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1 Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: identification
2 of a new colony morphology type and the role of SGI1 in biofilm formation

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1 **Abstract**

2 In this study we examined the extent of biofilm formation in field strains of *Salmonella*
3 *enterica* serovar Typhimurium (*S. Typhimurium*), an important foodborne pathogen. Ninety-
4 four field strains of *S. Typhimurium* were tested for their ability to form biofilm and
5 components contributing to its formation. Most *S. Typhimurium* strains were highly capable
6 of biofilm formation except for strains of phage type DT2 originating from pigeons. The most
7 efficient biofilm forming strains were those of phage type DT104 positive for *Salmonella*
8 genomic island 1 (SGI1). A comparison of SGI1 positive and negative strains indicated that
9 the increased biofilm formation of SGI1 positive strains was associated with the presence of
10 this genomic island. Finally, in 5 strains we found an alternative strategy of biofilm formation
11 independent of curli fimbriae and cellulose production but solely dependent on an
12 overproduction of capsular polysaccharide. Due to a mucoid and brown appearance on Congo
13 Red agar we designated these strains as belonging to the SBAM (Smooth Brown and Mucoid)
14 morphotype.

15
16 **Keywords**

17 Biofilm, *Salmonella Typhimurium*, morphotype, cellulose, curli fimbriae, extracellular
18 polysaccharide, DT104, SGI1

19

1 **Introduction**

2 *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) periodically colonizes
3 susceptible hosts or persists outside a host in the environment during its life cycle. To survive
4 in the environment, *S. Typhimurium* has evolved several distinct mechanisms (White et al.,
5 2006) and its ability to adhere to solid surfaces and to form biofilms belongs among the most
6 important. Extracellular structures contributing to biofilm formation include curli fimbriae
7 (Collinson et al., 1996; Romling et al., 1998b), cellulose (Zogaj et al., 2001; Solano et al.,
8 2002), capsular polysaccharide (de Rezende et al., 2005) and other polysaccharides like LPS
9 (Anriany et al., 2006).

10

11 Strains capable of biofilm formation frequently form colonies of typical RDAR (Red, Dry and
12 Rough) morphotype which is caused by the coexpression of curli fimbriae and cellulose
13 (Romling, 2005). Other morphotypes such as BDAR (Brown Dry and Rough) or SAW
14 (Smooth and White) have been described in experiments with deletion mutants in *csg* genes
15 required for curli fimbriae synthesis (Romling et al., 1998a, Romling et al., 2000) or both *csg*
16 and *bcs* genes coding for cellulose synthesis, respectively (Solano et al., 2002). The
17 contribution of the remaining biofilm components to adhesion is less obvious (De Rezende et
18 al., 2005).

19

20 In the mid 1980's, multidrug resistant strains of *S. Typhimurium* belonging to phage type
21 DT104 were detected in the UK (Threlfall et al., 1994). Strains of this clone are mostly
22 resistant to 5 antibiotics and have been reported to be capable of efficient biofilm formation
23 (Anriany et al., 2001; de Rezende et al., 2005). The reasons for the successful spread of this
24 clone are largely unknown. We speculated that the acquisition of the SGI1 resulted not only in
25 antibiotic resistance but also in increased biofilm formation which allowed the spread of this

1 clone worldwide. In this study we therefore investigated to what extent the ability to form
2 biofilm is common to field strains of *S. Typhimurium* and whether there is any difference in
3 biofilm production between SGI1 negative and positive *S. Typhimurium*.

4

5 **Material and Methods**

6 *Bacterial strains*

7 Ninety four *S. Typhimurium* strains originating from different hosts isolated in the Czech
8 Republic from 2004-2007 were used in this study (Tab. 1). All but 9 strains were phage typed
9 according to Anderson et al. (1977). All the strains were also classified by PCR specific for
10 the absence of the STM0517-STM0529 genes and the presence of SGI1 as described
11 previously (Matiasovicova et al., 2007). Additional 55 strains representing another 10
12 *Salmonella enterica* serovars were screened for biofilm formation as controls. These included
13 serovars Agona (n= 3), Derby (n= 9), Heidelberg (n= 2), Enteritidis (n= 19), Infantis (n= 8),
14 London (n= 4), Montevideo (n= 3), Saintpaul (n= 2), Virchow (n= 2) and Zanzibar (n= 3).

