is important in developing future antimicrobials, a matter of pressing importance given the rise of antimicrobial resistance across multiple pathogens against many different classes of antibiotic.

METHODS

Transposon sequencing

Y. pseudotuberculosis strain IP32953 was previously used to generate a transposon library of approximately 40 000 unique insertion mutants using the Ez-Tn*5* Kan2 transposome complex (Epicentre) as described and validated by Willcocks *et al.* [18].

We first inoculated 15 ml pre-warmed BAB medium at 28 °C from pooled frozen aliquots of the *Y. pseudotuberculosis* library and incubated this for 4 h before transferring 5 ml to 50 ml of fresh blood agar base 2 (BAB) liquid medium (ThermoScientific) for overnight incubation at 28 °C with shaking. From this medium, 3 ml was used to inoculate 50 ml pre-warmed BAB medium in the absence or presence of ciprofloxacin for a further 24 h before extraction of genomic DNA (gDNA). Published minimum inhibitory concentrations (MICs) of ciprofloxacin against *Y. pseudotuberculosis* vary [8, 19]; in our study, we treated the bacteria with 0.004 $\mu g\ ml^{-1}$, which is below the lowest reported MIC.

gDNA was extracted (ArchivePure DNA Extraction Kit, 5Prime) and pooled from triplicate technical repeats and from triplicate biological repeats performed independently. Library preparation for Illumina MiSeq sequencing was performed as described by Willcocks *et al.* [18] and reads were mapped

against the reference genome (European Molecular Biology Laboratory accession number: BX936398).

Curation of the list of ciprofloxacin tolerance genes

We sequenced bacteria from three different experimental conditions: directly from frozen stocks of the pooled transposon library (unpassaged control); after passage in the absence of antibiotic (untreated, passaged control); and after passage in the presence of antibiotic (antibiotic-treated condition). To obtain a list of ciprofloxacin tolerance genes, we adapted the methodology of Eckert *et al.* [20]. We initially normalized the number of reads for each transposon insertion site against the total number of reads within each condition. We then excluded any transposon mutants that were absent from the sequencing of the unpassaged control, as these represent essential fitness genes, regardless of treatment condition (this list was verified against the 488 published essential genes from our strain). We next calculated $\log_2$-fold change values for the untreated control against the unpassaged control, and excluded any genes that had lower than a −1 difference versus unpassaged control. Our preliminary list of ciprofloxacin tolerance genes was determined by identifying those transposon mutants that showed lower than −1 $\log_2$-fold change in the treated versus untreated control. For mutants with zero reads in the antibiotic-treated condition, we applied an arbitrary value of 0.5 reads to enable a $\log_2$ calculation. We next performed the non-parametric permutation statistical analysis test, also known as the randomization test, to assign *P*-values to each gene, as described previously [21, 22], removing any that were not significantly differently represented between the two conditions ($P<0.05$). For the main list of ciprofloxacin tolerance genes, we only included genes where we identified at least ten total reads per gene following passage in the untreated condition. We separately collated genes with a read count of 2–10; while this second list must be interpreted with more caution, we hypothesized that it may contain transposon mutants that suffered a general fitness handicap that is exacerbated under the additional stress of antibiotic treatment. A representative gene from each of these two categories of ciprofloxacin tolerance genes was chosen for subsequent mutagenesis and phenotypic analysis (*dksa* >10 reads; *hda* <10 reads).

Creation of targeted isogenic mutants

In order to create targeted mutants to validate our TraDIS findings, we used a lambda Red recombination approach, by which the gene of interest was replaced with a kanamycin resistance cassette (adapted from Datsenko *et al.* [23]). We have summarized the method in diagrammatic form in Fig. S1 (available in the online version of this article).

Y. pseudotuberculosis IP32956 pDK46 served as the recipient strain for mutagenesis (kindly provided by Professor Richard Titball, Exeter University). This strain was maintained at 28 °C in the presence of 0.2 % arabinose and ampicillin in BAB medium. The kanamycin resistance cassette was amplified from pKD4 by the PCR, with primers designed to incorporate the nucleotide sequence flanking the gene of interest (Table S1). The purified PCR product was subsequently *Dpn*I-digested and used to transform the recipient strain, integration being mediated by lambda Red-induced homologous recombination. These strains were subsequently maintained at 37 °C, at which temperature the temperature-sensitive pDK46 plasmid is lost, and were grown thereafter in the presence of 50 µg kanamycin ml^{-1}. After curing the pDK46 plasmid from the strain, we could ensure that kanamycin resistance was due to chromosomally integrated gene recombination. To confirm the null allele, PCR with primers for the gene of interest was conducted on gDNA extracted from individual colonies, which were subsequently confirmed by Sanger sequencing.

Bacterial culture conditions and *in vitro* assays

Growth curve analysis was conducted using a fresh aliquot from an overnight culture at 28 °C diluted 1 : 50 in prewarmed, sterile BAB medium. Optical density at 600 nm was determined at the time points described.

Antibiotic susceptibility of mutants to a fixed concentration of ciprofloxacin, moxifloxacin, chloramphenicol and tetracycline (0.05 µg ml^{-1}) was assessed using the alamar blue assay (Thermo Fisher Scientific). This assay is indicative of cell viability by virtue of reduction of the reagent by the metabolic processes of living cells, causing a quantifiable colour change. For absolute viability, we also performed a c.f.u. assay using a titration of ciprofloxacin (0–0.1 µg ml^{-1}). Fresh cultures were aliquoted into 96-well plates at 1×10^7 c.f.u. mL^{-1} final concentration in BAB medium with or without antibiotic supplementation. Alamar blue reagent was added at 1 : 20 (v/v) and the optical density was recorded at 540 nm and at 620 nm at selected time points. Relative metabolic activity was calculated first by normalizing 540 nm values against 620 nm values and the bacteria-free control samples as per the manufacturer's instructions, and finally by normalizing these values against the untreated bacteria condition. We confirmed the sensitivity of the strains to ciprofloxacin by measuring absolute viability through serial dilution of the bacteria in sterile PBS and enumerating colony-forming units from growth on Yersinia selective agar at 28 °C. Sensitivity of the mutants to hydrogen peroxide (H_2O_2), cumyl hydroperoxide and hydroxyurea (HU; all from Sigma) was examined by incubating bacteria at 5×10^6 c.f.u. ml^{-1} in a 96-well plate for 24 h with a titration of the chemical agents and assessing optical density at 540 nm.

