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Transcriptional control of RfaH on polysialic and colanic acid synthesis by *Escherichia coli* K92

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ABSTRACT

The transcriptional antiterminator RfaH promotes transcription of long operons encoding surface cell components important for the virulence of *Escherichia coli* pathogens. In this paper, we show that RfaH enhanced *kps* expression for the synthesis of group 2 polysialic acid capsule in *E. coli* K92. In addition, we demonstrate for the first time that RfaH promotes *cps* expression for the synthesis of colanic acid, a cell wall component with apparently no role on pathogenicity. Finally, we show a novel RfaH requirement for growth at low temperatures.

1. Introduction

Bacterial capsules are structures surrounding the cell surface which exhibit extraordinary diversity and confer important advantages upon micro-organisms. Nearly eighty capsular polysaccharides have been described in *Escherichia coli* alone [1]. *E. coli* K92 synthesizes two different capsular polysaccharides in a temperature-dependent manner [2]. When inside the host (37 °C), this bacterial strain synthesizes a Group 2 capsule known as polysialic acid (PA), a polysaccharide responsible for bacterial virulence [3,4]. The chromosomal loci responsible for PA biosynthesis is designated *kps* operon (Fig. 1A), has a conserved organization consisting of three regions [5] and its transcription is driven by two convergent temperature-regulated promoters located upstream of regions 1 and 3 [6,7]. Transcription in region 1 is driven by the region 1 promoter, whereas regions 2 and 3 are organized into one transcriptional unit under the control of the region 3 promoter. Unlike what happens in region 1, efficient transcription in regions 2 and 3 requires RfaH [8]. This acts as a transcriptional anti-terminator for large operons and its loss promotes transcriptional polarity without affecting initiation from the promoters [8]. RfaH-dependent operons share a short element termed *ops* (operon polarity suppressor) that is essential for rfaH function [8–10]. The *ops* sequence recruits RfaH and other factors to the RNA polymerase complex, increasing its processivity by reducing pausing and termination and allowing transcription to proceed over long distances [11].

The PA capsule in *E. coli* K92 is co-expressed with colanic acid (CA), an exopolysaccharide predominantly synthesized at low temperatures (20 °C), which provides protection against stressful conditions outside the mammalian host [12]. However, CA does not play a directly role in pathogenesis [13].

The *cps* CA operon (Fig. 1B) comprises one large transcription unit encoding proteins involved in colanic acid biosynthesis [14]. In addition, the *ugd* gene is located outside the *cps* CA operon but is also involved in colanic acid synthesis [5,14].

Previous findings suggest that the expression of the *cps* CA operon is mediated by RfaH [8]. Thus, this transcriptional anti-terminator is often required for the expression of long operons encoding bacterial capsules [8]. Furthermore, the *cps* CA operon in *E. coli* K-12 is preceded by *ops* elements [15]. RfaH also modulates the expression of *cps* encoding Group 1 K30 capsule with genetic organization and gene content is similar to *cps* CA [16]. However, despite the fact that the expressions of both *cps* K30 and *cps* CA operons share many regulatory characteristics, their regulatory mechanisms are quite different. Most notable are the facts that

Abbreviations: CA, colanic acid; Glc-Pro, glucose-proline; MM, minimal media; PA, polysialic acid; Xyl-Asn, xylose-asparagine

