Klebsiella pneumoniae yfiRNB operon affects biofilm formation, polysaccharide production and drug susceptibility

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Klebsiella pneumoniae is an opportunistic pathogen important in hospital-acquired infections, which are complicated by the rise of drug-resistant strains and the capacity of cells to adhere to surfaces and form biofilms. In this work, we carried out an analysis of the genes in the K. pneumoniae yfiRNB operon, previously implicated in biofilm formation. The results indicated that in addition to the previously reported effect on type 3 fimbriae expression, this operon also affected biofilm formation due to changes in cellulose as part of the extracellular matrix. Deletion of yfiR resulted in enhanced biofilm formation and an altered colony phenotype indicative of cellulose overproduction when grown on solid indicator media. Extraction of polysaccharides and treatment with cellulase were consistent with the presence of cellulose in biofilms. The enhanced cellulose production did not, however, correlate with virulence as assessed using a Caenorhabditis elegans assay. In addition, cells bearing mutations in genes of the yfiRNB operon varied with respect to the WT control in terms of susceptibility to the antibiotics amikacin, ciprofloxacin, imipenem and meropenem. These results indicated that the yfiRNB operon is implicated in the production of exopolysaccharides that alter cell surface characteristics and the capacity to form biofilms – a phenotype that does not necessarily correlate with properties related with survival, such as resistance to antibiotics.

INTRODUCTION

Healthcare-associated infections (HAIs) are a major public health problem worldwide and infections caused by multiresistant bacteria such as Klebsiella pneumoniae are of particular importance. This micro-organism, which can be found in the gut and nasopharynx, is also a major opportunistic pathogen within the family Enterobacteriaceae. K. pneumoniae causes a large percentage of community-acquired diseases and HAIs, such as pneumonia, septicemia, and urinary tract and wound infections, especially in infants, the elderly and in immunocompromised patients (Pan et al., 2013; Podschun & Ullmann, 1998). An important virulence factor is its capacity to adhere to and form biofilms on different surfaces, including indwelling medical devices. It has been reported that biofilms can be involved in ~65% of bacterial infections, allowing cells to persist, and leading to chronic infections and increased resistance to antibiotic treatment (Høiby et al., 2011).

Bacterial biofilms are surface-associated communities characterized by the production of an extracellular matrix that contains extracellular polymeric substances (EPSs) that differ in composition and physical properties, depending on the microbial species (Kostakioti et al., 2013). Biofilm formation in K. pneumoniae is affected by mutations in genes responsible for capsule biosynthesis and surface EPSs (Balestrino et al., 2008; Boddicker et al., 2006), genes for the synthesis of cell surface type 3 fimbriae (Di Martino et al., 2003; Langstraat et al., 2001), by mutations that affect quorum sensing (Balestrino et al., 2005) and by the intracellular levels of the second messenger c-di-GMP (Johnson & Clegg, 2010; Johnson et al., 2011; Wilksch et al., 2011). The cellular levels of c-di-GMP are modulated by diguanylate cyclases (DGCs) and phosphodiesterases, which catalyse its synthesis and degradation, respectively (Boyd & O’Toole, 2012; Römling et al., 2013). In K. pneumoniae, the c-di-GMP turnover enzymes YfIN and MrkJ have been shown to modulate type 3 fimbriae expression via the transcriptional regulator MrkH, which contains a c-di-GMP-binding PilZ domain (Johnson & Clegg, 2010; Johnson et al., 2011; Wilksch et al., 2011).

Abbreviations: DGC, diguanylate cyclase; EPS, extracellular polymeric substance; FRT, FLP recognition target; HAI, healthcare-associated infection; MBEC, minimum biofilm eradication concentration; qRT, quantitative real-time.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
The yfiN gene is part of an operon together with the upstream proposed negative regulator yfiR, which codes for a hypothetical protein of 177 aa, and yfiB, an apparent outer membrane lipoprotein proposed as a sensor of the YfIBNR system in *Pseudomonas aeruginosa* (Malone et al., 2012).