RAW 264.7 culture conditions

Infection of murine macrophage-like RAW 264.7 cells was used to assess the resistance of the mutants to innate immune killing. Cells were grown and maintained in Gibco RPMI 1640 Glutamax (Thermo Fisher Scientific) with 10 % FBS (Thermo Fisher Scientific) at 37 °C and 5 % CO_2. In total, 1×10^5 cells ml^{-1} were aliquoted into flat 96-well plates with the addition of 0.1 µg ml^{-1} recombinant murine IFNγ (RND Systems) and incubated overnight. Wild-type IP32956 and mutants were cultured overnight as previously described and inoculated at an m.o.i. of 0.1. RAW 264.7 cells with bacteria were incubated for 90 min to allow intracellular infection.

Media and extracellular bacteria were removed and cells were washed three times with sterile PBS before fresh medium was added containing 100 µg imipenem ml^{-1} (Sigma). After 16 h, cells were lysed with 0.1 % Triton X100 in sterile, molecular-grade water for 10 min before manual disruption by pipetting and serial dilution of the resultant lysate for the c.f.u. assay. As a marker of cell activation state, we harvested cell culture supernatant at various time points and inferred nitric oxide production through the quantification of nitrite by a Griess Assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Galleria mellonella larvae infection

Galleria mellonella larval infection is an increasingly popular tool for *in vivo* microbiological research [24]. Larvae were purchased from Livefoods Direct and stored at 10 °C before use. Bacteria were cultured overnight in Lysogeny Broth (LB) at 37 °C and 210 r.p.m. Cultures were diluted in PBS to 10^8 c.f.u. ml^{-1}, and 10 µl was injected into the foremost left pro-leg using a 25 µl syringe (Hamilton). Actual challenge dose in the input was established by retrospective viable counts. A control group was injected with 10 µl PBS to control for mortality caused by handling and injection. After injection of ten larvae per group, they were kept in sterile Petri dishes in the dark at 37 °C and survival was assessed at 24 and 48 h post-infection. The bacterial burden of survivors was determined by calculating the c.f.u. ml^{-1} contained in the larvae haemolymph after 48 h. Individual larvae were placed in microcentrifuge tubes and kept on ice until they were comatose. Haemolymph was collected by cutting off 2 mm of the tail tip of the larva and allowing the contents to drain into the microcentrifuge tube while still on ice. Haemolymph was diluted 1 : 10 in sterile PBS and serial ten-fold dilutions were performed. Dilutions were spread onto LB agar and incubated at 37 °C for 72 h, and c.f.u. ml^{-1} values were calculated.

RESULTS

Identification of ciprofloxacin tolerance genes

TraDIS detects at the genome level the genes required for survival and replication under defined experimental conditions. We have exploited this to identify genes of interest following treatment of a TraDIS library of *Y. pseudotuberculosis* with a sub-MIC concentration (0.004 µg ml^{-1}) of ciprofloxacin for 24 h. Bacteria carrying a transposon insertion in a gene that is required for survival are negatively selected from the pooled library, and have fewer reads upon next-generation sequencing of the pooled extracted gDNA.

The complete list of 326 ciprofloxacin tolerance genes with at least −1 log_2-fold change can be found in Table S2 (for mutants with at least ten reads in the untreated passaged condition) and Table S3 (for mutants with fewer than ten reads in the untreated passaged condition). For completion, we have provided the remaining dataset of 2510 genes that appeared to show no change, or positive selection, in Table S4; of these, 273 mutants showed fewer than ten reads. Genes that were highly represented (high proportion of reads) in the untreated condition and poorly represented or absent in the treated condition (low proportion of reads) represent genes encoding proteins whose functions are important in tolerating sub-MIC ciprofloxacin (Fig. 1a). Examples of such genes include: *manC*, encoding a mannose-1-phosphate guanylyltransferase; *gmd*, encoding a GDP-mannose 4,6-dehydratase; *fcl*, encoding a GDP-L-fucose synthase; and *wbyL*, encoding a probable glycosyltransferase.

Interestingly, we observed a high number of genes relating to lipopolysaccharide (LPS) lipidA, O-antigen and core biosynthesis as being negatively selected by ciprofloxacin treatment; these are highlighted and a full list of genes with a similar function is provided in Table 1.

When log_2-fold change scores are applied to the data, it can be seen that the majority of genes are neither significantly positively nor negatively selected (0–1 log_2-fold change) by the treatment condition (Fig. 1b). The ranked log_2-fold change score for every gene in the genome is displayed graphically in Fig. 1(c), and particular genes of interest are highlighted.

While mutants that show a large drop in read count due to ciprofloxacin treatment exhibit a large log_2-fold change, to only focus on these targets is to overlook transposon mutants that had a low read count following culture in the untreated condition. These may include genes involved in tolerance to ciprofloxacin, but also in general fitness as well, especially when cultured in competition with bacteria carrying a functional copy of the gene. Examples of such genes from this study include: *folD*, encoding a bifunctional protein; *yptb2743*, encoding an uncharacterized protein; and *secF*, encoding a protein-export membrane protein. This is not the only explanation for a low read count, and hence we tabulated these genes separately (Table S3), and any conclusions regarding their role in antibiotic tolerance requires experimental confirmation. Similar caution must be applied to interpretation of genes displaying apparent positive selection, which may be a consequence of a proportional increase resulting from loss of negatively selected mutants from the pool.v

We therefore sought to further validate some of the TraDIS-identified genes representing both high read-count and low read-count groups after untreated passage that still displayed negative selection under ciprofloxacin stress. We chose the genes: *dksa* (*yptb0734*) with −5.07 log_2-fold change, and *hda* (*yptb2792*) with −2.61 log_2-fold change. These genes were chosen as they are both predicted to have roles that are relevant to the mechanism of action of ciprofloxacin, namely in DNA repair and replication, and are 100 % conserved at the amino-acid level with *Y. pestis*. Replacement of the wild-type genes with the kanamycin resistance cassette via lambda Red recombination was confirmed by PCR (Fig. 2). To ensure there were no polar effects resulting from the mutations that might affect the expression of flanking genes, we conducted RNA extraction and cDNA synthesis to use as template for PCR and gel electrophoresis and found that the flanking genes adjacent to *dksA* (*sfsA* and *gluQ*) and *hdA* (*yptb2793* and *arsC*) were expressed normally (Fig. S2).

