Pathogens and Disease, 79, 2021, ftaa079

doi: 10.1093/femspd/ftaa079
Advance Access Publication Date: 22 December 2020
Research Article

RESEARCH ARTICLE

Induction of the reactive chlorine-responsive transcription factor RclR in *Escherichia coli* following ingestion by neutrophils

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One sentence summary: The reactive chlorine-responsive transcription factor RclR is induced in *E. coli* following ingestion by neutrophils.

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ABSTRACT

Neutrophils generate hypochlorous acid (HOCl) and related reactive chlorine species as part of their defence against invading microorganisms. In isolation, bacteria respond to reactive chlorine species by upregulating responses that provide defence against oxidative challenge. Key questions are whether these responses are induced when bacteria are phagocytosed by neutrophils, and whether this provides them with a survival advantage. We investigated RclR, a transcriptional activator of the *rclABC* operon in *Escherichia coli* that has been shown to be specifically activated by reactive chlorine species. We first measured induction by individual reactive chlorine species, and showed that HOCl itself activates the response, as do chloramines (products of HOCl reacting with amines) provided they are cell permeable. Strong RclR activation was seen in *E. coli* following phagocytosis by neutrophils, beginning within 5 min and persisting for 40 min. RclR activation was suppressed by inhibitors of NOX2 and myeloperoxidase, providing strong evidence that it was due to HOCl production in the phagosome. RclR activation demonstrates that HOCl, or a derived chloramine, enters phagocytosed bacteria in sufficient amount to induce this response. Although RclR was induced in wild-type bacteria following phagocytosis, we detected no greater sensitivity to neutrophil killing of mutants lacking genes in the *rclABC* operon.

Keywords: neutrophil; phagocytosis; hypochlorous acid; myeloperoxidase; *Escherichia coli*; RclR transcription factor

Received: 30 September 2020; **Accepted:** 18 December 2020

INTRODUCTION

Immune cells generate reactive oxygen species (ROS) as a component of mammalian antimicrobial defence. Neutrophils provide the first line of attack and their armoury depends to a large extent on ROS production (Winterbourn, Kettle and Hampton 2016). For oxidative killing to be successful, neutrophil ROS need to overcome bacterial antioxidant defences. An important question is whether the bacteria can upregulate these defences sufficiently to protect against neutrophil oxidants and thus give themselves a survival advantage.

Neutrophils respond to bacteria by ingesting them into phagosomes. At the same time they assemble their NADPH oxidase (NOX2) on the phagosomal membrane and release myeloperoxidase and other proteins from intracellular granules into the phagosomal space (Klebanoff *et al.* 2013; Winterbourn, Kettle and Hampton 2016; Nauseef 2019). NOX2 converts oxygen to superoxide which dismutates to give hydrogen peroxide (H_2O_2). Myeloperoxidase uses the H_2O_2 to oxidise chloride to hypochlorous acid (HOCl), the most bactericidal of the neutrophil oxidants. HOCl can further react with amine groups on small molecules or proteins to generate chloramines (R-NHCl) which retain the oxidising capacity and selectivity for sulfur centers of HOCl but are less reactive (Fig. 1). In media or biological fluids, multiple amino acids will react with HOCl and chlorine exchange between amines will occur so a range of reactive chlorine species will be generated (Peskin and Winterbourn 2001; Peskin *et al.* 2005; Pattison and Davies 2006; Ashby *et al.* 2020).

The best known bacterial response to ROS is the upregulation of genes responsive to the transcription factor OxyR. Homologues of OxyR are widely distributed but it is best characterised in *Escherichia coli* (Imlay 2015). PerR plays a similar role to OxyR in Firmicutes. Although they operate via different mechanisms, both OxyR and PerR respond particularly to H_2O_2 and increase expression of many of the same protective genes. OxyR contains a thiol switch that is sensitive to low concentrations of H_2O_2, and is activated by oxidation to a disulfide. In *E. coli* this increases expression of about two dozen genes, including antioxidant systems involving a catalase (KatG), a peroxiredoxin system (AhpC/AhpF), thioredoxin and glutaredoxin A (Antelmann 2015; Imlay 2015). It is also well established that bacteria upregulate the SoxRS transcriptional system in response to compounds that generate superoxide (Imlay 2015). However, this appears to be primarily an indirect response to the redox active compounds, which need to be intracellular. Therefore a superoxide response may not be relevant where superoxide is produced by the neutrophil in the phagosomal space and is unable to cross membranes.

