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Null mutations in the essential gene *yrfF* (*mucM*) are not lethal in *rcsB*, *yojN* or *rcsC* strains of *Salmonella enterica* serovar Typhimurium

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Abstract

Insertion of factor MudJ in the intergenic region between divergent genes *yrfF* and *yrfE*, at centisome 76 in the genome of *Salmonella enterica* serovar Typhimurium LT2, confers the characteristics recently described for *mucM* mutants, i.e. mucoidy and resistance to mecillinam. Cloning of the intergenic region plus either the *yrfF* or the *yrfE* gene in a multicopy plasmid showed that only the plasmid carrying the *yrfF* gene complemented *mucM* mutants, thus suggesting that *mucM* mutations are in fact *yrfF* mutations. A null *yrfF* mutation obtained by insertion of a kanamycin cassette into the *yrfF* open reading frame (*yrfF28*::Kan) produced abortive colonies when transduced to a wild-type strain but was normally accepted by *rcsB, rcsC* or *yojN* strains. Neither mutations preventing synthesis of the capsular exopolysaccharide colanic acid (*cps*, *galE*) nor *rcsA* mutations, which reduce expression of *cps* genes, conferred tolerance to the lethal *yrfF28*::Kan mutation. Spontaneous suppressor mutations arose very frequently in abortive *yrfF28*::Kan colonies, and all of them affected either *rcsC*, *yojN*, or *rcsB* genes. Thus, the lethal effect caused by inactivation of gene *yrfF* appears to be mediated by a function that is dependent on the *rcsC-yojN-rcsB* phosphorelay system but does not involve synthesis of colanic acid.

Keywords: *yrfF*; *mucM*; *igaA*; Essential gene; Mucoid mutant; *rcsC-yojN-rcsB* phosphorelay

1. Introduction

It has recently been reported that the most frequent type of mucoid mutant isolated by resistance to mecillinam in *Salmonella enterica* serovar Typhimurium is affected in a new gene, tentatively called *mucM*, located at about centisome (Cs) 76, between markers *aroB* and *envZ* [1]. Mucoidy and resistance of *mucM* mutants to mecillinam have been assigned to increased activity of gene *rcsB*. *rcsB* product is the positive effector of a complex phosphorelay system formed by genes *rcsC*, *yojN*, and *rcsB* that regulates the transcription of a cluster of 19 genes (*cps* genes) involved in the synthesis of capsule exopolysaccharide colanic acid [2,3]. Moreover, RcsB activates transcription of the cell division genes *ftsZ* and *ftsA* by acting specifically on one of the promoters (*ftsA1p*) governing expression of the *ftsQAZ* cluster [4]. Increased transcription of *cps* genes in *mucM* mutants would lead to mucoidy while overexpression of FtsZ and FtsA proteins would result in resistance to mecillinam [1]. In this paper, *mucM* was found to be the gene designated by McClelland et al. [5] as *yrfF*, and results demonstrating that the *yrfF* gene product is essential to wild-type *S. typhimurium* but is not required for survival of *rcsB*, *yojN*, or *rcsC* mutants are presented.

2. Materials and methods

2.1. Bacterial strains, phage and media

All the strains used were derivatives of *S. typhimurium* LT2 unless otherwise specified; their origins and relevant genetic properties appear in Table 1. All the strains carrying plasmids were grown in LB broth containing the appropriate antibiotic to keep the plasmid; strains carrying

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plasmid pCC74 were grown at 25°C for better maintenance of the plasmid. Transductions were mediated by phage P22 HT105/1 *int-201* as previously described [1]. Complete medium was LB broth and LB agar, and E medium was used as minimal medium [6]. MacConkey agar was used to test fermentation of lactose. Antibiotics were used at the following concentrations: mecillinam, 1 μg ml^{-1}; kanamycin, 20 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; ampicillin, 100 μg ml^{-1}. Mecillinam was a kind gift of Leo Pharmaceutical Products (Denmark).

2.2. *Isolation and characterization of* rcsB, yojN *and* rcsC *insertion mutants*

Insertional mutations in genes *rcsB*, *yojN* and *rcsC* were obtained by transducing strain DA2026 (*yrfF1 wcaJ1*::MudJ) with phage grown on a pool of random Tn*10d*Tet insertions. Tetracycline-resistant (Tet^r) transductants that produced white colonies on MacConkey plus tetracycline agar were characterized by transduction and polymerase chain reaction (PCR). Insertions that showed cotransduction with marker *gyrA* were identified as affecting the *yojN-rcsB-rcsC* group. DNA of those mutants was used for amplification with the following primers: UrcsB (5′-GAA GAG ATT CCC GCC TCC C-3′) and LrcsB (5′-GTG TAT GCC GAG CGG GTA CG-3′) for *rcsB*, UyojN (5′-CGG CTG ATT TAT GCT ACC TG-3′) and LyojN (5′-GTA CAA TCG GGT GGT CAT CG-3′) for *yojN*, and UrcsC (5′-CGC TTT TAT GTT ACC CAG CC-3′) and LrcsC (5′-GTT ATT GCT GTG CGA GGG-3′) for *rcsC*. Size modification and change in the restriction pattern of the amplified fragments due to insertion of the 2910-bp transposon allowed identification of the gene affected by Tn*10d*Tet insertion. Among five strains analyzed, one *rcsB*, one *rcsC* and three *yojN* mutants were identified.

