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Curli Fibers Are Highly Conserved between *Salmonella typhimurium* and *Escherichia coli* with Respect to Operon Structure and Regulation

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Mouse-virulent *Salmonella typhimurium* strains SR-11 and ATCC 14028-1s express curli fibers, thin aggregative fibers, at ambient temperature on plates as judged by Western blot analysis and electron microscopy. Concomitantly with curli expression, cells develop a rough and dry colony morphology and bind the dye Congo red (called the rdar morphotype). Cloning and characterization of the two divergently transcribed operons required for curli biogenesis, *csgBA(C)* and *csgDEFG*, from *S. typhimurium* SR-11 revealed the same gene order and flanking genes as in *Escherichia coli*. The divergence of the curli region between *S. typhimurium* and *E. coli* at the nucleotide level is above average (22.4%). However, a high level of conservation at the protein level, which ranged from 86% amino acid homology for the fiber subunit CsgA to 99% homology for the lipoprotein CsgG, implies functional constraints on the gene products. Consequently, *S. typhimurium* genes on low-copy-number plasmids were able to complement respective *E. coli* mutants, although not always to wild-type levels. *rpoS* and *ompR* are required for transcriptional activation of (at least) the *csgD* promoter. The high degree of conservation at the protein level and the identical regulation patterns in *E. coli* and *S. typhimurium* suggest similar roles of curli fibers in the same ecological niche in the two species.

Proteinaceous, filamentous appendices on bacterial surfaces, called fimbriae or pili, enable the bacterial cell to make contact with inanimate surfaces and eukaryotic or prokaryotic cells. Tight contact, called adherence, precedes, e.g., colonization of surfaces and invasion of eukaryotic cells by the bacteria. Fimbriae are best studied in the family *Enterobacteriaceae*, particularly in *Escherichia coli* and *Salmonella enterica* (49), in the context of pathogen-host interactions (44). Related species, subspecies, and even particular strains can have a specific set of fimbrial genes which are often located on pathogenicity islands on the chromosome or on plasmids (28, 31, 43, 45). The need for flexibility in the strategy of adhesion in order to overcome the host immune system, for example, has also led to a variability in fimbrial genes derived from a common ancestor. The immunogenic and adhesive properties of these fimbriae, which can be encoded either by the fimbrial subunit gene, as in the case of K88 fimbriae, or by separate genes, as in the case of the Pap pili, can be exchanged as gene cassettes in the context of a common frame (45). Therefore, fimbrial genes often do not appear to fit the phylogenetic classification of the bacterium but are shared by more distantly related organisms occupying the same ecological niche (45, 63).

Most of the fimbriae identified in *Salmonella enterica* subsp. *enterica* serotype Typhimurium (in this paper, referred to as *S. typhimurium*) have been described only phenotypically; the few whose genes have been cloned and sequenced (6, 15, 16, 28, 56) are unique to *S. enterica* or a subset of its subspecies (5, 56). However, curli fibers, thin aggregative fibers, seem to be present and expressed in almost all *Salmonella* spp. and *E. coli* (5, 17, 23) and maybe also in other *Enterobacteriaceae*, such as *Shigella*, *Citrobacter*, and *Enterobacter* spp. (23). So far, two nomenclature systems exist (20, 34). The genes for curli biogenesis (*csg*) in *E. coli* are called *agf* (thin aggregative fibers) in *Salmonella enteritidis*. For convenience, we use one nomenclature system in this communication.

Curli fibers detected, for example, on *S. typhimurium* strains causing acute salmonellosis in pigeons (32) and on *E. coli* isolates causing bovine mastitis (54) mediate binding to fibronectin (54), a variety of other human serum and tissue matrix proteins (7, 54, 62), and the dye Congo red (CR) (18). In *E. coli* MC4100, two divergently transcribed operons, *csgDEFG* and *csgBA(C)*, which are separated by a 513-bp intergenic region are required for the biogenesis of curli fibers (34). Transposon insertions in the *csgD* gene, which encodes a transcriptional regulator belonging to the LuxR family as identified by the sequence similarity of the DNA binding helix-turn-helix motif, completely abolished transcription of the *csgBA* operon (34). Assembled by the extracellular nucleation-precipitation pathway, the secreted fiber subunit CsgA is polymerized on the surface-exposed nucleator CsgB (35), which, in addition, is present along the filament in minor amounts (8). CsgA and CsgB show 49% similarity and contain repeat regions whose interaction triggers polymerization of CsgA (8, 35). The outer-membrane-located lipoprotein CsgG is required to protect CsgA and CsgB from proteolysis (48). The roles of *csgE* and *csgF* are just beginning to be elucidated. *csgE* is required for the fibronectin and CR binding properties of curli fibers but does not significantly affect polymerization of the fiber subunit (36). The nucleation function is impaired in a *csgF* mutant, in which CsgA is released into the growth medium (37). Curli expression in *E. coli* MC4100 and YMel is highly regulated by environmental conditions; it is restricted to low temperature on plates containing medium with a low salt concentration.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
S. typhimurium		
ATCC 14028-1s	wt1[a]	American Type Culture Collection
SR-11	wt1[a]	R. Curtiss III
LB5010	*metA22 metE551 ilv-452 leu-3121 trpD2 xyl-404 galE856 hsdLT6 hsdSA29 hsdSB121 rpsL120*	12
146	*polA-2 zig214*::Tn*10* (Tet^r)	M. Rhen
SF1005	*rpoS*::pRR10(Δ*trfA*)(Amp^r)	D. Guiney
UMR1	ATCC 14028-1s Nal^r	This study
UMR3	SR-11 Nal^r	This study
MAE1	UMR1 Δ*csgA101*::Km^r	This study
MAE2	UMR3 Δ*csgA101*::Km^r	This study
MAE5	UMR1 Δ*csgA101*	This study
INK1	SR-11 *zcg-101*::Km^r	This study
MAE40	SF1005 × UMR1; UMR1 *rpoS*::pRR10(Δ*trfA*)(Amp^r)	This study
MAE29	SF1005 × SR-11; SR-11 *rpoS*::pRR10 (Δ*trfA*)(Amp^r)	This study
MAE46	UMR1 Δ*ompR101*::Amp^r	This study
MAE34	UMR3 Δ*ompR101*::Amp^r	This study
E. coli K-12		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*lacZ*Δ*M15*)	Laboratory collection
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Laboratory collection
XL1 Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
HLO7	MC4100 Δ(*csgG-csgC*)::Km^r	47a
MHR204	MC4100 *csgA2*::Tn*105* Cm^r	34
MHR210	MC4100 *csgG1*::Tn*105* Cm^r	34
MHR261	MC4100 *csgB2*	35
MHR426	MC4100 *csgF4*	36
MHR480	MC4100 Δ*csgE3*	37
MHR503	MC4100 *csgD6*	This study

[a] wt1, wild type 1.

The alternative sigma factor RpoS (σ^S) is a global regulator controlling the expression of a large number of genes during starvation and other stress conditions in *E. coli* (52) and *S. typhimurium* (26). *rpoS*-deficient *S. typhimurium* strains are impaired in their virulence in the mouse model for typhoid fever (22, 26); the *rpoS* deficiency also seems to be the cause of attenuation of common laboratory derivatives of strain LT2 (65, 68). Transcription by the RNA polymerase containing σ^S at different promoters can include complex interactions with additional regulators (25). The stationary-phase-induced transcription of the genes for curli biogenesis is dependent on σ^S in *E. coli*. It has not been resolved whether *rpoS* is needed only for transcription from the *E. coli csgD* promoter or also affects the CsgD-dependent *csgBA* promoter (34). Absence of H-NS has been shown to make at least the *csgD* promoter independent of *rpoS*, suggesting an efficient repression of *rpoD*-dependent transcription by *hns* (2, 34).