The present work analysed in more detail the genes of the yfiRNB operon in *K. pneumoniae* and their effect on biofilm formation, and showed that this operon also affected production of EPS cellulose, in addition to the previously identified effect on fimbriae. Mutations in genes of this operon therefore resulted in changes in the extracellular matrix that had unexpected effects on cell properties, such as resistance to antibiotics.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids used are shown in Table 1. Cells were grown either in liquid Luria–Bertani (LB), brain heart infusion (BHI; Becton Dickinson) supplemented with 2 mM MOPS (Sigma-Aldrich) or Mueller Hinton (MH; Oxoid) at 37 °C with agitation at 200 r.p.m. Solid medium was made by adding 1.5% agar (Difco). When needed, antibiotics were added at the following concentrations: 100 μg ampicillin ml⁻¹, 50 μg kanamycin m⁻¹, 20 μg tetracycline ml⁻¹ and 20 μg chloramphenicol ml⁻¹ (Sigma-Aldrich). The concentrations of the antibiotics imipenem, amikacin, ciprofloxacin and trimethoprim/sulfamethoxazole and meropenem (Sigma-Aldrich) varied depending on the assay (diluting stock concentrations), and were determined following the protocols of the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013).

Phenotypes on solid media were analysed by diluting 18 h overnight cultures 1/100 in fresh medium and spotting 10 μl onto LB agar without salt, supplemented with 40 μg Congo red ml⁻¹ and 20 μg Coomassie blue ml⁻¹, and on LB agar with 0.2 μg ml⁻¹ calcofluor (fluorescent brightener 28; Sigma-Aldrich) to observe cellulose fluorescence under UV light (Solano et al., 2002; Zogaj et al., 2003). Phenotypes were analysed after growth for 18 h at 37 and 28 °C.

**Biofilm formation assay.** *K. pneumoniae* mutants defective in biofilm formation were identified as described previously by screening a miniTn10Km insertion mutant library of strain CG217 (Suésçün et al., 2006). All biofilm assays were done by growing cells in LB medium or BHI + MOPS for 18 h at 37 °C, in 96-well, flat-bottom, non-tissue-culture-treated polystyrene plates (Microtest 96; BD Falcon). Biofilms were stained with 0.1% crystal violet and quantified by measuring OD595, as described previously (O’Totoole & Kolter, 1998). All assays were done using three biological replicas and repeated independently at least once.

**Mutant construction and complementation analysis.** Deletions of yfiR, yfiN and yfiB genes were constructed in WT *K. pneumoniae* LM21gfp, which bears an egfp marker, by allelic replacement with a KmR gene using pKOBEG199 (Balestrino et al., 2005). Briefly, the KmR cassette was PCR-amplified from plasmid pKD4 with 60 nt forward and reverse primers specific for each target gene that included sequences homologous to the KmR gene and FLP recognition target (FRT) sites, as reported previously (Balestrino et al., 2008; Datsenko & Wanner, 2000). Each PCR fragment (100 ng), purified using a QIAquick PCR purification kit (Qiagen), was transformed into *K. pneumoniae* LM21gfp cells harbouring the Red recombinase expression plasmid pKOBEG199 and mutants were selected at 37 °C on LB agar containing kanamycin. In order to avoid polar effects, the KmR cassette was excised via the FRT sites using the FLP helper plasmid pCP20 (Cherepanov & Wackernagel, 1995). Gene deletions were verified by PCR amplification and sequence analysis. All primers used are listed in Table S1 (available in the online Supplementary Material).

For complementation analysis, genes were PCR-amplified from WT *K. pneumoniae* LM21gfp using primers bearing restriction sites (Table S1), cloned by restriction and ligation, first into pCR2.1TOPO vector (Invitrogen), and then released by restriction enzyme digestion and ligated to pBAD18-Cm (Guzman et al., 1995), generating pBAD18-yfiR.

<table>
<thead>
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<th>Table 1. Strains and plasmids used in this study</th>
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<td>Strain/plasmid</td>
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<td><strong>Strains</strong></td>
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<td><em>Klebsiella pneumoniae</em></td>
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<td>CG217</td>
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<td>CG217 M39</td>
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pBAD18-yfiN and pBAD18-yfiB (Table 1). The constructs were confirmed by PCR and sequence analysis, and then transformed into LM21gfp and each deletion mutant. All restriction enzymes were from New England Biolabs.