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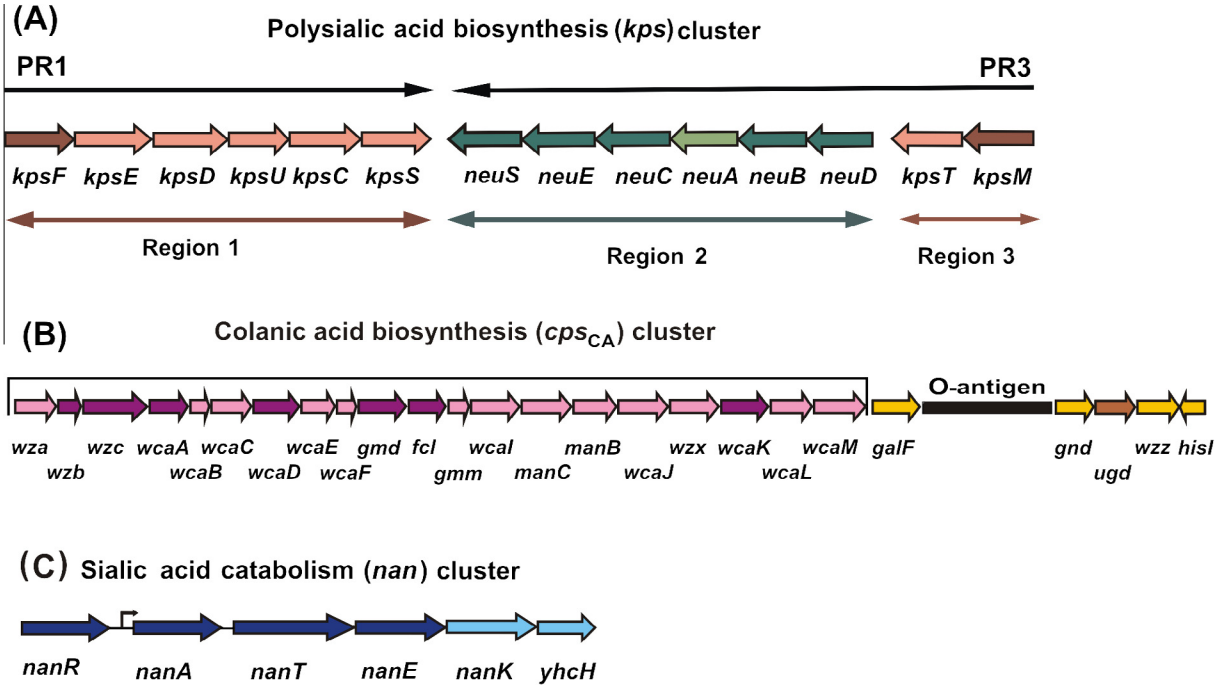

Fig. 1. Genetic organization of *E. coli* polysialic acid and colanic acid metabolism clusters: (A) Polysialic acid synthesis (*kps*), (B) colanic acid synthesis (*cps* CA) and (C) polysialic acid catabolism (*nan*). Dark arrows indicates the genes used in this study. PR1 and PR3: promoters located upstream of regions 1 and 3 in the *kps* cluster.

the virulent K30 antigen is expressed at 37 °C, whereas CA is mainly synthesized at low temperatures and that the Rcs phosphorelay controls *cps* CA gene expression [17] in contrast to *cps* K30 expression [16].

This report investigates the function of RfaH in *E. coli* K92 in controlling *kps* and *cps* gene expression for PA and CA biosynthesis, respectively, an RfaH-deficient mutant having been used for this purpose.

2. Materials and methods

2.1. Strains, culture media, and growth conditions

The strains and plasmids used in this study are shown in Table 1. Bacterial cultures were grown in Luria–Bertani (LB) complex medium, LA (LB supplemented with 2% w/v agar) and Xylose-Asparagine (Xyl-Asn) or Glucose-Proline (Glc-Pro) minimal media (MM) for *E. coli* K92. We chose Xyl-Asn or Glc-Pro MM because they induce maximal PA [19] and CA production in *E. coli* K92, respectively [18]. When indicated, Glc-Pro MM was supplemented with agar 2% (w/v). During the allelic exchange experiments, LA medium (LB supplemented with 2% (w/w) agar), with the addition of with 5% (wt/vol) sucrose, and without NaCl, was used to select plasmid excision from the chromosome [20]. When required, the following supplements were added to the culture media: rifampicin (25 and 10 μg/ml for liquid and solid media, respectively), ampicillin (100 μg/ml), and chloramphenicol (30 μg/ml).

2.2. DNA manipulations, RNA isolation and qRT-PCR

Routine molecular biology techniques, including the use of restriction enzymes, plasmid DNA and RNA isolation, mobilization of plasmids between *E. coli* strains, DNase treatment, reverse transcription and qPCR (qRT-PCR), were performed as previously

Table 1
Strains, plasmids and constructions used in this work.

