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Secreted single-stranded DNA is involved in the initial phase of biofilm formation by *Neisseria gonorrhoeae*

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Summary

Neisseria gonorrhoeae is an obligate human pathogen that colonizes the genital tract and causes gonorrhoea. *Neisseria gonorrhoeae* can form biofilms during natural cervical infections, on glass and in continuous flow-chamber systems. These biofilms contain large amounts of extracellular DNA, which plays an important role in biofilm formation. Many clinical isolates contain a gonococcal genetic island that encodes a type IV secretion system (T4SS). The T4SS of *N. gonorrhoeae* strain MS11 secretes ssDNA directly into the medium. Biofilm formation, studied in continuous flow-chamber systems by confocal laser scanning microscopy (CLSM), was strongly reduced, especially in the initial phases of biofilm formation, in the presence of Exonuclease I, which specifically degrades ssDNA or in a $\Delta traB$ strain that does not secrete ssDNA. To specifically detect ssDNA in biofilms using CLSM, a novel method was developed in which thermostable fluorescently labelled ssDNA- and ss/dsDNA-binding proteins were used to visualize ssDNA and total DNA in biofilms and planktonic cultures. Remarkably, mainly dsDNA was detected in biofilms of the ssDNA secreting strain. We conclude that the secreted ssDNA facilitates initial biofilm formation, but that the secreted ssDNA is not retained in mature biofilms.

Introduction

Biofilms are surface-attached microbial communities embedded in a self-produced extracellular matrix of polymeric substances (Costerton *et al.*, 1999). About 80% of the world's microbial biomass resides in biofilms, and more than 75% of human microbial infections are assisted by the formation of stable biofilms (Davies, 2003). During biofilm formation bacteria undergo a transition from a planktonic to an aggregated form that is embedded in an exopolymeric matrix. During this transition, planktonic bacteria first attach to the surface. This attachment is initially reversible and becomes irreversible once the bacteria start to produce extracellular polymeric substances like exopolysaccharides, secreted proteins, membrane vesicles and extracellular DNA (eDNA) (Dunne, 2002). The extracellular polymeric substance is essential for maturation of the biofilm and can constitute up to 90% of its biomass (Lemming *et al.*, 2000). Extracellular polymeric substances produced by different bacteria is diverse in its composition, but for many organisms, like *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002), *Streptococcus pneumoniae* (Moscoso *et al.*, 2006), *Enterococcus faecalis* (Thomas *et al.*, 2008), *Staphylococcus aureus* (Rice *et al.*, 2007), *Staphylococcus epidermidis* (Qin *et al.*, 2007), *Haemophilus influenzae* (Izano *et al.*, 2009), *Shewanella oneidensis* (Godeke *et al.*, 2011a), *Neisseria meningitidis* (Lappann *et al.*, 2010) and *Neisseria gonorrhoeae* (Steichen *et al.*, 2011), it was demonstrated that eDNA is an important component of the biofilm. A common mechanism by which eDNA is released into the biofilm is autolysis. Different mechanisms that induce autolysis have been identified. For example, in *P. aeruginosa*, a part of the population lyses under the control of a quorum sensing system (Allesen-Holm *et al.*, 2006), while in *E. faecalis* autolysis occurs via a fratricidal mechanism (Thomas *et al.*, 2009). In *S. aureus*, autolysis originates from a programmed cell death similar to apoptosis of eukaryotic cells (Yang *et al.*, 2006). DNA can also be released via active secretion, as has been observed in *P. aeruginosa* (Hara and Ueda, 1981) and in *N. gonorrhoeae* (Hamilton *et al.*, 2005). The release of vesicles-containing DNA (Kadurugamuwa and Beveridge, 1996) and prophage-mediated lysis of a subpopulation of cells (Webb *et al.*, 2003; Godeke *et al.*,

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2011a) are further possible sources of eDNA. The amount of DNA is further regulated by nucleases (Mann *et al.*, 2009; Godeke *et al.*, 2011b; Steichen *et al.*, 2011).

Thus, DNA is known as one of the major structural components of many bacterial biofilms (Mulcahy *et al.*, 2008; Flemming and Wingender, 2010; Fuxman Bass *et al.*, 2010). The presence of DNA was demonstrated using different fluorescent probes that bound to DNA. None of the fluorescent probes used in these studies like, e.g., propidium iodide, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one (DDAO) (Allesen-Holm *et al.*, 2006), SYTOX Orange (Harmsen *et al.*, 2010), ethidium bromide (Allesen-Holm *et al.*, 2006) and PicoGreen (Steinberger and Holden, 2005) can discriminate between ssDNA and dsDNA. The importance of DNA was also demonstrated by assessing the effect of DNase I treatment. Treatment with DNase I often results in significant inhibition of biofilm formation and release of biomass from the formed biofilms (Whitchurch *et al.*, 2002; Harmsen *et al.*, 2010; Lappann *et al.*, 2010). However, also DNase I treatment degrades both ssDNA and dsDNA. Thus, it is currently unknown whether different forms of DNA, such as e.g. ssDNA or dsDNA play specific and/or different roles in biofilm. The amounts of the different forms of DNA have also not been assessed before.

N. gonorrhoeae is an obligate human pathogen that primarily infects superficial mucosal surfaces lined with columnar epithelium such as urethra, cervix, rectum, pharynx and conjunctiva (Edwards and Apicella, 2004). As a sexually transmitted disease agent, the gonococcus normally colonizes the genital tract (Seifert, 1992). Most of the gonococcal infections in males are inflammatory and pyogenic infections of the urogenital tract, whereas about 50% of the infections in woman are asymptomatic (Pariser, 1972). Microscopic examination of human cervical tissue demonstrated that gonococci are capable to form biofilms during natural cervical infection (Steichen *et al.*, 2008). Further studies revealed that *N. gonorrhoeae* can form biofilms both over glass, and over primary urethral and cervical epithelial cells and in continuous flow-chamber systems (Greiner *et al.*, 2005). The structure of *N. gonorrhoeae* biofilms structurally resembled that of biofilms of other studied bacterial species (Greiner *et al.*, 2005), although *N. gonorrhoeae* lacks genes capable of producing exopolysaccharides (Ferretti *et al.*, 1997). Remarkably, networks of bacterial membranes, most likely derived from blebbing of the gonococcal outer membrane, were observed within the gonococcal biofilms (Greiner *et al.*, 2005; Steichen *et al.*, 2008).

As observed for many well-studied bacterial biofilms, the *N. gonorrhoeae* biofilms contain large amounts of DNA (Steichen *et al.*, 2011). The possible sources of eDNA in gonococcal biofilms are diverse. Gonococci are

well known to be highly autolytic (Elmros *et al.*, 1976; Chan *et al.*, 2012). For *N. meningitidis*, it was demonstrated that the release of DNA in biofilms was mediated by lytic transglycosylases, cytoplasmic N-acetylmuramyl-L-alanine amidases and, at later stages of biofilm formation, by outer membrane phospholipase A (Lappann *et al.*, 2010). Furthermore, the release of membrane blebs, which are associated with large amounts of DNA (Dorward *et al.*, 1989), is also important for gonococcal biofilm formation (Steichen *et al.*, 2008). Biofilm formation and remodelling is also influenced by the *nuc* nuclease, which has homology with the staphylococcus-secreted thermonuclease (Steichen *et al.*, 2011). Another source of DNA within the biofilm might be the DNA secreted by the unusual type IV secretion system (T4SS) within a horizontally acquired region, the gonococcal genetic island (GGI) (Dillard and Seifert, 2001). The 57 kb large GGI is horizontally acquired and inserted in the *dif* site at the replication terminus (Hamilton *et al.*, 2001; 2005). It is found in approximately 80% of the *N. gonorrhoeae* strains and encodes a T4SS (Dillard and Seifert, 2001). The T4SS of *N. gonorrhoeae* strain MS11 secretes single-stranded chromosomal DNA into the extracellular milieu in a contact-independent manner (Hamilton *et al.*, 2005). The DNA secreted through the T4SS is taken up via the natural transformation system and contributes to the spread of genetic information (Ramsey *et al.*, 2011).

