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The TCA cycle is not required for selection or survival of multidrug-resistant *Salmonella*

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Objectives: The initial aim of this study was to use a systems biology approach to analyse a ciprofloxacin-selected multidrug-resistant (MDR) *Salmonella enterica* serotype Typhimurium, L664.

Methods: The whole genome sequence and transcriptome of L664 were analysed. Site-directed mutagenesis to recreate each mutation was carried out, followed by phenotypic characterization and mutation frequency analysis. As a mutation in the TCA cycle was detected we tested the controversial hypothesis regarding the bacterial response to bactericidal antibiotics, put forward by Kohanski *et al*. (*Cell* 2007; **130**: 797–810 and *Mol Cell* 2010; **37**: 311–20), that exposure of bacteria to agents such as ciprofloxacin produces reactive oxygen species (ROS), which transiently increase the mutation rate giving rise to MDR bacteria.

Results: L664 contained a mutation in *ramR* that conferred MDR. A mutation in *tctA* affected the TCA cycle and conferred the inability to grow on minimal agar. The virulence of L664 was not attenuated. Ciprofloxacin exposure produced ROS in L664 and SL1344 (*tctA::aph*), but it was reduced and occurred later. There were no significant differences in the rates of killing or mutations per generation to antibiotic resistance between the strains.

Conclusions: Whilst we confirm production of ROS in response to ciprofloxacin, we have no data to support the hypothesis that this leads to selection of MDR strains. Our results indicate that the mutations in *tctA* and *glgA* were random as they did not pre-exist in the parental strain, and that the mutation in *tctA* did not provide a survival advantage or disadvantage in the presence of antibiotic.

Keywords: TctA, RamR, efflux pumps, genome sequences

Introduction

Infections caused by multidrug-resistant (MDR) Gram-negative bacteria are a serious medical concern of the 21st century as there are few treatment options and few new drugs are being developed. For instance, antibiotic-resistant *Salmonella enterica* infections have been associated with increased risk of extra-intestinal infections, hospitalization and longer duration of illness, compared with infections due to susceptible isolates.[1–4] Bacteria resistant to quinolone antibiotics (e.g. nalidixic acid) and fluoroquinolones (e.g. ciprofloxacin and norfloxacin) can be selected with single or several mutations in one or more chromosomal genes. Two types of mutant can be selected. Firstly, there are those with mutations that affect the interaction of the drug with the target topoisomerase proteins, DNA gyrase and DNA topoisomerase IV, encoded by *gyrAB* and *parCE*, respectively.[5] Such mutants are only resistant to quinolones and fluoroquinolones. Secondly, MDR bacteria can be selected. Typically these contain mutations in the local repressor gene of a transcriptional activator, such as *Escherichia coli marA*[6] or *S. enterica* serotype Typhimurium *ramA*,[7–12] which is then overexpressed and in turn confers over-production of a resistance-nodulation-cell division (RND) type MDR efflux pump such as AcrAB-TolC.[13]

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In the absence of a mutagen, mutation in bacteria has been classically considered to be a spontaneous random event, the consequences of which will only be detected under suitable selective conditions; for instance, an antibiotic-resistant sub-population of bacteria is only detected when the population is grown on antibiotic-containing agar. However, mutation rates can be affected by external influences, such as exposure to a mutagen. It has also been proposed that mutation occurs when an organism is exposed to a growth-limiting condition,[14] and in 1997 we documented the occurrence of ciprofloxacin-resistant *E. coli* after prolonged antibiotic exposure.[15] The phenomenon of so-called 'late arising', or 'adaptive' mutants, and the mechanisms by which such mutations occur, have been largely studied using the *E. coli* Lac system (Lac− to Lac+),[16] and it has been suggested that under conditions of stress a sub-population of bacteria becomes transiently hypermutable,[16,17] and that this is *rpoS* dependent.[16] However, Koskiniemi *et al*.[18] found that different levels of error-prone translesion polymerases and RpoS in *Salmonella* Typhimurium did not affect the mutation rate.

Antibiotic exposure is stressful to bacteria, and there have been several studies showing that exposure to a fluoroquinolone antibiotic gives rise to altered expression of hundreds of genes.[19,20] Exposure to high concentrations of fluoroquinolone decreased expression of *rpoS*,[20] whereas low concentrations decreased expression of *sirA* (which affects RpoS stability).[21] Dwyer *et al*.[19] also found three clusters of genes that responded to fluoroquinolone exposure that had not been described previously: (i) an iron uptake and utilization cluster; (ii) iron–sulphur cluster synthesis; and (iii) genes that respond to oxidative damage. They hypothesized that in response to fluoroquinolone exposure highly destructive hydroxyl radicals are produced. The same team[21] also showed that after 30 min of exposure to a fluoroquinolone, a transient ≥5-fold increase in the ratio of NAD^+ over NADH was seen, leading to their suggestion that there was a surge in NADH consumption upon antibiotic exposure. This induced a burst in superoxide generation via the respiratory chain and they postulated that this promoted destabilization of iron–sulphur clusters, stimulated a Fenton reaction and caused cell death. As NAD^+ is reduced to NADH by tricarboxylic acid (TCA) cycle activity, it was proposed that loss of TCA cycle components would reduce the available pool of NADH, decrease superoxide generation and lead to increased survival in the presence of bactericidal antibiotics. Using *E. coli* mutants defective in components of the TCA cycle they found that loss of isocitrate dehydrogenase activity led to increased survival following norfloxacin treatment, whereas inactivation of genes that encode enzymes later in the TCA cycle had no effect. Most recently, Kohanski *et al*.[22] have shown that there was a significant increase in mutation rate after exposure to a fluoroquinolone, and that there was significant correlation between the fold change in mutation rate and the peak signal when determining reactive oxygen species (ROS) formation. Thiourea, which mitigates the effects of hydroxyl radical damage, significantly reduced the mutation rate to near untreated levels. These data led Kaufmann and Hung[23] to suggest that ROS transiently increase the bacterial mutation rate by generating DNA damage that is repaired in an error prone fashion. This led to the suggestion that bactericidal antibiotics, e.g. fluoroquinolones, behave as if they are mutagens. This could allow antibiotic-resistant bacteria to be selected at an elevated frequency whilst under pressure from antibiotics, and selection of resistant bacteria may occur in a few steps rather than many.