15

16 *Colony morphology and expression of cellulose and curli fimbriae*

17 Bacterial colony morphology was analyzed on LB agar without salt supplemented with Congo
18 Red (40 $\mu\text{g ml}^{-1}$) and Coomassie brilliant blue (20 $\mu\text{g ml}^{-1}$). Cellulose production was
19 determined on the same agar supplemented with calcofluor (20 $\mu\text{g ml}^{-1}$). To assess colony
20 morphology and cellulose production, 18-hour old liquid bacterial cultures were serially
21 diluted and plated on appropriate agar plates. After 96 hours at 28 °C, the colony morphology
22 was determined on plates where tens of colonies were growing. For the detection of cellulose,
23 colony fluorescence was measured visually under 366 nm UV light.

24

25 *Biofilm formation*

1 Eighteen-hour-old bacterial cultures of *S. Typhimurium* grown in minimal medium (M9) were
2 500x diluted and inoculated into 200 μ l M9 medium in a flat bottomed 96-well polystyrene
3 microtitre plate. The plates were incubated statically at 28 °C. After 96 hours, the microplates
4 were processed according to the standard procedure (Genevaux et al., 1996) using safranin to
5 stain the adherent bacteria. The experiment was repeated on 3 separate occasions.

6

7 *Capsular polysaccharide detection in bacterial culture*

8 Enzyme-Linked Lectinsorbent Assay (ELLA, Leriche et al., 2000) was adapted for the
9 quantification of mannose and glucose residues in planctonic bacterial culture. After a 48 hour
10 cultivation, OD_{600nm} was determined. Bacterial culture (200 μ l) from each well of the
11 microplate was transferred to microtubes and 2 μ l of concanavalin A labeled with peroxidase
12 (1g ml⁻¹ stock solution) were added. After 1 hour the bacteria were pelleted by centrifugation
13 at 10 000g for 1 min and processed as described by Leriche et al. (2000) for the adherent
14 bacteria. The final absorbance of each culture was measured at 405 nm and divided by
15 OD_{600nm} to obtain capsular polysaccharide production per cell.

16

17 *Scanning electron microscopy*

18 To visualize biofilms attached to the glass slides, sterile rectangular slides were incubated in
19 test tubes containing bacterial culture in M9 medium in a vertical position for 96 hours at
20 28°C. The slides with adherent bacteria were fixed in 3% glutaraldehyde in phosphate-
21 buffered saline pH 7.4 and serially dehydrated with acetone. All samples were critical-point
22 dried, coated with gold alloy and examined with a Vega/LMU microscope under an 30 kV
23 acceleration and 12 000x magnification.

24

25 *Statistical analysis*

1 The comparison of biofilm formation measured by safranin staining between strains differing
2 in the presence or absence of SGI1 was performed by a T-test. ANOVA followed by
3 Tuckey's test was used for the comparison of capsular polysaccharide production among
4 different morphotypes. All statistical calculations were carried out using SPSS v.14.0
5 statistical software.

6

7 **Results**

8 *Colony morphology, cellulose and capsular polysaccharide production determine adhesive*
9 *abilities of isolates*

10 Four different colony morphotypes were detected among the investigated strains. In 17 strains
11 we detected the previously described SAW morphotype, in 10 strains the BDAR morphotype
12 was identified and the RDAR morphotype was found in 62 strains. In 5 strains we found a
13 morphotype that had not been described previously. The strains of this morphotype grew on
14 Congo Red agar in smooth brown colonies and were mucoid (Fig. 1), similar to the growth of
15 *lon* mutants (Torres-Cabassa and Gottesman, 1987). The colonies did not produce cellulose
16 but overproduced capsular polysaccharide as determined by the ELLA assay. The average
17 amount of capsular polysaccharide in SBAM strains, expressed as absorbance measured by
18 the ELLA assay, reached value 2.42 ± 0.40 while the same values in SAW, BDAR and RDAR
19 strains were 1.12 ± 0.73 , 1.32 ± 0.38 and 1.69 ± 0.45 , respectively. This difference was confirmed
20 as significant by ANOVA followed by a posthoc Tuckey's test. We designated this
21 morphotype as SBAM (Smooth Brown and Mucoid).