The different physicochemical properties of ssDNA might have a different effect on biofilm formation than dsDNA. Here, the effect of ssDNA secreted by the T4SS on biofilm formation was studied in continuous flow-chamber systems by confocal laser scanning microscopy (CLSM). Furthermore, to specifically detect ssDNA in these biofilms, a novel method was developed in which thermostable fluorescently labelled ssDNA and ss/dsDNA binding proteins were used to visualize ssDNA and total DNA. It is demonstrated that the secreted ssDNA plays an important role in the initial phases of biofilm formation but the ssDNA is not retained in mature biofilms.

Results

Exonuclease I inhibits the initial stages of biofilm formation

To study whether the secreted ssDNA plays a role in biofilm formation, biofilm formation by *N. gonorrhoeae* strain MS11 was investigated in a continuous flow chamber system in the presence and absence of Exonuclease I, an enzyme which specifically degrades ssDNA. Purified Exonuclease I was highly specific for ssDNA in the medium used for the continuous flow experiments (see Fig. S1). Purified Exonuclease III, which normally is specific for dsDNA, degraded dsDNA efficiently, but in the medium used for the continuous flow experi-

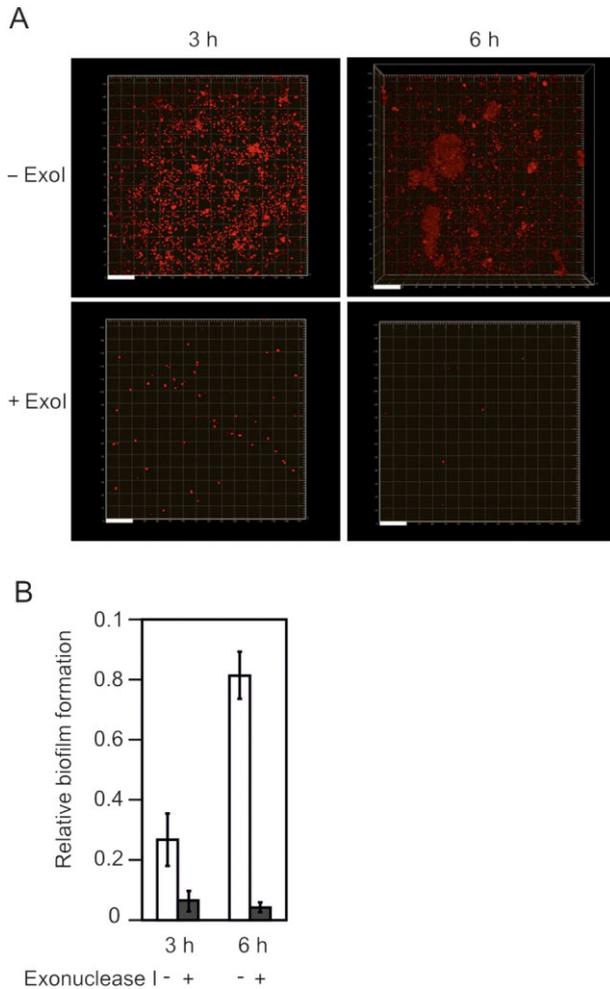


Fig. 1. The effect of Exonuclease I on initial attachment. Confocal microscopy of continuous flow chambers inoculated with *N. gonorrhoeae* strain MS11 imaged 3 and 6 h after inoculation. The flow chambers contained minimal medium with or without Exonuclease I. The enzyme was refreshed every 2 h. A. The biofilm was stained with SYTO62 (red) and visualized by CLSM. Micrographs represent three-dimensional images. The bar is 20 μ m in length. B. Quantification of the amount of biofilm in the images shown in (A). Images were processed and the biofilm mass was determined as the total number of voxels with IMARIS Software. Voxel numbers from five distinct channels were determined and averaged. The standard deviations between the channels are depicted as error bars. Biofilm formation is depicted relative to the amount of biofilm formed by MS11 after 24 h in the absence of Exol.

ments also showed significant activity against ssDNA (data not shown), and therefore could not be used to specifically degrade dsDNA. Biofilm development was monitored by CLSM after addition of the cell-permeant fluorescent nucleic acid stain SYTO62 after 3 and 6 h (see Fig. 1A). The quantification of the relative biofilm formation is shown in Fig. 1B. Almost no attached cells could be detected when Exonuclease I was added. This demon-

strates that the presence of Exonuclease I affects biofilm formation by *N. gonorrhoeae*.

DNA secretion facilitates biofilm formation

Because biofilm formation was influenced by the addition of Exonuclease I, and thus most likely by the presence of ssDNA, it was tested whether the ssDNA secreted via the T4SS encoded within the GGI facilitates biofilm formation. Therefore, biofilm formation of *N. gonorrhoeae* strain MS11 and a MS11 strain in which the *traB* gene was deleted (MS11 Δ *traB*) were compared. The *traB* gene encodes a component of the T4SS core complex spanning the inner and outer membrane, and deletion of this gene results in the abolishment of DNA secretion (Ramsey *et al.*, 2011). The *traB* mutation does not affect the growth rate (data not shown). To demonstrate that any observed effects were caused by the deletion of the *traB* gene, a strain was created in which the *traB* gene was restored (MS11 Δ *traB*::*traB*). Because biofilm formation is strongly affected by the presence and absence of type IV pili (O'Toole and Kolter, 1998) and the type IV pili of *N. gonorrhoeae* can undergo both phase and antigenic variation (Seifert *et al.*, 1988), piliation of the three strains was compared by comparing colony morphology after growth on plates, by sequencing the *pilE* gene, and by determining the expression levels of PilE by Western blotting with a PilE antibody. No differences between the three mutants were observed (data not shown).

Biofilms of the three strains were grown for 3 days in a continuous flow chamber system. After 48 and 72 h, most of the surface was covered by strain MS11, and the formation of distinct three-dimensional structures was observed (see Fig. 2A). The quantification of biofilm formation is shown in Fig. 2B. Biofilm formation after 48 and 72 h was strongly reduced in MS11 Δ *traB*. The MS11 Δ *traB*::*traB* complementation strain showed similar biofilm formation as observed for strain MS11 after 48 and 72 h, with coverage of most of the surface and formation of distinct three-dimensional structures (Fig. 2). This demonstrated that the MS11 Δ *traB* mutant is strongly reduced in biofilm formation. This suggests that either the presence of the T4SS or a substrate secreted by the T4SS facilitates biofilm formation and contributes to the stabilization of the biofilm.