Carbohydrate measurement and cellulase treatment. The polysaccharide content was measured using the anthrone–sulfuric acid method, with some modifications (Laurentin & Edwards, 2003; Raunkjær et al., 1994). Overnight cultures grown in LB broth were used to spot 20 μl colonies onto LB agar plates. After incubation at 37 °C for 48 h, cells were scraped and resuspended in 300 μl 1 N NaOH, heated at 80 °C for 30 min and centrifuged at 10,000 r.p.m. at 4 °C for 15 min. Aliquots of 40 μl of each supernatant were added to 100 μl anthrone reagent (0.2% anthrone in 75% H2SO4; Sigma Aldrich) placed in 96-well plates (Corning Costar) and incubated for 5 min at 92 °C, followed by 15 min at room temperature and 15 min at 45 °C. A550 was measured. A glucose standard curve was used for quantification, providing an estimate of hexose sugar concentration in each sample. Sugar concentration was standardized based on soluble protein concentration determined using 800 μl of each cell suspension and the Bradford method (A660) (Barnhart et al., 2013).

Assays were performed with three biological replicates and repeated independently at least once.

The presence of cellulose in biofilms was estimated by assessing the effect of cellulase treatment on biofilms, as described previously (Solano et al., 2002). Briefly, biofilms were formed by growing cells in LB for 24 h in 96-well Calýbio Device plates (Nunc-Immuno TSP). After incubation, the lid pegs with biofilms were removed, washed in PBS, placed in 0.05 M citrate buffer, pH 4.6, with 0.1% cellulase (Sigma Aldrich) and incubated for 48 h at 45 °C. After digestion, biofilms were quantified by staining with crystal violet and measuring OD595.

Quantitative real-time (qRT)-PCR assays. Total RNA was extracted from cultures grown in LB broth for 6 and 10 h using a Direct-zol RNA MiniPrep kit (Zymo Research), treated with RQ1 RNase-Free DNase (Promega), quantified by spectrometry (NanoDrop 2000; Thermo Scientific) and used to synthesize cDNA (three per RNA) using SuperScript III (Invitrogen). cDNA was quantified using SYBR Green (Life Technologies) and 150 ng was used for qRT-PCR analysis in a LightCycler 1.5 (Roche) by preheating at 95 °C for 10 min, followed by 40 cycles of amplification and quantification: 95 °C for 10 s, annealing at 62 °C for 10 s, extension at 72 °C for 60 s and quantification at 83 °C for 60 s. Relative expression was obtained using the 16S rRNA housekeeping gene for K. pneumoniae and the equation proposed by Pfaffl (Bustin, 2010; Pfaffl, 2001).

Caenorhabditis elegans killing assay. Assays were performed as described previously (Fuursted et al., 2012) by spreading 50 μl drops of overnight cultures of either Escherichia coli OP50 or K. pneumoniae LM21gfp WT, yfiIR, yfiN and yfiB mutant strains on nematode growth media (NGM) agar plates (Stiernagle, 2006). After drying, 30 adult (stage L-4) C. elegans N2 (WT) were added to each plate. The plates were incubated at 20 °C and scored daily for dead nematodes using a stereoscope (SMZ168 BN; Motic). Nematodes were age-synchronized with bleach and transferred every 2 days to fresh plates to eliminate overcrowding by progeny until they laid no further eggs (Bialek et al., 2010; Fuursted et al., 2012; Srinivasan et al., 2012). Worm mortality was scored over time for ~20 days, with death defined when a worm no longer responded to touch. The experiments were performed in triplicate at least three times on separate days. Bacterial colonization of C. elegans was determined at 12 h after feeding by picking at least five worms, placing them on an agar plate containing 100 μg gentamicin ml−1 to remove surface bacteria and then washing them in 5 ml M9 buffer. Worms were placed on microscope slides in 2% agarose with sodium azide as an anaesthetic, a coverslip was placed over the agarose and worms were examined by fluorescence microscopy using an Axioskop 40 (Zeiss) microscope. Images were obtained using a × 5/1.3 objective.

MIC and minimum biofilm eradication concentration (MBEC) assays. MICs were determined by microdilution in 96-well plates (Corning Costar) (CLSI, 2013). Briefly, overnight cultures were grown in MH broth at 37 °C with shaking at 200 r.p.m., and then each inoculum was adjusted to 0.5 McFarland standard, diluted 1/100 into fresh medium with antibiotic in each well of a 96-well microtitre plate and incubated at 37 °C for 18 h without agitation; all assays were performed in duplicate. The MIC was defined as the lowest concentration of antibiotic that inhibited bacterial growth.