Increasing osmolarity has been shown to shut off expression of curli genes at the transcriptional level (53) but to increase the levels of RpoS (42). Therefore, other regulators must also influence the transcription from the *csgD* and *csgBA* promoters. OmpR is a transcriptional regulator which was studied mainly for its role in regulating transcription of the outer membrane proteins OmpF and OmpC in response to surrounding osmolarity sensed by EnvZ in *E. coli* (55). Besides OmpF and OmpC, a tripeptide permease (TppB) is known to be regulated by OmpR in *S. typhimurium* (30). *ompR* mutants of *S. typhimurium* are attenuated in vivo (24) and unable to kill macrophages in vitro (47). *ompR* has been reported to be required for transcriptional activation of both the *csgBA* and the *csgDEFG* promoters in *E. coli* (40); however, no experimental data have been reported so far.

In this paper, we report the cloning and characterization of the two operons for curli biogenesis from *S. typhimurium* SR-11. The highly conserved genes displayed the same arrangement in the same chromosomal context as in *E. coli*. Consequently, *S. typhimurium*-derived genes on plasmids could functionally replace their *E. coli* counterparts, although not always to the wild-type levels. Regulation of curli biogenesis in *S. typhimurium* SR-11 and ATCC 14028-1s was reminiscent of *E. coli* MC4100 and YMel. Curli biogenesis was restricted to ambient temperature on plates, and transcription from the *csgDEFG* and *csgBA* promoters required *rpoS* and *ompR*. The conservation of genes and of the regulation pattern implies an important role of curli fibers in the lifestyle of *E. coli* and *Salmonella* spp. in the same ecological niche.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used and constructed in this study are given in Tables 1 and 2, respectively. In the beginning, *S. typhimurium* SR-11 was used for the analysis of curli biogenesis. However, the initial lack of tools for genetic analysis led to a subsequent shift to strain ATCC 14028-1s, a virulent derivative of the well-characterized LT2 strain. Curli expression in *E. coli* and *S. typhimurium* was monitored by growth on solid Yesca (35) and Luria-Bertani (LB) medium without salt, respectively. Media were supplemented with CR (40 μg/ml) and Coomassie brilliant blue (20 μg/ml) to judge colony morphology and color (34, 35). However, CR slightly inhibits the growth of *S. typhimurium*; therefore, the colony morphology develops later than on medium without CR. Recombinant clones were grown on LB medium supplemented with recommended concentrations of antibiotics (4), if required.

DNA techniques. Isolation of plasmid, cosmid, and chromosomal DNAs and all enzymatic manipulations (restriction digestion, ligation, phosphorylation, and PCR) were carried out by standard protocols (4, 58) with enzymes from Boehringer Mannheim or Biolabs. Southern blotting was done as described previously (57). Individual PCR fragments were purified with a Quiaquick PCR purification kit (Quiagen); otherwise, a Geneclean II kit (Bio 101, Inc.) was used after electrophoresis. After polyethylene glycol precipitation (1), plasmids were sequenced with a Cycle Sequencing Ready Reaction kit (Perkin-Elmer). All se-

TABLE 2. Plasmids used and constructed in this study

Plasmid	Characterization	Reference
pLAFR3	Tet^r RK2 *oriT*	64
pMAK700	Cm^r temperature-sensitive replicon derived from pSU101	33
pMAK705	pMAK700 containing pUC19 polylinker	33
pRK2013	Km^r ColE1 *mob tra*	27
pUC4K	Amp^r Km^r cassette	Pharmacia
pWSK29	Amp^r pSC101 *ori*	67
pWKS30	Amp^r pSC101 *ori*	67
pUMR2b	pMAK700 Δ*csgA101*; Cm^r	This study
pUMR2c-1	pMAK700 Δ*csgA101*::Km^r; Cm^r Km^r	This study
pUMR10-7	pBS, *Nsi*I-*Bam*HI-cut CSGD1-CSGB3 from SR-11; Amp^r	56a
pMU1	pUC18, *Pst*I fragment of *zcg-101*; Amp^r	This study
pMU2	pUC18, *Hin*dIII-*Pst*I fragment of *zcg-101*; Amp^r	This study
pMU3a	pMAK705, *Hin*dIII-*Pst*I fragment of *zcg-101*; Cm^r	This study
pMU3b-1	pMAK705, *zcg-101*::Km; Cm^r Km^r	This study
pMU4	pLAFR3 containing a 39-kb *Pst*I fragment harboring the *csg* genes; Tet^r Km^r	This study
pCSGA	pWKS30, *Pst*I-*Bam*HI-cut CSGB2-CSGC; Amp^r	This study
pCSGB	pWKS30, *Hin*dIII-*Pst*I-cut CSGBD-CSGA1; Amp^r	This study
pCSGD	pWSK29 (*Sma*I/*Eco*RI), *Eco*RI-cut SP2-SP5; Amp^r	This study
pCSGE	pWSK29 (*Sma*I/*Bam*HI), *Bst*YI-cut SP5-SP9; Amp^r	This study
pCSGF	pWKS30 (*Eco*RI/*Sma*I), *Eco*RI-cut SP6-SP17; Amp^r	This study
pCSGEFG	pWSK29 (*Pst*I/*Eco*RI), *Nsi*I-cut CSGD2-SP21; Amp^r	This study
pUMR7b-2	pMAK705 Δ*ompR101*::Amp^r; Cm^r Amp^r	This study

quence analyses were performed with the Genetics Computer Group package, version 8 or 9 (GCG, University of Wisconsin).

Cloning of the genes for curli biogenesis from *S. typhimurium* SR-11. The curli genes from *S. typhimurium* SR-11 were cloned in the following way. First, a DNA fragment downstream of the *csgC* gene was sought. Therefore, plasmid pCurli, containing a 3.1-kb *Hin*dIII fragment (analogous to the fragment described in reference 17) from the non-curli-producing strain *S. typhimurium* SL2965 in pUC18, was integrated into the chromosome of a *polA2* derivative of strain LB5010. After determination of appropriate restriction sites by hybridization with the vector, genomic DNA isolated from an integrant was cut with *Pst*I and ligated under conditions favoring the intramolecular reaction (pMU1). A *Hin*dIII/*Pst*I digest with subsequent subcloning depleted pMU1 from any DNA fragments containing curli sequences (pMU2).

As the second step, a resistance marker was placed downstream of *csgC*. The 6.6-kb *Hin*dIII/*Pst*I fragment of pMU2 was cloned into pMAK705 (pMU3a), and a Km^r cassette was introduced into a single *Nsi*I site (pMU3b-1). The Km^r marker was placed on the chromosome of SR-11 by a procedure described below (see "Strain construction"), thereby creating the *zcg-101*::Km^r allele on the chromosome.

