Genes involved in *Cronobacter sakazakii* biofilm formation

Running title: Analysis of biofilm formation of *Cronobacter sakazakii*

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Abstract

*Cronobacter* spp. are opportunistic food-borne pathogens that can cause severe and sometimes lethal infection in neonates. In some outbreaks, the sources of infection were traced to contaminated powdered infant formula (PIF) or contaminated utensils used for PIF reconstitution. In this study, we investigated biofilm formation in *Cronobacter sakazakii* strain ES5.

To investigate the genetic basis of biofilm formation in *Cronobacter* on abiotic surfaces, we screened a random transposon mutant library of strain ES5 for reduced biofilm formation using a polystyrene microtitre assay. Genetic characterization of the mutants led to the identification of genes that are associated with cellulose biosynthesis and flagellar structure and biosynthesis, genes involved in basic cellular processes and virulence as well as several genes of currently unknown function. In two of those, hypothetical proteins ESA_00281 and ESA_00282, a strong impact on flow cell biofilm architecture was observed and their contribution to biofilm formation was confirmed by genetic complementation.

In addition, adhesion of selected biofilm formation mutants to Caco-2 intestinal epithelial cells was investigated. Our findings suggest a contribution of flagella, the hypothetical proteins ESA_00281 and ESA_00282, but not cellulose, to adhesion of *Cronobacter* to this biotic surface.
Introduction

Biofilms are interface-associated consortia of microorganisms that are typically embedded in an endogenous slimy matrix referred to as extracellular polymeric substance (EPS). It is generally accepted that growth as biofilm is the predominant microbial lifestyle in nature. Biofilms display several phenotypic characteristics that clearly set them apart from planktonic cultures, most notably increased resistance to a variety of environmental influences (16), which makes their eradication more difficult. Microbial biofilms are of special concern to the food industry, as biofilms on raw materials or food contact surfaces represent possible sources of product contamination with spoilage or pathogenic microorganisms (for a recent review, see 4).

Cronobacter spp. are opportunistic food-borne pathogens that can cause severe disease in neonates which may present as septicemia, meningitis or necrotizing enterocolitis (NEC). In several outbreaks, the source of infection could be traced to contaminated powdered infant formula (PIF) or to spoons and blenders used in its preparation (8, 10). The genus Cronobacter currently comprises six species: Cronobacter sakazakii, Cronobacter dublinensis, Cronobacter turicensis, Cronobacter malonaticus, Cronobacter muytjensii and Cronobacter genomospecies 1 (20). Cronobacter spp. display a remarkable desiccation resistance in comparison to other Enterobacteriaceae (7), which possibly contributes to their long-term survival in PIF and on surfaces. A few studies on biofilm formation of Cronobacter spp. have been conducted to date. The ability of certain strains to form biofilms on glass, stainless steel, PVC, polycarbonate, silicone and enteral feeding tubes in different media has been observed (19, 25, 28). As in other bacteria, biofilm formation differs between strains and is highly dependent on the medium and surface used. Furthermore, C. sakazakii survival in biofilms under different environmental conditions was investigated (23) and increased resistance of Cronobacter biofilms to
disinfectants has been demonstrated (25). Cellulose was described as a component of the Cronobacter extracellular matrix (15, 28, 53).

In this study, we performed a genetic analysis of biofilm formation in strain Cronobacter sakazakii ES5, a clinical isolate, by random transposon mutagenesis and subsequent screening of a mutant library for altered biofilm phenotype in a microtitre assay system. In addition, biofilm structure of the wild type and selected mutants was investigated in a continuous culture flow cell system by confocal laser scanning microscopy. Finally, we tested in selected mutants whether the defects in biofilm formation observed on the abiotic surface also had an influence on the adherence capacity of C. sakazakii to Caco-2 intestinal epithelial cells.

Materials and Methods

Bacterial strains and plasmids.

All bacterial strains and plasmids used in this study are listed in Tables 1 and 3. C. sakazakii ES5 is a clinical strain that exhibited strongest biofilm formation on polystyrene surfaces in preliminary experiments with a panel of Cronobacter strains.

Media and growth conditions.

Media ingredients were obtained from BD (Franklin Lakes, NJ, USA), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) and Sigma (Buchs, Switzerland). C. sakazakii and Escherichia coli were routinely grown in modified LB broth (3) at 37°C with vigorous agitation unless otherwise stated. Media were solidified by addition of 12 g/L agar. When required, antibiotics were added to the following final concentrations: ampicillin 100 µg/mL; kanamycin 50 µg/mL. For blue/white selection, 5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside (X-Gal) and Isopropyl-β-D-1-thiogalacto-pyranoside were added to final concentrations of 40 µg/mL and
0.2 mM, respectively. Congo red (CR) agar and Calcofluor (Fluorescent brightener 28) agar were prepared as described in (38) and (15), respectively. Growth in liquid cultures was monitored by measuring OD$_{600}$ with an Ultrospec II spectrophotometer (Biochrom, Cambridge, UK) or by viable cell count of 10-fold serial dilutions on Plate Count Agar (Oxoid, Basingstoke, UK).

