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Fluoropyrimidines affect *de novo* pyrimidine synthesis impairing biofilm formation in *Escherichia coli*

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ABSTRACT

Antivirulence agents are considered a promising strategy to treat bacterial infections. Fluoropyrimidines possess antivirulence and antibiofilm activity against Gram-negative bacteria; however, their mechanism of action is yet unknown. Consistent with their known antibiofilm activity, fluoropyrimidines, particularly 5-fluorocytosine (5-FC), impair curli-dependent surface adhesion by *Escherichia coli* MG1655 via downregulation of curli fimbriae gene transcription. Curli inhibition requires fluoropyrimidine conversion into fluoronucleotides and is not mediated by *c*-di-GMP or the *ymg-rcs* envelope stress response axis, previously suggested as the target of fluorouracil antibiofilm activity in *E. coli*. In contrast, 5-FC hampered the transcription of curli activators RpoS and stimulated the expression of Fis, a curli repressor affected by nucleotide availability. This last observation suggested a possible perturbation of the *de novo* pyrimidine biosynthesis by 5-FC: indeed, exposure to 5-FC resulted in a ca. 2-fold reduction of UMP intracellular levels while not affecting ATP. Consistently, expression of the *de novo* pyrimidine biosynthesis genes *carB* and *pyrB* was upregulated in the presence of 5-FC. Our results suggest that the antibiofilm activity of fluoropyrimidines is mediated, at least in part, by perturbation of the pyrimidine nucleotide pool. We screened a genome library in search of additional determinants able to counteract the effects of 5-FC. We found that a DNA fragment encoding the unknown protein D8B36_18,480 and the *N*-terminal domain of the penicillin-binding protein 1b (PBP1b), involved in peptidoglycan synthesis, could restore curli production in the presence of 5-FC. Deletion of the PBP1b-encoding gene *mrcB*, induced *csgBAC* transcription, while overexpression of the gene encoding the D8B36_18,480 protein obliterated its expression, possibly as part of a coordinated response in curli regulation with PBP1b. While the two proteins do not appear to be direct targets of 5-FC, their involvement in curli regulation suggests a connection between peptidoglycan biosynthesis and curli production, which might become even more relevant upon pyrimidine starvation and reduced availability of UDP-sugars needed in cell wall biosynthesis. Overall, our findings link the antibiofilm activity of fluoropyrimidines to the redirection of at least two global regulators (RpoS, Fis) by induction of pyrimidine starvation. This highlights the importance of the *de novo* pyrimidines biosynthesis pathway in controlling virulence mechanisms in different bacteria and makes the pathway a potential target for antivirulence strategies.

1. Introduction

The spreading of antibiotic resistance among bacteria makes common infections harder to treat and more prone to develop into life-threatening diseases [1]. The use of compounds that act on essential components of the cells significantly fosters the development of antibiotic resistance. Different strategies have been devised as both alternatives and adjuvants of classic antimicrobials. For instance, using bacteriophages in hard-to-treat infections is a blooming field of research, showing promising yet limited results [2].

Antivirulence and virulence-modulating strategies aim to interfere with determinants for bacterial infection without killing the

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microorganism, thus lowering the chance of developing resistance to the compound. Antivirulence compounds can either target single virulence determinants or hamper complex behaviours, such as biofilm formation, quorum sensing, and antibiotic resistance itself [3]. Although directly interfering with virulence determinants might represent the most straightforward approach, acting on cell metabolism is becoming an appealing solution to modulate bacterial pathogenesis. A strong association exists between metabolite levels and the control of structures involved in virulence. Sulphate metabolism and its intermediates modulate virulence in *Pseudomonas aeruginosa* [4] and biofilm formation in *Escherichia coli* [5,6].

Similarly, the disruption of nucleotide metabolism results in the loss of virulence mechanisms in different bacteria. Transposon insertions in genes involved in purine and pyrimidine biosynthesis hinder biofilm formation in *Streptococcus sanguinis* [7], while uracil and the nucleoside analogue 5-fluorocytosine affect quorum sensing and additional virulence determinants in *P. aeruginosa* [8,9].

In *E. coli*, perturbation of pyrimidine and purine biosynthesis results in abrogation of the production of curli fibres, type I fimbriae, and other virulence determinants in the MG1655 laboratory strain [10–12], as well as in clinical isolates [13,14].

In the laboratory strain MG1655, curli fibres are the primary adhesion determinant and, together with cellulose and other exopolysaccharides, one of the major components of the biofilm matrix. Curli and cellulose are also critical for the adhesion and persistence of different *E. coli* pathotypes, such as uropathogenic strains in urinary tract infections [15,16] and the Shiga toxin-producing *E. coli* O104:H4 strain [17]. In contrast, loss of curli seems to be an important pathoadaptive mechanism in the epidemic strain O157:H7 [18]. Thus, biofilm formation and curli are potential targets in specific antivirulence strategies.

Curli and cellulose are often co-expressed through the activity of the CsgD transcriptional regulator. CsgD activates the transcription of curli-encoding operons *csgDEFG/csgBAC* (*csg* operons) and the *dgcC* gene, which codes for the DgcC diguanylate cyclase, which induces cellulose production via the synthesis of the second messenger *c*-di-GMP [19,20]. Overall, the regulation of curli expression is exceptionally complex. It integrates environmental and physiological cues via the activity of many regulatory circuits, such as the alternative sigma factor RpoS, Fis, OmpR, IHF, H-NS, CpxR, Crl, CRP [20–23]. In addition, the expression of CsgD is controlled at the post-transcriptional level by at least seven non-coding RNAs [24].

The second messenger *c*-di-GMP is a critical regulator of biofilm formation in several Gram-negative bacteria and is synthesised and degraded by enzymes known as diguanylate cyclases (DGCs) and phosphodiesterase (PDEs), respectively. In *E. coli*, *c*-di-GMP controls the production of exopolysaccharides (EPS), such as cellulose and poly-*N*-acetylglucosamine, acting on the EPS biosynthetic machinery [25]. Similarly, transcription of genes involved in curli fibres production is regulated by *c*-di-GMP via a signalling cascade in which *c*-di-GMP controlled by the DGC/PDE pair DgcE/PdeH regulates the activity of the DgcM/PdeR pair, and the MlrA transcriptional regulator [26].

Therefore, *c*-di-GMP signalling is another attractive target for antivirulence strategies. 6-mercaptopurines such as azathioprine, an anti-inflammatory drug used in the treatment of Crohn's disease, inhibit *c*-di-GMP biosynthesis in *E. coli*, probably via perturbation of the intracellular purine nucleotide pool [11]. Consistent with this observation, 6-mercaptopurines hamper curli production in adherent-invasive *E. coli* (AIEC), a pathotype associated with Crohn's disease, and prevent AIEC invasion and replication in human cells [27,28], suggesting that their therapeutic efficacy in Crohn's disease is at least in part mediated via their antivirulence activity. Finally, specific sensing of the relative ratio between an intermediate in the *de novo* pyrimidine pathway and its final product, UTP, affects cellulose production via direct modulation of the DgcQ diguanylate cyclase [29]. Targeting nucleotide metabolism thus represents another excellent way to control and modulate *E. coli* biofilm formation, virulence, and persistence.

In this work, we evaluated the potential antibiofilm activity of three pyrimidine analogues, namely 5-fluorouracil (5-FU), 5-fluorocytosine (5-FC) and 5-fluorouridine (5-FUr), which showed antivirulence and antibiofilm properties in different bacteria. We provide evidence that, in *E. coli*, 5-FC inhibits *de novo* pyrimidine biosynthesis, suggesting that 5-FC impairs biofilm and virulence factor production by interfering with the bacterial cell's primary metabolism. We also performed a genetic search for determinants able to counteract 5-FC antibiofilm effects and identified penicillin-binding protein 1b (PBP1b) and a small protein encoded by a gene in the same locus as new regulatory factors in curli production, unravelling a direct link between peptidoglycan biosynthesis and curli expression.

2. Materials and methods

2.1. Bacterial strains, plasmid construction, and growth conditions

The strains used in this work are described in Table S1. *E. coli* MG1655 mutant derivatives were obtained through homologous recombination [30] using primers listed in Table S2.

