Effects of Norspermidine and Spermidine on Biofilm Formation by Potentially Pathogenic *Escherichia coli* and *Salmonella enterica* Wild-Type Strains

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Polyamines are present in all living cells. In bacteria, polyamines are involved in a variety of functions, including biofilm formation, thus indicating that polyamines may have potential in the control of unwanted biofilm. In the present study, the effects of the polyamines norspermidine and spermidine on biofilms of 10 potentially pathogenic wild-type strains of *Escherichia coli* serotype O103:H2, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *S. enterica* serovar Agona were investigated. We found that exogenously supplied norspermidine and spermidine did not mediate disassembly of preformed biofilm of any of the *E. coli* and *S. enterica* strains. However, the polyamines did affect biofilm production. Interestingly, the two species reacted differently to the polyamines. Both polyamines reduced the amount of biofilm formed by *E. coli* but tended to increase biofilm formation by *S. enterica*. Whether the effects observed were due to the polyamines specifically targeting biofilm formation, being toxic for the cells, or maybe a combination of the two, is not known. However, there were no indications that the effect was mediated through binding to exopolysaccharides, as earlier suggested for *E. coli*. Our results indicate that norspermidine and spermidine do not have potential as inhibitors of *S. enterica* biofilm. Furthermore, we found that the commercial polyamines used contributed to the higher pH of the test medium. Failure to acknowledge and control this important phenomenon may lead to misinterpretation of the results.

The ability to form complex multicellular communities such as biofilms has been linked to persistence and survival of pathogens in food and feed processing environments (1–3). Due to the increased biocide tolerance of bacteria in biofilms, finding molecules that can control biofilm attracts considerable interest (4). Recent publications have indicated that certain polyamines may possess such properties.

Polyamines are small aliphatic hydrocarbon molecules with quaternary nitrogen groups that have a net positive charge at physiological pH. They are present in all living cells. In bacteria, polyamines are involved in a variety of functions, including intercellular signaling, stress resistance, and RNA and protein synthesis, as well as motility and virulence (5–13). Some polyamines have also been shown to play an essential role in biofilm formation, i.e., putrescine in *Yersinia pestis* (14) and *Escherichia coli* (15), spermidine in *Bacillus subtilis* (16), and norspermidine in *Vibrio cholerae* (17). Burell et al. suggested that a role in biofilm formation may be an ancient physiological function of polyamines in bacteria (16).

In addition to *de novo* polyamine synthesis systems, bacteria have transport systems that allow uptake of extracellular polyamines. Interestingly, exogenous spermidine has been reported to inhibit biofilm formation by *Vibrio cholerae* (18) and *Neisseria gonorrhoeae* (19). Kolkodin-Gal et al. reported that *Bacillus subtilis* produced biofilm disassembly factors, one of which was norspermidine (20). Furthermore, they reported that exogenous norspermidine inhibited biofilm formation by one strain each of *Bacillus subtilis*, *E. coli*, and *Staphylococcus aureus*. However, the results for *Bacillus subtilis* were recently disputed by Hobley et al. (21).

Preparation and processing of food are considered an important route for cross-contamination with pathogens (22), leading to contaminated products and subsequently consumer illness. Potentially pathogenic *E. coli* and *Salmonella enterica* subsp. *enterica* (*S. enterica*) are important foodborne bacteria that have been

shown to form biofilm under food processing conditions on a number of different surfaces, as well as biofilm floating on liquid (a pellicle) (3, 23, 24). Diarrheagenic strains of *E. coli* are the most common bacterial pathogens implicated in diarrhea worldwide, causing disease ranging from mild diarrhea to hemorrhagic colitis. *E. coli* O157 is the serogroup most frequently identified in human outbreaks. However, six other serogroups, namely, O26, O45, O103, O111, O121, and O145, which are often referred to as “the big six,” have also attracted significant attention because they have been increasingly associated with serious outbreaks (25). In Norway, several nationwide outbreaks caused by *E. coli* O103 have been registered. Nontyphoidal serovars of *S. enterica* cause gastroenteritis estimated at over 90 million cases globally each year, with more than 150,000 deaths (26). *S. enterica* serovars Typhimurium and Enteritidis are those most commonly associated with human disease. *S. enterica* serovar Agona, which has been shown to persist in Norwegian production facilities for years (3), is also capable of

