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Glycolysis and pyrimidine biosynthesis are required for replication of adherent–invasive *Escherichia coli* in macrophages

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Adherent–invasive *Escherichia coli* (AIEC) have been implicated in the aetiology of Crohn's disease (CD), a chronic inflammatory bowel condition. It has been proposed that AIEC-infected macrophages produce high levels of pro-inflammatory cytokines thus contributing to the inflammation observed in CD. AIEC can replicate in macrophages and we wanted to determine if bacterial replication was linked to the high level of cytokine production associated with AIEC-infected macrophages. Therefore, we undertook a genetic analysis of the metabolic requirements for AIEC replication in the macrophage and we show that AIEC replication in this niche is dependent on bacterial glycolysis. In addition, our analyses indicate that AIEC have access to a wide range of nutrients in the macrophage, although the levels of purines and pyrimidines do appear to be limiting. Finally, we show that the macrophage response to AIEC infection is indistinguishable from the response to the non-replicating glycolysis mutant (Δ*pfkAB*) and a non-pathogenic strain of *E. coli*, MG1655. Therefore, AIEC does not appear to subvert the normal macrophage response to *E. coli* during infection.

INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease that affects millions of people around the world, particularly in North America and Europe (Molodecky *et al.*, 2012). The disease is characterized by chronic inflammation anywhere in the gastrointestinal tract (from the mouth to the anus) but is most associated with lesions in the ileum and colon of patients. There is a genetic component to CD and recent genome-wide association studies have identified polymorphisms in a number of host genes that are strongly associated with CD, e.g. *NOD2*, *ATG16L1* and *IRGM* (Khor *et al.*, 2011). However the relatively rapid, and recent, increase in the incidence of CD also points to a significant role for environmental factors in the aetiology of this disease. In recent years, a group of *Escherichia coli* called adherent–invasive *Escherichia coli* (AIEC) have been found to be present in the guts of CD patients at a higher frequency than in healthy subjects, leading to the suggestion that AIEC may be involved in initiating and/or maintaining the inflammation associated with CD (Negroni *et al.*, 2012; Rhodes, 2007; Rolhion & Darfeuille-Michaud, 2007). AIEC have also been found associated with CD-like diseases in other mammals, e.g. granulomatous colitis in boxer dogs (Simpson *et al.*, 2006).

AIEC can be distinguished from other strains of *E. coli* by the presence of two phenotypes. Firstly, AIEC have the ability to invade epithelial cells, and secondly, AIEC have the ability to replicate in a variety of macrophages, including primary cells such as human monocyte-derived macrophages (Boudeau *et al.*, 1999; Glasser *et al.*, 2001; Martin *et al.*, 2004; Subramanian *et al.*, 2008). Molecular studies using the AIEC strain LF82 have identified factors that are important for each of these phenotypic properties. For example, invasion of epithelial cells by LF82 requires the expression of both Type 1 fimbriae and flagella (Barnich *et al.*, 2003; Boudeau *et al.*, 2001). It has also been previously shown that LF82 requires acidification of the phagosome to replicate in the macrophage and it is not surprising, therefore, that the *htrA (degP)* and *dsbA* genes, both encoding proteins required for the bacteria to survive stress, are required for replication in this niche (Bringer

Abbreviations: AIEC, adherent–invasive *Escherichia coli*; CD, Crohn's disease; Kn, kanamycin; SCV, *Salmonella*-containing vacuole.

Nine supplementary figures and a supplementary table are available with the online Supplementary Material.

et al., 2005, 2007, 2012). However, all of the genes so far implicated in the AIEC phenotype are also found in non-AIEC strains of *E. coli*. Indeed recent genomic studies have failed to identify any AIEC-specific genes that can adequately explain the ability of this group of *E. coli* to invade epithelial cells and replicate in macrophages (Clarke *et al.*, 2011; Krause *et al.*, 2011; Miquel *et al.*, 2010; Nash *et al.*, 2010; Vejborg *et al.*, 2011; Zhang *et al.*, 2015). Nonetheless epidemiological evidence does suggest that *E. coli* are enriched in the guts and macrophages of the lamina propria of CD patients compared to healthy controls (Conte *et al.*, 2014; Darfeuille-Michaud *et al.*, 2004; de Souza *et al.*, 2012; Elliott *et al.*, 2015; Martinez-Medina *et al.*, 2009).

We wanted to identify the metabolic requirements of AIEC replication in the macrophage. We also wanted to determine whether AIEC replication was achieved by, or resulted in, any alteration to the normal macrophage response to infection. In this study we confirm that our strain of AIEC replicates within acidified vacuoles in the macrophage. Moreover we show, using genetics, that AIEC replication requires glycolysis and is largely independent of the TCA cycle, the glyoxylate pathway, the pentose-phosphate pathway and gluconeogenesis. Furthermore our data reveal that AIEC replication in the macrophage does have a requirement for *de novo* nucleotide biosynthesis. Finally we report that the response of the macrophage to infection with wild-type AIEC, the non-replicating glycolysis mutant or the non-pathogenic strain MG1655 appears to be identical, suggesting that AIEC do not subvert the normal macrophage response to infection in order to establish their replicative niche.

METHODS

Bacterial strains, plasmids and cell lines. The strain of AIEC used in this study was HM605, isolated from the colon of a patient with CD (Martin *et al.*, 2004). *E. coli* K-12 strain MG1655 was used as a non-pathogenic control. All bacteria were routinely cultured in LB broth or M9 minimal medium [supplemented with 0.2 % (w/v) glucose unless otherwise indicated] at 37 °C with or without shaking, as indicated. Antibiotics were added as required at the following concentrations: ampicillin (Amp) 100 μg ml^{-1}, kanamycin (Kn) 50 μg ml^{-1}, and chloramphenicol (Cm) 25 μg ml^{-1}. Murine macrophage-like J774A.1 cells were purchased from ATCC and were maintained in a 5 % (v/v) CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM), high glucose, Glutamax (Gibco) supplemented with 10 % (v/v) FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Gentamicin protection assays. To measure the intramacrophagic replication of HM605 we used a gentamicin protection assay described previously (Subramanian *et al.*, 2008). Approximately 4×10^5 J774A.1 cells were seeded into each of the wells of a 24-well tissue culture plate. After 20 h cells were infected, at an m.o.i.of 10 bacteria per cell, with the appropriate bacteria which had been cultured overnight at 37 °C in LB broth without shaking. Bacterial cultures were centrifuged and cell pellets were re-suspended in DMEM to the appropriate cell density before infecting the macrophages. After 2 h at 37 °C the medium was removed and replaced with an equal volume of DMEM containing gentamicin (100 μg ml^{-1}), to kill any extracellular bacteria. After incubation for 1 h, the cells were either washed three times with PBS and lysed with 500 μl^{-1} of 1 % (v/v) Triton X-100 to release the intracellular bacteria (this is equivalent to T_0) or fresh DMEM with gentamicin was added for further incubation for the appropriate time. Released bacteria were enumerated by plating on LB agar containing ampicillin because HM605 is naturally resistant to this antibiotic.