For strain construction and manipulation, plasmid propagation and extraction bacteria were routinely grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) or LB-agar medium (LB medium with 15 g/L agar) at 37 °C in shaking conditions (200 rpm). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml. To test the effect of fluoronucleotide analogues, 5-Fluorocytosine (5-FC; stock concentration: 10 mg/ml in Milli-Q H_2O), 5-Fluorouracil (5-FU; stock concentration: 10 mg/ml in 50 % DMSO), and 5-Fluorouridine (5-FUr; stock concentration: 10 mg/ml in Milli-Q H_2O) were added at specified concentrations. For induction of gene expression from the pUC19 vector, 0.5 mM IPTG was used. NaCl was prepared as a concentrated stock, filter sterilised using 0.22 μm syringe filters, and added at the required concentrations to induce hyperosmotic stress.

2.2. Molecular biology techniques and gene expression analysis

Gene expression levels were measured using quantitative real-time RT-PCR as previously described [6]. 16 S RNA levels, which were always comparable in all samples, were used as a reference. Total RNA was extracted from cultures grown in YESCA medium (10 g/L casamino acids, 1.5 g/L Yeast extract) or YESCA medium supplemented with fluoropyrimidines in shaking conditions (200 rpm) at 30 °C during the early stationary phase (8 h of incubation; $OD_{600} \sim 2.0$). The complete list of primers used for amplification is reported in Table S2. For constructing the pUC19 plasmids, target genes were amplified via PCR using the genomic DNA of the *E. coli* MG1655 strain as a template. PCR products were cloned into the pUC19 vector after digestion with specific restriction enzymes. If not otherwise stated, experiments were performed at 30 °C.

2.3. Phenotypic assays and β-glucoronidase assay

Determination of the inhibitory effects of fluoropyrimidines on cell growth was performed using the standard 2X broth dilution method in liquid YESCA medium in 96-well plates and a starting inoculum normalised to an OD_{600} of 0.025, corresponding to 10^7 CFU/ml. OD_{600} of each well was determined at the time of inoculum ($OD_{600\text{-I}}$) and after 24 h of incubation at 30 °C in static conditions ($OD_{600\text{-F}}$). The difference in growth $\Delta OD_{600} = OD_{600\text{-F}} - OD_{600\text{-I}}$ was calculated for each well and expressed as a percentage of the ΔOD_{600} obtained in the untreated control condition (Cell density - % of untreated control). The effect of selected concentrations of fluoropyrimidines (5-FU, 5-FC, and 5-FUr), and osmotic stress and their combination on bacterial growth and cell viability were determined using growth curve analysis and viable cell

enumeration by agar plate counting method, respectively. Briefly, overnight cultures were normalised to an OD_{600} of 0.025 (10^7 CFU/ml) in 96-well plates and incubated in static conditions for 24 h at 30 °C. OD_{600} was measured every 60/120 min using a microtiter plate reader (AMR-100 T, Hangzhou Allsheng Instruments Co., Ltd.). After 24 h, cultures were serially diluted and plated on LB plates to determine the total viable cells.

Congo red (CR)-binding phenotypic assays were essentially carried out as previously described [5]: overnight cultures normalised to an OD_{600} of 1.00 were spotted on YESCA-agar medium (YESCA medium with 2 % agar) supplemented with 0.004 % Congo red and 0.002 % Coomassie blue (CR-medium) after autoclaving. Plates were incubated for 24 h at 30 °C and then, before image acquisition, stored for 48–72 h at 4 °C to further enhance colour development.

For static biofilm adhesion assays, overnight *E. coli* cultures grown in YESCA medium were normalised to $OD_{600} = 0.025$ and incubated in the YESCA medium in a 96-well flat bottom plate in static conditions for 24 h at 30 °C. Next, adhesion was determined using crystal violet staining, as previously reported [10].

For β-Glucuronidase assays, bacterial cultures of the reporter strain AB12 (MG1655 csgA*::uidA-kan*) [21] or its derivatives were grown in YESCA medium at 30 °C overnight in shaking conditions (200 rpm), normalised to an $OD_{600} = 0.05$ in 20 ml of YESCA medium and grown at 30 °C in shaking conditions (200 rpm). Growth was monitored by measuring OD_{600} every 60 min, and β-glucuronidase activity was measured after 8 h of growth (late exponential phase, $OD_{600} \sim 1.0$) when *csgBAC* operon expression was maximal. β-glucuronidase activity was assayed on 200 μl bacterial culture measuring the hydrolysis of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol at 405 nm, as previously described [31].

2.4. Genomic library preparation and screening for genes suppressing 5-FC-dependent curli inhibition

Genomic DNA (gDNA) was extracted from overnight cultures of the MG1655 strain grown in LB medium using the GeneElute Bacterial Genomic DNA kit (Merck). A total of 1 μg of gDNA was partially digested with 1 μl of the 4 bp-cutter FastDigest Bsp143I (Thermo Fisher Scientific) diluted 1:30 in the FastDigest buffer in a final volume of 30 μl. The reaction was incubated 15 min at 37 °C and inactivated for 10 min at 60 °C. Partial digestion was monitored by running the digestion on 1 % agarose gel. The gDNA fragments ranging from 500 to 2000 bp were purified from the agarose gel using a QIAquick Gel Extraction kit (Qiagen). 1 μg of the pUC19 vector was digested using 1 μl of FastDigest BamHI (Thermo Fisher Scientific) in a 30 μl reaction, then purified using a QIAquick PCR Purification Kit (Qiagen). Purified pUC19 and the gDNA fragments were ligated using 5U of T4 DNA ligase (New England Biolab). The ligation mixture was transformed into ultracompetent *E. coli* DH5α cells [32], and transformants were selected on LB/Amp plates after overnight incubation at 37 °C. All the colonies were scrapped from the plates using sterile inoculation loops and resuspended in LB medium. The plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen), transformed in electro-competent *E. coli* MG1655 cells, plated on LB/Amp plates and incubated overnight at 37 °C. The colonies were individually picked and resuspended in 100 μl of LB/Amp in separate wells of multiple 96-well plates. The resuspended colonies were spotted on YESCA/CR/Amp agar plates supplemented with 1.0 μg/ml 5-FC, using a replica titre apparatus. Plates were incubated at 30 °C for 24 h. Plasmids were isolated from colonies showing a red phenotype in the presence of 5-FC, *i.e.*, harbouring a gDNA fragment counteracting the compound effects. The phenotypic switch was confirmed by transforming the MG1655 strain with the extracted plasmids and spotting the transformants on YESCA/CR media supplemented with 1.0 μg/ml and 2.5 μg/ml 5-FC. The gDNA fragment in the plasmid was identified by Sanger sequencing using universal M13/pUC sequencing primers (M13/pUC_frd and M13/pUC_rev).

2.5. Nucleotide determination by HPLC analysis

Frozen cell pellets were resuspended in 0.4 M $HClO_4$, sonicated (30 s at 50 W with 0.5 s pulses), and centrifuged (20,000×*g*) for 5 min at 4 °C. Supernatants were neutralized by adding 1 M K_2CO_3 (1:7 v/v) and frozen to precipitate proteins. Samples were thawed before analysis and centrifuged again (20,000×*g*) to remove $KClO_4$ precipitate. Pellets were resuspended in formic acid by pipetting thoroughly and used for protein determination via Bradford assay. For HPLC analysis, 50 μl of clear cell extracts were analysed via UV C18-HPLC as previously reported [33]. Column temperature was set to 4 °C, to allow concurrent separation of UMP and ATP. Peaks of interest were identified by UV spectral analysis and co-elution with appropriate standards. For quantitation, integrated peak areas, expressed by the software LabSolution in μAU*seconds, were converted to pmol by dividing with the extinction coefficient (mM) at 260 nm, as previously described [33].