**Crystal violet (CV) microtitre biofilm assay**

Quantification of biofilms grown in microtitre dishes was performed as described in (18). AB minimal medium (2 g/L (NH$_4$)$_2$SO$_4$, 6 g/L Na$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 3 g/L NaCl, 2 mM MgCl$_2$, 0.1 mM CaCl$_2$, 3 µM FeCl$_3$ x 6 H$_2$O) supplemented with 0.4% (w/v) maltose was used as growth medium and cultures incubated at 37°C for 45 h. After CV-staining, absorbance at 570 nm measured using a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Construction of the transposon mutant library**

A random transposon mutant library of strain *C. sakazakii* ES5 was constructed using the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (EPICENTRE, Madison, WI) following the manufacturer’s instructions. Mutants were picked to the wells of polystyrene microtitre dishes (Nunc, Denmark) containing modified LB supplemented with 7.5% glycerol and 50 µg/mL kanamycin, grown overnight at 37°C and stored at -20°C.

**DNA extraction, cloning, transformation and sequencing**

All kits for DNA purification were obtained from QIAGEN (Hilden, Germany) and handled following the manufacturer’s instructions. Unless otherwise stated, chromosomal DNA was purified using the DNeasy® Blood and Tissue Kit. Plasmids were extracted with the QIAprep® Spin Miniprep or Plasmid Midi kits. DNA fragments from PCR reactions, restriction digests and agarose gels were purified using the MinElute PCR Clean-up and the MinElute Gel purification
Kits, respectively. Concentration of nucleic acids was determined using a Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Cloning, restriction analysis and transformation of *C. sakazakii* and *E. coli* were performed essentially as described in (40). Restriction enzymes and corresponding buffers were obtained from Roche (Mannheim, Germany) and T4 DNA ligase from New England Biolabs (Ipswich, MA, USA). All sequencing was performed by Microsynth (Balgach, Switzerland).

**Southern analysis and identification of transposon insertion sites**

Southern analysis was performed as described previously (21). For insertion site identification by the subcloning procedure, chromosomal DNA of the transposon mutants was digested with SphI and the fragments ligated into pUC18NotI (Table 1) digested with the same enzyme. The ligation mixture was electroporated into *E. coli* XL1-Blue and transformants carrying a plasmid containing the transposon sequence selected on modified LB containing 50 µg/mL kanamycin. Plasmids were extracted from the selected clones and transposon-flanking regions sequenced with primer KAN-2 FP1 (Table 2).

For the single primer PCR, DNA fragments were amplified from chromosomal DNA of the transposon mutants using primer Tn5PCRF (Table 2) with the GoTaq Green PCR System (Promega, Madison, WI, USA) and the reaction conditions described in (22). PCR reactions were purified and used as template in a sequencing reaction using primer KAN-2 FP2.

Transposon insertion sites were determined by blastn search (1) against the genome of *C. sakazakii* BAA-894 (accession numbers NC_009778, NC_009779, NC_009780) or the NCBI nucleotide collection (nt/nt). In case of missing annotations or gene names, these were added manually by blastx (1) search with the sequence obtained from the transposon mutants and backed up by blastp (1, 2) search with the corresponding ORF sequence of BAA-894 against the NCBI non-redundant protein sequences. All blast searches were carried out using default
parameters. Cellular localization of the affected proteins was predicted by Psortb v.2.0 (12) and cello (51).

**Construction of complementation plasmids**

Putative operon ESA_00281-ESA_00280 and ORF ESA_00282, both including flanking sequences, were amplified from wild type chromosomal DNA using primer pairs 281f1/280r4 and 282f2/282r1, respectively. PCR products were ligated into pCR2.1 (Table 1) using the TA-cloning® kit (Invitrogen, Carlsbad, CA, USA) and inserts were verified by sequencing using primers M13 and M13r (Table 2). Inserts were recovered from the plasmids by HindIII or BamHI (Roche, Mannheim, Germany) digestion, gel-purified and cloned into pUC18NotI digested with the same enzyme to yield plasmids pP1b and pR8b.

**Electron microscopy**

Bacteria were grown as overnight liquid cultures at 37°C with agitation in AB minimal medium supplemented with 0.8% lactose and investigated by EM as previously described (48).

**Cultivation of biofilms in flow cells and confocal laser scanning microscopy (CLSM).**

Biofilms were grown in flow cells fitted with 24 x 50 mm #1 glass cover slips (Menzel Gläser, Braunschweig, Germany) as substratum in modified LB diluted 1:20 with distilled water. Ampicillin (100 µg/mL) was added to the medium when required. Flow cell system components were obtained from DTU (Lyngby, Denmark; flow cells and bubble traps) and Omnilab (Mettmenstetten, Switzerland; silicone tubes). The flow cell system was operated with a Watson-Marlow 205S peristaltic pump (Watson-Marlow, Wilmington, MA, USA) at a flow rate of 0.7 mm/s.