Lysotracker Red-DND99 staining. J774A.1 were seeded on no. 1.5 glass coverslips in 24-well plates (Corning) 20–24 h before infection. GFP-tagged HM605 and MG1655 were added to cells at an m.o.i. of 10; plates were then centrifuged at 300 ***g*** for 5 min to synchronize infections, followed by incubation at 37 °C/5 % CO_2 in a humidified incubator for 20 min. Cells were then washed twice with warm 1× PBS and resuspended in growth medium supplemented with 50 μg ml^{-1} gentamicin. Cells were incubated as before for a further 30 min and this was taken as time T_0. Cells were washed with 1× PBS and 250 nM Lysotracker Red-DND99 (Molecular Probes) diluted in gentamicin-supplemented culture medium was added to sample wells for 30 min. Cells were then fixed in 4 % paraformaldehyde (pH 7.4) for 15 min at room temperature in the dark and washed extensively. Coverslips were then mounted on microscope slides with DAKO mounting medium (DAKO) and incubated at room temperature in the dark overnight. Samples were visualized with a Zeiss LSM 5 laser scanning confocal microscope using a Plan-Apochromat ×63/1.40 oil DIC M27 lens. For GFP, samples were excited with an argon laser at 488 nm and emitted light was acquired using a bandpass emission filter of 505–550 nm. For Lysotracker visualization, samples were excited with laser light at 543 nm and emitted light acquired through a bandpass filter at 560–615 nm. Sample images were taken using Zen 2008 SP2 software (Zeiss). Cells were scored as positive for co-localization if green bacteria-containing vacuoles overlapped with red Lysotracker vesicles. Slides were stored at 4 °C in the dark until analysed and all analysis was performed within 1 week. All experiments were carried out in triplicate.

Transposon mutant library. A transposon mutant library of HM605 was constructed using the EZ-Tn5 <KAN-2> insertion kit, following the manufacturer's instructions (Epicentre). After screening, transposon insertion sites were determined by amplifying the genomic region flanking the transposon using two rounds of arbitrarily primed PCR. In the first PCR (PCR 1), the random primers ARB1 (5′-GGCCACGCG TCGACTAGTTACNNNNNNNNNNGATAT-3′) and ARB6 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3′) were paired with an external Tn5 specific primer, Tn5ext (5'-CCTGA TTGCCCGACATTATCGCG-3′). The products from PCR 1 were purified and used as a template for the second PCR (PCR 2), using the internal Tn*5* specific primer, Tn5int (5′-GAATATGGCTCATAA-CACCC-3′) and the random primer ARB2 (5′-GGCCACGCGTCGAC TTAGTTAC-3′). PCRs were carried out using the Velocity PCR kit (Bioline) and PCR products were purified using the QIAquick PCR purification kit (QIAGEN). After PCR 2, the amplicons were purified and sequenced, using Tn5int as the primer, by GATC Biotech (Germany). DNA sequences were then compared to the HM605 genome sequence in order to determine the exact location of the transposon (Clarke *et al.*, 2011).

Construction of metabolic mutants by P1 transduction. Mutants were constructed by P1 transduction using strains from the KEIO library as donors. The KEIO library is a library of single gene knockouts in all non-essential genes in *E. coli* K-12 strain BW25113 (Baba *et al.*, 2006). Each gene is deleted and replaced with a Kn resistance cassette flanked by FLP recognition target (FRT) sites. All HM605 mutants constructed in this way were phenotypically tested for veracity (see text for details). For the construction of the Δ*pfkAB* double mutant, the Kn resistance cassette was excised from the Δ*pfkA*::Kn mutant using a yeast FLP recombinase expressed from pFLP3 (Choi *et al.*, 2005). The Δ*pfkB*::Kn allele was then transduced into the Δ*pfkA* background, resulting in Δ*pfkA* Δ*pfkB*::Kn (represented as Δ*pfkAB* for simplicity).

Measuring cytokine production. The production of cytokines was measured using Meso Scale Discovery technology, according to the manufacturer's instructions (MSD Meso Scale Discovery). Macrophages were infected with the appropriate bacteria (as described) and cell culture supernatants were taken at the indicated times for analysis. For detection of cytokines 25 μl^{-1} of each supernatant was dispensed into separate wells of the MSD plate. The plate was then sealed and incubated for 2 h with vigorous shaking at room temperature before 25 μl^{-1} of the 1× Detection Antibody Solution was dispensed into each well. The plate was again sealed and incubated for 2 h with vigorous shaking at room temperature. Finally, each well was washed three times with PBS + 0.05 % (v/v) Tween 20 followed by the addition of 150 μl^{-1} 2× Read Buffer T to each well of the plate. The plate was then immediately analysed using the SECTOR Imager and cytokine concentrations were determined using a standard curve.

RNA isolation and microarray analysis. J774A.1 macrophages were seeded in 6-well tissue culture plates (Sarstedt) at a density of 1×10^6 cells and infected at an m.o.i. of 10 with the appropriate bacteria, as described above. At 6 h post-gentamicin treatment, the macrophages were washed with PBS, scraped from the well surface and pelleted. Cells were lysed using RLT lysis buffer (QIAGEN) and total RNA was isolated using RNeasy Mini kit (QIAGEN) as per manufacturer's instructions. Before further analysis the quality and purity of the RNA was analysed using a Nanodrop spectrophotometer. Total RNA was then shipped to Beckman Coulter for microarray analysis using their SurePrint G3 Mouse GE 8×60K microarrays. Each array contains oligonucleotide probes for 39 340 murine Entrez gene RNAs and 16 251 lincRNAs, and hybridizations were carried out in triplicate using independent total RNA preparations. For analysis, each array was corrected for background, quantile normalized and log-transformed (log2) using Bioconductor packages in the R software environment (Gentleman *et al.*, 2004). Differential expression was carried out using functions from the LIMMA package in Bioconductor (Smyth, 2004). *P* values were adjusted to correct for multiple testing by using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