Treatment of biofilms with Exonuclease I only affects strains that secrete ssDNA

To test whether the effects of the mutation in *traB* and the addition of Exonuclease I are both caused by the absence of ssDNA in the medium or on the surface of the cell, it was tested whether these two effects are dependent or independent. Twenty-four-hours-old biofilms of MS11 and

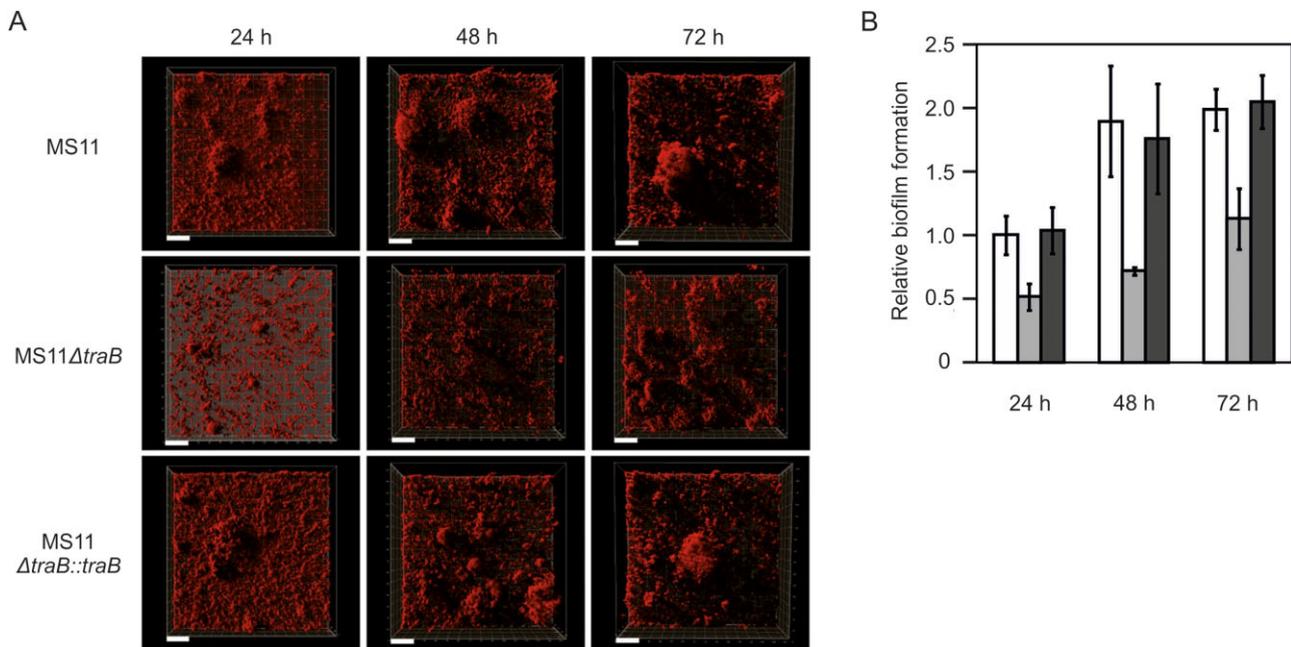


Fig. 2. Deletion of the *traB* gene results in a strong decrease of biofilm formation. Confocal microscopy of continuous flow chambers inoculated with *N. gonorrhoeae* strain MS11, MS11Δ*traB* and MS11Δ*traB*::*traB* imaged 24, 48 and 72 h after inoculation.

A. The biofilm was stained with SYTO62 (red) and visualized by CLSM. Micrographs represent three-dimensional projections. The bar is 20 μm in length.

B. Quantification of the amount of biofilm formed in (A). Images were processed as described in Fig. 1. The white, light grey and dark grey columns represent respectively the MS11, MS11Δ*traB* and MS11Δ*traB*::*traB* strains. Biofilm formation is depicted relative to the amount of biofilm formed by MS11 after 24 h.

the non-secreting mutant MS11Δ*traB* were perfused for 1 h with medium with and without Exonuclease I (see Fig. 3A and B). Because 24 h old biofilms of MS11 contain both single cells and/or microcolonies and mature three-dimensional structures, the effects on both initial and later phase of biofilm formation can be studied at this timepoint. Again, biofilm formation was affected both in the MS11Δ*traB* strain and by the addition of Exonuclease I. Treatment of 24 h old biofilms formed by strain MS11 resulted in the loss of part of the single cells and/or microcolonies, whereas no effects were observed on the distinct three-dimensional structures. Biofilm formation by the MS11Δ*traB* strain was not significantly affected by additional treatment with Exonuclease I. Thus, the effects of Exonuclease I treatment and the Δ*traB* mutation are not additive, demonstrating that the effects observed in the MS11Δ*traB* strain are caused by the absence of ssDNA. Remarkably, the effects of Exonuclease I were only observed for the single cells in the initial phases of biofilm formation but not for the distinct three-dimensional structures observed later in biofilm formation, suggesting that ssDNA plays a role in the initial phase of biofilm formation but not in mature biofilms. DNA has previously been proposed to function as a cell-to-surface and/or cell-to-cell adhesion during the initial phase of biofilm formation (Das *et al.*, 2010). Based on the observations that biofilm for-

mation by *N. gonorrhoeae* is affected by Exonuclease I treatment and by the Δ*traB* mutation, and that these effects are not additive, we propose that the ssDNA secreted via the T4SS plays an important role in biofilm development.

Specific visualization of ssDNA in the presence of dsDNA

Because our experiments provided evidence for a role of ssDNA in biofilm formation of *N. gonorrhoeae* strains, we set out to develop a technique to specifically visualize ssDNA. However, many different methods and stains are available to fluorescently detect DNA, such as ethidium bromide, propidium iodide, DDAO, SYTO60, SYBR Green, OliGreen and many others. To our knowledge, all currently available stains detect both ssDNA and dsDNA. In contrast, ssDNA-binding proteins (SSBs) bind to ssDNA with high specificity but without clear sequence specificity, and bind to dsDNA only with much lower affinity (Lohman and Ferrari, 1994). After initial attempts with purified SSBs from *N. gonorrhoeae* (SsbB) and *Sulfolobus solfataricus* (SSB), we set out to use TteSSB2 of *Thermoanaerobacter tengcongensis* to specifically detect ssDNA. This protein is highly stable and binds to ssDNA with high affinity (~ 150 nM in the medium used for

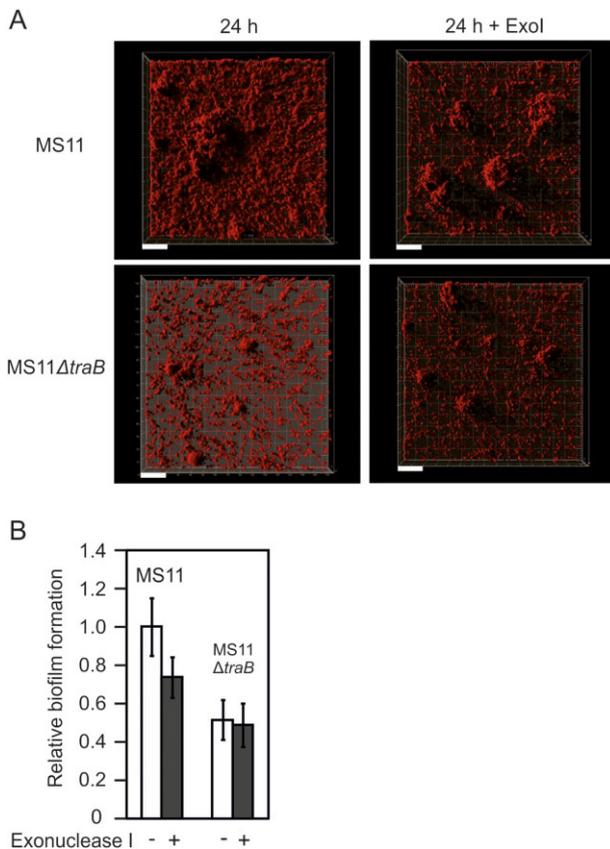


Fig. 3. The effect of Exonuclease I on 24 h old biofilms. Confocal microscopy of continuous flow chambers inoculated with *N. gonorrhoeae* strain MS11 and MS11ΔtraB. Continuous flow chambers were inoculated with the strains, and the system was perfused with medium for 24 h. After 24 h, the chambers were incubated for 1 h with medium with or without Exonuclease I. After this incubation, the chambers were perfused with medium and imaged.

