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Extracellular DNA Plays an Important Structural Role in the Biofilm of the Plastic Degrading Actinomycete *Rhodococcus ruber*

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ABSTRACT

Biofilms, the preferred bacterial mode of living and survival, are employed by most microorganisms—which tend to attach to surfaces—to gain physical support, increase nutrient utilization and availability, and augment their resistance against anti-bacterial agents. *Rhodococcus ruber* (C208) has been shown to form a dense biofilm on polyethylene surfaces while degrading them. Bacterial biofilms comprise bacterial cells embedded in self-secreted extracellular polymeric substances (EPS) whose main components are polysaccharides, proteins and nucleic acids. Revealing the roles of these components will enable further insight into biofilm development and, therefore, the EPS structure-function relationship. The current study focuses on contribution of extracellular DNA to biofilm formation and stability. This was approached by investigating the influence of nucleases on biofilm formation via degradation of their corresponding substrates within the biofilm of C208. RNase application to cultures of C208 decreased biofilm formation. Degradation of biofilm DNA by DNase reduced early-stage biofilm formation by 20% - 25% but had no significant effect on established, mature biofilm. Likewise, the addition of DNA to cultures significantly enhanced early-stage biofilm formation by 50% - 100%. RAPD-PCR analysis revealed different band patterns from intra-cellular DNA and extra-cellular DNA and also between the supernatant and biofilm fractions of extra-cellular DNA, indicating that perhaps only certain DNA molecules are utilized as part of the biofilm.

Keywords: Biofilm; Extracellular DNA; DNase; RNase; RAPD-PCR

1. Introduction

Polyethylene (PE), the most commonly used synthetic polymer, is highly inert and virtually non-biodegradable. Current global production of PE stands at about 140 million tons per year. In the absence of safe disposal procedures, plastic waste accumulates in the environment posing an ever increasing ecological threat to terrestrial and marine wild life [1].

Biodegradation has been suggested as the best approach for degrading polyethylene (Reviewed by Sivan [1]). The formation of a biofilm on the surface of plastic macro-molecules may accelerate their biodegradation. As a common mode of living adopted by microorganisms is to attached to almost any surface-water interface; biofilms protected communities that allow survival in challenging environments [2-5]. This form of life enables

vital interaction between the microorganisms and the surface to which they are attached and markedly increases the survival potential of the microbial community. Biofilms promote utilization of the surface either as support and/or as a substrate.

After initial biofilm establishment, cell-to-cell communication (*i.e.*, quorum sensing) via extracellular signaling molecules regulates further modification and development of the biofilm [6]. In Gram-negative bacteria, the specificity of these signals is mediated by homoserine lactones [7-9], while in Gram-positive bacteria, diverse peptides with limited specificity control of biofilm development [10-12]. Although microbial biofilms often exert various deleterious effects in natural, clinical or industrial environments, they may be beneficial from the biodegradation of complex synthetic polymers, such as

plastics.

The preferred mode of growth of plastic-degrading bacteria seems to be based on the formation of a biofilm on the plastic surface. Since plastic polymers such as polyethylene and polystyrene are hydrophobic, the bacterial surface must be also hydrophobic to form a stable biofilm [13]. Sakharovskii *et al.* [14] showed that a correlation exists between carbon starvation and bacterial hydrophobicity. Similarly, Sanin *et al.* [15] reported changes in bacterial surface hydrophobicity in response to carbon starvation, noting that decreased hydrophobicity was accompanied by reduced bacterial attachment capabilities.

Recently, we isolated a strain of *Rhodococcus ruber* (designated C208) that utilizes polyethylene as a sole carbon and energy source and that degrades the polymer. This Gram-positive bacterium is an actinomycete that showed a high level of hydrophobicity [16], enabling it to form a dense biofilm on the polyethylene surface. However, little is known about the structure/function relationships in the biofilm. Thus, revealing C208 biofilm composition may provide insight into its mode of action and facilitate its use in biodegradation processes.