Our initial aims and objectives were to determine the genome and transcriptome of a ciprofloxacin-selected MDR *Salmonella*. However, following the discovery of the *tctA* mutation, its known role in the TCA cycle and subsequent links to survival during antibiotic exposure, we then used this mutant to explore the hypotheses of Kohanski *et al*.,[21,22] which have been offered as a paradigm for the responses of bacteria to antibiotics in general. We hypothesized that exposure of *Salmonella* Typhimurium SL1344 to ciprofloxacin causes the generation of ROS, and that this transiently increases the mutation rate so that an MDR (L664) mutant containing mutations in three genes is selected. We further postulated that the mutation in *ramR* confers MDR, the mutation in *tctA* affects the TCA cycle (but allows survival of the mutant during elevated ROS levels) and mutation in *glgA* has no effect on antibiotic susceptibility or selection of resistant bacteria. Using mutants in which the mutations in L664 were introduced by site-directed mutagenesis, mutation experiments were carried out to test these hypotheses.

Materials and methods

Bacterial strains, mutant selection and determination of susceptibility to antibiotics, dyes, detergents and disinfectants

Mutant *Salmonella* Typhimurium SL1344[24] with decreased susceptibility to ciprofloxacin were selected as described previously.[11,25] L664 *ramR::aph* mutants were created by P22 transduction from L1007.[26] The SL1344 mutant lacking a functional *tctA* gene was constructed using the method of Datsenko and Wanner.[27] The gene-inactivated mutants were complemented with their respective wild-type genes (amplified from SL1344 and cloned into pUC19). The site-directed substitution of glycine at position 109 of TctA with a serine was carried out with a commercial kit (QuikChange™ site-directed mutagenesis kit, Stratagene) (Table S1, available as Supplementary data at *JAC* Online). The MIC of each agent was determined by the standardized agar doubling-dilution method as described previously by the BSAC (http://www.bsac.org.uk).[28]

Total genome sequencing, analysis of sequence data and single nucleotide polymorphism (SNP) detection

Paired-end whole genome sequencing was performed on SL1344 and L664 at the University of Liverpool, using a 454 Life Sciences GS-FLX sequencer (Roche). The nucleotide sequence and gene predictions for *Salmonella* Typhimurium SL1344 NCTC 13347 were retrieved from the Sanger Centre web site (http://www.sanger.ac.uk/Projects/Salmonella/). Eight reads were obtained and the gene predictions were assigned a provisional functional annotation through homology searches: the best hit for BLASTP searches of each gene product prediction against the predicted *Salmonella* Typhimurium LT2 proteome. The read data were aligned against the annotated SL1344 sequence using xBASE-NG (http://www.ncbi.nlm.nih.gov/pubmed/17984072), which in turn uses the 'runMapping' component of Newbler (Roche). Newbler version 1.1.03.24 was used with default settings. High confidence differences as defined by Newbler were used, briefly: (i) at least three reads differing from the reference sequence; and (ii) at least one read aligned in the forward and reverse directions (see the manufacturers' instructions for details). These high-confidence differences were subsequently subjected to sorting and filtering by read depth and percentage coverage. The

effects of individual nucleotide variations on predicted protein sequence were determined using xBASE-NG.SL1344 and L664 *ramR* and *tctA* were amplified by PCR, and the DNA was sequenced at the Functional Genomics Laboratory at the University of Birmingham.

RNA extraction and transcriptional analyses

Overnight cultures of *Salmonella* Typhimurium SL1344 and the test strain were grown in MOPS minimal medium (Teknova, USA) supplemented with histidine at 37°C, and the microarray experiments were carried out exactly as described by Webber *et al.*[29] using the Pan-*Salmonella* Generation IV array generated at the Wellcome Trust Sanger Institute (Hinxton, UK). The Microarray dataset for L664 has been deposited in ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) with the experiment identifier E-MEXP-2696.

Growth kinetics and phenotype microarray (PM) system

The rate of growth in Luria–Bertani (LB) broth and MOPS minimal medium (Teknova, USA) supplemented with histidine for all strains, with and without the MIC of ciprofloxacin for each strain, were determined over 24 h at 37°C using FLUOstar OPTIMA (BMG Labtech, UK). All tests with the PM system were performed as described previously.[30]

Virulence assays, protein purification and western blotting of Sip proteins

Adhesion by, and intracellular survival within eukaryotic macrophages (RAW 264.7) by *Salmonella* Typhimurium SL1344 (and strains derived therefrom) were assessed exactly as described previously.[29] A two-tailed Student's *t*-test was used to assess significance, using $P<0.05$ as the cut-off. *Caenorhabditis elegans* survival assays were carried out exactly as described by Bailey *et al.*[26] Protein purification and western blotting was performed exactly as described previously.[29]

Analysis of cell envelope components, and motility assays

Outer membranes were prepared by differential centrifugation, sonication and sarkosyl extraction, as described by Piddock *et al.*[31] The ability of strains to migrate through (swimming) or across (swarming) semi-solid agar was determined exactly as described by Webber *et al.*[29]

Measurement of glycogen accumulation

The method described by Morán-Zorzano *et al.*,[32] was followed. In brief, SL1344 and L664 were sub-cultured on LB agar supplemented with 50 mM glucose and incubated aerobically overnight at 37°C. Following overnight incubation, colonies were stained with iodine by overlaying the agar plates with 5 mL of iodine.

