

# Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose

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## Summary

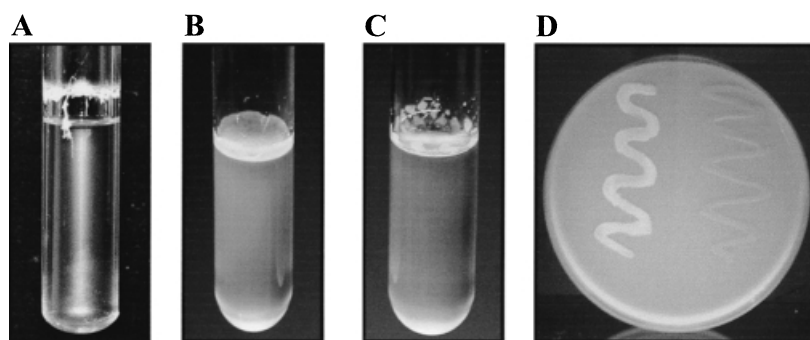
**We report here a new screening method based on the fluorescence of colonies on calcofluor agar plates to identify transposon insertion mutants of *Salmonella enteritidis* that are defective in biofilm development. The results not only confirmed the requirement of genes already described for the modulation of multicellular behaviour in *Salmonella typhimurium* and other species, but also revealed new aspects of the biofilm formation process, such as two new genetic elements, named as *bcsABZC* and *bcsEFG* operons, required for the synthesis of an exopolysaccharide, digestible with cellulase. Non-polar mutations of *bcsC* and *bcsE* genes and complementation experiments demonstrated that both operons are responsible for cellulose biosynthesis in both *S. enteritidis* and *S. typhimurium*. Using two different growth media, ATM and LB, we showed that the biofilm produced by *S. enteritidis* is made of different constituents, suggesting that biofilm composition and regulation depends on environmental conditions. Bacterial adherence and invasion assays of eukaryotic cells and *in vivo* virulence studies of cellulose-deficient mutants indicated that, at least under our experimental conditions, the production of cellulose is not involved in the virulence of *S. enteritidis*. However, cellulose-deficient mutants were more sensitive to chlorine treatments, suggesting that cellulose production and biofilm formation may be**

**an important factor for the survival of *S. enteritidis* on surface environments.**

## Introduction

*Salmonella enteritidis*, an important food-borne enteric pathogen, has been shown to form biofilms on materials of different nature and under different growth conditions (Korber *et al.*, 1997; Austin *et al.*, 1998; Solano *et al.*, 1998; Bradshaw and Marsh, 1999). However, nothing was known about the genetic requirements of this pathogen during the biofilm formation process until very recently, when Zogaj *et al.* (2001) demonstrated that natural *S. enteritidis* isolates showed the multicellular and aggregative behaviour [rdar (red, dry and rough) morphotype] previously described in *Salmonella typhimurium* (Romling *et al.*, 1998a,b; 2000; Romling and Rohde, 1999). *S. typhimurium* rdar cells have been shown to absorb the Congo red dye, to adhere to plastic surfaces and glass in an assay used to analyse biofilm formation (Romling *et al.*, 1998b) and to form a pellicle with a tight bacterial network at the air–liquid interface when incubated in rich medium in standing culture at room temperature (Romling and Rohde, 1999). It has been shown that knocking out the gene encoding for the subunit for thin aggregative fimbriae, AgfA, results in pink colony formation (the pdar morphotype; Romling *et al.*, 1998b; Romling and Rohde, 1999). The cells in the pdar colony were still connected in an elastic fashion, but no pellicle was formed in standing culture, indicating that thin aggregative fimbriae are one of the compounds of the extracellular matrix (Romling *et al.*, 1998b; Romling and Rohde, 1999). On the other hand, insertional mutation on *adrA*, a gene encoding a putative transmembrane protein, changed the rdar morphotype to a brown colony (the bdar morphotype). In this case, the absence of an unknown extracellular substance caused the pellicle formed in standing culture to be much more fragile (Romling *et al.*, 2000). Multicellular behaviour has been shown to be positively regulated at the onset of the stationary phase by *agfD*, a gene encoding a putative response regulator of thin aggregative fimbriae expression (Romling *et al.*, 2000). Deletion of *agfD* changed the red, wrinkled, dry and spreading colony of the rdar morphotype to a white and smooth colony (the saw morphotype). Such mutants completely lacked all forms of multicellular behaviour. In addition, the sigma factor *rpoS* and OmpR have been reported to be required

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**Fig. 1.** Biofilm formation phenotypes of natural *S. enteritidis* isolates.

A. The biofilm in ATM was visualized as a ring of cells adhered to the glass wall at the air–liquid interface after 4 h of incubation at 37°C under strong shaking conditions.

B. Biofilm formation in standing LB broth was visualized after 96 h of incubation at room temperature as a floating pellicle at the air–broth interface that totally blocked the surface of the culture and could not be dispersed by shaking.

C. A few natural isolates from the environment formed a pellicle of fragile appearance in LB at the air–broth interface, which could easily be disrupted by shaking.

D. Biofilm-forming strains in LB fluoresced on calcofluor agar plates (left), whereas none of the strains that did not form a biofilm in LB fluoresced under similar conditions (right).

for transcription of the *agf* operons (Romling *et al.*, 1998a,b). *rpoS* is also involved in the production of the unknown extracellular substance (Romling *et al.*, 2000).

Although most of the work relating to *S. typhimurium* multicellular morphotypes has been directed to knocking out specific genes, a more generalized approach based on the generation of random transposon insertion libraries has traditionally been used to screen for ‘biofilm genes’ by detecting the ability of mutant clones to adhere to the wells of microtitre plates. This approach has been used successfully with a number of Gram-negative bacteria, including *Escherichia coli* (Genevaux *et al.*, 1996; Pratt and Kolter, 1998), *Pseudomonas aeruginosa* (O’Toole and Kolter, 1998a), *Pseudomonas fluorescens* (O’Toole and Kolter, 1998b), *Pseudomonas putida* (Espinosa-Urgel *et al.*, 2000) and *Vibrio cholerae* (Watnick and Kolter, 1999), as well as Gram-positive bacteria such as *Staphylococcus epidermidis* (Muller *et al.*, 1993; Heilmann *et al.*, 1997), *Staphylococcus aureus* (Cucarella *et al.*, 2001) and *Streptococcus gordonii* (Loo *et al.*, 2000). The results obtained with Gram-negative bacteria have shown that biofilm formation is impaired by mutations in genes involved in flagellar-mediated motility, twitching motility, synthesis of exopolysaccharides, quorum sensing, outer membrane adhesins as well as global regulators of gene expression (for a review, see O’Toole *et al.*, 2000).

In the present work, we have carried out a random transposon mutagenesis and *S. enteritidis* biofilm-deficient mutants have been selected using a screening method based on the fluorescence of colonies on calcofluor agar plates. During the course of this study, Zogaj *et al.* (2001) showed that cellulose is a component of

the matrix produced by biofilm-forming *S. typhimurium* cells in LB and identified an operon, named *bcsABZC*, responsible for the synthesis of cellulose. Consistent with this, the results obtained in the present study demonstrate the significance of not only *bcsABZC* but also another operon that we named *bcsEFG*. Cellulose deficiency does not affect *S. enteritidis* virulence but increases the sensitivity to chlorine treatments, strongly suggesting that biofilm formation may be an important factor in the survival of *S. enteritidis* on surface environments. In addition, we identified genes already described for the modulation of multicellular behaviour in *S. typhimurium* and other species and also the involvement of a wide range of genetic pathways, which strongly suggests that our screening is covering novel aspects of the biofilm formation process.