	Description	Reference or source
E. coli strains		
DH5α′	F$^-$ *Δlac*U169 □80d*lac*Z1M15 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thy*-1 λ$^-$ *rel*A1 *sup*E44 *deo*R	[35]
S17λpir	λ*pir recA thi pro hsdR* M$^+$, RP4:2-Tc::Mu::km Tn7 Tp^r Sm^r	[36]
K92	Wild type	ATCC 35860
K92*ΔrfaH*	K92*ΔrfaH::cat*; constructed using pDS132-UD	This work
K92*ΔrfaHprfaH*	*E. coli* K92 harboring plasmid pMCrfaHxp	This work
Plasmids and constructions		
pGEM-TEasy	Ap^r *ori*ColE1 *lacZ*α$^+$ SP6 T7 *lac* promoter, direct cloning of PCR products	PROMEGA
pDS132	R6K ori mobRP4 cat sacB	[37]
pGEM-U	*rfaH* upstream sequences PCR amplified with primers rfaHup5′ and rfaHup3 cloned into pGEMT-easy; Ap^r	This work
pGEM-D	rfaH downstream sequences PCR amplified with primers rfaHdown5′ and rfaHdown3 cloned into pGEMT-easy; Ap^r	This work
pGEM-UD	*ΔrfaH*; *rfaH* upstream sequence from pGEM-U removed with EcoRI and ligated with *rfaH* downstream sequence from pGEM-D removed with EcoRI; Ap^r	This work
pDS132-YZ	ΔrfaH sequences from pGEM-UD removed with SacI and SphI and inserted into pDS132 digested with the same enzymes; Cat^r	This work
pGEM*rfaHxp*	*rfaH* sequences PCR amplified with primers rfaHxpup and rfaHxpdown cloned into pGEMT-easy; Ap^r	This work
pBBR1MCS-3	Broad host range-cloning vector, Tc^R	[21]
pMC*rfaHxp*	*E. coli* K92*ΔrfaH* cloned into pBBR1MCS-3	This work

Table 2
Primers used in this work.

	Name	Sequence (5′ → 3′)
rfaH deletion	rfaHup5′	CTCACGCCAAAGCCATCATCC
	rfaHup3′	GAAAGACATTGTCGATCCGGC
	rfaHdown5′	CAGGGTGATCATCGGTGCCAG
	rfaHdown3′	CTGCAAGAAGTCGCGTAAATCG
rfaH complementation	rfaHxpup	GTTAAACTAGTGAACTCTGACGG
	RfaHxpdown	GGATGCTAGAGCTCAAAACACTG

described [18]. For deletion experiments, PCR products were generated by using the *E. coli* K92 ATCC 35860 genome as a template, together with the primers described in Table 2. The relative gene expression levels were calculated as previously described [18]. The qPCR data represent the average change (n-fold) determined from two independent experiments containing three biological replicates each.

2.3. Deletion of the rfaH gene from E. coli K92

The 0.3-kb upstream and downstream sequences included in the *rfaH* locus were PCR amplified using primers rfaHup5′ and rfaHup3′, rfaHdown5′ and rfaHdown3′ (Table 2). These 0.3-kb amplicons were individually cloned into pGEM-T Easy, yielding plasmids pGEM-U and pGEM-D, respectively. The cloned sequences were excised using *Eco*RI. Downstream and upstream sequences were ligated and the product was amplified using primer pairs rfaHup5′ and rfaHdown3′, and cloned again into pGEM-T Easy yielding pGEM-UD. The *rfaH* upstream–downstream DNA sequence (*ΔrfaH*) was excised from pGEM-UD with *Sac*I and *Sph*I enzymes and cloned into pDS132 previously digested with the same enzymes, yielding plasmids pDS132-UD. The suicide vector carrying *ΔrfaH* was electroporated into *E. coli* S17-1*λpir* for biparental conjugation into *E. coli* K92. The deletions were recombined into the chromosome of *E. coli* K92 by using the standard two-step sucrose-resistance-assisted allelic exchange method previously described [20]. The correct allelic exchange of the wild-type allele for each mutant allele was confirmed by PCR using primers rfaHup5′ and rfaHdown3′. The *ΔrfaH E. coli* K92 mutant was named *E. coli* K92*ΔrfaH*.