2.3. *Recombinant DNA techniques*

Plasmids used in this work are listed in Table 1. Con-

Table 1
Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Origin
Strain		
DA1468	*argC95*	[1]
DA1736	*cysB484*	Laboratory collection
DA1952	*zhg-7131*::Tn*10d*Tet	[1]
DA2023	*recA1 zfj-623*::Tn*10d*Cam	Laboratory collection
DA2025	*yrfF1*	[1]
DA2026	*yrfF1 wcaJ1*::MudJ	[1]
DA2032	*yrfF9*::MudJ *wcaJ1*::MudJ	This work
DA2044	*wcaJ1*::MudJ	[1]
DA2069	*yojN1*::Tn*10d*Tet	This work
DA2070	*rcsC37*::Tn*10d*Tet	This work
DA2092	*aroB25*	Laboratory collection
DA2095	*yrfF9*::MudJ *rcsC29*	This work
DA2103	*yrfF28*::Kan *rcsC37*::Tn*10d*Tet	This work
DA2108	*rcsB15*::Tn*10d*Tet	This work
DA2109	*yrfF1 rcsB15*::Tn*10d*Tet	This work
DA2112	*yrfF28*::Kan *rcsB30*	This work
DA2118	*yrfF1 yojN1*::Tn*10d*Tet	This work
DA2119	*yrfF1 rcsC37*::Tn*10d*Tet	This work
DA2120	*cps-4*::Tn*10d*Tet	This work
SH7241	*ompC396*::Tn*10*	[16]
TT5371	*zef-754*::Tn*10*	[16]
TT10289	*hisD9953*::MudJ *his-9949*::MudI	[13]
TT15258	*cysA1586*::MudQ	[9]
TT15265	*envZ1005*::MudP	[9]
Plasmid		
pGEM-T Easy	Cloning vector, Amp-R	Promega
pWSK29	Cloning vector, Amp-R	[10]
pUC4K	Source of Kan-R cassette	Pharmacia
pCC14	pGEM-T Easy+$yrfE^+$	This work
pCC23	pGEM-T Easy+$yrfF^+$	This work
pCC24	Derived from pCC23, *yrfF28*::Kan	This work
pCC25	pGEM-T Easy+$rcsB^+$	This work
pCC26	pWSK29+$rcsB^+$	This work
pCC74	pGEM-T Easy+$yojN^+$	This work
pFAB4	Transcriptional fusion *ftsA1p-lacZ*	[4]

All the DA strains are derivatives of DA1468 and contain the *argC95* mutation.

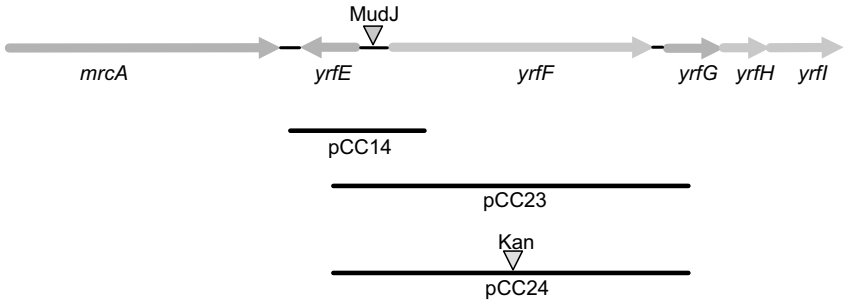

Fig. 1. Schematic representation of the *S. typhimurium yrfE-yrfF* region. Arrows represent the different ORFs and the orientation of transcription. Plasmids constructed for this study are represented as black lines indicating the DNA region carried by each plasmid. The location of the MudJ factor in the *yrfF9* mutant and the restriction site used for insertion of the Kan cassette in the *yrfF* gene (*yrfF28*::Kan) are indicated by inverted triangles.

ventional recombinant DNA techniques were used for manipulation of DNA, amplification by PCR and plasmid construction [7]. Primers used for PCR were designed on the basis of the genome sequence of *S. typhimurium* LT2 [5]. *Escherichia coli* DH5α (Bethesda Research Laboratories) was used as recipient strain for plasmid constructions that were, subsequently, transformed into *S. typhimurium* DA2023 (*recA1*), and then transferred by transduction to other *S. typhimurium* strains.

