Modulation of Biofilm-Formation in *Salmonella enterica* Serovar Typhimurium by the Periplasmic DsbA/DsbB Oxidoreductase System Requires the GGDEF-EAL Domain Protein STM3615

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Abstract

In *Salmonella enterica* serovar Typhimurium (S. Typhimurium), biofilm-formation is controlled by the cytoplasmic intracellular small-molecular second messenger cyclic 3',5'-di- guanosine monophosphate (c-di-GMP) through the activities of GGDEF and EAL domain proteins. Here we describe that deleting either *dsbA* or *dsbB*, respectively encoding a periplasmic protein disulfide oxidase and a cytoplasmic membrane disulfide oxidoreductase, resulted in increased biofilm-formation on solid medium. This increased biofilm-formation, defined as a red, dry and rough (rdar) colony morphology, paralleled with enhanced expression of the biofilm master regulator CsgD and the biofilm-associated fimbrial subunit CsgA. Deleting *csgD* in either *dsb* mutant abrogated the enhanced biofilm-formation. Likewise, overexpression of the c-di-GMP phosphodiesterase YhjH, or mutationally inactivating the CsgD activator EAL-domain protein YdiV, reduced biofilm-formation in either of the *dsb* mutants. Intriguingly, deleting the GGDEF-EAL domain protein gene STM3615 (*yhjK*), previously not connected to rdar morphotype development, also abrogated the escalated rdar morphotype formation in *dsb* mutant backgrounds. Enhanced biofilm-formation in *dsb* mutants was furthermore annulled by exposure to the protein disulfide catalyst copper chloride. When analyzed for the effect of exogenous reducing stress on biofilm-formation, both *dsb* mutants initially showed an escalated rdar morphotype development that later dissolved to reveal a smooth mucoid colony morphology. From these results we conclude that biofilm-development in S. Typhimurium is affected by periplasmic protein disulphide bond status through CsgD, and discuss the involvement of selected GGDEF/EAL domain protein(s) as signaling mediators.

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Introduction

Infections with *Salmonella enterica* (*S. enterica*) represent a major health problem and a significant burden on food industry [1]. In the European Union alone, infections caused by *S. enterica* serovar Typhimurium (S. Typhimurium) stand as the second most prevalent cause of food-borne acute gastroenteritis [2]. In human, infections related to non-typhoidal serovarants, such as S. Typhimurium, are usually seen as an acute self-healing infection [3]. In contrast, typhoid fever, caused by *S. enterica* serovar Typhi (S. Typhi), represents a severe and potentially lethal systemic infection [3].

Marine salmonellosis as caused by S. Typhimurium [4], as well as cell culture infections using the pathogen, have been used as models for dissecting the details of invasive systemic salmonellosis. In this, several virulence factors have been identified as instrumental in steering the acute systemic infection [3]. A hallmark of salmonellosis, whether non-typhoidal or typhoidal, is the establishment of carrier states [5,6]. In fact, 3 to 5% of the convalescent typhoid fever cases are converted to asymptomatic carriers [7,8] while animals, such as reptiles, may harbor and shed non-typhoidal *Salmonella*ae for prolonged periods of time [9]. Such carrier states are likely responsible for transmission and continuous outbreaks of salmonellosis [5,7]. Details governing this very important step of salmonellosis have remained much less explored. However many virulence factors identified to act in the acute phase of the infection also contribute to persistency, including factors mediating tolerance to oxidative stress [10]. In addition, the formation of a so-called biofilm on cholesterol-rich gallstones is believed to promote persistent carriage both in marine infection models, as well as in man [11].

Bacterial biofilms are complex communities consisting of microorganisms embedded in a self-produced extracellular matrix. In this matrix, microbes grow on either biotic or abiotic...
surfaces, attaching to the surface and each other, conferring resistance to both immunity-related as well as pharmaceutical antimicrobials [12].

Apart from being a probe for microbial pathogenesis, *Salmonella Typhimurium* is a well-defined model organism for detailing events in bacterial biofilm-formation. For *S. Typhimurium*, biofilm-formation is characterized by a red dry and rough (rdar) morphotype when grown on low-osmolarity nutrient agar plates supplemented with the diazo dye Congo red [12]. Formation of the rdar morphotype much relies on the production of the extracellular matrix components cellulose and so-called curli fimbrae consisting of the CsgA as the major protein subunit [13].

The transition into an rdar morphotype relies on the biofilm master gene regulator protein CsgD. CsgD activates the csgBAC operon with accompanied increased production of the curli fimbral CsgA and CsgB subunits [14,15]. Further, CsgD indirectly increases cellulose production by activating adrA that codes for a di-guanylate cyclase [15]. The small molecule cyclic di-guanosine monophosphate (c-di-GMP) generated by AdrA is a ubiquitous secondary messenger found in almost all bacterial species [16–18]. The AdrA-mediated increase in c-di-GMP enhances expression of the cellulose synthetase BcsA, which in turn increases cellulose production [14,19].