The MBEC was evaluated by adjusting overnight cultures, grown in MH broth at 37 °C with shaking at 200 r.p.m., to 0.2 McFarland standard and inoculating 150 μl of this suspension into each well of a Calgary Biofilm Device plate (Thermo Nunc). After incubation for 24 h at 37 °C, the lid with pegs was removed, washed in MH broth, transferred to fresh medium with antibiotic and incubated for 8 h at 37 °C. After washing again, the lid was transferred to recovery medium without antibiotic. The lowest concentration of antimicrobial agent that prevented visible growth in the recovery medium was defined as the MBEC (Harrison et al., 2010; Singla et al., 2013).

Microscopy and image analysis. Biofilms were formed by placing microscope slides (Menzer Glaser; 1.0 mm thick, 26.5 × 76 mm) in 50 ml Falcon tubes containing strains LM21 ΔyfiB, ΔyfiN and ΔyfiB, and the WT LM21gfp control, grown in 25 ml LB broth at 37 °C for 18 h without shaking. Slides with biofilms were analysed by microscopy with an Olympus U-TR/90 confocal microscope equipped with an argon laser, and detectors and filter sets for monitoring egfp fluorescence (excitation 488 nm, emission 517 nm). Images were obtained using a × 60/1.35 objective and three representative images were taken for analysis. Image visualization was obtained using the software DAIME (Digital image Analysis In Microbial Ecology) (Daims et al., 2006). Quantification of biofilm biomass was performed using COMSTAT software (Heydorn et al., 2000) by taking ~20–50 image stacks (three image stacks from each slide), acquired randomly for each strain.

Bioinformatics analyses. Analysis of the original transposon insertion site, of the yfiRNB operon and of each sequenced mutant allele was done using BLAST_N and TBLAST_N against the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Putative bcsA sequences were identified by first doing an in silico search for PILZ domain-containing sequences. A PILZ domain profile was generated using HAMMER (http://hammer.janelia.org) and sequences reported in the Pfam database, and this profile was then used to search for domains in four K. pneumoniae genomes: Kp342 (GenBank accession number NC_011283), MGH 78578 (GenBank accession number NC_000913.2), NTUH-K2044 (GenBank accession number NC_012731) and HS11286 (GenBank accession number NC_011284). The two bcsA sequences identified and the corresponding operon genes were further analysed by multiple alignments of these regions using the same four K. pneumoniae genomes. BLAST_P and TBLAST_N searches were carried out with all the genes from the cellulose operon in order to determine orthologous and possible paralogous genes in other K. pneumoniae strains and other bacteria belonging to the Klebsiella clade using the following genomes: E. coli K-12 strain MG1655 (GenBank accession number NC_000913.2) and Salmonella enterica subsp. enterica serovar Enteritidis (GenBank accession number NC_011294.1) and Salmonella enterica subsp. enterica serovar Typhimurium str. LT2 (GenBank accession number NC_003197.1). Homologous proteins were aligned and phylogenies were reconstructed using Geneious software (http://www.geneious.com).

K. pneumoniae yfiRNB genes affect biofilms and survival
Statistical analyses. All statistical analyses were performed using the SPSS statistical package (IBM). Data were analysed by ANOVA and Student’s t-test. \( P<0.05 \) was considered significant.

RESULTS

Mutations in the yfiRNB operon affect cellulose production

The yfiRNB operon (Fig. 1a) has been previously implicated in biofilm formation in *K. pneumoniae* due to mutations that affect the synthesis of c-di-GMP, which is involved in the regulation of type 3 fimbriae expression (Johnson & Clegg, 2010; Johnson et al., 2011; Wilksch et al., 2011). Consistent with this, we identified a mutant via transposon mutagenesis of *K. pneumoniae* clinical isolate CG217 (CG217 M39) (Suescun et al., 2006) that mapped to the yfiR gene, which negatively controlled the YfiN DGC protein, and thus formed a biofilm more robust than that of the parental strain (Fig. 2). This mutant strain also showed differences in cell morphology when grown on indicator media. It had a rugose and dark red colony morphology when grown on solid LB containing Coomassie blue + Congo red, and growth on solid LB supplemented with calcofluor led to fluorescent colonies distinct from the parental strain and suggestive of cellulose overproduction in the extracellular matrix (Fig. S1) (Solano et al., 2002; Zogaj et al., 2003).