2.4. Inverse PCR

DNA flanking the left end of *yrfF9*::MudJ was obtained as described by Ahmer et al. [8]. Genomic DNA of strain DA2095 was isolated and digested with enzymes *Taq*I or *Alu*I. Different aliquots of the restricted DNA were treated with T4 DNA ligase and inverse PCR was performed using these ligations as templates. DNA digested with *Taq*I was amplified with primers MudTaq and MudOut [8]; DNA digested with *Alu*I was amplified with primers MudAlu and MudOut [8]. An amplification product of 386 bp, obtained with *Taq*I-digested DNA, was cloned into plasmid pGEM-T Easy (Promega) and sequenced. The same procedure was used with strain DA2044 (*wcaJ1*::MudJ) to identify the *cps* gene affected in the fusion formerly called *cps-1*::MudJ [1]. In this case, sequence analysis of an amplification product of 650 bp obtained with *Alu*I-digested DNA showed MudJ to be inserted in gene *wcaJ*.

2.5. Construction of plasmids

Genes *yrfF* and *yrfE* were obtained by PCR amplification of DNA from strain TT15265 enriched in the Cs 76 chromosomal region of *S. typhimurium* [9]. The primers used were: UyrfF (5′-CGA CGG TTT CCA CTT TCA GA-3′) and LyrfF (5′-TCC AGC AAA ACG GTA TCC AC-3′) for amplification of *yrfF*, and UyrfE (5′-ATT GTG TTT AGC GGA TGA CG-3′) and LyrfE (5′-CGG GTG ATG GAG TGG GTT AG-3′) for amplification of *yrfE*. The 2615-bp (*yrfF*) and 1243-bp (*yrfE*) amplification fragments were cloned into pGEM-T Easy, to obtain plasmids pCC23 and pCC14, respectively. The intergenic region between *yrfF* and *yrfE* was amplified from genomic DNA of the *yrfF1* strain DA2025 using primers UyrfF and LyrfE and was cloned into pGEM-T Easy. For the construction of the *yrfF28*::Kan mutant, plasmid pUC4K (Pharmacia) was used as the source of a kanamycin resistance cassette (Kan) by digestion with *Bam*HI. Introduction of the Kan cassette into the unique *Bam*HI site of the *yrfF* gene in pCC23 produced plasmid pCC24 (Fig. 1).

Primers UrcsB and LrcsB (see above) were used for amplification of the *rcsB* gene from genomic DNA of strain DA1468, the standard wild-type strain used in this laboratory [1]. The 1100-bp amplification product was cloned into multicopy vector pGEM-T Easy to obtain plasmid pCC25. Plasmid pCC25 produced mucoidy in all the strains tested, including wild-type DA1468. Therefore, to have gene *rcsB* in a low copy number plasmid, pCC25 was digested with *Eco*RI and the fragment containing *rcsB* was cloned into the *Eco*RI site of plasmid pWSK29 [10] to obtain plasmid pCC26. Primers UyojN and LyojN (see above) were used for amplification of gene *yojN* from DNA of strain TT15258 enriched in the Cs 50 chromosomal region of *S. typhimurium* [9]. The amplification fragment of 3015 bp obtained was cloned into vector pGEM-T Easy to produce plasmid pCC74.

2.6. Other methods

β-Galactosidase activity of *wcaJ-1*::MudJ and pFAB4 strains was assayed in stationary cultures grown in LB broth as described by Miller [11] on cells treated with sodium dodecyl sulfate and chloroform. Specific activities are expressed in Miller units referred to OD_{650}.

The minimum inhibitory concentration (MIC) of mecillinam was determined by plating cells from stationary cultures on LB plates containing twofold serial dilutions of the antibiotic [12].

3. Results

3.1. Isolation of mutant mucM9::MudJ

About 70% of the mucoid mutants isolated by resistance to mecillinam belong to the new class *mucM* [1]. Many attempts to isolate *mucM* mutants by insertion of the Tn*10d*Tet transposon into the *mucM* gene were carried out but all of them failed. Attempts to isolate *mucM* mutants by insertion of the MudJ factor [13] also failed until a mucoid mutant was finally obtained when strain DA1736 treated with a lysate grown on strain TT10289 [13] was plated on kanamycin plates and filter paper disks containing 1 μg of mecillinam were placed on the surface. There was no growth on a zone of about 3 cm diameter around the disk while on the rest of the plate, where only kanamycin was present, many non-mucoid MudJ transductants appeared. In just one case, a mucoid colony was observed in the inhibition zone near the mecillinam disk. This isolate displayed resistance to 80 μg ml^{-1} of mecillinam; its kanamycin resistance (Kan^r) marker was 100% linked to the mucoid phenotype and was cotransduced with markers *aroB* (69%), *cysG* (33%), and *zhg-7131*::Tn*10d*Tet (91%). It was concluded that the mutation was caused by insertion of MudJ into the *mucM* gene, and it was named *mucM9*::MudJ. No increase in β-galactosidase-specific activity due to the MudJ factor was observed in *mucM9*::MudJ strains; so, either the insertion was not in frame or the gene was not expressed.