The cellular levels of c-di-GMP are maintained by GGDEF and EAL/HD-GYP domain proteins, which act as diguanylate cyclases and phosphodiesterases respectively [20–23]. Contrary to sessility, motility is inhibited by increased levels of c-di-GMP [23]. Hence, increased cellular levels of c-di-GMP promote a sessile growth of bacteria [20,21]. Activation of motility is also regarded as initiating egression from biofilm-formation to allow for further colonization of new habitats [24].

Interestingly, in a number of bacteria a substantial number of genes that are affected during switches between planktonic and sessile growth are connected to oxidative stress tolerance [25–27]. Relevant for the transition into an rdar morphotype when grown on low-osmomolarity nutrient agar plates without salt for two days at 28°C until a rugous morphotype development (Fig. S1). Hence, we focused on the strain pair proficient or deficient in pSLT showed an identical additional mutants, none of these deviations were comparable in degree with that of the AdsbA and AdsbB mutants (Fig. S1). Also, the strain pair proficient or deficient in pSLT showed an identical rdar morphotype development (Fig. S1). Hence, we focused on dsbA and dsbB in further study.

The rdar morphotype development in a AdsbA-dsbB double mutant behaved as either of the single dsbA or dsbB deletion mutants (Fig. 1A), implying that the dsbA and dsbB acted through a convergent rather than through divergent pathways in suppressing rdar morphotype development. To exclude any genetic downstream effect of the mutations introduced, we showed restoration of wild type rdar formation by providing a cloned dsbA or dsbB gene into the respective single mutant (Fig. 1B).

In parallel, somewhat surprisingly, we noted that both the AdsbA and AdsbB mutant failed to create pellets in static salt-less LB broth culture at the air-liquid interface another type of biofilm mediated by the rdar morphotype (Fig. 1C and 1D) [51]. Combined, these observations implied that biofilm-formation as assayed by rdar morphotype or by pellet formation differentially relied on the DsbA/DsbB redox-shuffling system.

**Results**

**Oxidoreductases and rdar morphotype development in *S. Typhimurium***

To map the role of oxidoreductases of the thioredoxin superfamily in *S. Typhimurium* in biofilm-formation, we started by collecting and constructing individual *AdsbA, AdsbB, AdsbC, AdsbD, AdsbH, AdsbL, AgsA, AgsB, AtrxB* and *AtrxB* mutants in the *S. Typhimurium* strain pair proficient or deficient in pSLT background, thus generating a set of mutants formally defective in any of the three oxidoreductase systems introduced above. In contrast to laboratory *E. coli* strains, *S. Typhimurium* codes for the additional thioredoxin-like SacsABCD proteins [49,50]. Finally, several strains of *S. Typhimurium* carry a “virulence-associated” plasmid (pSLT) that codes for the *dsbA* parologue *SrgA* [40]. Therefore, we included a *AoscABCD* mutant and an isogenic *S. Typhimurium* strain pair carrying or lacking pSLT into the strain panel.

Next, each mutant was compared with the wild type for rdar morphotype development on congo red (CR) agar plates. In this, the *AdsbA* and *AdsbB* mutants revealed a marked escalation in rdar morphotype development (Fig. 1A and S1). While we noted minor alterations in rdar morphotype development for some of the additional mutants, none of these deviations were comparable in degree with that of the *AdsbA* and *AdsbB* mutants (Fig. S1). Also, the strain pair proficient or deficient in pSLT showed an identical rdar morphotype development (Fig. S1). Hence, we focused on dsbA and dsbB in further study.

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**DsbA and DsbB affect expression of CsgA and CsgD**

The rdar morphotype of *S. Typhimurium* mainly develops through the expression of surface-located curli fimbrae and cellulose in the extracellular matrix [12]. CsgA (AgA) is the major structural subunit of these fimbrae [52,53], the expression of which is positively regulated by CsgD, the major transcriptional activator of biofilm-formation in *E. coli* and *S. enterica* [54]. Therefore, we extended our study to determine the effect of *AdsbA* and *AdsbB* mutations on CsgA and CsgD expression.