3. Results

3.1. Fluorinated pyrimidine nucleosides and nucleobase analogues inhibit E. coli biofilm formation via downregulation of curli fibres expression

Fluoropyrimidines reduce biofilm formation in *E. coli* [13] and are considered promising antivirulence compounds. Curli fibres are arguably the main adhesion factor in *E. coli* laboratory strains. We tested inhibition of curli production by 5-fluorouracil (5-FU), 5-fluorocytosine (5-FC), a 5-FU pro-drug, and 5-Fluorouridine (5-FUr), which can all be converted to pyrimidine nucleotides analogues via pyrimidine salvage pathway (Fig. S1). We first identified a range of fluoropyrimidine concentrations with sub-inhibitory effects on *E. coli* MG1655 growth, evaluating the cell density yield after 24 h of incubation (Fig. S2A). 5-FU was the most effective on bacterial growth, showing >50 % inhibition (IC_{50}) at concentrations ≥1 μg/ml, while 5-FUr showed an $IC_{50} > 4$ μg/ml. For 5-FC, the effect on growth was less marked ($IC_{50} \geq 32$ μg/ml), with negligible effects in the range of 0.625–2.5 μg/ml (<20 % growth reduction). We further analysed the effect of a selected range of concentrations (0.25, 0.5, 1.0, 2.5, 25 μg/ml) on *E. coli* MG1655 growth in liquid medium (Fig. S2B) and viability (Fig. S2C). While 5-FU and 5-FUr showed inhibition of cell viability even at the lowest concentrations tested (Figs. S2B and S2C), 5-FC did not affect growth and cell viability in the concentration range of 0.25–2.5 μg/ml (Figs. S2B and S2C).

We evaluated the effect of the three compounds on biofilm formation by *E. coli* MG1655 in growth conditions favouring curli production (30 °C, low osmolarity medium). In addition to the wild-type strain, we tested the curli-deficient strain AM70 [25] and the curli-overproducing strain PHL628 [34]. All compounds significantly inhibited the adhesion of the wild-type strain in a dose-dependent manner (Fig. 1A). Inhibition was also observed in the curli-overexpressing strain PHL628, in which bacterial adhesion was reduced to wild-type levels at the highest tested concentration (Fig. 1A). No differences were detected in the curli-deficient strain AM70, which cannot adhere to polystyrene [25]. 5-FU and its precursor 5-FC showed the most potent inhibitory effects, significantly reducing adhesion at the lowest concentration tested (0.125 μg/ml). In contrast, 5-FUr required at least four times higher concentration to obtain the same effect (Fig. 1A). To confirm that reduced adhesion was caused by suppression of curli production, we assessed curli production by growing *E. coli* MG1655 on YESCA plates supplemented with the Congo Red (CR) dye (YESCA/CR), *i.e.,* plates on which a red or white colony phenotype can distinguish curli-proficient and curli-deficient strains, respectively (Fig. 1B). Addition of 5-FC, 5-FU, and 5-FC to YESCA/CR resulted in a progressive loss of the red phenotype in the wild-type strain. 5-FC showed a more consistent and concentration-dependent reduction, as observed in the adhesion assay (Fig. 1A). At the same time, the reduction was less pronounced in the curli-overproducing strain PHL628, where a white phenotype was

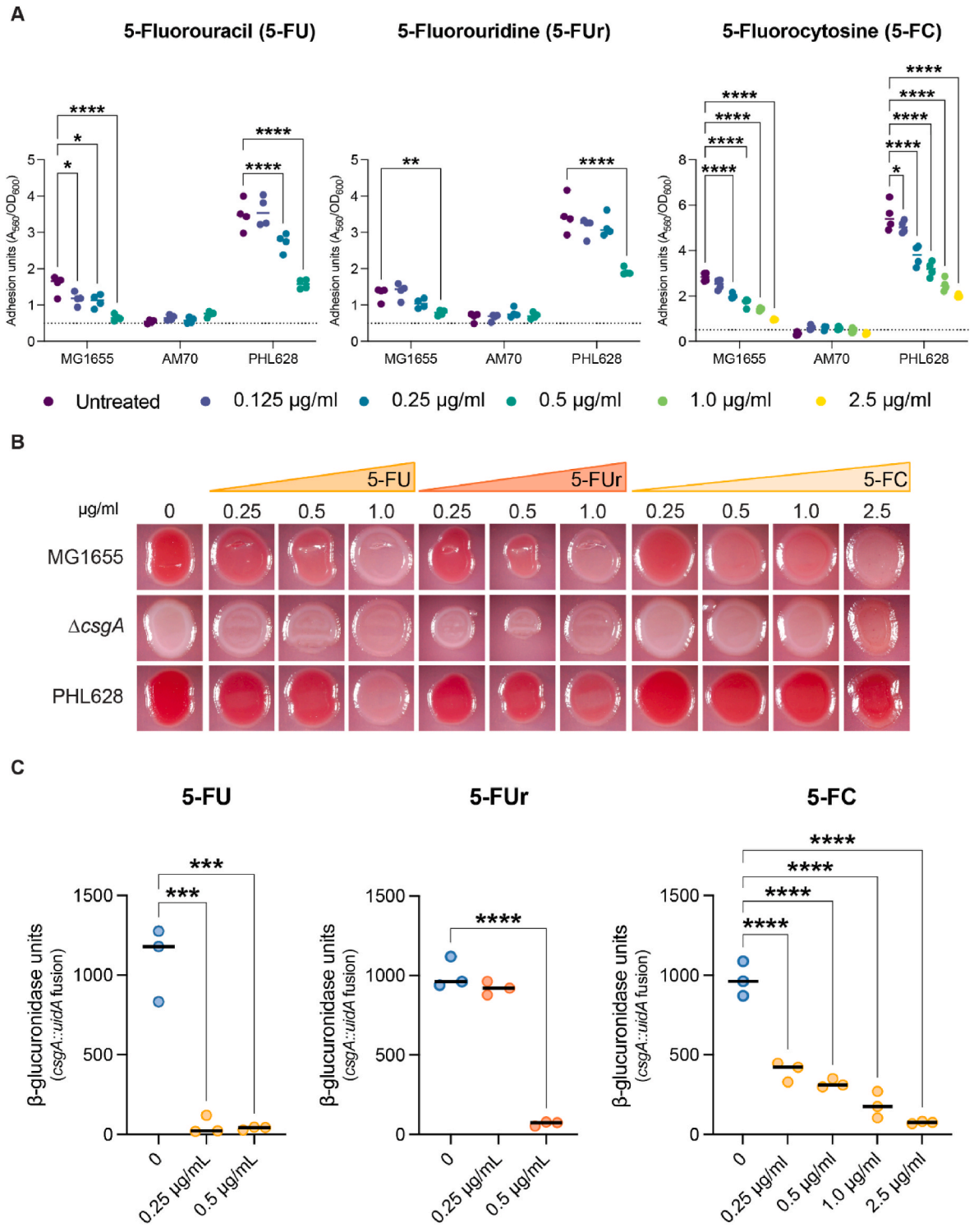

Fig. 1. Fluoropyrimidines effect on biofilm formation in MG1655 strain. **(A)** Biofilm adhesion, **(B)** colony phenotypes on YESCA/CR plates, and **(C)** β-glucuronidase activity of the *csgA::uidA* transcriptional fusion in wild-type (MG1655), the curli-deficient (AM70), and the curli-overproducing (PHL628) strains in the presence of increasing concentrations of 5-fluorouracil (5-FU), 5-fluorouridine (5-FUr), and 5-fluorocytosine (5-FC). The control without compounds contains 0.005 % DMSO. In panel A, the dotted line represents the average adhesion of the curli-deficient AM70 strain. Representative images or results of 3–4 independent replicates and medians are shown. *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; ****, p-value <0.0001, two-way ANOVA with Dunnett's test for multiple comparisons.

detectable only at 0.5 µg/ml with 5-FU and 5-FUr, but not with 5-FC, likely due to residual curli production.

Since phenotypic assays on YESCA/CR medium can only provide a qualitative evaluation of curli production, as CR can bind with less specificity to other extracellular structures [25,35], we directly measured the expression of the *csgA* gene, which codes for the major structural subunit of curli, using a strain harbouring a chromosomal replacement of the gene coding sequence with the reporter gene *uidA* (AB12/MG1655*csgA::uidA-kan*) [21] (Fig. 1C). All compounds reduced *csgA* expression by 15–20 times, thus confirming that reduced adhesion is dependent on lack of curli fibres production. While for 5-FC the effect is independent of growth inhibition (Fig. S2), this cannot be ruled out for

5-FU and 5-FUr, which affect bacterial viability at the tested concentrations (Fig. S2).