3.2. Identification of gene mucM

In order to identify gene *mucM*, inverse PCR was performed using genomic DNA of DA2095, a *mucM9*::MudJ strain (Section 2). The MudJ factor was found to be inserted in the intergenic region between genes *yrfE* and *yrfF*, 166 bp from the *yrfE* origin and 123 bp from the *yrfF* origin (Fig. 1). As *yrfE* and *yrfF* are divergent genes whose transcription starts from the same intergenic region [5], *mucM9*::MudJ insertion could affect one or both of the two genes. To discern which of these genes was responsible for the *mucM* phenotype, *yrfE* and *yrfF* sequences, both of them including the intergenic region, were amplified by PCR and independently cloned in multicopy plasmid pGEM-T Easy (Fig. 1).

The plasmid carrying the wild-type *yrfF* gene (pCC23) was found to complement the mucoid phenotype of several *mucM* mutants of *S. typhimurium* (*mucM1*, *mucM5*, *mucM8*, *mucM9*::MudJ) and also of a *mucM* mutant of *E. coli* K-12. Transcription of the *cps* cluster in a *mucM1* mutant carrying fusion *wcaJ1*::MudJ (DA2026) was also normalized by pCC23 (Table 2). A similar result was observed when the *mucM9*::MudJ strain (DA2032) was tested, although in this case the level of β-galactosidase activity remained somewhat higher than the wild-type level. On the other hand, the plasmid carrying the $yrfE^+$ gene (pCC14) not only did not modify the mucoidy of *mucM* mutants, but promoted a significant increase in the β-galactosidase activity of *wcaJ1*::MudJ derivatives of *mucM1* and *mucM9*::MudJ strains. This effect was due to plasmid pGEM-T Easy, because the plasmid with no insert also increased the activity of the enzyme but only in strains whose β-galactosidase activity was already increased (Table 2). The reason for this behavior is not known.

On the basis of the complementation observed with the plasmid carrying the $yrfF^+$ insert, the name of *mucM* mutants was changed to *yrfF* mutants.

To find out whether other *yrfF* mutants also carried alterations in the *yrfE-yrfF* intergenic region, the intergenic region of the *yrfF1* mutant was amplified by PCR and sequenced. No change was observed with respect to the wild-type sequence. It seems, then, that *yrfF1* does not affect the *yrfF* regulatory region but rather the *yrfF* open reading frame (ORF) since the pCC23 plasmid also complemented it (Table 2).

Table 2
Effect of plasmids pCC23 ($yrfF^+$) and pCC14 ($yrfE^+$) on the expression of fusion *wcaJ1*::MudJ in *mucM* strains

Strain	Plasmid	Insert in plasmid	β-Galactosidase activity
yrfF1	–	–	384 ± 34
yrfF1	pGEM-T Easy	–	825 ± 13
yrfF1	pGEM-T Easy	$yrfF^+$	1.6 ± 0.2
yrfF1	pGEM-T Easy	$yrfE^+$	803 ± 69
yrfF9::MudJ	–	–	302 ± 43
yrfF9::MudJ	pGEM-T Easy	–	482 ± 52
yrfF9::MudJ	pGEM-T Easy	$yrfF^+$	21 ± 2.4
yrfF9::MudJ	pGEM-T Easy	$yrfE^+$	458 ± 25
$yrfF^+$	–	–	2.1 ± 0.1
$yrfF^+$	pGEM-T Easy	–	3.2 ± 1.5
$yrfF^+$	pGEM-T Easy	$yrfF^+$	2.3 ± 0.2
$yrfF^+$	pGEM-T Easy	$yrfE^+$	2.6 ± 0.8

The strains used were: DA2026 (*yrfF1*), DA2032 (*yrfF9*::MudJ) and DA2044 ($yrfF^+$); all the strains carried fusion *wcaJ1*::MudJ (formerly called *cps-1*::MudJ [1]). β-Galactosidase activity is expressed in Miller units referred to OD_{650} ± S.D. [11]. All the assays were performed in duplicate and the results are averages of at least three independent experiments.