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or source	Morphotype ^a	Source or reference
Strains			
<i>S. enterica</i> serovar Typhimurium			
ATCC 14028		rdar	ATCC
LLN5	14028 Δ rhoS::Cm	saw	This study
<i>S. enterica</i> serovar Agona			
2000-01-2168-10	Wild-type fish meal isolate	rdar	3
LKV5	2000-01-2168-10 Δ rhoS::cm	saw	This study
2002-01-71-3	Wild-type feed factory isolate	rdar	3
2000-01-1454-1	Wild-type feed factory isolate	bdar	3
2000-01-1701	Wild-type feed factory isolate	bdar	3
2000-01-2168-6	Wild-type human clinical isolate	bdar	3
<i>E. coli</i> serotype O103:H2			
2006-22-1153-55-2	Wild-type sheep feces isolate	rdar	23
2006-60-21402-51-2	Wild-type sheep feces isolate	rdar	23
2006-22-1242-54-2	Wild-type sheep feces isolate	bdar	23
2006-22-1271-55-2	Wild-type sheep feces isolate	bdar	23
Plasmids			
pKD46	λ red recombinase system, Amp ^r , temp-sensitive replication		7
pKD3	FRT ^b -flanked <i>cat</i> cassette, Amp ^r Cm ^r		7

^a Morphotype on Congo red or Coomassie blue agar: rdar, red, dry, and rough; bdar, brown, dry, and rough; saw, smooth and white (i.e., no biofilm).

^b FRT, FLP recombination target.

causing food poisoning and was in 2011, e.g., linked to a large multistate outbreak of salmonellosis in the United States caused by the ingestion of contaminated papaya (<http://www.cdc.gov/salmonella/agona-papayas/>).

The biofilm matrix of *S. enterica* and *E. coli* consists mainly of exopolysaccharides (e.g., cellulose) and proteinaceous compounds (e.g., curli fimbriae) (27–30). Most *S. enterica* wild-type strains display curli fimbriae in the matrix, but the percentage of strains with cellulose varies from 40 to 100, depending on the serovar and the source of isolation (31, 32). Variations between *E. coli* strains have also been observed (unpublished results). The presence of cellulose has a major effect on the biofilm structure (31).

The aim of the present study was to investigate whether the polyamines norspermidine and spermidine may have potential as biofilm-controlling substances against potentially pathogenic *E. coli* and *S. enterica* strains. Consequently, we studied the effect of norspermidine and spermidine on biofilm production and disassembly in 10 wild-type strains of *S. enterica* serovar Typhimurium, *S. enterica* serovar Agona, and *E. coli* serotype O103:H2. We included strains both with and without cellulose in their biofilm matrix, as Klodkin-Gal reported that norspermidine interacted directly and specifically with exopolysaccharide (20). Two non-biofilm-producing *S. enterica* Δ rhoS mutants were used as controls. Furthermore, we investigated whether the two species themselves produced any biofilm disassembly factors.