Bacteria were subcultured from fresh overnight cultures in modified LB for 4 h at 37°C with shaking before they were diluted in 0.85% NaCl to an OD<sub>600</sub> of 0.1. Flow cell channels were inoculated in duplicate with 350-400 µL of cell suspension and attachment allowed for 1 h at
room temperature without flow. After that, the flow cell system was incubated at 30°C with constant flow. At 24 h or 45 h after incubation start, flow cells were rinsed with 0.85% NaCl for 20 min. To stain the biofilms for CLSM, 350-400 µL of a 10 nM SYTO 9 solution (Invitrogen, Carlsbad, CA, USA) was injected into the flow cell channels and incubated for 15 min in the dark. After rinsing with 0.85% NaCl for 10 min, biofilms were observed with a Leica TCS SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) at 400x magnification. Image scans were performed using a 488 nm argon laser for excitation and a 490-690 nm emission band for detection. At least two representative image stacks were captured per flow cell channel.

**Adhesion to Caco-2 cells, giemsa staining and microscopy of cell monolayers**

Caco-2 intestinal epithelial cells were routinely maintained in Gibco Minimal Essential Medium (MEM) containing 100 µM MEM non-essential amino acids, 4 mM GlutaMAX™-I, 10% (v/v) fetal calf serum (FCS) and 200 µg/mL gentamicin at 37°C in a 5% CO₂ atmosphere. All cell culture media, additives and PBS were obtained from Invitrogen (Carlsbad, CA, USA) with the exception of fetal calf serum, which was obtained from PAA (Pasching, Austria). To assess adhesion of *C. sakazakii* ES5 and derivatives to intestinal epithelial cells, 10⁵ Caco-2 cells/well were seeded in 24-well cell culture dishes (TPP, Trasadingen, Switzerland) containing 1 mL test medium (Gibco Iscove’s Modified Dulbecco’s Medium supplemented with 10% (v/v) FCS) per well and allowed to grow for 72 h at 37°C 5% CO₂. Bacteria were subcultured from fresh overnight cultures in modified LB for 4 h at 37°C with agitation and subsequently diluted in test medium to 10⁷ CFU/mL. Caco-2 cells were infected by replacing the medium with 1 mL bacteria suspension, resulting in an infection dose of 10⁷/well (MOI approx. 10:1). Infected cells were incubated for 3 h at 37°C 5% CO₂. After washing 5 times with 1 mL PBS/well, Caco-2 cells were lysed by addition of 1 mL 0.2% Triton X-100 in PBS/well and subsequent incubation at 37°C for
30 min. Total cell associated bacteria were enumerated by serially diluting the lysates in PBS, plating on Plate Count Agar.

Giemsa staining of Caco-2 monolayers was performed as previously described (30) and slides investigated at 1000x magnification using an Olympus VANOX-S microscope (Olympus, Hamburg, Germany) fitted with a ZEISS Axiocam digital camera using the ZEISS Axioview software (Carl Zeiss AG, Germany).

**Statistic analysis**

All comparisons were performed using a ranked t-test for unequal variances (Welch test) using a script for the statistical computing software R (35).

**Results**

**Isolation of mutants with altered biofilm formation**

A random transposon mutant library of strain *C. sakazakii* ES5 comprising 3328 clones was generated. These were screened for reduced biofilm phenotype on polystyrene using a crystal violet microtitre assay. Only mutants that retained wild type growth phenotype but displayed more than 15% reduction in biofilm formation were genetically characterized. Based on these criteria 21 transposon mutants impaired in biofilm formation (Figure 1, Table 3) were selected.

**Genetic characterization of the isolated mutants**

Southern hybridisation confirmed the presence of only one transposon in all of the mutants. Transposon insertion sites were determined using two different approaches: by a subcloning procedure or by single primer PCR if the subcloning procedure failed. Using standard homology searches, the transposon insertion sites were mapped to the genome of strain *C. sakazakii* BAA-894 and functions were assigned to the defective genes. While 16 of the identified mutations affected genes located on the chromosome of strain BAA-894, three were found in sequences that
are plasmid-borne in BAA-894 or other organisms. The genes with transposon insertions were grouped into functional classes as reported in Table 3. Mutations were found in genes involved in cellulose biosynthesis, motility, basic cellular functions, virulence, and in genes that are of unknown function. Two groups of “sibling” mutants with identical transposon insertion sites were discovered: three mutants carrying the transposon in the flhE gene (identical to mutant BF2) and two in a putatively plasmid-encoded sequence (representative mutant BF17). In two pairs of mutants (BF3/BF10 and BF11/BF15), the same gene, but at different positions, was mutated.

In total, four mutants were defective in the cellulose biosynthesis pathway. Biofilm formation in these mutants was reduced to 15-34% respective to the wild type as quantified in the CV-microtitre assay (Figure 1, Table 3).