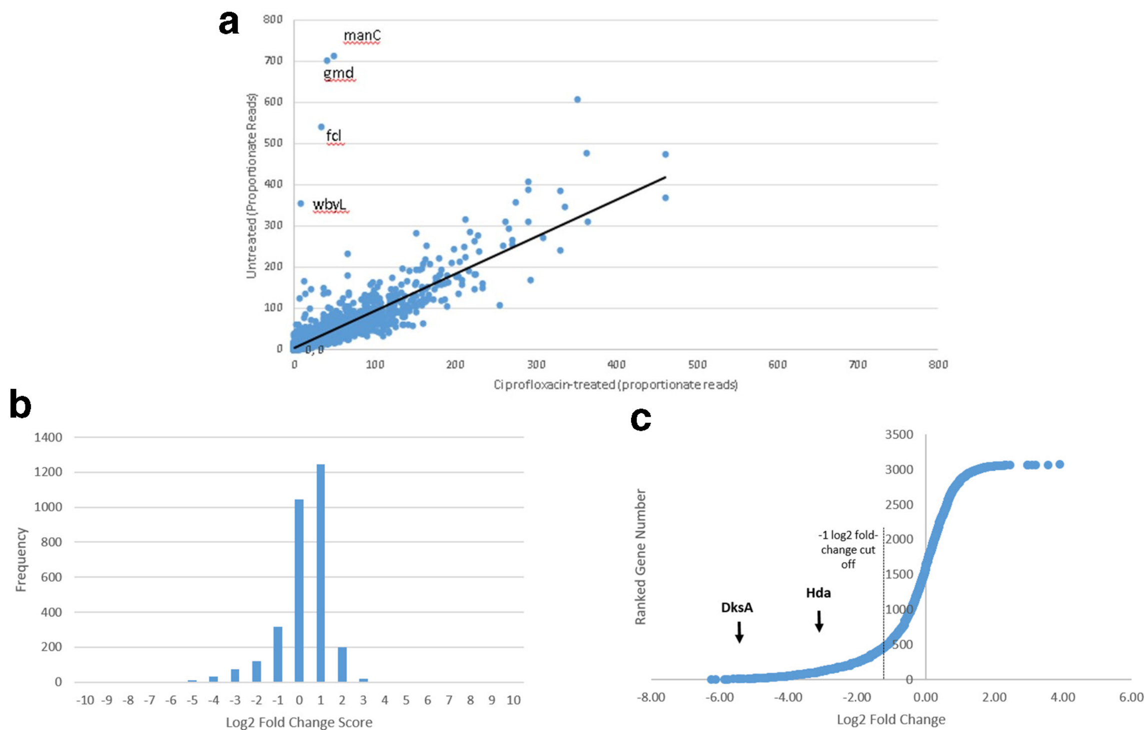

Fig. 1. Graphical representation of *Y. pseudotuberculosis* transposon mutant library selection by ciprofloxacin treatment. Read counts for each gene following TraDIS were expressed as a proportion of the total read count across the entire genome either with or without ciprofloxacin treatment – selected genes showing negative selection by ciprofloxacin treatment are highlighted (a); $\log_2$-fold change ratios were calculated for every gene and their frequency was plotted to demonstrate the distribution of values, with most genes accounting for within a range of −1 and +1 fold change from the untreated control (b); the $\log_2$-fold change values for every gene in the genome were ranked and selected genes highlighted (c).

In vitro phenotypic characterization of *dksA* and *hda* mutants

The growth rates of both mutants at 28 °C in shaking culture in BAB medium was measured up to 24 h and demonstrated a statistically significant reduction in exponential growth rate relative to the wild-type, although this recovered by late exponential to early stationary phase (Fig. 3). This confirmed that the genes are not essential for survival of *Y. pseudotuberculosis* under *in vitro* growth conditions, as suggested by our previously published TraDIS data [18].

Upon treating with different antibiotics, we found that the IP32956 Δ*dksa*::kanR and Δ*hda*::kanR mutants were significantly more sensitive to ciprofloxacin compared with the wild-type (Fig. 4). Furthermore, this was an antibiotic-specific effect, given that the mutants and wild-type did not show any significant difference in sensitivity to a related fluoroquinolone, moxifloxacin, nor to two non-quinolone antibiotics, chloramphenicol and tetracycline, at equivalent concentrations. Exposure to either H_2O_2 or cumyl hydroperoxide showed the IP32956 Δ*dksa*::kanR mutant to be significantly more susceptible to chemically induced oxidative stress (Fig. 5a and b), while Δ*hda*::kanR was significantly more susceptible to hydroxyurea, a DNA replication antagonist (Fig. 5c).

To broadly assess the sensitivity of the mutants to innate immune factors, murine IFNγ-activated macrophage-like RAW cells were infected at an m.o.i. of 0.1 for up to 24 h before bacterial c.f.u. was quantified from lysed cells (Fig. 6a). Both isogenic mutants were attenuated for survival intracellularly compared with the wild-type, although this was not affected by the activation state of the host cell. Confirmation of the activation state of the cells was assessed by use of the Griess assay to measure nitrite, an indicator of nitric oxide production during cell culture (Fig. 6b).

