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# Characterization of Hydrogen Peroxide-Induced DNA Release by *Streptococcus sanguinis* and *Streptococcus gordonii*[▽]

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**Extracellular DNA (eDNA) is produced by several bacterial species and appears to contribute to biofilm development and cell-cell adhesion. We present data showing that the oral commensals *Streptococcus sanguinis* and *Streptococcus gordonii* release DNA in a process induced by pyruvate oxidase-dependent production of hydrogen peroxide ($H_2O_2$). Surprisingly, *S. sanguinis* and *S. gordonii* cell integrity appears unaffected by conditions that cause autolysis in other eDNA-producing bacteria. Exogenous $H_2O_2$ causes release of DNA from *S. sanguinis* and *S. gordonii* but does not result in obvious lysis of cells. Under DNA-releasing conditions, cell walls appear functionally intact and ribosomes are retained over time. During DNA release, intracellular RNA and ATP are not coreleased. Hence, the release mechanism appears to be highly specific for DNA. Release of DNA without detectable autolysis is suggested to be an adaptation to the competitive oral biofilm environment, where autolysis could create open spaces for competitors to invade. Since eDNA promotes cell-to-cell adhesion, release appears to support oral biofilm formation and facilitates exchange of genetic material among competent strains.**

The release of bacterial DNA into the environment is of recent interest since this polymer is now recognized to stabilize cell-to-cell adherence and biofilm architecture (1, 35, 37). Treatment of extracellular DNA (eDNA) with DNase results in reduced intercellular stickiness, consistent with an adhesive function for eDNA. Furthermore, eDNA from *Neisseria meningitis* appears to have sufficient structural integrity to transform competent strains (11), indicating chromosomal origin. Since the abundance of eDNA is influenced by growth conditions, DNA release can also be regulated (40).

DNA release is typically a consequence of cell lysis. Linked to DNA release, genetic transformation is the natural ability of competent bacterial species to take up DNA from the environment (13, 34, 42). During competence development, *Streptococcus pneumoniae* DNA is released by lysis of a subpopulation of cells (30, 42). Cell lysis and DNA release are controlled in a cell density-dependent signal transduction process. The *S. pneumoniae comX* regulon, carrying late competence genes, also includes the murein hydrolase genes *lytA* and *cbpD* (19, 42). Murein hydrolases digest structural components of the peptidoglycan, contributing to remodeling, recycling, and daughter cell separation. Furthermore, murein hydrolases trigger autolytic cell wall digestion, leading to release of DNA and other cellular content into the environment (36). The autolysis of bacterial cells as part of a regulated death program seems to be an important source for eDNA in diverse species, including *Staphylococcus aureus* (4, 36, 37), *Staphylococcus epidermidis* (35), *Enterococcus faecalis* (44), and *Pseudomonas aeruginosa* (1). In these species, the eDNA contributes to biofilm formation as a component of the extracellular biofilm matrix (35, 37, 44).

Unlike for cell lysis-dependent release, the oral streptococci appear to induce eDNA release by a novel mechanism. In dual-species cultures, the oral commensals *Streptococcus sanguinis* and *Streptococcus gordonii* release eDNA in a manner dependent on pyruvate oxidase (Pox) generation of hydrogen peroxide ($H_2O_2$) under the control of ambient oxygen (23). In this report, we now provide direct evidence of selective $H_2O_2$-induced eDNA release by these oral commensal streptococci.

## MATERIALS AND METHODS

**Bacterial strains and media.** *S. sanguinis* SK36 (48) and *S. gordonii* DL1 (32), and their isogenic Pox$^-$ mutants JKH1 and JKH2 (23), were routinely grown anaerobically (90% $N_2$-5% $CO_2$-5% $H_2$) or aerobically (5% $CO_2$) at 37°C in brain heart infusion broth (BHI; Difco, Sparks, MD) or on BHI agar plates. When required, cultures of JKH1 and JKH2 were supplemented to 500 μg ml$^{-1}$ spectinomycin.

**eDNA and eRNA quantification.** To determine the eDNA and extracellular RNA (eRNA) release kinetics during growth, *S. sanguinis*, *S. gordonii*, and their isogenic Pox$^-$ mutants were grown overnight in BHI (mutants supplemented with 500 μg spectinomycin). The overnight cultures were washed twice with fresh BHI medium to remove contaminating extracellular nucleic acids, diluted to an absorbance at 600 nm ($A_{600}$) of approximately 0.05 in 20 ml BHI and incubated aerobically on a rocking platform for maximal $H_2O_2$ production (23). Under these conditions, neither *S. sanguinis* nor *S. gordonii* forms a biofilm due to shear forces generated by rocking. At the indicated times, cells were removed by centrifugation for 2 min at 16,000 × *g* at 4°C. Supernatants (1 ml) were transferred to new tubes containing 0.5 ml TE buffer (Tris-EDTA, pH 7.8 to 8.2) saturated with premixed phenol-chloroform–isoamyl alcohol (25:24:1) (saturated phenol at 25°C, pH of 6.5 to 6.9), and the mixture was vortexed for 30 s. To precipitate DNA, the mixture was centrifuged at 4°C for 5 min at 16,000 × *g*. The aqueous phase (0.8 ml) was removed and mixed with 80 μl of 3 M sodium acetate, pH 5.2, and 500 μl of 100% 2-propanol. The mixture was then centrifuged for 10 min at 16,000 × *g*, the supernatant was decanted, and the precipitated sample was air dried and suspended in 25 μl of deionized nuclease-free $H_2O$ (Fisher Scientific; Fair Lawn, NJ). Total DNA from 1 ml of cell suspension was extracted by mechanical disruption of cells as described earlier (50). DNA was precipitated in the same way as described for eDNA. To visualize nucleic

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TABLE 1. Primers used in this study

| Primer | Function[a] | Sequence (5′ to 3′) | Source or reference[b] |
|---|---|---|---|
| 16S strep F | DNA release quantification | AAGCAACGCGAAGAACCTTA | 23 |
| 16S strep R | DNA release quantification | GTCTCGCTAGAGTGCCCAAC | 23 |
| EUB 338 | Fluorescent FISH probe | GCTGCCTCCCGTAGGAGT | 2 |
| Ss gyrB F | | ATGGAATGGAGCAGGTCAAG | |
| Ss gyrB R | | TTCAACCGTAATGTCGTCCA | |
| Ss ccpA F | | TGGCGACAGTCAGTCGAGTA | |
| Ss ccpA R | | AGAACTTCGCGTTCTTCCAA | |
| Ss clpX F | | TGGGAAGAACCAAGATGAGG | |
| Ss clpX R | | AGCATCCTCGTCAAATTCAAG | |
| Ss ppsA F | | ACTTCAGCGGTGTCTGCTTT | |
| Ss ppsA R | | CCGGCTTGTACCAAGGAATA | |
| Ss LDH F | | TTGCTTTGGCTCGTATCACA | |
| Ss LDH R | | TACGATACCGTGAGCACCAA | |
| Sg ccpA F | | TGAAGCAGGGGTTTCTATGG | |
| Sg ccpA R | | TTCCAACTCTTCCTTGTGCAT | |
| Sg gyrA F | | CCAAACCTTTTGGTCAATGG | |
| Sg gyrA R | | CCCAGGCAAAACTTCCATAA | |
| Sg LDH F | | GCTCTTGGCTAGTGGAGGTG | |
| Sg LDH R | | CCGATTTCCAGCCTTCTACA | |
| Sg ppsA F | | CAAGCGACCTTGCTCTCTCT | |
| Sg ppsA R | | CCCTTGGGATTAGCTGTGAA | |
| Sg clpX F | | CCGTGAAGAATTAGCCGAAG | |
| Sg clpX R | | TCCATCATGGTTTCTTCGAT | |
| Sg sspA 1 | | CTAAAAACGATCCTGAACTTGGTAAATAC | |
| Sg sspA 2 | | CCTAAAACTCCTAGTAAAGCCGCAAGAC | |
| Ss 0243 1 | | CTTGCCTAAAATATCAGAAACATAGC | |
| Ss 0243 2 | | CCTGCAAGTCCGGTCATGACAAGTC | |

[a] The function is eDNA characterization unless otherwise noted.
[b] The source is this work unless otherwise noted.

acids, 1-μl samples were electrophoresed on 1% agarose gels and stained with ethidium bromide (1 μg/ml).

