Bacteria Present in Carotid Arterial Plaques Are Found as Biofilm Deposits Which May Contribute to Enhanced Risk of Plaque Rupture

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ABSTRACT **Atherosclerosis, a disease condition resulting from the buildup of fatty plaque deposits within arterial walls, is the major underlying cause of ischemia (restriction of the blood), leading to obstruction of peripheral arteries, congestive heart failure, heart attack, and stroke in humans. Emerging research indicates that factors including inflammation and infection may play a key role in the progression of atherosclerosis. In the current work, atherosclerotic carotid artery explants from 15 patients were all shown to test positive for the presence of eubacterial 16S rRNA genes. Density gradient gel electrophoresis of 5 of these samples revealed that each contained 10 or more distinct 16S rRNA gene sequences. Direct microscopic observation of transverse sections from 5 diseased carotid arteries analyzed with a eubacterium-specific peptide nucleic acid probe revealed these to have formed biofilm deposits, with from 1 to 6 deposits per thin section of plaque analyzed. A majority, 93%, of deposits was located proximal to the internal elastic lamina and associated with fibrous tissue. In 6 of the 15 plaques analyzed, 16S rRNA genes from *Pseudomonas* spp. were detected. *Pseudomonas aeruginosa* biofilms have been shown in our lab to undergo a dispersion response when challenged with free iron *in vitro*. Iron is known to be released into the blood by transferrin following interaction with catecholamine hormones, such as norepinephrine. Experiments performed *in vitro* showed that addition of physiologically relevant levels of norepinephrine induced dispersion of *P. aeruginosa* biofilms when grown under low iron conditions in the presence but not in the absence of physiological levels of transferrin.**

IMPORTANCE **The association of bacteria with atherosclerosis has been only superficially studied, with little attention focused on the potential of bacteria to form biofilms within arterial plaques. In the current work, we show that bacteria form biofilm deposits within carotid arterial plaques, and we demonstrate that one species we have identified in plaques can be stimulated *in vitro* to undergo a biofilm dispersion response when challenged with physiologically relevant levels of norepinephrine in the presence of transferrin. Biofilm dispersion is characterized by the release of bacterial enzymes into the surroundings of biofilm microcolonies, allowing bacteria to escape the biofilm matrix. We believe these enzymes may have the potential to damage surrounding tissues and facilitate plaque rupture if norepinephrine is able to stimulate biofilm dispersion *in vivo*. This research, therefore, suggests a potential mechanistic link between hormonal state and the potential for heart attack and stroke.**

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Atherosclerosis is a syndrome in which arterial elasticity is reduced due to the accumulation of fatty deposits and calcium within an arterial wall. Atherosclerosis is the principal underlying cause of coronary artery disease, peripheral arterial disease, and stroke (1). It is currently accepted that atherosclerosis develops gradually, with low-density lipoprotein (LDL) and cholesterol from plasma collecting beneath the endothelium of arterial walls, resulting in an atheromatous plaque (2). The principal danger associated with atherosclerosis is the sudden rupture of a stable atheroma, leading to a life-threatening atherothrombotic lesion (3). The importance of plaque stability is underscored by the finding that 76% of all fatal coronary thrombi arise from arterial plaque rupture (4).

There is mounting evidence that arterial plaques typically contain bacteria or signature prokaryotic biomarkers (1, 5–13). We believe the association of bacteria with arterial plaques may be an indication of colonization by biofilms, which are localized aggregations of bacteria that are refractory to antimicrobial treatment and are associated with a chronic infection state (14). The potential of biofilms to influence the progression of atherosclerosis has not been previously addressed. However, we hypothesize that if biofilms are present within atherosclerotic lesions, they may be susceptible to induction of a dispersion response with the potential of affecting the stability of the plaque deposit. Biofilm dispersion is characterized by the coordinated release of degradative enzymes by bacteria to liberate individual cells from the biofilm matrix and a transition to higher growth rates (15–21). Interestingly, it has been shown under *in vitro* conditions that when exposed to a sudden increase in a limiting nutrient, biofilm bacteria will respond by mounting a dispersion event (15). We believe that in an atheroma, a biofilm dispersion event could result from a sudden increase in the availability of free iron, an essential nutri-

ent, released from its bound state by elevated levels of norepinephrine. The induction of a biofilm dispersion response within an atheroma may, therefore, have the potential to induce collateral tissue damage resulting from the localized release of degradative enzymes by the participating bacteria. This response, in turn, could influence the integrity of the surrounding arterial tissues, leading to an enhanced risk of plaque rupture and thrombogenesis.