Metabolomics. J774A.1 macrophages were infected with either WT, the Δ*pfkAB* mutant or the Δ*pyrF* mutant at an m.o.i. of 10, as previously described, and total cell lysates were collected 6 h post-gentamicin treatment. An uninfected macrophage control was also included and all samples were prepared in triplicate. Macrophages (approx. 8×10^7 cells per sample) were rapidly cooled by submersion in a dry ice/ethanol bath. All subsequent steps were carried out at 4 °C. Cells were collected by centrifugation at 1000 ***g*** for 10 min and supernatant was discarded. The cell pellet was resuspended in cold medium and transferred to an Eppendorf tube. The cells were pelleted by centrifugation at 2500 ***g*** for 5 min and the supernatant was completely removed. The cell pellet was resuspended in 200 µl chloroform/methanol/water (1 : 3 : 1) at 4 °C and cells were lysed by vortexing at 4 °C. The lysate was centrifuged at 13000 ***g*** for 3 min to remove debris and the supernatant (approx. 180 µl) was retained and stored at −80 °C until further analysis. Samples were analysed by hydrophilic interaction liquid chromatography–mass spectrometry (UltiMate 3000 RSLC; Thermo Fisher) with a 150×4.6 mm ZIC-pHILIC column running at 300 µl min^{-1} and Orbitrap Exactive (Thermo Fisher) analysis. Buffers consisted of (A) 20 mM ammonium carbonate in H_2O, and (B) acetonitrile. The gradient ran from 20 % A, 80 % B to 80 % A, 20 % B in 15 min, followed by a wash at 95 % A, 5 % B for 4 min, and equilibration at 20 % A, 80 % B for 6 min. Raw mass spectrometry data were processed using a standard pipeline, consisting of XCMS (for peak picking), MzMatch (for filtering and grouping) and IDEOM (for further filtering, post-processing and identification) (Creek *et al.*, 2012; Scheltema *et al.*, 2011; Smith *et al.*, 2006). Metabolites listed as identified were validated against a panel of unambiguous standards by mass and retention time and may be designated $HRMS^1_aRT_a$ (Sumner *et al.*, 2014). Additional putative identifications were assigned by mass and predicted retention time and are designated $HRMS^1_{lib}$ (Creek *et al.*, 2011). Means and SEM errors of the mean were generated for all groups of picked peaks and the resulting data were uploaded to KEGG for pathway analysis.

Statistical analysis. All statistical analysis was performed using GraphPad Prism v6.0e for Mac OS X software. All experiments were performed at least three times and the data were tested for statistical significance using Student's *t*-test (for direct comparisons) or one-way ANOVA with Dunnett's post-test correction for multiple comparisons.

RESULTS

Intramacrophagic replication of AIEC strain HM605 occurs in an acidified phagosome

AIEC are characterized by their ability to replicate in macrophages, including the J774A.1 murine macrophage-like cell line. In this study we used a strain of AIEC, called HM605, isolated from a biopsy taken from the colon of a patient with CD (Martin *et al.*, 2004). Using a gentamicin protection assay we showed that, over a 6 h period, HM605 replicated inside the J774A.1 macrophage whilst a non-pathogenic strain of *E. coli*, MG1655, did not exhibit any replication (Fig. 1a). Moreover, using bacterial strains that were tagged with GFP under the control of a constitutive promoter, we could clearly see an increase over time in the number of HM605 cells/macrophage compared to MG1655 (Fig. 1b, c). Using transmission electron microscopy we showed that, inside the macrophage, HM605 cells were located within vacuoles (Fig. S1, available in the online Supplementary Material). Using Lysotracker Red-DND99, we could show that, 6 h post-gentamicin addition, >60 % of HM605 cells co-localized with acidified vacuoles (Fig. 1b, d). Interestingly this is similar to the level of co-localization that was observed for MG1655, the non-pathogenic strain of *E. coli*, suggesting that HM605 does not subvert the normal phagocytic response of macrophages (Fig. 1b, d). Finally, addition of bafilomycin A1 (an inhibitor of the phagosomal ATPase proton pump) to infected macrophages prevented HM605 replication, showing that acidification of the phagosome is required for AIEC replication (Fig. 1e). Importantly bafilomycin A did not affect bacterial growth during culturing in growth medium (Fig. S2). HM605 replication was also blocked when NH_4Cl [an inhibitor of phagosome–lysosome fusion (Hart & Young, 1991)] was added to the macrophages (Fig. S3). Our data are in good agreement with previous work using the prototypical AIEC strain LF82, where it was shown that bacterial replication requires the acidification of phagosomes (Bringer *et al.*, 2006). Therefore, we conclude that HM605 replicates within acidified vacuoles, most likely phagosomes, in J774A.1 macrophages.

Glycolysis is required for replication of HM605 in the macrophage

HM605 exhibited rapid growth within the phagosome, suggesting that the bacteria have ready access to nutrients (see