One mutant (BF12) carried the transposon in bcsA, which codes for the catalytic subunit of the cellulose synthase. Two non-isogenic mutants were defective in bcsC (BF3 and BF10), which encodes the cellulose biosynthesis operon protein C, a putative oxidoreductase. The remaining mutant was defective in bcsG, whose product is not yet characterized.

A total of five mutants were defective in flagellum-associated genes. In one mutant (BF5), the transposon was inserted in fliD, which encodes the flagellar capping protein, and in another (BF13) in flgJ, a muramidase gene involved in the biosynthesis process of the flagellum. In the three remaining mutants (identical to BF2), the transposon was found in a flagellar gene (flhE) whose function is still unknown, at an identical insertion position. In the fliD and flhE mutants, the biofilm in the CV-assay was dramatically reduced (9% and 20% relative to the wild type), while the biofilm of the flgJ mutant was only reduced to 84% relative to the wild type.

In three mutants, the transposon was inserted into genes that are involved in fundamental cellular processes, such as cell division (ftsK, involved in chromosome segregation, mutant BF1) and energy metabolism (cyoD, fourth subunit of the cytochrome o ubiquinol oxidase (mutant BF8),...
and the acetolactate synthase gene alsS, (mutant BF9) involved in mixed acid fermentation). As these mutations cause severe cellular defects or retarded growth due to impaired energy metabolism, the observed reductions in biofilm formation as compared to the wild type (by 93%, 81% and 82%, respectively) have to be regarded as unspecific in the context of biofilm formation.

In mutant BF17, the transposon was inserted in mgtB, a gene encoding a P-type Mg²⁺ transport ATPase that has been described in the context of invasion of epithelial cells and macrophages. The gene is plasmid-encoded in C. sakazakii BAA-894. The disruption of mgtB resulted in 77% less biofilm relative to the wild type in the CV-microtitre assay.

A total of six mutants were affected in hypothetical proteins. One mutant (BF4) carried the transposon in ORF ESA_04103, whose product is a hypothetical protein with unknown function. This mutant biofilm displayed a markedly different appearance when assessed visually in the microtitre assay. This translated into a 31% reduction in the CV quantification relative to the wild type. Only one similar sequence, from Enterobacter sp. 638, could be found by blastp search against the NCBI non-redundant protein database. The subcellular localisation of this protein was predicted to be in the inner membrane. The biofilm architecture of mutant BF4 was further investigated in the flow cell system. However, no difference with respect to the wild type could be observed (data not shown).

Two identical mutants (representative BF16) were isolated in which the transposon insertion site could not be mapped to any sequence in the genome of BAA-894. However, the nucleotide sequence obtained from the mutants was 89-90% similar to the nucleotide sequences of certain cryptic plasmids of different E. coli strains (see Table 3 for details). On two of these plasmids (pMG828-5 and p6148), the insertion site was located in intergenic regions, while in plasmid pE2348-2, the insertion site was mapped to a putative ORF encoding a hypothetical protein. The
defect in this putatively plasmid-borne region led to a biofilm reduction of 66% as compared to the wild type in the CV microtitre assay (Figure 1, Table 3).

Finally, a total of three mutants were found to carry the transposon in two further genes encoding hypothetical proteins, ORFs ESA_00281 and ESA_00282. In one mutant (BF14) the transposon was inserted into ESA_00281 and in two (BF 11 and BF 15) it was inserted into ESA_00282, however, at different sites. The mutant biofilms had a clearly different appearance in the microtitre assay and bound 73% (mutant BF14) or 60% (mutant BF15) of the CV-amount trapped by the wild type biofilm (Figure 1, Table 3). The mutated genes are located next to each other in the genome of C. sakazakii BAA-894, but oriented divergently. ORF ESA_00281 is organized in a putative operon with ORF ESA_00280, which is also not characterized to date. Similar proteins are also encoded in the Salmonella, Enterobacter and Citrobacter genomes sequenced so far. In E. coli, the genes seem to be absent with the exception of strain O157:H7 EC4024.

**Phenotypic characterisation of the mutants**

The mutants were further characterized using suitable phenotypic assays. As the extracellular matrix is known to play an important role in bacterial biofilm formation, the wild type and all mutants were streaked on CR agar and on calcofluor agar, which both contain cellulose-binding dyes. The former also contains Coomassie brilliant blue to indicate proteinaceous components of the extracellular matrix. The wild type formed pink, rugose colonies similar to the pdar (pink, dry and rough) morphotype defined in Salmonella, which indicates the presence of cellulose but absence of curli fimbriae in the cellular matrix ((38); Figure 3A). All mutants displayed the wild type phenotype on both CR and CF agar, except mutants BF14 (ESA_00281::Tn), BF15 (ESA_00282) and, as expected, the four cellulose mutants (Table 3, Figure 3A). In mutants BF14 and BF15, an interesting phenotype could be observed, which was identical in both mutants. The colonies were of the same pink color as the wild type, but they displayed a smooth colony edge.
The typical wrinkled structure observed in wild type colonies on CR agar was restricted to the colony center (Figure 3A). The cellulose mutants formed, as expected, smooth, whitish colonies resembling the saw (smooth and white) morphotype of *Salmonella* (38), which indicates that neither cellulose nor curli fimbriae are present (Figure 3B). On calcofluor agar, the wild type colonies were brightly fluorescent under UV light, indicating cellulose presence. Among the mutants, all were equally fluorescent, except for the cellulose mutants, whose colonies showed only weak fluorescence (data not shown).