A. Biofilm was stained with Syto62 (red) and visualized by CLSM. Micrographs represent three-dimensional images. The bar is 20 μm in length.

B. Quantification of the amount of biofilm formed in (A). Images were processed as described in Fig. 1. Biofilm formation is depicted relative to the amount of biofilm formed by MS11 after 24 h.

the continuous flow experiments, see Fig. S2C) in a sequence independent manner (Zhang *et al.*, 2009). As a control for DNA binding, also the thermostable Sac7d protein from *Sulfolobus acidocaldarius* was overproduced and purified to homogeneity. Sac7d is a highly stable protein that was shown to bind dsDNA with high affinity in a sequence independent manner (Gao *et al.*, 1998). However, in the medium used for the continuous flow experiments, Sac7d bound only slightly better to dsDNA (~ 4 μM, see Fig. S3C) than to ssDNA and therefore can only be used to image total DNA. Thus in culture medium, TteSSB2 binds sequence specific, but with high affinity to ssDNA specifically whereas Sac7d bound sequence specific to ssDNA and dsDNA. Because TteSSB binds to ssDNA with a 20-fold higher affinity than Sac7d to

ss/dsDNA, the sensitivity for the detection of ssDNA with TteSSB should be higher than the sensitivity for the detection of ss/dsDNA with Sac7d. TteSSB2 and Sac7d were fluorescently labelled on introduced cysteines. A detailed description of the purification, the DNA binding specificity, the optimization of fluorescent labelling for the different cysteine mutants and the characterization of the fluorescence properties of TteSSB2 and Sac7d is provided in the Supplementary Materials and Methods. Based on these experiments, TteSSB2 labelled at the cysteine introduced at the position of Trp55 (TteSSB2-W55C) and Sac7d labelled at the cysteine introduced at the position of Ser18 (Sac7d-S18C) were used to detect ss- and dsDNA respectively.

ssDNA and dsDNA can be visualized in biofilms of the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* using fluorescently labelled TteSSB2 and Sac7d

To demonstrate that ssDNA and total DNA could indeed be visualized in biofilms using CLSM and fluorescently labelled TteSSB2 and Sac7d, the presence of ssDNA and dsDNA in biofilms of the crenarchaeon *S. acidocaldarius* was studied. It has previously been shown that 3 days old biofilms of *S. acidocaldarius* contain eDNA (Koerdts *et al.*, 2010). This DNA is found at positions where cellular aggregates are visible and was sensitive to DNase I treatment. However, removal of the DNA had no effect on the structure of the biofilms, which suggested that eDNA does not play an important structural role in biofilms of *S. acidocaldarius* (Koerdts *et al.*, 2010). Based on the conditions under which the *S. acidocaldarius* biofilms were grown (76°C and pH 3.0), we assumed that the extracellular DNA was present as a mixture of denatured ssDNA and dsDNA, and that therefore this system was well suited to test whether the fluorescently labelled TteSSB2 and Sac7d could be used to visualize DNA within the biofilm. In a first experiment, a 3 days old static biofilm of *S. acidocaldarius* was imaged by CLSM. The 3 days old biofilm was washed with Brock media of pH 5.0 and imaged by CLSM after incubation with DAPI (to visualize DNA inside and outside the cells), DDAO (to visualize the external DNA), IANBD-labelled TteSSB2-W55C (to visualize ssDNA) and Texas Red-labelled Sac7d-S18C (to visualize total DNA). As has been described previously, *S. acidocaldarius* readily formed biofilms, which contained a high density of cells and large aggregates, forming towering structures above the surface of attached cells. Labelling with DDAO showed the presence of external DNA which was associated with the large aggregates forming towering structures above the surface (Fig. 4A). The labelling with IANBD-labelled TteSSB2-W55C showed a very similar labelling pattern as DDAO,

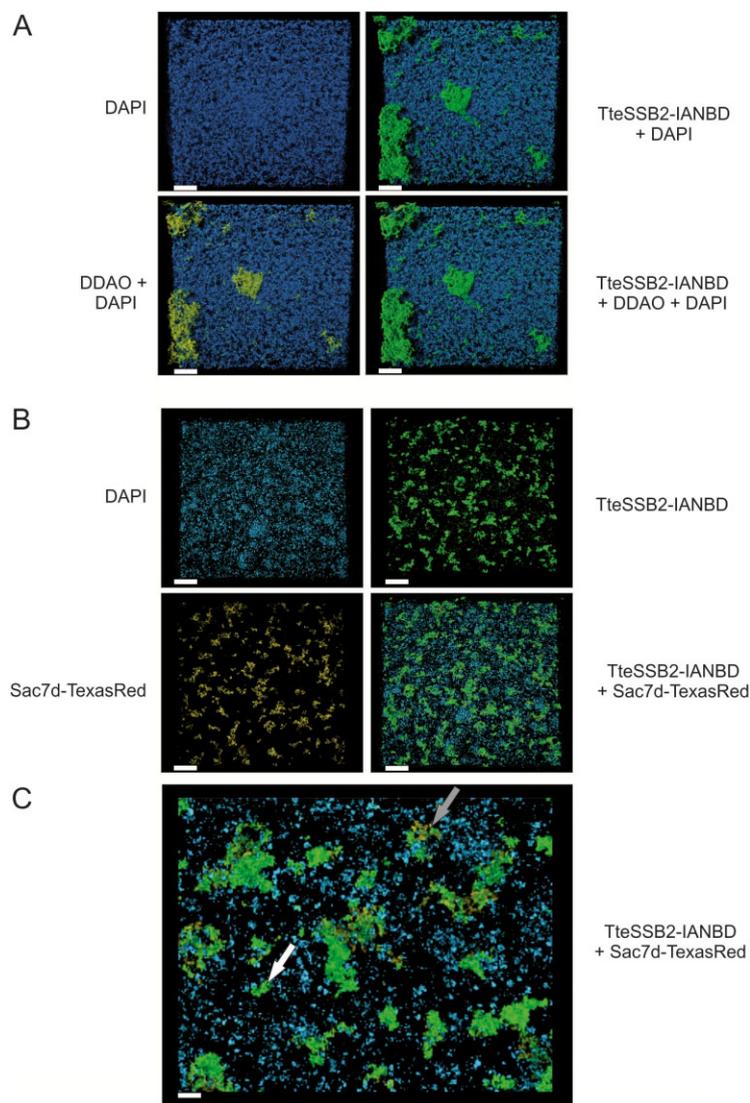


Fig. 4. Visualization of DNA in static biofilms formed by *S. acidocaldarius*. Three-day-old biofilms formed by *S. acidocaldarius* were labelled with (A) DAPI (blue), DDAO (yellow) and IANBD-labelled TteSSB2-W55C (green) or with (B) DAPI (blue), Texas Red-labelled Sac7d-S18C (yellow) and IANBD-labelled TteSSB2-W55C (green) and visualized by CLSM. The lower right panels show an overlay of the signals. (C) Enlargement of a part of the overlay shown in (B) is shown. Micrographs represent three-dimensional projections. The bar in (A) and (B) is 20 μm in length, while the bar in (C) is 5 μm in length. White and grey arrows depict positions where respectively ssDNA or dsDNA are labelled.