Measurement of production of ROS following antibiotic exposure

To establish whether ROS were produced following exposure to subinhibitory levels of ciprofloxacin, the method described by Kohanski *et al.*[2] was followed, except that oxidation of the fluorescent reporter dye 3′-(*p*-hydroxyphenyl) fluorescein (HPF) was detected with a fluorescent plate reader (BMG FluoSTAR) and a fluorospectrophotometer (LS-30) with an excitation setting of 492 nm and an emission setting of 520 nm. Early logarithmic cultures ($OD_{600}=0.3$) were exposed to 0.5× MIC of ciprofloxacin and 5 μM HPF simultaneously, then 100 μL of each culture was pipetted into black 96-well plates, the plates were placed in the FluoSTAR and a reading was taken every 3 min for 6 h. When using the LS-30 fluorospectrophotometer, 3 mL samples were taken every hour for 6 h, then washed three times in PBS prior to reading.

Results

MDR Salmonella Typhimurium are easily selected after exposure to ciprofloxacin

In this study we used ciprofloxacin as it is in widespread clinical use and is associated with the selection of MDR bacteria *in vivo*.[33] Five separate, identical experiments were carried out to select mutants from SL1344 growing on agar containing ciprofloxacin. There was no significant difference (Kruskal–Wallis $P=0.406$) between the frequencies of mutation to resistance or mutation rates, and MDR mutants were always selected (Table S2). L664 was one of the single colonies randomly selected in experiment 2 after 18 h of exposure of SL1344 on agar to double the MIC (0.06 mg/L) of ciprofloxacin. L664 was MDR with decreased susceptibility to a range of antibiotics of different classes, dyes, detergents and biocides (Table S3).[11,25]

L664 contains an SNP within ramR (SL0568) and tctA (SL2772)

Following whole genome sequencing of L664 and comparison with the genome of the parental SL1344 strain, four SNPs were found in L664 (Table S4). Two of the four SNPs conferred predicted amino acid substitutions in ORFs; these were in the genes *ramR* (SL0568) and *tctA* (SL2772). The mutations in L664 *ramR* and *tctA* were confirmed by PCR and DNA sequencing. Mutation in these genes in the SL1344 parental strain was never detected despite testing multiple stored cultures (freeze-dried, −20°C, beads and slopes). Inactivation of *ramR* in L664 and complementation with wild-type *ramR* conferred multidrug susceptibility as seen for the parental strain, SL1344. The *ramR* mutation resulted in a substitution, T50P, within a helix-turn-helix motif, so the mutation is likely to affect DNA binding at the operator region of *ramA*. The second SNP was found in *tctA* (SL2772). TctA is part of the tripartite system TctABC in *Salmonella* Typhimurium that transports tricarboxylates of the TCA cycle.[34] The tricarboxylates citrate, isocitrate and cis-aconitate can be utilized by salmonellae as carbon and energy sources under aerobic and anaerobic conditions. A pre-requisite for metabolism is that the tricarboxylates are transported into the cells across the cytoplasmic membrane, and this is performed by the TctABC transport system. To establish the role, if any, of the SNP found in *tctA*, the observed mutation (G109S) was introduced into SL1344 by site-directed mutagenesis, to create L1207. The introduction of this mutation had no effect on antibiotic susceptibility (Table S3).

L664 also contains a 1 bp deletion in glgA (SL3502)

glgA encodes glycogen synthase. Morán-Zorzano *et al.*[32] showed that mutants with a deletion of this gene lacked glycogen, so colonies of SL1344 and L664 growing on LB agar supplemented with 50 mM glucose were stained with iodine. SL1344 stained brown whereas L664 stained yellow, indicating that the mutant did not accumulate glycogen. To establish the role of

GlgA, if any, in ciprofloxacin resistance, *glgCAP* was inactivated in SL1344 to give L1316. This mutation had no affect on susceptibility to antibiotics (Table S3).

The transcriptome of L664 revealed significant differential gene expression

Of the 386 genes (Table S5) for which expression was altered compared with the parental strain SL1344, 143 (37%) had increased expression and 243 (63%) had decreased expression. RT–PCR confirmed the expression pattern changes of 15 representative genes detected by microarray experiments, although, as found in other studies,[26,29] the magnitude of the fold changes was different.

The expression of genes encoding components of drug efflux pumps was altered: expression of *acrA* and *acrB* was increased 2- and 5-fold, respectively, and expression of *emrA*, which encodes a major facilitator superfamily (MFS)-type transporter, was increased 1.5-fold. Expression of *ompC*, which encodes a porin, was decreased 5-fold (Table S4) and electrophoresis of outer membrane protein extracts by SDS–PAGE revealed the lack of OmpC in L664. The AraC-XylS family transcriptional activators MarA, SoxS and Rob, and local regulator AcrR, are proteins that influence expression of multidrug transporters (such as AcrB) and outer membrane proteins (such as OmpF and OmpC). However, no differential expression of *marA*, *soxS* or *rob* was observed in L664. Likewise, there was no change in the expression of *envZ* or *ompR*, the products of which also regulate the expression of *ompF* and *ompC*. However, compared with SL1344, expression of *ramA*, which encodes RamA and is also a member of the AraC-XylS family, was increased 8.7-fold in L664. Previous data[26] suggest that porin genes are part of the regulon of RamA.