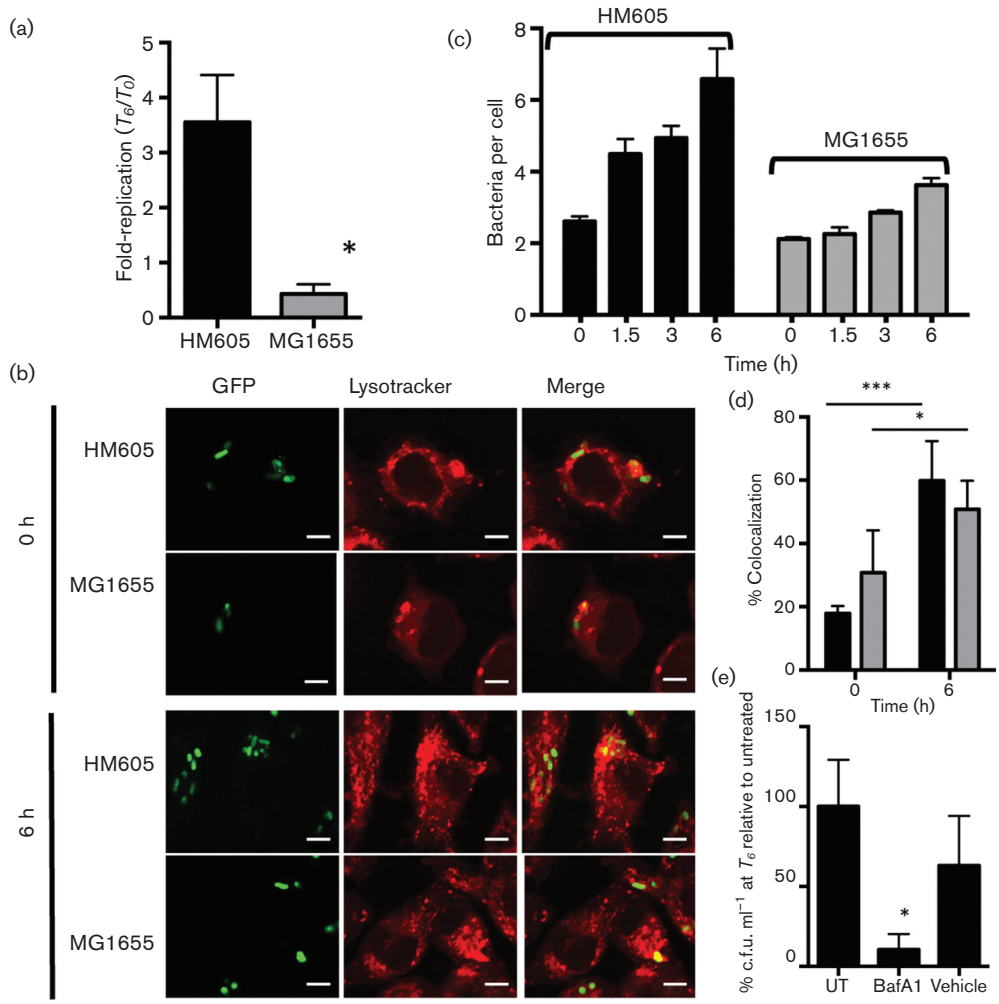

Fig. 1. HM605 replication in J774A.1 macrophages. (a) J774A.1 cells were infected with HM605 or MG1655 at an m.o.i. of 10 and intracellular bacterial numbers were assayed at the indicated times using the gentamicin protection assay. The fold-replication was calculated by dividing the number of bacteria inside the macrophage at T_6 by the number of bacteria at T_0. (b) J774A.1 macrophages were infected with GFP-tagged HM605 or GFP-tagged MG1655. Macrophages were stained with Lysotracker Red-DND99 (as described in Methods) and samples were analysed using a Zeiss LSM 5 confocal microscope. Images are representative of three independent biological replicates. Bars, 5 μm. (c) Bacterial number per macrophage as measured by directly counting the number of GFP-labelled bacteria per macrophage (n=100) at the time indicated. (d) Percentage of bacteria co-localizing with Lysotracker Red-DND99 at T_0 and T_6. Macrophages were infected and cells fixed and stained at the indicated times. At least 100 bacteria-containing macrophages were counted per replicate and data shown are the mean of three independent replicates. (e) J774A.1 macrophages were infected with HM605 and treated with 50 nM bafilomycin A (BafA1) to prevent phagosome acidification. The numbers of intracellular bacteria were measured at T_6 and are presented as percentage c.f.u. ml^{-1} relative to untreated HM605-infected macrophages. All experiments were carried out in triplicate and the error bars represent SD. Statistical significance was determined using the Student t-test; ***P<0.0005, *P<0.05.

Fig. 1). We were interested in characterizing the metabolic pathways required by HM605 during replication in this niche. To do this we constructed mutations in genes involved in the major metabolic pathways of glycolysis (Δ*pfkAB*), the TCA cycle (Δ*icd*), the glyoxylate pathway (Δ*aceA*), the Entner–Doudoroff pathway (Δ*eda*), the pentose-phosphate pathway (Δ*gnd*) and gluconeogenesis (Δ*fbp*). The disruption in the expected pathway was verified by growing the mutants in M9 minimal medium supplemented with glucose, glycerol, acetate or gluconate as the sole carbon source (see Figs S4 and S5). Although a null mutation in *gnd* does not have a significant effect on the growth of *E. coli* it has been shown that a Δ*gnd* mutation does block the pentose-phosphate pathway and this results

in a significant rerouting of carbon flux in *E. coli* (Zhao *et al.*, 2004). Each of the mutants (Δ*pfkAB*, Δ*icd*, Δ*aceA*, Δ*eda*, Δ*gnd* and Δ*fbp*) was then checked for its ability to replicate in J774A.1 macrophages relative to HM605. Interestingly, the Δ*pfkAB* mutant showed the most significant defect in its ability to grow in the macrophage (6.1±1.1 % of wild-type levels; P=0.0026) suggesting an important role for glycolysis during the *in vivo* replication of HM605 (Fig. 2). Importantly replication was restored when the Δ*pfkAB* mutant was complemented by the expression of either *pfkA* or *pfkB* from a plasmid (Fig. 2b). The *pfkA*- or *pfkB*-expressing plasmids did complement the growth of the Δ*pfkAB* mutant on glucose, suggesting that the partial complementation observed in the macrophage is likely due to expression levels and/or plasmid retention during infection (Fig. S6). There also appears to be a role, albeit more limited, for the pentose-phosphate pathway during *in vivo* replication as the Δ*gnd* mutant showed a significant reduction in growth in the macrophage relative to HM605 (43 ± 17.1 % of wild-type levels; P=0.0024) (Fig. 2). The remaining mutants grew as well as HM605 in the macrophage, suggesting no significant requirement for the TCA cycle, gluconeogenesis, the glyoxylate pathway or the Entner–Doudoroff pathway during intracellular growth.

Phenotypic analysis of the Δ*pfkAB* mutant

The carbon assimilation profile of the Δ*pfkAB* mutant was phenotypically characterized using the Biolog phenotypic array. This analysis confirmed that the Δ*pfkAB* mutant was unable to assimilate carbon sources that would normally be metabolized through glycolysis, e.g. D-glucose, D-mannose, *N*-acetyl-glucosamine, trehalose (data not shown). The phagosome is also known to be a stressful environment and it is possible that the growth defect of the Δ*pfkAB* mutant is as a result of increased sensitivity to one (or all) of the stresses encountered in this niche. However, analysis, using both Biolog phenotypic arrays and other independent assays, revealed that the Δ*pfkAB* mutant does not appear to be more sensitive to phagosome-relevant stresses, such as exposure to acidic pH (pH 5.5), reactive oxygen species (H_2O_2) and reactive nitrogen species (acidified sodium nitrite), than the wild-type (data not shown).

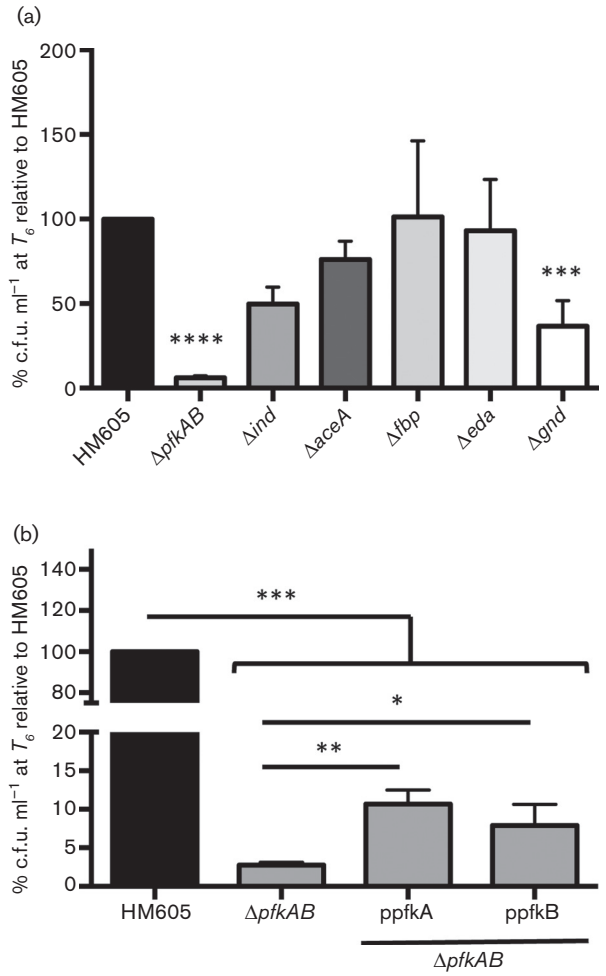

Fig. 2. Glycolysis is required for intramacrophagic replication of HM605. (a) J774A.1 macrophages were infected with HM605 and different metabolic mutants at an m.o.i. of 10 and the numbers of intracellular bacteria were measured 6 h after the addition of gentamicin (T_6). The data are presented as percentage c.f.u. ml^{-1} relative to HM605-infected macrophages. (b) Complementation of the Δ*pfkAB* mutation by the *in trans* expression of *pfkA* or *pfkB*. Statistical significance was determined using one-way ANOVA (with Dunnett's post-test correction for multiple comparisons); *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Phagosome is limiting in nucleotides