More recently, it has been recognised that bacteria respond to sublethal doses of HOCl. Focussing on *E. coli*, upregulation of genes responsive to the transcription factors, HypT (Drazic *et al.* 2013; Drazic *et al.* 2014), RclR (Parker *et al.* 2013) and SoxRS (Dukan *et al.* 1996), inactivation of the transcriptional repressor, NemR (Gray *et al.* 2013, 2015), induction of a heat shock type response (Dukan *et al.* 1996) and formation of polyphosphate (Gray *et al.* 2014) have been observed. In contrast, activation of OxyR by HOCl was not detected (Dukan *et al.* 1996). Activation of HypT involves oxidation of a Met residue, whereas NemR and RclR responses require Cys oxidation. Bacteria lacking RclR (Parker *et al.* 2013) or OxyR (Gundlach and Winter 2014) showed increased sensitivity to HOCl and the NemR mutant was more resistant (Gray *et al.* 2013). NemR also responds to electrophiles, but of the reagents that have been tested, only HOCl and chloramines activated HypT and RclR. Therefore, to study how *E. coli* respond to HOCl when ingested into neutrophil phagosomes, we have focussed on RclR.

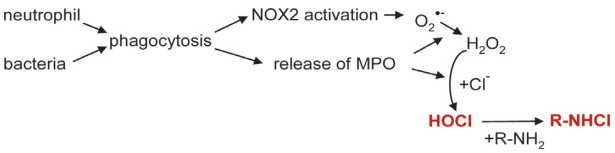

Figure 1. Scheme showing the steps to reactive chlorine species production following phagocytosis. MPO is released from cytoplasmic granules and catalyses the dismutation of superoxide and its conversion to HOCl. As in other biological media, one reaction is with amine groups present to form chloramines (Winterbourn *et al.* 2006).

RclR is a transcription factor that on oxidation activates the *rclABC* operon for expression of the proteins RclA, RclB and RclC (Parker *et al.* 2013). RclA has recently been identified as a flavoprotein with Cu(II) reductase activity (Baek *et al.* 2020). RclB and RclC are postulated to act synergistically with RclA (Derke *et al.* 2020), although this still requires direct confirmation. Although initial observations suggested that *E. coli* lacking RclR were more sensitive to killing by reactive chlorine species (Parker *et al.* 2013), recent analyses indicate that altered sensitivity is more evident for the time of recovery after sublethal doses (Derke *et al.* 2020).

The response of the RclR system has not been investigated in *E. coli* phagocytosed by human neutrophils. The one in depth study of oxidant-responsive changes in mRNA expression was carried out by Staudinger *et al.* (2002). They observed strong induction of a number of genes following phagocytosis, in particular those under the control of OxyR. They also showed that an *oxyRS* mutant strain of *E. coli* was more sensitive to oxidant-dependent neutrophil-mediated killing. At that time, a full gene array for the strain of bacteria used was not available, and changes in HOCl-responsive genes (which were not known at the time) were not reported.

We have investigated whether the HOCl-responsive transcription factor RclR is upregulated following phagocytosis by measuring expression of *rclB* using qRT-PCR, as described previously (Parker *et al.* 2013). We show that *E. coli* are exposed to sufficient HOCl in the phagosome to cause rapid induction of *rclB* expression and thus mount a defence to HOCl. Although this response should provide a survival advantage, we were not able to detect enhanced killing of mutant *E. coli* lacking genes in the rclABC operon following neutrophil ingestion, suggesting the presence of additional, compensatory systems.

MATERIALS AND METHODS

Bacteria and reagents

Null mutations were generated in wild-type *E. coli* MG1655 (F^-, λ^- and *rph-1 ilvG*$^-$ *rfb-50*) for the knockout strains Δ*rclA* (MJG046) and Δ*rclABC* (MJG901) as previously described (Parker *et al.* 2013). Bacteria were stored and grown under standard conditions, using Miller's Luria–Bertani or lysogeny broth (LB, ThermoFisher, Waltham MA) unless stated otherwise, such as when using MOPS minimal medium (Teknova Inc, Hollister CA). M9 minimal medium was made to the following formulation: 42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7 mM NH_4Cl, 17.1 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.4% glucose was used. Fresh HOCl stock solutions were prepared daily from sodium hypochlorite (household bleach, Pental, Melbourne, Australia) by measuring A_{292} at pH 12 (ε_{292} 350/M/cm) and then dilutions were made in phosphate buffered saline (PBS) or Hank's buffer

(Sigma H8264, St Louis Mo). Chloramines were prepared immediately prior to use by mixing HOCl with excess of ammonium sulfate, glycine or taurine (Thomas, Grisham and Jefferson 1986). The myeloperoxidase inhibitor, 2-thioxanthine derivative TX1 (3-isobutyl-2-thioxo-7*H*-purin-6-one) was gifted by AstraZeneca; all other reagents were of the highest purity commercially available and were from Sigma-Aldrich unless stated otherwise.

Neutrophil isolation

Heparinised peripheral blood was collected from healthy adult volunteers, with informed consent approved by the Health and Disability Ethics Committee, New Zealand. Neutrophils were freshly isolated by dextran (MW 200–300 000, MP Biomedicals, Irvine CA) sedimentation followed by Ficoll-PaquePLUS (GE Healthcare, Chicago Il) centrifugation and hypotonic lysis of contaminating erythrocytes (Segal, Dorling and Coade 1980). Preparations of neutrophils were approximately 95% pure, and were finally resuspended at 1×10^7 per mL in Hank's buffer containing 10% serum.