L664 has altered expression of numerous virulence genes, but is not attenuated

The ability of a bacterium, such as *Salmonella*, to infect the host is multifactorial and the expression of several virulence genes was different in L664 compared with SL1344. L664 had decreased expression (2–8-fold) of genes that encode proteins required for *Salmonella* to colonize and infect its host. These included 8 genes within salmonella pathogenicity island (SPI)-1 and 13 genes in SPI-2, as well as 5 genes in SPI-3, 4, 5 and 6 (Table S6). However, western blotting revealed that production of the secreted SPI-1 proteins SipA, SipB and SipC by L664 was similar to that by SL1344. It has been suggested that motility may be important in the virulence of *Salmonella* Typhimurium.[35] Expression of flagella and chemotaxis genes was decreased in L664 (Table S6) suggesting that the MDR mutant was less motile than the susceptible parental strain, SL1344. The ability of L664 to migrate through or over semisolid agar was also evaluated. Compared with SL1344, L664 showed a significant ($P<0.000016$) decrease in its ability to swim in 0.25% minimal agar. However, no significant difference was observed in its ability to swarm over 0.5% minimal agar. There was also no significant difference in the ability of L664 to adhere to, or survive in, mouse macrophage RAW 264.7 cells compared with SL1344 (Figure S1a). Furthermore, no attenuation of L664 was observed in the *C. elegans* infection model (Figure S1b). More detailed analysis of the expression of genes encoding proteins involved in virulence revealed that *invF* and *hilA* were not differentially expressed in L664. HilA is a member of the ToxR/OmpR family and is known to activate the *sip* operon, as well as to activate the transcriptional regulator InvF, which also induces the expression of the secreted proteins of the *sip* operon,[36] which are important in invading host cells. InvF also has functions independent of HilA and is known to control expression of the effector protein, SopB.[37] There was also no decrease in the expression of *sopB*. Furthermore, there was no decrease in the expression of the two-component regulator, BarA/SirA, which is known to control invasion.[38] Taken together, despite changes in the level of transcription of some virulence genes, this was apparently insufficient to confer a distinct phenotype, and the ability of MDR L664 to infect the host was equal to that of its wild-type parental strain, SL1344.

Mutation in ramR confers a smaller effect on the transcriptome than inactivation of ramR

To determine which aspects of the L664 phenotype and subset of the transcriptome were due to mutation in *ramR*, the transcriptome of L664 was compared with that of a mutant in which *ramR* had been inactivated, L1007.[26] Expression of the efflux pump genes *acrA/B* was similar in L1007 and in L664. However, compared with SL1344, expression of *ramA* was 25-fold higher in L1007, whereas L664 was ~9-fold greater. These data suggest that the mutant RamR in L664 retained partial repression of *ramA*. There were other differences in the transcriptomes and phenotypes between L664 and L1007. For instance, L1007 had no differential expression of SPI genes, increased (not decreased) expression of flagellar and chemotaxis genes and had reduced virulence in various models of infection.

The transcriptome of L664 resembles that of E. coli exposed to the bactericidal fluoroquinolone, norfloxacin

Transcriptional analysis of L664 showed a decrease in expression of 45 genes that encode proteins involved in cellular respiration and other energy-releasing pathways (Table S6). Pathway Tools[39] analysis revealed that expression of 45 genes encoding enzymes involved in energy-producing pathways, such as glycolysis and the TCA cycle, was significantly different from that in SL1344 (Figure 1). The number of metabolic genes with differential expression was much lower in L1007 (*ramR::aph*), and where changes were seen they were opposite to those in L664. For instance, expression of *glpBCF*, *frdABD*, *pckA* and *ilvM* was increased in L1007 but decreased in L664. Kohanksi *et al.*[21] also noted increased expression of *nuo* genes after fluoroquinolone treatment. These were also differentially expressed by L664; however, in the MDR mutant, expression of *nuo* genes was decreased.

Due to the similarities between the transcriptome of L664 and data published by Kohanski *et al.*[21] it led us to hypothesize that the response to ciprofloxacin had been 'fixed' by mutation. Therefore, to further characterize the involvement of the mutation observed in *tctA* of L664 (G109S), expression of four representative metabolic genes, *acnA*, *aceF*, *accA* and *pykF*, was determined for a mutant in which *tctA* had been inactivated (L1207), along with its complement (L1269) and another

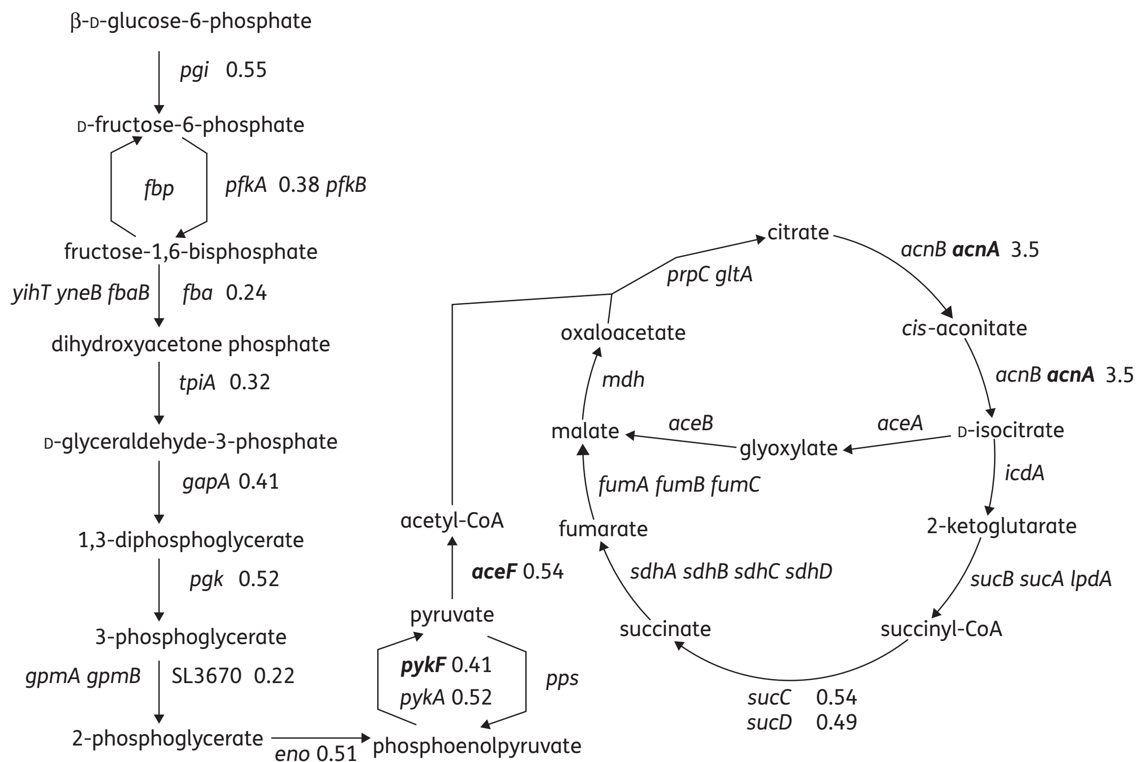

Figure 1. Pathway Tools output of gene expression data of L664 relative to SL1344. Values show the altered expression of 14 genes in the glycolysis and TCA cycle pathways. Where no value is shown, no change in expression was detected. Values in bold text indicate those where differential expression was verified by RT–PCR.