Bacterial biofilms are encased within an extracellular matrix consisting of polysaccharides, proteins and nucleic acids [17]. Until recently, polysaccharides were considered as the main component of the biofilm matrix [18-20]. However, recent findings have demonstrated that extracellular proteins and DNA contribute to biofilm stability and regulation. Studies showed that degradation of biofilm proteins by proteases [21,22] and degradation of extracellular DNA (eDNA) by DNase [23,24] reduced the formation and expansion of biofilms. Indeed, eDNA plays a major role in biofilm development, providing structural stability [24,25] and protection against antimicrobial agents [26,27]. Recently, eDNA was found to be involved in coordination of bacterial movements during biofilm expansion and mediation of intercellular connectivity within the expanding biofilm of *Pseudomonas aeruginosa* [28].

Gram-positive bacteria release eDNA to the biofilm, apparently by autolysins (bacterial murein hydrolases). These molecules were reported to be involved in biofilm formation, mediating bacterial lysis in *Enterococcus faecalis* and *Staphylococcus epidermidis* [23,27,29,30].

Here we further assess the role of *Rhodococcus ruber* C208 eDNA in biofilm formation and stability.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Cultures of *Rhodococcus ruber* C208 were cultured in 100 ml nutrient broth (NB; Difco), incubated in flasks (250 ml) on a rotary shaker (120 rpm) at 30°C or on nutrient agar (Difco) Petri dishes at 30°C. Cultures of *Bacillus cereus* and *Pseudomonas aeruginosa* from our laboratory collection were maintained in 100 ml Luria Broth (LB) and incubated as above or on LB agar Petri dishes as above.

Experiments were performed in a minimal salts medium (SM; [16]) supplemented with 0.1% (w/v) mannitol (designated SMM) in polystyrene (PS) microplates (Kartell, Spain) for the biofilm assays. Twenty-four-hour cultures grown in NB medium served as inoculums. Cultures were diluted to reach an OD_{595nm} of about 0.1 in the SMM medium. Medium without inoculum served as negative control.

2.2. Enzymatic Treatment of Bacterial Cultures

Aliquots from diluted cultures were transferred to PS microplates (190 µl per well) and incubated for the desired time at 30°C. When required, DNase I (RNase-free) or RNase A (DNase and protease-free) (Fermentas, Vilnius, Lithuania) was added to the wells, at time of seeding or after 24 h of growth, to achieve a final concentration of 15 U/ml or 50 mg/ml, respectively. DNase I heat-inactivated samples (15-min incubation at 65°C) served as control. Prior to processing, the turbidity of the culture in the wells was determined at 595 nm with a plate reader (Biotek ELx808, Winooski, VT, USA) and serial diluted for CFU counts.

2.3. Metabolic and Viability Tests of the Bacterial Biofilm

The metabolic activity of the bacterial biofilm was determined by metabolism monitoring with CTC- Fluorescent Redox Probe (monitoring reduction of CTC (5-cyano-2,3-ditolyl tetrazolium chloride) to CTCF (Formazan violet) by bacterial respiration. [31,32].

2.4. Biofilm Quantification

Treated and untreated cultures grown in the PS microplates were decanted and the wells were washed twice with distilled water and air-dried. Afterward, 200 µl crystal violet (0.2% w/v) (Sigma-Aldrich) were added to the wells, which were incubated for 15 min and then washed twice with distilled water to remove loosely attached biomass, and the wells were air-dried again. Crystal violet retained by the cells was subsequently re-dissolved in 200 µl ethanol (99% w/v), and after incubation of 1 h, absorbance was determined with the plate reader at 595 nm [33].