We wanted to identify additional nutritional requirements of HM605 during growth in the macrophage. To do this we firstly identified HM605 auxotrophic mutants by screening a transposon mutant library for cells that were unable to grow on M9 minimal medium agar plates with glucose as the sole carbon source. In total 28 mutants (out of 5513 screened) were isolated and subjected to arbitrarily-primed PCR, resulting in the identification of independent insertions in 20 genes (see Table S1). Some of the identified genes encode proteins in the same metabolic pathway (e.g. *ilvE* and *ilvD* are both involved in the biosynthesis of isoleucine, leucine and valine). Therefore, 12 mutants, representing unique interrupted metabolic pathways, were chosen for further analysis: *purM* (purine biosynthesis), *pyrF* (pyrimidine biosynthesis), *argB* (arginine/ornithine biosynthesis), *aroC* (shikimate pathway, biosynthesis of aromatic amino acids), *trpB* (tryptophan biosynthesis), *proB* (proline biosynthesis), *metB* (methionine biosynthesis), *ilvE* (isoleucine/leucine/valine biosynthesis), *panC* (biosynthesis of pantothenate), *cysG* (siroheme biosynthesis), *pdxB* [pyridoxine 5-phosphate (vitamin B6) biosynthesis] and *nadC* (NAD biosynthesis). Where possible the nature of the auxotrophy in each of these mutants was confirmed by supplementing the growth medium with the required nutrient

(see Fig. S7). The mutants were then tested for their ability to replicate inside J774A.1 macrophages, using the gentamicin protection assay. The *purM* and *pyrF* mutants were defective in intracellular growth compared to HM605 suggesting that nucleotides are not available in sufficient quantities to support normal growth of HM605 in the phagosome (Fig. 3a). Moreover, we have confirmed that growth of the *pyrF* mutant is complemented by the expression of the *pyrF* gene from a plasmid (Fig. 3b). However, the remaining auxotrophic mutants were able to grow in macrophages to a level that was similar to WT (Fig. 3a). Therefore, whilst nucleotides appear to be limiting, HM605 does appear to have access to sufficient concentrations of a wide variety of amino acids and other growth factors during growth in the phagosome.

Pyrimidine biosynthesis intermediates accumulate in macrophages infected with the Δ*pyrF* mutant

To examine whether the Δ*pyrF* mutant was experiencing pyrimidine auxotrophy during growth in the macrophage we undertook a metabolomic analysis of infected macrophages. J774A.1 macrophage cells were infected with the WT, Δ*pyrF* or Δ*pfkAB* mutant and, 6 h post-gentamicin addition, we lysed the macrophages and analysed the intracellular metabolites using liquid chromatography–mass spectrometry (LC-MS). The *pyrF* gene encodes orotidine-5-phosphate decarboxylase, an enzyme involved in the last essential step in *de novo* pyrimidine biosynthesis, i.e. the conversion of orotidine-5′-phosphate (OMP) to UMP (see Fig. 4a). Both the bacteria and the macrophage can synthesize pyrimidines and we could detect all pyrimidine biosynthetic intermediates in the uninfected macrophage, with the exception of OMP (Fig. 4a). However, a metabolite with the same predicted mass and retention time as OMP is clearly accumulating in macrophages infected with the Δ*pyrF* mutant (Fig. 4a). Indeed, we could detect a significant accumulation of all pyrimidine biosynthesis intermediates in macrophages infected with the Δ*pyrF* mutant compared to uninfected macrophages or macrophages infected with either WT or the Δ*pfkAB* mutant, indicative of a block in pyrimidine biosynthesis in this mutant (Fig. 4a). Therefore, the Δ*pyrF* mutant is unable to synthesize pyrimidines in the macrophage.

Although we were measuring the total metabolite pool from both the bacteria and the macrophage, it is possible to annotate bacteria-specific metabolites in our metabolomic datasets. N2Succinyl-L-arginine ($HRMS^1_{lib}$) is a bacteria-specific metabolite produced by arginine succinyltransferase, an enzyme encoded by *astA*. This is the first step in arginine degradation via the arginine succinyltransferase (AST) pathway. *S*-D-Ribosyl-L-homocysteine ($HRMS^1_{lib}$) is generated by the cleavage of *S*-adenosyl-L-homocysteine by the 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase enzyme (encoded by *mtn*) during the regeneration of S-adenosylmethionine. S-D-Ribosyl-L-homocysteine is subsequently cleaved by the product of the *luxS* gene, resulting in the production of AI-2, a quorum sensing signalling molecule. As expected, neither N2-succinyl-L-arginine nor S-D-ribosyl-L-homocysteine could be detected in uninfected macrophages (Fig. 4b). However, we could detect both metabolites in cell extracts from macrophages infected with either WT or *pyrF* bacteria, implying that these bacteria are metabolically active in the macrophage. Interestingly N2-succinyl-L-arginine is not produced when HM605 is cultured in DMEM, suggesting that arginine degradation is specifically induced in the macrophage (data not shown). It should also be noted that there was a significantly lower level of *S*-D-ribosyl-L-homocysteine in the Δ*pyrF* mutant compared to WT, suggesting that pyrimidine biosynthesis is not the only metabolic defect in this mutant. Interestingly we could not detect either of these metabolites in macrophages infected with the Δ*pfkAB* mutant, suggesting that this mutant has a more complete block in metabolism (see Fig. 4b and Fig. S8). Therefore, our metabolomics analysis suggests that the Δ*pyrF* mutant is unable to replicate due to a block in pyrimidine biosynthesis in the macrophage. However, it does appear that the Δ*pyrF* mutant is metabolically active, albeit to a lesser extent than the WT.

Tacrophage response to infection is not affected by replication of HM605

We wanted to know if the ability of AIEC to replicate inside macrophages might alter the normal macrophage response to *E. coli* and, for example, contribute to the inflammation associated with CD. In this instance AIEC replication might be useful as a therapeutic target for the treatment of some cases of CD. Therefore, we compared the transcriptional response of macrophages infected with either HM605, the Δ*pfkAB* mutant or the non-pathogenic strain, MG1655. We selected the Δ*pfkAB* mutant as our previous analyses had suggested that this mutant was severely crippled in its ability to replicate in the macrophage (see Fig. 2). Macrophages were incubated with bacteria in the usual way and total RNA was isolated 6 h after the addition of gentamicin. Analysis of the resulting transcriptional profiles revealed that there was no significant difference in the transcriptional response of the macrophage to infection with either HM605, the Δ*pfkAB* mutant or MG1655 (see Fig. S9). Therefore, HM605 does not produce a protein (or any other factor) that interferes with the normal macrophage response to *E. coli*, at least not under the conditions tested here. The Gram-negative cell-wall contains LPS, an important MAMP (microbe-associated molecular pattern) that is recognized by TLR4, a membrane-localized signalling protein that controls the activity of important transcriptional regulators such as NF-κB. Not surprisingly many of the macrophage genes that exhibited the largest increase in expression in our experiments are known to be NF-κB regulated, e.g. *Tnf*, *Il6* and *Il12a*, encoding the important cytokines TNF-α, IL-6 and IL-12, respectively (Fig. 5a). To confirm the increase in transcription we measured the levels of TNF-α, IL-6 and IL-12 produced from infected macrophages at T_6 and we observed that the levels of these cytokines were very similar regardless of which bacteria infected the macrophage (Fig. 5b). Macrophages also respond to infection through the production of nitric oxide (NO) by inducible

nitric oxide synthase, encoded by *NOS2*. Both WT and the Δ*pfkAB* mutant bacteria induced similar levels of expression of *NOS2* and we could also detect equivalent levels of a metabolite annotated as *N*-ω-hydroxyarginine ($HRMS^{1}_{lib}$) in macrophages infected with either WT or the Δ*pfkAB* mutant (Fig. 5c). *N*-ω-Hydroxyarginine is an intermediate in the production of NO from arginine. Therefore, our data suggest that AIEC replication does not alter the response of the macrophage to infection.

