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Mycobacterium tuberculosis DNA repair in response to subinhibitory concentrations of ciprofloxacin

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***Objectives*: To investigate how the SOS response, an error-prone DNA repair pathway, is expressed following subinhibitory quinolone treatment of *Mycobacterium tuberculosis*.**

***Methods*: Genome-wide expression profiling followed by quantitative RT (qRT)–PCR was used to study the effect of ciprofloxacin on *M. tuberculosis* gene expression.**

***Results*: Microarray analysis showed that 16/110 genes involved in DNA protection, repair and recombination were up-regulated. There appeared to be a lack of downstream genes involved in the SOS response. qRT–PCR detected an induction of *lexA* and *recA* after 4 h and of *dnaE2* after 24 h of subinhibitory treatment.**

***Conclusions*: The pattern of gene expression observed following subinhibitory quinolone treatment differed from that induced after other DNA-damaging agents (e.g. mitomycin C). The expression of the DnaE2 polymerase response was significantly delayed following subinhibitory quinolone exposure.**

Keywords: SOS repair, mutation, gene expression

Introduction

Quinolones are commonly used for the treatment of a wide range of bacterial infections. Resistance has emerged rapidly in important bacterial pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA)[1] largely through transmission, *Pseudomonas aeruginosa*[1] and *Streptococcus pneumoniae*.[2] Resistance emerging in *Mycobacterium tuberculosis* could threaten the development of these agents for the treatment of susceptible and multidrug-resistant disease that is currently underway.[3]

Fluoroquinolones act on DNA gyrase resulting in lethal double-stranded DNA breaks.[4] In response to DNA damage, there is potent induction of the SOS regulon: a set of genes involved in DNA repair, recombination and mutagenesis.[5] The first step in the pathway depends on activation by *recA* and repression by *lexA*. Quinolones are less mutagenic in cells that cannot mount an SOS response (for example, stationary-phase cells, $RecA^-$ mutants[6] and *gyrA* mutants[5]). The low fidelity polymerases induced during the SOS response enhance survival through the emergence of quinolone resistance mutations.[7]

Members of the quinolone family with C-8 position substituents, for example, moxifloxacin, have increased efficacy at killing resistant gyrase and topoisomerase IV (a quinolone target in bacteria other than mycobacteria) mutants.[8] This is important as the bactericidal action of the quinolones with C-8 substituents will not only kill mutants but will prevent further mutation. While ciprofloxacin has been part of the regimen for treatment of multidrug-resistant tuberculosis (TB), the superior activity of moxifloxacin (and the probability that it is less likely to select resistance) against pulmonary TB has led to its further evaluation in clinical studies.[9]

We have shown that exposure of *Mycobacterium fortuitum* to subinhibitory concentrations of quinolone increases the mutation rate.[10] When cultures were exposed to 1/2 MIC ciprofloxacin, there was a 73–120-fold increase in mutation rate. This effect was found to be dose-dependent with a smaller increase associated with 1/4 and 1/8 MIC. The increase in mutation rate was found for all selecting agents including rifampicin, erythromycin, gentamicin and moxifloxacin, suggesting that the mutagenic effect of fluoroquinolones is across the whole genome.

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These data are in accord with studies that showed that subinhibitory doses of fluoroquinolones result in an increased mutation rate in *Escherichia coli*, MRSA and *P. aeruginosa*.[1,4,5]

The implications for anti-TB therapy are clear. The prolonged use of fluoroquinolones in an anti-TB regimen may risk treatment with sub-optimal doses, increasing the selection pressure to resistance.[11] To characterize the mechanism behind the induction of mutation by subinhibitory fluoroquinolone exposure in mycobacteria, the induction of the error-prone SOS system was investigated by whole genome expression profiling and quantitative RT (qRT)–PCR in *M. tuberculosis*.

Materials and methods

Quinolone treatment of cultures

Exponentially growing *M. tuberculosis* H37Rv (NCTC 7416) ($\sim 10^6$ cfu/mL) cultured in Middlebrook 7H9 broth, containing 0.2% Tween 80 (BDH) and 10% albumin dextrose catalase enrichment (BD), was exposed to 0.25 mg/L (1/2 MIC) or 0.125 mg/L (1/4 MIC) of ciprofloxacin (Cellgro Herndom) over a period of 24 h. Untreated culture controls were included at 0 h where sterile distilled water was added instead of ciprofloxacin. As a positive control of the SOS response, a sample was also treated with 0.2 mg/L mitomycin C (Sigma Aldrich) for 24 h. This experiment was repeated three times.