## Results

### *Biofilm formation capacity and cellulose production is widespread among natural isolates of S. enteritidis*

In order to study the frequency of biofilm formation capacity of natural isolates of *S. enteritidis* and to select a strong biofilm producer strain for further genetic studies, we analysed the ability of 204 natural *S. enteritidis* isolates of different origins to produce a biofilm in both a nutrient-deficient medium (ATM) at 37°C and a rich medium (LB) at room temperature. The biofilm formation phenotypes were visualized as a ring of cells adhered to the glass wall at the air–liquid interface in ATM (Fig. 1A) and as a pellicle containing a tight bacterial network on the air–broth interface in LB (Fig. 1B). The results summarized in Table 1 show that, irrespective of their origin, nearly all (97%) the *S. enteritidis* strains tested were able

**Table 1.** Biofilm formation phenotypes of natural *S. enteritidis* isolates.

Origin	No. of isolates	Biofilm in ATM <sup>a</sup>	Phenotype		
			Pellicle in LB <sup>b</sup>	Morphotype <sup>c</sup>	Calcofluor binding <sup>d</sup>
Environmental	54	+ (89%) – (11%)	Rigid	rdar (50%)	+
			Absence	saw (39%)	–
			Fragile	bdar (5.5%)	+/-
Food	31	+ (100%)	Absence	saw (5.5%)	–
			Rigid	rdar (65%)	+
Animal	56	+ (100%)	Absence	saw (35%)	–
			Rigid	rdar (71%)	+
Clinical	63	+ (100%)	npc <sup>e</sup> (18%)	saw (11%)	–
			Absence	rdar (76%)	+
			Rigid	saw (24%)	–
Total	204	+ (97%)	Absence	rdar (66%)	+
			Rigid	npc <sup>e</sup> (5%)	+
			Fragile	bdar (1.5%)	+/-
			Absence	saw (27.5%)	–

a. Biofilm attached to the wall at the air–liquid interface after incubation in ATM at 37°C for 4 h.

b. Pellicle at the air–liquid interface formed in standing rich culture was examined visually after growth in 5 ml of LB at room temperature for 96 h.

c. Morphotype on CR plates at 28°C.

d. The relative level of calcofluor binding was assessed qualitatively, using clinical wild-type strains of known biofilm-forming capacity as reference (Solano *et al.*, 1998).

e. Morphotype not characterized previously.

to produce a biofilm in ATM (ATM+). The few isolates unable to produce a biofilm in ATM (ATM–) came from the environment. With respect to LB biofilm formation, 71% of the isolates tested produced a rigid pellicle in LB (LB+). Interestingly, although most of them (93%) showed the expected rdar morphotype on Congo red agar, the rest displayed a smooth coloured colony morphology not characterized previously. Three environmental isolates were characterized by the presence of a pellicle of fragile appearance at the air–broth interface, which could easily be disrupted by shaking (Fig. 1C) and was related to the bdar morphotype. Finally, 27.5% of the isolates tested were characterized by the total absence of a biofilm (LB–) and presented the saw morphotype on Congo red agar plates. It is important to stress that a natural isolate of the pdar morphotype was never found.

#### *Calcofluor fluorescence phenotype is associated with S. enteritidis biofilm formation*

Calcofluor white has been widely used to identify mutants defective in the production of exopolysaccharides in different bacterial species such as *Rhizobium meliloti* (Leigh *et al.*, 1985), *Myxococcus xanthus* (Ramaswamy *et al.*, 1997) and *Agrobacterium tumefaciens* (Thomashow *et al.*, 1987). We therefore analysed the calcofluor phenotype of the 204 natural *S. enteritidis* isolates under study. The results summarized in Table 1 showed that all LB+ strains fluoresced strongly under long-wave UV light on calcofluor agar plates (Fig. 1D), whereas strains that formed a fragile pellicle in LB (bdar) fluoresced with lower intensity, this difference being significant enough to dif-

ferentiate them. None of the LB– strains (saw) was able to fluoresce under similar conditions. Based on this strong correlation, we concluded that calcofluor fluorescence phenotype could be a novel screening method for isolating biofilm-deficient mutants of *S. enteritidis*.

#### *Screening for S. enteritidis mutants defective in biofilm formation*

*Salmonella enteritidis* 3934, a clinical isolate capable of forming a biofilm in both ATM (Solano *et al.*, 1998) and LB, was selected to be subjected to transposon mutagenesis. A collection of 10 000 kanamycin-resistant MudJ insertion mutants was screened for their ability to bind calcofluor on agar plates. Mutants defective in calcofluor-binding activity (did not fluoresce or fluoresced with lower intensity than the wild-type strain) were grown in LB broth, and only strains exhibiting growth rates indistinguishable from the wild type are discussed further. To confirm that each mutant arose from an independent insertion event, a Southern blot of *Pst*I-digested total DNA from each mutant was probed with a digoxigenin (DIG)-labelled MudJ transposon fragment. In addition, to exclude fortuitous problems, such as spontaneous mutations, each MudJ insertion was back-crossed into the wild-type strain 3934 by P22 transduction. As shown in Table 2, mutants were classified into three groups according to their deficiency in biofilm formation in LB and/or ATM. Group I includes mutants that were totally unable to form a biofilm in both LB and ATM (saw morphotype). Group II includes mutants that were either unable to produce a biofilm or formed a very thin pellicle in LB but remained able to

**Table 2.** Phenotypes and molecular analysis of *S. enteritidis* biofilm-deficient mutants.

Group	Phenotype			Genes already described/Reference <sup>d</sup>	Accession no.	Homologous protein	BLAST description		Organism
	Pellicle in LB <sup>a</sup>	Biofilm in ATM <sup>b</sup>	Morphotype <sup>c</sup>				Function of the homologous protein	BLAST description	
I	Absence	-	saw	Loo <i>et al.</i> (2000)	I55076	Pgm	Polysaccharide biosynthesis Phosphoglucosyltransferase	Polysaccharide biosynthesis Phosphoglucosyltransferase	<i>E. coli</i>
II	Absence	+	npc <sup>e</sup>		Q05878	CyaA	Adenylate cyclase	Polysaccharide biosynthesis	<i>S. typhimurium</i>
	Absence	+	saw		P09200	Fbpase	Fructose-1,6-bisphosphatase	Polysaccharide biosynthesis	<i>E. coli</i>
	Absence	+	npc	Danese <i>et al.</i> (2000)	P32054	Gmd	GDP-D-mannose 4,6-dehydratase	Polysaccharide biosynthesis	<i>E. coli</i>
	Absence	+	npc		AAG24816	Wcal	Colanic acid biosynthesis fucosyl transferase	Polysaccharide biosynthesis	<i>S. typhimurium</i>
	Thin pellicle	+	saw		P11537	Pgi	Phosphoglucose isomerase	Polysaccharide biosynthesis	<i>E. coli</i>
	Absence	+	npc	Makin and Beveridge (1996); Williams and Fletcher (1996)	AAF33462	WeeE	Lipopolysaccharide and enterobacterial common antigen biosynthesis	Lipopolysaccharide and enterobacterial common antigen biosynthesis	<i>S. typhimurium</i>
	Absence	+	npc		AAF33461	WzxE	LPS and ECA biosynthesis protein	LPS and ECA biosynthesis protein	<i>S. typhimurium</i>
	Absence	+	rdar		AAF33465	RffG'	dTDP-Glucose 4,6-dehydratase	LPS and ECA biosynthesis protein	<i>S. typhimurium</i>
	Absence	+	npc		AA23893	SdhC	Energy metabolism	Energy metabolism	<i>E. coli</i>
	Absence	+	npc		AA23896	SdhB	Succinate dehydrogenase small subunit	Succinate dehydrogenase small subunit	<i>E. coli</i>
III	Absence	+	npc		AA23900	SucD	Succinyl-CoA synthetase alpha-subunit	Succinyl-CoA synthetase alpha-subunit	<i>E. coli</i>
	Absence	+	saw	Romling <i>et al.</i> (1998b)	O54294	AgfD	Adhesins	Adhesins	<i>S. typhimurium</i>
	Absence	+	rdar	O'Toole <i>et al.</i> (2000)	P26609	FliS'	Motility function	Motility function	<i>S. typhimurium</i>
	Absence	+	saw	Romling <i>et al.</i> (2000) Adams and McLean (1999)	AA61628	RpoS	Regulatory functions	Regulatory functions	<i>S. typhimurium</i>
	Thin pellicle	+	npc		AAD38080	CytR	RNA polymerase $\sigma$ factor	RNA polymerase $\sigma$ factor	<i>S. typhimurium</i>
	Thin pellicle	+	npc		AAF34823	BarA	Transcriptional regulator	Transcriptional regulator	<i>S. typhimurium</i>
	Absence	+/-	rdar		P14846	CarB'	Sensor kinase	Sensor kinase	<i>S. typhimurium</i>
	Absence	+/-	npc	Ausubel <i>et al.</i> (1990)	P29847	CysE'	Amino acid and pyrimidine biosynthesis	Amino acid and pyrimidine biosynthesis	<i>S. typhimurium</i>
	Fragile	-	bdar	Zogaj <i>et al.</i> (2001)		BcsABZC BcsEFG	Carbamoylphosphate synthase large chain Serine acetyl transferase Cellulose synthesis Cellulose biosynthesis	Carbamoylphosphate synthase large chain Serine acetyl transferase Cellulose synthesis Cellulose biosynthesis	<i>S. typhimurium</i>

a. Pellicle at the air-liquid interface formed in standing rich culture was examined visually after growth in 5 ml of LB at room temperature for 96 h.

b. Biofilm attached to the wall at the air-liquid interface after incubation in ATM at 37°C for 4 h.

c. Morphotype on CR plates at 28°C.

d. Homologous genes or functions in which they are involved have already been described as playing a role in the biofilm formation process of different microorganisms.

e. Morphotype not characterized previously.

f. These strains show a medium level of calcofluor binding, using the wild-type strain *S. enteritidis* 3934 as reference.

produce a biofilm in ATM (these mutants showed a variety of morphotypes on Congo red agar, including the *rdar* and *saw* morphotype and a smooth coloured colony not characterized previously). Group III includes mutants that formed a fragile pellicle in LB (*bdar* morphotype) and were unable to produce a biofilm in ATM.