Table 1. Generation time after growth in minimal medium and fold expression of *accA*, *aceF*, *acnA* and *pykF* observed in L664 and of *tctA* mutants compared with SL1344

		Fold change[a] relative to SL1344			
Strain	Generation time (min)	*accA*	*aceF*	*acnA*	*pykF*
SL1344	88.78 ± 6.3	1	1	1	1
L1007 (SL1344 *ramR::aph*)	93.43 ± 9.4	(1)	(1)	(1)	**(1.49)**
L664	**128.7 ± 8.5**	**1.45**	**0.67**	**2.43**	**0.65**
L1316 (SL1344 *glgCAP::aph*)	89.26 ± 8.2	ND	ND	ND	ND
L1207 (SL1344 *tctA::aph*)	**131.3 ± 6.2**	**1.63**	**0.57**	**2.05**	**0.59**
L1269 [SL1344 *tctA::aph*/pGEM-*tctA*(WT)]	91.28 ± 5.3	1.01	0.99	0.97	1.18
L1208 [SL1344 *tctA::aph*/pGEM-*tctA*(SDM G109S)]	**122.8 ± 8.6**	**1.55**	**0.55**	**1.95**	**0.64**

WT, wild-type; SDM, site-directed mutagenesis; ND, not determined.
Bold text indicates statistically significant differences.
[a]Data are from RT–PCR; values in brackets are from microarray data.

mutant in which the L664 *tctA* mutation had been introduced by site-directed mutagenesis (L1208; Table S3). Similar changes in expression of the four metabolic genes were seen in L664, L1207 and L1208 (Table 1). Biolog PM plates 1 and 2 were used to compare the ability of L664 with its parental strain, SL1344, to use 192 single carbon sources (Figure 2). SL1344 could utilize all; however, L664 was less able to utilize 65 (34%). L664 was not better able than SL1344 to utilize any of the tested carbon sources.

The growth kinetics of SL1344, L664, L1316 (*glgCAP::aph*), L1207 (*tctA::aph*), L1269 (*tctA::aph*/pGEM-*tctA*) and L1208 [*tctA::aph*/pGEM-*tctA*(G109S)] were compared after growth of the bacteria in LB and minimal medium. No difference was observed in growth in liquid LB medium, or in time-to-grow