Strain ES5 wild type, flagellar mutants BF2 (*flhE::Tn*), BF5 (*fliD::Tn*) and BF13 (*flgJ::Tn*) as well as mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn) were examined by electron microscopy to investigate flagellar structure. Examination of the wild type and flagellar mutants with respect to flagellar motility remained unsuccessful due to immobility of the wild type strain under standard motility assay conditions, suggesting that swimming motility might not be critically important for *C. sakazakii* biofilm formation. EM analysis showed that ES5 wild type was peritrichously flagellated, as is typical for most Enterobacteriaceae. However, the *flgJ* mutant (BF13) was aflagellate, and the *fliD* mutant (BF5) had shorter flagella that seemed to be more brittle, as unattached flagellum fragments could be observed in the sample (Figure 3B). No difference compared to the wild type could be observed in mutants BF2, BF14 and BF15 with respect to structure or average number of flagella per cell.

**Complementation of mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn)**

Because of these interesting phenotypes and the adjacent localisation of the mutant genes on the BAA-894 genome, we decided to perform genetic complementation experiments with these mutants. The complemented mutants BF14 (pP1b) and BF15 (pR8b) and ES5 wild type (pUC18NotI) were first investigated in the CV microtitre assay. However, the presence of high copy cloning vector pUC18NotI alone reduced biofilm formation of ES5 wild type by 43%
The medium copy plasmid pBBR1MCS (27) was also investigated as a possible alternative, but its negative effect on the wild type biofilm was even greater, inducing a 60% reduction in biofilm formation respective to the wild type (data not shown). Therefore, we decided to use pUC18NotI for our complementation experiments. OD$_{600}$ measurements indicated a lower growth rate of the transformants compared to the wild type (data not shown), possibly as a result of the increased metabolic load caused by plasmid presence. Replication of the plasmid and constitutive expression of plasmid-borne antibiotic selection markers drains the cell’s resources in terms of energy and building blocks (14).

The complemented mutants BF14 (pP1b) and BF15 (pR8b) were investigated on CR agar, in the CV microtitre assay and in the flow cell system. As controls, strains BF14 (pUC18NotI), BF15 (pUC18NotI) and ES5 wild type (pUC18NotI) were included in these experiments. In the CV microtitre assay, the presence of pUC18NotI reduced the biofilm of mutant BF15 to 19% compared to the wild type, which corresponded to a further reduction of 60%. In mutant BF14, this plasmid-induced additional reduction was less pronounced: Mutant BF14 produced 68% of wild type biofilm in the presence of pUC18NotI, corresponding to a 14% further reduction. Despite these effects, expression of ORFs ESA_00280-ESA_00281 or ORF ESA_00282 from the complementation plasmids restored biofilm as quantified in the CV microtitre assay to 108% and 56% of wild type level, respectively, showing a clearly positive effect of in-trans expression of the mutated genes (Figure 4). In the flow cell system (see below) and on CR agar, no effects of pUC18NotI were apparent in ES5 wild type or mutants BF14 or BF15. Wild type phenotype could be restored in both mutants in the presence of the respective complementation plasmids both on CR agar (data not shown) and in the flow chamber system (Figure 2B).

**Biofilm architecture in a flow cell system**
Flagellar mutant BF2 (flhE::Tn) and mutants disrupted in uncharacterized genes BF4 (ESA_04103::Tn), BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn) were further characterized by comparison of their biofilms with wild type biofilms grown in a continuous culture flow cell system. After 24 hours of incubation, a basal layer of cells of about 20 µm could be observed in the wild type, with microcolonies protruding to about 30 µm. After 45 h, wild type biofilm thickness in the basal layer remained unchanged but microcolony height increased slightly to about 40 µm (Figure 4A).

In mutants BF2 (flhE::Tn) and BF4 (ESA_04103::Tn), no difference to wild type biofilm architecture was apparent (data not shown). Mutants BF14 (ESA_00281::Tn) and BF15 (ESA:00282::Tn), however, displayed an altered biofilm architecture, which was identical in both strains. Only microcolonies could be observed after 24 h of incubation that were not interconnected by a homogenous layer of bacteria as observed in the wild type at this stage. Only single attached cells were detected on the glass surface between the microcolonies (data not shown). After 45 hours, clonal growth of the singly adherent bacteria and the bacteria in microcolonies seemed to have occurred, however, patches of uncolonized surface were still visible between the microcolonies (Figure 4B), which was also in contrast to the wild type.