2.5. DNA Extraction and Treatment

Intra-cellular DNA was extracted from 24-h liquid cultures in SM supplemented with polyethylene films using the UltraClean^TM Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's

instructions. Two fractions of eDNA were extracted: S—supernatant DNA from cell-free supernatants [by centrifugation at 10,000 × g for 10 min at 4°C and filtration (0.2 μm)] of cultures grown in liquid SM medium supplemented with polyethylene films, and B—biofilm DNA obtained from the washed biofilms adhered to the surface of the polyethylene films. The biofilm was gently scraped, re-suspended in sterile deionized water and vortexed. The suspension was then centrifuged (10,000 × g for 2 min at 4°C) and filtered, as above. This resulted in a cell-free fraction for DNA isolation.

Short fragments of C208 genomic DNA were produced by the amplification of two 16S rRNA gene fragments. The resultant amplicons were confirmed by agarose gel electrophoresis to be about 300 bp and 500 bp (forward primer 8F, reverse primers 341R and 518R, respectively). The DNA fragments were added to the growth medium at increasing concentrations, and biofilm formation was compared to that obtained in the presence of intact genomic DNA.

Genomic DNA (3 μg/ml) in Ultrapure® water was used to pre-coat the wells of the PS microplate under incubation for 20 h at 4°C. The solution was decanted and the wells were air-dried prior to inoculation with the bacteria and assessment of biofilm mass (Modified from [34]). Coating was verified by propidium iodide (Invitrogen, USA) staining according to the manufacturer's instructions.

2.6. Random Amplified Polymorphic DNA (RAPD)-PCR Analysis

Profiling of intra- and extra-cellular DNA fractions (obtained as detailed above) was performed by RAPD-PCR using ten mer primers. A primer mixture of 208-ACGGCCGACC/228-GCTGGGCCGA was used, *Bacillus* DNA served as control and gel was run in TAEx1 buffer; Or Primer 241-GCCCGAGCGG was used with *Pseudomonas* DNA as control and gel run in TBEx1 buffer [35-38].

3. Results

3.1. External DNA and Biofilm DNA

Extracellular DNA was obtained from two fractions, supernatant DNA (S) and biofilm DNA (B). RAPD-PCR analysis was conducted in an attempt to distinguish between the eDNA sources. The analysis showed different patterns of PCR products between the eDNA fractions and the cellular DNA (C) (**Figure 1**). This indicates that the eDNA did not originate exclusively from the chromosomal DNA of lysed cells and that possibly it was actively secreted by the bacterium. Moreover, the pattern for eDNA from the biofilm fraction (B) differs from those of the supernatant DNA (S) and cellular DNA (C), which may indicate that only certain DNA molecules are

being utilized as part of the matrix EPS (**Figure 1**).

Extracellular DNA found as part of the bacterial EPS is a novel finding and its presence and characterization in *Rhodococcus ruber's* biofilm was first investigated in our lab.

3.2. Effect of Nucleases on the Biofilm

To further elucidate the role of eDNA in our strain, we amended cultures of C208 with DNase I and RNase A. All biomass quantifications of the biofilm were conducted by crystal violet (CV) staining as detailed in the Materials and Methods chapter. First we verified that the bacteria were not damaged by the nucleases. Respiration test showed no harm to their viability (**Figure 2**).

The enzymes were added at inoculation time or to 24-h old biofilms. In **Figure 3** it can be seen that biofilm development with the addition of nucleases follows the same pattern of the untreated control (Oscillation of attachment-detachment), only its biomass is reduced. The addition of inactivated DNase had no effect on the biofilm indicating that the observed effect is caused by the nucleolytic activity.