The relative quantity of chromosomal DNA (chDNA) was determined using a standard curve of dilutions (1:1, 1:5, 1:25, 1:125, and 1:625), using universal primers for the 16S rRNA genes (16S strep F and 16S strep R) (23). *S. gordonii* and *S. sanguinis* each have four chromosomal copies of the 16S rRNA gene. DNA from the standard dilutions and samples from *S. sanguinis* and *S. gordonii* were amplified by real-time PCR as described previously (23). The quantity of DNA was calculated relative to the cycle threshold values of the DNA dilutions. Real-time PCR was performed using FullVelocity SYBR green quantitative PCR master mix (Stratagene) in accordance with the manufacturer's instructions. The concentration of RNA was determined using a Quant-iT RNA assay kit (Invitrogen; Carlsbad, CA), which is based on RNA-specific binding by a fluorescent dye (5), and read using a Qubit fluorometer (Invitrogen).

**ATP determinations.** At the indicated times, culture supernatants (1 ml) were removed and cells were cleared by centrifugation as described above. The ATP concentration was measured with a luciferase-based ATP determination kit (Invitrogen) in accordance with the manufacturer's instructions. Luciferase activity was measured using an Orion microplate luminometer (Berthold Detection Systems, Pforzheim, Germany).

**Autolysis assay.** As described previously (35), cells from overnight static cultures in BHI (10 ml) were grown to mid-log phase, harvested by centrifugation, and washed twice with chilled water. After resuspension in autolysis buffer (0.05 M Tris-HCl, pH 7.2, 0.05% [vol/vol] Triton X-100), cells were incubated at 37°C in 5% $CO_2$ as static cultures. Cells were resuspended and absorption ($A_{600}$) was measured at the indicated times. To test the effect of $H_2O_2$ on cell lysis, BHI cultures were harvested from logarithmic phase ($A_{600}$, ~0.7), washed with phosphate-buffered saline (PBS) (pH 7.4), and resuspended in PBS to give an $A_{600}$ of ~0.3 in PBS supplemented with the indicated concentrations of $H_2O_2$. Absorption ($A_{600}$) was measured using a Bioscreen C analyzer, version 2.4 (Oy Growth Curves AB, Ltd., Finland). Programmed to shake the samples periodically to simulate conditions used to determine eDNA concentrations, this analyzer kinetically measured the turbidity in multiple cultures simultaneously.

**Ampicillin treatment.** Overnight cultures were washed twice in BHI, inoculated in fresh BHI, and grown at 37°C in 5% $CO_2$ for approximately one generation on a rocking platform for maximal $H_2O_2$ production. The cultures were split, ampicillin was added to give a final concentration of 8 μg/ml, and incubation continued. Samples were removed at the indicated times for DNA, RNA, and ATP quantification as described above.

**PCR.** PCR was performed with a Mastercycler thermocycler (Eppendorf, Westbury, NY) in accordance with the manufacturer's protocol. GoTaq-DNA polymerase was obtained from Promega. Primer sequences (Table 1) were designed using sequence data obtained from the Los Alamos National Laboratory Oral Pathogens Sequence Database (http://www.oralgen.lanl.gov/) and synthesized by Integrated DNA Technologies (Coralville, IA). Chromosomal DNA (chDNA) was isolated by following standard procedures, using lysozyme digestion of cells, chloroform-phenol extraction, and isopropanol precipitation, essentially as described previously (28). PCR products were run on a 1% agarose gel and stained with ethidium bromide.

Semiquantitative PCR was performed with precipitated eDNA samples by using the same protocol. Briefly, 0.5 μl of eDNA (suspended in 25 μl of deionized nuclease-free $H_2O$) was added to 24.5 μl PCR master mix. PCR was run for 20, 23, and 26 cycles with the 16S rRNA-specific oligonucleotides, and the products were resolved on an agarose gel as described above.

**DNase and RNase.** For RNase and DNase treatment of nucleic acids, samples were treated for 1 h at 37°C with either 5 μl of 0.4-mg/ml RNase A (Sigma-Aldrich) or 5 units of RQ1 DNase (Promega) in 25 μl (final volume).

**Cell aggregation.** For cell aggregation, cells from overnight cultures were washed twice in BHI and resuspended in 2 ml BHI (5-ml tubes) to give an $A_{600}$ of 0.05. Cells were incubated at 37°C for 1 h on a rocking platform, $A_{600}$ was measured, and RQ1 DNase (15 units) (Promega) was added. Cells were then incubated for 1 h at 37°C, and the final $A_{600}$ was recorded.

**Fluorescence microscopy and fluorescent labeling of bacteria.** Cells were grown in BHI on a rocking platform at 37°C to late logarithmic phase ($A_{600}$, ~0.6 to 0.7). To differentiate between bacteria with compromised or intact membranes, a Live/Dead *Bac*Light bacterial viability kit was used in accordance with the manufacturer's recommendations (Invitrogen). The dyes were added directly to the growth medium and incubated for 10 min prior to microscopy. The kit contains two different dyes, propidium iodide (PI) conferring red fluorescence and SYTO9 conferring green fluorescence. PI diffuses only into cells with compromised membranes and binds to DNA, whereas intact cells can be stained only with SYTO9 (8).

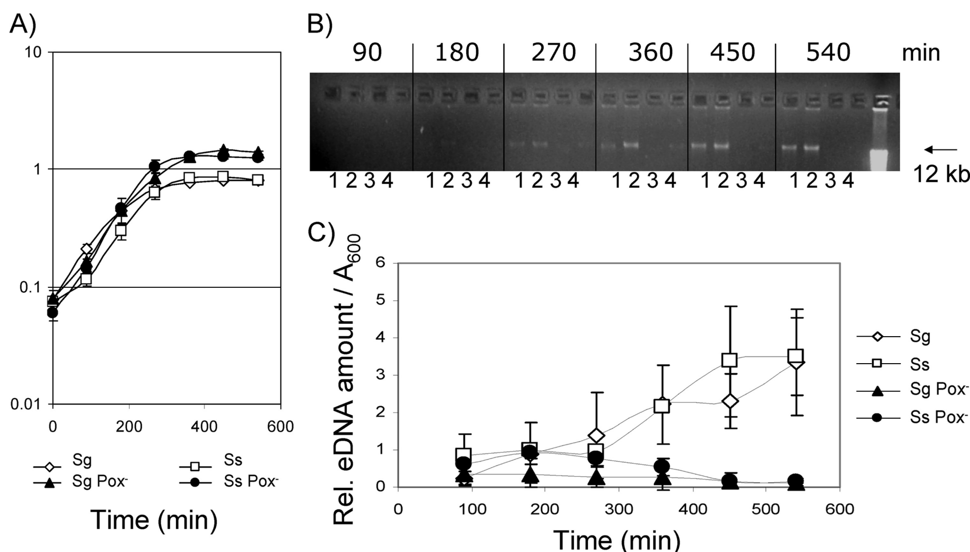


FIG. 1. Growth curves and eDNA release kinetics for *S. sanguinis* (Ss) and *S. gordonii* (Sg) wild-type strains and Pox$^-$ mutants. (A) Growth curves in BHI medium. (B) Agarose gel electrophoresis (1%) of high-molecular-weight DNA stained with ethidium bromide (1 μg/ml) as described in Materials and Methods. The photograph is representative of three independent experiments with similar results. Lanes: 1, *S. gordonii*; 2, *S. sanguinis*; 3, *S. gordonii* Pox$^-$; 4, *S. sanguinis* Pox$^-$. (C) Quantitative real-time PCR of eDNA, using the 16S rRNA genes as an amplification template. The relative quantities of eDNA in the streptococcal supernatants were calculated in comparison to the cycle threshold values of a dilution series of isolated chDNA. The values were adjusted to the $A_{600}$ levels of the bacterial cultures. Data presented are the means ± SD of results from two independent experiments done in duplicate on different days.