An additional microtitre plate assay was performed in order to correlate these results with the standard procedure for studying biofilm formation. We found a very strong correlation between pellicle formation in LB and the ability to form a biofilm on polyvinyl chloride (PVC), as mutants in group I and II, with the exception of *rpoS*, *rffG* and *barA* mutants, were unable to form a biofilm in microtitre wells. In contrast, mutants in group III displayed a slightly weaker capacity to adhere to the microtitre plate compared with the wild-type strain, suggesting that, if we had only used the standard method for the screening of *S. enteritidis* biofilm-deficient mutants, we would probably have failed to notice mutants in group III (see *Supplementary material*).

Sequencing the regions adjacent to the transposon insertion site and homology searches using the BLAST 2.0 program at the NCBI server or unfinished *S. typhimurium* genome sequence programs showed that only a mutation in the gene encoding for phosphoglucomutase rendered strain 3934 unable to form a biofilm in both LB and ATM (group I, Table 2). In contrast, mutations in genes involved in multiple pathways included in polysaccharide, lipopolysaccharide, enterobacterial common antigen, adhesins, amino acid and pyrimidine biosynthesis, energy metabolism, regulatory functions and motility rendered *S. enteritidis* 3934 unable to form a biofilm or pellicle in LB, although the biofilm-forming capacity in the nutrient-deficient medium, ATM, remained intact (group II, Table 2). It is worth noting that thin aggregative fimbriae,

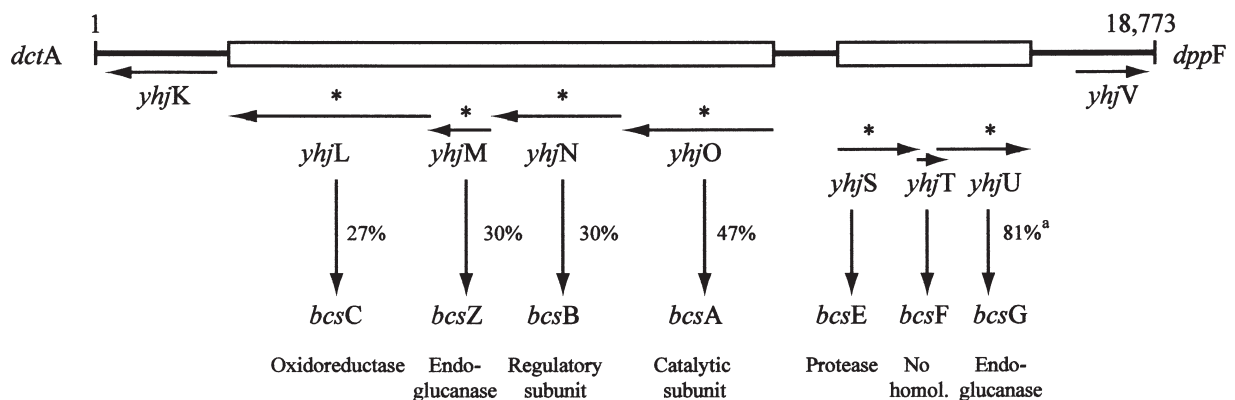
which have already been described as essential for *S. typhimurium* multicellular and aggregative behaviour (Romling *et al.*, 2000), are not required for biofilm formation in ATM.

On the other hand, all the mutants that formed a fragile pellicle in LB (*bdar* morphotype) and did not produce a biofilm in ATM presented mutations along the same chromosomal region, which was showed to be highly homologous to the *dctA*–*dppF* intergenic region of *Escherichia coli* (group III, Table 2). Our screening was validated by the identification of genes and subsequent structures that have already been described as being involved in the biofilm formation process of different microorganisms, namely phosphoglucomutase (Loo *et al.*, 2000), serine acetyltransferase (Yildiz and Schoolnik, 1999), RpoS (Adams and McLean, 1999; Romling *et al.*, 2000), colanic acid (Danese *et al.*, 2000), lipopolysaccharide (Makin and Beveridge, 1996; Williams and Fletcher, 1996), flagella (for a review, see O'Toole *et al.*, 2000) and fimbriae (Romling *et al.*, 1998b; Romling and Rohde, 1999).

#### Two operons located in the *dctA*–*dppF* region affect cellulose synthesis and biofilm formation in *S. enteritidis*

DNA sequence analysis comparisons demonstrated that the *dctA*–*dppF* intergenic regions of *E. coli* K-12 (Sofia *et al.*, 1994) and *S. typhimurium* LT2 (Genome Sequencing Center, Washington University School of Medicine, MO, USA) share a very high level of similarity (79% identity at the nucleotide level) throughout the entire region, with comparable open reading frames (ORFs). The genes are in the same order and organized into two operons transcribed in opposite directions (Fig. 2).

Analysis of the genomic database sequence revealed that genes in the first operon, *yhjONML*, show high homol-



**Fig. 2.** Structure of the bacterial cellulose synthesis operons, *bcsABZC* and *bcsEFG*. The two gene clusters have been represented to scale. The putative functions of genes in the first operon were deduced from the homology of the *yhj* genes to genes in the bacterial cellulose operon of *A. xylinum* ATCC53582 (X54676) and to an endoglucanase-encoding gene of *A. xylinum* strain BPR2001 (BAA31461). The percentages of amino acid identities are shown. The putative endoglucanase function in the second operon was deduced from the homology at the nucleotide level (\*) between 311 nucleotides at the end of the sequences of *yhjU* and the endoglucanase gene of *C. uda* (M36503). The asterisks indicate the genes in which the MudJ element in cellulose-minus mutants was inserted.

ogy to genes of the bacterial cellulose synthase (*bcs*) operon (Saxena *et al.*, 1990) and an endoglucanase-encoding gene (cellulase family D; Nakai *et al.*, 1998) of *Acetobacter xylinum*. In this organism, *bcsA* encodes for the catalytic subunit of the cellulose synthase and polymerizes uridine 5'-diphosphate glucose to cellulose. *bcsB* encodes for the cellulose synthase regulatory subunit that binds to the activator, cyclic-di-GMP (Ross *et al.*, 1991), and *bcsC* has been shown to be required for maximal cellulose synthesis. On the other hand, the endoglucanase endohydrolyses 1,4-beta-D-glucosidic linkages in cellulose (Standal *et al.*, 1994). Similarly, a database homology search of genes in the second operon showed that *yhjS* encodes for a putative protease, *yhjT* displayed insufficient homology to known proteins to be assigned a putative function, and *yhjU* showed homology at the nucleotide level to an endoglucanase gene (cellulase family D) of *Cellulomonas uda* (Nakamura *et al.*, 1986; 81% of identity between 311 nucleotides at the end of both sequences), although significant homology was not found between these two proteins at the amino acid level. Two additional genes, *yhjK*, which had no significant similarity to any protein or ORF in the databases, and *yhjV*, encoding a putative transporter protein, flank the operons described. Interestingly, the MudJ element in our *bdar* mutants was independently inserted three times in *yhjO*, once in *yhjN*, once in *yhjM* and four times in *yhjL*. On the other hand, the MudJ element in *bdar* mutants was also found to be independently inserted three times in *yhjS* and twice in *yhjU*. All these mutants formed a fragile pellicle in LB, lacked the ability to produce a biofilm in ATM, fluoresced with low intensity on calcofluor plates and showed the *bdar* morphotype.

To investigate the role of both operons in the biofilm formation process and to exclude polar effects on downstream genes, one-step insertional mutations were carried out in *yhjL*, *yhjS*, *dctA* and *dppF*. The resulting strains, 3934 *yhjL*-km and 3934 *yhjS*-km verified by Southern blot and polymerase chain reaction (PCR), formed a fragile pellicle in LB, lacked the ability to produce a biofilm in ATM, fluoresced with low intensity on calcofluor plates and showed the *bdar* morphotype. On the other hand, mutant strains 3934 *dctA*-km and 3934 *dppF*-km showed exactly the same phenotypes as the wild-type strain. Finally, to prove that the cellulose minus phenotype (*bdar*) resulted from the disruption of both operons, mutants 3934 *yhjL*-km and 3934 *yhjS*-km were complemented with plasmids pUC:*yhjL* and pGEM:*yhjSTU* respectively. As a result, the complemented strains gained the ability to form a rigid pellicle in LB and a biofilm in ATM, fully fluoresced on calcofluor agar plates and showed the *rdar* morphotype.

As mentioned before, Zogaj *et al.* (2001) have recently described how *yhjONML* is responsible for cellulose

biosynthesis in *S. typhimurium*. Thus, these authors renamed this operon *bcsABZC* (bacterial cellulose synthesis). Similarly, based on the analysis described above, we renamed the genes in the second operon of the *dctA*-*dppF* intergenic region, *yhjSTU*, as *bcsEFG*.