Deletion mutants were constructed for each of the genes of the yfiRNB operon in the *K. pneumoniae* WT GFP-tagged strain, LM21 gfp (Balestrino et al., 2008). The resulting mutants, LM21 ΔyfiR, LM21 ΔyfiN and LM21 ΔyfiB, were confirmed by PCR amplification and sequence analysis of each locus. As seen in Fig. 2, strain LM21 ΔyfiR showed enhanced biofilm formation, consistent with the original CG217 M39 insertion mutant strain. In contrast, the biofilms produced by mutants LM21 ΔyfiN and LM21 ΔyfiB were similar to that of the parental WT strain. Complementation with WT genes sequences cloned in pBAD18-Cm (Guzman et al., 1995) largely restored the biofilm formation phenotype of the strain lacking yfiR and resulted in slightly enhanced biofilms when yfiN was introduced for complementation of the corresponding deletion strain – the latter probably due to an increase in gene copy number when present on pBAD18-Cm (Fig. 2). The empty vector control did not have any impact on biofilm formation in the strains tested and the various mutations did not affect cell growth (data not shown). A deletion of the entire yfiRNB operon also resulted in reduced biofilm formation with respect to the parental strain, as has been reported previously (not shown) (Wilksch et al., 2011). In addition to changes in biofilm formation, strain LM21 ΔyfiR also showed colony morphologies consistent with the observations for the CG217 M39 mutant when plated on LB with either Coomassie blue + Congo red or calcofluor, again indicative of changes in the extracellular matrix due to the presence of cellulose (Fig. S1).

CLSM was used to analyse 18 h biofilm structures. The ΔyfiR mutant produced a more robust, highly compact and dense structure when compared with the WT control cells (Fig. 3). The ΔyfiN mutant showed decreased cell surface adherence and fewer attached bacteria, consistent with biofilm measurements (Fig. 2). Finally, the ΔyfiB mutant

![Fig. 1. Schematic representation of the *K. pneumoniae* (a) yfiRNB and (b) cellulose operons. Arrows indicate direction of transcription; the small bent arrow indicates a putative promoter, the inverted triangle shows the transposon insertion in mutant M39 (yfiR gene) and numbers below the line indicate gene coordinates. The map was produced using SnapGene (http://www.snapgene.com/).](image-url)
showed a uniform but thin biofilm relative to the WT strain. Images were analysed using COMSTAT (Heydorn et al., 2000) for biomass distribution, mean thickness, roughness coefficient and substratum coverage, and the data obtained supported the above descriptions (Table S2).

Cellulose as part of the extracellular matrix in *K. pneumoniae*

The observed phenotypes on solid media prompted us to look for the presence of cellulose in the extracellular matrix. The amount of EPS was therefore quantified using the anthrone–sulfuric acid reaction (Laurentin & Edwards, 2003; Raunkjær et al., 1994), which measured total carbohydrates by the amount of hexose released. Cells lacking the yfiR gene released more glucose than both the WT strain LM21gfp or the ΔyfiB and ΔyfiN mutant strains, indicative of more polysaccharide production. The complemented strains bearing plasmids with WT alleles were similar to one another and even higher than the WT strain, particularly for the strain complemented with the DGC gene yfiN (Fig. 4a). The high levels observed for the yfiR-complemented strain could be due to altered expression that led to imbalances in control and appropriate function of the operon, as has been proposed (Malone et al., 2010). The presence of cellulose in biofilms was also assessed by treating 24 h biofilms with cellulase as an indirect measurement of extracellular matrix composition (Solano et al., 2002). In all cases, biofilms were affected by treatment with cellulase, as could be seen by the decrease in crystal violet staining when compared with untreated controls (Fig. 4b). The reduction in biofilm staining was slightly greater for cellulase-treated biofilms of the WT and yfiN-complemented strains — an observation that contrasted with the apparent increase of cellulose in yfiR cells. This was probably due to the enzyme’s activity being limited to the outermost surface cells and its restricted accessibility to the interior of the more highly compact multicellular structures of the yfiR mutant biofilms.