Cells with high metabolic activity were fluorescently labeled with CellTracker Green CMFDA in accordance with the manufacturer's instructions (Invitrogen). Cells were incubated for 1 h at 37°C in the presence of 5 μM dye (15) and then examined microscopically.

Fluorescence in situ hybridization (FISH) was performed essentially as described earlier (15). The oligonucleotide probe for 16S rRNA (EUB338) was 5′ labeled with Alexa Fluor 488 *N*-hydroxysuccinimide ester and synthesized by Integrated DNA Technologies (Coralville, IA). To perform FISH, planktonic cells in 40 μl of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl, 20% formamide, 0.01% sodium dodecyl sulfate) containing 100 ng of the oligonucleotide probe were incubated for 4 h at 46°C in a water bath. Cells were washed twice with washing solution (0.18 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate), and total bacteria in the FISH sample were stained with 1 ng DAPI (4′,6-diamidino-2-phenylindole) (Merck, Darmstadt, Germany) for 10 min. Upon binding to DNA, the DAPI fluoresced intensely and cells were observed by microscopy.

Bacterial cell walls were stained by incubating cells for 1 h with 1 μg per ml Alexa Fluor 555-labeled wheat germ agglutinin (WGA; Invitrogen) and washed twice with PBS. For microscopy, 8 μl of cell suspension was applied to a microscope slide, covered with a coverslip, and sealed with clear nail polish to avoid evaporation. Microscopy was performed with a Nikon Eclipse E800 microscope and a 60×/1.40-numerical-aperture or 100×/1.40-numerical-aperture Plan Apo oil lens. Pictures were processed with MetaMorph software (Molecular Devices, Sunnyvale, CA). Contrast and brightness for the entire image were adjusted nonselectively.

**Statistical analysis.** Descriptive statistics, including the mean and standard deviation (SD), were calculated. Statistical analysis of data was performed with QuickCalcs online calculators (http://www.graphpad.com/quickcalcs/index.cfm) using *t* test software to compare the means of results from two groups. Data were considered significantly different if the two-tailed *P* value was ≤0.05.

## RESULTS

**Characterization of eDNA release kinetics of *S. sanguinis* and *S. gordonii*.** *S. sanguinis* and *S. gordonii* wild-type strains and Pox$^-$ mutants showed similar generation times in BHI medium, but the Pox$^-$ mutants yielded higher cell densities in stationary phase (Fig. 1A). To determine the kinetics of release, the relative amount of eDNA in the medium was analyzed using agarose gel electrophoresis (Fig. 1B) and quantified with real-time PCR (Fig. 1C). By 270 min of incubation, the wild-type strains of both species released DNA resolving as a high-molecular-weight band (Fig. 1B, lanes 1 and 2), which appeared to increase in intensity by 540 min. At all times, the Pox$^-$ mutants appeared to release less high-molecular-weight DNA (Fig. 1B, lanes 3 and 4). The kinetics of eDNA presence in the medium was confirmed by quantitative PCR (Fig. 1C). By 540 min (stationary phase), the wild-type strains released about 10-fold more DNA than the Pox$^-$ mutants (Fig. 1C). When normalized for cell density, the wild-type strains showed increased release of eDNA and the Pox$^-$ mutants showed decreased release of eDNA over time (Fig. 1C). These results suggest that *S. sanguinis* and *S. gordonii* release DNA in a Pox-dependent manner during growth.

**Total amount of DNA versus eDNA.** To determine the ratio of eDNA to the total amount of cellular DNA, cells were grown to late logarithmic growth phase and the concentrations of DNA in the supernatant and whole-cell lysates were measured. Total DNA from *S. sanguinis* was 5,121 ± 813 ng/ml and eDNA 953 ± 49 ng/ml (mean ± SD for duplicate samples from two independent experiments). For *S. gordonii*, total DNA was 7,319 ± 804 ng/ml and eDNA 1,012 ± 100 ng/ml, suggesting that eDNA represents about 15 to 20% of the total cellular DNA.

**$H_2O_2$ dependence of eDNA release.** To determine whether eDNA release is stimulated by $H_2O_2$, we added $H_2O_2$-degrading catalase to wild-type cultures of *S. sanguinis* and *S. gordonii* grown under $H_2O_2$-producing conditions as described previously (23). Catalase reduced eDNA detected in overnight cultures more than 10-fold (Fig. 2A), to levels similar to those in

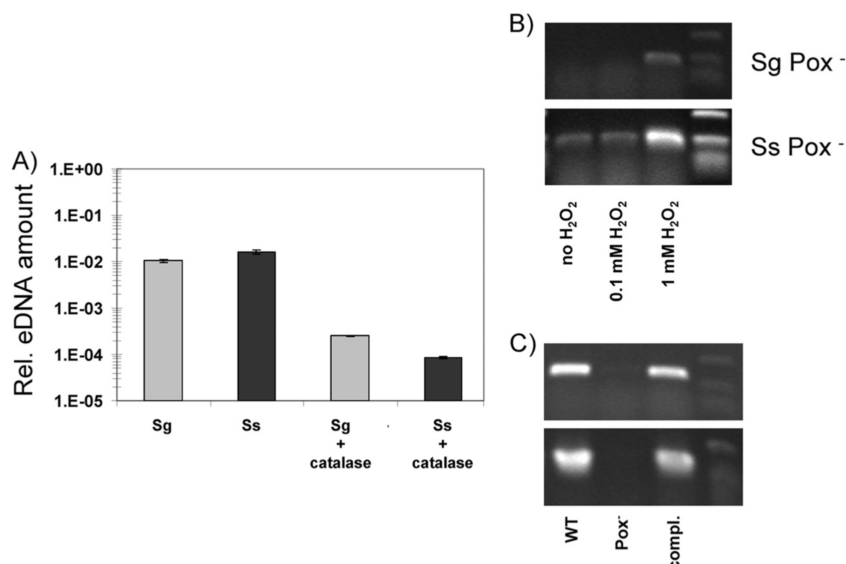


FIG. 2. Influence of catalase and $H_2O_2$ addition on eDNA amounts of *S. sanguinis* (Ss) and *S. gordonii* (Sg) cultures. (A) The relative eDNA amounts in culture supernatants were measured with real-time PCR and standardized to 16S rRNA of isolated chDNA by using different dilutions. Data presented are the means ± SD of results from two independent experiments done on different days. (B) Effect of $H_2O_2$ addition on eDNA release of the $Pox^-$ mutants. Semiquantitative PCR was performed on precipitated eDNA. (C) Complementation of the $Pox^-$ mutants reinstalled eDNA release. Semiquantitative PCR was performed on precipitated eDNA. Pictures are representatives of at least two experiments. WT, wild type.

anaerobic mixed cultures of these bacterial strains (no $H_2O_2$ production) (23). Addition of $H_2O_2$ (final concentration = 1 mM) to mid-logarithmic phase ($A_{600}$, ~0.4) $Pox^-$ mutants increased eDNA (Fig. 2B). Complementing $Pox^-$ mutants rescued eDNA release to wild-type strain levels (Fig. 2C). $H_2O_2$ appeared to be directly involved in DNA release (eDNA) under aerobic conditions.