RNA extraction

RNA was extracted from each culture at 0, 4, 12 and 24 h using the method of Mangan *et al.*[12] and the Fast RNA Pro-Blue Kit (Q-Biogene Inc.). Samples were purified using the RNeasy MiniElute Cleanup Kit for bacteria (Qiagen Ltd). The RNA was quantified using the Agilent 2100 Bioanalyser System (Agilent Technologies Inc.).

Microarray protocol

M. tuberculosis whole genome arrays, consisting of PCR products representing 3924 genes from *M. tuberculosis* H37Rv, were supplied by the Bacterial Microarray Group at St George's Hospital, University of London (ArrayExpress accession number: A-BUGS-2; http://bugs.sgul.ac.uk/A-BUGS-2). Aliquots of 5 μg (equivalent to $\sim 2–5 \times 10^8$ bacteria) of total RNA were used as a template for cDNA synthesis and fluorescent dye analogues incorporated during reverse transcription using Cy3-dCTP. This was hybridized against Cy5-labelled *M. tuberculosis* H37Rv genomic DNA (provided by Colorado State University). For each sample, two technical replicates were performed. Arrays were scanned using an Affymetrix™ 428 array scanner (MWG). All images were overlaid for feature extraction in ImaGene 5.5 (BioDiscovery Inc.) and further processed with the MAVI Pro 2.6.0 software package (MWG Biotech). Data were entered into GeneSpring GX™ 7.2 (Agilent Technologies) software to perform normalization and statistical analysis.

Statistical analysis

The ratios of Cy3/Cy5 intensities were median normalized using only the data flagged as present or marginal by ImaGene. These ratios were further normalized to the control samples to provide a more meaningful expression ratio that reflected up- or down-regulation compared with the control. Differentially expressed genes were identified by the ANOVA-based approach using a parametric test in which variances were not assumed equal (Welch *t*-test) with a false discovery rate of <0.05.

Fully annotated microarray data has been deposited in BμG@Sbase (accession number: E-BUGS-63; http://bugs.sgul.ac.uk/E-BUGS-63) and also ArrayExpress (http://www.ebi.ac.uk/microarray-as/aer/entry; accession number: E-BUGS-63).

qRT–PCR

qRT–PCR was performed using primers and dual-labelled probes to amplify *dnaE2* from Boshoff *et al.*,[13] *recA* (forward primer, aatgaccggcgcgctga; reverse primer, cgttgaagttctacgcgtc; probe, ttcggg caccacggcgatcttcat), *lexA* (primers from Brooks *et al.*;[14] probe, tcttc ccgctgccgcgtgagc) and *sigA* (forward primer and probe from Hampshire *et al.*;[15] reverse primer, ttctcgacctgatccaggaag).

RNA (5 μL of ~1 pg/reaction) was added to 1× QuantiTect Probe RT–PCR Master Mix (Qiagen) and made up to a final volume of 25 μL with RNase-free water (Qiagen). The reaction was performed on a Rotorgene™ 3000 thermal cycler (Corbett Research). The reaction components and the cycling conditions were all as outlined in the manufacturer's instructions in the QuantiTect™ Probe RT–PCR Handbook. Each sample was processed in triplicate, including a control without RT for each sample also performed in triplicate. All qRT–PCR experiments included a positive control of RNA from *M. tuberculosis* H37Rv. The threshold level was set to 0.1, which was above the background fluorescence of the 'no template' control in the exponential phase of the curve. The efficiency of each probe and primer pair was >99%. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method,[16] normalizing all genes to *sigA*, which is considered to be constant under stress conditions.[17]