Biomass quantification of the biofilm after 5 days showed that both enzymes reduced its formation without affecting culture growth or vitality (**Figure 4**). Addition of Bovine Serum Albumin (BSA), caused a significant increase in growth rate, while almost no biofilm was formed (**Figure S1**). This indicates that the
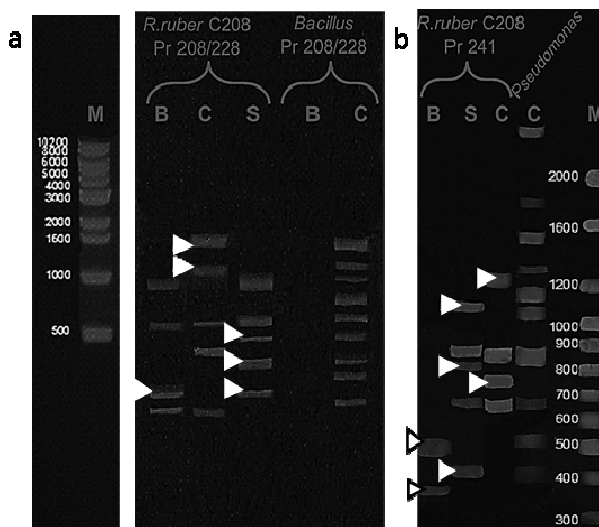

Figure 1. Random Amplified Polymorphic DNA-PCR with 10 mer primers of three fractions of the bacterial culture grown in synthetic medium supplemented with polyethylene. S = supernatant; B = biofilm (EPS); C = cellular; M = DNA ladder. a) Primer mixture of 208-ACGGCCGAC/228-GCTGGGCCGA; *Bacillus* DNA served as control and gel was run in TAEx1 buffer; b) Primer 241-GCCCGAGCGG; *Pseudomonas* DNA as control and gel run in TBEx1 buffer. ▷ Indicates a dissimilar band.
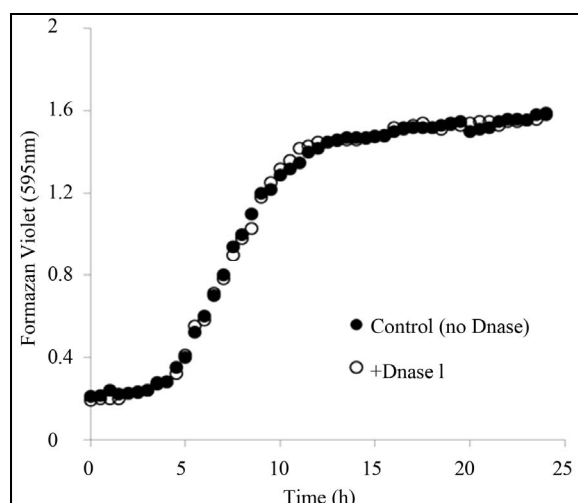
Figure 2. Respiration of *Rhodococcus ruber* C208 incubated with and without DNase measured by reduction of Tetrazolium to Formazan violet by the cellular electron transport chain.
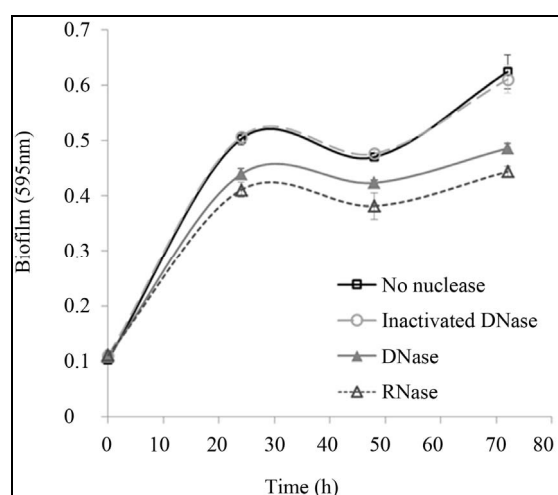

Figure 3. Biofilm formation of C208 during 3 days of growth. Cultures were grown on PS microplates, in SMM supplemented with nucleases (0.2 mg/ml). Error bars are standard error of the means (n = 6).

bacteria preferred the planktonic mode when food was abundant. Addition of DNase I and RNase A to 24-h old biofilms had very little effect (data not shown). These findings suggest that the nucleic acids are important to the formation of C208 biofilm mainly in the initial stages of its development.