**Adhesion of selected mutants to Caco-2 intestinal epithelial cells**

To investigate whether genes identified in the screening for defects of biofilm formation on polystyrene also play a role in adhesion to a biotic surface, we assessed adhesion capacity of selected mutants to confluent monolayers of Caco-2 cells by measuring total cell associated bacteria after 3 hours of incubation. Results are summarized in Figure 5. The flhE-mutant (BF2), the mgtB-mutant (BF17) and the cellulose-negative bcsA-mutant (BF12) showed no difference in comparison to the wild type (data not shown). In the aflagellate flgJ-mutant (BF13; data not shown), adhesion was reduced almost to the level of negative control E. coli K-12, in which the
number of recovered bacteria was approximately two orders of magnitude lower than that of the wild type. The numbers of bacteria recovered from the Caco-2 cells was reduced by one order of magnitude in comparison to the wild type in both the ESA_00281 and the ESA_00282 mutants, BF14 and BF15. Total cell associated bacteria were also determined for mutants BF14 and BF15 harboring either pUC18NotI or complementation plasmids pP1b and pR8b, respectively, and an ES5 wild type control containing pUC18NotI (Figure 5A). The presence of pUC18NotI in the wild type reduced the number of recovered bacteria by approx. 0.1 log CFU/mL, which was statistically significant. In the two mutants, the presence of the empty vector did not lead to a further significant reduction in the number of recovered bacteria. In the complemented ESA_00282 mutant (BF15 (pR8b)), the number of total cell associated bacteria returned to the level of the wild type control containing pUC18NotI. In complemented mutant BF14 (pP1b), this number was restored to wild type level.

The wild type adhered in clusters with diffusive adhesion visible between the clusters (Figure 5B). In the flhE-mutant (BF2) and the mgtB-mutant (BF17), no differences to the wild type could be observed. However, in mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn), the clusters of bacteria were absent (Figure 5B), and the occasional mutant cluster was much smaller than in the wild type (not shown). Diffusively adherent bacteria appeared unchanged. Upon complementation, the wild type pattern, with bacteria both adhering diffusively and in clusters, was restored in both mutants.

Discussion

Cellulose biosynthesis mutants

Bacterial cellulose biosynthesis is encoded in two operons, bcsABZC (yhjONML) and bcsEFG (yhjSTU), which are present in E. coli and Salmonella (9, 42, 53) and which were also recently
characterized in *C. sakazakii* ES5 (15). Biofilm formation defects in cellulose biosynthesis operon mutants of *Salmonella* and *E. coli* have been reported previously (9, 42). Our screening also identified mutants defective in genes located in both operons, thereby confirming once more a role of **bcsG** from the as yet uncharacterized operon **bcsEFG** in cellulose biosynthesis and biofilm formation (42). In all these mutants, the absence of cellulose from the extracellular matrix could be phenotypically confirmed on CR and calcofluor agar. The colony morphologies were similar to those described in cellulose operon mutants in *Salmonella* and *E. coli.* (9, 53).

It is surprising that we did not detect any mutations in curli fimbriae-associated genes, as their role in biofilm formation and as adhesins in both *E. coli* and *Salmonella* is well established (19, 38, 53). However, the structural genes for curli fimbriae are not present in the BAA-894 genome, and *C. sakazakii* ES5 wild type formed rugose pink colonies on CR agar, similar to the pink dry and rough (pdar) morphotype of *Salmonella,* in which this morphotype indicates the presence of cellulose but absence of curli in the extracellular matrix (38, 53). This implies an important difference to other Enterobactericeae in adhesion to abiotic as well as biotic surfaces and biofilm formation.

**Flagellar mutants**

Flagella seem to play a role in the initial phases of biofilm development, in attachment as well as further development (5). Our screening identified mutations in three flagellar genes, **flgJ,** **fliD** and **flhE.**

EM investigations of the mutants defective in the flagellar muramidase FlgJ (mutant BF5; (34)) and the flagellar capping protein FliD (mutant BF2; (49)), respectively, revealed defects that are in agreement with previous reports in of other species (26, 34).

FlhE is a flagellar protein of currently unknown function and is present in several proteobacterial genera (43). Its cotranscription with flagellar export apparatus genes **flhAB** as part of the **flhABE**
operon has been confirmed in *Salmonella* Typhimurium (57). FlhA and FlhB are required for flagellar biosynthesis (29) and therefore both swimming and swarming motility. However, a *flhE* mutant of *Salmonella* Typhimurium was only defective in swarming and was observed to be unaffected in flagellar structure, which is in line with our EM results in the *Cronobacter flhE* mutant BF2 (43). Furthermore, the *Salmonella* Typhimurium mutant also displayed other interesting phenotypes such as enhanced biofilm formation on PVC, reduced calcofluor binding, and altered morphology on CR agar. Our results obtained with the *C. sakazakii* ES5 mutant, although not always in agreement with the phenotypes described in the corresponding *Salmonella* mutant, support the hypothesis of Stafford and Hughes that FlhE plays a role in extracellular matrix composition (43).