3.2. Inhibition of curli fibres expression requires the conversion of fluoropyrimidines into fluoronucleotides

We reasoned that the effect of 5-FU, 5-FC, and 5-FUr on curli

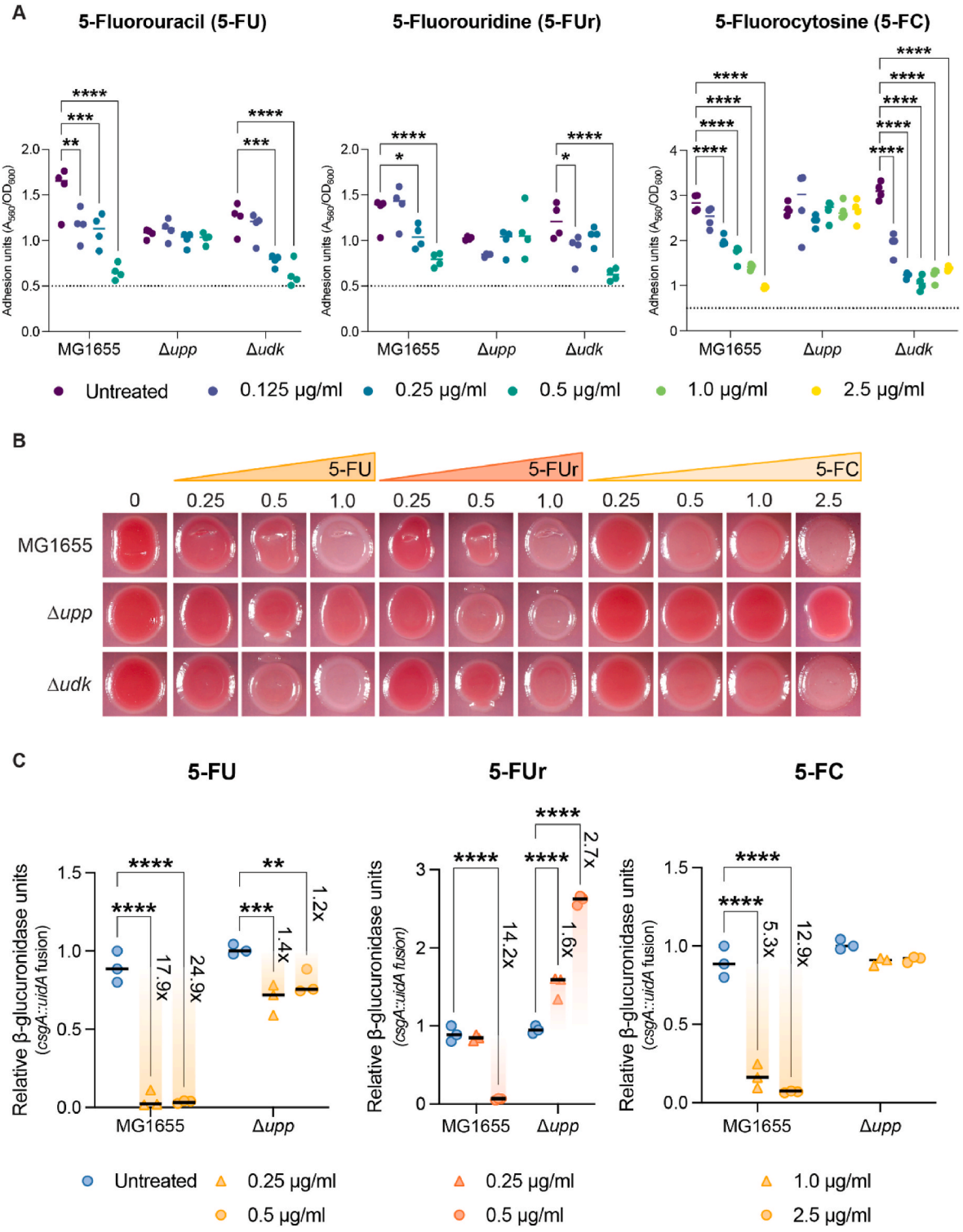

Fig. 2. Fluoropyrimidines effect on biofilm in strains lacking the pyrimidine salvage pathway. **(A)** Bacterial adhesion, **(B)** representative colony phenotypes on CR-agar plates, **(C)** β-glucuronidase activity of the *csgA::uidA* transcriptional fusion. Assays were performed using the MG1655, MG1655Δ*upp*, and MG1655Δ*udk* strains in the presence of increasing concentrations of fluoropyrimidines (5-FU, 5-FUr, 5-FC). The control without compounds contains 0.005 % DMSO. In panel A, the dotted line represents the average adhesion of the curli-deficient AM70 strain. In panel C, values are expressed as relative units, setting the untreated control to 1. Results of 3–4 independent replicates and medians are shown in dot plots. *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; ****, p-value <0.0001, two-way ANOVA with Dunnett's test for multiple comparisons.

expression might either take place through direct interaction with a cellular target or following metabolic activation, *i.e.*, their conversion into fluoronucleotides by the pyrimidine salvage pathway (Fig. S1) [10]. To assess their main route of action, we generated isogenic *upp* and *udk* mutants of the MG1655 strain, unable to directly convert cytosine, uracil, and uridine to UMP (Fig. S1). At first, we evaluated the effect of gene inactivation on the sensitivity to the three compounds. Inactivation of the *udk* gene did not affect the cytotoxicity of any tested compounds, suggesting that this gene is not involved in the cytotoxic effect (Fig. S2A). Similarly, the inactivation of neither *udk* nor *upp* gene could prevent 5-FUr toxicity, consistent with the notion that 5-FUr can be converted to 5-FUMP by both Udk and Upp enzymes (Fig. S1). In contrast, the inactivation of the *upp* gene led to an 8-fold increase in 5-FU IC_{50} and to the complete abrogation of the cytotoxic effects of 5-FC at all tested concentrations (Fig. S2A). The finding that inactivation of *upp* did not fully abrogate 5-FU cytotoxicity indicates that the toxic effect of this metabolite might be due to additional mechanisms that are *upp*-independent. The conversion of 5-FU to 5-fluoro-2′-deoxyuridine (FdUr) and then to FdUTP (Fig. S1), which inhibits thymidylate synthase activity causing damage to DNA, could be one of such mechanisms [36].

Based on these results, we evaluated whether the inactivation of *upp* and *udk* could interfere with the inhibitory effect on adhesion and curli expression. Inactivation of *udk* did not alleviate biofilm inhibition by the three compounds (Fig. 2A). In contrast, deletion of the *upp* gene completely restored bacterial adhesion (Fig. 2A) and red phenotypes on YESCA/CR plates (Fig. 2B) even in the presence of 5-FU and 5-FC. We also observed a substantial reduction in the red colour of the MG1655Δ*upp* strain when treated with 5-FUr, however, accompanied