DISCUSSION

In this study we have shown that AIEC strain HM605 co-localizes with acidified vacuoles in the macrophage. This high level of co-localization strongly suggests that bacterial replication is also occurring in this niche. The nature of these vacuoles remains to be fully determined but it is likely that they are phagosomes. Preliminary work in our laboratory indicates that HM605 is initially taken up into EEA1-containing vacuoles and it has been reported previously that AIEC replicates in LAMP1-positive vacuoles that have been identified as phagosomes (Bringer *et al.*, 2006; Lapaquette *et al.*, 2012; our unpublished data). We have also shown that treatment of J774A.1 macrophages with bafilomycin A or NH_4Cl (compounds known to interfere with phagosomal maturation and/or acidification) completely blocks the intramacrophagic replication of HM605. Therefore acidification of the phagosome appears to be a requirement for AIEC replication, an observation that has also been published elsewhere for a different strain of AIEC (Bringer *et al.*, 2006; Subramanian *et al.*, 2008). Several intracellular pathogens are known to establish their replicative niche within acidified vacuoles derived from the phagosome. For example *Coxiella burnetii*, the causative agent of Q fever, establishes a niche in an acidified, parasitophorous vacuole that develops through interactions between early phagosomes and the autophagic machinery (Voth & Heinzen, 2007). A Type IV secretion system has been implicated in the control of these trafficking events (Beare *et al.*, 2011; Winchell *et al.*, 2014). Similarly *Salmonella enterica* replicates in a modified phagosome called the *Salmonella*-containing vacuole (SCV) and acidification of this phagosome is necessary as a signal for the appropriate transcriptional response of *Salmonella* (including the expression of *S. enterica* virulence factors) (Arpaia *et al.*, 2011; Thompson *et al.*, 2011). *Klebsiella pneumoniae* and *Brucella suis* are additional bacterial pathogens for which acidification of the phagosome has been shown to be required for bacterial survival within macrophages (Cano *et al.*, 2015; Porte *et al.*, 1999). Both *Coxiella* and *Salmonella* secrete effector proteins to control the development of their replicative niches and the capsule of *K. pneumoniae* has been shown to be required to prevent lysosomal fusion with the acidified phagosome (Cano *et al.*, 2015). However our data suggest that AIEC strain HM605 does not modify the normal macrophage response to infection. Moreover comparative analysis of several AIEC genomes has failed to identify any AIEC-specific genes that would explain the ability of this group of bacteria to replicate in the macrophage. Therefore the molecular details underpinning the interaction between AIEC and the phagosome, and the reasons underlying the requirement of phagosomal acidification for replication are not yet understood.

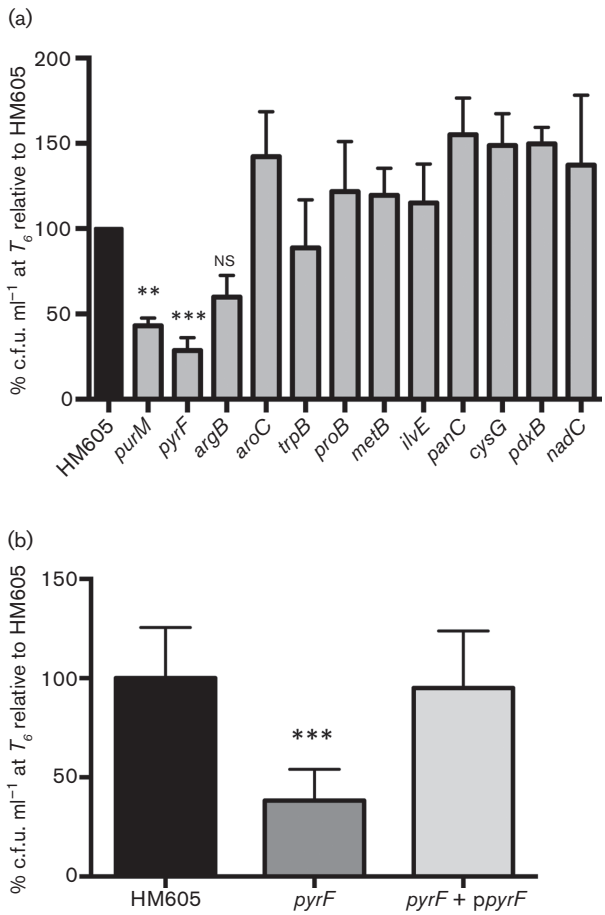

Fig. 3. Purine and pyrimidine biosynthesis is required for intramacrophagic replication of HM605. (a) J774A.1 macrophages were infected with HM605 and different metabolic mutants at an m.o.i. of 10 and the numbers of intracellular bacteria were measured 6 h after the addition of gentamicin (T_6). The data are presented as percentage c.f.u. ml^{-1} relative to HM605-infected macrophages. Statistical significance was determined using one-way ANOVA (with Dunnett's post-test correction for multiple comparisons); $^{**}P<0.01$, $^{***}P<0.001$. (b) Complementation of the Δ*pyrF* mutation by the *in trans* expression of *pyrF*. Data was analysed using the Student *t*-test: $^{***}P<0.001$; NS, not significant.