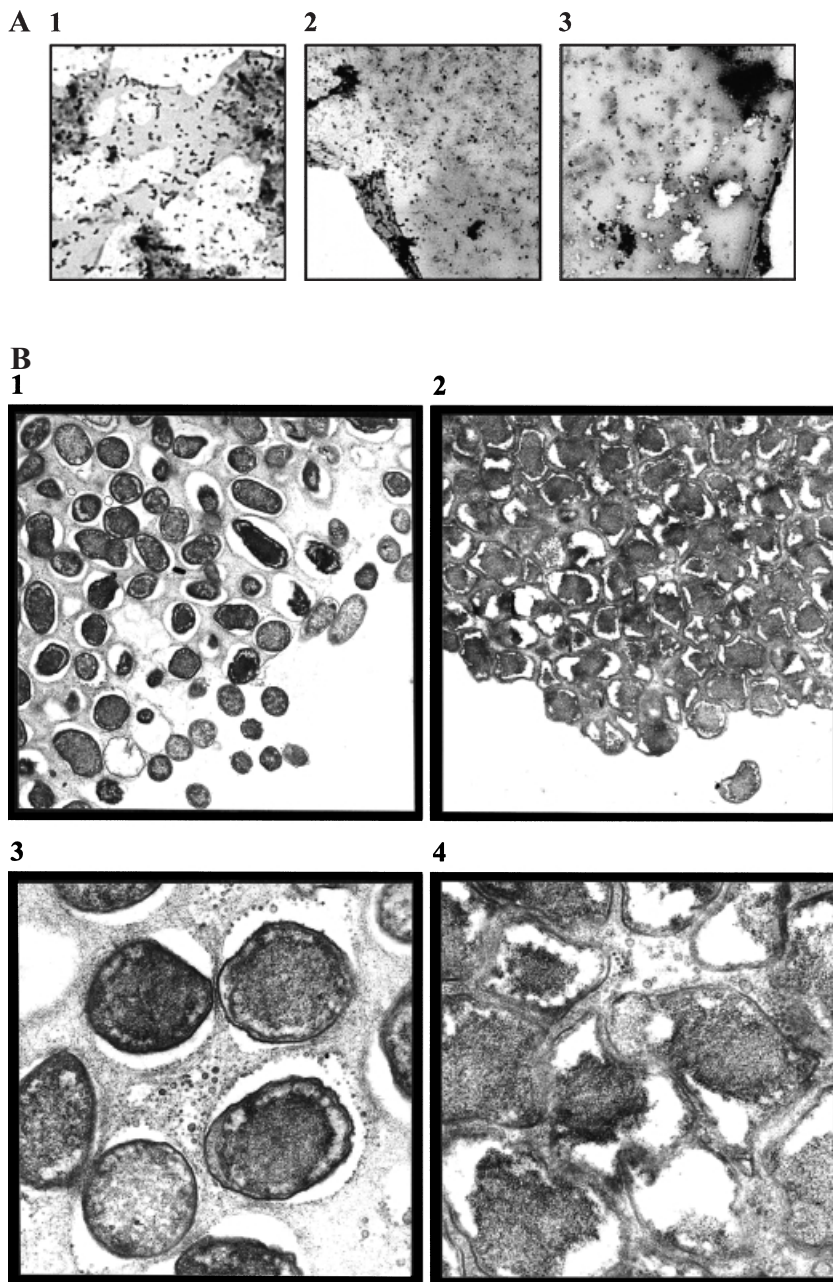
*Salmonella typhimurium cellulose production and biofilm formation are also affected by bcs mutations*

*bcsC* (formerly *yhjL*) and *bcsE* (formerly *yhjS*) mutations were moved from *S. enteritidis* 3934 to strain 5996, another *S. enteritidis* clinical isolate, and to three *S. typhimurium* biofilm-forming strains of clinical origin via P22 transduction. The resulting mutant strains, verified by Southern blot and PCR, formed a fragile pellicle in LB, lacked the ability to produce a biofilm in ATM, fluoresced with low intensity on calcofluor plates and showed the *bdar* morphotype.

*Cellulose-minus mutants are still connected by an uncharacterized material of polysaccharide nature when incubated in LB broth*

The data presented above suggested that the biofilm formed by *S. enteritidis* in ATM was mainly composed of cellulose, whereas additional components were taking part in the biofilm formed in LB. Alcian blue staining and direct observation by phase-contrast microscopy of the biofilms formed by the wild-type strain *S. enteritidis* 3934 in ATM and LB as well as the fragile pellicle formed by its mutants, *S. enteritidis* 3934 *yhjL*-km and *yhjS*-km, in LB revealed the presence in all cases of a close bacterial network embedded in a matrix stained with the dye (Fig. 3A). Ultrathin sections were stained with ruthenium red and analysed by transmission electron microscopy. This method, which preferentially stains acidic polysaccharides (Patterson *et al.*, 1975), revealed an abundant, ruthenium red-positive matrix between bacteria in all samples. The stained matrix presented a more organized pattern in the case of the wild-type strain than in the case of cellulose mutants (Fig. 3B). As expected, mutations in either bacterial cellulose operon (*bcsABZD* and *bcsEFG*) present in the *dctA*-*dppF* intergenic region led to the same matrix structure.

Cellulase treatment assays were carried out to confirm these results further. As expected, the treatment of a formed biofilm with pure cellulase partially digested the rigid structure in LB and totally disrupted the biofilm formed in ATM. In addition, the presence of cellulase in the media during the biofilm formation process in the wild-type strain *S. enteritidis* 3934 led to the formation of a low-consistency biofilm in LB and a total absence of a biofilm in ATM.



**Fig. 3.** Microscopic analyses of polysaccharides present in the biofilm. A. Direct observation by phase-contrast microscopy after staining the samples with Alcian blue 8GX revealed in all cases a tight bacterial network embedded in a polysaccharide matrix. 1, biofilm formed by the wild-type strain *S. enteritidis* 3934 in ATM; 2, biofilm formed by the wild-type strain *S. enteritidis* 3934 in LB; 3, fragile pellicle formed by its mutants, *S. enteritidis* 3934 *yhjL*-km and *yhjS*-km, in LB. B. Ruthenium red-stained thin sections of: 1 and 3, the biofilm formed by the wild-type strain *S. enteritidis* 3934 in LB; 2 and 4, fragile pellicle formed by its mutants, *S. enteritidis* 3934 *yhjL*-km and *yhjS*-km, in LB. Electron micrographs demonstrated the presence of closely packed bacteria and a stained matrix between cells in all samples. Magnification, 1 and 2  $\times$  7500; 3 and 4  $\times$  30 000.

#### Virulence studies of cellulose mutants

In order to test whether the lack of production of cellulose might confer a virulence defect on cellulose-minus mutants, several *in vitro* and *in vivo* studies were carried out. As a result, no differences were found between the wild-type and the cellulose mutant 3934 *yhjL*-km strains after performing survival assays in BALB/c mice infected orally or intraperitoneally (i.p.) and in 1-day-old chickens infected i.p. (Table 3). Studies carried out with the same strains using the ligated ileal loop co-infection model confirmed these results. Furthermore, no differences were detected in the ability of the wild-type and mutant strains

incubated under several environmental conditions to adhere to and invade HEp-2 cells during either standard or competition assays (data not shown). Overall, these results indicate that, at least under our experimental conditions, the production of cellulose is not involved in the virulence of *S. enteritidis* strains.

#### Cellulose mediates chlorine survival

To learn whether cellulose might be responsible for chlorine resistance and therefore the survival of *S. enteritidis* strains in water supplies and food-processing chains, we carried out survival experiments of the wild-type strain

Animal model	Infective dose (cfu)	Dead animals/inoculated animals	
		3934	3934 <i>yhjL-km</i>
Orally infected mice	10 <sup>4</sup>	1/5	1/5
	10 <sup>5</sup>	3/5	3/5
i.p. infected mice	10 <sup>0</sup>	3/5	2/5
	10 <sup>3</sup>	5/5	5/5

Animal model	Infective dose (cfu)	LD <sub>50</sub>	
		3934	3934 <i>yhjL-km</i>
i.p. infected chickens	2 × 10 <sup>1</sup> –2 × 10 <sup>6</sup>	3 × 10 <sup>3</sup>	7.4 × 10 <sup>2</sup>

**Table 3.** Lethality of a cellulose-minus mutant in three animal models.

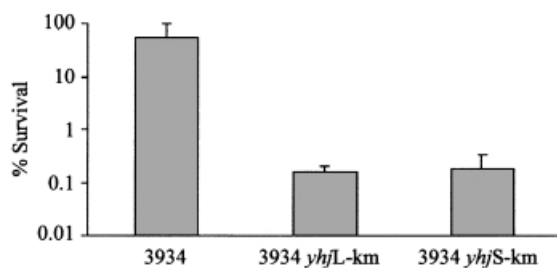
and cellulose-minus mutants after incubation in ATM. We used a concentration of NaOCl (30 p.p.m.) that is 100- to 200-fold higher than the free chlorine concentrations typically obtained in municipal water supplies and is into the concentration range used as a sanitizer for food plants. After a 20 min exposure period, 75% of the wild-type cells survived NaOCl exposure. In contrast, only 0.3% of cellulose-deficient mutant cells survived under the same experimental conditions (Fig. 4).