**Characterization of eDNA.** The genomic structures of eDNA and chDNA were compared. In each DNA sample, selected genes were amplified from different loci on the chromosome. The chDNA genes *ppsA*, *clpX*, *ccpA*, *ldh*, and *gyrA* (*gyrB* for *S. sanguinis*) could be amplified from eDNA from all wild-type strains and $Pox^-$ mutants (Fig. 3A and B). The primers for *clpX* and *ccpA* encompassed a region of 1 kb, whereas the other primers amplified targets of about 0.12 kb, indicating that the wild-type and mutant eDNAs were similar to each other and chDNA. We were also able to amplify larger segments (over 4 kb) from both species (Fig. 3C), suggesting that the genomic sequence is intact over large sections of eDNA.

**Detection of eRNA.** eDNA levels began to increase over baseline at about 270 min of growth and continued to increase during log phase until plateauing at 540 min. To determine if leaky cells released eDNA, the kinetics were compared to those of eRNA. During electrophoresis of eDNA, bands that

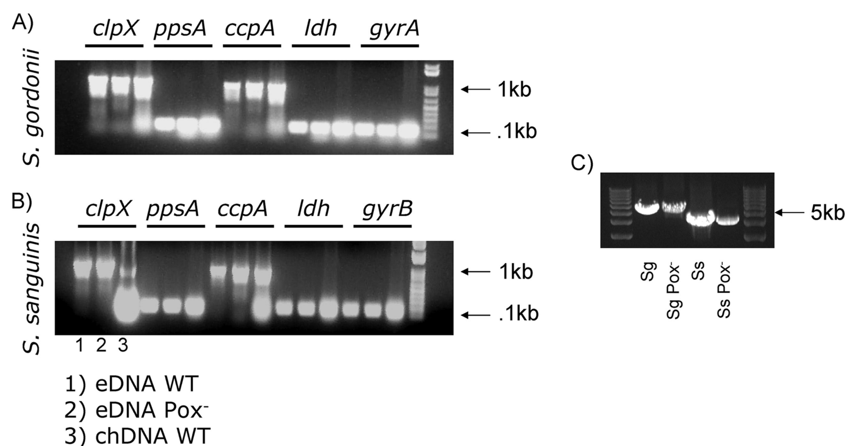


FIG. 3. Characterization of eDNA. eDNA was isopropanol precipitated from wild-type (WT) and $Pox^-$ mutant cell supernatants and compared to purified chDNA. PCR amplification was performed over 32 cycles by using primers targeting genes located on different positions on the chromosome. PCR products were resolved on 1% agarose gels. (A) *S. gordonii* with primers for *clpX*, *ppsA*, *ccpA*, *ldh*, and *gyrA*. (B) *S. sanguinis* with primers for *clpX*, *ppsA*, *ccpA*, *ldh*, and *gyrB*. Lanes: 1, eDNA wild type; 2, eDNA $Pox^-$ mutant; 3, chDNA wild type. (C) PCR amplification of products greater than 4 kb. Sg, *S. gordonii*; Ss, *S. sanguinis*.

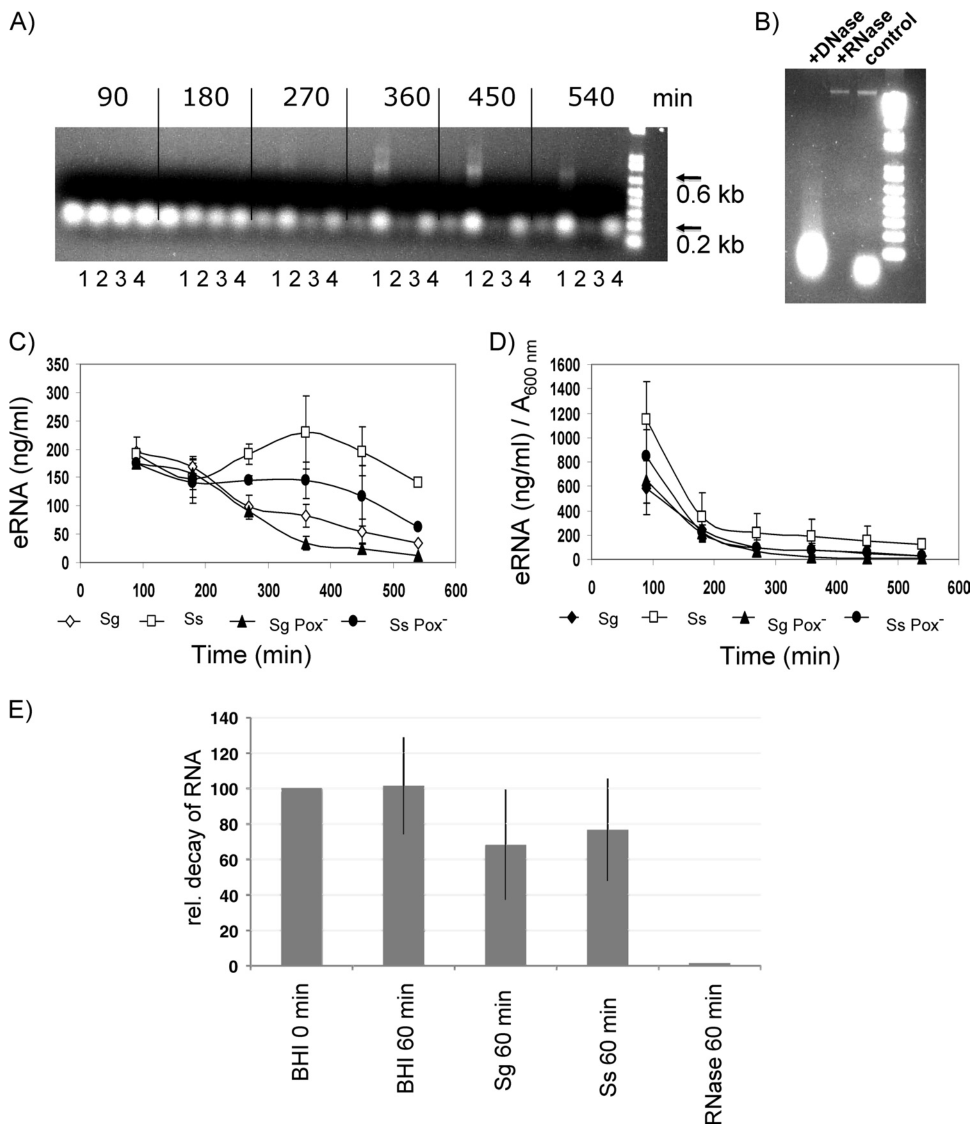


FIG. 4. Characterization of eRNA. (A) Agarose gel electrophoresis (1%) of RNA stained with ethidium bromide (1 μg/ml) as described in Materials and Methods. The photograph is representative of three independent experiments with similar results. Lanes: 1, *S. gordonii*; 2, *S. sanguinis*; 3, *S. gordonii* Pox$^-$; 4, *S. sanguinis* Pox$^-$. (B) Precipitated nucleic acids from *S. gordonii* were digested with DNase RQ1 and RNase A, resolved by agarose gel electrophoresis (1%), and stained with ethidium bromide (1 μg/ml). (C) Quantification of eRNA by use of an RNA-specific fluorescent dye with the Quant-iT RNA assay. Sg, *S. gordonii*; Ss, *S. sanguinis*. (D) Relative amounts of RNA normalized to cell density measured at $A_{600}$. Data presented are the means ± SD of results from three independent experiments done on different days. (E) eRNA degradation. Total RNA from *S. mutans* (2 μg/ml) was added to the cultures. After 60 min of incubation, a 1-ml aliquot was removed. RNA was precipitated and analyzed for decay on an agarose gel by comparing pixel intensities of RNA bands with ImageJ software (http://rsb.info.nih.gov/ij/). Shown are means ± SD of results from at least two independent experiments with BHI, with the 0-min arbitrary level set to 100%.

appeared to correspond to 16S rRNA, 23S rRNA, and low-molecular-weight nucleic acids usually seen during RNA isolation from S. *sanguinis* and *S. gordonii* were detected (Fig. 4A). To verify that RNA is present in the media, precipitated nucleic acids from *S. gordonii* were treated with RNase or DNase and analyzed using agarose gel electrophoresis (Fig. 4B). The low-molecular-weight band disappeared upon treatment with RNase but not DNase (Fig. 4B, lane 2). In contrast, DNase digested the high-molecular-weight band (Fig. 4B, lane 1).