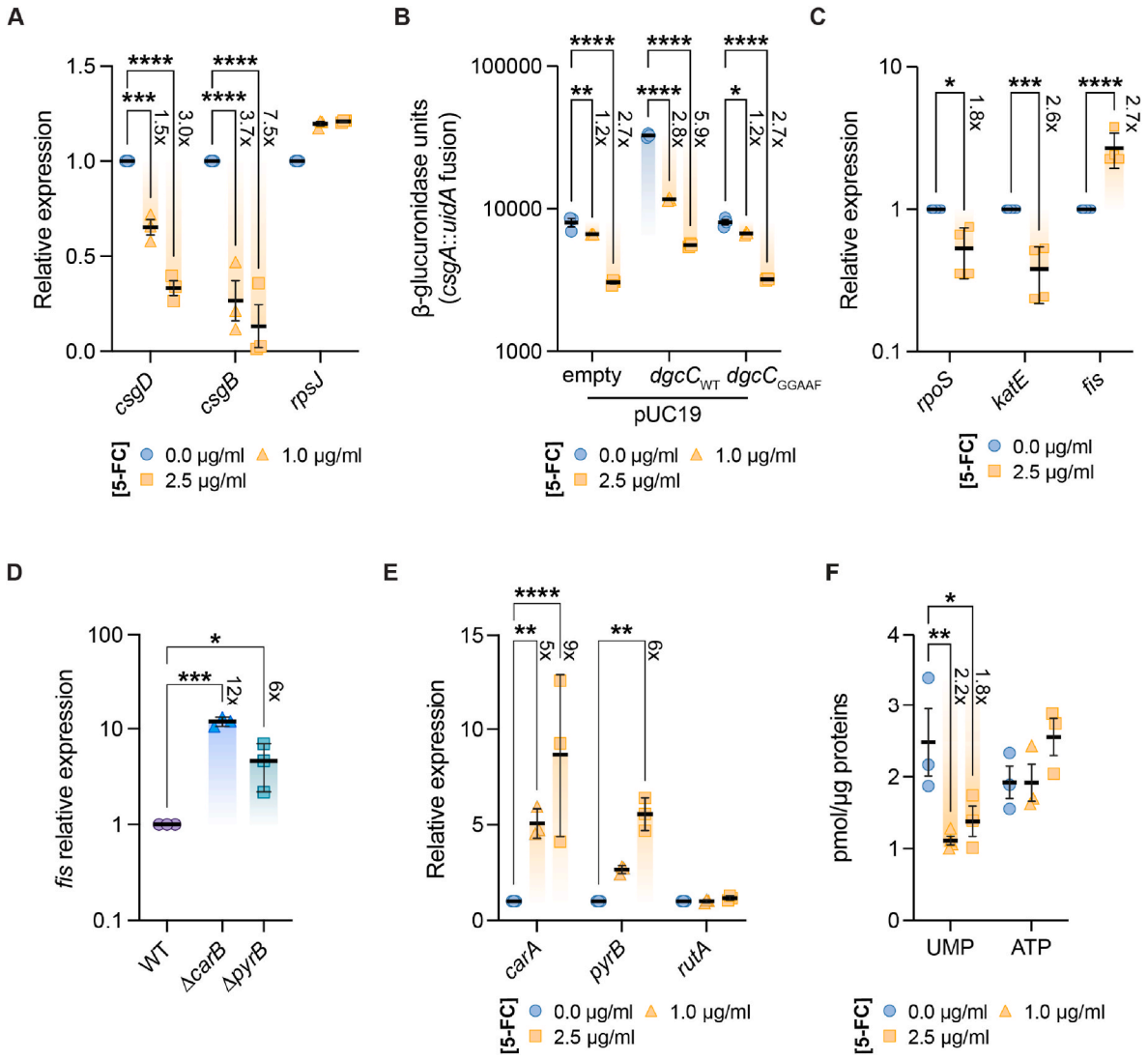

Fig. 3. Effects of 5-FC on curli regulators and pyrimidine nucleotide pool. **(A)** Relative expression of the *csgD, csgB,* and *rpsJ* genes determined by RT-qPCR analysis on RNA extracted from the MG1655 strain in the presence or absence of 5-fluorocytosine (5-FC). Values are expressed as arbitrary units, with the untreated control set to 1. **(B)** β-glucuronidase activity of the *csgA::uidA* transcriptional fusion in the MG1655 strain carrying the empty vector pUC19, or plasmids harboring the wild-type (pUC19-$dgcC_{WT}$) or the catalytically inactive (pUC19-$dgcC_{GGAAF}$) alleles of the *dgcC* gene in absence or presence of 5-FC. **(C)** Relative expression of the *rpoS*, *katE* and *fis* genes determined by RT-qPCR analysis in the MG1655 strain in the presence or absence of 5-FC. Values are expressed as arbitrary units, with the untreated control set to 1. **(D)** Relative expression determined by qRT-PCR of the *fis* gene in wild-type strain MG1655, and in the MG1655Δ*carB* and MG1655Δ*pyrB* mutant strains. Values are expressed as arbitrary units, with levels in the WT set to 1. **(E)** Relative expression determined by qRT-PCR of the *carA, pyrB* and *rutA* genesin the wild-type strain MG1655 in the presence or absence of (5-FC). Values are expressed as arbitrary units, with the untreated control set to 1. **(F)** Determination of intracellular levels of UMP and ATP in wild-type strain MG1655 in presence or absence of 5-FC by HPLC analysis. Values are expressed as pmol/μg protein. Results of 3–4 independent replicates and medians are shown. *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001; ****, p-value <0.0001, two-way ANOVA with Dunnett's test for multiple comparisons.

by a reduction of colony size and thickness. This result indicates a more substantial inhibitory effect of growth on solid media, again suggesting a broader toxic effect of this compound than 5-FC or 5-FU.

Consistent with the results of adhesion assays and CR phenotype, all compounds failed to substantially inhibit *csgA* gene expression in the MG1655Δ*upp* strain (Fig. 2C), with 5-FUr even leading to an up to 2.5-fold increase.

Overall, these results show that inhibition of curli fibres expression and cell adhesion requires the conversion of fluoropyrimidines to 5-FUMP.

3.3. 5-Fluorocytosine alters the expression of global regulators via perturbation of the de novo pyrimidine biosynthetic pathway

Conversion of fluoropyrimidines into fluorinated nucleotides is necessary to inhibit curli gene expression through repression of the *csgBAC* operon. This indicates that all pyrimidine analogues tested may act through a similar mechanism. To characterize their mechanism of curli inhibition, we focused on 5-fluorocytosine (5-FC), which showed the lowest antimicrobial activity in *E. coli* (Fig. S2), and it is also the most used fluoropyrimidine in clinical therapy due to its low toxicity against human cells [37].

The main driver of *csgBAC* operon expression is the transcriptional regulator CsgD, which is transcribed from the second curli-encoding operon *csgDEFG* [20,38]. At first, we evaluated whether 5-FC affected only the *csgBAC* operon expression, or its activity was directed towards the expression of the *csgD* gene. We measured the expression of *csgB* and *csgD* genes in bacterial cultures treated with 5-FC. As a control, we analysed the *rpsJ* genes, which codes for the small ribosomal subunit protein S10. Expression of *rpsJ* was unaffected, but both *csgB* and *csgD* genes were significantly repressed in a concentration-dependent manner between 1.5 and 7 times (Fig. 3A). These results indicate that 5-FC affect curli expression through the CsgD regulator and that the effect is not due to a general disruption of transcription.

Previous studies by Attila and colleagues indicated that the inhibitory effect on biofilm formation of 5-FU, the antimetabolite to which 5-FC is converted, depends on the induction of the *ariR* (*ymgB*) gene [13], potentially through the RcsC/RcsD/RcsB two-component phosphorelay system that represses CsgD expression via the RprA sRNA [39–41]. However, independent inactivation of *ymgB*, *rcsB, rcsC, rcsD,* and *rprA* genes was not able to counteract the 5-FC mediated inhibition of biofilm formation (Fig. S3), suggesting that 5-FC activity on curli expression occurs independently of the *ymg-rcs-rprA* regulatory circuit.

The main regulatory pathways controlling the *csgD* expression are the second messenger *c*-di-GMP and the alternative sigma factor RpoS. High levels of *c*-di-GMP lead to an increase in *csgD* transcription, while low levels lead to the opposite effect [42], a condition that might be promoted by 5-FC treatment. Therefore, we evaluated the effect of artificially increasing *c*-di-GMP via overexpression of the diguanylate cyclase DgcC (AdrA) in combination with 5-FC treatment on the expression of the *csgA* gene. As a control, we expressed a variant of DgcC with an inactive catalytic domain ($DgcC_{GAAF}$). The wild-type DgcC allele (pUC19/$dgcC_{WT}$) led to a 4-fold increase in *csgA* levels, in agreement with a previous report [12]. The effect relies on *c*-di-GMP biosynthesis, as it is abrogated in the strain harbouring the catalytically inactive variant of DgcC (pUC19/$dgcC_{GAAF}$) (Fig. 3B). Treatment with 5-FC inhibited *csgA* expression regardless of the presence of the $dgcC_{WT}$ allele (Fig. 3B), leading to expression levels comparable to the control. We obtained the same results with the ectopic expression of the WpsR diguanylate cyclase of *Pseudomonas aeruginosa* (Fig. S4), suggesting that 5-FC affects curli independent of *c*-di-GMP.

Then, we evaluated the potential impact of 5-FC on RpoS and its regulon, assessing the expression of the *rpoS* gene and the RpoS-dependent gene *katE*. We included in our analysis another global regulator, the Fis protein, which also negatively controls *csgD* and *katE* expression [22]. The *rpoS* gene showed a slightly significant reduction in transcript levels (1.8-fold) upon treatment with 2.5 μg/ml 5-FC (Fig. 3C), suggesting that decreased RpoS levels might partially account for the inhibition of curli operons expression [26]. In agreement, transcription of the *katE* gene was reduced by 2.6-fold (Fig. 3C). In contrast, *fis* was upregulated 2.7 times in response to 5-FC (Fig. 3C). Fis acts as a negative regulator of *csg* and *katE* genes, and its inactivation results in induction of their expression (Fig. S5A). However, the deletion of the *fis* gene did not reverse the effect of 5-FC on adhesion (Fig. S5B), *csgA* expression (Fig. S5C), and YESCA/CR plates (Fig. S5D).