2.4. Complementation experiment

For complementation studies we obtained a DNA fragment (620-kb) containing *rfaH* gene by PCR amplification using rfaHxpup and rfaHxpdown primers (see Table 1) and *E. coli* K92 chromosomal DNA as template. The *Spe*I and *Sac*I sites (underlined) were incorporated for future directional cloning. The PCR gene product was cloned into pGEM-T Easy vector to yield pGEM*rfaHxp*. The rfaH fragment was excised from pGEMrfaHxp with *Spe*I and *Sac*I and directionally cloned into pBBR1MCS-3 plasmid previously digested with the same enzymes [21] yielding pMC*rfaHxp*. pMC*rfaHxp* were transferred to *E. coli* K92*ΔrfaH* by biparental mating using *E. coli* S17-1*λpir* as donor strain to obtaining the complemented strain *E. coli* K92*ΔrfaHprfaH*.

2.5. Quantification of exopolysaccharides

Quantitative determination of PA [19] and CA [2] production by *E. coli* K92 cultures was carried out as previously described. Briefly, cell-free supernatants from bacterial cultures were dialyzed against 1000 vol of distilled water for 24 h at 4 °C. Dialyzed supernatant samples were used for quantitative determination of PA and CA using the resorcinol and orcinol colorimetric assays, respectively. Data represent the average of three replicates.

2.6. Statistical analysis

The results are presented as means ± S.E. Significant differences between means were calculated with Student's *T* test. *P* values of 0.05 or less were considered statistically significant.

3. Results

3.1. RfaH is required for growth of E. coli K92 at low temperatures

To study the role of RfaH in the expression of CA and PA capsular polysaccharides in *E. coli* K92, gene deletion experiments were performed so as to obtain an *E. coli* K92 *ΔrfaH* mutant strain lacking the *rfaH* gene. The loss of the DNA fragment was confirmed by PCR and the absence of rfaH expression was confirmed by qRT-PCR.

Parallel cultures of wild-type and *rfaH* mutant strains were established at 37 °C and 19 °C in both MM Xyl-Asn and Glc-Pro with aeration to ensure maximum PA [19] and CA production by the wild-type strain, respectively [2]. Deletion of *rfaH* did not significantly affect the growth of *E. coli* K92 at 37 °C in either medium (Fig. 2). In contrast, the *rfaH* mutant suffered a growth delay of approximately 80 h when growing in MM Xyl-Asn at 19 °C (Fig. 2A), and failed to grow in MM Glc-Pro (Fig. 2B).

3.2. RfaH regulates PA metabolism at a transcriptional level

RfaH acts as a transcriptional anti-terminator involved in the expression of *E. coli* group 2 capsule operon [22]. To test this role in PA synthesis by *E. coli* K92, wild-type and *rfaH* mutant strains were grown at 37 °C and polymer production was analysed after 120 h. As expected, deletion of *rfaH* completely suppressed PA production at 37 °C both in MM Xyl-Asn (Fig. 3A) and in MM Glc-Pro (Fig. 3B). The gene encoding the *rfaH* was PCR-amplified, cloned in

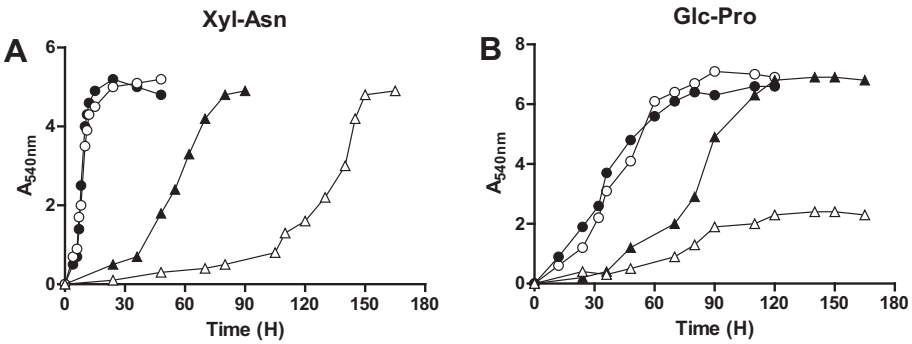

Fig. 2. Growth of *E. coli* K92 (black) and *E. coli* K92*ΔrfaH* (white) incubated in MM containing Xyl-Asn (A) or Glc-Pro (B) at 19 °C (triangles) or 37 °C (circles).