A maximum of 230 ng per ml eRNA was recovered from *S. sanguinis* wild-type supernatant, higher than the level for *S. gordonii* or either Pox$^-$ mutant (Fig. 4C). When normalized to cell density, however, the highest concentration of eRNA was recovered during initial logarithmic growth and then declined for all four strains (Fig. 4D), which contrasted with the release kinetics of eDNA (Fig. 1C). To determine whether RNA is degraded after release, total RNA from *Streptococcus mutans* was isolated and then added to *S. sanguinis* and *S. gordonii* logarithmic-phase cells. At 60 min, the relative amount of *S. mutans* RNA recovered after incubation with *S. sanguinis* and *S. gordonii* was reduced by 30 to 40% more than when recovered from sterile BHI medium (Fig. 4E). Nuclease activity might account for decreasing RNA levels in log phase.

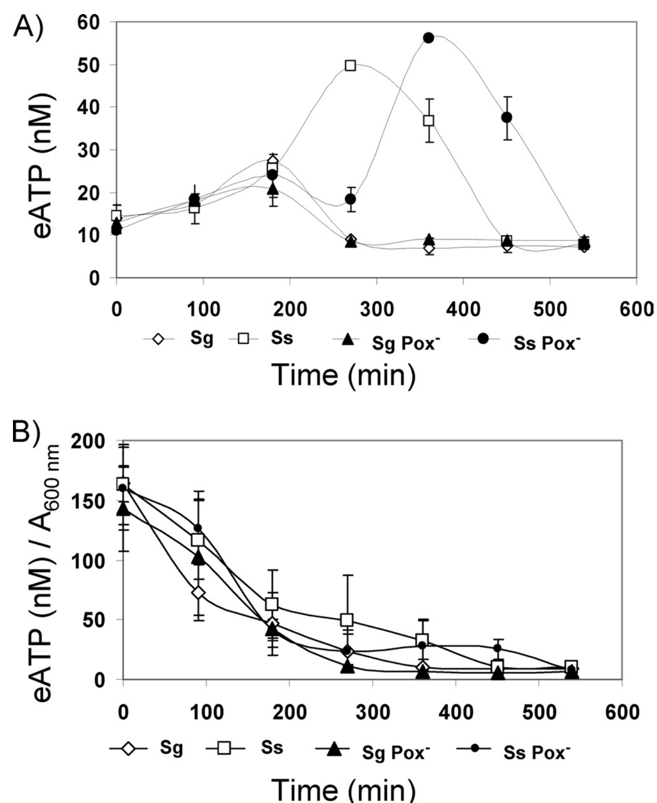


FIG. 5. ATP release from *S. sanguinis* (Ss) and *S. gordonii* (Sg) wild-type strains and Pox$^-$ mutants. (A) Quantification of ATP by use of a luciferase assay as described in Materials and Methods. The concentration relative to an ATP dilution standard curve was determined. (B) Relative ATP amount normalized to cell density measured at 600 nm. Data presented are the means ± SD of results from three independent experiments.

**Release of ATP.** To learn whether eDNA is associated with release of other cellular content, we measured the concentrations of ATP in the supernatants of *S. sanguinis* and *S. gordonii* wild-type strains and Pox$^-$ mutants grown under $H_2O_2$-producing conditions. ATP has been used as indicator of nisin-induced cell lysis (45). The *S. gordonii* wild type and the Pox$^-$ mutant yielded similar amounts of extracellular ATP (eATP), maximizing at 180 min (Fig. 5A). For *S. sanguinis*, the kinetics of eATP appearance differed (Fig. 5A). After 180 min, the *S. sanguinis* wild type and the Pox$^-$ mutant released increasing amounts of ATP to reach maximal concentrations about twice that of *S. gordonii*. Compared to the wild type, the *S. sanguinis* Pox$^-$ mutant yielded a maximum eATP concentration about 60 min later. When normalized to cell densities, all four strains showed similar continuous decreases in eATP concentration over time (Fig. 5B), suggesting that lysis-induced release of ATP did not occur. When added to mid-logarithmic-phase cells, ATP appeared to be relatively stable, and eATP remained undegraded after release from cells (data not shown).

**Cell viability and integrity.** To learn whether the level of eDNA was associated with membrane compromised cells, late-logarithmic-growth-phase cells grown under $H_2O_2$-producing conditions (300 min) ($A_{600}$ for wild-type strains, ~0.9; $A_{600}$ for Pox mutants, ~1.2) were stained with PI and SYTO9 to ascertain cell viability. PI would be excluded from cells with intact membranes. *S. sanguinis* and *S. gordonii* wild-type strains showed higher proportions of nonviable red fluorescent cells than the Pox$^-$ mutants (Fig. 6A). On the basis of enumeration of PI-stained red cells, about 10% of the wild-type cell population was membrane compromised (*S. gordonii*, 9.6% ± 0.29%; *S. sanguinis*, 10.9% ± 0.08%). The Pox$^-$ mutant population, however, contained less than 1% compromised cells (*S. gordonii*, 0.7% ± 0.52%; *S. sanguinis*, 0.95% ± 0.43%). These data appeared to be consistent with the approximately 10-fold difference in eDNA release between wild-type and Pox$^-$ mutant cells in late logarithmic to early stationary phase (Fig. 1C).

To determine the metabolic activity of the PI-stained red fluorescent cells, cells were counterstained using CellTracker Green dye (Fig. 6B). Cells staining with PI showed only faint green fluorescence in comparison to the surrounding cells, indicating weak, residual metabolic activity (Fig. 6B). To determine whether cell wall lysis had occurred, we probed wall integrity by using WGA, which selectively binds to *N*-acetylglucosamine (GlcNAc) of the peptidoglycan. Nonviable and viable *S. sanguinis* (Fig. 6C) and *S. gordonii* (data not shown) cells showed similar levels of cell wall integrity.

If $H_2O_2$ adversely affected cell envelope integrity, we would expect ribosomes to be lost. Wild-type cells were grown under $H_2O_2$-producing conditions, and ribosome content was assessed using FISH with the oligonucleotide probe EUB338 (2) for eubacterial 16S rRNA (15). All cells hybridizing with the 16S rRNA probe (bright green fluorescence) also counterstained with the DNA fluorescent dye DAPI (blue fluorescence), indicating no obvious loss of ribosome content (Fig. 6D).

**Autolysis of *S. sanguinis* and *S. gordonii*.** Although the data suggested that cells maintain wall integrity and ribosomes in the presence of $H_2O_2$, eDNA could be released from an otherwise undetectable portion of autolysing cells. To learn whether cells undergo autolysis during prolonged incubation in BHI, absorption was measured over several days as an indicator of cell density. Over time, there was no obvious loss in absorption (Fig. 7A). Since *E. faecalis* was recently described as autolytic during eDNA generation (44), we monitored *S. sanguinis* and *S. gordonii* under autolytic conditions for other firmicutes and for comparison to *E. faecalis* strains TX5179 (49) and JH2 (18). Initially, $A_{600}$ decreased 10% in all strains, consistent with cell shrinkage after transfer into autolysis buffer (Fig. 7B). After 80 min, the $A_{600}$s of *S. sanguinis* and *S. gordonii* remained constant through overnight incubation (data not shown). In contrast, the two strains of *E. faecalis* showed cell lysis at different rates (Fig. 7B). Since $H_2O_2$ triggers DNA release, we determined whether $H_2O_2$-induced release of DNA is the result of cell lysis. *S. sanguinis* and *S. gordonii* were incubated in the presence of 1 mM and 10 mM $H_2O_2$. No autolysis was detected (Fig. 7C). These data suggest that both streptococcal species do not lyse substantially under conditions known to cause lysis of other firmicutes.