Interestingly, the *fis* gene responds to intracellular GTP/CTP ratio and is activated in mutants unable to carry out *de novo* pyrimidine biosynthesis [43]. We wanted to confirm these findings in our experimental conditions and could indeed measure a 6 to 12-fold induction of *fis* transcription in isogenic *carB* and *pyrB* mutants of MG1655, respectively (Fig. 3D). Induction of *fis* in *carB* and *pyrB* mutants pointed to a possible inhibition of *de novo* pyrimidine biosynthesis by the fluoropyrimidines. Indeed, exposure to 2.5 μg/ml 5-FC activated transcription of the *carB* and *pyrB* genes, encoding the enzymes responsible for the first two steps in the *de novo* pyrimidine biosynthesis by up to 10-fold (Fig. 3E). Conversely, the expression of the *rutA* gene, which is involved in uracil utilization as a nitrogen source, was not affected (Fig. 3E). Finally, in *E. coli* cultures treated with or without 5-FC, we measured the intracellular concentration of uridine monophosphate (UMP), *i.e.,* the main product of pyrimidine *de novo* and salvage pathways. We also quantified total ATP to evaluate whether exposure to 5-FC could also have a broader effect on nucleotide biosynthesis, altering the cell's energy state. While ATP levels were not significantly affected by 5-FC treatment (Fig. 3F), UMP levels decreased 1.8–2.5 times. This indicates that the nucleobase analogue at the tested concentrations specifically affects UMP levels rather than disrupting the cell physiology and energy state.

3.4. Identification of genes counteracting 5-FC dependent curli inhibition

Our results suggest that 5-FC act on curli fibres transcription by interfering with the pyrimidine nucleotide pool, potentially affecting the expression of at least two global regulators, i.e. RpoS and Fis. However, reduction of intracellular UMP levels by 5-FC seems to affect curli gene expression via additional regulators (Fig. S5). To identify additional determinants involved in curli inhibition, we generated *E. coli* MG1655 genomic library by cloning random genomic DNA fragments sized 500–2000 bp into the pUC19 vector. Clones expressing the genomic library were screened on YESCA/CR medium supplemented with 1.0 μg/ml 5-FC. Out of the 25,000 colonies screened, we identified 7 clones showing a red phenotype on YESCA/CR medium supplemented with 5-FC. All clones were subsequently tested to verify that the phenotypic reversion was preserved at higher 5-FC concentrations. A single clone maintained the red phenotype when grown on YESCA/CR supplemented with 2.5 μg/ml 5-FC (Fig. 4A, clone C1). Sequencing of the plasmid identified five coding sequences grouped into two distinct fragments ligated together (Table S3): a fragment contained the sequences coding for the initial 179 amino acids of the penicillin-binding protein 1b (PBP1b/MrcB), the terminal domain of the *hrpB* gene, *i.e.* the ATP-dependent RNA helicase HrpB, and the complete coding sequence of a protein of unknown function (D8B36_18,480); the second fragment contained the beginning and the end of the *mukE* and *mukF* genes, respectively. To verify which sequence was responsible for counteracting the effect of 5-FC treatment, we tested the ability of each encoded fragment, and of their corresponding full-length genes, cloned into the pUC19 vector, to reverse the 5-FC effect on YESCA/CR medium (Fig. 4A). Only the strain expressing the fragment coding for the first 179 amino acids of the penicillin-binding protein 1b showed a consistent red phenotype when treated with 5-FC (Fig. 4A; $mrcB_{short}$).

Interestingly, we also observed that the expression of the D8B36_18,480 coding sequence led to a white phenotype even in the untreated condition (Fig. 4A), indicating a potential role of this protein

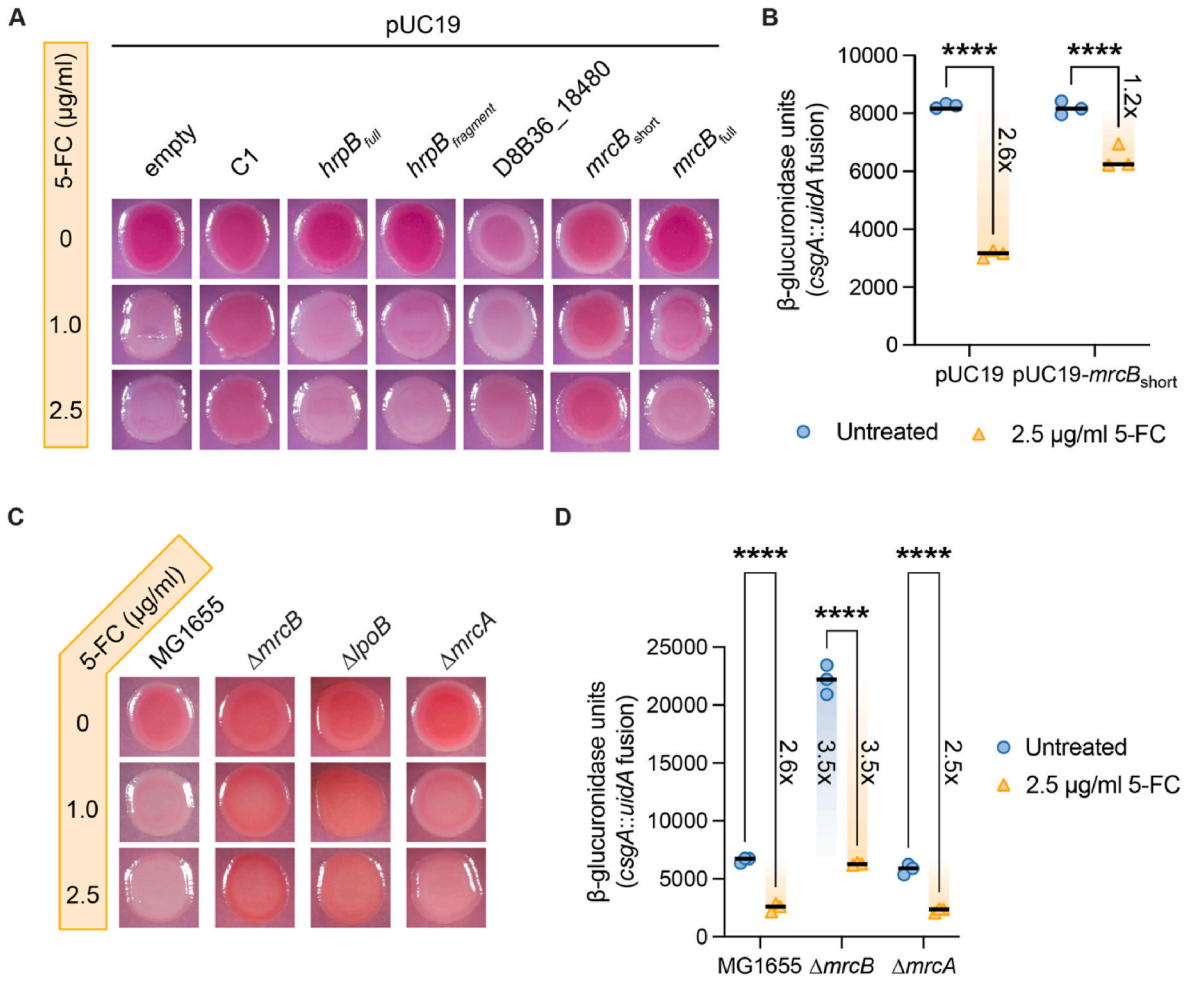

Fig. 4. Genomic library screening and analysis of genetic determinants counteracting 5-FC inhibitory effects. **(A)** Colony phenotypes on YESCA/CR plates of the MG1655 strain carrying the empty vector pUC19, pUC19 from clone C1, pUC19 harboring the fragments or full-length coding sequences of the *hrpB*, D8B36_18,480 and *mrcB* genes, in the absence or presence of 5-FC. **(B)** β-glucuronidase activity of the *csgA::uidA* transcriptional fusion in the MG1655 strain carrying the pUC19 or pUC19-$mrcB_{short}$ vectors in the absence or presence of 2.5 μg/ml 5-FC. **(C)** Colony phenotypes on YESCA/CR plates of wild-type MG1655, MG1655Δ*mrcB*, MG1655Δ*lpoB,* and MG1655Δ*mrcA* strains in the absence or presence of 5-FC. **(D)** β-glucuronidase activity of the *csgA::uidA* transcriptional fusion in wild-type MG1655, MG1655Δ*mrcB,* and MG1655Δ*mrcA* strains in the absence or presence of 2.5 μg/ml 5-FC. Representative images or results of three independent replicates and medians are shown. ****, p-value <0.0001, two-way ANOVA with Dunnett's test for multiple comparisons.

in inhibiting curli fibres production. In agreement with the phenotypes observed on YESCA/CR, ectopic expression of the $mrcB_{short}$ resulted in almost complete abrogation of the effect of 5-FC on *csgA* expression (Fig. 4B). Likewise, D8B36_18,480 expression resulted in an 18-fold reduction in *csgA* expression in the untreated conditions (Fig. S6).