The streptomycin mouse model has been used to show that a number of central metabolic pathways, e.g. the Entner–Doudoroff pathway and glycolysis are required for *E. coli* colonization of the mouse gut (Chang *et al.*, 2004). Indeed different strains of *E. coli* appear to have different metabolic requirements for colonization, and *E. coli* strains with similar metabolic requirements appear to compete to occupy the same niche in the gut of the host (i.e. the restaurant hypothesis) (Conway & Cohen, 2015; Maltby *et al.*, 2013). Previous work has also shown that gluconeogenesis and the TCA cycle are important for the growth of uropathogenic *E. coli* (UPEC) in urine (Alteri *et al.*, 2009). We have analysed the

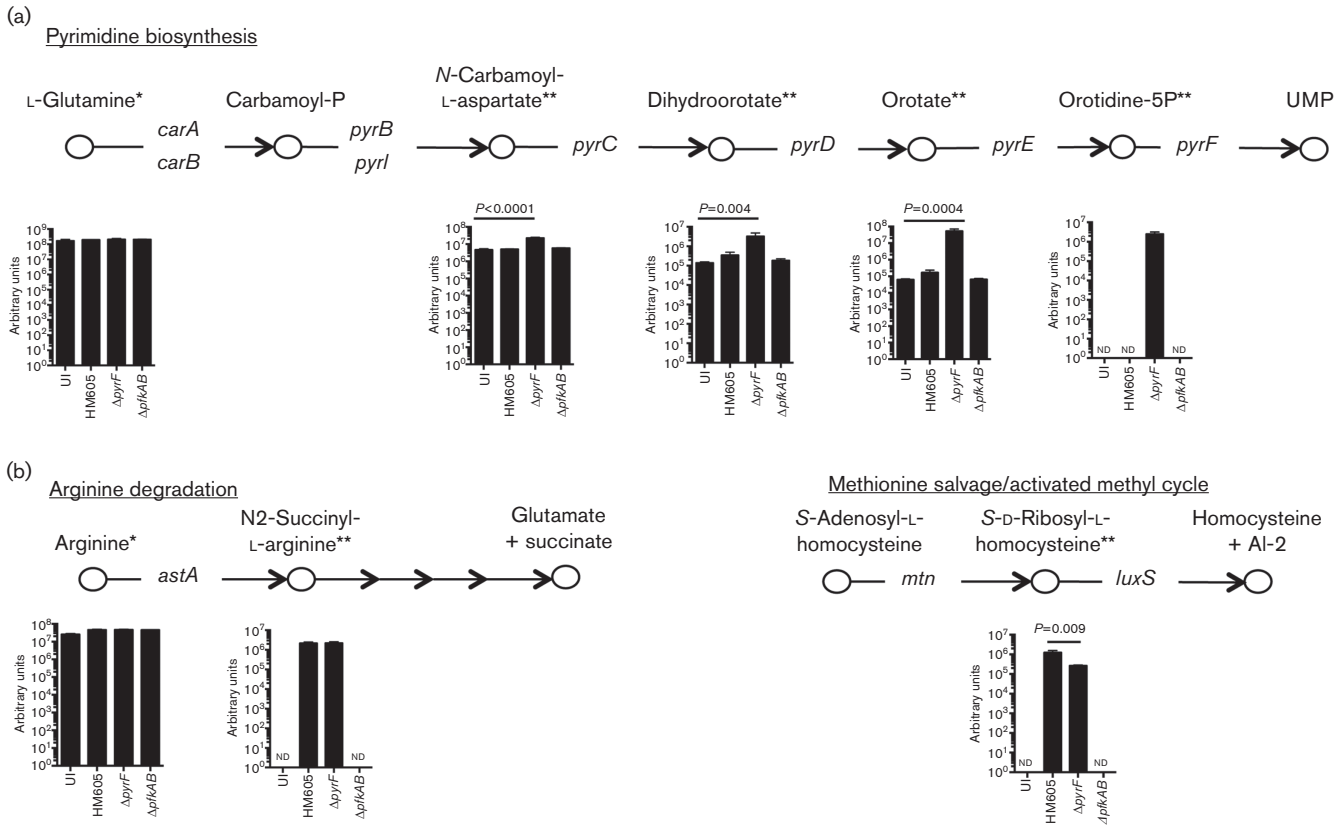

Fig. 4. Metabolomic analysis of macrophages infected with HM605, the Δ*pfkAB* mutant or the Δ*pyrF* mutant. Macrophages were infected (m.o.i. of 10) and total cell extracts were prepared for metabolomic analysis 6 h post-gentamicin addition. (a) Macrophages infected with the HM605 Δ*pyrF* mutant accumulate intermediates in pyrimidine biosynthesis. (b) Annotated bacteria-specific metabolites can be detected in macrophages infected with HM605 WT and the Δ*pyrF* mutant. Values shown are the means of three independent experiments and the error bars represent SD. Data were analysed for statistical significance using the Student *t*-test. *, Identified metabolites ($HRMS^1_aRT_a$); **, annotated metabolites ($HRMS^1_{lib}$).

ability of a number of metabolic mutants in AIEC to replicate in macrophages and we have shown that glycolysis has a significant role during growth in the acidified phagosomal niche. This is similar to what has recently been reported for *S. enterica* serovar Typhimurium where the same mutation (Δ*pfkAB*) prevented intracellular replication of *S.* Typhimurium in murine RAW264.7 macrophages (Bowden *et al.*, 2009). The Δ*pfkAB* mutant in *Salmonella* was also attenuated in virulence and this mutant showed significantly less colonization of BALB/c mice after either intravenous or intraperitoneal injection (Bowden *et al.*, 2009; Paterson *et al.*, 2009). Glycolysis has also been shown to be required for the replication of *Shigella* within the cytoplasm of infected epithelial cells (Waligora *et al.*, 2014). In AIEC, glycolysis appears to be the most important metabolic pathway as mutations in the other major central metabolic pathways involved in carbon assimilation (the TCA cycle, the pentose-phosphate pathway and the Entner–Doudoroff pathway) had much smaller effects on bacterial replication in the macrophage (see Fig. 2). In contrast mutations in the TCA cycle actually increased the ability of *Salmonella* to survive within macrophages (Bowden *et al.*, 2010). Therefore, whilst there are clearly similarities in the intracellular metabolic requirements of *E. coli* and *Salmonella* there also appear to be significant differences (Eisenreich *et al.*, 2010; Götz *et al.*, 2010).

Our analysis of auxotrophic mutants in HM605 suggests that the phagosome is relatively rich in amino acids and other nutrients. Indeed, none of the amino acid auxotrophs exhibited a significant decrease in replication, suggesting that these nutrients are available in adequate amounts in the phagosome. This is in agreement with a previous study that analysed *S.* Typhimurium gene expression during replication in the macrophage and concluded that the SCV is rich in amino acids (Eriksson *et al.*, 2003). However, our study did reveal a requirement for *de novo* purine and pyrimidine biosynthesis during the intramacrophagic replication of HM605. Therefore, mutations in either *purM* or *pyrF* were significantly affected in their ability to replicate in the macrophage (see Fig. 3). The *pyrF* gene encodes the enzyme required for the conversion of OMP to UMP and