3.3. *Effects of a null* yrfF *mutation*

In order to investigate the role of the *yrfF* gene, a null *yrfF* mutation was constructed by inserting the Kan cassette of plasmid pUC4K in the unique *Bam*HI site of the *yrfF* ORF carried by plasmid pCC23 (Fig. 1). Transfer of the new plasmid, pCC24, to several *yrfF* mutants (*yrfF1*, *yrfF5*, *yrfF8*, *yrfF9*::MudJ) demonstrated that the modified *yrfF* gene (*yrfF28*::Kan) had lost the capacity to complement the mucoid phenotype of those mutants. To achieve integration of *yrfF28*::Kan into the chromosome, phage grown on a strain carrying the pCC24 plasmid was used to select for *aroB*$^+$ Kan^r transductants with appropriate *aroB25* recipients, taking advantage of the close linkage between *aroB* and *yrfF*. It was observed that when the *aroB25* recipient (DA2092) carried the wild-type *rcsBC* alleles, all of the *aroB*$^+$ Kan^r transductants were also resistant to ampicillin due to the presence of plasmid pCC24. However, when the *aroB25* recipient also carried an *rcsB* or *rcsC* mutation, *aroB*$^+$ Kan^r transductants displaying sensitivity to ampicillin and containing no plasmid were easily obtained. Thus, in the latter case allelic exchange occurred and the *yrfF28*::Kan marker was integrated in the chromosome. All those *yrfF28*::Kan transductants grew quite normally and were non-mucoid. Phage grown on a *yrfF28*::Kan *rcsB30* strain (DA2112) was used to transduce the *yrfF28*::Kan mutation to different recipient strains by selection for Kan^r transductants. It was found that transduction of the *yrfF28*::Kan mutation to wild-type recipients produced a few normal-sized non-mucoid colonies and a large number of very tiny, almost transparent, colonies that failed to grow when restreaked on the same medium (Fig. 2a). Only large normal colonies were observed when the recipient of the *yrfF28*::Kan marker was an *rcsC* strain (Fig. 2b). Since both *rcsB* and *rcsC* are components of the phosphorelay system controlling transcription of the *cps* cluster [2], the capacity of *rcsB* and *rcsC* mutations to suppress the lethality of *yrfF28*::Kan could be due to their blocking effect on colanic acid synthesis. To test this possibility, strain DA2120 carrying insertion *cps-4*::Tn*10d*Tet, which prevents colanic acid synthesis, was used as recipient for the *yrfF28*::Kan mutation. The *cps-4*::Tn*10d*Tet mutation not only did not suppress *yrfF28*::Kan lethality but decreased even more the size of the abortive colonies (Fig. 2c). Similar results were observed when synthesis of colanic acid was stopped by a *galE* mutation (*galE1922*), and also when the recipient strain carried an *rcsA* mutation (*rcsA16*::Tn*10d*Tet) that reduced expression of the *cps* cluster [14] (not shown). In all these cases, the simultaneous presence of an *rcsC* mutation in the recipient normalized the number and size of *yrfF28*::Kan transductants demonstrating that colanic acid synthesis per se was not responsible for lethality.

Abortive *yrfF28*::Kan colonies presented cellular heterogeneity: spherical cells, short filaments, as well as empty envelopes, and cellular debris. In many cases, dark round bodies could be seen at both ends of elongated and almost lysed cells (Fig. 2d). Cells from *yrfF28*::Kan *cps-4*::Tn*10d*Tet colonies displayed severe anomalies too, and their general aspect was similar to that of *yrfF28*::Kan colonies, but there were many large ovoid cells and short lysed filaments containing regularly spaced dark bodies (Fig. 2f). Cells from *yrfF28*::Kan *rcsC37*::Tn*10d*Tet colonies were normal bacilli (Fig. 2e).