demonstrating that IANBD-labelled TteSSB2-W55C was colocalizing with the DNA. Thus, large amounts of ssDNA can be detected in biofilms of *S. acidocaldarius*. In a second experiment, a 3 days old static biofilm of *S. acidocaldarius* was imaged after incubation with DAPI, Texas Red-labelled Sac7d-S18C and IANBD-labelled TteSSB2-W55C (Fig. 4B). Because the fluorescence spectra of DDAO and Texas Red overlap, these fluorophores could not be used at the same time. Both Texas Red-labelled Sac7d-S18C and IANBD-labelled TteSSB2-W55C specifically stained the large aggregates forming towering structures above the surface. Remarkably, although the labelling patterns of Sac7d-S18C and TteSSB2-W55C overlapped in many positions, several positions were only labelled with Sac7d-S18C or TteSSB2-W55C, demonstrating that the biofilm of *S. acidocaldarius* contained regions containing mainly ssDNA or dsDNA (Fig. 4C). Thus fluorescently labelled

TteSSB2 and Sac7d can be used to detect either ssDNA or total DNA in biofilms using CLSM.

Most extracellular DNA in biofilms of N. gonorrhoeae MS11 is double-stranded

Because ssDNA might play an important role in the initial attachment of single cells to the surface, we tried to visualize ssDNA and total DNA in a 10 h old biofilm of MS11 (Fig. 5). At 10 h after inoculation, the formation of the distinct three-dimensional structures has started, but also single attached cells are still observed. Furthermore, in 10 h old biofilms, the single attached cells were still sensitive to Exonuclease I (data not shown). Thus under these conditions, we expect to be able to visualize ssDNA. To enable comparison of the amounts of ssDNA and total DNA, biofilms were incubated with IANBD-labelled Sac7d-S18C or IANBD-labelled TteSSB2-W55C

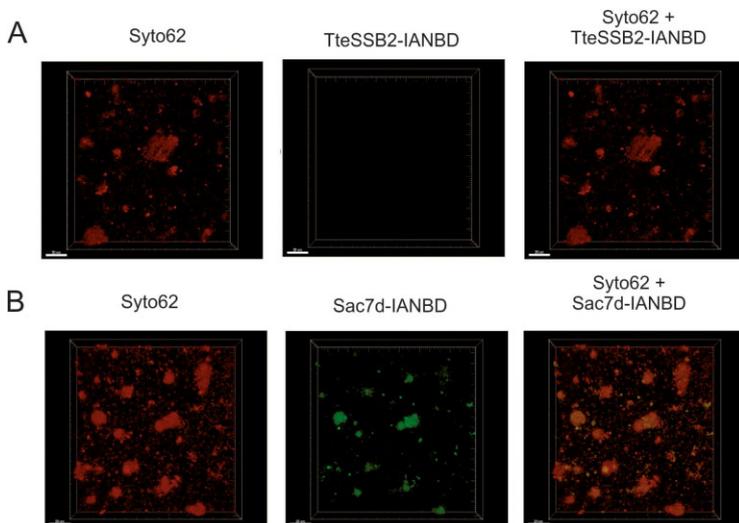


Fig. 5. Detection of ssDNA and dsDNA in 10 h old biofilms of *N. gonorrhoeae*. Confocal microscopy of continuous flow chambers inoculated with *N. gonorrhoeae* strain MS11 imaged 10 h after inoculation. The biofilm was stained with (A) Syto62 (red) and IANBD-labelled TteSSB2-W55C (green) or (B) Syto62 (red) and IANBD-labelled Sac7d-S18C (green) and visualized by CLSM. Micrographs represent three-dimensional projections. The bar is 20 μ m in length.

(Fig. 5). Both proteins showed approximately equal fluorescence intensities. Interestingly, a fluorescent signal was only found to colocalize with the distinct three-dimensional structures after incubation with IANBD-labelled Sac7d-S18C but not after incubation with IANBD-labelled TteSSB2-W55C. Thus, only the protein that had affinity for dsDNA associated with the cells, whereas the protein with affinity for ssDNA remained in the medium (Fig. 5). Because we were not able to detect ssDNA associated with the biofilm under continuous flow conditions, we attempted to visualize ssDNA in planktonic cultures. IANBD-labelled Sac7d-S18C or IANBD-labelled TteSSB2-W55C was added to planktonic cultures followed by fluorescence microscopy (Fig. 6). After incubation with IANBD-labelled Sac7d-S18C, again a strong fluorescent signal was observed associated with the cells (Fig. 6A). After incubation with IANBD-labelled TteSSB2-W55C, the fluorescent signal was found mainly in the medium, and not associated with cells (Fig. 6B). This shows that secreted ssDNA is not found associated with the cells, but is found in the medium, whereas dsDNA released from lysed cells remains mainly attached to the cells. Incubation of the cells with DNase I before incubation with IANBD-labelled Sac7d-S18C strongly reduced the labelling of the cells, demonstrating that the labelled DNA is accessible. To test whether externally added ssDNA would bind to the cells, cells were incubated with M13-derived ssDNA. At high concentrations of ssDNA, small amounts of IANBD-labelled TteSSB2-W55C were located at the cells, but most of IANBD-labelled TteSSB2-W55C was still found in the medium (Fig. 6C). Thus, both in planktonic cultures and under continuous flow conditions, dsDNA is found mostly associated with cell surfaces, while the ssDNA is detected mainly in the medium. Because biofilm formation was strongly reduced in the presence of Exonuclease I and in a $\Delta traB$ strain that does

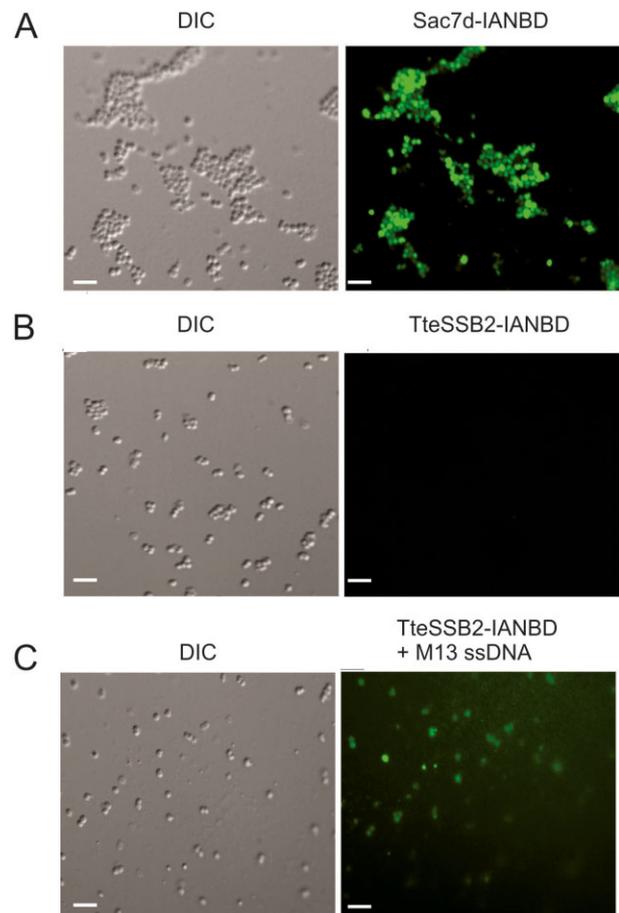


Fig. 6. Detection of ssDNA and dsDNA in an exponentially growing culture of *N. gonorrhoeae*. *Neisseria gonorrhoeae* MS11 was grown exponentially in minimal medium. The cells were incubated with (A) IANBD-labelled Sac7d-S18C, (B) IANBD-labelled TteSSB2-W55C, or (C) incubated with IANBD-labelled TteSSB2-W55C after incubation with M13 ssDNA. The cells were visualized by DIC (left side) or CLSM (right side). Micrographs represent three-dimensional projections. The bar is 20 μ m in length.

not secrete ssDNA, especially in the initial phases of biofilm formation we propose that initially, under conditions where still low amounts of dsDNA are available, the secreted ssDNA facilitates biofilm formation. At later stages, this function is performed by the dsDNA that remains better associated with bacterial cell surfaces.