**Magnesium uptake and biofilm formation**

One mutant was found to be defective in a homologue of *mgtB*, which encodes a magnesium-transporting P-type ATPase. Although MgtB has been shown to mediate Mg\(^{2+}\) influx, it is presently not clear if this is its primary physiological function, as constitutive Mg\(^{2+}\) uptake in bacteria is usually mediated by CorA (41), which is also present in the BAA-894 genome (ORF ESA_03744). The fact that the *mgtB* homologue is plasmid-encoded in BAA-894 further supports the notion of an ancillary magnesium uptake system. Interestingly, *mgtE*, a Mg\(^{2+}\)-channel, was found in a biofilm screening in *Vibrio cholerae* (33) and a screening in *Aeromonas hydrophila* for reduced adherence to Hep2-cells (31). The recovered *A. hydrophila mgtE*-mutant also showed reduced adherence in a microtitre biofilm assay. Both *V. cholerae* and *A. hydrophila* possess CorA, so *mgtE* might also not be the main route of Mg\(^{2+}\) uptake in these organisms.

**Genes of unknown function**

A total of three mutants were disrupted in uncharacterized ORFs ESA_00281 and ESA_00282, which are conserved in different Enterobacteriaceae but not *E. coli*. Apart from the CV-microtitre
assay, the corresponding mutants BF14 and BF15, respectively, also showed phenotypes different from the wild type on CR agar, in flow cell biofilm architecture and adhesion to a biotic surface (see below). In each case, the phenotypes were identical in both mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn), suggesting the involvement of the two genes in the same process. Furthermore, all wild type phenotypes could, at least partly, be restored by complementation. In

*Salmonella* Typhimurium ORFs STM3154-3156 corresponding to ESA_00280-ESA_00282 were described as motility genes (11, 46) and deficiencies in swimming and swarming were reported in their mutants. The „microcolonies-only“ biofilm architecture observed in both mutants, BF14 and BF15, is consistent with this notion, as similar phenotypes have been reported for motility mutants in *P. aeruginosa*, in which microcolonies arise through clonal growth of an immotile subpopulation while the substratum surface is fully colonized by a motile subpopulation (5). In addition, the mutant colony morphology on CR agar also suggested a role in the context of extracellular matrix composition.

**Adhesion of selected mutants to Caco-2 intestinal epithelial cells**

As some genetic factors involved in attachment to abiotic surfaces also play a role in adhesion to both plant and animal tissues (47, 50), we analysed adhesion to Caco-2 cells in selected mutants. In these experiments, we observed that the absence of flagella (*flgJ* mutant BF13) greatly reduced adhesion capacity, suggesting that flagella are important for *Cronobacter* adhesion to biotic surfaces, as has been shown for other Enterobacteriaceae (13, 37). Absence of cellulose had no effect on adhesion capacity under our experimental conditions, which, moreover, is in line with results in *Salmonella* cellulose biosynthesis mutants reported by others (42). Also mutants BF14 and BF15, defective in ORFs ESA_00281 and ESA_00282, respectively, showed, in addition to an altered adhesion pattern (only diffusively adherent bacteria versus diffusively adherent bacteria and bacteria adhering in clusters), a significantly reduced adhesion capacity. However,
adhesion was still considerably higher than that of the aflagellate flgJ mutant BF13. As the
Salmonella homologues of ESA_00281 and ESA_00282 were reported in the context of motility
and flagella are known to be involved in surface and bacterial cell-to-cell adhesion, it could be
assumed that these phenotypes are a consequence of dysfunctional or altered flagella. However,
the Cronobacter flhE mutant BF2, which is similar with respect to flagellum-related phenotypes
(intact structure, swarming defects in a Salmonella mutant (43)) showed no difference to the wild
type in both adhesion capacity or adhesion pattern. Therefore, it is tempting to speculate that the
mutations in ORFs ESA_00281 and ESA_00282 affect adhesion factor(s) other than flagella that
are involved in adhesion to and aggregation on the Caco-2 cells.
In conclusion, this study implicates that factors important for biofilm formation in other
Enterobacteriaceae, cellulose and flagella, also contribute to biofilm formation in C. sakazakii.
However, our results also point to possible differences such as the absence of curli fimbriae, at
least in the isolate investigated in this study and strain C. sakazakii BAA-894, whose genome is
fully sequenced.

Acknowledgments
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We would like to thank Maya Grimm and Elisabeth Schraner for technical assistance, Susan
Schönmann, Stefanie Heller, Chantal Loepfe and Carmen Kaiser for experimental support and
Taurai Tasara and Peter Wild for helpful advice.

References


Figure Legends

Figure 1: Quantification of biofilm. Biofilm of ES5 wild type and mutants measured in the CV microtitre assay. Values are means from at least two independent experiments with 2-8 replicates each. Error bars indicate one standard deviation. The dashed line indicates the level of ES5 wild type (100%). All mutant values were significantly different from the wild type (p <0.001, ranked Welch test).