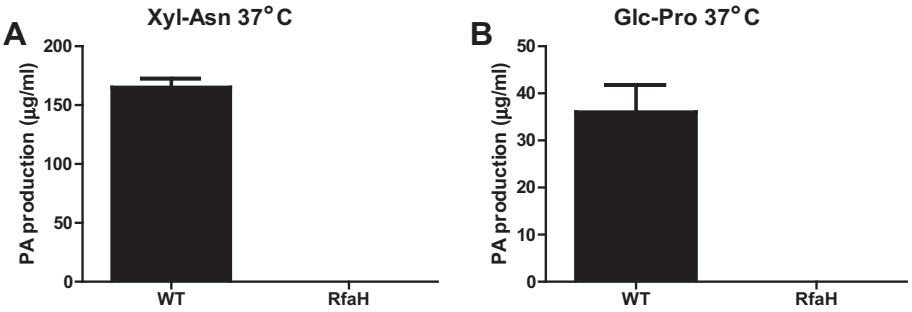

Fig. 3. PA production by *E. coli* K92 (wt) and *E. coli* K92*ΔrfaH* (mutant) grown in MM containing Xyl-Asn (A) or Glc-Pro (B) after 120 h at 37 °C.

pBBR1MCS-3 [21] and used to transform *E. coli* K92*ΔrfaH*. The recombinant strain was still able to produced PA when was cultured in MM Xyl-Asn at 37 °C (data not shown). The expression of several genes belonging to the *kps* operon in both strains growing in MM Xyl-Asn at 37 °C was also analysed by qRT-PCR. Despite the differences in bacterial growth (see Fig. 2), RNA samples were harvested from the mid-exponential phase (around thirty generations after inoculation). Hence, the expression of the *kspF* gene belonging to region 1 was not affected in the *rfaH* mutant (Table 3). Deletion of *rfaH* resulted in a diminished expression of all the genes tested that belonged to *kps* regions 2 and 3. Thus, in region 2, *neuD* expression was reduced sixteen-fold, *neuB* and *neuC* expressions were reduced between seventy- and eighty-fold, and *neuE* and *neuS* expressions diminished up to around three hundred-fold. Finally, the expression of the *kpsM* gene belonging to region 3 was reduced ten-fold.

The expression of several genes involved in PA catabolism was also analysed. The results showed that the expression of *nanATE* genes was greatly reduced (Table 3), between one hundred and one hundred and seventy times approximately, in the *rfaH* mutant. To our knowledge, we show for the first time a role of RfaH regulating, directly or indirectly, the expression of *nan* operon. Finally, the expression of *nanR*, a repressor of *nan* operon transcription, was up-regulated by 1.7 times.

3.3. RfaH promotes cps CA transcription mainly at low temperatures

It has been established that rfaH promotes *cps* transcription for K30 capsule production [16]. To test whether RfaH is able to enhance *cps* CA transcription aimed at CA production, *E. coli* K92 wild-type and *rfaH* mutant strains were grown in MM Xyl-Asn and MM Glc-Pro at 19 °C, and CA production was analysed after 180 h of growth. At this temperature it was observed that deletion of *rfaH* had dramatically decreased CA production (by around 90%) in both media (Fig. 4). The requirement of RfaH to synthesize CA was determined when the mutant phenotype was complemented with a plasmid pMC*rfaHxp* carrying the sequence wild type rfaH (data not show). Similarly to the *kps* operon, the expression of several genes belonging to *cps* CA operon was next analyzed by qRT-PCR. Wild-type and mutant strains were grown at 19 °C or 37 °C in MM Xyl-Asn. RNA samples harvested from the mid-exponential phase, after 15 and 18 h at 37 °C (wild-type), and after 55 and 130 h at 19 °C (mutant), were used to analyze the expression of genes. Concomitant with the decreased CA production, deletion of *rfaH* led to a great reduction, approximately between twenty-one and forty-seven times, in all *cps* CA genes (Table 4). Unexpectedly, the loss of RfaH did not seem to increase transcriptional polarity. Finally, the *rfaH* mutant showed a ten-fold reduction in the expression of the *ugd* gene.