Discussion

Extracellular DNA plays an important role in biofilm formation by *N. gonorrhoeae* (Steichen *et al.*, 2011). Cultures of *N. gonorrhoeae* strain MS11 contain ssDNA, which is secreted directly into the medium via the T4SS encoded within the GGI (Salgado-Pabon *et al.*, 2007). Biofilm formation of *N. gonorrhoeae* has previously not only been studied in MS11, a strain that contains the GGI, but also in *N. gonorrhoeae* strains 1291 and FA1090 which do not contain a GGI (Greiner *et al.*, 2005). All three of these strains were able to form biofilms, demonstrating that the presence of the T4SS is not essential for biofilm formation. However, biofilm formation of *N. gonorrhoeae* is affected by many different factors, like the ability to release blebs (Steichen *et al.*, 2008), the expression of Nuc, the extracellular thermonuclease (Steichen *et al.*, 2011) and the presence of type IV pili. Many gonococcal genes, like the *pilE* gene, undergo rapid variation (Seifert *et al.*, 1988), which complicates a direct comparison of different isolates. Because these other effects might mask an effect of the secreted ssDNA on biofilm formation, we have studied the effects of ssDNA secretion by comparing biofilm formation in MS11 with biofilm formation in a mutant and a complementation mutant directly derived from MS11, and show that actively secreted ssDNA facilitates biofilm formation of *N. gonorrhoeae*. Our data suggest that ssDNA plays an important role in the initial phases of biofilm formation and that this role is taken over by other components at later stages of biofilm formation. The $\Delta traB$ mutant that does not secrete ssDNA showed strongly reduced biofilm formation. The formation of the distinct three-dimensional structures observed in the wild type and complementation mutant was strongly reduced in the $\Delta traB$ mutant, and the stability of the formed biofilm was also strongly reduced. This is the first time that an effect of ssDNA on biofilm formation is demonstrated.

Lappann and colleagues (2010) demonstrated that in *N. meningitidis*, the capability to form biofilms differs between different lineages. In the most prevalent lineages, biofilm formation is dependent on DNA. These lineages form stable biofilms and show a very stable interaction with the host. Several less prevalent lineages do not use DNA for biofilm formation, and form much less stable biofilms. It was proposed that the lineages

that do not use DNA for biofilm formation and show poor colonization properties compensate for their poor colonization properties by higher transmission rates. Possibly, in *N. gonorrhoeae*, the secretion of ssDNA via the T4SS modulates the colonization and transmission rates.

In this study, a method was developed to specifically detect ss- and dsDNA, using fluorescently labelled thermostable ssDNA- and dsDNA-binding proteins. Both proteins bind to DNA in an essentially sequence unspecific manner (Gao *et al.*, 1998; Zhang *et al.*, 2009). The fluorescent detection of the labelled proteins was optimized by introducing the fluorescent probes at positions where their fluorescence increases upon ssDNA binding. This reduces the signal of the proteins that are not bound to DNA. Detection of ssDNA using TteSSB2 of *Thermoanaerobacter tengcongensis* was highly specific, and ssDNA could be specifically detected in biofilms of *S. acidocaldarius*. TteSSB2 could also be used to detect ssDNA bound to *N. gonorrhoeae* strains, when ssDNA had been added to the culture. The Sac7d protein from *S. acidocaldarius* bound to dsDNA with higher affinity, but most likely will detect both ssDNA and dsDNA, especially since even secreted ssDNA will contain many positions where the ssDNA will anneal and form stretches of dsDNA. Localization of the DNA using fluorescently labelled Sac7d in *N. gonorrhoeae* biofilms showed that DNA was mainly localized inside the mature mushroom-like structures. This resembled the localization patterns of DNA found for *N. gonorrhoeae* using other DNA stains. Also, the localization of DNA using fluorescently labelled Sac7d in biofilms of *S. acidocaldarius* showed a pattern similar to what has been observed before using other DNA stains. This demonstrated that the use of these fluorescent proteins is a valid method to detect the DNA. Our approach could differentiate between ssDNA and total DNA. In biofilms of *S. acidocaldarius*, both ssDNA and dsDNA could be detected, while in the biofilms of *N. gonorrhoeae*, only dsDNA was detected. DNA has been found in different patterns for different organisms. For example, the extracellular DNA of *P. aeruginosa* is present in the microcolonies and organized as a grid-like structure on the substratum (Allesen-Holm *et al.*, 2006), whereas the extracellular DNA in *Bacillus cereus* biofilms is evenly distributed over the surface, while in biofilms of *Haemophilus influenzae* and the gamma proteobacterium F8-5 eDNA occurs as filamentous strands (Bockelmann *et al.*, 2006; Jurecsek and Bakaletz, 2007). Whether this DNA is single or double stranded is currently unknown, but can now be easily assessed with our newly developed method.

In this study, we attempted to detect both ss- and dsDNA within biofilms of *N. gonorrhoeae*. During the initial phases in which cells attach to the surface, and where

experiments with Exonuclease I demonstrated that ssDNA plays an important role, neither ssDNA nor dsDNA could be detected. Most likely the amounts of ss- and dsDNA are too low for detection at these stages using the fluorescently labelled proteins. At later stages, the fluorescently labelled Sac7d protein detected DNA attached to the cells in the biofilms. Remarkably, at this stage, ssDNA, as detected by the fluorescent TteSSB2 protein, was found mainly in the medium. Thus, the ssDNA secreted via the T4SS plays a role in the initial stages of biofilm formation. At this stage, only very low amounts of DNA are present. At later stages, where large amounts of dsDNA are present, no ssDNA and no effects of ssDNA on biofilm formation could be detected.

Experimental procedures

Materials

Oligonucleotides and M13 ssDNA were purchased from Sigma-Aldrich (Steinheim, Germany). Restriction and, DNA-modifying enzymes were purchased from New England Biolabs (Frankfurt/Main, Germany). *N*-((2-(iodoacetoxy)ethyl)-*N*-Methyl)amino-7-Nitrobenz-2-Oxa-1,3-Diazole (IANBD-Ester), Fluorescein, Texas Red, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) and Syto62 were purchased from Invitrogen (Darmstadt, Germany).

Strains and growth conditions

Neisseria gonorrhoeae strain MS11 and derivatives thereof (MS11Δ*traB*, MS11Δ*traB*::*traB*) used in this study are described in Table S1. *Neisseria gonorrhoeae* strains were plated from glycerol stocks on GC agar (BD Difco, Heidelberg, Germany) containing Kellogg's supplements (Kellogg *et al.*, 1963) and grown overnight under 5% CO₂ at 37°C. When necessary, chloramphenicol and/or erythromycin were added at the final concentration of 10 μg ml⁻¹.