3.3. Effect of Endogenous and Exogenous DNA on Biofilm

Since eDNA degradation affected the biofilm we wondered if addition of excess DNA molecules would have any influence on it. DNA was extracted from C208 cells (endogenous DNA) and used at a final concentration of 3,

30 or 100 ng/ml. To rule out the effect of self DNA, the same experiments were repeated using salmon sperm DNA (exogenous DNA). As shown in **Figure 5**, incubation with DNA added at time of seeding enhanced biofilm development by up to 100%. Moreover, this effect was observed with both endogenous (self) and exogenous (foreign) DNA, indicating that this was due to an intrinsic property of the DNA molecule and independent of the exact nucleotide sequence and donor organism.

The addition of DNA to a 2-day-old biofilm enhanced biofilm formation, but cell growth was also improved due to the utilization of the DNA not only as a biofilm support, but also as a nutrient, and the net effect is unequivocal (**Figure 6**).
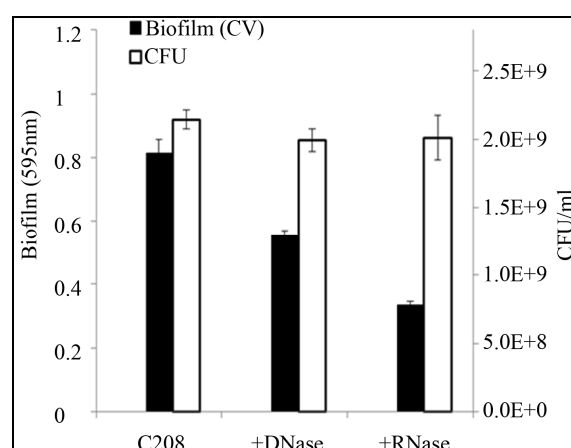

Figure 4. Effect of nucleolytic enzymes on biofilm formation and planktonic growth of C208. Biofilm formation (■) and planktonic growth (□) of C208 after 5 days. Cultures were grown on PS microplates, in SMM supplemented with nucleases (0.2 mg/ml). Error bars are standard error of the means (n = 6).
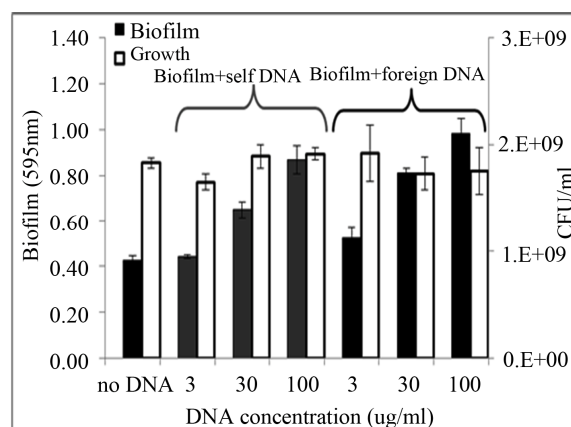

Figure 5. Effect of added DNA on initial biofilm formation and planktonic growth of C208. Biofilm formation (■) and planktonic growth (□) of C208 with DNA added to the SMM growth medium at time of seeding. (Foreign DNA—Salmon sperm DNA). Error bars are standard error of the means (n = 3).
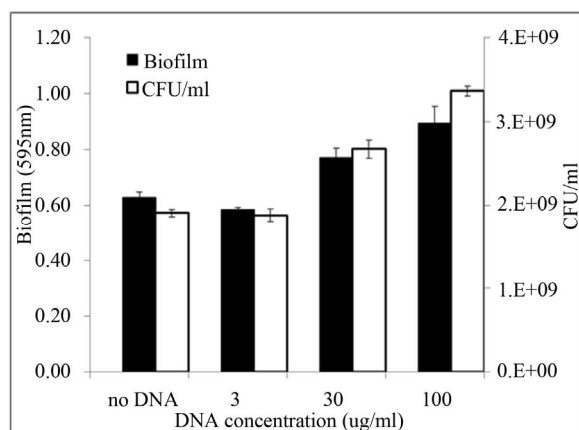
Figure 6. Effect of added DNA on mature biofilm and planktonic growth of C208. Biofilm formation and planktonic growth of C208 with DNA added to the SMM growth medium after 2 days of growth. Error bars are standard error of the means (n = 3).