In vivo phenotypic characterization of *dksA* and *hda* mutants

To assess a wider role in virulence for Hda and DksA, we utilized a *G. mellonella* larval infection model of *Y. pseudotuberculosis* pathogenesis [24]. Briefly, ten larvae per group were injected with 1×10^6 c.f.u. of each strain or sterile PBS. Both survival and bacterial burden in the haemolymph was assessed up to 48 h. The median survival of larvae infected with the Δ*hda*::kanR strain, but not the Δ*dksa*::kanR strain, was significantly greater than larvae infected with the IP32956 control, with more larvae surviving at 48 h (Fig. 7a). However, there was no significant difference in the mean bacterial burden (as quantified by c.f.u. assay) in the larval

Table 1. A selection of genes with related function required by *Y. pseudotuberculosis* to survive sub-MIC ciprofloxacin

Genes relating to LPS lipid A, O-antigen and core biosynthesis		
Locus iD	**Gene name**	**Predicted function**
YPTB0051	*kdtX*	Lipopolysaccharide core biosynthesis glycosyl transferase
YPTB0053	*rfaC*	Lipopolysaccharide heptosyltransferase-1
YPTB0054	*rfaF*	ADP-heptose-LPS heptosyltransferase II
YPTB0055	*rfaD*	ADP-L-glycero-D-manno-heptose-6-epimerase
YPTB0172	*rffG*	dTDP-glucose 4,6-dehydratase
YPTB0173	*rffH*	Glucose-1-phosphate thymidylyltransferase
YPTB0175	*rffA*	dTDP-4-amino-4,6-dideoxygalactose transaminase
YPTB0177	*WecF*	TDP-*N*-acetylfucosamine:lipid II *N*-acetylfucosaminyltransferase
YPTB0229	*livG*	ABC type branched-chain amino acid transport
YPTB0263	*rfaH*	Transcription antitermination protein
YPTB0775	*nlpD*	Lipoprotein
YPTB0998	*ddhD*	CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase reductase
YPTB1001	*ddhC*	Putative CDP-4-keto-6-deoxy-D-glucose-3-dehydratase
YPTB1002	*prt*	Paratose synthase
YPTB1003	*wbyH*	Putative exported protein
YPTB1004	*wzx*	Putative O-unit flippase
YPTB1005	YPTB1005	Uncharacterized protein
YPTB1006	*wbyJ*	Putative mannosyltransferase
YPTB1008	*wbyK*	Putative mannosyltransferase
YPTB1009	*gmd*	GDP-mannose 4,6-dehydratase
YPTB1010	*fcl*	GDP-L-fucose synthase
YPTB1011	*manC*	Mannose-1-phosphate guanylyltransferase
YPTB1012	*wbyL*	Probable glycosyltransferase
YPTB1453	*ompA*	Outer membrane protein
YPTB1622	*pagP*	Lipid A palmitoyltransferase
YPTB1914	YPTB1914	ABC sugar (sorbitol/trehalose/maltose) transporter, permease subunit
YPTB2014	YPTB2014	Putative ABC transporter
YPTB2327	*pmrJ*	Probable 4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase
YPTB3041	*ygeD*	Lysophospholipid transporter
YPTB3407	*rfaE*	Bifunctional protein - ADP-L-glycero-beta-D-manno-heptose biosynthesis
YPTB3955	YPTB3955	ABC amino acid transporter
YPTB3964	*glmS*	Glutamine-fructose-6-phosphate aminotransferase
YPTB3965	*glmU*	Bifunctional protein GlmU

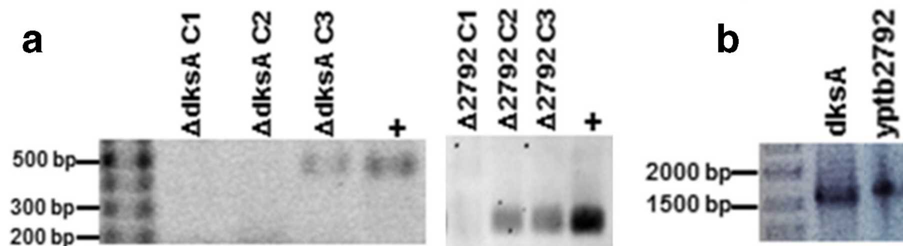

Fig. 2. *Y. Pseudotuberculosis* hda::kanR and dksa::kanR lack expression of wild-type *hda* and *dksa.* 1=*dksa* (a) or *hda* (b) -specific primers versus dksa::kanR or hda::kanR gDNA; 2=*dksa* (a) or *hda* (b) -specific primers versus wild-type IP32956 gDNA; 3=*kanR*-specific primers versus dksa::kanR or hda::kanR gDNA

haemolymph between the mutant and wild-type strains (Fig. 7b).

DISCUSSION

Ciprofloxacin tolerance genes identified by TraDIS

Resistance to antibiotics is complex and multifactorial. TraDIS is now established as a robust molecular tool that enables genome-wide interrogation of gene essentiality in a given experimental condition. By treating a *Y. pseudotuberculosis* transposon library with sub-MIC concentrations of ciprofloxacin, we have revealed many more genes that are involved in tolerance to this antibiotic beyond the recognized target, DNA gyrase. These data reveal more subtle effects of ciprofloxacin-induced stress that expand our understanding of the drug–pathogen interface.

Our screen detected the requirement of multiple genes that may rationally be expected to contribute to the processes that are disrupted by ciprofloxacin, namely DNA replication and repair [25]. These include, for example, *recB*/*C*/*G*, *ruvB*, *nrdA*/*B* and *holE*, some of which have been shown to be upregulated upon treatment with quinolones in other bacterial species [26, 27] and contribute to bacterial persistence in the presence of these antibiotics [28]. Functional loss of these genes combined with ciprofloxacin stress significantly harms the ability of the bacteria to cope with even sub-MIC concentrations of the antibiotic. Surprisingly, the importance of DNA repair mechanisms in tolerating quinolones has only relatively recently come to be appreciated, with the emphasis being point mutations at the DNA gyrase and topoisomerase target site [29–31].

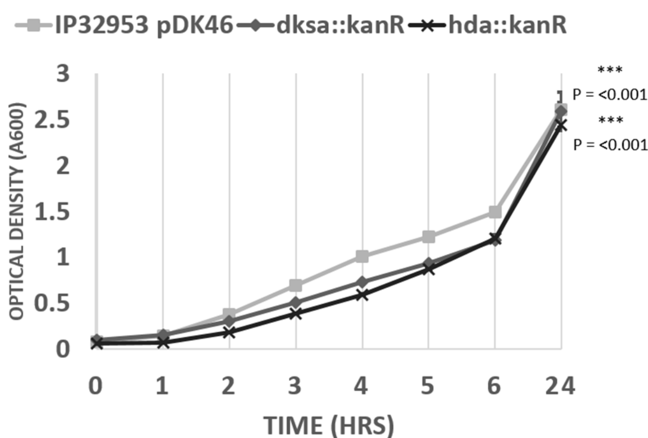

Fig. 3. Growth rate of hda::kanR and dksa::kanR mutants. Wild-type or mutant *Y. pseudotuberculosis* were grown in fresh BAB liquid culture at 28 °C and optical density (600 nm) was recorded over 24 h. Regression analysis was conducted and both hda::kanR (P<0.001, R^2=0.98) and dksa::kanR (P<0.001, R^2=0.97) strains were found to have significantly reduced growth rate.