Treatment of isolated *E. coli* for RclR induction

For treatment of bacteria suspended in MOPS minimal medium (experiments carried out in Birmingham), *E. coli* (wild-type MG1655) were grown overnight in MOPS , then 500 μL of the overnight culture was diluted into 9.5 mL fresh medium and grown with shaking at 37°C to mid-log phase (A_{600} = 0.3–0.4). Once mid-log phase was reached, 1 mL aliquots were put into microcentrifuge tubes for each treatment. Cells were treated by adding the indicated amounts of HOCl (diluted in metal-free water), then incubated at 37°C for 10 min. For gene expression analysis, cells were harvested and stored at −20°C in RNA*later* (ThermoFisher) for subsequent RNA purification using the RiboPure Bacteria RNA Purification kit (ThermoFisher). For survival assays, treated cells were immediately serially diluted in PBS and 5 μL aliquots were spotted on LB agar plates. Plates were dried and incubated at room temperature for 2 days for colony formation.

For the experiments done in comparison with neutrophil studies (in Christchurch), overnight cultures in LB of *E. coli* (wild-type strain ATCC 25922) were diluted into fresh LB or washed and resuspended in M9 medium as indicated for different experiments), then grown to OD ∼0.5, measured at 600 nm. Bacteria were immediately washed with PBS, resuspended at twice the desired final concentration in Hank's then rapidly mixed with an equal volume of stated concentrations of HOCl or premixed chloramines that were diluted in Hank's and incubated at 37°C. At stated times, 1 mM methionine was added to quench unreacted chloramines, then bacteria were spun down and RNA was purified using a NucleoSpin extraction kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions.

Incubation of *E.coli* with neutrophils for RclR induction

Wild-type *E. coli* (ATCC 25922) grown in LB medium to OD ∼0.5 were resuspended at 2×10^8 per mL in Hank's buffer (OD ∼0.35) and opsonised by incubating for 20 min at 37°C by the addition of 10% serum (pooled from several normal donors and heat-inactivated at 56°C for 20 min). Bacteria were then mixed with an equal volume of neutrophils in Hank's buffer to give a ratio of 20:1 and incubated at 37°C with rotation for the requisite time. For experiments using inhibitors, an addition was made (at twice the desired inhibitor concentration) to the neutrophil suspension and incubated for 10 min at 37°C prior to mixing with the bacteria. Incubations were stopped by placing tubes on wet ice and centrifuging with a swing-out rotor at 100 g for 5 min at 4°C to sediment the neutrophils but leave the non-ingested bacteria in the supernatant. The neutrophils containing ingested bacteria were washed twice with PBS using the same procedure. The pellet was sonicated in PBS with two 15 s bursts using a microtip probe (Omni International, Kennesaw Ga) to lyse the neutrophils and RNA was purified as above. A bacteria alone sample was carried through the procedure as reference.

rclB detection by qRT-PCR

Gene expression of *rclB* was measured by qRT-PCR using standard methodology and as described previously (Parker *et al.* 2013). Briefly, cDNA was generated from the RNA extracted from treated or ingested *E. coli* using a reverse transcription kit (either SuperScript IV VILO from ThermoFisher in Birmingham, or PrimeScript kit, Takara, Kusatsu, Japan in Christchurch) and qRT-PCR was performed using a BioRad CFX96 thermocycler (in Birmingham) or a Roche LightCycler 480II (in Christchurch). In both centres, *rclB* was amplified and normalised against 16S (*rrsD*) gene expression, and changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The details of the dye and primers were as follows: SsoAdvanced Universal SYBR Green system (Biorad, Hercules CA) was used in Birmingham with *rclB* primers 5′-GCA ACA CTA TTA TCT GGC GTT ATG-3′, 5′-CGA TGT GAC TTT CCA GGA TGA-3′ and *rrsD* primers 5′-GAG CAA GCG GAC CTC ATA AA-3′, 5′-TCC CGA AGG TTA AGC TAC CTA-3′; in Christchurch EvaGreen dye (Biotium, Hayward CA) was used with *rclB* primers 5′-CAC TAT TAT CTG GCG TTA TGG CAT T-3′, 5′-TAT ACA ATA CTG CTG TTG CGG TTG-3′ and *rrsD* primers 5′-AAG AAC TTA CCT GGT CTT GAC ATC-3′, 5′-CAG TTT ATC ACT GGC AGT CTC CTT-3′.