CsgA was isolated from cultures grown on Luria agar (LA) plates without salt for two days at 28°C until a rugous morphotype development was evident. CsgA was then extracted and depolymerized with the use of formic acid before the preparations were run on SDS-PAGE gels to compare CsgA yields. In this analysis, the CsgA yields were around 10 times higher in the *AdsbA* mutant and around 15 times higher in the *AdsbB* mutant as compared to the isogenic wild type control (Fig. 2A). Thus, the increase in CsgA levels was in accordance with the *rdar* morphotype of the mutants on CR plates (Fig. 1A). Similarly, the CsgD levels, as determined by immunoblot analysis from the cellular fraction of same cultures, were about 2–4 times higher in both the *AdsbA* and *AdsbB* mutants.
Figure 1. Biofilm-formation on solid media and liquid media. The formation of rdar morphotype in wild type, single and double dsb mutants grown for 48 hours on Congo red plates at 28°C is illustrated in panel (A). This escalated rdar morphotype development can be reverted to wild type level by trans-complementation with corresponding cloned dsb genes, or by introducing a cloned yhjH gene or depleting csgD (B). VC indicates the vector control, pBAD30. C) Crystal violet staining of biofilm adherent to polystyrene tubes as an indicator of biofilm-formation at liquid-air interface. The ΔdsbA and ΔdsbB mutants fail to make a pellicle at air-liquid interface in static LB without salt culture at 28°C 24 hours post inoculation. D) Quantification of adherent biofilm measured as retained Crystal violet in biofilm. Error bars indicate SEM. *** = p≤0.001. doi:10.1371/journal.pone.0106095.g001
mutants (Fig. 2B). However, we could not detect CsgA or CsgD from the static liquid cultures, whether using bacterial extracts from wild type or dsb mutants (data not shown).

To test whether dsbA or dsbB affected CsgD levels at the transcriptional or post-transcriptional level, we measured the promoter activity of csgD by using a plasmid-carried csgD-lacZ transcriptional promoter fusion [55]. In this analysis, we could not detect any substantial differences in csgD promoter activity from salt-free LA plate cultures at any time point for either mutant (Fig. 2C). These results suggest that on plates, ΔdsbA and ΔdsbB mutants affect CsgD expression at a post-transcriptional level. Surprisingly though, in broth cultures under shaking conditions, we noted a decrease in csgD-lacZ activity for either dsb mutant that is consistent with the decreased pellicle formation of the dsb mutants (Fig. 2D).

CsgD- and c-di-GMP-dependency of ΔdsbA and ΔdsbB mutant rdar morphotypes

As stated above, rdar morphotype development is stimulated by c-di-GMP [54], while GGDEF and EAL domain proteins in turn balance the levels of c-di-GMP [54, 55]. Moreover, recent reports show that selected GGDEF/EAL domain proteins in E. coli activate the promoter of the csgBAC operon independent of CsgD [58, 59]. As DsbA and DsbB appeared to affect the levels of CsgD (Fig. 2B), we first asked whether the effect of the ΔdsbA and ΔdsbB mutations on rdar morphotype development required CsgD. Hence, to probe for a role of CsgD in the escalated rdar morphotype development in the dsb mutants, we deleted dsbA or dsbB in a ΔcsgD mutant background. Both double mutants expressed a red color, but failed in rdar morphotype development on the Congo red (CR) plates (Fig. 1A). This characteristic Congo red binding of ΔdsbA-csgD and ΔdsbB-csgD double mutants most likely implicates residual production of the extracellular matrix component cellulose (Fig. 1A and 3).

Next, we determined whether the enhanced rdar morphotype formation of the ΔdsbA or ΔdsbB mutants was released from c-di-GMP-mediated control. To address this, we used an indirect approach. The ΔdsbA and ΔdsbB mutants were transformed with a cloned plasmid containing the phosphodiesterase gene yhjH, under an arabinose inducible promoter. When the ΔdsbA and ΔdsbB mutants were grown under conditions inducing yhjH, formally depleting the bacterial cells of c-di-GMP, the increased rdar morphotype development of the two dsb mutants were decreased while no such effect was observed using the vector control (Fig. 1B). Thus, the escalated rdar morphotype development of ΔdsbA or ΔdsbB mutants was not due to a plain disconnection from CsgD or c-di-GMP-mediated control.

GGDEF/EAL domain proteins and rdar morphotype development

To date 20 GGDEF/EAL domain proteins have been identified in S. Typhimurium [60]. As selected housekeeping functions in S. Typhimurium affect rdar morphotype development through selected GGDEF/EAL domain proteins such as CsgD, and as the elevated rdar morphotype development could be down-regulated by the c-di-GMP phosphodiesterases YhjH in both the ΔdsbA or ΔdsbB mutant, we investigated to identify GGDEF/EAL domain protein(s) are required for the up-regulated rdar morphotype in ΔdsbA/B mutant. To this end, we created double mutants all of the twenty GGDEF/EAL domain protein genes in the ΔdsbA or ΔdsbB mutant backgrounds. Subsequently, we compared rdar morphotype development in single and all combinations of double mutants (Table 1, Fig. 3).

The GGDEF/EAL domain protein STM1703 (yciR) and GGDEF domain proteins STM3611 (yhjH) and STM4264 (yjcC)
each inhibit expression of CsgD and hence rdar morphotype development [57]. Accordingly, as also noted here, plain yciR, yhjH and yjcC mutants revealed an escalated rdar morphotype development. In parallel, we noted a further escalation in rdar morphotype development for corresponding D dsbA and D dsbB mutants in the D yciR or D yhjH mutant backgrounds at 48 hours post inoculation, while D dsbA-yjc and D dsbB-yjc double mutants much behaved as single dsb mutants (Table 1, Fig. 3).