**Antibiotic-induced release of DNA, RNA and ATP.** Ampicillin can inhibit bacterial cell wall synthesis and induce cell leakage and lysis in streptococci (17). *S. sanguinis* and *S. gordonii* wild-type cells were grown under $H_2O_2$-producing conditions with ampicillin and monitored for release of DNA,

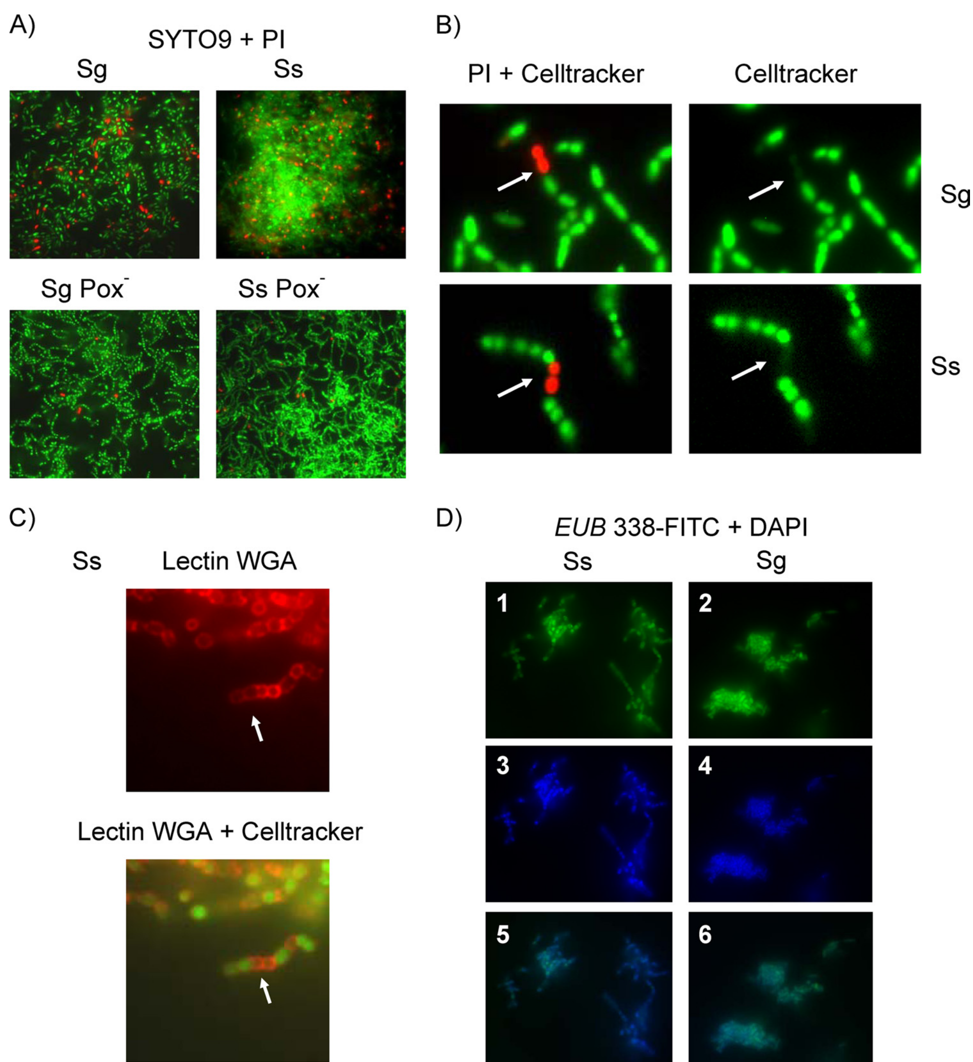


FIG. 6. Cell integrity of *S. sanguinis* (Ss) and *S. gordonii* (Sg). Fluorescence microscopy was performed with a Nikon Eclipse E800 microscope at ×60 or ×100 magnification. Pictures were processed with MetaMorph software (Molecular Devices, Sunnyvale, CA). (A) Fluorescence micrograph of live (green; SYTO9 staining) and membrane-compromised (red; PI staining) cells grown as planktonic cultures. (B) Fluorescence micrograph of metabolically active (green; CellTracker Green) and membrane-compromised (red; PI) cells. (C) Fluorescence micrograph localizing the *N*-acetylglucosamine of the peptidylglycan of the streptococcal cell wall (red; WGA lectin staining) and metabolic activity (green; CellTracker Green). (D) Fluorescence micrograph showing ribosomal (green; EUB338-specific FISH probe; images 1 and 2) and DNA (blue; DAPI staining; images 3 and 4) contents of wild-type strains grown as planktonic cells and an overlay of the green and blue channels (images 5 and 6).

RNA, and ATP. Ampicillin caused immediate growth arrest of the cells (Fig. 8A). When normalized to cell density, eDNA was found to be threefold greater in ampicillin-treated than in untreated *S. sanguinis* cells at 300 min ($P \leq 0.003$) (Fig. 8B). *S. gordonii* eDNA was consistently greater in response to ampicillin, but the difference was not significant. With ampicillin, *S. sanguinis* eRNA increased about threefold ($P \leq 0.003$) (Fig. 8C) and *S. gordonii* eATP was about fivefold higher ($P \leq 0.0001$) (Fig. 8D). *S. sanguinis* did not release significantly more ATP when treated with ampicillin. The releases of DNA, RNA, and ATP were increased compared to the levels for untreated cells, but the increase was not always significant. In response to ampicillin, *S. sanguinis* and *S. gordonii* cell walls lost staining with WGA lectin at 300 min (data not shown). Hence, DNA, RNA, and ATP are released when lysis is induced by ampicillin, and all accumulate in the medium.

**eDNA promotes *S. sanguinis* aggregation.** When grown under aerobic, $H_2O_2$-producing conditions, the *S. sanguinis* wild type forms visible aggregates, which precipitate to the bottom of the tube over time (Fig. 9A). Under the same conditions, the Pox$^-$ mutant did not aggregate or precipitate (Fig. 9A). To determine whether aggregation is mediated by eDNA, DNase was added to cultures growing under aerobic, $H_2O_2$-producing conditions. The DNase-treated cells showed reduced aggregation (Fig. 9B). *S. sanguinis* aggregation was also induced by $H_2O_2$ in a dose-dependent manner (Fig. 9C). Under the same conditions, *S. gordonii* did not form aggregates.