MATERIALS AND METHODS

Strains, media, and reagents. In all, six *S. enterica* and four *E. coli* wild-type strains, as well as two *S. enterica* Δ rhoS mutants, were included (Table 1). The strains displayed variations in the composition of the biofilm matrix, visualized as different morphotypes on Congo red or Coomassie brilliant blue agar, as described previously (31). The morphotypes were

categorized as follows: (i) rdar (red, dry, and rough), indicating matrix with curli fimbriae and cellulose; (ii) bdar (brown, dry, and rough), indicating biofilm matrix with curli fimbriae and without cellulose; and (iii) saw (smooth and white), indicating no biofilm formation. The construction of Δ rhoS mutants was carried out according to the protocol of Datsenko and Wanner (7) with the primers described in Table S1 in the supplemental material. The two mutants were used as controls as they do not form biofilm. All strains were stored at -80°C in brain heart infusion broth (BHI) (Difco, BD, Franklin Lakes, NJ) supplemented with 15% glycerol (Merck KGaA, Darmstadt, Germany) and recovered on blood agar (sheep blood) at $37.0 \pm 1.0^{\circ}\text{C}$ overnight. Overnight working cultures were obtained by static incubation at $37.0 \pm 1.0^{\circ}\text{C}$ in Luria-Bertani (LB) broth (Merck KGaA, Darmstadt, Germany). All biofilm assays were performed at $20 \pm 1.0^{\circ}\text{C}$ in LB broth without NaCl (LB^{wo}/NaCl) (10 g/liter Bacto-tryptone, 5 g/liter yeast extract) or LB agar^{wo}/NaCl (also containing 15 g/liter agar). Norspermidine (Sigma-Aldrich, St. Louis, MO) and spermidine (Sigma-Aldrich) were dissolved in sterile distilled water to a 0.1 M stock solution.

Biofilm at the liquid-air interface. To study biofilm formation at the liquid-air interface (i.e., pellicles), glass tubes containing 10 ml LB^{wo}/NaCl were inoculated with 100 μl of an overnight culture and incubated statically for 4 weeks. The strains were visually examined regularly as described previously (31). The experiment was performed twice. To see if norspermidine could make the strains dissolve their pellicle, 50 μl of 5,000 μM norspermidine in LB^{wo}/NaCl or 50 μl of LB^{wo}/NaCl (control) was carefully placed on each pellicle, and the pellicles were visually examined daily for 3 days.

Biofilm in microtiter plates. The crystal violet binding assay was performed in microtiter plates as described earlier, with minor modifications (3); 4 μl overnight culture and 196 μl LB^{wo}/NaCl with the polyamine to be tested (or an equal amount of sterile distilled water [SDW]) were added to each well in 96-well polystyrene microtiter plates (Nunc, Nuncleon, Roskilde, Denmark). The optical density at 595 nm (OD₅₉₅) was measured directly with a Multiscan mass spectrometer (MS) (Thermo Fisher Scientific, Inc., Waltham, MA) after incubation and used as an indication

of bacterial planktonic growth, and the OD₅₉₅ of the dissolved crystal violet was used as a measure of biofilm formation. Tests were performed in triplicate in at least three independent experiments.

Biofilm on agar plates. To study the effects of norspermidine and spermidine on biofilm production on LB agar ^{wo}/NaCl, 10 µl overnight culture was inoculated on agar plates containing the polyamine to be studied or equal amounts of SDW (for controls). After 5 days of incubation at 20°C, the morphology of the colonies was visually inspected, and the diameters were measured using a conventional ruler.

Statistics. The effect of different concentrations of polyamines was tested with a two-sided, paired Student's *t* test using Excel (Microsoft, Redmond, WA). Differences were considered statistically significant when *P* was ≤0.05.

RESULTS

Biofilm at the liquid-air interface. All of the wild-type strains formed biofilm at the liquid-air interface in the form of a pellicle within 7 days of incubation. All pellicles covered the entire surface of the broth. The *Salmonella* strains with the rdar morphotype produced rather flat pellicles with a wrinkled pattern, whereas those with the bdar morphotype produced pellicles with a more fluffy or “cotton-like” appearance. The *E. coli* strains displayed a lower production rate than the *S. enterica* strains. If the tubes were carefully shaken, cracks appeared in the pellicles of the *E. coli* strains and the *S. enterica* strains with the bdar morphotype, but not in those of the *S. enterica* rdar strains. However, new biofilm grew into and covered the cracks when the tubes with the pellicles were left undisturbed for a few days. After 4 weeks of incubation, all wild-type strains still had pellicles. The $\Delta rpoS$ mutants did not form pellicles at any time.