Results and discussion

Gene expression analysis by microarrays following exposure to subinhibitory concentrations of ciprofloxacin revealed differential expression across the whole genome. The 4 h treatment with 1/2 MIC ciprofloxacin resulted in the greatest number of genes differentially expressed. The expression of genes involved in DNA repair, mutagenesis and recombination in *M. tuberculosis* was further analysed [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. One hundred and ten genes were identified using the gene list presented by Mizrahi and Andersen,[18] and a search of the Entrez Genome, NCBI database of the COG (clusters of orthologous groups of proteins) classification DNA replication, recombination and repair. The microarray data indicated that, following subinhibitory concentrations of ciprofloxacin, 16/110 genes involved in DNA repair, mutagenesis and recombination were up-regulated (Table 1) and 11/110 genes were down-regulated (data not shown). Following this microarray analysis, we investigated further the expression of *recA* and *lexA*, representing the primary genes involved in the SOS response, and *dnaE2*, the polymerase thought to be associated with mutagenesis.[13] For these experiments, ciprofloxacin exposure was extended to 24 h and mitomycin C, a DNA-damaging agent known to induce the SOS response, was included as a control.

A 2-fold up-regulation of *recA* was observed by microarray analysis after 4 h of treatment with 1/4 MIC ciprofloxacin and 4-fold after 12 h of treatment with 1/2 MIC ciprofloxacin, indicating induction of the SOS response (Table 1). This

Table 1. Up-regulated genes from DNA repair, recombination and mutagenesis gene list (110 genes) with statistically significant differences among time and MIC for 4 and 12 h treatments with 1/2 and 1/4 MIC ciprofloxacin including the *t*-test *P* value

Gene name	Rv no.	Normalized expression (*P* value)	Treatment	Predicted function
tagA	Rv1210	1.44 (3.10E−02)	12 h 1/2 MIC	DNA-3-methyladenine glycosidase I
recF	Rv0003	1.50 (1.22E−03)	4 h 1/4 MIC	DNA replication and SOS induction
	Rv3202c	1.51 (6.95E−03)	12 h 1/2 MIC	possible ATP-dependent DNA helicase
uvrD2	Rv3198c	1.51 (3.40E−03)	4 h 1/4 MIC	putative UvrD
		1.65 (1.16E−02)	12 h 1/2 MIC	
nei	Rv3297	1.60 (1.66E−02)	12 h 1/2 MIC	probable endonuclease VIII
dnaB	Rv0058	1.66 (2.79E−02)	12 h 1/4 MIC	DNA helicase (contains intein)
	Rv2896c	1.68 (1.89E−05)	4 h 1/2 MIC	predicted Rossmann fold nucleotide-binding protein involved in DNA uptake
		2.11 (3.92E−03)	4 h 1/4 MIC	
mutT4	Rv3908	1.76 (8.24E−04)	12 h 1/2 MIC	mutator protein MutT
recA	Rv2737c	1.78 (5.16E−04)	4 h 1/4 MIC	recombinase (contains intein)
		4.13 (8.31E−06)	12 h 1/2 MIC	
gyrA	Rv0006	1.87 (4.59E−03)	12 h 1/4 MIC	DNA gyrase subunit A
dut	Rv2697c	1.89 (5.34E−03)	4 h 1/2 MIC	deoxyuridine triphosphatase
		2.00 (1.99E−05)	4 h 1/4 MIC	
xthA	Rv0427c	2.11 (9.16E−06)	4 h 1/4 MIC	exodeoxyribonuclease III
		4.30 (1.33E−02)	12 h 1/2 MIC	
		3.13 (1.96E−03)	12 h 1/4 MIC	
radA	Rv3585	2.14 (1.24E−02)	12 h 1/2 MIC	probable DNA repair RadA homologue
dnaE1	Rv1547	2.72 (2.43E−03)	12 h 1/2 MIC	DNA polymerase III, [alpha] subunit
	Rv2821c	2.84 (6.83E−05)	4 h 1/2 MIC	uncharacterized protein predicted to be involved in DNA repair (RAMP superfamily)
		2.27 (7.26E−06)	4 h 1/4 MIC	
xthA	Rv0427c	3.13 (1.96E−03)	12 h 1/4 MIC	exodeoxyribonuclease III

Genes are ranked by ascending levels of expression when compared with the untreated control at time 0 h.