Neutrophil killing assay

The survival of *E. coli* Δ*rclA* and Δ*rclABC* mutants was compared to the parent wild-type MG1655 after phagocytosis by neutrophils. Pre-warmed human neutrophils (final concentration 5×10^6/mL) were mixed at 1:7 ratio with opsonised bacteria in final volumes of 500 μL Hank's buffer containing 10% heat-inactivated pooled or autologous serum, and end-over-end rotated (6 rpm) in 2 mL size Eppendorf tubes at 37°C. The neutrophil bacterial killing assay has been described in detail elsewhere (Magon *et al.* 2020). In short, uptake of bacteria by neutrophils was measured after a timed period of phagocytosis, and a differential spin step followed by careful washing of the neutrophils allowed isolation of only the bacteria that had been phagocytosed. Neutrophils (1.25×10^6 per condition) were lysed in 1 mL ice-cold PBS containing 0.05% saponin (Fluka, Buchs) to release ingested bacteria for viability testing, using 10-strokes of a small tube and grinder glass homogeniser (Kimble). Bacterial viability was quantified by standard colony counting of dilution series that were spread on Columbia sheep blood agar plates (Fort Richard Labs, Auckland) and incubated overnight at 37°C.

Two procedures were used to test for differences in bacterial susceptibility to killing over a short and longer term incubation period. In the first protocol, bacteria were harvested at mid-growth phase and incubated with neutrophils for 10 min. Then ice-cold PBS was used to quench phagocytosis, and all subsequent handling until plating was at 4°C or below. The survival of intracellular bacteria from the lysed neutrophils is expressed as a percentage of the measured number of bacteria that were

phagocytosed by 10 min (inferred by the bacterial count of the neutrophils' supernatant and washes). In the second protocol, neutrophils were allowed to phagocytose stationary phase bacteria for 20 min, after which the differential spin and wash steps to remove extracellular bacteria were carried out at room temperature using warmed (37°C) PBS. By 40 min from the start, the washed neutrophils were returned to fresh Hank's buffer with 10% serum at 37°C, until 60 min from the initial mixing, when they were pelleted and lysed as above. Intracellular survivors at 60 min are expressed as a percentage of the number of bacteria phagocytosed at the 20 min time point.

Statistical analyses

Data analysis was carried out using GraphPad Prism software. Statistical significance was determined using appropriate tests as described in the figure legends.

RESULTS

Activation of RclR in isolated bacteria by HOCl and chloramines

Before carrying out the neutrophil studies, we examined the specificity of RclR activation in isolated *E. coli* by different reactive chorine species. In line with most other studies of transcriptional responses to oxidants, the HOCl in the original study of RclR was added to the bacteria in media (Parker *et al.* 2013). We first replicated the original experiments (Parker *et al.* 2013) by treating *E. coli* MG1655 (~5 × 10^8/mL) with HOCl in MOPS medium, and measured the expression of the RclR-responsive gene *rclB* by qRT-PCR. As shown in Fig. 2A, RclR responded measurably to 5–10 μM HOCl, with a steady increase in *rclB* expression up to about 1 mM, past which induction went down. Addition of 0.4 mM HOCl to bacteria in M9 medium also increased expression 700-fold relative to unexposed bacteria, but the same concentration added to the bacteria suspended in inorganic Hank's buffer gave only a modest (4–14-fold) increase (data not shown). This difference is related to toxicity: plating assays showed that at 0.4 mM HOCl in Hank's buffer all the bacteria were dead, whereas in MOPS medium killing only became apparent at 1 mM HOCl and a lack of induction associated with complete toxicity required 10 mM (Fig. 2B). As observed in other situations (Groitl *et al.* 2017, Ashby *et al.* 2020) these results show that culture media constituents affect the response of the bacteria to HOCl. Different media contain a wide range of biological compounds that react with HOCl. Some of these will quench the oxidant, whereas amino acids and other amines will react to generate chloramines (R-NHCl; Peskin and Winterbourn 2001; Peskin *et al.* 2005; Pattison and Davies 2006; Ashby *et al.* 2020). Both MOPS and M9, although minimal media, contain ammonium ions, and these plus MOPS buffer itself (the tertiary amine, 3-(N-morpholino)propane sulfonic acid), will form these reactive chlorine species (Prutz 1998; Ashby *et al.* 2020). Therefore, to investigate whether HOCl alone is capable of activating RclR, we studied the bacteria in Hank's buffer and titrated the HOCl down to concentrations where most bacteria remained viable: 0.2 mM for 5 × 10^8/mL bacteria grown in LB, and 0.05 mM for those grown to similar density in M9 medium. When these concentrations of HOCl,were added to bacteria suspended in Hank's buffer, strong activation of *rclB* expression (280–30,000-fold increase, $n = 3$) was observed. These results show that HOCl itself is able to activate the RclR response.