To find out whether the size of the DNA molecule is significant for supporting biofilm formation we added short DNA fragments obtained from C208 DNA, as detailed in the Materials and Methods. Our results showed that biofilm development was similar to that of biofilms grown in the absence of additional DNA (Data not shown).

Next, we sought to evaluate whether the DNA-driven enhancement of biofilm formation was apparent only during the initial steps of biofilm establishment (initial cell attachment) or whether the presence of DNA was also required during the subsequent early phases of biofilm development. C208 DNA was used to pre-coat the wells of the PS plates (verified by propidium iodide staining), and the bacteria were incubated in DNA-free medium. The similarity in biofilm masses of the coated well and the uncoated control (**Figure 7**) Indicates that the observed DNA effect has no bearing on initial cell adherence.

To further understand the contribution of eDNA in biofilm formation, we tried to eliminate the influence of DNase by adding excess DNA to the same growth medium (**Figure 8**). However, the addition of even large amounts of DNA did not prevent the effect of the DNase, and biofilm formation remained low.

4. Discussion

Biofilms are highly structured communities of microorganisms attached to a surface. It has been proposed that all bacterial biofilms have a number of functionally conserved components in common, including the production of an extracellular polysaccharide matrix, extracellular DNA and proteins.

The present study shows that DNA contributes to the development of the biofilm of *Rhodococcus ruber* C208.
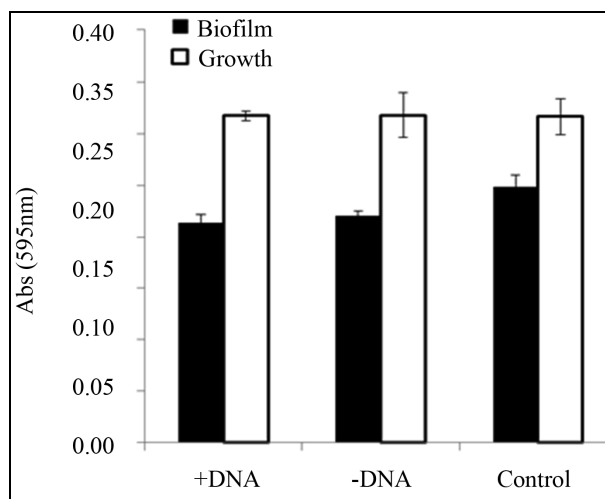

Figure 7. Biofilm formation and planktonic growth of C208 with pre-coating or w/o (control) of genomic DNA. Prior to biofilm inoculation, 3 ng/µl of C208 DNA were incubated in the wells for 20 h at 4°C (modified from [34]). Error bars are standard error of the means (n = 3).
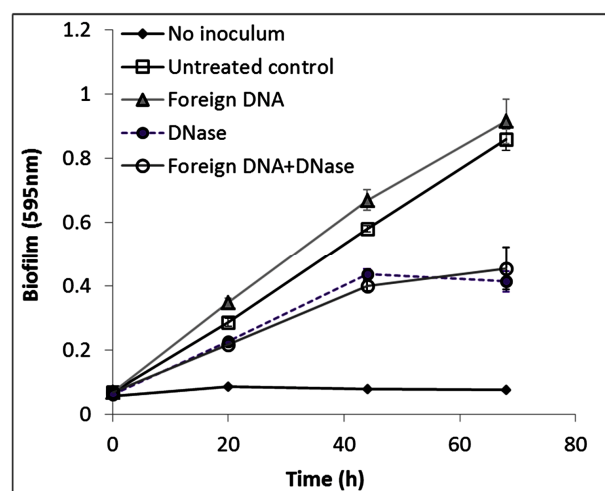

Figure 8. Biofilm formation of C208 during 3 days in SMM supplemented with DNase or DNA or both, compared to untreated control. Foreign DNA = salmon DNA. Error bars are standard error of the means (n = 3).