We also identify a number of genes likely to encode efflux transporters, such as *rosA*, *qacE*, *mepA* and *yptb3307*, that are probably involved in preventing cytoplasmic accumulation of the drug [32].

As well as reinforcing current understanding of resistance to ciprofloxacin, our TraDIS screen identified genes that might point to novel or under-appreciated mechanisms of resistance. For example, we find multiple genes relating to the synthesis and function of molybdenum-containing enzymes. These have hitherto had no reported role in resistance to antibiotics, but have various roles relating to oxidative and reductive catalytic activity [33]. We furthermore identify genes involved in urease synthesis, enzymes whose activity has variously been reported to be both enhanced [34] and inhibited [35] by ciprofloxacin in different species. An interesting finding is the requirement for *dps*, encoding DNA protection during starvation protein. This has been reported to bind non-specifically to the chromosome and protect nucleotides from oxidative-stress-induced strand breakages [36]. The requirement of genes encoding Xer tyrosine recombinases (*yptb3167* and *yptb0192*) may reflect their ability to resolve dimeric chromosomes arising from stalled replication that may be more common in the presence of ciprofloxacin. Supporting this, we also find a requirement for *ftsK*, a gene encoding a cell division protein that has recently been described as an accessory to Xer recombinases [37]. We also identify several genes of unknown function whose transposon mutants are strongly negatively selected by ciprofloxacin treatment.

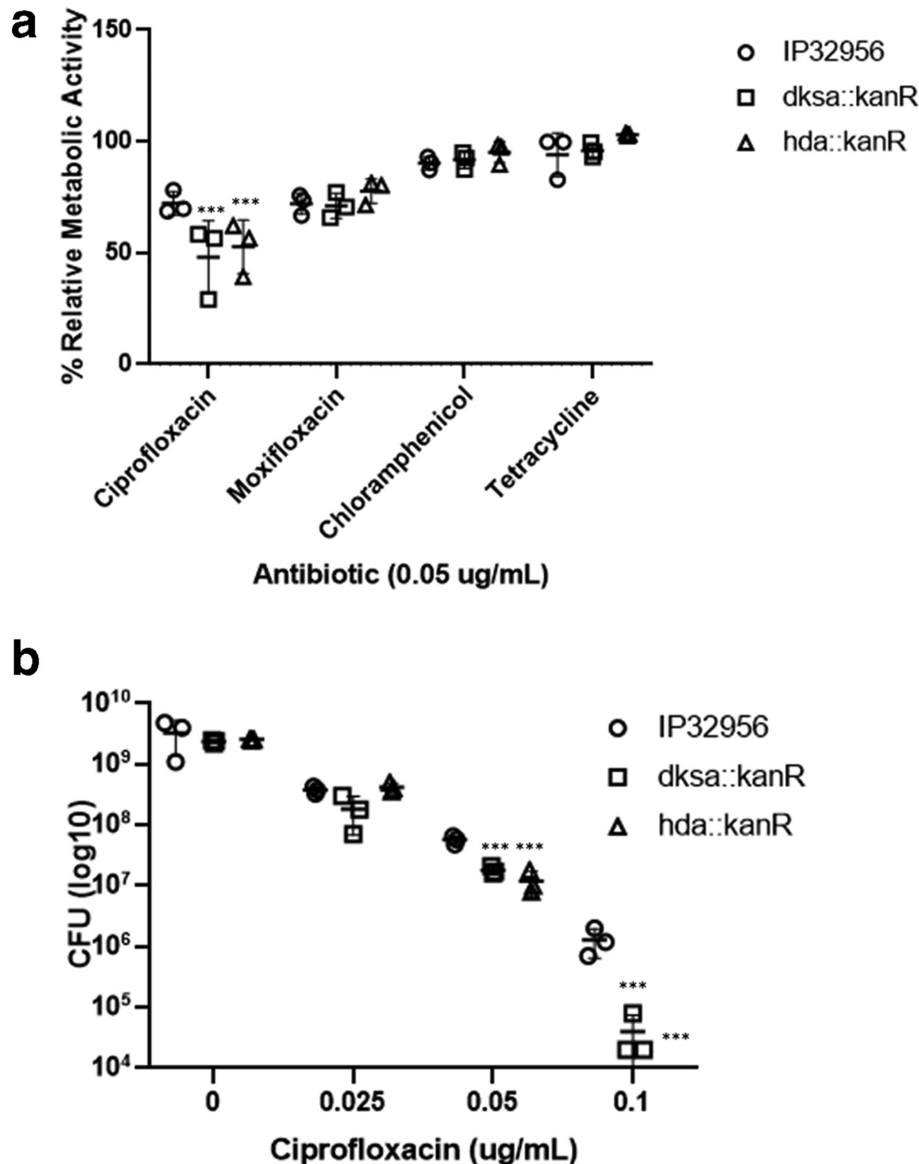

Fig. 4. *Y. Pseudotuberculosis* hda::kanR and dksa::kanR are significantly more sensitive than the wild-type to ciprofloxacin. *Y. pseudotuberculosis* wild-type strain IP32956 and mutants identified as ciprofloxacin-sensitive by TraDIS were treated with ciprofloxacin or alternative antibiotics for 16 h and then subjected to alamar blue metabolic assay (a) or plated for c.f.u. enumeration (b). For metabolic assays, values are all normalized relative to the untreated condition for each strain. All statistical comparisons are Student's *t*-test versus the wild-type strain for the same antibiotic treatment condition (*P<0.01)

Sub-MIC ciprofloxacin causes broad cell envelope stress

It was observed as early as 1987 that sub-MICs of quinolone antibiotics could alter the outer membrane (OM) composition of bacteria, modifying the phospholipid to amino acid ratio [38]. We can now report evidence in support of this at the genomic level.