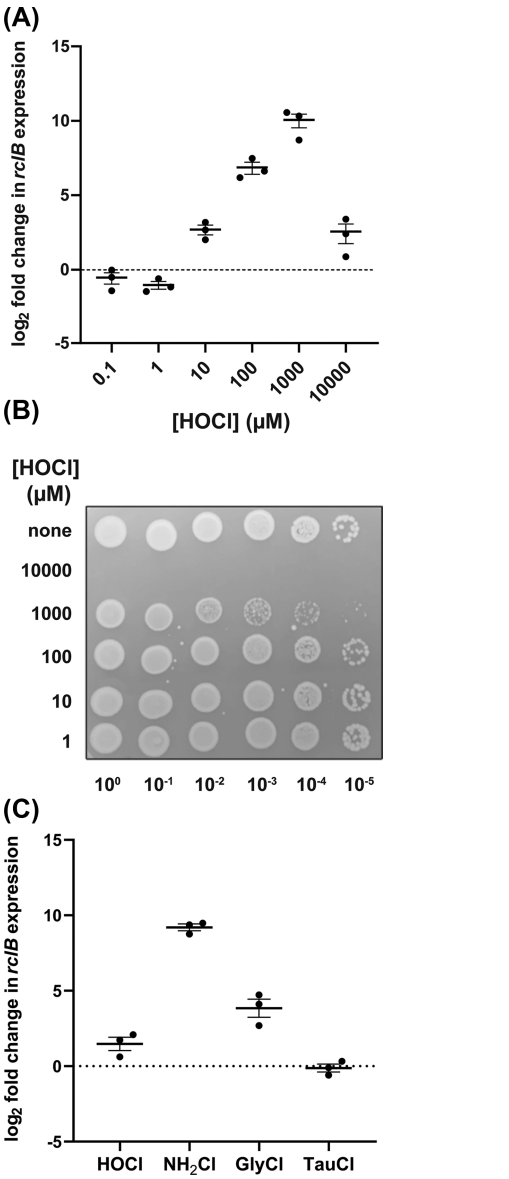

Figure 2. Induction of RclR response by HOCl and chloramines. (A, B) HOCl was added at the indicated concentrations to aliquots of *E. coli* cultured to mid-log phase in MOPS minimal medium. After 10 min at 37°C, bacteria were harvested for measurement of *rclB* expression by qRT-PCR **(A)** and analysis of cell survival by colony formation **(B)**. Means ± S.E.M. from three experiments are plotted in (A) and a mixed test using restricted maximal likelihood (without Geisser-Greenhouse correction) and Tukey multiple comparison showed HOCl at 10, 100 and 1000 μM caused significantly increased *rclB* expression ($P < 0.03$). (B) shows a representative survival assay, also from three replicates. See methods sections for more details. **(C)** RclR induction in *E. coli* after 20 min at 37°C in Hank's buffer containing HOCl (5 μM), NH_2Cl (25 μM), GlyCl (100 μM) and TauCl (150 μM) at 5 × 10^7 bacteria/mL. Reactions were stopped by adding 1 mM methionine and RNA extracts were tested for *rclB* expression by qRT-PCR, determined as fold induction relative to untreated *E. coli*. Results are from three independent experiments and show significant induction by NH_2Cl and GlyCl ($P < 0.001$) by one way ANOVA with Tukey test for multiple comparison) but no significant effect of TauCl. Note that because the oxidant to bacteria ratio determines lethality, the lower bacterial density than for the experiments described in the text required less oxidant to retain viability and give activation.

The observation that RclR is activated in media that would rapidly scavenge HOCl suggests that chloramines could be responsible for the response. We therefore tested ammonia chloramine (NH_2Cl), and the chloramines of two amino acids, glycine and taurine. GlyCl is slightly more reactive than TauCl but the main difference is that NH_2Cl and GlyCl enter cells whereas TauCl has very low membrane permeability (Midwinter *et al.* 2004, Peskin *et al.* 2005). Concentrations were selected at which most of the bacteria survived and the bacteria were suspended in Hank's buffer. NH_2Cl and GlyCl both induced *rclB* expression, but consistent with its low permeability, TauCl caused no significant increase (Fig. 2C). Induction by HOCl was lower than in the experiments described in the previous paragraph. This reflects the greater variability seen with HOCl and is probably due to the narrow concentration window between induction and toxicity, and sensitivity to factors such as exact bacterial numbers and speed of mixing.

Activation of RclR in phagocytosed bacteria

To investigate whether phagocytosed bacteria respond to the reactive chlorine species they are likely to be exposed to within the phagosomal environment, opsonised *E. coli* were added to isolated neutrophils. At various times after mixing, the neutrophils plus ingested bacteria were separated from non-ingested bacteria, and extracts were analysed for *rclB* expression. As shown in Fig. 3A, there was a rapid increase in *rclB* expression in the phagocytosed bacteria, consistently evident by 15 min. Bacteria extracted from the neutrophils gave a much higher response than those collected from the supernatant: for analyses made at 15 min, the fold-increase in *rclB* expression of the bacteria collected in the supernatant was only 15 ± 14 (mean ± S.E.M of three experiments), compared to the mean of 8358-fold increase in expression for the phagocytosed bacteria (Fig. 3A, 15 min). To establish oxidant involvement in RclR activation, diphenylene iodonium (DPI) was added to inhibit NOX2, and azide and 2-thioxanthine TX1 (Tiden *et al.* 2011) to inhibit myeloperoxidase. The much lower induction of *rclB* expression in the presence of these inhibitors (Fig. 3B) shows oxidant-dependence and is a strong indication that HOCl production by myeloperoxidase was responsible for the response.