Figure 2: Confirmation of mutant phenotype. 2A: Colony morphology of ES5 wild type, cellulose biosynthesis mutants BF3 (bcsC::Tn), BF6 (bcsF::Tn) and BF12(bcsA::Tn) and mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn) on CR agar.

2B: electron micrographs of ES5 wild type and flagella mutants BF2 (flhE::Tn), BF5 (fliD::Tn) and BF13 (flgJ::Tn). The arrow indicates an unattached flagellar fragment.
**Figure 3:** Complementation of mutants BF14 and BF15. Quantification of biofilm in ES5 wild type, ES5 (pUC18NotI), mutants BF14 (ESA_00281::Tn), BF15 (ESA_00282::Tn) and complemented mutants in the CV microtitre assay. Values are means of at least three independent experiments with at least six replicates each. Error bars indicate one standard deviation. The dashed line denotes the level of ES5 (pUC18NotI). All values were compared to the wild type, and one asterisk (*) indicates a significant difference (p < 0.05, Welch test). BF14 (pUC18NotI), BF14 (pP1b), BF15 (pUC18NotI) and BF15 (pR8b) were also compared to wild type (pUC18NotI). Significant differences (p < 0.01, Welch test) are indicated by two asterisks (**).

**Figure 4:** CLSM image stack profiles of flow cell biofilms. The large panel represents a horizontal cross section of the flow cell-grown biofilm, while the smaller panels to its sides represent vertical cross sections. The white dashed lines on the panels indicate the planes of the respective other two cross sections. 4A: *Cronobacter sakazakii* strain ES5 wild type at 24 hours and 45 hours after incubation start; BL: basal layer; MC: microcolony. 4B: Mutants BF14 (ESA_00281::Tn), BF15 (ESA_00282::Tn) and their respective complemented mutants at 45 hours after incubation start. All images were taken at 400x magnification.

**Figure 5:** Adhesion to Caco-2 intestinal epithelial cells. 5A: Total cell associated bacteria of ES5 wild type, ES5 (pUC18NotI), mutants BF14 (ESA_00281::Tn) and BF15 (ESA_00282::Tn) and their respective complemented mutants. One asterisk (*) indicates a significant difference to ES5 wild type (p < 0.01, Welch test), while two asterisks (**) indicate a significant difference to ES5 wild type (pUC18NotI) (p < 0.001, Welch test).
5B: Micrographs of Giemsa stained Caco-2 monolayers with adherent bacteria (ES5 wild type, BF14 and BF15) taken at 1000x magnification. Nuclei stained red-violet, bacteria stained dark blue. Cl: cluster of bacteria; CP: cytoplasm; N: nucleus; S: single adherent bacterium.
### TABLE 1. Bacterial strains and plasmids used in this study.

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<th>Strain or plasmid</th>
<th>Genotype/characteristics</th>
<th>Source or reference</th>
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<td></td>
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<td>F’endA1 recA1 hsdR17 (r6k-, mK+) supE44 thi-1 gyr96 relA1 Φ80lacZΔM15 Δ(lacZY A-argF) U169 λ</td>
<td>Invitrogen</td>
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<td><strong>Plasmids and transposons</strong></td>
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<td>This study</td>
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<tr>
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<td>ESA_00281 and ESA00_280</td>
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### TABLE 2: Primers used in this study. Recognition sequences of restriction enzymes are underlined.
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This study
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<th>Description</th>
<th>Accession&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Positives&lt;sup&gt;4&lt;/sup&gt; (aa)</th>
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<td>15, 38, 53</td>
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<td>15.5 ± 5.9</td>
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<td>ND&lt;sup&gt;j&lt;/sup&gt;</td>
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<sup>a</sup>Annotation

<sup>b</sup>Locus tag is denoted by (Gene ID).

<sup>2</sup>Conserved hypothetical protein.

<sup>3</sup>Accession number.

<sup>4</sup>Reference values include the mean ± standard deviation.

<sup>i</sup>Biological variation in repeat experiments.

<sup>j</sup>ND: Not determined.

<sup>k</sup>ES4. 659

<sup<l>Functional group

<sup>3</sup>Cellulose biosynthesis

<sup>4</sup>Biofilm

<sup>5</sup>CV-assay, CR, agar (EM)
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Transposon-containing derivative of C. sakazakii ES5


based on blastp search with BAA-894 protein sequences against the NCBI non-redundant protein sequences on January 15, 2010

C. sakazakii ES5 sequence from BAC-clone (15)

not applicable

blastn search with sequence obtained from ES5 mutant against the nucleotide collection (nr/nt) on January 15, 2010

wild type; biofilm in CV-assay: 100±9.2%

standard deviation

smooth and white (38)

pink, dry and rough (38)

pink, dry and rough, smooth edge (this study)

not determined

35