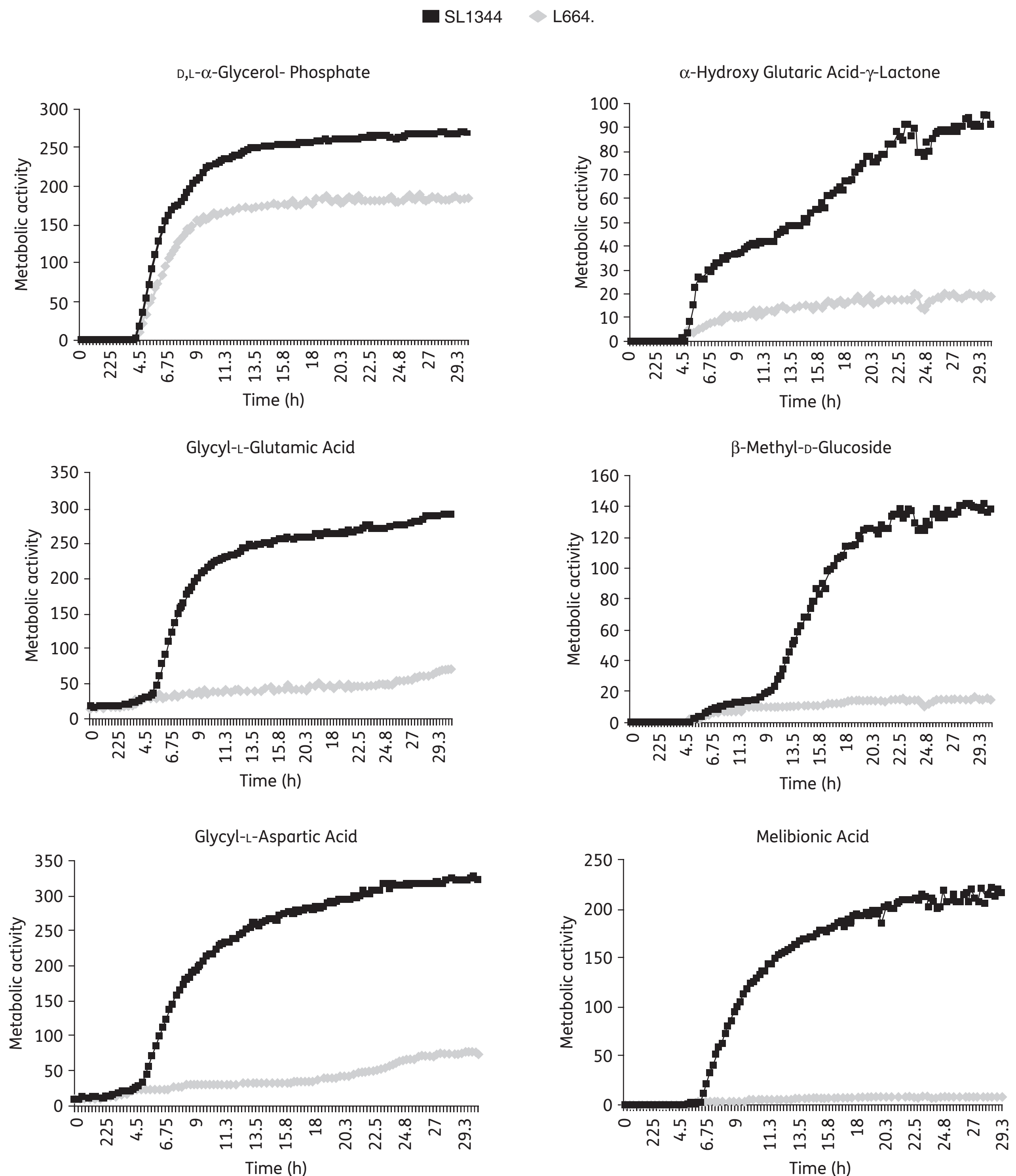

Figure 2. Selected carbon substrates that L664 poorly utilizes, as assayed on the Biolog PM.

or size of colonies on LB agar (Figure S2a). L664 and the mutants that contained a mutation in *tctA* (or had the gene inactivated) grew poorly in minimal liquid medium compared with those mutants that had wild-type *tctA* (Figure S2b). These mutants did not form colonies on minimal agar even after 72 h of incubation. Inactivation of *ramR* or *glgCAP* had no effect upon growth in minimal medium (Table 1). These data suggest that the growth defects of L664 are entirely due to the mutation in *tctA* and the consequent metabolic effects.

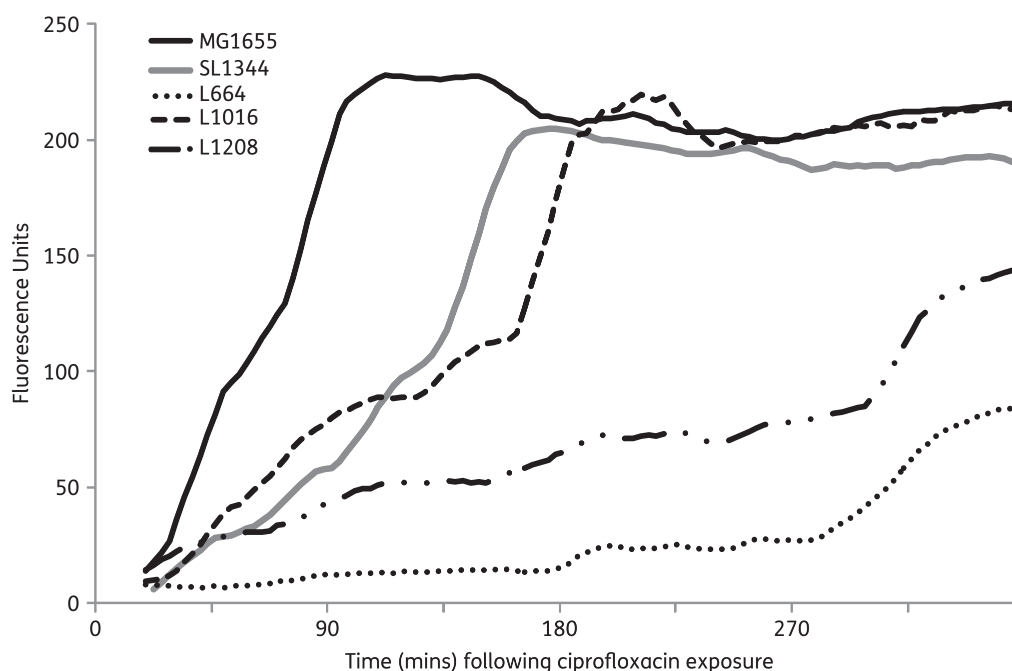

Figure 3. Production of ROS (as indicated by fluorescence) in *E. coli* (MG1655), *Salmonella* Typhimurium (SL1344), L664, L1016 and L1208 following exposure to subinhibitory levels of ciprofloxacin (0.5× MIC).

Is L664 hypermutable?

As L664 contained three mutations, the transcriptome of L664 was interrogated for expression of genes in which mutations or altered expression has been previously associated with conferring an altered mutable state; these comprised *dinI*, *dinF*, *lexA*, *mutL*, *recA*, *rpoS*, *sulA*, *uvrB*, *uvrD*, *uvrA*, *umuD* and *umuC*. Only *lexA* had altered expression: half that of SL1344. *mutS* was not on the microarray. Furthermore, the frequencies of mutation to resistance and mutations per generation in the presence of 100 mg/L rifampicin were not significantly different (frequency 5.6×10^{-9} versus 9.7×10^{-8}, and 0.32×10^{-7} versus 0.48×10^{-7} mutations per generation for SL1344 and L664, respectively; $P=0.317$). Taken together these data indicated that L664 was not hypermutable. Therefore, the mutations had arisen spontaneously and had become fixed in the population because they conferred an advantage (or no disadvantage) under the conditions used to select L664.

Does mutation in tctA or glgA confer a survival advantage in the presence of antibiotic?

It has been suggested that: (i) mutants defective in components of the TCA cycle have increased survival during norfloxacin[19,21,22] and nalidixic acid exposure;[40,41] and (ii) exposure to a fluoroquinolone gives rise to ROS that transiently increase the rate of mutation. Exposure of SL1344, L664, L1016 and L1208 to subinhibitory levels of ciprofloxacin gave rise to the production of ROS when compared with *E. coli* MG1655 (Figure 3). However the onset of ROS production was delayed by approximately 3 h for *tctA* mutants compared with wild-type *E. coli* (MG1655) and *Salmonella* Typhimurium (SL1344). Based on these findings it was hypothesized that, compared with SL1344 and L1269 (*tctA*::*aph*/pGEM-*tctA*), L664 and L1208 [*tctA*::*aph*/pGEM-*tctA*(G109S)] would have increased survival to ciprofloxacin. However, at the MIC, 2× MIC, 5× MIC and 10× MIC of ciprofloxacin for each of SL1344, L664, L1208 and L1269 there was no difference in the rate of killing by the antibiotic over 18 h (Figure S3).