In accordance with prevailing literature, single mutants lacking STM1344 (ydiV), STM2123 (yegE), STM2672 (yfiN), STM3388 or STM4551 expressed a strongly reduced rdar morphotype development (Table 1, Fig. 3). Still, when the D dsbA or D dsbB deletions were introduced into the D yegE or D yfiN mutant backgrounds we did not note any substantially reduced rdar morphotype development compared to single dsb mutants (Table 1). In contrast, the D dsbA-ydiV and D dsbB-ydiV double mutants revealed a delayed rdar morphotype development in comparison to plain D dsbA or D dsbB mutants (Table 1, Fig. 3), notably at 24 hours post inoculation. Intriguingly, while not affecting rdar morphotype development in a wild type background, deleting STM3615 (yhjK) in either the D dsbA or D dsbB mutant resulted in a delayed rdar morphotype development (Table 1, Fig. 3).

When assaying for CsgD expression in the D ydiV and D yhjK mutants, we noted decreased CsgD levels in the D ydiV, D dsbA-ydiV and D dsbB-ydiV mutants as compared to the wild type and single dsb mutants (Fig. 4). In contrast, a plain D yhjK mutant did not reveal any obvious alteration in CsgD expression, while the double mutants particularly D dsbB-yhjK double mutant did reveal a decrease in CsgD levels. Furthermore, the CsgD expression and rdar morphotype in the D yhiV-yhjK and D yhjK-dsbA/B mutants was also reduced to the respective wild type and single dsb mutants (Fig. 4, Fig S2). However, the D yhjH and D yhjH-dsbA/B mutants showed induced CsgD levels as compared to the wild type (Fig. 4). Thus, the CsgD levels closely correlated with rdar morphotype development of respective strains.

**Figure 3. Effect of GGDEF/EAL protein gene mutations on dsb associated biofilm-formation.** The development of rdar morphotype in wild type, single and double mutants on Congo red plates at 28°C after 24 and 48 hours of incubation. doi:10.1371/journal.pone.0106095.g003

Oxidative and reductive stresses disperse rdar morphotype development in D dsbA and D dsbB mutants

Bacterial biofilm-formation responds to oxidative and nitrosative stress [34,35,61], and relies on periplasmic oxidoreductase activity (Fig. 1A). Consequently, we tested whether the enhanced rdar morphotype development of D dsbA or D dsbB mutants could be affected by providing exogenous oxidative stress. Thus, we first supplemented the CR plates with the disulphide bond catalyst copper chloride (CuCl₂) at non-lethal concentrations. Alternative-
ly, sterile filter paper discs soaked with 1M CuCl₂ were put at the very edge of bacterial inoculum streaked on CR plates. In this analysis, S. Typhimurium lost its ability to generate rdar morphotype upon CuCl₂-exposure in a dose dependent manner, whether being proficient or deficient in dsbA or dsbB (Fig. S3). We next tested the reductant dithiothreitol (DTT) at a 5 mM final non-lethal concentration in the CR plates, after which we followed rdar morphotype development. When grown on such plates, the dsb mutants again showed enhanced rdar morphotype development at day one post inoculation, at which time the wild type strain still exhibited a rather smooth colony morphotype (Fig. 5A). At the second day post inoculation, both dsb mutants had lost their rdar morphotype, and converted into a highly mucoid morphotype three days post inoculation (Fig. 5A).

Complementation of either ΔdsbA or ΔdsbB mutant, with respective gene on a recombinant plasmid, under reductive stress, resulted in enhanced rdar morphotype development resembling the wild-type pattern (data not shown). Interestingly, the mucoid morphotype was enhanced in both the ΔdsbA and ΔdsbB mutant upon by knocking out csgD or upon expressing YhjH from the recombinant plasmid after 48 hours (Fig. 5A).

Accompanying the altered rdar morphotype development, the levels of CsgA and CsgD were reduced in ΔdsbA and ΔdsbB mutants as compared to the wild type strain under DTT stress (Fig. 5B and 5C). Still, we could not detect any effect of DTT stress on the CsgD promoter activity (Fig. 5D and 5E).