22

23 The vast majority ($n = 76$) of strains formed biofilms on plastic microplates as well as on
24 glass slides under the conditions tested and this ability correlated with the colony
25 morphology. Sixteen strains of SAW morphotype did not adhere at all. The remaining one

1 SAW strain isolated from cattle adhered poorly (Fig. 2). Strains of SBAM (average biofilm
2 formation 0.72 ± 0.27) and BDAR (average biofilm formation 0.60 ± 0.34) morphotypes formed
3 intermediate levels of biofilm. Except for a single strain, 61 RDAR strains (average biofilm
4 formation 1.21 ± 0.32) were highly proficient biofilm producers (Fig. 2). In control strains of
5 other serovars we found a similar distribution of different morphotypes and correlation
6 between the morphotype and biofilm formation (not shown).

7

8 *Scanning electron microscopy and biofilm formation on glass slides*

9 The electron microscopy of biofilms adhered to glass slides confirmed the ability of BDAR,
10 RDAR and SBAM strains to form differentially organized biofilms. Cells of the BDAR
11 morphotype were organized in microcolonies connected with a fragile extracellular matrix
12 which did not allow the formation of mature biofilms (Fig. 3a). RDAR strains were the most
13 efficient in biofilm formation and cells of this morphotype were enclosed in a compact
14 meshwork of curli fimbriae and cellulose (Fig. 3b). SBAM strains formed voluminous
15 biofilms on glass slides with cells embedded in a dense glycocalyx composed of capsular
16 polysaccharides (Fig. 3c).

17

18 *Biofilm formation and presence of SGI1*

19 We have described recently that multidrug resistant SGI1 positive *S. Typhimurium* strains are
20 characterized by the absence of STM0517-0529 genes for anaerobic allantoin utilization.
21 Similar if not identical strains which lack the SGI1 can be found also among antibiotic
22 sensitive strains (Matiasovicova et al., 2007). Consequently, we hypothesized that the
23 comparison of STM0517-0529 genes negative strains differing only in the presence of SGI1
24 as determined by PCR might allow us to assess the role of SGI1 in the biofilm formation.
25 Nineteen STM0517-0529 genes and SGI1 negative strains formed biofilms at an average

1 value of 0.88 ± 0.47 while forty seven STM0517-0529 genes negative but SGI1 positive strains
2 formed biofilm at an average value of 1.16 ± 0.41 . When these values were compared by the T-
3 test, they were found to be significantly different ($P<0.05$) confirming the contribution of
4 SGI1 to biofilm formation.

5

6 **Discussion**

7 In this study, we investigated in particular *S. Typhimurium* field strains for their ability to
8 form biofilms and to produce the extracellular structures essential for adhesion to solid
9 surfaces. Since multiple authors have described a correlation between colony morphology and
10 biofilm formation (Gerstel and Romling, 2003; Romling, 2005; Kim and Wei, 2007) in *S.*
11 *Typhimurium* as well as in other *Salmonella* serovars, this was used as a main phenotypic
12 criterion for strain characterization. In *S. Typhimurium* four different morphotypes were
13 detected in all. Three of them (SAW, BDAR and RDAR) have already been described in
14 different defined mutants (Romling et al., 2000) as well as in strains isolated from various
15 sources (Solano et al., 2002; Jain and Chen 2006). The last one, SBAM, has not been
16 described in detail so far. Strains belonging to the SBAM morphotype were capable of
17 biofilm formation despite the fact that they produced neither cellulose nor curli fimbriae.
18 Their ability to adhere was solely dependent on the overproduction of capsular polysaccharide
19 as confirmed by ELLA assay (Leriche et al., 2000) and electron microscopy. The electron
20 microscopy of SBAM strains confirmed the presence of capsular polysaccharide in the form
21 of condensed granules and filaments typical for highly hydrated structures like colanic acid
22 (Prigent-Combaret et al., 2000). Although the capsular polysaccharide has been described as a
23 common component of *Salmonella* biofilms (Anriany et al., 2001, de Rezende et al., 2005), its
24 role has been considered as less important. Despite this, strains forming smooth mucous
25 colonies with adhesive abilities and which fluoresced poorly on calcofluor plates, i.e. similar

1 to the SBAM strains of *S. Typhimurium*, have also been described in *S. Enteritidis* (Solano et
2 al., 2002). We also observed strains of SBAM morphotype in three strains of serovar
3 Montevideo and one strain belonging to serovar Derby.