We previously reported that *E. coli* K92 produces CA mainly at low temperatures (19 °C), as compared to body temperatures (37 °C), but in contrast, *cps* CA expression between both temperatures remains almost unchanged [23]. Thus, we sought to analyze both CA production and *cps* expression mediated by RfaH at 37 °C. Deletion of *rfaH* also reduced CA production in both media (Fig. 4). At this temperature, the reduction in *cps* CA gene expression was much more modest (between around two- and six-fold) than what was observed at 19 °C (Table 4). Finally, *ugd* expression barely changed.

4. Discussion

RfaH acts together with the *ops* element and regulates the expression of genes involved in the synthesis of the cell surface,

Table 3
Expression levels differences measured by qPCR of genes involved in metabolism and regulation of the PA between *E. coli* K92 and *E. coli* K92*ΔrfaH* grown at 37 °C in MM Xil-Asp.

Function	Gen[a]	Product[b]	*E. coli* K92*ΔrfaH*/*E. coli* K92[c]
Sialic acid biosynthesis	*kpsF*	Sialic acid biosynthesis	+1, 0
	neuB	NeuNAc synthase	−80, 2
	neuC	UDP-GlcNAc epimerase	−71, 3
	neuD	Acyltransferase	−16, 0
	neuE	Sialic acid transport and polymerization	−305, 5
	neuS	Polysialyltransferase	−271, 0
	kpsM	ABC-transporter	−10, 0
Sialic acid catabolism	*nanA*	N-acetyl neuraminatelyase	−113, 8
	nanE	N-acetyl manosamine-6-P epimerase	−105, 6
	nanT	Sialic acid transporter	−171, 0
Regulation of sialic acid catabolism	*nanR*	Transcriptional dual regulator	+1, 7

[a] Genes involved in metabolism and regulation of PA.
[b] Description of the products encoded by genes.
[c] The relative levels of gene expression were calculated as described in the Section 2, and then transformed to relative change using the formula $2^{-\Delta\Delta CT}$. As a control gene we used the housekeeping gene *gapdh*.