**Table 1. Effect of GGDEF/EAL proteins on dsb associated biofilm-formation.**

| Mutation | Background | WT(dsba’ dsbb’) | ΔdsbA | ΔdsbB |
|----------|------------|-----------------|--------|--------|
|          |            | 24hr | 48hr | 24hr | 48hr | 24hr | 48hr |
| WT       | +/-        | +    | +    | 2+   | +    | 2+   | +    |
| Δstrom1142 (csgD) |        | -    | -    | -    | -    | -    | -    |
| Δstrom0385 (adrA) |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom1283 (yeaA) |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom1967 (yedD) |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom2672 (yflN) |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom4551 |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom2123 (yegE) |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom3388 |        | -    | -    | +    | 2+   | +    | 2+   |
| Δstrom1697 |        | -    | +    | 2+   | +    | 2+   | +    |
| Δstrom3375 (cstD) | +/- | +    | +    | 2+   | +    | 2+   | +    |
| Δstrom1344 (ydiV) |        | -    | -    | +    | 1.5+ | +    | 1.5+ |
| Δstrom3615 (yhjK) | +/- | +    | +/- | 1.5+ | +/- | 1.5+ | +/- |
| Δstrom0343 | +/- | +    | +    | 2+   | +    | 2+   | +    |
| Δstrom0468 (ydaB) | +/- | +    | +    | 2+   | +    | 2+   | +    |
| Δstrom1827 |        | +    | 2+   | 2+   | 3+   | 2+   | 3+   |
| Δstrom2215 (rtn) |        | +    | 2+   | 2+   | 3+   | 2+   | 3+   |
| Δstrom4264 (yjcC) |        | +    | 2+   | 2+   | 3+   | 2+   | 3+   |
| Δstrom3611 (yijhH) |        | +    | 2+   | 2+   | 4+   | 2+   | 4+   |
| Δstrom1703 (yebB) |        | 2+   | 3+   | 2+   | 4+   | 2+   | 4+   |
| Δstrom2410 (yfaA) |        | +    | 2+   | +    | 2+   | +    | 2+   |
| Δstrom2503 (yfgF) |        | +    | 2+   | +    | 2+   | +    | 2+   |
| Δstrom1344–3615 | +/- | +    | +/- | +    | +    | +/- | +    |

---/+/+/+ etc. represents the degree of the biofilm-formation compared to the WT.

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![Figure 4. Effect of ydiV, yhjK and yhjH mutations on dsb associated biofilm-formation.](image-url)
Discussion

Several lines of evidence link redox stress with formation on bacterial biofilms [44–46,61]. Here we demonstrate a role of the periplasmic Dsb oxidoreductase system in biofilm generation of S. Typhimurium. More precisely, we present that DsbA and DsbB negatively regulate biofilm-formation on solid growth medium but were required for the pellicle formation at the air-liquid interface in static broth, and that DsbA and DsbB prevent biofilm dispersion under reductive stress. We further indicate that ΔdsbA and ΔdsbB mutations affect expression of the major biofilm-activator protein CsgD and concomitantly expression of the biofilm extracellular matrix component CsgA.

Our results corroborate previous works in the sense that DsbA associates with biofilm-formation in E. coli O157 and Pseudomonas putida. For E. coli this dependence on DsbA materialized for biofilm-formation at the air liquid interface [45]. On the other hand, a dsbA mutant of P. putida expressed enhanced biofilm-formation in liquid medium [46]. Thus, the role DsbA for biofilm-formation of E. coli and S. Typhimurium in liquid media appears to contrast the requirement of DsbA for biofilm-formation in P. putida under similar conditions.

DsbA functions as a periplasmic disulphide bond catalyst, itself being reduced in the process. In E. coli, DsbA is returned into its oxidized state through DsbB [62]. As a pair, DsbA and DsbB are accounted responsible for no less than 40% of the E. coli envelope protein disulfide bond formation [63]. To the best of our knowledge, we could not find any report about the role of DsbB protein in biofilm-production. In our study, mutants deficient in either DsbA or DsbB exhibited an equal loss of biofilm-formation in broth, and in a comparable enhanced rdar morphotype development on plates that associated with an increased expression of CsgA and CsgD. As ΔdsbA-csgD and ΔdsbB-csgD double mutants failed to express any rdar morphotype, the increased CsgD expression in plain dsb mutants might well explain their enhanced rdar morphotype.

DsbA is not absolutely restrained in its substrate specificity. In fact, the non-specific disulphide catalyst CuCl2 can in part replace DsbA [64]. Therefore, we continued by testing the effect of CuCl2 on rdar morphotype development. CuCl2 totally

Figure 5. Effect of reducing stress on biofilm. A) Upon reductive stress (5 mM DTT) ΔdsbA and ΔdsbB mutants first shows an escalated rdar morphotype development, where after the appearance becomes smoother and mucoid. Overexpression of YhjH and deletion of csgD enhances slime production in ΔdsbA and ΔdsbB mutants under DTT reductive stress. Coomassie blue stained SDS-PAGE (B) and immunoblot (C) show decrease in the amounts of respectively CsgA and CsgD under 5 mM DTT stress from 18 hours at 28 °C. The numbers underneath the lanes indicate relative amounts. ns = non-significant compared to respective wild type.

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abolished biofilm-development in S. Typhimurium whether the bacteria were proficient in dsbA or dsbB, or not. This observation adjusts well with an idea stating that a high periplasmic oxidation potential suppresses rdar morphotype development in S. Typhimurium. However, we also noted that reducing stress, in the form of sublethal concentrations of DTT, affected the rdar morphotype of the dsb mutants. While initially revealing an enhanced rda morphotype upon DTT stress, the biofilm of the mutants soon dispersed, and eventually converted to a highly mucoid morphotype.