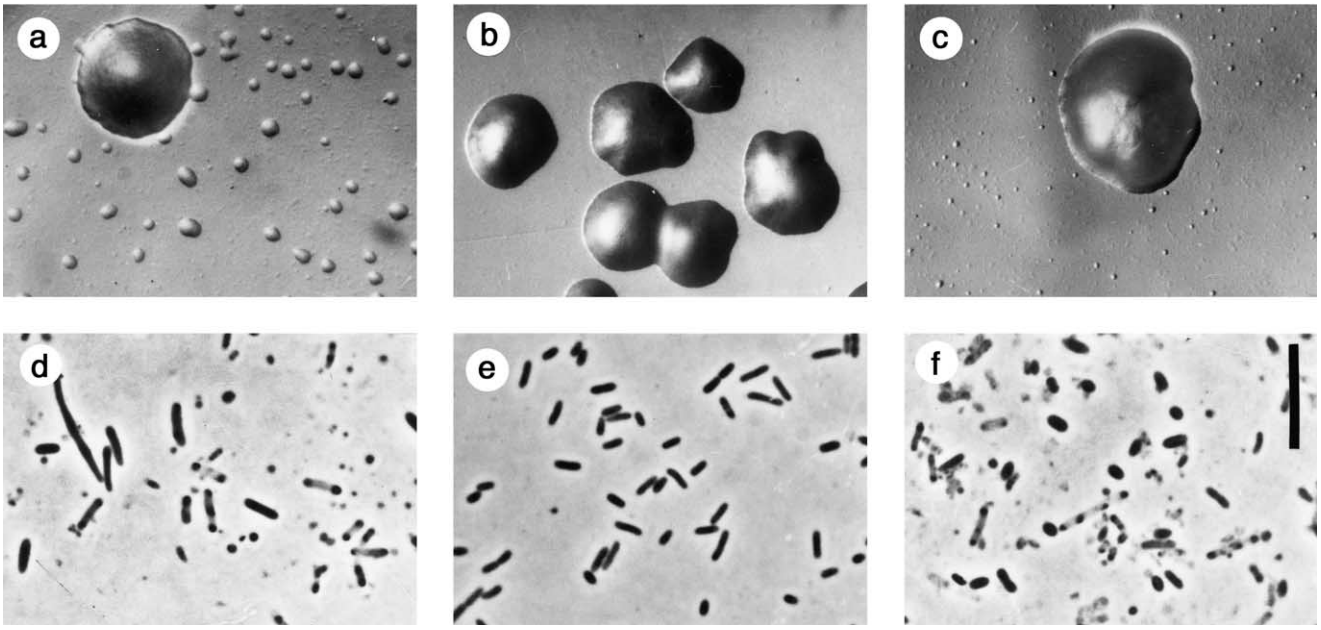

Fig. 2. Colony and cell morphology of *yrfF28*::Kan derivatives. Insertion *yrfF28*::Kan was transduced to different recipients strains and the colonies that appeared on the transduction plates were photographed (a–c). Cells from those colonies were suspended in saline solution and phase contrast photomicrographed (d–f). Wild-type recipient (DA1468): a and d; *rcsC37*::Tn*10d*Tet recipient (DA2070): b and e; *cps-4*::Tn*10d*Tet recipient (DA2120): c and f. Bar represents 10 μm (d–f).

yrfF is the first ORF in a presumptive operon formed also by three other genes: *yrfG*, *yrfH* (*hslR*), and *yrfI* (*hslO*), the latter two encoding heat shock proteins [5,15]. To verify that lethality was due to inactivation of *yrfF* rather than to a polar effect on transcription of downstream genes, wild-type recipients carrying plasmid pCC23 were transduced with *yrfF28*::Kan phage. It was found that the *yrfF*$^+$ gene contained in plasmid pCC23 allowed those strains to produce normal colonies in contrast with the abortive colonies produced by the same strains lacking the plasmid. Thus, lethality was specifically due to loss of the *yrfF* function.

3.4. *Effect of* rcsB, rcsC, *and* yojN *mutations on the phenotype of* yrfF *mutants*

The effect was tested of mutations produced by insertion of the Tn*10d*Tet transposon in genes *rcsB* (*rcsB15*::Tn*10d*Tet), *rcsC* (*rcsC37*::Tn*10d*Tet), and *yojN* (*yojN1*::Tn*10d*Tet) on the phenotype of strains carrying either the *yrfF28*::Kan mutation or the viable *yrfF1* allele. It was found that the three types of mutation allowed survival of *yrfF28*::Kan derivatives. In relation to *yrfF1* strains, the three mutations behaved alike. They abolished mucoidy and reduced *cps* expression in the *wcaJ1*::MudJ *yrfF1* strain from 384 units of β-galactosidase activity to 1.4 units in the corresponding *rcsB*, *rcsC*, or *yojN* derivatives. They also restored the sensitivity to mecillinam of the *yrfF1* strain (MIC of mecillinam on the *yrfF1* strain: 40 μg ml^{-1}; MIC on any of the three derivatives: 0.08 μg ml^{-1}) and normalized the expression of *ftsZ* and *ftsA* genes from the *ftsA1p* promoter (β-galactosidase activity of stationary cultures of strain *yrfF1* carrying plasmid pFAB4 was 6900 units while the activity of derivatives of the same strain containing any one of the three suppressors was about 830 units). Plasmid pFAB4 carries a *lacZ* reporter gene fused to a fragment of the *ftsQAZ* promoter region comprising *ftsA1p*, the *ftsZ* promoter subjected to RcsB stimulation [4]. These results confirm that all those characteristics are dependent on every one of the three components of the *rcsC-yojN-rcsB* phosphorelay system.

3.5. *Suppressors of* yrfF28*::Kan lethality*

As described before, transduction of the *yrfF28*::Kan mutation to a wild-type recipient produced many abortive colonies and a few large ones. Further incubation of the transduction plates allowed spots of outgrowth to appear in many of the tiny colonies. Both large colonies and outgrowth spots produced normal non-mucoid colonies when restreaked on the same medium and appeared to be caused by secondary mutations which arose spontaneously and suppressed *yrfF28*::Kan lethality.