Escherichia coli strains used for cloning and overexpression (Table S1) were grown in Luria-Bertani (LB) medium at 37°C with the appropriate antibiotics; ampicillin (100 μg ml⁻¹), erythromycin (500 μg ml⁻¹) or chloramphenicol (34 μg ml⁻¹).

Sulfolobus acidocaldarius (DSM639) (Table S1) was grown in Brock medium at 76°C, with pH adjusted to 3.0 using sulphuric acid and supplemented with 0.1% (w/v) tryptone (Brock *et al.*, 1972).

Construction of *Neisseria gonorrhoeae* strains

An overview of plasmids created to construct *N. gonorrhoeae* strains is shown in Table S2. Primers used are described in Table S3. To generate a knockout of *traB*, flanking regions of *traB* were amplified by polymerase chain reaction (PCR) using the GGI-87F and GGI-88R, GGI-89F and GGI-90R primers on chromosomal DNA of *N. gonorrhoeae* strain MS11. These fragments were digested by *Hind*III and *Kpn*I

and with *Eco*RI and *Sac*I, respectively, and ligated into vector pSH001, resulting in vector pEP015_1. To construct pSH001, two PCR products amplified with primers ForwardDUS and ReverseDUS and primers ForwardErmC and ReverseErmC using pIDN1 as template were digested with *Eco*RI and *Hind*III and ligated. PCR product of *traB* fragment created with primers GGI-89F and GGI-90R was cloned in *Eco*RI and *Sac*I sites of pEP015_1, resulting in pEP015_2. Plasmid pEP015_2 was transformed to *N. gonorrhoeae* strain MS11 by natural transformation (Spence *et al.*, 2008) resulting in strain MS11Δ*traB* in which *traB* is replaced by an ermC cassette. The replacement of *traB* by the ermC cassette was confirmed by PCR analysis and by sequencing of the PCR products. For the construction of the complementation strain MS11Δ*traB*::*traB*, the deletion mutant MS11Δ*traB* was transformed with genomic DNA of MS11. Transformants were selected for sensitivity to erythromycin. The presence of the insertion was determined by PCR and confirmed by sequencing of the region.

Piliation state of *N. gonorrhoeae* strains

Piliation of different strains was first evaluated visually after growth on plates. The expression levels of PiiE in the different *N. gonorrhoeae* strains were further assayed by Western blot analysis. *Neisseria gonorrhoeae* colonies were inoculated from over night grown plates in 3 ml of prewarmed GCB medium, grown at 37°C with continuous shaking for 2 h and diluted in fresh medium to an OD₆₀₀ of 0.2. Growth was continued until an OD₆₀₀ of 0.75 was reached and then cells were collected by centrifugation. The TriFast reagent (PEQLAB Biotechnologie GMBH, Erlangen, Germany) was used for total protein isolation. Protein isolation was performed according to the manufacturer's protocol. Isolated protein fractions were solubilized in 100 μl SDS-Sample buffer, incubated at 75°C for 15 min and loaded on the SDS-Gel for further analysis. Western blotting was performed by transferring the protein from the gels on polyvinylidene difluoride membranes and incubating with 1:5000 dilution of PiiE primary AB and anti-mouse-AP conjugate secondary antibody (IBA). The chemiluminescence signal was obtained using the CDP-star substrate (Roche Diagnostics, Mannheim, Germany) on a LAS-4000 imager (GE Healthcare Europe, Freiburg, Germany).

Construction of plasmids used for overexpression

The construction of the plasmids used for overexpression in this study is described in Table S1.

Expression and purification of TteSSB2, Sac7d, ExoI and ExoIII

All proteins were overexpressed in *Escherichia coli* strain BL21pLysS. Cells were grown in 1 l of LB medium supplemented with 1% glucose at 37°C to an OD₆₀₀ of 0.5 and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. After 3 h, the cells were harvested by centrifugation, resuspended in 30 ml of buffer A (50 mM NaPO₄, 300 mM NaCl, pH 8.0) and stored at -80°C. Prior to purification,

frozen cell pellets were thawed on ice. After thawing, the solution was supplemented with 1 tablet of protease inhibitor cocktail (Roche) and 1 mg of DNase I (Roche), and the cells were disrupted by passing them three times through a high-pressure cell disrupter (constant cell disruption systems) at 2300 bar. Cell debris was removed by centrifugation at 10 000 r.p.m. in an F10S-6x500y rotor (Thermo Fischer Scientific, Waltham, MA, USA), and the supernatant was filtered through a 0.45 µm filter. Purifications were performed on an ÄKTA-Purifier system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

His-tagged Exol, ExoIII and Sac7d were purified by loading the clarified supernatant on a 1 ml Hi-Trap Chelating column (GE Healthcare) preloaded with 0.1 M NiSO₄ and equilibrated in buffer A. The column was washed with 10 column volumes of buffer A and bound proteins were eluted with a linear gradient of buffer A supplemented with 400 mM imidazole. Peak fractions containing proteins were pooled and diluted with two volumes of buffer B (10 mM Tris-HCl pH 8.0, 10 mM NaCl). This sample was loaded on a Hi-Trap Q column (GE Healthcare) equilibrated with buffer B, and the protein was eluted with a linear gradient up to 1 M NaCl in buffer B. Fractions containing proteins were concentrated to 2 ml using Amicon Ultra – 10K Concentrators (Millipore, Billerica, MA, USA). Finally, these fractions were loaded on a Superdex SD200 gel filtration column (GE Healthcare), equilibrated with buffer C containing 150 mM NaCl and 20 mM Tris-HCl pH 8.0. Fractions containing proteins were pooled and frozen in liquid N₂ until further use.

To purify TteSSB2, the disrupted cell suspension was heated for 15 min at 75°C, followed by centrifugation at 10 000 r.p.m. in an F10S-6x500y rotor (FiberLite). The supernatant was filtered through a 0.45 µm filter. The filtrate was loaded on Hi-Trap Q column (GE Healthcare) equilibrated with buffer B, and the protein was eluted with a linear gradient up to 1 M NaCl in buffer B. Fractions containing proteins were concentrated to 2 ml using Amicon Ultra – 10K concentrators (Millipore). Finally, these fractions were loaded on a Superdex SD200 gel filtration column (GE Healthcare), equilibrated with buffer C containing 150 mM NaCl and 20 mM Tris-HCl pH 8.0. Fractions containing proteins were pooled, and frozen in liquid N₂ until further usage.

Site-specific labelling of proteins via cysteines

Prior to labelling of the cysteines, purified proteins were reduced by incubation with 10 mM tris[2-carboxyethyl] phosphine for 10 min on ice. Two microlitres of 50 mM stock solution of the fluorescent probe was added to a total volume of 200 µl containing the protein of interest at a concentration of 100 µM ml⁻¹ in buffer C. Labelling was performed in the dark for 2 h at room temperature. The reaction was stopped by addition of 10 mM glutathione. Access of dye and glutathione was removed by separation of the reaction solution on a 2 ml PD-column (GE Healthcare) equilibrated with buffer C. Fractions containing the labelled protein were frozen in liquid N₂ until further use.