## Discussion

Studies initiated several decades ago started to appreciate that, in most natural environments, growth as a biofilm is the prevailing microbial lifestyle (Costerton *et al.*, 1978). Accordingly, in this report, we have demonstrated that the great majority of the *S. enteritidis* isolates tested, regardless of their origin, were able to form a biofilm. Therefore, the biofilm seems to comprise a normal and favourable phenotype for the microbial life of *S. enteritidis* in the environment. Initially, we analysed biofilm formation ability in different laboratory conditions as, consistent with other authors (for a review, see Sutherland, 2001), we consider that any single biofilm formed under any specific set of parameters may well be unique to that single environ-

ment. In support of this fact, we observed that some of the *S. enteritidis* strains capable of forming a biofilm in ATM were unable to form one in LB and that the macroscopic appearance of the biofilm in ATM and LB differed from each other. In order to determine the genetic requirements to produce the polymeric matrix of the biofilm formed by *S. enteritidis* in both laboratory conditions, we carried out a transposon mutagenesis on a clinical isolate and tested the mutants for deficiency of fluorescence on calcofluor agar plates. This fluorescent dye was chosen because it binds  $\beta$ -glucans, and it is well established that mutants unable to synthesize exopolysaccharides (EPS) are unable to form biofilms (O'Toole *et al.*, 2000; Sutherland, 2001).

We found that mutants affected in several previously uncharacterized ORFs of two operons located in the *dctA–dppF* intergenic region formed a fragile pellicle in LB and did not produce a biofilm in ATM. Interestingly, the protein sequence deduced from the ORFs of the first operon showed significant homology with the cellulose biosynthesis operon of *A. xylinum* (Saxena *et al.*, 1990) and an endoglucanase encoded in an operon 3.0 kb upstream of the *bcs* operon (Nakai *et al.*, 1998). Treatment of the biofilm produced by wild-type bacteria with pure cellulase rendered the same fragile pellicle produced by these mutants in LB and abolished biofilm formation in ATM, strongly suggesting that those mutants were deficient in the production of cellulose. Our results indicate that not only *bcsABZD* but also *bcsEFG* is involved in the cellulose biosynthesis process, as mutants in any of those operons yielded a similar phenotype. In order to confirm these results, we used a simple and highly efficient method for one-step inactivation of chromosomal genes and complementation experiments to demonstrate that both operons are responsible for cellulose biosynthesis in *S. enteritidis* and *S. typhimurium*. The role of the *bcsEFG* operon in the biosynthesis of cellulose remains unclear, as sequence databank searches only yielded striking similarities at the nucleotide level at the end of *bcsG* and the endoglucanase gene (cellulase family D) of *C. uda* (Nakamura *et al.* (1986). As stated above, in the



**Fig. 4.** Differences in resistance to the bactericidal activity of chlorine between wild type and cellulose-deficient mutants. The surviving bacteria were enumerated by viable plate counts, and their numbers were compared with that of control bacteria that had not been incubated with NaOCl. This number was defined as 100% survival. Bars represent the mean values, and error bars represent the standard errors of the means ( $n = 3$ ).

case of *A. xylinum*, the endoglucanase gene is encoded outside the cellulose synthase operon. In contrast, this gene is part of the operon in *A. tumefaciens* and *Rhizobium leguminosarum* (Matthysse *et al.*, 1995; Ausmees *et al.*, 1999). It is remarkable that, in the case of *S. typhimurium*, one endoglucanase is part of the cellulose synthase operon, and a second endoglucanase is located in a different operon, both of them being required for cellulose synthesis.

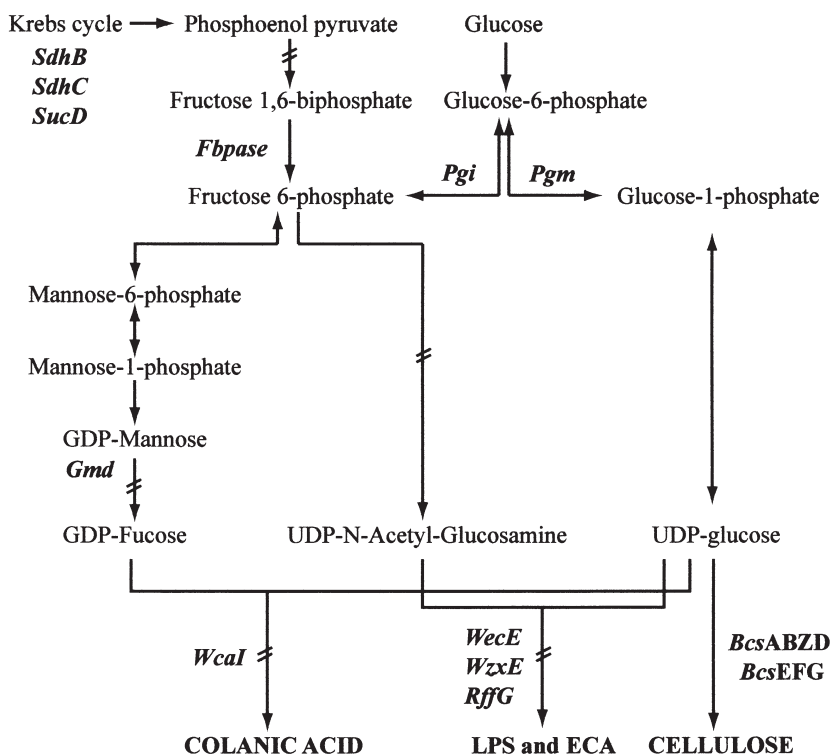
Enzymes leading to EPS formation can be roughly divided into four groups: enzymes responsible for the initial metabolism of a carbohydrate; enzymes involved in sugar nucleotide synthesis and interconversion; glycosyl-transferases that form the repeating unit attached to the glycosyl carrier lipid; and translocases and polymerases that form the polymer. Our results showed that, under LB growth conditions, several mutations affecting enzymes included in these four groups and involved in the synthesis of colanic acid, lipopolysaccharide, the enterobacterial common antigen and cellulose rendered *S. enteritidis* unable to form a biofilm fully (Fig. 5).

In contrast to the complexity of polysaccharide components required for biofilm formation in LB, our study has shown that, in ATM, only mutants affected in cellulose production or the phosphoglucumutase gene were unable to produce a biofilm and, therefore, cellulose seems to be the main component of the biofilm produced in these conditions. It would be interesting to study further how and why mutations included in group II affect cellulose

production in LB conditions but not in ATM. Furthermore, whereas regulation of cellulose synthesis in LB depends on AgfD, RpoS, CytR and BarA, these regulators do not affect the process in ATM, as the capacity to produce a biofilm remained intact in the respective mutants. These results are significant because, consistent with Romling *et al.* (2000), we have observed that mutations in *agfD* and *rpoS*, which regulate transcription of fimbrial operons, and *adrA* yield *Salmonella* unable to form a biofilm in LB. In contrast, *agfD* and *rpoS* mutants are not affected in their capacity to produce a biofilm in ATM. Therefore, in ATM, fimbriae are not a component of the biofilm, and cellulose production does not depend on the interaction of AdrA with Bcs proteins.

Cellulose production in a strictly nutrient-deficient medium such as ATM supports numerous reports demonstrating that the presence of excess available carbon substrate and limitations in other nutrients, such as nitrogen or phosphate, promote the synthesis of EPS (Sutherland, 2001). Thus, in *A. xylinum* washed cells, deprived of a nitrogen source, the production of cellulose continues when supplied with an adequate supply of carbon substrate and does not depend on net protein synthesis. Similarly, cellulose synthesis in *A. tumefaciens* has also been demonstrated to occur in resting cells (Ross *et al.*, 1991).

Bacterial cellulose appears to fulfil a structural role in the sense that it may confer mechanical, chemical or biological protection within the natural habitat, as in *A.*



**Fig. 5.** Generalized scheme illustrating the role of different enzymes in the pathways leading to the synthesis of polysaccharides involved in the biofilm phenotype. Interrupted lines represent pathways in which intermediate steps are not shown. Colanic acid, lipopolysaccharide, enterobacterial common antigen and cellulose are all required for a fully expressed biofilm in LB. Of these, only cellulose contributes to the biofilm formed in ATM. LPS, lipopolysaccharide; ECA, enterobacterial common antigen; SdhB, succinate dehydrogenase small subunit; SdhC, succinate dehydrogenase cytochrome *b556*; SucD, succinyl-CoA synthetase alpha-subunit; Pgm, phosphoglucumutase; Pgi, phosphoglucose isomerase; Fbpase, fructose-1,6-bisphosphatase; Gmd, GDP-D-mannose 4,6-dehydratase; WcaI, colanic acid biosynthesis fucosyl transferase; WecE and WzxE, LPS and ECA biosynthesis protein; RffG, dTDP-glucose 4,6-dehydratase; Bcs, bacterial cellulose biosynthesis.