It was investigated whether survivors were double transductants which received both the *yrfF28*::Kan mutation and the permissive marker from the donor. To this purpose, wild-type strain DA1468 was transduced to Kan^r with phage grown on a *yrfF28*::Kan *rcsC37*::Tn*10d*Tet donor (DA2103) so that double transductants carrying the *rcsC* mutation of the donor would be marked by the resistance to tetracycline of the *rcsC37*::Tn*10d*Tet insertion. Among 140 viable colonies that included large colonies appearing early and outgrowth spots appearing until 5 days after plating, only four (3%) carried the donor *rcsC* mutation and so were double transductants. Thus, the suppressor mutations carried by most of the survivors were not due to double transduction but to fresh mutations present in the recipient strain or produced on the selection plates.

Lethality suppressors from 43 *yrfF28*::Kan Tet^s transductants were subjected to genetic analysis to find out if they also affected *rcsBC* genes. To that purpose, the 43 strains were transduced to Tet^r with phage grown on strains carrying either insertion *ompC396*::Tn*10* (SH7241) or *zef-754*::Tn*10* (TT5371), both of which are located at Cs 50 [16], very close to the *yojN-rcsB-rcsC* region. All 43 survivors produced Tet^r transductants with *zef-754*::Tn*10* phage but only 60% did the same with the *ompC396*::Tn*10* phage, suggesting that in the latter case the wild-type allele of the lethality suppressor was so close to the selective marker that most of the *ompC396*::Tn*10* transductants received it and died. Phage grown on Tet^r derivatives of the 43 strains (either *ompC396*::Tn*10* or *zef-754*::Tn*10*) was used to transduce the Tet^r marker to the mucoid *yrfF1* strain DA2025. In all cases, 50–90% of Tet^r transductants became non-mucoid, demonstrating that the survivors carried mutations in the *yojN-rcsB-rcsC* region that suppressed expression of the *cps* cluster.

In order to identify the genes affected by the suppressor mutations, the complementing behavior of plasmids pCC26 (*rcs*B$^+$, low copy) and pCC74 (*yojN*$^+$, multicopy) was tested with *yrfF*$^+$ and *yrfF1* derivatives of *rcsB15*::Tn*10d*Tet, *yojN1*::Tn*10d*Tet and *rcsC37*:: Tn*10d*Tet mutants (Table 1). Plasmid pCC26 produced mucoidy in all the strains tested except in the wild-type strain DA1468 and in *yrfF*$^+$ derivatives of *rcsB* mutants that remained non-mucoid. Plasmid pCC74 produced mucoidy in strain DA1468 as well as in *yojN* mutants, either *yrfF*$^+$ or *yrfF1*, but *yrfF*$^+$ and *yrfF1* derivatives of *rcsB* and *rcsC* mutants remained non-mucoid. Thus, low-level RcsB turned *cps* expression on in *yrfF*$^+$ *rcsC* and *yrfF*$^+$ *yojN* mutants but not in *yrfF*$^+$ *rcsB* strains. On the other hand, high-level YojN protein led to mucoidy in *yrfF*$^+$ *yojN* strains but not in *yrfF*$^+$ *rcsB* or *yrfF*$^+$ *rcsC* mutants.

To obtain *yrfF*$^+$ derivatives of the 43 suppressed strains, removal of the *yrfF28*::Kan mutation was effected by cotransducing the *yrfF*$^+$ allele with close by insertion *zhg-7131*::Tn*10d*Tet present in strain DA1952. Plasmids pCC26 (*rcsB*$^+$) and pCC74 (*yojN*$^+$) were then transduced to *yrfF*$^+$ derivatives of the 43 strains. On the basis of the behavior of the two plasmids in well characterized *rcsB*,

yojN and *rcsC* mutants and their behavior in each of the 43 strains, 14 (33%) of the suppressors were identified as *yojN* mutations, two (5%) were *rcsB* mutations, and 20 strains (46%) were probably *rcsC* mutants. The remaining seven suppressors (16%) produced atypical responses and appeared to be complex mutants carrying more than one mutation. Most of the strains producing few or no transductants with insertion *ompC396*::Tn*10* carried mutations in gene *yojN*, the closest to that marker.

4. Discussion

The results presented in this paper identify gene *yrfF* as responsible for the *mucM* phenotype and demonstrate that it performs an essential function since its inactivation in wild-type strains leads to cell death. Insertion of MudJ in the intergenic region between the *yrfF* and *yrfE* genes produced mutation *yrfF9*::MudJ that displays the typical characteristics of *mucM* mutations even though only the level of transcription of the *yrfF* gene would be expected to be modified by the insertion. As full loss of the *yrfF* product is lethal for wild-type strains, it can be assumed that all the *yrfF* mutants isolated by resistance to mecillinam are partial mutants that maintain at least the minimal level of *yrfF* product or function compatible with survival. Although mucoidy makes *yrfF* mutants very conspicuous and facilitates isolation, the connection of *rcsB*, *rcsC*, and *yojN* genes with the essential role of YrfF appears to be unrelated to their known function as regulators of capsule synthesis. Since the lethality caused by *yrfF* loss was not relieved by mutations interfering with colanic acid synthesis nor by inactivation of RcsA, a short-lived protein required to optimize RcsB-positive regulation of *cps* genes [14], it can be concluded that *cps* genes do not participate in *yrfF* lethality. The same conclusion can be drawn from the absence of *cps*, *galE*, *malA*, and *rcsA* mutations among spontaneous suppressors of *yrfF* lethality. In fact, spontaneous suppressors included, almost exclusively, *rcsC* and *yojN* mutations. This leaves one to wonder if only selective advantage promoted the amazing abundance of these two types of mutants.