Fluorescence titrations

Titrations of 50 nM fluorescently labelled protein were performed with increasing concentrations [0–200 nM] of

M13 ssDNA (TteSSB2) or M13 dsDNA (Sac7d). Experiments were performed on a temperature-controlled PC1 spectrofluorometer (ISS, Champaign, IL, USA) with a cooled photomultiplier. The excitation wavelength was set to 472 nm and the emission wavelength to 536 nm for IANBD. The slit widths for the excitation and the emission beam were set to 1 and 2 nm respectively. Experiments were performed in a 60 µl cuvette at 8°C in a buffer containing 20 mM Tris pH 7.5 and 1 mM dithiothreitol, and samples were allowed to equilibrate for 90 s between measurements.

Electrophoretic mobility shift assays

To determine the binding efficiency of purified TteSSB2 and Sac7d to ssDNA and dsDNA, a dT₉₀ oligonucleotide labelled with Cy3 at the 5' end and a 468 bp PCR product obtained with an unlabelled primer (407R-GGI) and a Cy3-labelled primer (790R-GGI) were used. 20 nM of labelled dT₉₀ oligonucleotide or the PCR product was mixed with increasing concentrations (0–5000 nM) of TteSSB2 or Sac7d in SBA buffer (10 mM NaOH, 2 mM EDTA, titrated to pH 7.5 with boric acid) in a total volume of 12 µl. The reaction solutions were incubated at 4°C for 15 min after which the reaction was mixed with 3 µl 40% sucrose. The aliquots were analysed by electrophoresis on 7.5% native polyacrylamide gels in SBA buffer. The fluorescently labelled DNA was visualized on a LAS-4000 imager (Fujifilm).

Activity assays for specificity of Exol

The specificity of Exol for ssDNA and dsDNA was tested using a dT₉₀ oligonucleotide labelled with Cy3 at the 5' end, and a 468 bp PCR product obtained with an unlabelled primer (407R-GGI) and a Cy3-labelled primer (790R-GGI). The reactions were performed in the growth medium used in the continuous flow experiments (Graver–Wade medium diluted 1:5 with phosphate buffered saline). Shortly, 150 ng of the dT₉₀ oligonucleotide or the PCR product was mixed with increasing concentrations of Exol (0–5000 nM) in a total volume of 12 µl. The reaction was incubated at 37°C for 1 h after which the reaction was mixed with 3 µl 40% sucrose and 10% SDS. The aliquots were analysed by electrophoresis on 7.5% native polyacrylamide gels using an SBA buffer system. The fluorescently labelled DNA was visualized on a LAS-4000 imager (Fujifilm).

Fluorescent light microscopy

To perform light microscopy, *N. gonorrhoeae* colonies were scraped from overnight grown plates and diluted in Graver–Wade medium (Wade and Graver, 2007) supplemented with Kellogg's supplements and 0.042% NaHCO₃. Cultures were grown at 37°C constantly shaken at 140 r.p.m. Cells were grown to an OD₆₀₀ of 0.8, collected by centrifugation and diluted in fresh medium. This procedure was repeated at least three times, until most DNA derived from lysed cells was removed. Samples were collected directly after the last dilution. When the indicated enzymes (1 µM Exol) or M13 ssDNA (final concentration 0.0625 µg ml⁻¹) were added, incubation was continued for 15 min. Two microlitres of the cell suspen-

sion was loaded on the glass slide covered with 1% agarose. Microscopy and image acquisition was performed on a Zeiss Imager M1 (Zeiss, Göttingen, Germany). Image data obtained were processed with Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

Confocal laser microscopy

For continuous-flow chamber experiments with *N. gonorrhoeae*, a Graver–Wade medium supplemented with Kellogg's supplements and 0.042% NaHCO₃ diluted 1:5 with PBS was used. Colonies from three plates grown overnight were collected and inoculated in 3 ml prewarmed Graver–Wade medium. Cultures were grown to an OD₆₀₀ of 0.8; diluted 1:1000 with fresh Graver–Wade medium and 200 µl of the diluted cell suspension was inoculated in the continuous-flow chamber. Flow chamber experiments were performed as described previously (Lappann *et al.*, 2006). Strains were grown in three separate channels. From each channel, at least six image stacks were acquired. For a continuous incubation with Exol, Exol was added to a final concentration of 1 µM to the medium used to perfuse the continuous-flow chamber. The perfusion medium was exchanged every 2 h with medium with freshly thawed Exol. When the effect of Exol on 24 h old biofilms was studied, Exol was injected directly into the channels. The flow rate was stopped for 1 h, and continued after the incubation. To exclude the effect of stopped flow, one channel was inoculated with medium not containing enzymes and compared afterwards with the channels containing enzymes. Microscopy and image acquisition was performed on a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany). Images were processed, and for the quantification of the biofilm mass, the total number of voxels was calculated with IMARIS software (Bitplane, Zurich, Switzerland). Voxel numbers from five distinct channels were determined. An average number and an error bar from five calculations are used for the final picture. At least two fully independent experiments were performed. Biofilm experiments with different *Sulfolobus* spp. were performed as described previously (Koerdt *et al.*, 2010).

Visualization of ssDNA/dsDNA in the biofilms

For visualization of DNA in the biofilms, either 500 nM labelled TteSSB2 or 500 nM labelled Sac7d was inoculated into the channels. After 10 min, the flow was resumed to wash away unbound protein.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Purification and characterization of Exonuclease I. His-tagged Exonuclease I was purified by Ni-NTA affinity, anion exchange and gel filtration chromatography. (A) His-tagged Exonuclease I was essentially pure as assayed by Coomassie staining of an SDS-PAGE of the purified protein. (B) Exonuclease I eluted as a monomer from the SD200 gel filtration column. The positions of the various markers for both the gel filtration and the SDS-PAGE are indicated. To test the specificity of the purified Exonuclease I, 150 ng of a Cy3-labelled dT90 oligonucleotide (C) or 150 ng of a Cy3-labelled PCR product (D) were incubated with an increasing concentrations of Exonuclease I for 1 h at 37°C. The fluorescently labelled DNA was analysed after separation on a 7.5% polyacrylamide gels using a LAS-4000 imager.

Fig. S2. Purification and specificity of the ssDNA binding protein TteSSB2. TteSSB2 was overproduced in *E. coli* and

purified by a heat-step followed by anion exchange and gel filtration chromatography. (A) Coomassie staining of an SDS-PAGE of the purified protein. (B) TteSSB2 eluted as a tetramer from the SD200 gel filtration column and was essentially pure. The positions of the various markers for both the gel filtration and the SDS-PAGE are indicated. Electromobility shift assays were performed with incubating either a Cy3-labelled dT90 oligonucleotide (C) or a Cy3-labelled PCR product (D) with increasing concentration of TteSSB2. The fluorescently labelled DNA was analysed after separation on 7.5% polyacrylamide gels using a LAS-4000 imager.

Fig. S3. Purification and specificity of the DNA binding protein Sac7d. His-tagged Sac7d was overproduced in *E. coli* and purified by Ni-NTA affinity, anion exchange and gel filtration chromatography. (A) Coomassie staining of an SDS-PAGE of the purified protein. (B) Sac7d eluted as a monomer from the SD200 gel filtration column. The positions of the various markers for both the gel filtration and the SDS-PAGE are indicated. Electromobility shift assays were performed with incubating either a Cy3-labelled dT90 oligonucleotide (C) or a Cy3-labelled PCR product (D) with increasing concentration of Sac7d. The fluorescently labelled DNA was analysed after separation on 7.5% polyacrylamide gels using a LAS-4000 imager.

Table S1. Strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Primers used in this study.

Table S4. Increase in fluorescence upon binding to DNA for TteSSB2 and Sac7d labelled at different positions.

Supplementary Material and Methods: includes supplementary methods, figures and tables.

Supplementary References: contains references cited in supplementary material and methods.