*xylum* and *Sarcina ventriculi*, or facilitate cell adhesion processes necessary for symbiotic or infectious interactions, as in *Rhizobium* and *Agrobacterium* species (Ross *et al.*, 1991). In the case of *S. enteritidis*, we were not able to demonstrate any role for cellulose in the virulence of the bacteria. On the other hand, the chlorine survival experiment described in this study suggests that cellulose is directly responsible for the chlorine resistance of the wild-type strain, that is when using a chlorine concentration in the range authorized by the US Department of Agriculture for use on equipment surfaces in food-processing plants. Similarly, Yildiz and Schoolnik (1999) have described how the production of an exopolysaccharide, EPS<sup>ETr</sup>, by *Vibrio cholerae* O1, biotype El Tor, plays a role in the survival of the organism within aquatic habitats, as it confers chlorine resistance and biofilm formation capacity. Thus, cellulose production and biofilm formation by *S. enteritidis* could represent an essential phenotype of the organism's life cycle as, presumably, the better adapted a bacterium is to survival in its environment, the more likely it is to colonize an animal host.

In addition to the polysaccharides described, a defect in fimbriae or flagella affected the biofilm formation process only in LB. The role of thin aggregative fimbriae in the biofilm formed by *S. typhimurium* cells in LB has already been demonstrated (Romling *et al.*, 1998b). On the other hand, one of the mutants obtained in this study presented a transposon insertion in the *fljS* gene. It has been shown that the number of flagella produced in an *S. typhimurium fljS* mutant was about twice as many as those in the wild-type strain, as *fljS* is involved in the negative regulation of FlgM export, which acts as an anti-sigma factor (Yokoseki *et al.*, 1996). Thus, FlgM depletion causes enhanced expression of the whole flagellar regulon, resulting in the overproduction of flagellar structures. Also, the *fljS* mutant showed impaired flagellin export and produced flagellar structures with short filaments. Furthermore, in several microorganisms, evidence supports the fact that flagellum and exopolysaccharide synthesis are inversely regulated (Garrett *et al.*, 1999; Prigent-Combaret *et al.*, 1999; Watnick *et al.*, 2001). For instance, in *E. coli*, biofilm-associated cells repress the transcription of flagellar genes and increase the transcription of colanic acid biosynthetic genes (Prigent-Combaret *et al.*, 1999). This suggests that, in the *fljS* mutant obtained in this study, the impairment in producing a biofilm is probably caused by the overproduction of short filament flagellar structures together with the repression of transcription of genes involved in exopolysaccharide production.

The results presented in this study provide an overview of the important functions required for biofilm formation in *S. enteritidis*. Although a detailed analysis of the genes identified and their specific roles is still in progress,

several conclusions can be drawn from this study: (i) correlation with the standard microtitre plate assay and the isolation of mutants defective in colanic acid biosynthesis, lipopolysaccharide, fimbriae, flagella, *rpoS*, phosphoglucomutase and serine acetyltransferase validates our screening, as these genes and structures have been implicated in biofilm formation on abiotic surfaces in other microorganisms; (ii) cellulose is one of the main components of the biofilm produced by *S. enteritidis* and *S. typhimurium*; (iii) two operons, *bcsABZD* and *bcsEFG*, are required for cellulose biosynthesis; (iv) biofilm composition and regulation depends on environmental conditions, and (v) cellulose does not play any role in *Salmonella* virulence under the experimental conditions tested, but may enable the survival of *Salmonella* within environmental surface habitats.

## Experimental procedures

### *Bacterial strains, plasmids and culture conditions*

A total of 204 *S. enteritidis* and three *S. typhimurium* natural isolates were used in this study. They were isolated in Navarra, Spain, from the environment, dairy products or infected patients. The most relevant bacterial strains and plasmids used and constructed in this study are listed in Table 4. *E. coli* XL1-Blue, *S. enteritidis* and *S. typhimurium* cells were grown in Luria-Bertani (LB) broth, in trypticase soy broth (TSB) and on LB agar or trypticase soy agar (TSA) (Pronadisa) with appropriate antibiotics at the following concentrations: kanamycin (Km), 30 µg ml<sup>-1</sup>; ampicillin (Am), 100 µg ml<sup>-1</sup>.

### *DNA manipulations*

Routine DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989; Ausubel *et al.*, 1990) unless otherwise stated. Plasmid DNA from *E. coli* was purified with a Quantum Prep plasmid kit (Bio-Rad). Plasmids were transformed into *E. coli* and *S. enteritidis* by either heat shock or electroporation. Transformants carrying the Red helper plasmid were made electrocompetent with the following protocol. Cells were grown overnight in LB broth Am at 30°C and then used to inoculate 500 ml of LB broth Am that was incubated with aeration at 30°C to an OD<sub>600</sub> of 0.2. Then, L-arabinose (Sigma) was added to a final concentration of 0.08%, and incubation continued until the OD<sub>600</sub> reached 0.7. The suspension was cooled down on ice for 15 min, and cells were made electrocompetent by washing twice with the same volume and then once with 40 ml of ice-cold 10% glycerol. Cells were finally resuspended in 1.5 ml of ice-cold 10% glycerol. Restriction enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. Oligonucleotides were obtained from Life Technologies (Table 4). Phage P22 HT105/1 int-201 (Schmieger, 1972) was used to carry out transductions between strains according to recommended protocols (Maloy *et al.*, 1996). Random MudJ insertions in *S. enteritidis* 3934 were carried out as described previously (Hughes and Roth, 1988). Pools of

Table 4. Strains, oligonucleotides and plasmids used in this study.

Strain, oligonucleotide and plasmid	Relevant characteristics				Morphotype <sup>d</sup>
	Genotype	Pellicle in LB <sup>a</sup>	Biofilm in ATM <sup>b</sup>	Calcofluor binding <sup>c</sup>	
<b>S. enteritidis</b>					
3934	Clinical isolate (Km <sup>s</sup> )	Rigid	+	+	rdar
3934 <i>yhjL</i> -km	3934 $\Delta$ <i>yhjL</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3934 <i>yhjS</i> -km	3934 $\Delta$ <i>yhjS</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3934 <i>dciA</i> -km	3934 $\Delta$ <i>dciA</i> ::Km <sup>r</sup>	Rigid	+	+	rdar
3934 <i>dppF</i> -km	3934 $\Delta$ <i>dppF</i> ::Km <sup>r</sup>	Rigid	+	+	rdar
5996	Clinical isolate (Km <sup>s</sup> )	Rigid	+	+	rdar
5996 <i>yhjL</i> -km	5996 $\Delta$ <i>yhjL</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
5996 <i>yhjS</i> -km	5996 $\Delta$ <i>yhjS</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
<b>S. typhimurium</b>					
5210	Clinical isolate (Km <sup>s</sup> )	Rigid	+	+	rdar
5210 <i>yhjL</i> -km	5210 $\Delta$ <i>yhjL</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
5210 <i>yhjS</i> -km	5210 $\Delta$ <i>yhjS</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3643	Clinical isolate (Km <sup>s</sup> )	Rigid	+	+	rdar
3643 <i>yhjL</i> -km	3643 $\Delta$ <i>yhjL</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3643 <i>yhjS</i> -km	3643 $\Delta$ <i>yhjS</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3647	Clinical isolate (Km <sup>s</sup> )	Rigid	+	+	rdar
3647 <i>yhjL</i> -km	3647 $\Delta$ <i>yhjL</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
3647 <i>yhjS</i> -km	3647 $\Delta$ <i>yhjS</i> ::Km <sup>r</sup>	Fragile	-	+/-	bdar
<b>E. coli</b>					
MC4100 hssc:km	Used as a template for amplification of the kanamycin resistance gene (kindly provided by P. Deleplaire)				
XL1Blue	Used for cloning assays				
<b>Oligonucleotide</b>					
<i>yhjL</i> 3	GCGCCAGCGGAGCGCGGCTGCATGTTAATAGAGGTGATTGGCAGGAGGCGCGCCGAAAGCCACGGTTGTGCTCAA'			3771-3830	+
<i>yhjL</i> 4	AAGCGGAAGCGTGTACGTCAGCAGCCGCTTACCGTAACTGGCGGGCTGAGGCTGCGCTCGTG'			3981-4040	-
<i>yhjS</i> 1	GATATATGACCATCGCGGCAACCCCTGGTCTGTTTATCTGCGGGTCAACAAGCCACGTTGTGCTCAA'			14359-14418	+
<i>yhjS</i> 2	TACGACCCAGATGCGCCATCGTTCGGCATTTAATACCGGGTCCGGCGGTTTGCCAGCGCTGAGGCTGCGCTCGTG'			14570-14629	-
<i>dciA</i> 1	GTAATCTCCGGATAGCAATAAATTAATAATGCTAAAGCCGTTACCCGTGCAAAAAGCCACGTTGTGCTCAA'			-781 to -722	+
<i>dciA</i> 2	GCCAGCTCAATTTCAATGATGATTGAGAGTTTTCCGAGGTCATTTCCGATCATCAATGCGCTGAGGCTGCGCTCGTG'			-570 to -511	-
<i>dppF</i> 1	CGCGCACCCAGACGTCGCGCGGAGCCGGTTCGTGGCAATCACTACGTCGCGGATCAAAGCCACGTTGTGCTCAA'			19195-19254	+
<i>dppF</i> 2	CTACGGTTCCTGAAATCCACCAAGAAAGTGGGGCAATTCGGAAGAGCCGTTGCTGAGCGCTGAGGCTGCGCTCGTG'			19406-19465	-
<i>yhjL</i> 5	GACGAATCGGTGAATTTACCTGA (EcoRI site underlined)			5893-5909	-
<i>yhjL</i> 6	GGAAGCTTTACAGTCAGCGTAA (HindIII site underlined)			2338-2353	+
<i>yhjS</i> 10	CAGGAGCGGCCATCTTTT			12699-12716	+
<i>yhjJ</i> 1	GGAAGCTTACTGCGGGTAAGGC ( <i>Hind</i> III site underlined)			16528-16544	+
<i>yhjL</i> 11	CATTGAGATCCTGCTGA			3456-3472	-
<i>yhjL</i> 12	AGCAGAAAGCCAAACGAT			4228-4244	-
<i>yhjS</i> 11	CTATCCCATCAGGAGGA			13738-13754	+
<i>yhjS</i> 12	GTCGTCGAGCAATCGCA			14642-14658	+
<i>dciA</i> 11	AACCGGTAATGGCCTA			-1098 to -1082	+
<i>dciA</i> 12	AAAGCATGAAAGCGGTG			-210 to -194	+
<i>dppF</i> 11	TACTCTTTAAGCTGCGG			18841-18857	-
<i>dppF</i> 12	CACATCCGGTGAAGAA			19711-19727	-
MuL	CGAATAATCCAAATGTCCTCC				-
<b>Plasmids</b>					
pUC: <i>yhjL</i>	Vector for <i>yhjL</i> complementation experiments (this study)				
pGEM: <i>yhjSTU</i>	Vector for <i>yhjS</i> complementation experiments (this study)				
pKOBEGA	Vector for recombination experiments (Chaveroche <i>et al.</i> , 2000)				
pGFPmut3	Vector for GFP expression in ileal loop co-infection experiment (Cormack <i>et al.</i> , 1996)				