Among the transposon mutants that showed the greatest loss of fitness during ciprofloxacin treatment are multiple genes relating to LPS lipid A, O-antigen and core biosynthesis. These include the O-antigen operon itself, as well as associated glycosyltransferases, transcriptional regulators, ATP synthases, ABC transporters, and sugar and lipoprotein biosynthetic genes. A role for the O-antigen might suggest that different isotypes or species respond differently to the antibiotic, considering this is hypermutable region of the genome, and has been deactivated entirely in *Y. pestis*.

Since ciprofloxacin is hydrophilic, the core region of LPS represents an impermeable barrier, and therefore it is logical that maintenance of the OM is required to exclude the antibiotic, restricting its entry to porin channels, which can in turn be regulated by the bacteria. Yet besides porin-mediated

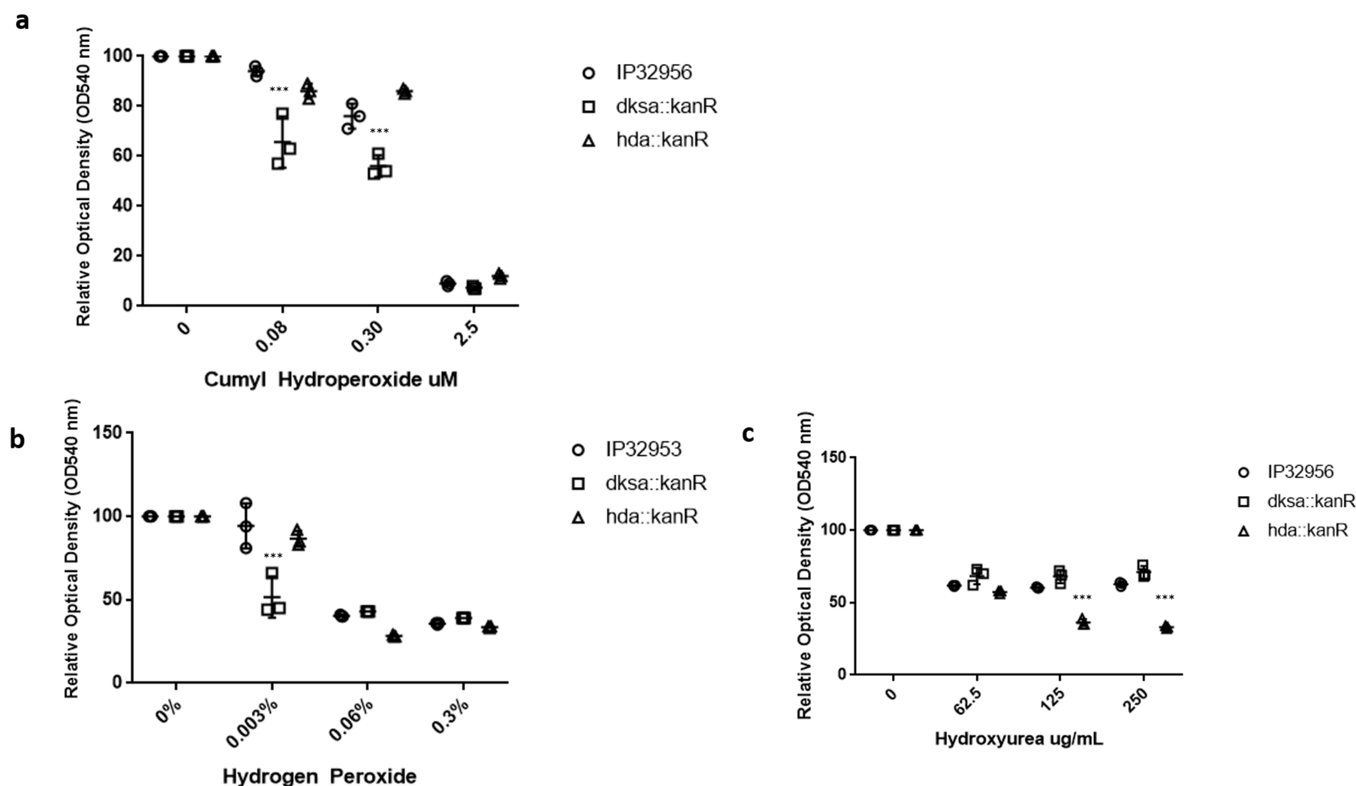

Fig. 5. *Y. Pseudotuberculosis* dksa::kanR and hda::kanR are sensitive to oxidative stress and inhibition of DNA replication, respectively. Bacterial strains were incubated with a titration of either cumyl hydroperoxide (a), hydrogen peroxide (b) or hydroxyurea (c) in fresh BAB liquid culture at 28 °C for 24 h prior to assessment of bacterial growth by optical density. Values are all normalized relative to the untreated condition for each strain. All statistical comparisons are Student's *t*-test versus the wild-type strain for the same antibiotic treatment condition (**P*<0.01).

uptake, quinolones have been reported to permeabilize the OM directly via displacement of divalent cation linkages in the LPS, similar to the action of polymyxin B. Resistance to this includes modification of the lipid A charge, for example by esterification with amino-arabinose. Interestingly, we observe the requirement of a deformylase, *pmrJ*, likely to be responsible for the attachment of modified arabinose to lipid A [39]. A further example of lipid A modification is its palmitoylation by the transferase pagP (a pseudogene in *Y. pestis*), which we also identified as a ciprofloxacin tolerance gene. The additional reported role of palmitoylation of lipid A in resistance to host immune effectors highlights it as part of general adaptation to myriad stressors, including antibiotics, and make it an attractive target for new antimicrobial design.

Also indicative of OM stress is the requirement of two genes that have been associated with PagP activity, and which have been found to be upregulated following perturbation of the O-antigen: the σ^E regulatory factor and *cpx*, part of a two-component system. Cpx is reportedly able to upregulate efflux systems, and may also be required for optimal type III secretion in *Yersinia* species [40].