For cloning of the curli operons, after the position of the fragment containing the curli genes and the Km^r marker was checked by Southern hybridization of a *Pst*I-digested chromosomal DNA, the DNA fragments of respective size were isolated from a gel by the Freeze-Squeeze method (66) and ligated with *Pst*I-cut pLAFR3. The partial cosmid library was packed (Gigapack III Gold; Stratagene) and amplified in *E. coli* XL1 Blue MR in liquid culture with kanamycin resistance as an additional selection marker. The cells were plated for individual colonies to be examined further, and subsequently one cosmid clone (pMU4) which complemented HLO7 [Δ(*csgG-csgC*)] was chosen for sequencing. Sequence identity between SR-11 and ATCC 14028-1s was confirmed for *csgD* and the intergenic region.

Strain construction. Phage P22 HT105/1 *int-201* (60) was used for transduction of LT2 strains and SR-11 according to the recommended protocol (13, 50). In order to detect lytically infected cells, LT2 strains were streaked on green plates. Lysogens were detected by streaking LT2 derivatives against phage H5. Since SR-11 does not support the propagation of P22, the purification procedures were skipped. DNA translocation into bacteria was also done by using competent cells (41), electrocompetent cells (9), and conjugation by triparental mating. A deletion in *csgA* was constructed as follows. Primers CSGBD (dACG AAAGCTTGCACTGCTGTGGGTTG [*Hin*dIII restriction site underlined]), CSGA1 (dCGTCTGCAGGATTGCTGCGAATGCTGC [*Pst*I site underlined]), CSGA2 (dCGTCTGCAGTGGAACGCTAAAAACTC [*Pst*I site underlined]), and CSGC (dCGAGGATCCGGCCATTGTTGTGATAAA [*Bam*HI site underlined]) were used to create fragments PCR1 (CSGBD↔CSGA1) and PCR2 (CSGA2↔CSGC). After restriction digestion, PCR1 and PCR2 were directly cloned into pMAK700 cut with *Hin*dIII and *Bam*HI, which resulted in plasmid pUMR2b. Cloning of the Km^r cassette of pUC4K into the single *Pst*I site of pUMR2b resulted in pUMR2c-1. The plasmids were passaged through LB5010 and finally electroporated into *S. typhimurium* wild-type strains. After propagation of the strains at 28°C, the temperature was shifted to 44°C in order to integrate the pMAK derivative into the chromosome. For pUMR2c-1, for which a selectable marker was available, individual colonies were streaked on chloramphenicol and kanamycin plates in order to screen for a double crossover event. With pUMR2b, 10 individual integrants were selected and incubated at 28°C in order to resolve the plasmid again. Strains harboring a deletion were selected by their white color on CR plates. All constructs were checked by Southern hybridization and/or PCR. A deletion in *ompR* was created in the same way. The *ompR* primers OMPR1 (dTGGAAGCTTTGTTTGAGTGTT TCGT [*Hin*dIII site underlined]), OMPR2 (dCTTAGATCTCTCTTGCATTGT CTGT [*Bgl*II site underlined]), OMPR3 (dCCTGAGATCTGTCTTTGTAC CGGAC [*Bgl*II site underlined]), and OMPR4 (dGGCTCTAGAACTTCTAC CTGAAACCAG [*Xba*I site underlined]) were selected on the basis of sequence X12374 (EMBL database [46]). After restriction digestion, PCR fragments PCR3 (OMPR1↔OMPR2) and PCR4 (OMPR3↔OMPR4) were cloned into *Xba*I/*Hin*dIII-digested pMAK705, yielding pUMR7a. Cloning of the ampicillin resistance gene from pWKS30 (*Bgl*II/*Bam*HI digested) into the single *Bgl*II site resulted in pUMR7b-2.

Plasmid construction. Individual genes from the curli operon were cloned into low-copy-number vector pWSK29 or pWSK30. *csgA* was amplified by using primers CSGB2 (dCGTCTGCAGTGCAGAAACAGTCGCA [*Pst*I site underlined]) and CSGC (dCGAGGATCCGGCCATTGTTGTGATAAA [*Bam*HI site underlined]), and for *csgB* primers CSGBD (*Hin*dIII) and CSGA1 (*Pst*I) were used. The restriction sites at the primer ends were used to clone the fragments into pWKS30, yielding plasmids pCSGA and pCSGB, respectively. *csgD* was amplified with primers SP2 (dTTTCTCTTTCTGGATAATGGG) and SP5 (dT GTTTAACACGCATGACAGC), *csgE* was amplified with primers SP5 and SP9 (dCCTGACGATTATCCCTACC), and *csgF* was amplified with primers SP6 (dGATTGTTAACCGACCATACC) and SP17 (dGCAGGTAAGTGCGTCAA ATC). The ends of the PCR fragments were treated with Klenow polymerase. Primer pair SP2-SP5 was digested with *Eco*RI, SP5-SP9 was digested with *Bst*YI, and SP6-SP17 was digested with *Eco*RI. SP2-SP5 and SP5-SP9 were cloned into pWSK29 digested with the respective restriction enzymes, yielding pCSGD and pCSGE, respectively, and SP6-SP17 was cloned into pWKS30 in order to create pCSGF. The gene sequences for *csgEFG* were also amplified by using primers CSGD2 (dTGGATGCATACCCAGGCAGTTTCATGG [*Nsi*I site underlined]) and SP21 (dGCTTTGTCGTATTCATCAGG) and cloned into pWSK29, yielding pCSGEFG.

RNA techniques. Total RNA was prepared from 10 mg of *S. typhimurium* cells by the hot-phenol method. Cells were resuspended in 300 μl of 0.3 M sucrose–0.01 M sodium acetate (pH 4.5) and the same amount of 0.01 M sodium acetate (pH 4.5)–2% sodium dodecyl sulfate (SDS). After being mixed with an equal amount of hot acidic phenol, the cells were incubated at 65°C for 5 min. The extraction was repeated once with hot acidic phenol and twice with cold acidic phenol. After precipitation, the remaining DNA was digested with 10 U of RQ1 DNase (Promega) for 30 min in 0.05 M Tris (pH 7.5)–0.05 M NaCl–0.01 M $MgCl_2$, and phenol-$CHCl_3$ extraction was carried out twice. The concentration of the RNA dissolved in water was determined spectroscopically. A 10-μg sample of RNA was loaded on a 1.2% morpholine propanesulfonic acid (MOPS)-formaldehyde gel (4) which was run for 4 h at 4 V/cm with a 0.24- to 9.5-kb RNA ladder (Life Technologies) as a standard. After the gel was soaked in H_2O twice (20 min each), the RNA was transferred to an Amersham Hybond-N membrane overnight by capillary blotting with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (4). Single-stranded probes complementary to the RNA template on the blot were constructed by an asymmetric PCR with primers CSGB2 and SP2 on symmetric PCR templates spanning the region of the *csgA* (primers CSGB2 and SP8 [dCTAAATTAATACTGGTTGA]) and *csgD* (primers SP2 and SP24 [dTAACTCTGCTGCTACAATCC]) genes, respectively, and labeled with the RadPrime Labelling System (Life Technologies) using 30 μCi of [α-^{32}P] dCTP (3,000 Ci/nmol; Amersham). Hybridization (using less than 6 ng of probe per ml) and washing of blots were carried out according to standard procedures (4). The quality of transfer to the membrane was checked by probing with part of the 16S RNA sequence from plasmid pKK3535 (11) cut by *Hin*dIII. Signals were analyzed with a radioisotope imaging system (PhosphorImager 445SI; Molecular Dynamics) and quantified by integration over all bands detected by a single probe.