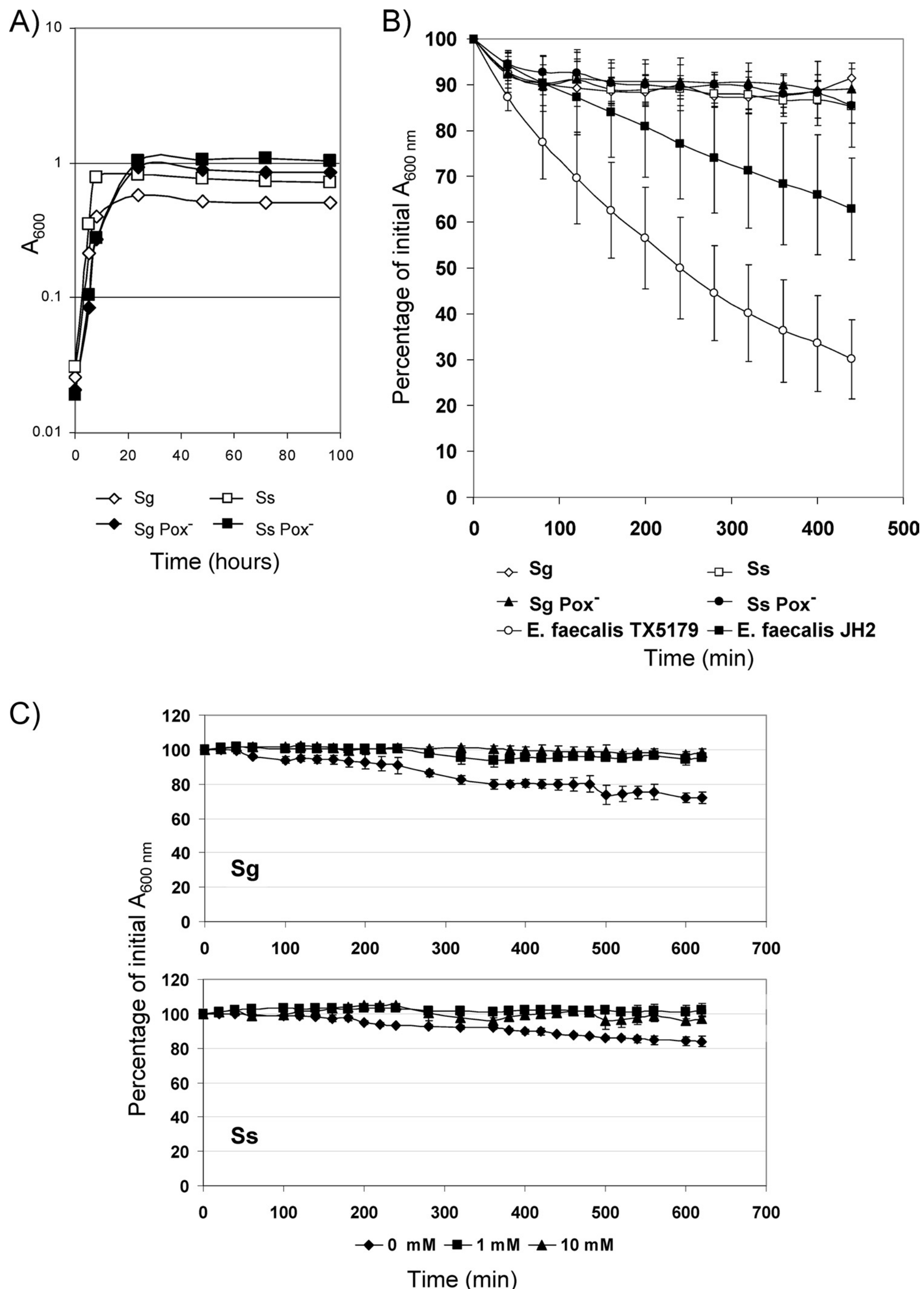


FIG. 7. Autolysis of *S. sanguinis* (Ss) and *S. gordonii* (Sg) wild-type strains and Pox⁻ mutants. (A) Cell density of cultures during prolonged incubation in BHI. (B) Cells were resuspended in specific autolysis buffer. Autolysis was estimated as the decrease in absorption ($A_{600}$) was recorded over time. Autolysis-positive *E. faecalis* strains were used as positive controls. Data presented are the means ± SD of results from three (streptococci) and two (enterococci) independent experiments done on different days. (C) Autolysis assay in the presence of 1 mM and 10 mM $H_2O_2$.

## DISCUSSION

This is the first report showing that eDNA is produced in response to the streptococcal virulence factor $H_2O_2$. The $H_2O_2$-dependent release of DNA without autolysis may reflect a special adaptation to the biofilm environment of *S. sanguinis* and *S. gordonii*, where autolysis could create open spaces where competitors would invade. During early oral biofilm formation, the concentration of oxygen is sufficient for *S. sanguinis* and *S. gordonii* to produce $H_2O_2$ (29). The production of $H_2O_2$ would help both species to compete efficiently with other hydrogen peroxide-sensitive early colonizers. Since eDNA promotes cell-to-cell adhesion, release of DNA appears to support oral biofilm formation and may facilitate exchange of genetic material among competent strains. In addition, the release of

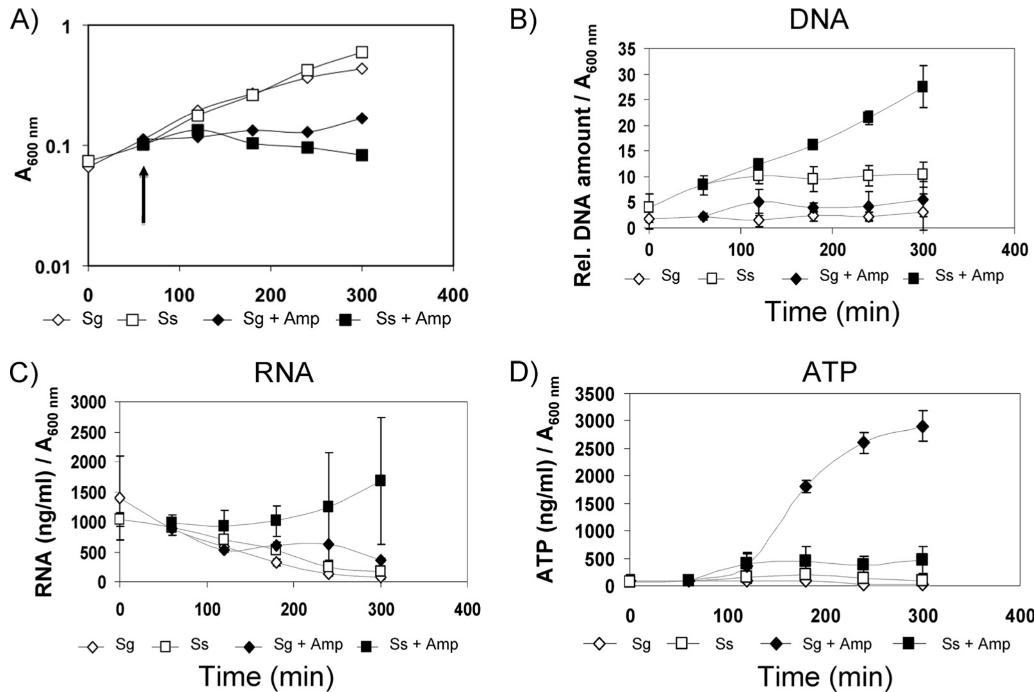


FIG. 8. Ampicillin-induced lysis of *S. sanguinis* (Ss) and *S. gordonii* (Sg). (A) Representative growth curve demonstrating the effect of ampicillin addition (arrow depicts time of addition). (B to D) Release of DNA, (B) RNA (C), and ATP (D) into the medium after induced lysis with ampicillin (8 μg/ml) was measured over time. The amounts of DNA, RNA, and ATP were determined as described for the other experiments and normalized to the cell density at $A_{600}$. Data presented are the means ± SD of results from three independent experiments done on different days.

DNA might promote *S. sanguinis* and *S. gordonii* adhesion to the tooth surface. For *S. sanguinis*, eDNA appeared to contribute to intercellular adhesion since DNase treatment reduces cell aggregation in aerobic cultures. eDNA contributes to intercellular interactions in other species since DNase treatment also prevents clumping of *P. aeruginosa* cells (1) and cell-to-cell adhesion of *P. aeruginosa* and *Rheinheimera baltica* (1, 6).

As reported previously for other species (12, 13, 24, 41), *S. sanguinis* and *S. gordonii* eDNA is characterized as chDNA. With the use of PCR to show the integrity of the eDNA, streptococcal chromosomal regions up to 5 kb could be amplified. Moreover, eDNA and high-molecular-weight DNA (>12 kb) showed similar mobilities on agarose gels, and no obvious degradation was seen. Hence, streptococcal eDNA is strongly suggested to be largely intact chDNA.