As $mrcB_{short}$ was the only genetic sequence able to counteract the effect of 5-FC, we further analysed its role. The $mrcB_{short}$ fragment coded for the amino acids 1–179 of the penicillin-binding protein 1b (PBP1b), encompassing a short cytoplasmic NH2 domain (residues 1–63), a single hydrophobic transmembrane segment (residues 64–87) and part of the UB2H (UvrB domain 2 homolog) domain which interacts with the outer membrane protein LpoB. The truncated protein lacks the transglycosylase and transpeptidase catalytic domains. LpoB interaction with PBP1b via the UB2H domain is essential for stimulating PBP1b activity [44]. We hypothesised that the truncated MrcB protein could interfere with the interaction between MrcB and LpoB, titrating LpoB and resulting in an inactive MrcB protein. Thus, we created isogenic mutants lacking either *mrcB* or *lpoB* genes to mimic the same condition genetically. The MG1655Δ*mrcB* and MG1655Δ*lpoB* strains showed a red phenotype on YESCA/CR even when treated with 5-FC at 2.5 μg/ml (Fig. 4C), suggesting that inactivation of *mrcB* or inhibiting its activity through the inactivation of the *lpoB* gene can counteract the effect of 5-FC on curli production. While *mrcB* is mainly implicated in cell division, *E. coli* has a second bifunctional peptidoglycan synthase, which exerts its activity mostly during cell elongation and is encoded by the *mrcA* gene [44,45]. To verify whether the effect of *mrcB* inactivation on 5-FC repression of curli was specific or due to a general defect in peptidoglycan biosynthesis, we created a knockout mutant in the *mrcA* gene (Fig. 4C). The MG1655Δ*mrcA* mutant showed a wild-type phenotype on YESCA/CR when treated with 5-FC (Fig. 4C), suggesting a specific effect of the PBP1b protein.

However, when we tested the effect of the *mrcB* and *mrcA* deletion on *csgA* expression upon 5-FC treatment, we observed that, while the Δ*mrcA* mutants showed expression levels like the wild-type (Fig. 4D), deletion of the *mrcB* gene resulted in a 3.5-fold increase in *csgA* expression in the untreated condition itself (Fig. 4D).

Nevertheless, 5-FC treatment reduced curli expression in the MG1655Δ*mrcB* to a level similar to the parental strain in untreated conditions. Interestingly, while the inactivation of *mrcB* stimulates curli expression, the lack of PBP1b protein completely abrogated MG1655's ability to adhere (Fig. S7A), likely due to multiple effects of the *mrcB* deletion on other cell surface determinants [46,47]. The same phenomenon could be observed when the short fragment of *mrcB* was expressed from the pUC19 plasmid (Fig. S7B), suggesting that the

ectopic expression of the $mrcB_{short}$ fragment effectively mimics the inactivation of the *mrcB* gene.

We investigated whether increased *csgA* expression in the MG1655Δ*mrcB* strain might depend on *c*-di-GMP, by overexpressing the *c*-di-GMP phosphodiesterase PdeH. PdeH expression decreases *csgA* expression by 13-fold both in the WT and the *mrcB* mutant, thus confirming dependence of curli gene expression on *c*-di-GMP even in the *mrcB* strain background. However, inactivation of PBP1b resulted in a 3.4-fold increase in *csgA* levels in both the WT and Δ*mrcB* backgrounds, regardless of the ectopic expression of PdeH (Fig. S8A). Although the 3.4-fold difference was not statistically significant in the *mrcB* strain, it seems to suggest that a *c*-di-GMP-independent pathway might also be involved.

Similarly, curli induction in a strain lacking PBP1b maintained its dependence on growth temperature: *csgA* stimulation in the MG1655Δ*mrcB* strain could be observed only at 30 °C, but not at 37 °C (Fig. S8B).

Identifying a link between curli and peptidoglycan biosynthesis led us to hypothesize that 5-FC can act by interfering with cell wall synthesis. Indeed, 5-FU, the precursor of 5-FC, is known to disrupt peptidoglycan production [48,49]. We grew the MG1655 strain under different osmotic conditions with or without 2.5 μg/ml 5-FC to verify this hypothesis. While at the highest salt concentration (500 mM), the growth of the strain was slightly affected, the addition of 5-FC did not lead to an additional defect in growth (Fig. S9A) and cell viability (Fig. S9B), excluding any significant defect in cell wall biosynthesis.

Overall, our results suggest that although the activity of PBP1b is linked explicitly with curli expression, the reversion of 5-FC inhibition observed in the MG1655Δ*mrcB* strain, and thus in the strain overexpressing the $mrcB_{short}$ fragment, was most likely due to an increase in *csg* operon expression rather than the increased expression of a direct target of 5-FC.

4. Discussion

Targeting biofilm formation and its microbial determinants as alternatives to antibiotics has been proposed as a possible strategy for developing new antimicrobial agents. Biofilm confers a higher tolerance towards antimicrobials [50] and protects bacteria from the immune system [51]. Antibiofilm and antivirulence strategies acquire even more relevance in the context of the spreading of antibiotic resistance and the lack of development of new antimicrobials. To this aim, researchers have primarily focused on drug repurposing, *i.e.,* screening the old drugs for those with antimicrobial or antivirulence activities. Antimetabolite drugs and nitrogen base analogues can inhibit virulence traits in several Gram-positive and Gram-negative bacteria, as well as in fungi, at concentrations sub-inhibitory for growth [8,9,11–13,52–54]. Pyrimidine nucleosides and nitrogen base analogues, namely 5-fluorouracil (5-FU), 5-fluorocytosine (5-FC), and 5-fluorouridine (5-FUr) were shown to inhibit a variety of virulence factors and impair biofilm formation in different pathogenic microorganisms [9,13,36]. However, whether a single mechanism mediates these effects is yet to be made clear.

In this work, we showed that 5-FU, 5-FUr, and 5-FC affect biofilm formation via the reduction of transcription of curli fibres encoding operon *csgBAC*, thus blocking the production of *E. coli* main adhesion factor (Fig. 1). Inhibition takes place at the level of *csgD* transcription (Fig. 3A) and requires fluoropyrimidines conversion to fluoronucleotides as inactivation of the *upp* gene blocks the conversion of 5-FU or 5-FC to FUMP interfering with the inhibitory effects of the drugs. The need for metabolic activation of 5-FC is consistent with previous observation [9] and points to a possible common mechanism in different bacteria.

In the search for a mechanism of action behind fluoropyrimidines antibiofilm activity we focused on 5-FC, an antimycotic well tolerated in humans due to the lack of cytosine deaminase [55], which prevents its conversion to the metabolically active and toxic 5-FU [56].

A potential mechanism was previously suggested by Attila and co-workers [13]. Biofilm repression upon 5-FU treatment seems to depend on the overexpression of the YmgB/AriR gene. YmgB modulates the Rcs signalling system [40], which inhibits curli expression via the RprA small RNA in response to envelope and peptidoglycan stress [39,57]. In our conditions, neither AriR nor any of the genes belonging to the Rcs signalling pathway seem to be implicated in the repression of curli fibres upon 5-FC treatment (Fig. S3). This discrepancy is likely due to the different growth conditions: Attila *et al.* performed their adhesion assay at high temperature and rich medium, where curli are not expressed, and other adhesion factors mediate biofilm formation.