The observed mucoid phenotype implied increased presence of an extracellular organic capsular-like polymer. A possible candidate would be cellulose already comprised in the biofilm. Yet, that genetically inactivating the cellulose-synthesis activator gene csgD in dsb mutant backgrounds rather increased the mucoid phenotype that formally speaks against this option. At the other hand we noted that AdshA-csgD and AdshB-csgD double mutants, while not revealing a rda morphotype, still did bind the dye Congo red implicative of cellulose expression. Thus the AdshA/B-csgD double mutants could either express structures apart from curli or cellulose ploymers binding the dye, or having alternative CsgD/AdrA independent activation of cellulose synthesis. Indeed, it has been reported that cellulose production can be activated independently of the CsgD/AdrA regulatory pathway in a mutant of c-di-GMP specific phosphodiesterase YjcC or under alternative growth conditions [57].

Apart from cellulose, many organisms contain extracellular DNA (eDNA) as an essential component of their biofilm-matrix [65–69]. The release of eDNA has been proposed by three mechanisms; membrane vesicles packed with DNA [70], by lysis of a fraction of bacterial population [66] and by the secretion through secretory machineries [13]. However, when exposing the mucoid colonies to deoxyribonucleases, we did not record any reducing effect in sliminess (data not shown). As S. Typhimurium does not express capsular Vi antigen nor poly-N-acetyl-glucosamine, respectively expressed by S. Typhi or E. coli, our observations would leave us with the slimy morphotype being derived from over-expression of colanic acid. A forthcoming approach will be to solve whether this would be the case, in order to solve whether in fact periplasmic oxidoreductase activity connect to colonic acid synthesis via CsgD.

The fact that DsbA is a defined periplasmic protein raises the question on how it affects expression of either csgD or the CsgD protein located in the cytoplasm. A plausible explanation comes from a model provided by Prigent-Combaret and co-workers [71]. In this model, the CpxA/CpxR response-regulator becomes activated upon accumulation of miss-folded cell envelope proteins. Such a situation could well manifest in the absence of periplasmic disulphide bond formation, such as in the absence of DsbA. As a consequence, CpxR would become phosphorylated and start inhibiting expression of csgD as well as csgA.

The PhoQ/PhoP response regulator pair responds to a number of factors, including DsbA [72]. PhoQ/PhoP in turn regulates expression of RsaA/RsaB, another response regulator pathway affecting CsgD expression [73]. Thus, DsbA could reach CsgD expression through RsaA/RsaB by affecting PhoQ/PhoP. If so, divergent activities of the CpxA/CpxR, RsaA/RsaB and PhoQ/PhoP systems could explain the different contributions of dsbA and dsbB in regulating biofilm-formation under different growth conditions.

DsbA is reported to directly affect another phosphorelay system, the RsGCD system that responds to altered disulphide bond formation [74]. In this, the response regulator gene resB appears to suppress csgD expression [75]. However, we could not define the accompanying increase in csgD transcription in our dsb mutants. Rather, the increase in CsgD levels in our dsb mutants appeared to act at a post-transcriptional stage. Hence, the models listed above may not be able to directly explain the increased CsgD levels noted for the dsb mutants.

Parts of the cascades initiated by response regulators are relayed to CsgD through GGDEF/EAL domain proteins. Thus, we genetically probed for the potential role of c-di-GMP and GGDEF/EAL domain proteins in the enhanced rda morphotype formation exhibited by the dsb mutants. In these experiments, we noted that depletion of the cellular c-di-GMP lead to reappearance of mutant phenotype.

In E. coli, the yddV-dos genes code for an EAL-domain/sensor-protein complex that activate csgBAC expression with a heme-based redox-sensing ability [39]. Conceptually, such a complex could well fit to bridge CsgD with periplasmic redox activity through c-di-GMP signaling. However, S. Typhimurium lacks the yddV-dos genes [40]. We noted an enhanced rda morphotype development in AdshA/B-yjcC and AdshB-yjcC double mutants. In parallel, the enhanced rda morphotype development was retained in either dsb mutant background whether depleted for yegE (STM2123), yfiN (STM2672), STM4551 and yelQ (STM1987), all needed for rdar morphotype development in the wild type background. The only GGDEF/EAL domain protein genes that appeared necessary for the dsb mutant-associated enhanced rdar morphotype development were ydiV (STM1344) and yhjK (STM3615); with ydiV or yhjK deleted, the rdar morphotype development was more significantly down-regulated in the AdshA/B mutants than in the wild type strain.

However, interestingly, of note, YdiV is an EAL-domain-like protein that does not bind c-di-GMP, and elevates CsgD protein expression through repression of the activity of the master regulator of the flagella operon [76]. Through this, YdiV counteracts the inhibitory effect of EAL-domain proteins YciR and YhjH on CsgD expression. YhjK in turn has not previously been associated with rdar morphotype development in S. Typhimurium. However, a yhjK mutant was found severely attenuated in gut colonization in the streptomycin-treated mouse model [77].