When 50 µl of a 5,000 µM concentration of norspermidine or LB ^{wo}/NaCl (control) was carefully placed on pellicles from all wild-type strains, all pellicles remained intact during the observation period of 3 days, except for those of the *E. coli* strain 2006-22-1242-54-2 which broke immediately after placement of both norspermidine and the control.

Biofilm in microtiter plates. In all of the tests performed in microtiter plates, no statistically significant difference was observed between rdar and bdar strains within each species. All further presentations of results will therefore be related to species only. When the commercial solutions of the polyamines were diluted in LB ^{wo}/NaCl, the pH of the final test broth ranged from 7 at 100 µg/ml to 8.5 at 5,000 µg/ml. The pH in the test broth was therefore adjusted with HCl back to 7 for all concentrations of the polyamines. Interestingly, the *E. coli* and *S. enterica* strains reacted differently to the polyamines. For the *E. coli* strains, increasing concentrations of polyamines reduced biofilm production by up to approximately 90% (Fig. 1A). In contrast, polyamine concentrations from 100 to 1,000 µM caused a small, but statistically significant increase in biofilm production by all of the *S. enterica* strains, followed by a small decline in biofilm production at higher concentrations (Fig. 1A). Both species displayed decreased planktonic growth with the highest concentrations of norspermidine, and *E. coli* displayed such decreased growth also with spermidine (Fig. 1B).

We also tested the effect of non-pH-adjusted solutions of the two polyamines to see what would happen if one overlooked the pH-raising effect of the commercial solutions. Again *E. coli* displayed decreased biofilm production, but for *S. enterica*, the biofilm production increased more than what was observed when the pHs of all solutions were adjusted to 7 (see Fig. S1a in the supple-

mental material). The planktonic growth was reduced for both species by both polyamines (Fig. S1b). Interestingly, the same pattern was seen when we tested all strains in LB ^{wo}/NaCl adjusted to pHs of 8, 8.5, and 9 without addition of polyamines (see Fig. S2 in the supplemental material).

Biofilm on agar plates. When the strains were grown on agar with LB ^{wo}/NaCl, the *S. enterica* rdar strains all displayed rather flat biofilm colonies with highly structured wrinkled patterns (Fig. 2a), whereas the *E. coli* rdar strains produced more compact biofilm colonies with larger structures (Fig. 2b). The biofilm colonies of the bdar strains of both species were compact and slightly conical, with a “dry” appearance and with very little or no structure (Fig. 2c). The *rpoS* deletion mutants did not produce biofilm but grew in colonies with a smooth, shiny appearance (Fig. 2d). Changes in morphology were observed in the biofilms of *E. coli* rdar strains, which displayed less distinct structures at polyamine concentrations of 1,000 µM and higher, regardless of pH (Fig. 2e). We did not observe any morphological changes in the biofilms of the *S. enterica* rdar strains (Fig. 2f) or any of the bdar strains. However, changes in the structure of the bdar biofilms would have been difficult to see and may thus have been overlooked. On agar plates with the pH adjusted to 7, the only statistically significant changes in mean diameter were observed for *S. enterica* with the highest concentration of norspermidine and spermidine (*P* = 0.0005 and 0.028, respectively) (Fig. 3). On non-pH-adjusted agar plates, increasing concentrations of norspermidine and spermidine caused increased diameters of the biofilms of *S. enterica* rdar strains only, whereas the majority of the other strains displayed decreased diameters, including those of the nonbiofilm colonies of the *rpoS* deletion mutants (see Fig. S3 in the supplemental material). Similar trends were observed on agar plates with pHs ranging from 7 to 9 without polyamines (see Fig. S4 in the supplemental material).