up-regulation was confirmed by qRT−PCR. Expression of *recA* increased after 4 h by 2-fold and continued to increase to 4-fold after 12 h of treatment with 1/4 MIC ciprofloxacin (Figure 1). Differences in *lexA* expression during treatment were not identified by microarray analysis but a gradual increase in fold expression over 24 h was observed by qRT−PCR, from 1.8 to 2.3 to 6.8 and 2.9 to 4 to 7.4 with 1/4 and 1/2 MICs, respectively (Figure 1). Subtle shifts of gene expression may not be detected by microarray-based transcriptome analysis because of a smaller dynamic range than qRT−PCR so it is possible that more genes involved in DNA repair may be up-regulated.[19] Small changes in LexA expression have been observed in *M. tuberculosis* after various DNA-damaging treatments but only after extended periods of exposure.[20]

The expression of *dnaE2* was found unchanged in the microarray analysis; however, a delayed response, only observed after 12 h, was detected by qRT−PCR with an increase from 0.8-fold to 25.5-fold and 0.9-fold to 11.2-fold between 12 and 24 h, after 1/2 and 1/4 MIC treatments, respectively (Figure 1).

Following 24 h of treatment with mitomycin C, *recA*, *dnaE2* and *lexA* were overexpressed (Figure 1). The levels of *recA* expression (14.2-fold) were comparable to those found in other studies;[14,20] however, induction of *dnaE2* (7.7-fold) and *lexA* (1.6-fold) were lower than that reported previously,[13,21] but one of these studies used a 90 min exposure to mitomycin C.

The mechanism whereby quinolones increase the mutation rates is thought to be the error-prone SOS system.[22,23] Microarray-based analysis of *M. tuberculosis* gene expression response to sub-MIC of quinolones did not show a consistent pattern of SOS up-regulation. It was noted that both microarray and qRT−PCR analyses demonstrated a significant delay in DnaE2 response. These data suggest that the SOS response following subinhibitory quinolone exposure differs from that with other DNA-damaging agents. Further work is required to dissect these pathways in view of their critical role in the mechanism of hypermutability. The risk of selecting for resistance must be considered when introducing a quinolone to a TB treatment regimen, especially if sub-optimal dosing occurs.

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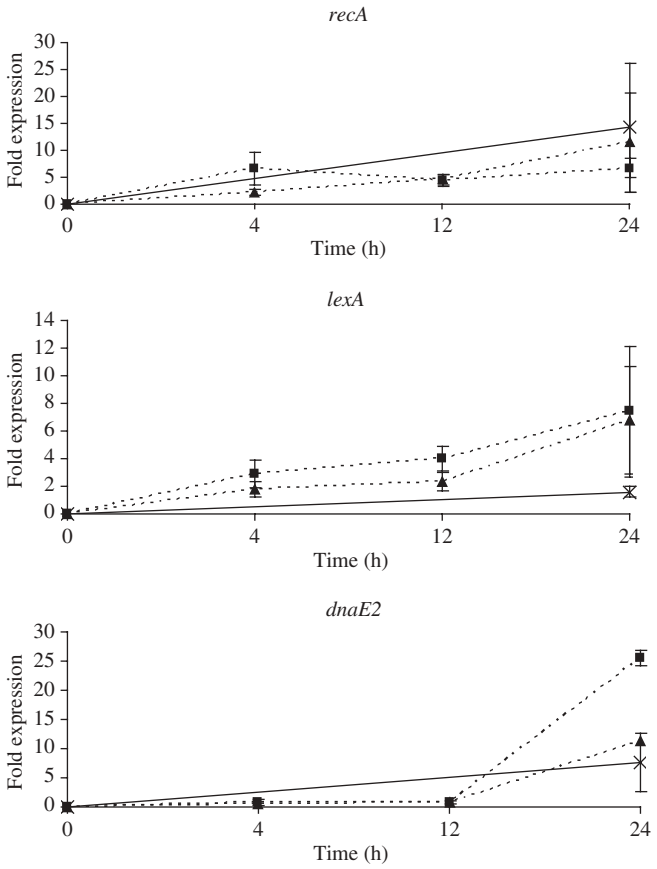

Figure 1. Relative fold change in expression of *recA*, *lexA* and *dnaE2* normalized to *sigA* calculated by the equation $2^{-\Delta\Delta Ct}$, after treatment with 1/4 MIC ciprofloxacin (triangles), 1/2 MIC ciprofloxacin (squares) or 0.2 mg/L mitomycin C (crosses) over 24 h. The values are the means of triplicate samples on at least two independent inductions; the error bars indicate the standard error of the mean.

Transparency declarations

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Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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