A peculiarity in our data is the finding that *ompA*, encoding a porin, is actually required to resist sub-MIC ciprofloxacin. However, unlike OmpF/C, OmpA does not form a highly porous trimeric channel, but rather a monomeric beta-barrel structure with much smaller pore size and lower permeability [41]. In fact, OmpA may instead have an essential role in maintaining LPS stability under stress conditions [42], potentially serving as an anchor between the OM and periplasmic peptidoglycan (PGN) [43]. This potentially mirrors the presence in our data of other major OM lipoprotein genes such as *lpp*, *yptb3358* and *yptb3313*, as well as members of the general secretion pathway (*gspJ*/*G*/*D*) encoding proteins that traverse the OM, periplasm and inner membrane. A secondary role for such uptake and secretory transmembrane proteins in ameliorating membrane disruption by conferring mechanical stability requires further study.

Taken together, our data show that even at sub-MIC, ciprofloxacin induces fundamental changes to the physiology of the bacterial LPS, which requires the collective activity of a multitude of host genes to cope with.

The DNA replication and repair genes, DksA and HDA, are required for ciprofloxacin tolerance

As described in our methodology, we stratified our TraDIS data into two distinct groups: ciprofloxacin-sensitive mutants

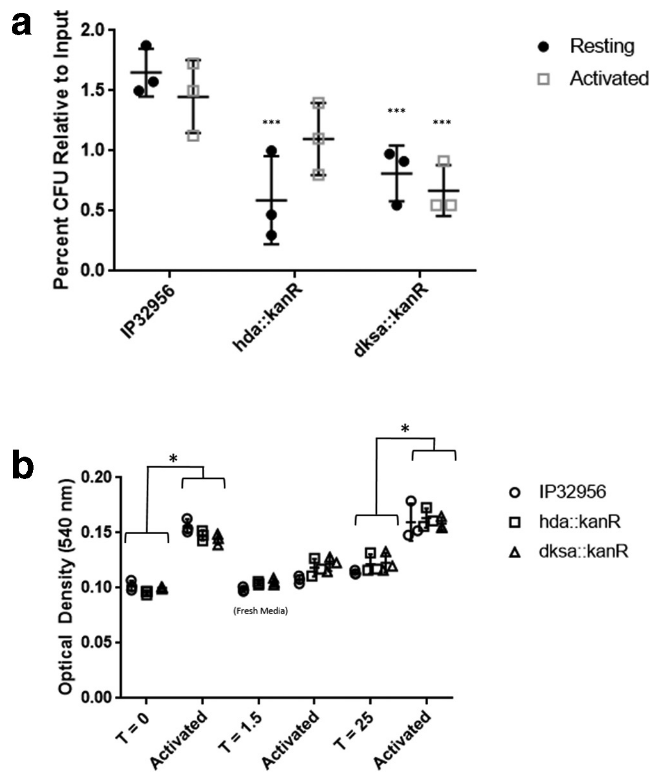

Fig. 6. *Dksa* and *hda* contribute to intracellular fitness. An m.o.i. of 0.1 was used to infect IFNγ-activated murine RAW 264.7 cells for 1 h, followed by washing (T=0) and lysis after 24 h (T=24) before enumeration by c.f.u. (a). Extracellular growth was controlled by using 100 µg imipenem ml^{-1}. Before and during cell infection, cells were treated or not with IFB and cell activation was measured indirectly by quantification of nitrite in culture supernatant by Griess assay (b). All statistical comparisons are Student's *t*-test versus the wild-type strain for the same antibiotic treatment condition (*P<0.01) (a) or between activated versus resting macrophages at each time point (b).

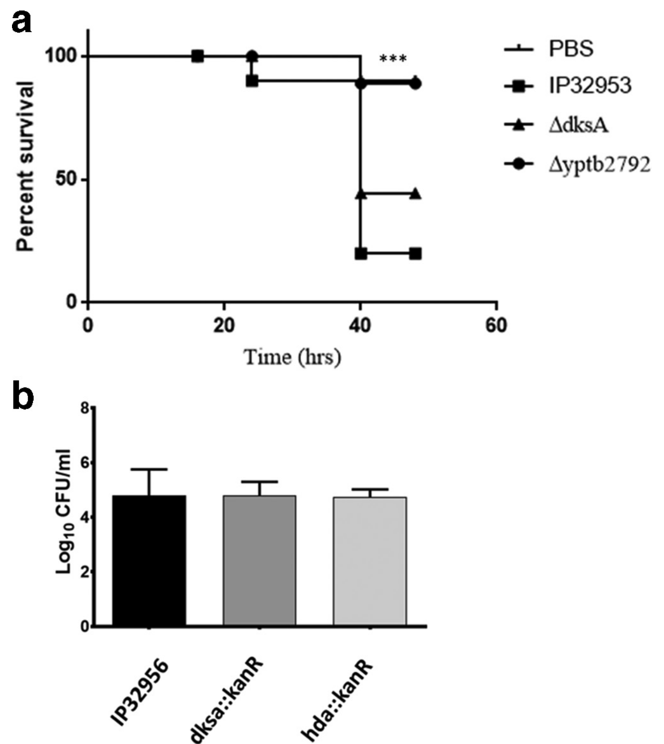

Fig. 7. hda::kanR and dksa::kanR have attenuated virulence *in vivo*. In total, 1×10^{6} *Y. pseudotuberculosis* wild-type, mutant bacilli or sterile PBS control were injected into *Galleria mellonella* larvae and survival was quantified over 48 h (a). Post-mortem, bacterial burden in the haemolymph was assessed and enumerated by a c.f.u. assay (b). All statistical comparisons are Student's *t*-test of survival groups for each condition at 48 h versus 24 h (*P<0.01).

that showed no defect in general fitness when cultured with the entire pooled transposon library; and ciprofloxacin-sensitive mutants that showed reduced fitness in co-culture with the transposon library without antibiotic. A representative gene from each group was examined for a potential role in ciprofloxacin sensitivity as confirmation of our TraDIS data.

Δ*dksA::kanR* and Δ*hda::kanR* were significantly more sensitive than wild-type *Y. pseudotuberculosis* to sub-MIC ciprofloxacin. To our knowledge, this is the first report to describe a role for either gene in ciprofloxacin tolerance. Novel compounds that inhibit the function of such genes may offer synergistic benefit when coupled with existing antibiotics, similar to the use of beta-lactamase inhibitors that increase the efficacy of penicillin-related compounds [44]. The precision of TraDIS is exemplified by the finding that the two mutants were sensitive to ciprofloxacin, but not to other antibiotics. Even though moxifloxacin is also a quinolone-class drug, it additionally targets ParC of topoisomerase IV rather than just GyrA; DksA and Hda may hence have a comparatively restricted role in tolerance to this antibiotic.