It was also hypothesized that during antibiotic exposure there would be a greater transient increase in the rate of mutation for a *tctA* mutant than for SL1344. Furthermore, if mutation in *tctA* offered a survival advantage during antibiotic exposure, mutation in this gene would be found more frequently in mutants selected by antibiotic exposure. It was also postulated that under these conditions no revertants containing wild-type *tctA* would arise from the TctA mutant. To explore these hypotheses, SL1344, L664 and L1208 were exposed on two separate occasions to no antibiotic or to 0.5× MIC (for each strain) of ciprofloxacin, ampicillin or rifampicin. As described by Kohanski *et al.*,[22] the strains were exposed to antibiotic for 4 h followed by a recovery period of 18 h in antibiotic-free medium. The mutation rates and frequencies of mutation to resistance were determined. There was no difference in the values for SL1344, L664 and L1208 exposed to ciprofloxacin, with or without prior exposure to ciprofloxacin or ampicillin (Table 2). Only the cultures exposed to ciprofloxacin gave rise to ampicillin-resistant colonies; all were MDR [resistant to chloramphenicol (8 mg/L), tetracycline (2 mg/L) and triclosan (0.25 mg/L)], suggesting they were *ramR* mutants. No rifampicin-resistant colonies were obtained irrespective of growth condition. Data for ciprofloxacin exposure, but not those for ampicillin exposure, were similar to those obtained by Kohanski *et al.*[22] In this experiment, none of the mutants arising from SL1344 had a mutation in *tctA*. Additionally, *tctA* revertants were selected from L664 and

Table 2. Frequency of mutation and mutations per generation of SL1344, L664, L1316 and L1208

		Mutation frequency ($\times 10^{-9}$)/mutations per generation ($\times 10^{-8}$) after plating onto agar containing:			
Strain	4 h of exposure to	CIP	AMP	RIF	reversion to wild-type *tctA*
After growth in the presence of antibiotic for 4 h and then 18 h recovery in antibiotic-free medium					
SL1344		1.9/0.075	0	0	—
	AMP	0	0	0	—
	CIP	3.3/0.22	4.4/0.27	0	—
L664		7.8/0.25	0	0	0
	AMP	9.2/0.28	0	0	0
	CIP	8.3/0.25	7.6/0.23	0	0.53/0.03
L1316		6.0/0.30	—	—	—
	CIP	2.5/0.19	—	—	—
L1208		9.9/0.25	0	0	0
	AMP	7.2/0.28	0	0	0
	CIP	5.9/0.22	8.9/0.3	0	0
After replica plating growth on antibiotic-free plates on to antibiotic-containing LB agar or minimal agar					
SL1344		1.6/0.06	0	0	—
	AMP	0	0	0	—
	CIP	3.3/0.22	5.2/0.31	0	—
L664		5.4/0.18	3.1/0.12	0	5.9/0.19
	AMP	6.5/0.21	0.77/0.04	0.46/0.03	4.5/0.15
	CIP	7.6/0.23	8.4/0.25	0.26/0.02	4.1/0.14
L1316		3.6/0.20	—	—	—
	CIP	2.5/0.19	—	—	—
L1208		7.9/0.22	0.47/0.027	0	6.1/0.19
	AMP	5.6/0.24	3.2/0.16	0	5.2/0.17
	CIP	5.1/0.20	8.4/0.29	0.27/0.03	3.2/0.12

AMP, ampicillin; CIP, ciprofloxacin; RIF, rifampicin.
—, not tested.
Concentrations of antibiotics to which strains were exposed for 4 h in broth and then on agar plates following replica plating: SL1344, ampicillin (1 mg/L), ciprofloxacin (0.007 mg/L), rifampicin (100 mg/L);
L664, L1316 and L1208, ampicillin (2 mg/L), ciprofloxacin (0.06 mg/L), rifampicin (100 mg/L).

L1208 after exposure to ciprofloxacin, but not after exposure to ampicillin. There were no statistically significant differences in the frequencies of mutation or mutations per generation for antibiotic-free cultures versus those exposed to ciprofloxacin, between SL1344, L664 and L1208. At this stage an additional experiment was added to those of Kohanksi *et al.*:[22] the colonies obtained on the antibiotic-free agar were replica-plated onto antibiotic-containing LB and minimal agar. Except for SL1344 and the culture derived from the 4 h ampicillin exposure, both ciprofloxacin- and ampicillin-resistant colonies were obtained. In addition, rifampicin-resistant colonies were obtained from L664 in the cultures arising from the initial 4 h of exposure to ampicillin and ciprofloxacin, and from L1208 after 4 h of exposure to ciprofloxacin. Irrespective of whether exposed to antibiotic or not, all cultures of L664 and L1208 contained *tctA* revertants. When tested, the revertants all contained wild-type *tctA*, indicating a point mutation reversing the spontaneous mutation in L664 and the engineered site-directed mutation in L1208.

Inactivation of *glgCAP* had no effect on the frequency of mutation to antibiotic resistance (Table 2).

Discussion

Although, after ciprofloxacin exposure, the MDR mutant L664 was selected at a frequency suggesting a mutation in a single gene,[25] whole genome sequencing of L664 revealed two SNPs: one in *ramR*, the second in *tctA* plus a 1 bp deletion in *glgA*. The mutation in *ramR* in L664 conferred increased expression of *ramA*, which resulted in MDR and organic solvent tolerance. L664 expressed less *ramA* than a *ramR::aph* mutant (L1007), and comparison of their transcriptomes suggested that the mutated RamR of L664 has retained some repressor activity. MDR and/or fluoroquinolone-resistant clinical isolates often contain multiple mutations.[33] Marcusson *et al.*[42] showed that in *E. coli* up to three mutations in a topoisomerase gene and *marRO* (homologous to *ramR*) conferred a fitness burden, but a fourth or fifth mutation in part ameliorated this effect. As virulence has been shown to be RamA-dependent,[26] it is possible that this lower level of RamA, whilst sufficient to cause MDR, was insufficient to attenuate L664. There are several hypotheses to explain how, during antibiotic exposure, L664 was selected to

contain three mutations. Firstly, one or both of the mutations was co-incidentally selected with the third, but these conferred no advantage or disadvantage to the mutant. Secondly, exposure to the antibiotic conferred conditions in which multiple mutations could occur more frequently than in antibiotic-free conditions. Thirdly, one of the mutations conferred antibiotic resistance, and another allowed survival of the mutant during antibiotic stress.