DISCUSSION

In the present study, exogenously supplied norspermidine and spermidine affected biofilm production but did not mediate disassembly of preformed biofilms. Interestingly, the two species *S. enterica* and *E. coli* reacted differently to the polyamines. In microtiter plates at pH 7, both polyamines inhibited biofilm formation by *E. coli* but tended to increase biofilm formation by *S. enterica*. Similarly on agar plates, reduction of biofilm structure was observed in *E. coli* strains but not in *S. enterica* strains. To the best of our knowledge, the effect of exogenous spermidine and norspermidine on biofilm production by *S. enterica* has not been reported earlier.

The effect of norspermidine on our *E. coli* strains was similar to that reported by Kolodkin-Gal (20), although much higher concentrations of norspermidine were required in our system. On the other hand, spermidine which also inhibited biofilm formation by our strains, did not affect the strain used by Kolodkin-Gal et al. (20). This may be due to differences between the strains tested. Another explanation may be the different test conditions used: i.e., our incubation in nutrient broth (LB ^{wo}/NaCl) at 20°C versus that by Kolodkin-Gal et al. in minimal medium M9 at 30°C. We also tried to use minimal medium M9 and incubation at 30°C with our strains, but the biofilm formation of our wild-type strains under these conditions was much too low to be used in any of our experiments, although growth was supported (results not shown). However, exogenous spermidine has previously been reported to

FIG 1 Mean biofilm production (A) and planktonic growth (B) as indicated by OD₅₉₅ in microtiter plates with or without norspermidine and spermidine. The pH is adjusted to 7.0 ± 0.1 . Error bars indicate the standard error of the mean. An asterisk indicates that the mean OD₅₉₅ is statistically different from the mean OD₅₉₅ of the control at $P \leq 0.05$.

inhibit biofilm formation by other species—e.g., *Vibrio cholerae* (18) and *Neisseria gonorrhoeae* (19).

The fact that the commercial solutions of the polyamines can raise the pH of the test broth is certainly something to be aware of,

as this will introduce at least two additional variables that change with increasing polyamine concentrations (i.e., the pH itself and the protonation state of the polyamines). This did not appear to influence our results obtained with the *E. coli* strains but had a

FIG 2 Biofilm morphology on LB^{w/o}/NaCl agar. Shown are the morphotypes *S. enterica* rdar (a), *E. coli* rdar (b), *E. coli* bdar (c), *S. enterica* saw (d), *S. enterica* rdar with 5,000 μ M norspermidine (e), and *E. coli* rdar with 5,000 μ M norspermidine (f). Magnification scale bars are 5 mm.

major impact on the results of the *S. enterica* strains. These strains produced up to twice as much biofilm when the polyamine concentrations, and thereby also the pH, increased ($P < 0.05$). This was probably not due to a specific polyamine action as a similar effect was observed with increasing pH without polyamines. Consequently, knowledge and control of the pH can be crucial to the interpretation of the results. However, this is usually not addressed in papers reporting effects of these commercial polyamines on biofilm, thereby making comparison of results difficult.

Kolodkin-Gal et al. suggested that norspermidine inhibited *E. coli* biofilm production by targeting negatively charged exopolysaccharides, possibly colonic acid (20). In our study, there were no indications that the biofilm formation inhibitory activity on the *E. coli* strains was mediated by binding to exopolysaccharides, as it

was not influenced by pH and thereby the protonation state of the polyamines or by differences in the composition of the matrix.

E. coli has several polyamine uptake systems, including the spermidine preferential system consisting of the PotA, PotB, PotC, and PotD proteins (33). The same conserved transport systems are also found in *S. enterica* serovar Typhimurium (33) and *S. enterica* serovar Agona (unpublished results). In *E. coli*, almost 90% of spermidine is associated with RNA, and it has been shown that polyamines through their binding to mRNAs alter their structure (34) and affect the translation efficiency of specific targets, including several important regulators (14, 33, 35–37). Thus, the observed polyamine effects on biofilm formation by *E. coli* and *S. enterica* in our study may be a result of the polyamines specifically targeting mechanisms involved in biofilm production.