4
5 Consistent with previous studies (White and Surette, 2006; Kim and Wei, 2007), the most
6 efficient biofilm formation was observed among the strains belonging to the phage type
7 DT104 forming a RDAR morphotype which correlates with biofilm formation in
8 *Enterobacteriaceae* in general (Romling, 2005). When all the strains were differentiated
9 according to the presence or absence of genes for anaerobic allantoin utilization (STM0517-
10 0529) and SGI1 (Matiasovicova et al., 2007), i.e. regardless of their phage-type, we observed
11 a positive effect of SGI1 on biofilm formation in *S. Typhimurium*. Our observations may
12 correlate with the findings of Golding et al. (2007) who reported that the acquisition of SGI1
13 led to the increased expression of *sthE*, a putative major fimbrial subunit in *S. Typhimurium*
14 LT2. The SGI1, besides the antibiotic resistance also increases the biofilm formation potential
15 of host strains. This improved ability can enhance the capability of these strains to survive in
16 the environment and consequently to more frequently infect new hosts.

17

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23

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1 **Figure legends**

2

3 **Fig. 1.** Morphotypes of *S. Typhimurium* after 96-hour cultivation at 28 °C on Congo Red LB
4 agar. A - RDAR, B - SAW, C - BDAR and D - SBAM morphotype, respectively.

5

6 **Fig. 2.** Correlation between morphotype and biofilm production determined after 96-hour
7 growth in microplate and biofilm detection by safranin staining (Y axis). Each spot represents
8 one strain of particular morphotype.

9

10 **Fig. 3.** Scanning electron microscopy of biofilms on glass slides; biofilm formed by BDAR
11 (A), RDAR (B) and SBAM strains (C) respectively. Strains of SAW morphotype are not
12 shown since they did not adhere to the glass slide. Bar represents 2 μm .

1 **Tab. 1.** List of *S. Typhimurium* field strains used in this study.

phage type	n ^{&}	original host [#]
DT1	3	poultry (2) wild boar
DT2	10	pigeon (9) duck
DT12	1	human
DT14	1	cattle
DT104	57	human (17) cattle (15) pig (14) poultry (5) environment (2) pigeon (2) cat food
DT114	1	human
DT120	3	cattle (2) human
DT166	1	pigeon
DT178	2	cattle (2)
DT193	1	cattle
DT195	1	pigeon
U	2	human parrot
RDNC	2	cattle pheasant
not known	9	cattle (2) pig (3) poultry laboratory strain (2) human

2

3 [&] number of strains

4 [#] numbers in brackets indicate number of strains if different from 1

5

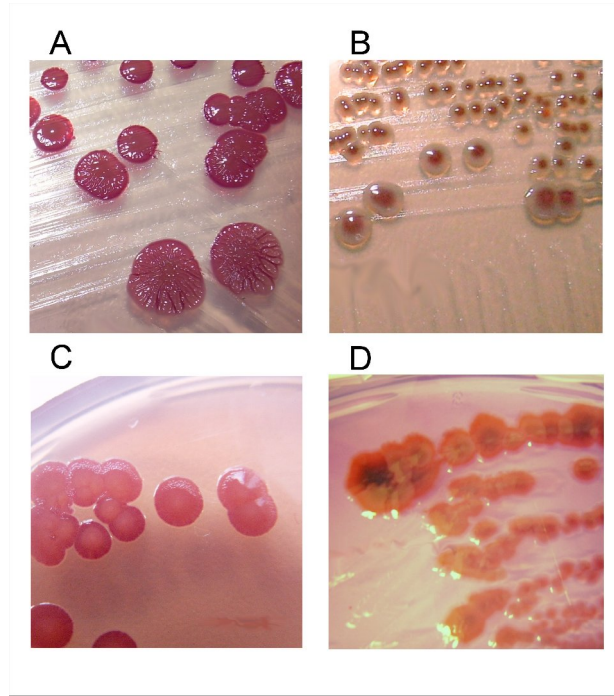
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9

- 1 **Fig. 1.** Malcova et al., Biofilm formation in field strains of *Salmonella enterica* serovar
- 2 Typhimurium: identification of a new colony morphology type and the role of SGI1 in
- 3 biofilm formation.



1 **Fig. 3.** Malcova et al., Biofilm formation in field strains of *Salmonella enterica* serovar
2 Typhimurium: identification of a new colony morphology type and the role of SGI1 in
3 biofilm formation.

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5