For primer extension, 10 μg of RNA and 2 pmol of primer PEXD1 (dTGA CAGATGTTGCACTGCTG) were diluted in 9 μl of 1× avian myeloblastosis virus (AMV) reverse transcriptase buffer (Boehringer Mannheim). A 10-pmol amount of PEXD1 had been labeled with 30 μCi of [γ-^{32}P]ATP (3,000 Ci/nmol; Amersham) by use of 10 U of polynucleotide kinase (Boehringer Mannheim). After incubations at 95°C for 5 min, 67°C for 3 min, and 50°C for 5 min, 5 U of AMV reverse transcriptase (Boehringer Mannheim) and 5 mM deoxynucleoside triphosphates (Pharmacia) were added, and primer extension was performed at 50°C for 1 h. The reaction mixture, containing 5 μl of formamide loading buffer, was heated at 95°C for 5 min and cooled on ice, and 6 μl was analyzed on a 7% denaturing polyacrylamide gel (4). A sequencing ladder generated with a T7 Sequencing kit (Pharmacia) using the PEXD1 primer and pUMR10-7 as template DNA was run as a standard.

Western blotting. Bacteria were grown for 48 to 60 h on plates at 28°C and for 17 to 24 h at 37°C. In order to depolymerize the curli fiber into subunits, the cells

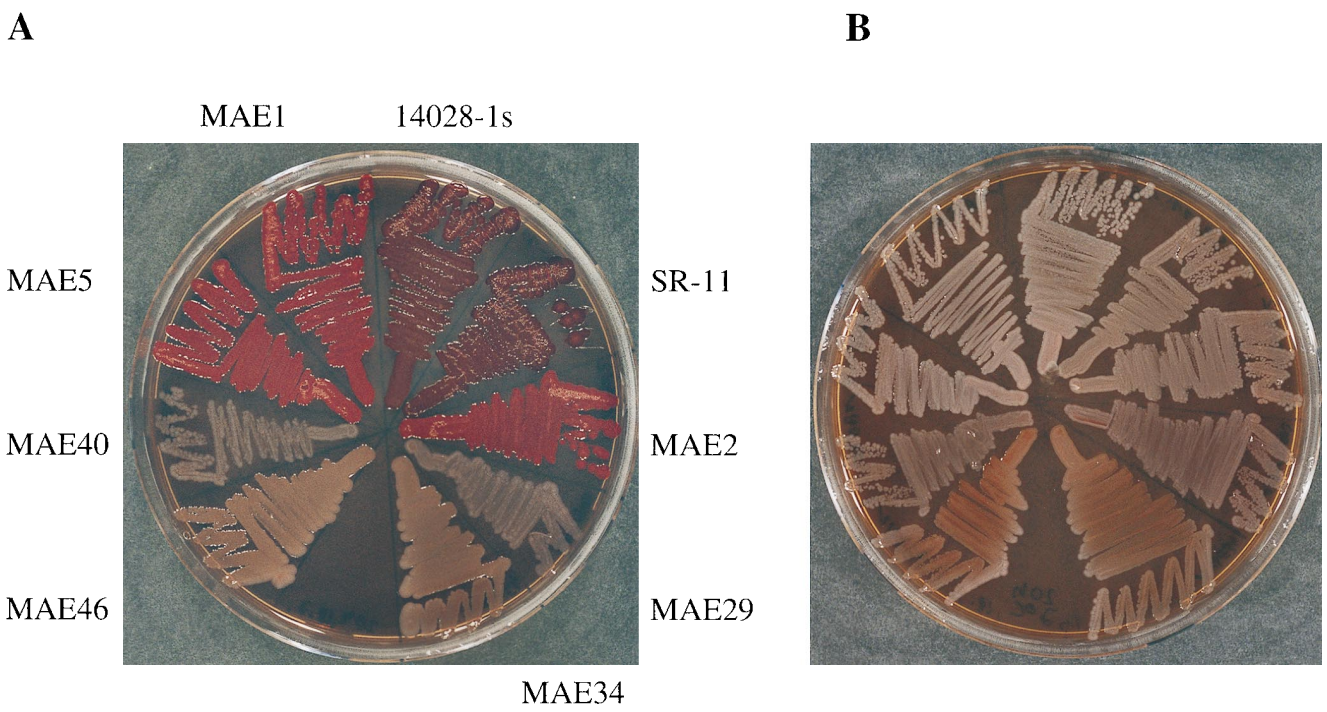

FIG. 1. Colony morphology and color of *S. typhimurium* ATCC 14028-1s and SR-11 and their respective derivatives. (A) Cells grown at 28°C on LB medium plates without salt containing the dye CR. SR-11 and ATCC 14028-1s (here shown as the Nal^r derivatives UMR3 and UMR1, respectively) developed a rough colony morphology with a dry surface and showed a deep red color by binding the dye CR, the rdar morphotype. The strains with deletions in *csgA* (MAE1, MAE2, and MAE5) were also rough but had a shinier surface. Binding of the dye CR led to a pinkish color of the colonies (the pdar morphotype). The strains with deletions in *rpoS* (MAE40 and MAE29) and *ompR* (MAE46 and MAE34) were white. (B) The same strains as in panel A but grown at 37°C. All cells were white and smooth, the saw morphotype.

have to be treated with strong acids (19). After resuspension of the cells in 100 μl of 99% formic acid and incubation for 10 min on ice, the liquid was removed by evaporation in a Speed Vac. The pellet was resuspended in 200 μl of SDS-polyacrylamide gel electrophoresis sample buffer (4), while 3 μl was loaded on a gel (15% separating gel with a 4% stacking gel, with a double concentration of buffer used in the separation gel and the running buffer). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). The membranes were blocked and incubated with a 1:4,000 dilution of an anti-*E. coli* CsgA antiserum (34); a secondary goat antibody against rabbit immunoglobulin G conjugated with horseradish peroxidase was used for detection according to the protocol of the manufacturer (Boehringer Mannheim).

Electron microscopy. Bacteria were grown on plates under the same conditions used for Western blotting. A concentrated bacterial suspension in water was allowed to adhere to a carbon-coated copper grid for 2 min. The liquid was removed by blotting, and staining was carried out for 30 s with 0.7% ammonium molybdate–150 μg of bacitracin per ml. The samples were examined with a Zeiss microscope at 80 kV.

Nucleotide sequence accession number. The nucleotide sequence of the genes for curli biogenesis has been submitted to the EMBL data library under accession no. AJ002301.

RESULTS

Detection of curli expression in *S. typhimurium* SR-11 and ATCC 14028-1s on plates. The mouse-virulent strains SR-11 and ATCC 14028-1s exhibited distinct colony morphologies when grown on CR plates at different temperatures; a white and smooth colony morphology was seen at 37°C, while a red, dry, and rough colony morphology was displayed at 28°C (Fig. 1). We called the two morphotypes saw$_{37}$ and rdar$_{28}$, respectively. Morphotypes similar to the rdar morphotype were previously described for *E. coli* MC4100 and YMel (34) and *Salmonella enteritidis* (18) and shown to be tightly linked to the expression of a thin aggregative fiber called curli fiber or SEF 17, respectively.