Genomic and transcriptional analysis

To investigate the possible mechanism underlying the observed changes in cellulose production associated with the ΔyfiR strain, we looked at expression of the DGC-encoding gene yfiN and of putative cellulose synthase genes present in the *K. pneumoniae* genome using qRT-PCR. Two loci containing putative cellulose synthesis genes were
first identified in the sequenced *K. pneumoniae* genomes by looking for PilZ domain-containing sequences (Fig. 1b). One of these consisted of two divergently oriented operons, bcsABZC and bcsEFG, and was found to be highly conserved in four *K. pneumoniae* genomes (Kp342, MGH 78578, NTUH-K2044 and HS11286) and in other enterobacteria examined, i.e. *E. coli* str. K-12 substrain MG1655, *Salmonella enterica* subsp. enterica serovar Enteritidis and *Salmonella enterica* subsp. enterica serovar Typhimurium str. LT2. The second locus was separated by 108 bases and consisted of an apparently duplicated copy of the bcsABZC operon, as it was found only in the four *K. pneumoniae* genomes and was absent from other enterobacteria analysed. Both operons contained copies of *bcsA* (named *bcsA1* and *bcsA2*), each harbouring a glycosyltransferase 2 family domain, a cellulose synthase domain and a c-di-GMP-binding PilZ

*Fig. 3.* Biofilm structure of *K. pneumoniae* LM21gf and isogenic mutants. Biofilms grown for 18 h were monitored by CLSM. Images indicate a representative 3D structure of each strain: (a) WT LM21gf, (b) LM21 Δ*yiR*, (c) LM21 Δ*yiN* and (d) LM21 Δ*yiB*. Image visualization was obtained using DAME (Daims et al., 2006).
domain – the latter characteristic of some effector proteins from the c-di-GMP signal transduction network (Römling et al., 2013).

Expression analysis of the DGC-encoding yfiN gene and both bcsA copies was carried out with RNA extracted from 6 and 10 h WT and mutant strain cultures, when cells were likely to express genes that could affect biofilm formation. As control, we also analysed a strain with a deletion of ycdT – another DGC-encoding gene in the K. pneumoniae genome (Cruz et al., 2012). The qRT-PCR results showed that in a strain lacking yfiR, the yfiN gene was overexpressed approximately seven and 12 times in 6 (not shown) and 10 h cultures, respectively, when compared with the WT strain (Fig. 5). Expression of yfiN was not altered significantly in either a strain bearing a deletion of ycdT or in the strain lacking yfiB. Similar results were obtained using 6 h cultures (not shown). The overexpression of yfiN in the absence of yfiR indicated regulation at the transcriptional level, and differed from expression of the putative cellulose synthase genes bcsA1 and bcsA2, which showed no significant difference with respect to the WT strain (Fig. 5). Together, these qRT-PCR results indicated that cells lacking the negative regulator yfiR resulted in overexpression of yfiN, but not of the bcsA genes putatively involved in cellulose synthesis.

Cell susceptibility and survival

The observed changes in cell surface characteristics raised the possibility that the yfiRNB operon could also alter the cell’s capacity to survive under adverse environmental conditions. Given that K. pneumoniae is a well-known opportunistic pathogen associated with HAIs and resistance to antibiotic treatment, we wanted to see if the yfiR mutant, which produced more robust biofilms in vitro and more cellulose, would also present enhanced resistance to antibiotics. We therefore assessed the resistance of the various strains to antibiotics, both during planktonic growth (MIC) and when grown as biofilms (MBEC). Antibiotics were chosen based on their different modes of action and included some that are commonly used to treat K. pneumoniae infections. Overall, resistance was greater in biofilms (MBECs) than for planktonic cells (MICs) for all antibiotics tested (Table 2). When compared with the WT

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\text{Fig. 4. Cellulose is present in the EPS. (a) Hexose sugars were quantified from cells grown as colony biofilms. (b) 24 h biofilms formed on lid pegs were treated with cellulose (hatched) or left untreated (black), and remaining attached cells were quantified using crystal violet staining. Bar, SD. Statistical significance was determined by ANOVA, compared with the WT strain. }^* P<0.05 \text{ was considered significant.}
\]
strain, the yfiR mutant showed decreased resistance in biofilms for most antibiotics tested, amikacin, ciprofloxacin, a second-generation fluoroquinolone that affects DNA gyrase, the broad-spectrum β-lactam antibiotics meropenem and imipenem that inhibit cell wall synthesis, and trimethoprim/sulfamethoxazole, which inhibits folate biosynthesis. Trimethoprim/sulfamethoxazole did not affect either the MIC or MBEC of any of the remaining strains when compared with WT LM21gfp (not shown). The yfiN strain was more sensitive to ciprofloxacin when grown planktonically (MIC), but more resistant in biofilms. Cells bearing yifB behaved differently and were more resistant to ciprofloxacin during planktonic growth, yet more susceptible to both imipenem and meropenem, the latter when in biofilms. Overall, susceptibility with respect to the WT strain was altered most with ciprofloxacin, in both planktonic cells and biofilms. Together, these results indicated that mutations in the yfiRN operon altered susceptibility to antibiotics in a way that was not consistent with the expected effect based on biofilm formation. The yfiN mutation increased resistance in biofilms at least in one case and contrasted with the increased sensitivity of yfiR in all cases assayed, despite the fact that mutation of this gene resulted in more robust biofilms.