FIG 3 Mean colony diameter (mm) on agar plates with LB^{w/o}/NaCl, with or without norspermidine and spermidine. The pH of the agar is adjusted to 7. Error bars indicate the standard error of the mean. An asterisk indicates that the mean OD₅₉₅ is statistically different from the mean OD₅₉₅ of the control at $P \leq 0.05$.

On the other hand, polyamines can be toxic in high concentrations: e.g., spermine at concentrations of 3 mM has been reported to inhibit growth of *E. coli* at pH 7 (38). Furthermore, high pH amplifies polyamine stress, and it had earlier been shown that spermidine decreases *E. coli* survival in extreme base (39), similar to what was observed in our experiments. In microtiter plates, planktonic growth at the highest concentrations of the polyamines was more reduced in broth with high pH than in broth adjusted to pH 7. Furthermore, the combination of polyamines and high pH also gave a greater reduction in planktonic growth than high pH alone. The same was seen on agar plates where the *rpoS* deletion mutants served as growth controls. Hobley et al. suggested that high concentrations of norspermidine inhibit the growth of planktonic cells of *B. subtilis*, which in turn leads to less biofilm produced (21). This may also be a possible explanation for what we observed in *E. coli* in our experiments, but a similar general correlation between biofilm formation and planktonic growth was not observed for *S. enterica* under the same conditions. For this species, both reduced biofilm production and increased biofilm production were observed together with reduced planktonic growth. However, the polyamines may have induced a type of stress where *E. coli* and *S. enterica* respond differently, like the stress situation observed when increasing pH from 7 to 9 causing *S. enterica* to increase and *E. coli* to reduce biofilm formation. The fact that the responses to norspermidine and spermidine were similar in our study may support the hypothesis that the observed effects on biofilm formation are results of a stress response. In *V. cholerae*, where the two polyamines specifically target biofilm formation, they exert opposite responses—i.e., biofilm formation is increased by norspermidine and reduced by spermidine (18, 40).

Kolodkin-Gal et al. reported full biofilm self-disassembly by their *Bacillus subtilis* strain just a few days after biofilm was produced (20). As the biofilm lifestyle is a survival strategy under suboptimal environmental conditions, we found these results puzzling. We have never observed any total self-disassembly by *S. enterica* or *E. coli*, although we have previously studied biofilm produced by large numbers of wild-type strains. In the present study, we extended the study period from our usual maximum of 8 days to 4 weeks. Not only did all of our wild-type strains produce pellicle biofilms that lasted the whole period, they even made the effort of maintaining the biofilm by quickly repairing cracks that occurred due to rough handling. Consequently, neither of our strains produced any general biofilm disassembly factors under these conditions. Kolodkin-Gal et al. claimed that the observed biofilm disassembly by *Bacillus subtilis* was mediated by self-produced factors, suggested to be D-amino acids and norspermidine (20, 41), but production of the latter was later disputed by Hobley et al. (21). As the *Bacillus subtilis* strain tested by Kolodkin-Gal et al. later was shown to have a mutation affecting its response to D-amino acids (42), the rapid biofilm self-disassembly reported may have been an isolated phenomenon by this particular strain, rather than a general trait.

In conclusion, exogenously supplied norspermidine and spermidine inhibited biofilm formation by *E. coli*, but not by *S. enterica*. Furthermore, the polyamines did not exhibit biofilm disassembly effects, and none of the *E. coli* and *S. enterica* wild-type strains produced any biofilm disassembly factors under the conditions used. Our results indicate that norspermidine and spermidine do not have potential as inhibitors of *S. enterica* biofilm. Furthermore, we found that the commercial polyamines used

contributed to higher pH of the test medium. Failure to acknowledge and control this important phenomenon may lead to misinterpretation of the results.

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