Both *S. typhimurium* strains were analyzed for expression of curli fibers by immunoblot analysis using a polyclonal antiserum against CsgA (34), detection of fibers on the surface of the cells by electron microscopy, and gene replacement of *csgA*, the fiber subunit gene, the sequence of which was taken from the one published for *S. enteritidis* (17). In accordance with the colony morphology, a signal for CsgA was detected only at 28°C in both strains (Fig. 2). By electron microscopy, an abundance of fibers was detected at 28°C (Fig. 3), while very few were occasionally seen at 37°C (data not shown). However, electron micrographs might not reflect the actual amount of

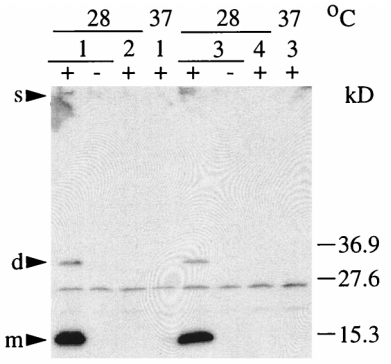

FIG. 2. Western blot analysis of fiber-derived CsgA from whole cells grown at 28 and 37°C. The cell pellets were immediately resuspended in SDS sample buffer (−) or treated with formic acid (+) as described in Materials and Methods. Minor signals for the fiber subunit which could vary in their intensities were regularly found in the slot (s) and in the gel corresponding to a dimer (d), while the major signal was consistent with the running behavior of a monomer (m). SR-11 and ATCC 14024-1s showed a signal for CsgA only at 28°C and not at 37°C. *csgA* knockouts did not show any signal at all. Lanes: 1, SR-11; 2, MAE2; 3, ATCC 14028-1s; 4, MAE1.

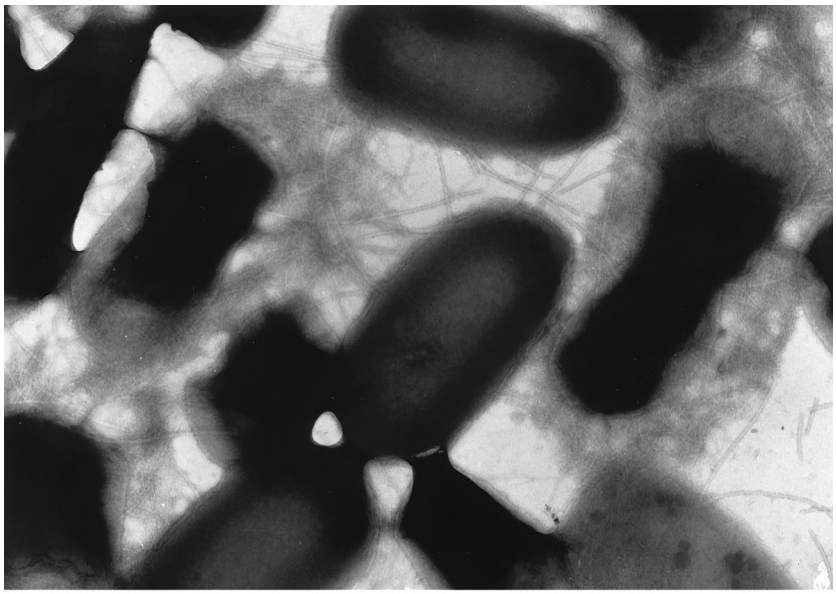

FIG. 3. Electron micrograph of negatively stained ATCC 14028-1s cells grown at 28°C on LB agar without salt. The cells which are surrounded by a thick layer of curli fibers show a different cell morphology. Bar, 1 μm.

curli fibers present on the cells at 28°C, since the cells clump together and only a fraction of them can be released from the tight extracellular matrix. Replacement of the curli subunit gene *csgA* with a Km^r cassette (MAE1 and MAE2) or an in-frame deletion of *csgA* (MAE5) abolished the $rdar_{28}$ phenotype. Instead of rdar, mutant strains of SR-11 and ATCC 14028-1s, MAE2 and MAE1, respectively, and MAE5 displayed a pink colony which developed a delayed roughness, the $pdar_{28}$ morphotype (Fig. 1). This phenotype seems to be more common in *Salmonella* spp., since it was also described for *S. enteritidis* 27655-3b after gene replacement of *csgA* (17), whereas *E. coli* MC4100 and YMel gave white colonies after knockout of the fiber subunit gene (34, 35). All these experiments showed that the curli fibers are expressed in SR-11 and ATCC 14028-1s in a temperature- and surface-dependent manner; therefore, the curli operon from SR-11 was cloned and characterized (see Materials and Methods). The organization of the two divergently transcribed *csg* operons, *csgDEFG* and *csgBAC*, is shown in Fig. 4.

Transcriptional analysis of the two curli operons. Primer extension analysis of the *csgD* operon revealed the nucleotide G 174 bp upstream of the putative translation start of the *csgD* transcript as the transcription start site in *S. typhimurium* ATCC 14028-1s (Fig. 5). Transcription initiation of the *E. coli csgD* operon takes place 2 bp further upstream (34). A signal was obtained only when RNA isolated from cells grown on plates at 28°C, and not at 37°C, was used. Primer extension

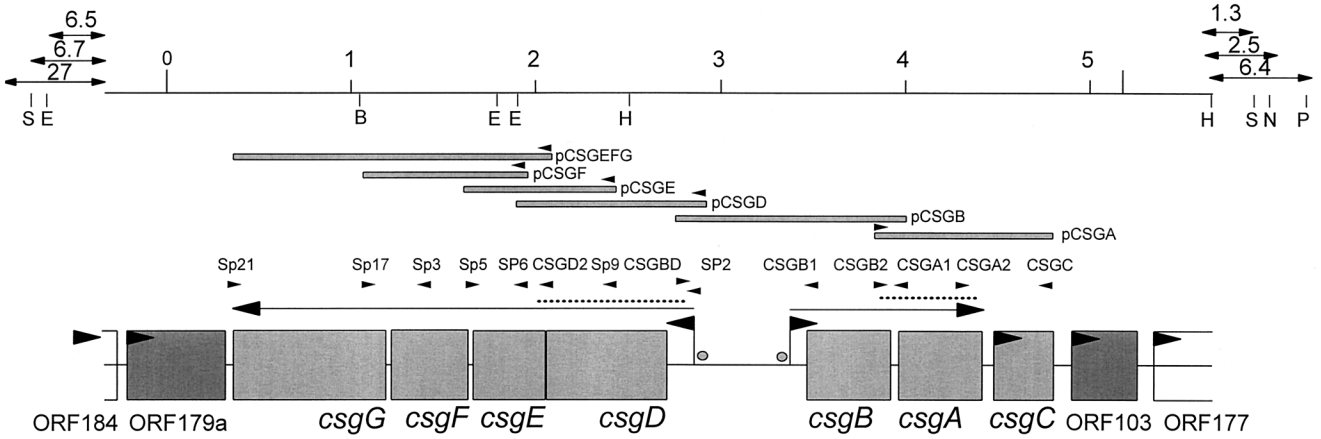

FIG. 4. Organization of the *csg* region on the *S. typhimurium* SR-11 chromosome. A restriction map containing sites important for the cloning and localization of the *csg* region is shown. The positions of the genes required for curli biogenesis, *csgDEFG* and *csgBAC* (boxes), the position of the transcriptional start sites and the direction of polymerization (flags), and the position of the promoter (circle) are indicated. Experimentally confirmed RNA full-length transcripts (arrows above gene boxes) and open reading frames for which transcriptional analysis had not been carried out or for which no transcript had been detected so far (boxes with arrowheads inside) are also shown. The DNA fragments used as probes in RNA transcript analysis (dotted lines), primers used for the PCR amplification of DNA fragments (arrowheads above dotted lines), and subclones used to complement *E. coli* isolates with mutations in the *csg* genes (pCSGA, pCSGB, pCSGD, pCSGE, pCSGF, pCSGG, and pCSGEFG) (bars with small arrows indicating the transcription from the *lacZ* promoter) are indicated. B, *Bst*YI; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; S, *Sac*I.