For most species investigated, eDNA appears upon autolysis of a subpopulation of cells, which requires the activity of

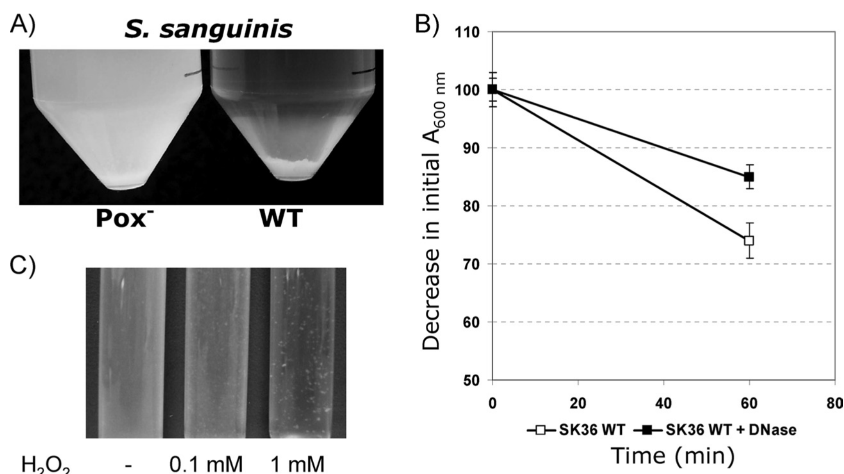


FIG. 9. Effect of aerobic growth and DNase treatment on the aggregation of the *S. sanguinis* wild type and the $Pox^-$ mutant. (A) Overnight cultures of the wild type and the $Pox^-$ mutant obtained after further static incubation has resulted in aggregation of the wild type but not the $Pox^-$ mutant strain. (B) Aggregation inhibition by DNase treatment of wild-type cells during anaerobic growth. Data presented are the means ± SD of results from two independent experiments done on different days. (C) $H_2O_2$-induced aggregation of the *S. sanguinis* $Pox^-$ mutant.

murein hydrolases (30, 36, 37, 42). Both *S. gordonii* and *S. sanguinis* encode putative *N*-acetylmuramidases (46), required for cell growth during cycles of controlled lysis, remodeling, and synthesis of new peptidoglycan building blocks (46). To what extent these murein hydrolases contribute to the release of DNA in oral streptococci is currently under investigation. Interestingly, earlier reports failed to detect peptidoglycan hydrolase activity in *S. sanguinis* (16, 17).

$H_2O_2$ addition did not result in measurable autolysis, although $H_2O_2$ can trigger the release of eDNA. Hence, there exists an eDNA-releasing mechanism for *S. gordonii* and *S. sanguinis* that does not appear to involve the generalized autolysis reported for other species, like *S. pneumoniae* (38). Consistent with this conclusion, DNA release appeared independent of release of other intracellular components, like ATP. ATP has been used to monitor nisin-induced lysis of lactobacilli (45). Interestingly, RNA was detected in the supernatant of *S. gordonii* and *S. sanguinis* during initial growth, but the concentration then declined over time. The decline might be caused by eRNA nuclease activity, since growing cells degraded added RNA. It is likely that *S. sanguinis* and *S. gordonii* release RNA only during initial growth. The release kinetics of RNA, thus, differed from those of eDNA. DNA, RNA, and ATP, however, did leak out of cells treated with ampicillin, which is known to cause cell wall damage (17). Hence, *S. sanguinis* and *S. gordonii* wild-type strains are strongly suggested to selectively release DNA in an $H_2O_2$-dependent manner.

Hydrogen peroxide production by *S. sanguinis* and *S. gordonii* promotes release of eDNA under aerobic conditions, as we have reported (23). Consistent with these findings, Pox-mediated production of $H_2O_2$ occurs only in aerobically grown cells (9, 10). As we now show, isogenic $Pox^-$ mutants produce significantly less eDNA during growth. Since catalase reduces eDNA from wild-type cells to $Pox^-$ mutant levels and $H_2O_2$ addition to $Pox^-$ mutants triggers eDNA release, $H_2O_2$ is the principal active agent. In the presence of catalase, wild-type cells showed significantly reduced but detectable eDNA, suggesting that *S. gordonii* and *S. sanguinis* may possess other $H_2O_2$- and Pox-independent eDNA release mechanisms.

*S. sanguinis* and *S. gordonii* are pioneer colonizers of oral biofilms (20), and about 80% of early colonizers are oral streptococci (31, 39). The oral streptococci share similar metabolic requirements, which in the nutritionally challenging oral environment promote fierce competition for space and nutrients. To compete with other species, *S. sanguinis* and *S. gordonii* produce hydrogen peroxide to antagonize peroxide-sensitive species, as we have reported (22). While *S. sanguinis* and *S. gordonii* produce $H_2O_2$, other oral streptococcal species are the sensitive targets. Although *S. sanguinis* and *S. gordonii* appear to resist autolysis in the presence of $H_2O_2$, we speculate that $H_2O_2$-compromised cell membranes cause the apparently specific release of DNA. We are currently investigating whether compromised membranes trigger partial autolysis and limited or incomplete digestion of cell wall peptidoglycan. Membrane-compromised cells show less metabolic activity (Fig. 6B), but the cell wall remains intact (Fig. 6C). The intact cell walls enable cells to retain intracellular components, including ribosomes (Fig. 6D). $H_2O_2$-producing *S. sanguinis* and *S. gordonii* strains grow to lower cell densities than their respective isogenic $Pox^-$ mutants. Wild-type cultures contain about 10-fold more membrane-compromised cells (Fig. 6A) than $Pox^-$ mutants and release correspondingly more eDNA. The eDNA represents about 15 to 20% of the total chDNA. While the extraction technique can underrepresent total DNA (7, 27, 51), we compared three different techniques (enzymatic digestion, boiling, and mechanical disruption) and reported only mechanical disruption as yielding the largest amount of DNA. Nonetheless, our results suggest that the membrane-compromised cells are likely responsible for the release of DNA without generalized lysis. While $H_2O_2$ compromises membrane integrity in a small fraction of streptococcal cells, leading to eDNA release, cells must be programmed to determine which cells actually functionalize (or resist) DNA release.

A developmental program for bacterial cell death and DNA release has been shown for several species, including streptococci (recently reviewed in reference 36). Under our experimental conditions, $H_2O_2$ should be uniformly distributed, preventing localized concentrations from reaching toxic levels. Individual cells in growing planktonic cultures, however, could be in different metabolic states, as proposed for biofilm cells (43). Less active cells could be more susceptible to $H_2O_2$ and trigger DNA release. In *S. mutans*, low concentrations of $H_2O_2$ inhibit glycolysis (3). In less metabolically active cells, $H_2O_2$ could further decrease metabolic activity and trigger eDNA release. Interestingly, the operon controlling cell lysis in *S. aureus*, *cidABC*, encodes a pyruvate oxidase, CidC (33). When excess glucose is available, stationary-phase *S. aureus* $CidC^-$ mutants show higher levels of cell viability than wild-type cells, indicating metabolic control of cell death. Whether the metabolic status of the cell determines the release of DNA in *S. sanguinis* and *S. gordonii* is currently under investigation.

The eDNA mediates the biofilm phenotype of diverse species and contributes to dispersal and colonization of the marine bacterium *Pseudoalteromonas tunicata* (25, 26). In oral streptococci, release of eDNA without obvious lysis of cells could provide an evolutionary advantage enabling gene selection during transformation, as suggested by mathematical modeling (14). For the pioneer oral streptococci, such as *S. sanguinis* and *S. gordonii*, $H_2O_2$-producing cells play a crucial role in preventing growth of competing bacteria in a highly competitive biofilm community (21, 23). Metabolically inactive cells are unable to contribute $H_2O_2$ and may not be competitors serving on behalf of the population. If these metabolically inactive cells are more susceptible to $H_2O_2$ and produce eDNA, however, these cells support the genetic fitness of the population. Cells adhere better in the presence of eDNA, as we show for *S. sanguinis*. Intraspecies aggregation could prevent spatial intrusion of competing bacteria. For aggregated oral streptococci, the overall negative charge of eDNA might also promote colonization of the positively charged enamel surface (47) and early dental plaque formation.

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