The effect of the observed changes in the extracellular matrix triggered by overproduction of YfiN DGC and cellulose was next evaluated using a C. elegans infection model (Bialek et al., 2010; Fuursted et al., 2012; Srinivasan et al., 2012). Age-synchronized, adult nematodes were fed LM21gfp WT cells or isogenic mutants lacking yfiR, yfiN or yfiB, and mortality was scored over time, as an indirect marker of virulence potential. All strains, including the WT strain, showed increased mortality (reduced survival) when compared with E. coli OP50 (P < 0.001). However, host survival curves were indistinguishable from one another and even worms fed K. pneumoniae LM21 ΔyfiR grew to maturity with no apparent adverse effects on viability compared with those fed the other LM21 strains (Fig. 6a). Likewise, no differences were observed in terms of bacterial colonization (Fig. 6b). This result was contrary to our expectations and indicated that an increase in cellulose production was not involved in virulence of K. pneumoniae in this assay.

**Table 2. Susceptibility to antibiotics of planktonic cultures and biofilms**

MIC and MBEC are shown for each strain incubated with the antibiotics indicated. Change indicates the change with respect to the WT strain under the same treatment and is shown in bold.

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<thead>
<tr>
<th>Strain</th>
<th>Ciprofloxacin</th>
<th>Amikacin</th>
<th>Imipenem</th>
<th>Meropenem</th>
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<tr>
<td></td>
<td>MIC</td>
<td>Change</td>
<td>MBEC</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>ΔyfiR</td>
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*No change in these MICs with respect to WT.
†Fold change with respect to WT was not calculated as the precise MBEC value was not obtained.
was consistent with other reports showing that the production of cellulose was not necessarily involved in virulence, such as in *Salmonella enteritidis* (Solano et al., 2002), and that virulence in many pathogens most likely requires fine regulation of c-di-GMP levels (Römling et al., 2013).

**DISCUSSION**

Our results show that *K. pneumoniae* strains with mutations that affect expression of the DGC-encoding gene yfiN have altered cell wall components that affect biofilm formation and cell survival. Previous reports had shown that the *K. pneumoniae yfiRNB* operon was involved in biofilm formation via production of the intracellular second messenger c-di-GMP and its regulation of type 3 fimbriae production (Wilksch et al., 2011). In the present study, specific deletion of the negative regulator yfiR increased expression of yfiN and resulted in altered cell matrix components. Colony phenotypes when plated on solid indicator media, as well as anthrone determination of the polysaccharides present in biofilms and the reduction in surface-attached cells upon treatment with cellulase, suggested that the *yfiR* mutant cells contained increased cellulose. The presence of cellulose as part of the *K. pneumoniae* EPS contrasts with reports analysing *K. pneumoniae* cell phenotypes when grown on solid indicator media (Zogaj et al., 2003). However, recent results have shown that high-molecular-weight exopolysaccharides from *K. pneumoniae* contain various glycosyl residues and linkages, amongst which were both glucose and 4-linked glucopyranosyl residues (Bales et al., 2013).