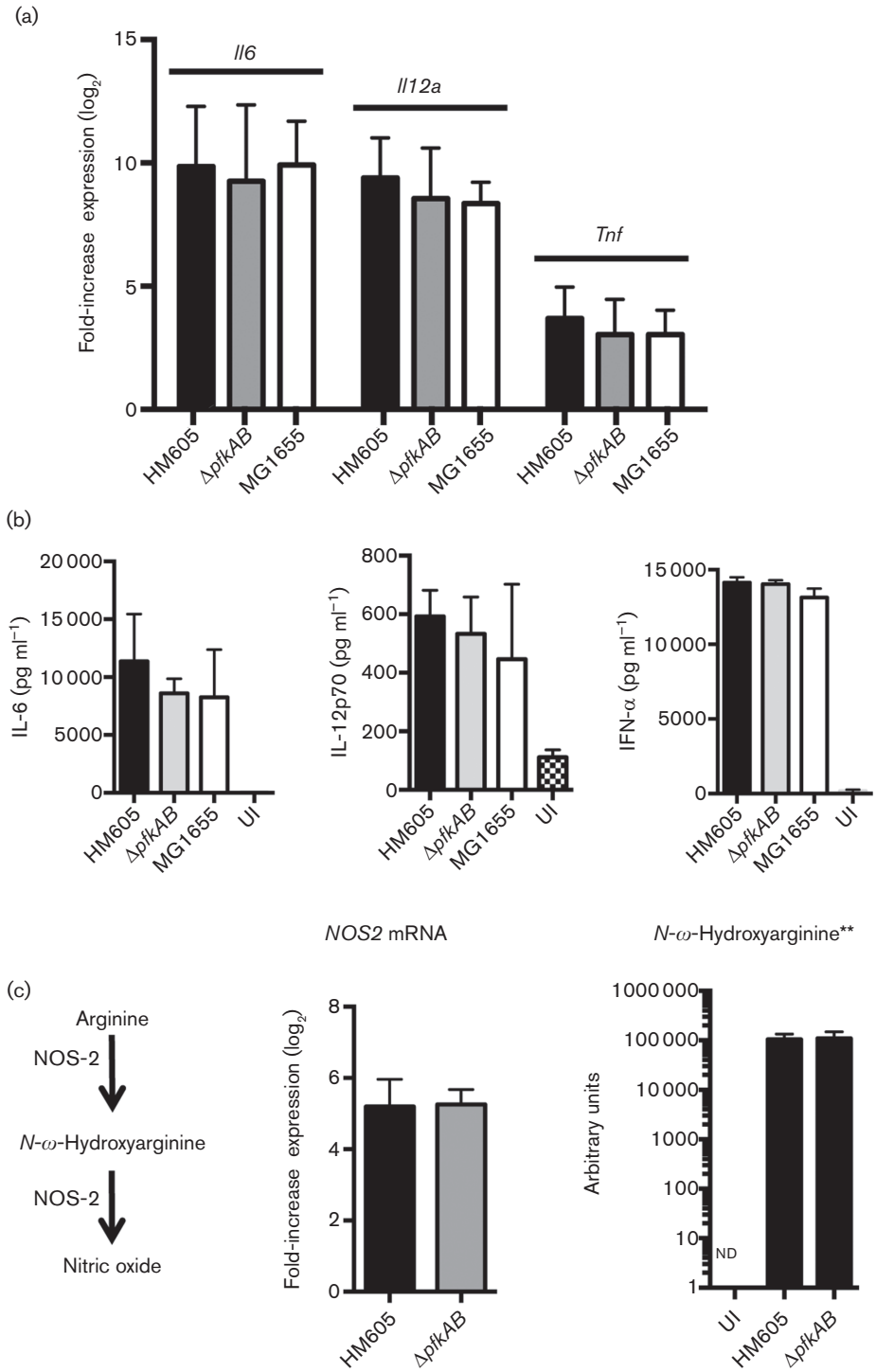

Fig. 5. The macrophage response to infection with HM605, the Δ*pfkAB* mutant or MG1655. (a) Cytokine production in macrophages infected with HM605 (black bars), the Δ*pfkAB* mutant (grey bars) or MG1655 (white bars). J774A.1 macrophages were infected with bacteria at an m.o.i. of 10 and, 6 h post-gentamicin treatment, total RNA was isolated from the macrophages and analysed, in triplicate, using the SurePrint G3 Mouse GE 8×60K microarray from Beckman Coulter. Expression of selected cytokine genes is shown as fold-increase (transformed by $\log_2$) in expression compared to uninfected macrophages. (b) Culture supernatants were also retained and assayed, in triplicate, for cytokine levels using ELISA. Uninfected (UI) controls were included to show baseline cytokine production. (c) The level of *NOS2* gene expression and *N*-ω-hydroxyarginine [**, annotated metabolite ($HRMS^1_{lib}$)] in macrophages infected with HM605 and the Δ*pfkAB* mutant. In all graphs the values shown are the mean of three independent experiments and the error bars represent SD. ND, Not detected.

metabolomics has confirmed that OMP accumulates in macrophages infected with the Δ*pyrF* mutant. Therefore, the Δ*pyrF* mutant is blocked in pyrimidine biosynthesis and appears to be experiencing pyrimidine auxotrophy in the macrophage. Interestingly, a Δ*pyrF* mutant in *Francisella tularenis* has been shown to be unable to replicate in the cytosol of infected macrophages, suggesting that sufficient levels of pyrimidines are limiting throughout the macrophage and not just in the phagosome (Horzempa *et al.*, 2010). The *purM* gene encodes an enzyme that catalyses the fifth step in the *de novo* biosynthesis of purines. Whilst there are no reports of any requirement for purine biosynthesis during bacterial intramacrophagic replication, it has been shown that purine biosynthesis-deficient mutants of *Burkholderia*, *Sinorhizobium* and *Photorhabdus* are defective in their ability to form symbiotic associations with insects, plants and nematodes, respectively (Ruisheng & Grewal, 2011; Buendía-Clavería *et al.*, 2003; Kim *et al.*, 2014). Moreover *Pseudomonas aeruginosa* must synthesize purines during both chronic and acute infections of animals (Turner *et al.*, 2014). Therefore the availability of purines would appear to be limiting in the host. Finally, although it was not deemed to be significant (P=0.086), there was an approximately 50 % reduction in the intramacrophagic growth of the *argB* mutant, suggesting that arginine levels may affect bacterial replication in the macrophage (see Fig. 3a). The reduced growth of the *argB* mutant may be a bona fide requirement for arginine, or the phenotype may be a consequence of the regulatory and metabolic links between arginine biosynthesis and both purine and pyrimidine biosynthesis (Caldara *et al.*, 2008; Cho *et al.*, 2011). Indeed we favour the latter as HM605 assimilates arginine during replication in the macrophage, suggesting that arginine is not limiting in this niche (see Fig. 4).

Recent work has implicated AIEC in the pathophysiology of CD and it has been shown that J774A.1 macrophages infected with AIEC strain LF82 produced up to three times more TNF-α compared with macrophages stimulated with LPS alone, and the production of TNF-α was dependent on the numbers and/or replication of LF82 in the macrophage (Bringer *et al.*, 2012; Glasser *et al.*, 2001). We have shown that J774A.1 macrophages infected with AIEC strain HM605 produce similar levels of TNF-α (and other cytokines) compared with macrophages infected with a non-pathogenic strain of *E. coli*, and we have shown that the production of TNF-α is similar regardless of whether the macrophage is infected with HM605 or a non-replicating mutant (see Fig. 5). Therefore it appears that the relationship between AIEC and the production of cytokines by macrophages may be strain-dependent, implying that different strains of AIEC may contribute to inflammation in different ways. This further highlights the heterogeneous nature of this group of *E. coli*.

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