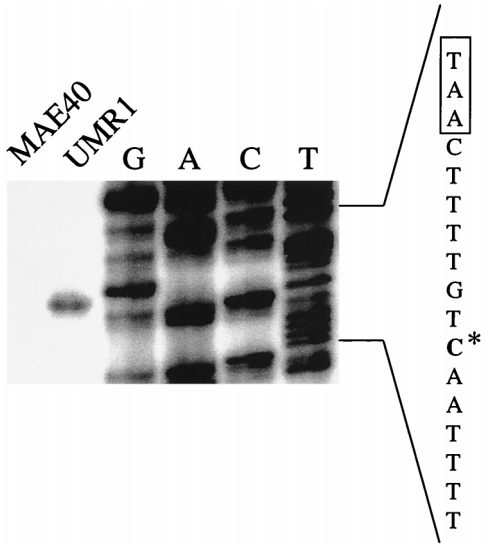

FIG. 5. Primer extension analyses for the determination of the transcriptional start site of the *csgDEFG* operon. RNA was prepared from strains UMR1 and MAE40 (the *rpoS* derivative of ATCC 14028-1s) grown at 28°C on plates, and primer extension was carried out as described in Materials and Methods. An extension product is seen for UMR1 but not for MAE40. Primer PEXD1, located 58 bp downstream of the *csgDEFG* start codon, was used for the extension reaction as well as for the sequencing reaction on pUMR10-7 as a template. The sequence derived from the PEXD1 primer on pUMR10-7 is complementary to the RNA template, so the coding strand is automatically shown. The transcriptional start site (asterisk) and bases belonging to the putative promoter sequences (boxed) are indicated.

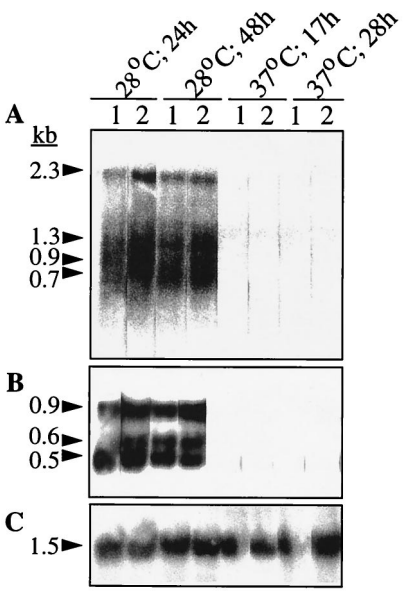

FIG. 6. Northern blot analysis of RNA transcripts of the *csg* region using *S. typhimurium* ATCC 14028-1s and SR-11 grown on LB medium plates without salt at 28 and 37°C for different periods. Probes covering the whole *csgD* and *csgA* genes were used; their locations are indicated in Fig. 4. Lanes: 1, UMR3; 2, UMR1. The sizes of the bands detected by the respective probes are shown on the left, calculated by using a 0.24- to 9.5-kb RNA ladder (Life Technologies) as a standard. (A) Hybridization with the *csgD* probe; (B) hybridization with the *csgA* probe; (C) control hybridization with 16S RNA as described in Materials and Methods.

analysis of the *csgBAC* operon confirmed previous results (2, 17; also data not shown).

In order to determine the expression state of both *csg* operons, analysis of the steady-state levels of the RNA transcripts of *csgD*, the transcriptional regulator, and *csgA*, the fiber subunit gene, was carried out by Northern blot analysis (Fig. 6). By using a probe encompassing the whole *csgD* gene, four major bands of 2.3, 1.3, 0.9, and 0.7 kb which could correspond to the transcripts *csgDEFG*, *csgDEF*, *csgDE*, and *csgD* (with theoretical sizes of 2.5, 1.6, 1.2, and 0.8 kb, respectively) were detected; the *csgA* probe hybridized to three bands of 0.9, 0.6, and 0.5 kb, as in *S. enteritidis* (17). Signals of the same intensity were detected for ATCC 14028-1s and SR-11 after the cells were grown at 28°C for 24 or 48 h on plates but not for cells grown at 37°C for 17 or 28 h. Therefore, detection of *csgD* and *csgA* transcripts is concomitant with the expression of CsgA at 28°C, and transcripts are present long after entrance into the stationary phase.

Comparative sequence analysis of the curli region. The *csg* genes are located at the same positions on the chromosomes of *E. coli* K-12 and *S. typhimurium* LT2 (26 centisomes [20]). In *S. typhimurium*, two divergently transcribed operons, *csgBAC* and *csgDEFG*, flank a 521-bp intergenic region (Fig. 4), a situation as in *E. coli* MC4100 (34). The nucleotide sequences of the two species showed an identity of 77.6%, a value which is below the average sequence conservation of 84.4% (61). The overall G+C contents in *S. typhimurium* and *E. coli* are similar; however, single genes show some variability in G+C content conservation (Table 3). The intergenic region between the transcriptional start sites has a low G+C content and is the least conserved (71%) (Table 3). However, the intergenic region has not homogeneously diverged but can be subdivided into four regions. Only four nucleotide substitutions occurred in the 60 bp upstream of the transcription start site of the *csgD* operon. The *csgD* promoter shares the characteristics of promoters transcribed by σ^D both in *S. typhimurium* and *E. coli*, with the *E. coli* −35 box being closer to the consensus sequence. The region upstream of the *csgD* promoter has a very low G+C content (21.2%) which is identical in *S. typhimurium* and *E. coli*, and a conspicuous peak of curvature at position −147 (data not shown) was found by calculation with the

TABLE 3. Properties of the *csg* genes and surrounding open reading frames

Gene or region	% G+C content (*S. typhimurium*/*E. coli*)	Sequence identity (%)[a]	Amino identity/similarity (%)[a]	K_S
ORF179a[b]	52.2/54.1	75.7	86.6/92.5	ND[e]
csg operon	43.5/43.2	77.6		
csgG	49.0/50.1	83.4	96.0/99.3	1.065
csgF	44.6/42.4	81.0	89.9/94.2	1.018
csgE	44.4/43.1	80.8	91.4/96.9	1.496
csgD	40.7/41.8	81.1	92.1/95.8	1.155
Intergenic region	33.7[c]/34.4	73.0[c]		
	30.6[d]/32.2	70.6[d]		
csgB	43.4/41.8	82.9	82.1/90.7	0.564
csgA	51.5/50.1	73.2	74.8/86.1	1.021
csgC	45.0/42.6	71.9	73.1/87.0	1.263
ORF103[b]	47.7/43.6	45.2	39.4/57.6	ND

[a] Between *S. typhimurium* SR-11 and *E. coli* MG1655.
[b] Nomenclature and sequence from *E. coli* MG1655 and W3110; EMBL files with accession no. ecae205 and ecae206 and ecd740 to ecd742 were used.
[c] Sequence between the translational start sites of *csgD* and *csgB*.
[d] Sequence between the transcriptional start sites of *csgD* and *csgB*.
[e] ND, not determined.