Evidence of the role of the *rcsB-rcsC-yojN* phosphorelay system in the regulation of other processes besides colanic acid synthesis is being gathered lately and has led to the proposal that it could act as a global regulator. Thus, its involvement in the synthesis of Vi antigen, flagellin, and invasion proteins in *Salmonella typhi* [17], in the control of swarming in *E. coli* and other bacteria [3], in the regulation of *osmC* gene expression [18], and in the recovery of cells treated with chlorpromazine [19] has been demonstrated. RcsB also works as a positive effector on *ftsA1p*, one of the promoters located upstream of the vital cell division gene *ftsZ* [4]. Results previously reported suggested that the resistance of *yrfF* mutants to mecillinam could be assigned to the overproduction of FtsZ and/or FtsA proteins promoted by increased RcsB activity. The abundance of small cells and short filaments observed in the abortive *yrfF28*::Kan colonies could perhaps reflect the increased level of FtsZ and FtsA cell division proteins caused by the enhanced activity of RcsB on *ftsA1p* promoter. It is well known that the FtsZ level is tightly regulated, and overproduction of this essential protein results in cell division anomalies [20]. A putative function as inner membrane protein has been assigned to the *yrfF* product on the basis of the identification of four hydrophobic transmembrane regions by sequence analysis [5]. Therefore, the observed severe structural damage and envelope fragility could also be assigned to the absence of YrfF from the inner cell membrane. However, as those anomalies did not appear in *yrfF28*::Kan strains lacking active RcsB even though their membranes were also deprived of YrfF, it is clear that the lethality of null *yrfF* mutations requires the action of the *rcsB-rcsC-yojN* group of genes to be manifested.

RcsC and YojN are inner membrane proteins [3,21] as YrfF is also supposed to be [5]. It has been proposed that the stimulus leading to activation of the *rcsC-yojN-rcsB* phosphorelay system is related to alterations of the cell envelope that are detected by RcsC [21], transmitted by YojN [3] and received by RcsB which, upon phosphorylation, becomes the positive effector of several regulatory processes. YrfF seems to act as some kind of negative regulator of RcsB activity, and the idea that RcsC (and/or YojN) could 'feel' environmental stimuli through interaction with YrfF, in such a way that mutations decreasing the YrfF amount or altering its properties would result in partial activation of RcsC whereas total loss of YrfF would cause full activation of the *rcsC-yojN-rcsB* system, is attractive. Lethality, in the latter case, would be evoked by RcsB action on some specific target. Only *rcsC*, *yojN*, and, at a much lower frequency, *rcsB* mutations were found among the spontaneous suppressors analyzed, and perhaps a more extensive screening might reveal infrequent types. Mutations rendering the target resistant to RcsB action could also be expected to act as suppressors of *yrfF* lethality, unless they themselves were lethal.

Similarity of YrfF to the UmoB protein of *Proteus mirabilis* has been reported (41% identity, 65% similarity) [22]. UmoB is a non-essential protein involved in the control of flagellar biogenesis, swarming and cell division [22]. Although no YrfF function is known yet, the fact that a *yrfF* mutant (named *igaA*) was obtained with a screen devised to isolate mutants of *S. typhimurium* able to proliferate in a fibroblast line [23] suggests a role connected with cell division. This role could be related to the increased activity of FtsZ in *yrfF* mutants [1].

Identification of *yrfF* as the gene responsible for the *mucM* phenotype correlates with observations reported in *E. coli*. Meberg et al. found that a deletion comprising *mrcA*, *yrfE*, and almost half of the *yrfF* gene caused mucoidy. That alteration was initially assigned to the deletion

of gene *mrcA* that encodes penicillin binding protein 1a, but it was recently found that a deletion affecting only *mrcA* did not display those characteristics [24]. The traits reported clearly denote *yrfF* damage; yet, it is surprising that such an extended deletion caused mucoidy but was not lethal. This leaves one to wonder if loss of the other two genes affected by the deletion (*mrcA* and/or *yrfE*) suppressed *yrfF* lethality or *yrfF* is not essential in *E. coli* K-12.

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