DksA acts as a regulator of RNA polymerase; in conjunction with RecN protein, it is able to sense and repair double-strand breaks in DNA [45] and prevent collisions occurring between the transcription elongation complex and the replication fork [33, 34]. Since ciprofloxacin stabilizes the gyrase–DNA complex at the replication fork [46], the likelihood of collisions with the transcription elongation complex increases, causing chromosomal damage [42]. DNA gyrase also relaxes torsion stress caused by the unwinding of supercoiled DNA by initiating double-strand breaks, which may increase after ciprofloxacin treatment [42, 47]. Therefore, DksA has an important housekeeping role that is especially sensitive to disruption by ciprofloxacin.

We confirmed the importance of DksA to DNA repair by modelling chemically induced oxidative stress. Aerobic prokaryotes have evolved to cope with naturally occurring endogenous reactive oxygen species (ROS) such as H_2O_2 [48]. H_2O_2 does not damage DNA directly, but upon reacting with cellular iron, generates radicals that can oxidize DNA and induce single-strand breaks that must be repaired by the Rec system [48]. Similarly, cumene hydroperoxide has been shown to induce DNA-repair mechanisms, and interestingly, also drug efflux proteins [49]. ROS have been shown to contribute

to quinolone-mediated cell death in *Escherichia coli* [50, 51], and there is emerging evidence that several other antibiotics may similarly induce lethal oxidative DNA damage [52]. This highlights the prominent role for genes that contribute to broad functions from housekeeping to managing oxidative and antibiotic stress.

While DksA plays a role in DNA repair, Hda (*yptb2792*) inactivates the DNA replication initiation factor, DnaA [53], and was also found to contribute to ciproflocaxin tolerance. An apparent reduction in exponential-stage growth in broth culture may partly account for diminished representation of the Δ*hda::kanR* mutant from the pooled, untreated transposon library. DnaA recruits replication machinery to the chromosomal origin of replication (OriC) site to form an initiation complex [41, 42]; without careful regulation, potentially lethal rounds of erroneous initiation cycles can occur [43, 54]. The importance of regulation of DNA replication to ciprofloxacin tolerance is also supported by our finding of the apparent requirement for both *diaA* (*yptb3495*) and *dam* (*yptb1863)*. DiaA regulates the timely recruitment of DnaA, preventing aberrant rounds of asynchronous initiation [55]. Dam has a similar role: DNA replication results in hemi-methylated strands of DNA that resist DnaA binding and prevent immediate re-initiation at the OriC; Dam restores full methylation at these sites, enabling DNA replication to proceed [55]. Temporal regulation of DNA replication by factors such as Hda, DnaA and Dam may contribute to ciprofloxacin tolerance by preventing collisions between the replication fork and the elongation complex, similar to DksA.

Pauses in the replication cycle are also necessary to enable DNA repair to take place, allowing the bacteria to cope with any ciprofloxacin-induced damage [56]. DNA replication and repair are naturally linked by a shared resource: dNTPs; thus, orderly DNA replication mediated by Hda may ensure a pool of dNTPs are present for utilization in DNA repair pathways. HU depletes dNTPs, preventing both their incorporation into the nascent DNA strand and their use in DNA repair [56]. The Δ*hda::kanR* mutant may be significantly more sensitive to HU because the replication fork is arrested, a similar outcome to ciprofloxacin treatment. Interestingly, HU-mediated cell death has been reported to replicate some of the same perturbations as caused by ciprofloxacin, including oxidative and cell-envelope stress [57].

Dksa and Hda contribute to virulence

Considering the significant roles of DksA and Hda in DNA replication and repair under ciprofloxacin and oxidative stress, we hypothesized that they may also be important in resistance to host-mediated innate immune stress. DksA has been proposed to sense ROS and reactive nitrogen species (RNS) in *Salmonella* sp. [32]. It controls 427 genes in response to stress related to aspects of cellular metabolism, and several antioxidants and oxidoreductases important in redox buffering [58]. Supporting our hypothesis, we found reduced survival of both mutants in murine macrophage-like RAW 264.7 cells. Interestingly, this was not affected by the activation state of the cell at the time of infection, despite demonstrating enhanced production of nitric oxide. Partly this may be due to the attenuation of the mutants even in resting cells; in addition, the resting cells still became activated over time simply due to the presence of the bacteria. Impaired intracellular growth of the *Yersinia* mutants may partly explain the reduced virulence of the Δ*yptb2792::kanR* strain in the *G. mellonella* larval infection model. Interestingly, however, there was no significant difference in overall bacterial burden post-mortem. This could be due to extracellular replication of bacteria that masked any differences in intracellular growth.

Conclusion

We have utilized TraDIS to identify novel genes with a role in tolerating sub-lethal concentrations of ciprofloxacin. In contrast to the notion that ciprofloxacin has a narrow target of action, our data highlight the broad stress induced by the antibiotic, especially with regard to cell envelope integrity. In particular, *Y. pseudotuberculosis* depends upon genes involved in DNA replication and repair to cope with ciprofloxacin treatment. Perturbation of two of these genes, *hda* and *dksA*, significantly enhanced the sensitivity of bacteria to ciprofloxacin. In principle, antagonistic compounds for targets such as these could be used to reduce the effective MIC of the antibiotic during therapeutic use.

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Author contributions

S.W.: investigation, data curation, methodology, formal analysis, manuscript writing; K.H.: investigation; R.S.: data curation, software, formal analysis, manuscript review; P.O.: conceptualization, manuscript review; A.S.: methodology, manuscript review; H.A.: conceptualization; B.W.: conceptualization, funding acquisition, manuscript review.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Data bibliography

1. Sequencing data (.BAM and .position files) may be obtained from www.ncbi.nlm.nih.gov GEO accession number GSE135236.

2. Reference genome may be obtained from the European Molecular Biology Laboratory, accession number: BX936398.

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