To summarize, we have observed that biofilm-formation is redox responsive. Oxidizing preferentially periplasmic proteins with sub-lethal concentrations of CuCl2 reduced rdar morphotype development. In contrast, genetically decreasing the periplasmic protein disulphide bond formation through dsbA or dsbB mutations enhanced the rdar morphotype development. This altered dsb-associated morphotype correlated with an increased CsgD and CsgA expression but relied on the GGDEF/EAL domain protein STM3615. Hence, the functional hierarchy of GGDEF/EAL domain proteins in S. Typhimurium could relate to redox despite a lack of an YddV-Dos system (Fig. 6). Such a parallel pathway would rely on periplasmic protein disulphide bond formation, in turn being dependent on intact DsbA/DsbB redox shuffling. As this system becomes disturbed, also the activity of selected GGDEF/EAL domain proteins, such as YdiV and YhjK, becomes altered leading to an altered CsgD activity.

Materials and Methods

Bacterial strains, plasmids and growth environment

The wild type S. Typhimurium 14028s strain (ATCC, Manassas, VA, USA) was used as reference strain in this study. All the strains and plasmids generated and used are listed in Table 2. For trans-complementation, the coding regions of dsbA and dsbB were cloned in pBAD30 under ara promoter with
ribosome-binding sites in front of the start codon by following the protocols as described earlier [78]. The primers used for cloning are listed in Table S1.

For strain propagation and cloning, bacteria were grown in ordinary Luria medium. To generate biofilm, cultures were prepared in Luria broth (LB) or on Luria agar (LA) plates without salt and grown at 28°C. When necessary, growth media were supplemented with ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin sulfate (50 μg/ml) or tetracycline (10 μg/ml). To activate the arabinose promoter in pBAD30, cultures were supplemented with L-arabinose to a final concentration of 0.1% (w/v). All antibiotics and L-arabinose were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned specifically.

**Construction of mutants**

Deletion mutations were carried out by one-step gene inactivation method as described earlier [79]. Mutants were selected on LA plates with antibiotic and confirmed by PCR with primers designed up- and downstream of the ORFs. The primers for mutation generation and confirmation are listed in Table S2 and Table S3 respectively. The mutations were transferred to clean 14028s S. Typhimurium background by P22 int transduc-

![Figure 6. DsbA/B regulatory pathway leading to rdar morphotype formation in S. Typhimurium.](image)

**Table 2. Strains and Plasmids.**

| Strains | Genotype/Property | Reference |
|---------|-------------------|-----------|
| MC5     | Wild Type         | ATCC 14028 |
| NA266   | 14028Δ(dsba)      | This study |
| NA264   | 14028Δ(dsbb)      | This study |
| Fia-569 | 14028Δ(dsbc)      | [41]       |
| NA-299  | 14028Δ(dsbd)      | This study |
| NA-268  | 14028Δ(dsbl)      | This study |
| NA-270  | 14028Δ(dsbb)      | This study |
| Fia-410 | 14028Δ(ttrxA)     | [41]       |
| Fia-412 | 14028Δ(ttrxB)     | [41]       |
| Fia-406 | 14028Δ(lyshA)     | [41]       |
| Fia-422 | 14028Δ(lyshA)     | [41]       |

| Plasmids | | Reference |
|----------|----------------|-----------|
| VC (pBAD30)| pBAD series vector control, Amp’ | [86]       |
| pdsba (pNA12) | The Δdsba (STM3997) ORF cloned in pBAD30, Amp’ | This study |
| pdsbb (pNA13) | The Δdsbb (STM1907) ORF cloned in pBAD30, Amp’ | This study |
| pUGE13 | pQF50 containing fragment +441/–685 of PcsgD1 | [55]       |
| pHyh (pRG51) | The yhyh (STM4264) ORF cloned in pBAD30, Amp’ | [23]       |

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tion [78] to rule out the possibility of secondary mutations’ effects. Deletion mutants were cured from their antibiotic resistance insert by using pCP20 plasmid as previously described [79]. The GGDEF/EAL and CsgD single (in wild type background) and double mutants (in ΔdsbA/B background) were created by P22 int phage transduction from the strains previously described [14,56,57,80]. The S. Typhimurium virulence plasmid pSLT was cured from SR11 background to generate pSLT deficient S. Typhimurium [81].

Biofilm-formation on solid and in liquid media

The bacteria were grown on LA plates overnight at 37°C and were re-suspended in PBS. The OD₆₀₀ were adjusted to 1.0 and 5 μl of spot inoculated on LA without salt plates supplemented with Congo red (40 μg/ml) and brilliant blue (20 μg/ml) for analyzing rdar morphotype development. The plates were supplemented with 5 mM diethiothreitol (DTT) and 1 mM, 2 mM or 3 mM CuCl₂ where stated. In addition sterile filter papers soaked with 10 μl of 1M CuCl₂ were applied on the plate to mimic oxidative stress. The growth was followed and pictures were taken at various time intervals post incubation.