Our findings confirm that extracellular DNA (eDNA) is an important structural component of C208 biofilm. Exposure of the biofilm to DNase I resulted in eDNA degradation and a subsequent reduction in biofilm formation. These results are supported by studies in other species where DNA was reported to be an essential component of the extracellular polymeric substances [24,27,39,40]. Whitchurch *et al.* [24] and Petersen *et al.* [41] have described eDNA as one of a major structural components of the *Pseudomonas aeruginosa* and *Streptococcus mutans* biofilm matrix, respectively. In *P. aeruginosa* the release of the eDNA macromolecules by the sessile bacteria induced the formation of strengthening linkages between

the cells within the biofilm [42]. According to Petersen *et al.* [41], in *S. mutans* biofilm, the presence of eDNA is associated with the export of a large quantity of competence-signaling peptides (CSP) to the medium.

Mulcahy *et al.* [26] and Guiton *et al.* [27] claimed that eDNA plays a major role in biofilm development also by protecting against antimicrobial agents in biofilms of the Gram-negative *P. aeruginosa* and the Gram-positive *Enterococcus faecalis*, respectively. However, the exact role of these extracellular molecules in the gram-positive and gram-negative biofilm development process is still under intensive research [28,42].

This structural role in C208 biofilm is further reinforced by the effect obtained when the bacteria were supplemented with DNA (either endogenous or foreign), which enhanced biofilm formation. It should be noted that the inhibitory effect of DNase I and enhancing effect of additional DNA occurred mainly during the early stage of biofilm development, while during maturation the effect was less obvious.

Incubation of RNase with cultures of C208 demonstrated a decrease similar to that obtained with DNase. Existence of extracellular RNA has been reported by Nishimura *et al.* [43] in the marine bacterium *Rhodovulum sulfidophilum*. However, the implication that this extracellular RNA is a part of the biofilm is a novel finding. Since significant size differences exist between the DNA and RNA molecules it is reasonable to assume that the similar effect that they exert on the biofilm is not structure-based. One may hypothesize that the mechanisms by which nucleic acids participate in the biofilm differ, such that DNA functions mainly as a structural component whereas RNA may play a more regulatory role, but this should be further explored. Interestingly, Izano *et al.* [44] showed that in *Staphylococcus aureus*, eDNA molecule size is important to its function within the biofilm. In agreement with that, the presence of short fragments of DNA (ca. 300 and 500 bp) in C208 cultures did not enhance biofilm formation and biofilm development was similar to that of biofilms grown in the absence of DNA.

We also found that DNA is not involved in the initial attachment stage since pre-treatment of the plastic substrate with DNA did not enhance biofilm formation. Similar results were obtained with the Gram-positive bacteria *Enterococcus faecalis* [27] and *Streptococcus pneumoniae* [34]. On the other hand, in other studies eDNA promoted biofilm attachment [23,45] .

Numerous studies indicate that some bacteria produce substantial quantities of extracellular DNA (eDNA) through mechanisms that are thought to be independent of natural cell death. In some cases, eDNA secretion may be mediated by the positive regulation of proteases, which contribute to biofilm formation via the regulation