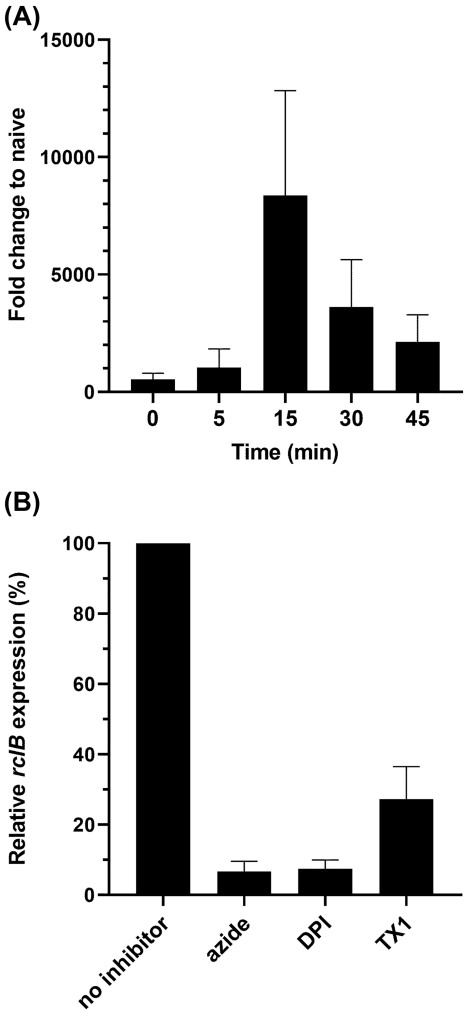

Figure 3. Induction of RclR response in phagocytosed bacteria. **(A)** Time course of *rclB* expression following the mixing of opsonised *E. coli* with neutrophils at 20:1 ratio and separating from non-ingested bacteria at indicated times. Means ± S.E.M from three individual experiments are shown. Fold induction is relative to bacteria not exposed to neutrophils. Values at 5–45 min are all significantly higher than control bacteria ($P < 0.01$ by ANOVA with Tukey multiple comparison on log transformed data from three experiments). Note that for the zero time point the bacteria were mixed with the neutrophils at 37°C before cooling and processing so some ingestion and neutrophil activation is likely. **(B)** Effects of inhibitors of NOX2 activation (10 μM diphenyleneiodonium chloride, DPI) and myeloperoxidase activity (1 mM sodium azide and 10 μM 2-thioxanthine TX1) on *rclB* expression at 15 min. Results are means ± S.E.M. from three experiments normalised to the no inhibitor response. Results with each inhibitor are significantly different from no inhibitor ($P < 0.001$ by ANOVA with Tukey multiple comparison).

Effect of *rcl* loci knockouts on killing of *E. coli* by neutrophils

We tested whether *E. coli* lacking *rcl* loci differ from wild-type (WT) in their sensitivity to phagosomal killing by neutrophils. We compared WT with one strain lacking RclA (Δ*rclA*) and another lacking RclA, RclB and RclC (Δ*rclABC*). Killing was measured after incubating the bacteria with neutrophils for 10 min. Over a range of experiments, 3–23% of the ingested bacteria remained viable, with no difference between the survival of WT *E. coli* and the *rcl* gene knockouts (Fig. 4A). Survival of ingested bacteria increased significantly to a mean of 53% in the presence of the NADPH oxidase inhibitor DPI ($P = 0.0011$ across all three strains in Fig. 4A by one-tailed paired *t*-test), indicating that there was a significant oxidative component in the killing mechanism. However, the effect of DPI was variable (range of survival 10–75%) and a difference between WT and the mutants could have been obscured. To test whether RclR upregulation protects a small fraction of the bacterial population that become evident after longer incubation with neutrophils, we separated phagocytosed bacteria from non-ingested bacteria after an initial phagocytosis period of 20 min, and monitored their survival at 1 h. The mean survival across all strains was 2.25 ± 0.87% (S.D.), again with no difference between WT and the *rcl* knockouts (Fig. 4B). These results suggest that either other RclR-independent compensatory systems exist that protect *E. coli* in the context of the phagosome, or that oxidative killing of *E. coli* is insufficient for protection by RclR to be noticed in our assays.