a. Pellicle at the air-liquid interface formed in standing rich culture was examined visually after growth in 5 ml of LB at room temperature for 96 h.  
 b. Biofilm attached to the wall at the air-liquid interface after incubation in ATM at 37°C for 4 h.  
 c. The relative level of calcofluor binding was assessed qualitatively, using wild-type strains as reference.  
 d. Morphotype on CR plates at 28°C.  
 e. Nucleotide sequence positions refer to the *dciA*-*dppF* intergenic region sequence deposited in the EMBL nucleotide sequence database under accession number AJ315148.  
 f. Priming sequence for the Km resistance gene underlined.

mutants were tested for histidine auxotrophy to eliminate mutants that had arisen by homologous recombination between the flanking *hisD* sequences of MudJ from the donor lysate and the corresponding sequence of the recipient strain. To identify genes containing MudJ fusions, chromosomal DNA from each mutant was purified, and the DNA sequence of the region adjacent to the transposon insertion site was determined at the DNA Sequencing Service of the University of Navarra (Spain), using primer MuL (Table 4) specific to the left arm of MudJ transposon as described previously (Cano *et al.*, 2001).

#### Screening of the mutants and phenotypic assays for biofilm formation

Screening of the kanamycin-resistant mutants was carried out by qualitatively assessing the level of calcofluor (fluorescent brightener 28; Sigma) binding of colonies grown on LB agar supplemented with 200 µg ml<sup>-1</sup> calcofluor at room temperature for 48 h. Fluorescence of the cells was observed under a 366 nm UV light source and compared with the wild-type strain, *S. enteritidis* 3934. The biofilm-forming assay in a nutrient-deficient medium, ATM, in which *Salmonella* cells do not grow, was carried out as described previously (Solano *et al.*, 1998). The biofilm formed in standing rich culture was examined visually after growth in 5 ml of LB broth at room temperature for 96 h. LB agar without salt supplemented with Congo red (40 µg ml<sup>-1</sup>) and Coomassie brilliant blue (20 µg ml<sup>-1</sup>) was used to judge colony morphology and colour (Romling *et al.*, 1998b).

Biofilm formation assay in microtitre wells was performed as described previously (O'Toole and Kolter, 1998a) with the following modifications. An overnight culture in LB was diluted 1:10 and used to inoculate PVC microtitre wells containing 90 µl of LB without NaCl but with 2% glucose. The plate was incubated at 28°C for 48 h. The biofilm formed by *S. enteritidis* was visualized at the interface between the air and the liquid medium (see *Supplementary material*).

#### One-step inactivation of chromosomal genes

For disruption of the *yhjL*, *yhjS*, *dctA* and *dppF* genes in *S. enteritidis* 3934, PCR-generated linear DNA fragments were used as described previously (Datsenko and Wanner, 2000) with some modifications. The Red helper plasmid pKOBEGA, an ampicillin-resistant derivative of pKOBEG (Chaverocche *et al.*, 2000), is a low-copy-number plasmid that contains an ampicillin resistance gene, a temperature-sensitive origin of replication and the Red system, which includes three genes expressing Exo, Bet and Gam functions of phage λ. Plasmid pKOBEGA was introduced into *S. enteritidis* 3934 by heat shock, and transformants were selected on LB agar Am after incubation for 24 h at 30°C. One transformant carrying the Red helper plasmid was made electrocompetent as described above. A selectable antibiotic resistance gene was generated by PCR from a freshly isolated colony of *E. coli* MC4100, using primer pairs of 80-nucleotide (nt)-long primers that included 60 nt homology extensions for the targeted locus and 20 nt priming sequences for the kanamycin resistance gene as template. Primer pairs *yhjL*3 and *yhjL*4,

*yhjS*1 and *yhjS*2, *dctA*1 and *dctA*2, and *dppF*1 and *dppF*2 (Table 4) were used for disruption of the *yhjL*, *yhjS*, *dctA* and *dppF* genes respectively. Electroporation (25 µF, 200 Ω, 2.5 kV) was carried out according to the manufacturer's instructions (Bio-Rad) using 50 µl of cells and 1–5 µg of purified and dialysed (0.025 µm nitrocellulose filters; Millipore) PCR product. Shocked cells were added to 1 ml of LB broth, incubated for 1 h at 30°C and then spread onto LB agar Km to select Km<sup>R</sup> transformants after incubation at 37°C for 24 h. Mutants were then grown on LB broth Km at 43°C for 24 h and incubated overnight on LB agar Km, Am at 30°C to test for loss of the helper plasmid.

#### Southern blot and PCR verification of the mutants

For Southern hybridization, chromosomal DNA was purified as described previously (Cano *et al.*, 2001), digested with *Pst*I, analysed by agarose gel electrophoresis and transferred onto nylon membranes using standard methods (Ausubel *et al.*, 1990). Labelling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labelling and chemiluminescent detection kit (Boehringer Mannheim). To confirm the disruption of *yhjL*, *yhjS*, *dctA* and *dppF* in *S. enteritidis* and *S. typhimurium* mutant strains, the following primers were used to generate the DNA probes: (i) *yhjL*11 and *yhjL*12; (ii) *yhjS*11 and *yhjS*12; (iii) *dctA*11 and *dctA*12; (iv) *dppF*11 and *dppF*12 (Table 4). Mutants were also tested for gain of the resistance gene by PCR using the primers described to generate the probes for Southern analysis. Control colonies, namely wild-type strains and *E. coli* MC4100 colonies, were always tested side by side.