of active cellular autolysis and DNA release [27,29,30]. Other mechanisms, especially in Gram-positive bacteria, involve murein hydrolases, which were reported to be involved in biofilm formation, mediating bacterial lysis in *Enterococcus faecalis* and *Staphylococcus epidermidis* [23,27,29,30,46,47]. Other reports demonstrate them as adhesins that contribute to bacterial attachment to polymeric surfaces and biofilm formation via the release of eDNA [23,48,49]. Elucidation of the role of eDNA in biofilm formation could be advanced by tracking its source. We observed sequence discrepancies between eDNA and cellular DNA in C208 in the RAPD-PCR analysis, a finding that was also confirmed by Nishimura *et al.* [43], Steinberger & Holden [50] and Allesen-Holm *et al.* [37]. These latter studies, however, did not distinguish between the two fractions of eDNA ("supernatant" and "biofilm"). The different band patterns obtained between the supernatant and the biofilm fractions may indicate that only certain DNA molecules are utilized as part of the EPS matrix. For instance, many *Rhodococcus* strains bear plasmids that may be favorably secreted and serve as eDNA. More evidence is accumulating on the proposed role of those plasmids in gene transfer and recombination [51]. In *Rhodococcus* sp. strain RHA1, the biphenyl/PCB degradative (*bph*) genes are distributed around the genome including the linear plasmids [52]. Sequencing of pRHL3—the 330 kb linear plasmid of RHA1—has shown that it is a typical actinomycete invertron, with more than 20% of its putative genes in catabolic clusters and four regions acquired by recombination. In strain *R. erythropolis* BD2 out of 99 putative genes, 23 have catabolic functions and 32 have possible transposition functions. The presence of both the isopropyl benzene degradation (*ipb*) and *bph* operons indicates that they were probably acquired via transposition and linear plasmid transfer. Other large linear plasmids of rhodococci encode genes for the catabolism of trichloroethene, naphthalene, toluene, alkylbenzene, biphenyl and chloroaromatic compounds. A plausible explanation for this is that a series of recombinations contributed to the possession of a large number of homologous genes scattered around a large genome comprised of linear plasmids and the chromosome [51]. This series of recombination events could enable the dispersal of newly acquired DNA in bacteria without requiring mobile genetic elements, as suggested by de Vries & Wackernagel [53] for *Acinetobacter*.

5. Conclusion

The findings indicate that eDNA is an important structural component involved in the biofilm formation of C208 to date, but more research is needed to fully elucidate its role.

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Appendix

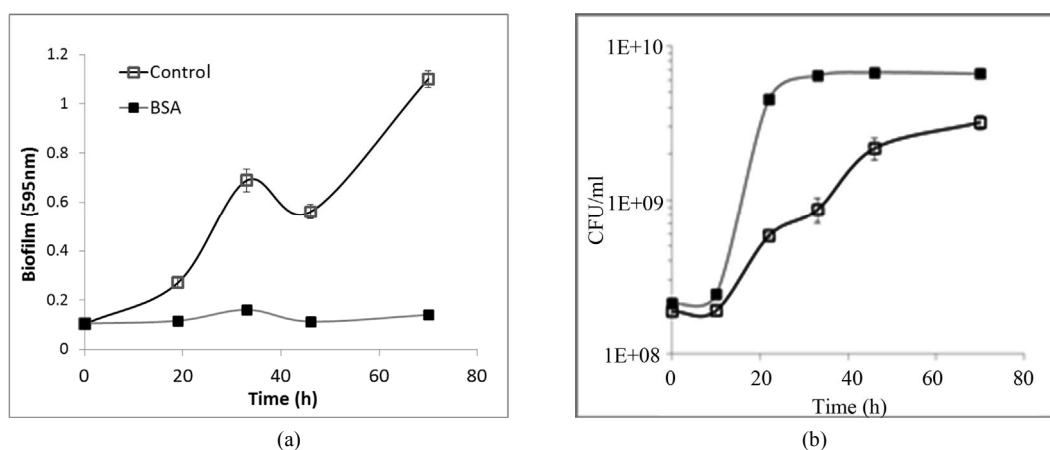(a)   (b)

Figure S1. Effect of non-nucleolytic protein on biofilm formation and planktonic growth of C208. Biofilm formation (a) and planktonic growth (b) of C208 over the course of three days. Cultures were grown on PS microplates in SMM with or without (*i.e.* control) supplementation of bovine serum albumin (BSA) (0.2 mg/ml). Error bars are standard error of the means (n = 6).