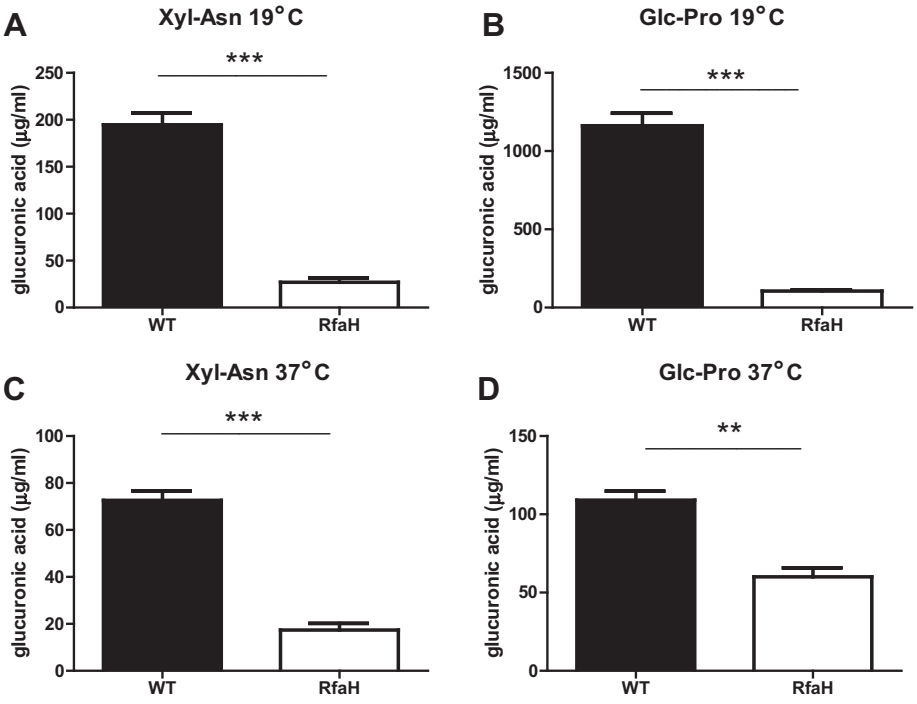

Fig. 4. CA production by *E. coli* K92 (wt) and *E. coli* K92*ΔrfaH* (mutant) growth in MM containing Xyl-Asn (A,C) or Glc-Pro (B,D) after 180 h at 19 °C (A and B) and after 120 h at 37 °C (C and D). $^{*}P < 0.005$, $^{***}P < 0.001$ by student's *t*-test.

Table 4
Expression levels differences measured by qPCR of genes involved on CA metabolism between *E. coli* K92 and *E. coli* K92*ΔrfaH* grown to mid-exponential phase at 19 °C and 37 °C in MM Xil-Asp.

Function	Gen[a]	Product[b]	*E. coli* K92*ΔrfaH*/*E. coli* K92 19 °C[c]	*E. coli* K92*ΔrfaH*/*E. coli* K92 37 °C[c]
CA synthesis	*wzb*	Tyrosine phosphatase	−31, 0	−3, 1
	wzc	Tyrosine kinase	−31, 1	−3, 6
	wcaA	Putative colanic acid glycosyltransferase	−25, 2	−1, 6
	wcaD	Colanic acid polymerase	−39, 4	−2, 8
	gmd	GDP-mannose 4,6-dehydratase	−47, 5	−3, 8
	fcl	GDP-fucose synthase	−47, 1	−6, 1
	wcaK	Putative colanic acid piruviltransferase	−21, 8	−1, 8
	ugd	UDP-glucose-6-dehydrogenase	−10, 5	+1, 4

[a] Genes involved in metabolism and regulation of CA.
[b] Description of the products encoded by genes.
[c] The relative levels of gene expression were calculated as described in the Section 2, and then transformed to relative change using the formula $2^{-\Delta\Delta CT}$. As a control gene we used the housekeeping gene *gapdh*.

including *kps* for the synthesis of *E. coli* group 2 and 3 capsules [7,24]. RfaH enhances the expression of *kps* region 2 genes by preventing the termination of region 3 transcripts, whereas the expression of region 1 genes remains unaffected [6]. Our results show that RfaH enhanced *kps* transcription for PA synthesis in *E. coli* K92 and its absence led to transcriptional polarity (Table 3), matching with the typical behaviour of RfaH-dependent operons [15]. In addition, the absence of RfaH greatly diminished *nan* operon expression, indicating that RfaH is important not only for PA synthesis but also for catabolism. It would appear that the *nan* operon lacks *ops* elements which are essential for the RfaH function, suggesting indirect control. On these lines, it was also observed that deletion of *rfaH* slightly increased the expression of *nanR* (Table 3), a transcriptional regulator which blocks the expression of the *nan* operon [25], providing a possible mechanism by which RfaH might regulate *nan* expression. On the other hand, NanR responds to sialic acid binding; in mechanical terms, the precursor of sialic acid (Neu5Ac) induces its own catabolic pathway by converting NanR to an inactive state (monomers) [26,27]. We suggest that the absence of sialic acid in an *rfaH* mutant would promote an active form of NanR (homodimers), repressing the *nan* operon transcription.