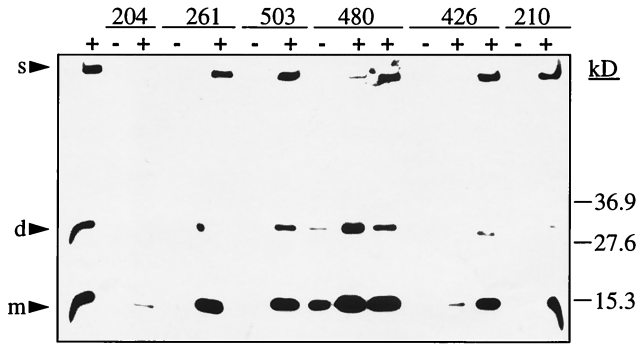

FIG. 7. Western blot analysis of the complementation of the *E. coli csg* mutants. Results for whole-cell preparations treated with formic acid are shown. The mutant (−) and the mutant harboring the respective complementing plasmid (+) were run next to each other. Except for MHR480 (Δ*csgE3*), none of the *E. coli* mutants showed a CsgA signal derived from polymerized fibers. MHR261 (*csgB2*) and MHR503 (*csgD6*) were complemented to wild-type levels by using plasmids with the single genes. MHR426 (*csgF4*) was complemented to wild type only when a plasmid carrying *csgEFG* was used. Faint signals of CsgA were detected when pCSGA was introduced into MHR204 (*csgA*::Tn*105*).

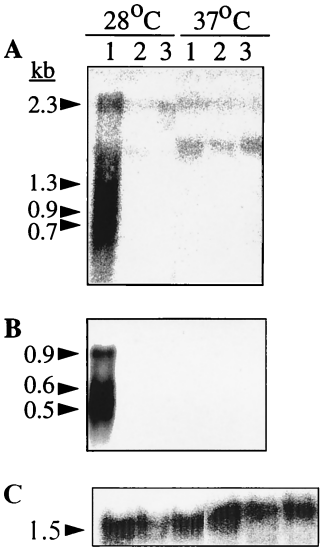

FIG. 8. Northern blot analysis of RNA transcripts from *S. typhimurium* ATCC 14028-1s and *rpoS* and *ompR* derivatives grown on LB medium plates without salt at 28 and 37°C. Probes covering the whole *csgD* and *csgA* genes were used; their locations are indicated in Fig. 4. Lanes: 1, UMR1; 2, MAE40 (*rpoS*); 3, MAE46 (*ompR*). The sizes of the bands detected by the respective probes are shown on the left, calculated by using a 0.24- to 9.5-kb RNA ladder (Life Technologies) as a standard. (A) Hybridization with the *csgD* probe; (B) hybridization with the *csgA* probe; (C) control hybridization with 16S RNA as described in Materials and Methods.

DNase I-based parameters using the bend.it server (29). Sequence identity drops to 42.7% between 165 and 443 bp upstream of the transcriptional start site of *csgD*. The *csgB* promoter has a −10 box, which resembles more the proposed consensus sequence of σ^S than that of σ^D (38). Two alternative −35 boxes have been found which both suggest an unusually wide spacing of 19 or 21 bp between the −35 and −10 boxes. Some promoters whose transcription can be initiated in the absence of specific −35 hexamer contacts contain an upstream extension of the −10 element (10) by 5′-Tgn-3′, a sequence which was not found at the *csgB* promoter.

The putative proteins encoded by the genes of the two operons showed variability in sequence conservation. With the exception of CsgC, whose role has not been unambiguously determined, the proteins for the fiber subunit, CsgA, and the nucleator, CsgB, displayed the lowest amino acid identities (74.8 and 82.1%, respectively). These values lie at the lower end of gene conservation between *S. typhimurium* and *E. coli*, which ranges from 100 to 74.3% amino acid identity (61), but are surprisingly high when homologies among fimbriae, even of common origin within a species, are considered (45). In addition, the amino acid identities of CsgA, CsgB, and CsgC were 100% when the proteins of *S. typhimurium* SR-11 and *S. enteritidis* 27655-36 were compared (see reference 17 for further characterization of the genes).

The degree of conservation of the CsgD, CsgE, CsgF, and CsgG proteins between *S. typhimurium* and *E. coli* is high and lies between 89.5 and 96% amino acid identity (Table 3). The lipoprotein CsgG, which has a putative molecular mass of 30 kDa, was most conserved and showed only conservative amino acid exchanges. The helix-turn-helix DNA binding motif of CsgD, the putative transcriptional regulator of 25 kDa, is completely conserved between the two species. Classified according to the sequence similarity of the DNA binding motif, CsgD belongs to the LuxR family. The closest sequence homology is to regulators belonging to a two-component sensory transduction system, such as DegU of *Bacillus subtilis* and NarP of *E. coli.*

Complementation of *E. coli csg* mutants with *S. typhimurium* genes. Considering the high homology of the Csg proteins between *S. typhimurium* and *E. coli*, functional complementation of gene products seemed likely. In order to test this hypothesis, PCR-generated gene fragments from *S. typhimurium* were cloned into the low-copy-number vectors pWSK29 and pWKS30 so that transcription occurred from the *lacZ* promoter (see Materials and Methods). All *E. coli csg* mutants were white and, with the exception of MHR480 (the *csgE* mutant), produced no curli fibers. MHR261 (*csgB2*) and MHR426 (*csgF4*) secrete CsgA in a soluble form (35, 36), but the soluble form of the protein was not detected in the whole-cell preparations used here (Fig. 7). Complementation was judged by the development of a red and rough colony morphology type and by Western blots detecting CsgA as an acid-sensitive polymer (Fig. 7 and Table 4). As seen in Table 4, all *S. typhimurium* genes complemented the respective *E. coli csg* mutants, although to different degrees. Single *csgB*, *csgD*, and *csgE* genes complemented the respective *E. coli* mutants to the wild-type phenotype. The *csgA* and *csgF* mutations could be only very poorly replaced by the copy on the plasmid. In the case of *csgA*, two signals of almost equal intensity were seen on an overex-

TABLE 4. Complementation of *E. coli csg* mutants with *S. typhimurium* genes

Mutation in *E. coli*	*E. coli* strain	Complementing plasmid	Colony morphology	Detection of CsgA derived from fibers
csgA2::Tn*105*	MHR204	pCSGA	Brown but not rough	Very weak signal
csgB2	MHR261	pCSGB	wt[a]	wt levels
csgD6	MHR503	pCSGD	wt	wt levels
Δ*csgE3*	MHR480	pCSGE	wt	wt levels
		pCSGEFG	wt	wt levels
csgF4	MHR426	pCSGF	Reddish but not rough	Weak signal
		pCSGEFG	wt	wt levels
csgG1::Tn*105*	MHR210	pCSGEFG	wt	wt levels

[a] wt, wild type.

posed Western blot, one of which has a slightly higher molecular weight than the wild-type signal and is most likely a premature form of CsgA (data not shown). In addition, the very low signal intensity could be explained by a lower specificity of the anti-*E. coli* CsgA antiserum against CsgA from *S. typhimurium*. MHR426, the *csgF* mutant used in this study, has a polar effect on *csgG* expression, leading to decreased amounts of CsgG (36). The reduced amount of CsgG might limit the full complementation of MHR426 by pCSGF, as occurs with the respective *E. coli* gene (36). Neither the vector control nor the *csgA*, *csgD*, *csgE*, and *csgF* genes cloned in the direction which would allow transcription from the T7 promoter gave a change in the color of the colonies or their morphology with respect to the wild type or in signal intensity of CsgA on Western blots (data not shown). We conclude from the available data that interspecies complementation of the *csg* genes is possible in principle.