The increase in biofilm formation and cellulose production of the *yfiR* mutant strain correlates with an increased...
transcription of yfiN, which has been shown previously to code for a protein with proposed DGC activity (Wilksch et al., 2011). However, transcription of the two putative cellulose synthase genes identified here in the K. pneumoniae genome (bcsA1 and bcsA2) did not change in a yfiR mutant background. This result indicates that the effect of c-di-GMP on cellulose production most probably occurs via the PilZ domains in both predicted Bcs proteins, as has been shown in Gluconacetobacter xylinus and several enterobacteria (Amikam & Galperin, 2006; Bhowmick et al., 2011; Raterman et al., 2013; Ryjenkov et al., 2006). Regulation of cellulose production by c-di-GMP has been studied extensively at the molecular level, and recent mechanistic insight has been obtained regarding enzyme activity based on analysis of the crystal structure of the Rhodobacter sphaeroides c-di-GMP-activated BcsA–BcsB complex (Morgan et al., 2014). The robust biofilms formed by K. pneumoniae yfiR cells are most probably due to enhanced polysaccharide production, triggered by local level c-di-GMP that can activate BcsA cellulose synthases. This is similar to what has been reported for both E. coli and P. aeruginosa, where homologues of yfiRN/NB have been shown to be involved in EPS production and biofilm formation (Malone et al., 2010; Raterman et al., 2013).

Microbial cellulose is synthesized during biofilm formation in species such as Salmonella typhimurium, Salmonella enteritidis and E. coli, and can have diverse functions. In Rhizobium and Agrobacterium species, it facilitates cell adhesion and promotes interaction with plant cells, it can confer protection to organisms such as Acetobacter xylinum and Sarcina ventriculi in their natural environments, and it is proposed to contribute to survival and persistence of Erwinia amylovora under stress (Ordax et al., 2010; Ross et al., 1991). Not much is known regarding cellulose production and its role in K. pneumoniae, yet the presence of the two putative synthase operons reported here for the first time indicates that this polymer could be important in certain environmental settings where its production could be triggered by niche-specific conditions not mimicked under the laboratory conditions used here.

Both the extracellular matrix and biofilm formation can promote resistance to antibiotic treatment and the adverse conditions within the host, and have been implicated in pathogenesis and long-term persistence in different microorganisms. Microbes defective in polysaccharide production can have increased sensitivity to antimicrobials and to the host immune system (Rendueles et al., 2013). In addition to their protective role, polysaccharides are involved in mediating cell surface and cell-to-cell interactions, and can even act as antibiofilm agents by inhibiting growth of other species (Kostakiot et al., 2013; Rendueles et al., 2013). Under our experimental conditions, enhanced K. pneumoniae biofilm formation in vitro was associated with an increase in cellulose production, but not necessarily with increased cell survival or virulence. The capacity to kill and colonize C. elegans was similar in all strains analysed, and indicated that cellulose is not involved in virulence in this model system – an observation consistent with reports for Salmonella (Solano et al., 2002), but different from the EPS-dependent resistance to ingestion observed for P. aeruginosa (Malone et al., 2010). Furthermore, robust biofilm formation and cellulose production did not necessarily lead to enhanced survival upon exposure to antibiotics. This was unexpected, as enhanced matrix production has been equated with increased biofilm survival (Van Acker et al., 2014). However, it is consistent with what has been found in P. aeruginosa where increased production of Pel EPS did not increase resistance to aminoglycosides (Khan et al., 2010), whilst the loss of EPS did – similar to our results with the K. pneumoniae yfiRN/NB mutant biofilms, which had increased resistance to ciprofloxacin. c-di-GMP is known to alter gene expression (Römling et al., 2013) and thus it is possible that the loss of the DGC YfiN could change expression of membrane proteins, such as efflux pumps or porins, that can influence drug susceptibility. It has been recently reported, for example, that a c-di-GMP-bound transcriptional factor plays an important role in the synthesis of mycolic acids in Mycobacterium smegmatis, and alters permeability and drug susceptibility (Li & He, 2012). Thus, imbalances in cellular pools of c-di-GMP can affect membrane components and transport across the membrane in our K. pneumoniae yfiRN/NB mutant strains. In addition, changes in expression of genes involved in protection against oxidative stress – a mechanism proposed to be important in protection against treatment with antibiotics (Van Acker et al., 2014) – could also render cells more susceptible to drug treatment, particularly in biofilms. Further analysis of possible changes in membrane protein profiles, gene expression or production of reactive oxygen species could help to understand the observed differences in these strains. As a consequence, and as has been observed for other organisms (Musaf et al., 2014), in vitro analysis of K. pneumoniae biofilm formation capacity does not necessarily correlate with drug resistance – an aspect that merits more detailed studies using clinical isolates given the importance of this pathogen in HAI infections.

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