It has been suggested that antibiotic-resistant bacteria are less fit than their antibiotic-susceptible counterparts.[43] Although counterintuitive, as the numbers of antibiotic-resistant bacteria isolated from infections in humans and animals continue to rise throughout the world, this has been explained not only by bacteria evolving to acquire mutations conferring antibiotic resistance, but also by various mechanisms that ameliorate the effects of such resistance.[43,44] These include acquiring compensatory mutations. There have been several reports associating fitness costs *in vivo* with antibiotic resistance mutations in *Salmonella* Typhimurium.[43–47] Giraud *et al*.[48] showed that highly ciprofloxacin-resistant mutants with one or more mutations in topoisomerase genes had longer generation times and were unable to colonize the gut of chickens. More recently, Wang *et al*.,[49] Fabrega *et al*.[50] and O'Regan *et al*.[51] have suggested a link between decreased expression of *Salmonella* SPI-1 genes and fluoroquinolone resistance, and that such resistant strains were less fit. All mutants in these studies had one or more mutations in a topoisomerase gene plus a mutation in *ramR*, and were selected after multiple exposures to ciprofloxacin, or from a strain with pre-existing topoisomerase gene mutations. Therefore, the contribution of each mutation to the phenotype is difficult to define, especially as mutations affecting *ramA* expression confer pleiotropic cellular effects,[26] as do those in topoisomerase genes (M. A. Webber and L. J. V. Piddock, unpublished data). We also previously showed that artificially engineered high-level expression of *ramA* in *Salmonella* Typhimurium conferred both MDR (due to enhanced efflux) and reduced fitness, and that this was associated with reduced virulence gene expression.[26] However, inactivation of *ramR* did not give rise to high-level *ramA* expression and had no effect on virulence *in vivo*.[26]

Data arising in this study indicate that ciprofloxacin selected for the mutant containing the mutation in *ramR,* as this gave a selective advantage for growth in the presence of this antibiotic. Without the genome sequencing data it is unlikely that the mutations in *tctA* and *glgA* would have been detected. Therefore, the transcriptional and phenotypic changes may well have been incorrectly attributed to the mutation in *ramR*. Furthermore, the metabolic gene expression changes due to inactivation of *ramR* suggest that it is unlikely that the mutation in *ramR* can ameliorate the energy costs of the mutation in *tctA*. These data provide evidence that caution must be taken in the interpretation of transcriptome data for antibiotic-resistant clinical isolates and spontaneous mutants.

The mutations in *glgA* and *tctA* were unexpected. However, inactivation of *glgCAP* had no effect on antibiotic susceptibility or on selection of antibiotic-resistant bacteria, and so it is thought that this mutation was selected coincidentally with those in *ramR* and *tctA*. It was hypothesized that mutation in *tctA* affected the conformation of TctA and thus hindered the flow of tricarboxylates into the cell. The SNP found in *tctA* had no effect on antibiotic susceptibility, suggesting that TctABC did not transport antibiotics. The differential expression of genes encoding enzymes in energy-producing pathways, and altered growth kinetics in L664, were shown to be due to the mutation in *tctA*. The Biolog PM data confirmed the metabolic changes and revealed that L664 was unable to utilize several carbon sources. Eng *et al*.[52] noted that carbon-source limitation in *E. coli* reduced the efficacy of killing by bactericidal drugs, including fluoroquinolones. Furthermore, Gruer *et al*.[40] and Kohanski *et al*.[21] found that *E. coli icdA*, *acnA* and *acnB* mutants had increased survival to nalidixic acid and fluoroquinolone treatment. However, despite the differential production of ROS due to antibiotic exposure, we found that the site-directed TctA mutant (L1208) was killed as rapidly as the parental strain (SL1344) by ciprofloxacin, and, in the absence of antibiotic in rich medium, had similar growth kinetics. L664 was also killed at the same rate as L1208 when exposed to the same multiple of the MIC. In addition, mutation in *tctA* did not give rise to a higher frequency of resistance or mutations per generation when L1208 was exposed to ciprofloxacin. Nonetheless, as found by Kohanksi *et al*.[22] with *E. coli* and norfloxacin, exposure of SL1344, L664 and L1208 to ciprofloxacin gave rise to ampicillin-resistant colonies; these were all MDR. As approximately 50% of the spontaneous mutants selected after exposure of SL1344 to ciprofloxacin were MDR, and ampicillin resistance is typically part of this phenotype, this was not a surprise. No rifampicin-resistant mutants were obtained; this agent is not usually part of the MDR phenotype.[8] Finally, spontaneous mutants with reversion to wild-type *tctA* were obtained with and without antibiotic pressure, and at similar frequencies.

While the hypothesis of Kohanski *et al*.[21,22] that fluoroquinolones increase ROS, allowing selection of MDR bacteria, is both plausible and attractive (and we confirm production of ROS), we have no data to support the theory that this leads to selection of MDR strains. Our results indicate that the mutations in *tctA* and *glgA* were random as they did not pre-exist in the parental strain, and that although the mutation in *tctA* affected energy-producing pathways, it did not provide a survival advantage or disadvantage in the presence of an antibiotic, and so there was no pressure for selection. These data question not only the model proposed, but whether data obtained with *E. coli* offers a paradigm for the responses of other bacterial species to antibiotics.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S6 and Figures S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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