#### Complementation studies

A 3572 bp fragment containing the promoterless *yhjL* coding sequence was amplified on *S. enteritidis* 3934 with high-fidelity thermophilic DNA polymerase (Dynazyme Ext; Finnzymes) using the primers *yhjL*5/*yhjL*6 (Table 4), containing *Eco*RI and *Hind*III restriction sites. The PCR-amplified fragment was cloned downstream of the β-galactosidase promoter of pUC18 to give pUC:*yhjL*. A 3846 bp fragment containing the *yhjSTU* and promoter coding sequence was amplified on *S. enteritidis* 3934 with high-fidelity thermophilic DNA polymerase (Dynazyme Ext; Finnzymes) using the primers *yhjS*10/*yhjU*1 (Table 4). The PCR-amplified fragment was cloned in pGEM-T Easy (Promega) to give pGEM:*yhjSTU*. Plasmids pUC:*yhjL* and pGEM:*yhjSTU* were transformed by electroporation into *S. enteritidis* 3934 *yhjL*-km and 3934 *yhjS*-km mutants, respectively, and the complemented strains were assessed for their ability to form a biofilm in ATM and LB as well as for fluorescence under UV light on LB agar supplemented with calcofluor, and for their morphotype on CR plates in the presence of 100 µg ml<sup>-1</sup> Am and 1 mM IPTG (in the case of *S. enteritidis* 3934, *yhjL*-km complemented strain).

#### Microscopic and enzymatic assays

Biofilms formed by the wild-type strain *S. enteritidis* 3934 in

ATM and LB as well as the fragile pellicle formed by its mutants, *S. enteritidis* 3934 *yhl*-km and *yhS*-km, in LB were stained with Alcian blue 8GX (1% Alcian blue in 95% ethanol), one of a family of copper pathalocyanin dyes that can be used to reveal acidic polysaccharides, using carbolfuchsin as the counterstain (Murray *et al.*, 1994). For ruthenium red staining assays, *S. enteritidis* 3934 and its mutants *S. enteritidis* 3934 *yhl*-Km and *yhS*-km were grown in LB broth for 96 h at room temperature, and bacteria embedded in the rigid and in the fragile pellicle, respectively, were processed sequentially as described previously (Yildiz *et al.*, 2001). The thin sections were visualized using a Zeiss EM10CR electron microscope. To confirm the role of cellulose in the biofilm formation process of the wild-type strain *S. enteritidis* 3934 in ATM and LB, the biofilm-forming assay was carried out in the presence of 0.1% cellulase (Sigma), and biofilms already formed in ATM and LB were digested with 0.1% cellulase in 0.05 M citrate buffer, pH 4.6, for 72 h at 45°C (Saxena and Brown, 1995).

### Virulence assays

**Preparation of bacterial inocula.** *Salmonella enteritidis* 3934 and the mutant strain 3934 *yhl*-km were retrieved from suspensions stored at -85°C, plated on TSA and incubated at 37°C overnight. Several colonies were then transferred to LB broth and, after 3 h (log phase) or 24 h (stationary phase) of incubation of bacterial liquid cultures at 37°C, the suspensions were adjusted with phosphate-buffered saline (PBS; pH 7.4) to an OD<sub>590</sub> of 0.125 ( $\approx 10^8$  cfu ml<sup>-1</sup>, based on viable cell counts on TSA). For the HEp-2 assays, additional environmental conditions were tested (static versus agitated broth cultures and aerobiosis versus anaerobiosis using gas pack jars; Becton Dickinson). Infective doses were estimated by plating the appropriate dilution of the stock suspension in sterile saline on TSA. TSB and TSA media were always supplemented with Km in the case of the mutant strain.

**Bacterial adherence and invasion assays.** HEp-2 cells, derived from a human epidermoid larynx carcinoma, were used. Confluent monolayers were prepared in 24-well tissue culture plates by adding  $2 \times 10^5$  cells per well and incubating them in a 5% CO<sub>2</sub> atmosphere in DMEM supplemented with 2 mM L-glutamine and 10% (v/v) fetal bovine serum (FBS). After washing with PBS, the monolayers were inoculated with  $10^7$  bacterial cells suspended in DMEM. Then, the tissue culture plates were centrifuged at 100 g for 5 min and incubated for 5 min in the case of adherence assays at 37°C. The monolayers were washed three times with PBS, treated with 0.2% Triton X-100 for 5 min at 37°C to release all bacteria that had adhered to the cells and counted after plating on TSA to determine the percentage adherence. Parallel-infected monolayers were incubated for 60 min to carry out invasion assays. Extracellular bacteria that remained after washing with PBS were killed by treatment with DMEM containing 100 µg ml<sup>-1</sup> gentamicin for 90 min. Wells were rinsed three times with PBS to remove residual gentamicin, and intracellular bacteria released by the addition of 1% Triton X-100 were quantified by plating on TSA plates to determine the percentage invasion. In competition

assays, HEp-2 monolayers were inoculated with a mixture of the parental and the *yhl*-km mutant strains ( $10^5$  cfu per strain). After adherence and invasion assays (see above), bacteria were spread onto TSA and TSA Km, which selected for one of the two competing strains. All the assays were validated using a non-invasive control *E. coli* EPEC E20513 strain. Two replicates were always included for each individual experiment. Experiments were repeated three times.

**Experimental infection.** Female BALB/c mice and 1-day-old Hy-line male chickens were used in experimental infections. Animals were housed and maintained in the animal facility at the University of Navarra in safety cages at constant humidity and temperature, receiving food and water *ad libitum*. Survival assays were accomplished in three different animal models: mice orally or i.p. infected and 1-day-old chickens i.p. infected. Female BALB/c mice, randomized in groups of six animals, were orally inoculated with  $10^4$  and  $10^5$  cfu *Salmonella* cells in 100 µl of PBS. Mice were prefed with 20 µl of 10% sodium bicarbonate 30 min before bacterial inoculation. Alternatively, mice were inoculated i.p. with  $10^0$ – $10^3$  cfu in 100 µl of PBS. The number of surviving mice was recorded at different times after infection. Newly hatched layer chicks, from *Salmonella*-free flocks, randomized in groups of six birds, were infected i.p. with  $2 \times 10^1$ – $2 \times 10^6$  cfu of the corresponding strain. The number of dead chickens was recorded every 24 h, and the LD<sub>50</sub> was calculated at day 3 after infection using the GRAFIT computer program (Erithacus Software, version 3.0). Experiments were repeated three times.

**Ileal loop co-infection experiment.** To examine the *in vivo* interaction of salmonella strains with murine intestinal epithelial cells, the ligated ileal loop co-infection model was used as described previously (Jones *et al.*, 1994) with modifications. Six-week-old BALB/c female mice were starved for 24 h and anaesthetized by i.p. injection of ketamine (100 mg kg<sup>-1</sup>) and xilacine (10 mg kg<sup>-1</sup>) before surgery. On practising a small incision, the small bowel was exposed, and an ileal loop was formed by ligating a piece of intestine containing a grossly identifiable Peyer's patch at a site proximal to the caecum. The blood supply to the loop was kept intact. PBS (100 µl) containing equal numbers of the parental and mutant strain ( $10^7$  cfu) was then injected through a 25-gauge needle. The bowel was returned to the abdomen and the incision stapled. Mice were kept alive for 3 h and then sacrificed. The ileal loop was scissed intact, opened longitudinally and placed into a tube with PBS. Extracellular bacteria were eliminated by three washes with PBS and a further incubation for 90 min in PBS containing gentamicin (100 µg ml<sup>-1</sup>). Samples were rinsed with PBS to remove residual gentamicin, and the whole ileal piece was treated in a stomacher (Seward Medical) using 2 ml of PBS. To determine intracellular parental and mutant bacteria, appropriate dilutions of the homogenized samples were spread on TSA and TSA Km plates. To validate the assay, plasmid pGFPmut3 (Cormack *et al.*, 1996) was introduced into *S. enteritidis* wild-type strain, which allowed us to trace invasion, and visualized by microscopy for the presence of fluorescent bacteria in the lymph follicles of the Peyer's patches in the ileal loop at 3 h after inoculation.

### Chlorine killing assay

For chlorine survival analysis, the wild-type strain *S. enteritidis* 3934 and its mutants 3934 *yhjL*-km and *yhjS*-km were assayed for biofilm formation in ATM. Then, the supernatant of the wild-type strain culture was discarded, leaving the biofilm formed coating the glass surface, whereas cultures of the mutants were centrifuged to pellet the cells. PBS (5 ml) containing 30 p.p.m. NaOCl was added to all the tubes and incubated for 20 min at 28°C. Control tubes were incubated with 5 ml of PBS. Surviving bacteria were enumerated by viable plate counts of bacteria that had been sonicated for 1 min to form a suspension of single cells.

### Nucleotide sequence accession number

The *dctA*–*dppF* intergenic region sequence has been deposited in the EMBL nucleotide sequence database under accession number AJ315148.

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### Supplementary material

The following material is available from <http://www.blackwell-science.com/products/journals/suppmat/mole/mole2802/mmi2802sm.htm>

**Fig. S1.** Biofilm formation phenotypes on PVC microtitre plates. A comparison of 48 h biofilms made by *S. enteritidis* wild type (3934) and representative mutants from group I (*pgm*<sup>-</sup>), group II (*cytR*<sup>-</sup>) and group III (*yhjL*<sup>-</sup>). The biofilm is concentrated at the interface between the air and the liquid medium (indicated by an arrow).

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