Analysis of the effect of *rpoS* and *ompR* mutations on colony morphology and transcription from the *csg* promoters. For *E. coli*, it was demonstrated that transcription of the *csgD* and *csgBA* operon requires σ^S. Transduction of the mutant *rpoS* allele from SF1005 into ATCC 14028-1s and SR-11 gave white and smooth colonies, a saw_{28} morphotype (Fig. 1). Transcriptional analysis with the *csgD* and *csgA* probes on RNA extracted from the *rpoS* mutant of ATCC 14028-1s (MAE40) grown at 28 and 37°C detected no signal for either probe (Fig. 8). In addition, no extension product was seen in the latter strain by primer extension analysis (Fig. 5). Therefore, *rpoS* is also required for transcription of the *csg* operons in *S. typhimurium* strains.

It is also known that *ompR* is necessary for transcription from both the *csgBA* and the *csgD* promoters in *E. coli* (40). An *ompR* mutant was constructed by introducing a deleted *ompR* gene carrying an Amp^r cassette instead of the major part of its open reading frame into the chromosome of ATCC 14028-1s and SR-11 by double crossover, yielding strains MAE46 and MAE34, respectively (Table 1; see Materials and Methods). The *ompR* mutants were white at 28 and 37°C (Fig. 1). No CsgA signal was detected for MAE46 in Western blots (data not shown). Northern blot analysis of RNA extracted from strain MAE46 grown at both temperatures gave no signal for *csgD* or *csgA* (Fig. 8). Since *ompR* was shown to be necessary for the transcription of the *csgD* and *csgBA* promoters, the intergenic region was examined for putative *ompR* binding sites. One nucleotide sequence which has one mismatch base pair to the recently proposed consensus sequence for independent binding was found (39). This sequence is centered at position −50.5 relative to the transcriptional start site of the *csgD* promoter. If this sequence is used for OmpR binding, it can be imagined that transcriptional regulation takes place mainly at the *csgD* promoter. CsgD may then act upon the *csgBA* promoter to initiate transcription there.

DISCUSSION

Although many fimbrial gene clusters have been isolated from *Salmonella* spp. and *E. coli*, variation in operon structure and genes encoding regulatory control features of pili from the same structural class suggests frequent remodeling of DNA sequences due to the necessity to respond flexibly to changing environmental conditions and various host environments (49). In contrast to this behavior of fimbrial operons, the operons for curli biogenesis embedded in the same context on the chromosome (reference 20 and this study) are remarkably conserved between *E. coli* and *S. typhimurium*. In addition, the sequence similarities at the amino acid level of all proteins in the operon, ranging from the fiber subunit CsgA (86%) to the transcriptional regulator CsgD (96%) and the lipoprotein CsgG (99%), are higher than the homologies for most functionally and sequentially related fimbrial gene products, even within a species (49, 51, 56). It is therefore suggested that the thin aggregative fibers in *Salmonella* be named curli fibers, as for the products of the homologous *E. coli* genes (*csg*) (59).

The sequence similarity at the amino acid level is reflected by the successful complementation of individual *csg* gene mutants of *E. coli* by *S. typhimurium* genes. The *S. typhimurium csgB* gene could complement the homologous *E. coli* gene to wild-type levels, showing that the four-repeat structure consisting of 22-amino-acid-long putative β-strand–turn–β-strand–turn units (35) which are all required for organelle assembly (8) functions between the species. It can be imagined that interspecies exchange of organelle subunits can occur in a natural environment where bacteria are tightly packed, such as the gut flora. The consensus sequence for the repeat structure of CsgB was determined to be NLA-I-Q-GS-N-A-I-Q-G--, where the underlined amino acids show 100% conservation between *S. typhimurium* and *E. coli*. The consensus motif for CsgA, which has five 23-amino-acid-long units, is NS--T-TQYG-GN-AT-DQTAA-. There may be several reasons for the lack of complementation to a full wild type for some genes, such as functional restriction of the proteins, imbalance of protein ratios, or instability of the RNA message created from the plasmid, which remain to be elucidated. Alternatively, polar effects of the *csg* mutations on downstream genes may prevent the full complementation to the wild-type phenotype.

The high degree of conservation at the protein level contradicts the low conservation at the nucleotide level. The nucleotide sequences encoding *csgDEFG* diverged more than average, and so does K_S (0.94 on the average), a value for the estimation of the number of synonymous substitutions per site (Table 3). *csgE* has a K_S value of 1.5 and is therefore almost saturated with substitutions. The high rate of nucleotide substitutions could reflect the chromosomal location of the *csg* operons proximal to the terminus of replication, where more nucleotide changes take place, and/or the low expression state of the genes from the *csgDEFG* operon (61). The high conservation of proteins could indicate a lack of selective pressures, such as the immune response in a host, on the fibers or functional constraints on the macromolecules and their specificities, which do not tolerate an evolution which develops too far from a common ancestor (45).

The two *csg* operons are flanked by the same open reading frames as in the fully sequenced *E. coli* MG1655 strain (EMBL accession no. ecae205, ecae206, and ecd740 to ecd742). The homologous ORF179a showed the same ambivalent conservation scheme as the curli genes, low conservation on the nucleotide level and high conservation of the amino acid sequence (Table 3); the intergenic region between *csgG* and ORF179a showed no homology. Downstream of *csgC*, the nucleotide homology already starts to decline at the end of *csgC* (the stop codon has changed with respect to *E. coli*) to a level of 60%, which continues within ORF103.

Another fiber, type 1 fimbria, which seems to be present in all *Salmonella* species, shares morphological and adhesive properties with *E. coli* type 1 fimbria. However, the different location on the chromosome in the two species and the high degree of sequence diversity and gene rearrangements (14), together with different regulation schemes having no overlapping regulating factors, suggest that these genes were acquired independently by *S. typhimurium* and *E. coli* and subject to evolutionary pressures (45). Therefore, curli genes are the only

fimbrial genes detected which were already present in a common ancestor of *E. coli* and *Salmonella* spp.

The regulation of the curli genes in *S. typhimurium* SR-11 and ATCC 14028-1s by temperature, *rpoS*, and *ompR* is identical to that in *E. coli* MC4100 and YMel (2, 3, 34, 40, 53), implying that fiber expression responds to the same environmental cues in general. The complementation to wild-type levels of the *E. coli csgD* gene by the respective *S. typhimurium* gene shows that the recognition sites for the transcriptional activator are also conserved between the species. The correspondence in regulation pattern in addition to gene conservation points to a similar or identical function of the curli fibers in the two species in the same ecological niche. The expression pattern on plates at low temperature and low osmolarity suggests a role for curli fibers primarily outside a host, on surfaces. A participation of curli fibers in a bacterial network such as a biofilm (21) is possible, considering the adhesive nature of the fibers and the colony morphology of cells. However, more environmental studies need to be performed before a firm role of curli fibers in biofilm formation can be concluded.

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