We tested several other regulators of curli gene expressions. 5-FC inhibition is independent of the global levels of the second messenger *c*-di-GMP. Indeed, 5-FC could counteract the stimulation of curli expression induced by the expression of the DgcC and WspR diguanylate cyclases. This also suggests that the mechanism behind 5-FC is different from what was suggested for compounds that act on purine biosynthesis [11].

However, 5-FC affected other global regulators: the alternative sigma factor *rpoS* was slightly repressed upon treatment, and the *fis* gene, a negative regulator of *csgD* expression [22], was expressed at higher levels in response to 5-FC (Fig. 3). The repression of the *csgD* gene by Fis is not the primary mechanism behind curli inhibition. Despite *csgA* gene induction in a Δ*fis* mutant, inactivation of the regulator does not counteract 5-FC repression, pointing to different or multiple concurrent mechanisms acting on *csg* genes.

Interestingly, the *fis* transcription regulation responds to GTP/CTP intracellular ratio and is enhanced in conditions that reduce pyrimidine intracellular pools [43]. The transcription of the *fis* gene, in our experimental conditions, is strongly activated when *de novo* pyrimidine synthesis genes, such as *carB* and *pyrB*, are inactivated.

Pyrimidine nucleotide starvation is known to control curli gene expression [10], and knockout mutants in genes belonging to the *de novo* pyrimidine biosynthesis pathway are curli deficient. Thus, we hypothesised that 5-FC could inhibit the curli gene expression via a similar mechanism, inducing a response connected with a specific reduction in pyrimidine nucleotide availability. Upon 5-FC treatment, we observed a strong induction of the *carAB* and *pyrBI* operons, whose expression is activated when intracellular concentrations of uracil and UTP are reduced [58]. Direct measurement of UMP intracellular pools, *i.e.*, the final product of the *de novo* pyrimidine biosynthetic pathway, showed a 2-fold reduction in the presence of 2.5 μg/ml 5-FC. The effect appears specific for pyrimidine biosynthesis, as ATP intracellular levels were unaffected (Fig. 3E), in agreement with the lack of a visible reduction in growth rate at this 5-FC concentration (Fig. S2).

Our data suggest that pyrimidine starvation's effect on CsgD expression occurs at the transcriptional level. However, in other bacteria, such as *Pseudomonas fluorescens*, pyrimidine limitation triggers an increase in ribosome biosynthesis and the production of the capsular polysaccharide colanic acid production due to competition between ribosomes and CsrA/RsmA proteins for the mRNA transcript of an activator of the polysaccharide synthesis [59].

Despite possible different mechanisms, the link between *de novo* pyrimidine biosynthesis and virulence factor production, and even with the response of innate immune response to pathogenic bacteria, is well established [8,10,14,60,61]. 5-FC, as well as the other fluoropyrimidines, might inhibit *de novo* pyrimidine biosynthesis upon conversion to a fluoronucleotide and acting as the allosteric inhibitor of CarB. This might take place by binding the allosteric domain of this enzyme with increased affinity than UMP, its natural effector, or by interfering with the complex attenuation mechanisms depending on UTP concentrations controlling several promoters of the *de novo* pyrimidine biosynthetic pathway [58].

Pyrimidine starvation induced by exposure to 5-FC might be relayed to biofilm formation and virulence factor production in a variety of different ways: direct interaction of regulatory proteins with pyrimidine nucleotides; incorporation into different RNA molecules possibly

altering their structure; or becoming part of fluorinated nucleotide-sugar complexes, mimicking the UDP-sugars precursors needed in cell wall and extracellular polysaccharide synthesis [36].

As an attempt to identify which of these mechanisms might relay inhibition of *de novo* pyrimidine biosynthesis to curli expression, we tested a genomic library looking for genes able to revert *csgA* transcription downregulation by 5-FC (Fig. 4). We found that a DNA fragment encoding the *N*-terminal domain of penicillin-binding protein 1b (PBP1b), the product of the *mrcB* gene, and another unknown protein, D8B36_18,480, could revert 5-FC inhibition of *csgA* transcription (Fig. 4). We observed that the ability to counteract the inhibitory effect might mainly depend on the induction of curli expression in the MG1655Δ*mrcB* strain rather than the expression of the specific target of 5-FC. Nevertheless, *mrcB* activity seems connected with the modulation of curli expression: the inactivation of either *mrcB* gene or the PBP1b activator LpoB stimulated the expression of the *csgA* gene (Fig. 4). The mechanism cannot overcome the control of the *csg* operons by both temperature and *c*-di-GMP. Yet, a slight induction of curli fibres is maintained in conditions of low *c*-di-GMP, indicating at least a partial independence from this second messenger, reiterating the complexity of curli gene regulation (Fig. S8). The effect is specific to the PBP1b protein as inactivation of the other bifunctional peptidoglycan synthase PBP1a has no significant effect on adhesion and curli production (Fig. 4).

Interestingly, although the inactivation of *mrcB* and *lpoB* stimulate *csgA* expression and results in a red phenotype on YESCA/CR, the strain cannot adhere to polystyrene plates, in agreement with previous reports [46,47]. How PBP1b relates to curli expression and why curli expression is not connected with adhesion is unknown. Still, it might be due to the induction of stress response caused by dysfunctional peptidoglycan biosynthesis in a specific growth condition. Although partially redundant, PBP1a and PBP1b activities are associated with cell elongation and cell division, respectively [44]. Lack of one of the two could be linked to a specific stress signal, which results in the stimulation of curli expression. However, the bacterium loses its ability to adhere to solid surfaces due to surface defects, as observed in mutants affected in LPS production [62], possibly due to more global effects on other cell surface determinants or the correct assembly of curli fibres at the outer-membrane [63].

From the same screening, we identified the gene D8B36_18,480 as an additional effector of curli expression. D8B36_18,480 encodes for a phylogenetically conserved small protein (68 amino acids) of unknown function. Its ectopic expression inhibits adhesion and curli gene expression (Fig. 4), independent of 5-FC treatment. No further information about D8B36_18,480 are available, but the gene localises in the genetic locus between *mrcB* and *hrpB* genes, potentially indicating an involvement of the protein in either peptidoglycan biosynthesis or RNA metabolism, two mechanisms associated with curli production and biofilm formation. Even if neither protein seems to be a target for 5-FC, the results of these experiments might shed additional light on how this molecule might impair curli gene expression. Our data suggest that 5-FC does not cause significant defects in the peptidoglycan. However, the observation that the ectopic expression of the *N*-terminal domain of the PBP1b protein can revert 5-FC downregulation of curli gene expression, would point to cell wall/envelope stress as the relay between perturbation of pyrimidine biosynthesis and inhibition of biofilm or virulence factors. In this respect, although through different mechanisms, our results would confirm the implication of envelope stress in 5-FU biofilm inhibition previously proposed by Attila and colleagues [13].

The effect of alteration in the pyrimidine pool on virulence factors goes beyond curli modulation. The expression of type I fimbriae in adherent-invasive *Escherichia coli* (AIEC) is reduced in mutants unable to synthesize UMP [14]. However, we can't determine whether 5-FC and biofilm inhibition are beneficial treatments for different *E. coli* pathotypes and strains. Strong curli-mediated adhesion to epithelial cells contributed to high incidence and mortality during the outbreak of Shiga toxin (Stx)-producing *Escherichia coli* O104:H4 in 2011 in Germany [17]. On the contrary, isolates of the epidemic strain O157:H7 are characterized by reduced curli production due to fine-tuning of gene expression [64] and genetic alteration in the *rpoS* and *mlrA* genes [18]. Loss of curli fibers seems to be an adaptation which foster the pathogen's success in the host and its dispersion in the environment. This observation clearly warns for tailored investigations of 5-FC effects in different *E. coli* pathotypes.

5. Conclusion

The finding that inhibition of *de novo* pyrimidine biosynthesis by 5-FC reiterates the importance of this pathway in presiding to virulence mechanisms in different bacteria and suggests that the enzymes involved in such pathway might be very interesting targets for novel antimicrobial agents targeting bacterial virulence.

CRediT authorship contribution statement

Srikanth Ravishankar: Writing – review & editing, Visualization, Investigation, Formal analysis. **Valerio Baldelli:** Investigation. **Carlo Angeletti:** Methodology, Investigation, Formal analysis. **Nadia Raffaelli:** Writing – review & editing, Supervision, Resources, Methodology. **Paolo Landini:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. **Elio Rossi:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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