Biofilm-formation at the air–liquid interface (pellicle formation) was carried out as described previously with slight modifications [82,83]. Briefly, 2 ml of LB without salt in 16 ml polystyrene tubes was inoculated with 10 μl of aforesaid bacterial suspension. The tubes were left undisturbed at 28°C and followed over time for biofilm-development and adherence to the tubes’ wall. Thereafter, the liquid phase was discarded by inverting the tubes carefully. The tubes were air dried and heat fixed at 60°C. Thereafter, the liquid phase was discarded by inverting the tubes in a draining tank. The tubes were air dried by inversion and photographed. For quantification of the adherent biofilm, the bound crystal violet was dissolved in 500 μl of 30% acetic acid. The suspension was boiled for 15–20 min in heat block at RT = 20°C for 15 min, with intermittent rotation at 5 min intervals to cover all the contents of adhered biofilm-mass with methanol. The methanol was replaced with 300 μl of crystal violet (1% solution in 50% methanol) and left for 10 minutes at RT with the rotations as described earlier. Finally, the stained contents were rinsed thoroughly with tap water by submerging the tubes in a draining tank. The tubes was inoculated with 10 μl of aforesaid bacterial suspension.

Curl fimbriae expression analysis

The main subunit of curli fimbriae, the CsgA protein, was detected by the formic acid enrichment technique [84] with slight modifications. Briefly, overnight LA without salt plate culture was re-suspended in TE buffer (10 mM Tris, 1 mM EDTA and 2% SDS; pH = 7.5) and boiled for 45 min at 95°C. The suspension was centrifuged at 14000 rpm and pellet was washed with dH₂O. After second centrifuged the pellet was re-suspended in H₂O, dried by Speed Vac for 1 hour and re-suspended in 100% formic acid. The suspension was boiled for 15–20 min in heat block at 95°C and samples were dried again in Speed Vac for 1 hour. The denatured pellet was dissolved in 20 μl of SDS reducing sample buffer, boiled for 15 min at 95°C and loaded on 15% SDS polyacrylamide gel. The band corresponding to CsgA was visualized by Coomassie staining of the SDS-PAGE gel. Relative protein contents were compared with the one obtained from the wild type by using Image Lab (Beta 2) version 3.0.11 (Bio-Rad Laboratories).

CsgD expression analysis

The expression of master regulator CsgD was analyzed by immunoblotting as described earlier [19]. Summarizing, overnight LA plate without salt cultures were harvested up to 5 mg (wet weight) and re-suspended in 200 μl of reducing SDS sample buffer. The samples were heated for 10–15 min at 95°C and ran on 12% SDS polyacrylamide gels. After checking the total protein contents on the Coomassie stained gel, the proteins were transferred to PVDF membrane (Amersham Hybond-P, GE Healthcare).

Detection CsgD was carried out by using a primary polyclonal peptide rabbit anti-CsgD antibody (1:5000) and a secondary HRP-conjugated goat anti-rabbit IgG antibody (1:10000). The chemiluminescent was detected by using ECL substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) and Bio-Rad Gel doc machine. The protein content was analyzed as described above.

Promotor-fusions and β-galactosidase measurements

The csgD promoter activity was determined from plasmid pUGE13 which contains a regulated csgD promoter (+441/−684 relative to the transcriptional start site) fused with lacZ [55]. Comconitant β-galactosidase activity was determined as described previously [85] from 7.5 and 18 hours LB without salt cultures and from 24, 48 and 72 hours LA without salt plate cultures and expressed as miller units.

Statistical analyses

All the experiments were repeated at least three times. The graphical presentation and analysis of data were done by using the PRISM (version 5.0) software. The data for Figs. 1D, 2C, 2D, 3B and 4B were analyzed by two sided independent sample t-test and mean values with standard errors of mean (SEM) are presented.

Supporting Information

Figure S1 Biofilm-formation for different members of oxidoreductase systems. The biofilm-formation was assessed on LA without salt plates supplemented with Congo red grown at 28°C. The pictures were taken 48 hours post inoculation. (TIF)

Figure S2 Effect of ydiV-yhjK gene mutations on dsb associated biofilm-formation. The development of rdar morphotype in wild type, single, double and triple mutants on Congo red plates at 28°C. The picture was taken 48 hours post inoculation. (TIF)

Figure S3 Effect of oxidative stress on biofilm. A) CuCl₂ induced oxidative stress generates a dose dependent suppression of biofilm-formation irrespective of genetic background of the strain. B) Effect of 1M CuCl₂ (soaked in sterile filter disc) on the rdar morphotypes of wildtype (WT) and dsb mutants grown on Congo red plate for 48 hours at 28°C. (TIF)

Table S1 Primers for cloning of dsb genes. (DOC)

Table S2 Primers for mutagenesis. (DOC)

Table S3 Primers for PCR verification of mutants. (DOC)
Author Contributions
Conceived and designed the experiments: SFR NA MR. Performed the experiments: SFR NA MR. Analyzed the data: SFR NA MR.

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