The regulatory role of RfaH in *Enterobacteriaceae* seems to be limited to operons encoding secreted and surface-associated cell components involved in the virulence of *E. coli* pathogens (LPS, K antigens, F pilus, exotoxins). However, it has now been demonstrated for the first time that RfaH also acts in enhancing *cps* transcription for the synthesis of CA, a cell wall component with apparently no role on pathogenicity, and that this occurs mainly at low temperatures, in accordance with its environmental protection role. RfaH also promoted *ugd* expression at low temperatures. Although *ugd* product is involved in many cellular processes of biological relevance at the host temperature, these results suggest that RfaH-mediated *ugd* transcription would be aimed at CA production. Surprisingly, despite the fact that the length of the *cps* CA cluster is 23 kb, deletion of RfaH did not lead to transcriptional polarity on these genes, or at least this was not clearly evident. Whether there exist any additional *cps* CA promoters active at

low temperatures which may explain this phenotype remains unknown.

RfaH was apparently not able to promote *cps* CA expression so efficiently at high temperatures (Table 4), suggesting that this protein is more important for *cps* CA expression at low temperatures. This phenotype is not likely to be attributable to differences in the amount of RfaH, since it has previously been reported that *rfaH* expression is around four times higher at 37 °C, as compared to 19 °C [23], suggesting higher protein levels at 37 °C. RfaH is composed of two domains which are tightly associated in free RfaH, whereas binding to *ops* elements triggers domain separation allowing RfaH binding to the RNA polymerase (RNAP) [28]. It may be speculated that temperature leads to a conformational change in RfaH, in some way modifying its capacity to enhance transcription. However, this would not explain, for example, why it enhances expression of *cps* K30 genes but not *cps* CA genes at 37 °C, or why it enhances *kps* genes but not *cps* CA genes in *E. coli* K92 at 37 °C.

Bacterial RNAP is a principal target for numerous accessory proteins, including sigma factors, which modulate gene expression profiles according to the cell's needs. Sigma factors compete for binding to RNAP and, when successful, directing it to a subset of sigma-specific promoters [29]. Sigma factors and RfaH share a common area for their recruitment to RNAP [11] suggesting a steric exclusion which may constitute an essential part of the RfaH regulatory function. However, the magnitude of this competition depends on the specific sigma factor. For example, sigma 70 (RpoD) binds more tightly to RNAP than sigma 38 (RpoS) [30], increasing the probability of sigma rebinding to the termination complex during elongation and consequent RfaH release. Analysis of DNA promoter sequences suggests that the RfaH-mediated transcription is devoid of RpoD recruitment. Thus, transcription from RpoD-dependent promoters covers the complete *kps* region 1 and no *rfaH* function is required [6,31]. Complete transcription of regions 2 and 3 requires rfaH but region 3 promoter lacks the RpoD 35 consensus sequence in *E. coli* K5 [7] and the role of RpoD has not been established. On the other hand, no evident RpoD promoter regions were found upstream of the rfaH-dependent *cps* operon in *E. coli* K-12 [15]. A putative RpoD promoter was found in *E. coli* K30 but it cannot drive transcription for detectable K30 CPS production. As has now been shown for *cps* CA transcription in *E. coli* K92, *cps* K30 transcription relies on RfaH, but this putative RpoD promoter lies downstream of the *ops* sequence, giving evidence of an RfaH-independent function [16].

Overall, an external signal, such as temperature, would determine the presence of specific sigma factors, and consequently, the expression of specific promoters and any RfaH requirement. One possible candidate responsible for the recruitment of RfaH to RNAP during *cps* CA transcription at low temperatures would be RpoS, which binds less tightly to RNAP, avoiding a possible RfaH release. In addition, a plasmid carrying *rfaH* restored the impaired haemolysin secretion, thanks to a mutation in RpoS [32], providing evidence of a functional connection. RpoS is mainly expressed at low temperatures, owing to the action of DsrA [33]. It has previously been shown that *DsrA* expression is up-regulated by around eight times at 19 °C, relative to 37 °C, in *E. coli* K92 [23], suggesting increased RpoS levels at 19 °C. Altogether, RpoS may orchestrate an rfaH-dependent *cps* CA expression at low temperatures, whereas *cps* CA transcription at 37 °C would be mediated by other sigma factors and/or accessory molecules and would be mostly independent of rfaH.

Finally, it has been shown that RfaH-dependent operons do not encode essential functions [34]. However, a hitherto unknown requirement for RfaH for growth at low temperatures has now been revealed. Further studies on these lines are needed to clarify this phenotype.

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