DISCUSSION

HOCl and related reactive chlorine species are potent antibacterial agents that are used by neutrophils as part of their defence against invading pathogens. A number of bacterial systems, including the RclR system, are induced by these species, and

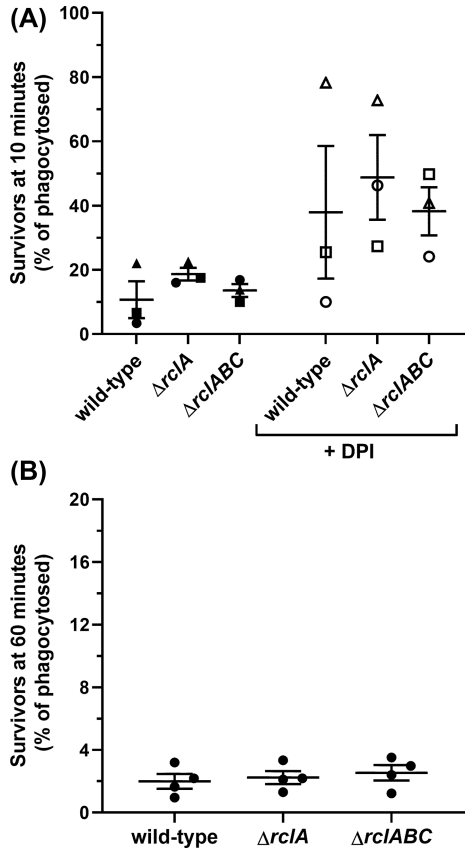

Figure 4. Neutrophil killing of wild-type and *rcl* mutants of *E. coli*. **(A)** Serum-opsonised bacteria were incubated with neutrophils at approximately 7:1 ratio for 10 min at 37°C with end-over-end rotation. The neutrophils were then gently pelleted by centrifugation and washed to remove extracellular bacteria, then lysed to release the phagocytosed bacteria. Viability was determined by spread-plating and colony counting. The starting number of bacteria and those remaining in the non-phagocytosed extracellular fraction at 10 min were also counted to determine the number of bacteria phagocytosed by 10 min (mean across all strains and three experiments 24.0 ± 10.9% (S.D.) of starting bacteria), and the viable bacteria measured in the lysed neutrophil fraction are expressed as a percentage of this. Results from incubations done in the presence of 10 μM DPI are plotted with hollow symbols, corresponding in shape with the solid symbol (no DPI) from the same experiment. **(B)** Protocol as above except the non-ingested bacteria were removed and measured at 20 min, and incubation of the neutrophils containing bacteria was maintained until they were lysed after 60 min from the start. The surviving bacteria in the neutrophils at 60 min are reported as a percentage of those phagocytosed by 20 min. The mean amount of bacteria phagocytosed after 20 min, across all strains and 4 experiments was 29.8 ± 5.8% (S.D.) of the starting bacteria. See methods sections for more details. The means ± S.E.M. are plotted from experiments carried out on separate days with different neutrophil donors; (A) $n = 3$, (B) $n = 4$. No differences between groups, $P > 0.05$.

appear to provide defence against further oxidant insult. The main objective of our study was to determine whether the HOCl generated by neutrophils following phagocytosis causes RclR activation in the ingested bacteria. We observed strong and rapid activation, detectable within 5 min of ingestion and still apparent after 40 min. The near complete suppression by inhibitors of NOX2 and myeloperoxidase indicates that activation was oxidative, and that it required myeloperoxidase activity. This is strong evidence that the HOCl generated by myeloperoxidase was responsible. The induction seen in ingested but not extracellular bacteria implicates HOCl generated in the phagosome.

Cysteine residues are the most favoured targets of HOCl, and activation of RclR requires cysteine oxidation. Widespread oxidation of thiol proteins has been observed in *E.coli* ingested by a neutrophil-like cell line (Degrossoli *et al.* 2018), and our results imply that RclR is one of these targets. RclR induction was seen within 5 min in isolated *E. coli* exposed to reactive chlorine species (Parker *et al.* 2013). We observed RclR induction in ingested bacteria over a similar time scale. This is consistent with the timing of assembly of the NADPH oxidase and superoxide production on the phagosomal membrane (Nauseef 2019). It is also the same period over which many of the ingested bacteria would be killed (Hampton, Vissers and Winterbourn 1994; Staudinger *et al.* 2002). However, sufficient numbers must retain metabolic activity for long enough to upregulate gene expression. Furthermore, although they are yet to be tested, other transcription factors that are activated by reactive chlorine species, including HypT (Drazic *et al.* 2013, 2014) and NemR (Gray *et al.* 2013, 2015), may also respond in this environment.

Related to our findings, Staudinger *et al.* (2002) observed rapid, NOX2-dependent activation of a number of genes in phagocytosed *E. coli*, in particular those regulated by OxyR. Although modest activation of some OxyR-responsive genes has been observed in *E. coli* following exposure to HOCl in rich broth (Wang *et al.* 2009), no activation of OxyR was detected when the bacteria were exposed to reagent HOCl in buffer (Dukan *et al.* 1996). This suggests that the H_2O_2 generated by NOX2 activation is more likely to be responsible for the OxyR activation following phagocytosis. Modelling studies predict that the myeloperoxidase present in the phagosomal space should consume most of the H_2O_2 and maintain its concentration in the low micromolar range (Winterbourn *et al.* 2006). The H_2O_2 is estimated to equilibrate into the bacterium (Winterbourn *et al.* 2006), and even this low concentration should be sufficient to achieve the > 200 nM shown to be capable of activating the OxyR response (Seaver and Imlay 2001).

HOCl could act directly to activate RclR in phagocytosed bacteria, or possibly via low molecular weight chloramines. The chemistry of HOCl in biological media is complex because of its wide range of reactions (Peskin *et al.* 2005, Pattison and Davies 2006). As previous studies on the effects of HOCl on the RclR system were carried out in media (Parker *et al.* 2013) where chloramines would be formed (Ashby *et al.* 2020), there was uncertainty as to which species initiated the response. Here, by treating bacteria in simple buffer, we have shown that HOCl itself, as well as individual chloramines, are effective inducers. However, RclR induction requires the chloramine to penetrate the bacteria, as demonstrated by a lack of response to TauCl. Although a positive response to TauCl was observed previously (Parker *et al.* 2013), this can be explained by chlorine exchange with other amines in the media giving rise to more membrane-permeable chloramines (Peskin *et al.* 2005). In MOPS medium, the most likely activator is NH_2Cl produced from the ammonia present. Such exchange is typical of what will occur when HOCl or any chloramine is added to media, or when HOCl generated by myeloperoxidase is released into complex biological fluids. In these situations, there will always be a range of reactive chlorine species, with their nature depending more on the amines present than the initial species added. These are likely to include HOCl and permeable chloramines capable of RclR activation.

The amount and fate of HOCl generated in the neutrophil phagosome is a matter of ongoing debate (Hurst 2012; Klebanoff *et al.* 2013; Winterbourn *et al.* 2016; Nauseef 2019). There is definitive evidence from several studies that HOCl is produced (Chapman *et al.* 2002; Palazzolo *et al.* 2005; Green, Kettle and Winterbourn 2014; Albrett *et al.* 2018). However, the efficiency of this process has been questioned (Levine *et al.* 2015; Levine and Segal

2016) and HOCl consumption by phagosomal proteins should restrict the amount that reaches ingested bacteria (Chapman *et al.* 2002). The current findings are proof of HOCl production, and take the evidence a step further to show that reactive chlorine species get into phagocytosed bacteria and react intracellularly. HOCl itself could be the reactive chlorine species responsible for RclR activation, or it could possibly be a low molecular weight chloramine such as NH_2Cl formed as a secondary product from the reaction of HOCl with amine groups on phagosomal proteins (Green *et al.* 2017).

If bacteria respond to ROS and reactive chlorine species by upregulating gene expression, a key question is whether this can protect against neutrophil oxidants and provide a survival advantage. For OxyR, this was found to be the case (Staudinger *et al.* 2002). A Δ*oxyRS* mutant strain of *E.coli* which cannot mount the antioxidant gene response observed following phagocytosis was significantly more sensitive to killing by neutrophils, and this was mostly due to an effect on NOX2-dependent oxidative killing. However, we did not find that △*rclA* or △*rclABC* strains were killed more readily than WT *E. coli,* either in the short or long term. Neutrophils kill *E. coli* rapidly, predominantly via non-oxidative mechanisms (Hampton, Vissers and Winterbourn 1994; Rosen *et al.* 1998). While this raises the possibility that our system was not discriminating enough to detect protection against oxidants, the slower killing we observed for all strains when NOX2 was inhibited indicates there was an oxidative component. One possible explanation relates to the mechanism of RclR protection and recent evidence that it may act by promoting recovery after sublethal doses of HOCl rather than on the initial hit (Derke *et al.* 2020). This may be less evident when killing is rapid. It may also explain why, in contrast to neutrophils, lack of RclA made *E. coli* more sensitive to killing by RAW macrophages (Baek *et al.* 2020). These cells contain less myeloperoxidase than neutrophils and kill much more slowly, and protection by *rclA* expression was apparent only after 24 h.

Thus, our results with *E. coli* should not rule out a possible role for the RclR system in protecting bacteria against neutrophils. It may be that it is more effective in organisms that are killed more slowly or where myeloperoxidase makes a greater contribution. In addition, release of myeloperoxidase from neutrophils and extracellular production of HOCl can occur at sites of infection and inflammation. If this results in upregulation of bacterial RclR, it could influence their ultimate fate. It is also feasible that other bacterial defence systems that respond to reactive chlorine species are upregulated in phagocytosed bacteria and it would be worthwhile testing whether these convey a survival advantage.

ACKNOWLEDGEMENTS

We are grateful to Dr Heather Parker for assistance with the neutrophil work and Professor Tony Kettle for helpful discussion. This research was supported by a Programme Grant from the Health Research Council of New Zealand and NIH grant R35GM12459 (MJG).

Conflicts of Interest. None declared.

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