It has long been appreciated that emotional or physical stress can act as a trigger for plaque rupture and thrombogenesis, even though the actual mechanism of destabilization may be poorly understood. In the Multicenter Investigation of Limitation of Infarct Size (MILIS) study, possible triggers of acute cardiovascular disease were identified in 48.5% of the population, with the most common being emotional upset (18.4%) and moderate physical activity (14.1%) (22). Associations with stress and anger have also been identified in both population-based studies of natural disasters (23) and clinical studies of hospital patients admitted with acute myocardial infarction (MI). Mittleman et al. (24) reported that the relative risk of acute MI more than doubled in the 2 h after an episode of anger, and similar effects have been reported for severe work-related stress (25).

One of the hallmarks of the onset of stress is an increase in the plasma concentration of norepinephrine (26). Among its other effects, norepinephrine has been shown to interact with the iron transport protein transferrin in serum, causing it to release free iron (27). Transferrin is used by the human body in part to inhibit the invasion and growth of microbial pathogens, restricting the amount of ionic iron available in body fluids to 10^{-18} M (13). This amount is insufficient for normal bacterial growth, requiring pathogens to produce their own iron-chelating agents to compete with transferrin (and other related chelators) for available iron (13, 28). A sudden increase in free iron, therefore, has the potential to impact the growth state or behavior of resident pathogens in an infected host.

In the current study, we hypothesized that in at least some cases, atherosclerosis may be a biofilm-associated chronic disease. To test this hypothesis, we examined atheromas within diseased human carotid artery explants for the presence of bacteria and performed microscopic analyses to determine whether these bacteria showed evidence of having developed into biofilm deposits. We also evaluated *Pseudomonas aeruginosa*, a bacterial species shown in this study to be present in atheromas, to determine whether it could be induced to undergo a dispersion response *in vitro* when norepinephrine was added to cultures of this organism grown as a biofilm in the presence of iron-bound transferrin.

RESULTS

PCR of 16S rRNA and *P. aeruginosa*-specific 16S rRNA gene sequences. Samples of diseased carotid arteries obtained from 15 patients with advanced atherosclerosis were examined by PCR amplification of the V2-V3 region of eubacterial 16S rRNA genes, followed by gel electrophoresis to determine the number of patients with bacterial DNA sequences associated with arterial plaque. All 15 samples were found to contain eubacterial 16S rRNA gene sequences (Fig. 1A). In order to determine if the single 16S rRNA band obtained represented multiple colonizing species, five random subsamples from this set were selected as representative data and analyzed to determine the number of unique bacterial 16S rRNA gene sequences in each sample via density gradient gel electrophoresis (DGGE). Each of the five carotid arteries tested was observed to have from 10 to 18 unique 16S rRNA gene signatures, indicating polymicrobial colonization (Fig. 1B). Comparison of the samples revealed a total of 8 bacterial 16S rRNA gene sequences that were common to all 5 patients and multiple additional sequences that were present in some patients but not others (Fig. 1B). The results from DGGE of the five samples proved to be unanimous for the presence of multiple strains in atherosclerotic plaques, and therefore no further samples were analyzed using DGGE.

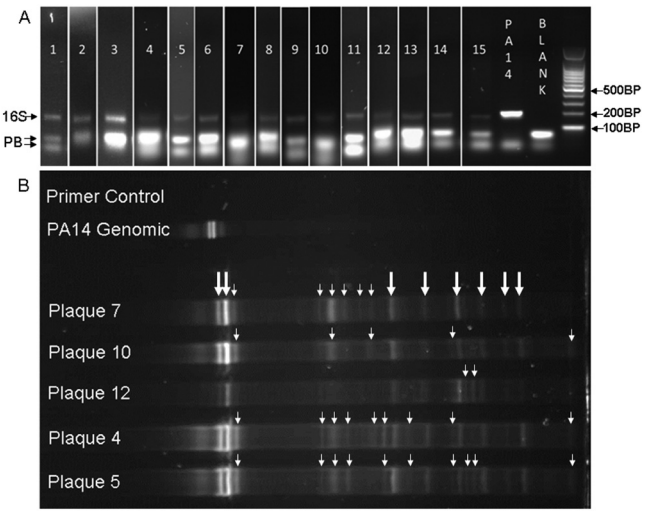

FIG 1 PCR and DGGE profiles from carotid artery explants of patients with atherosclerosis. (A) Eubacterial 16S rRNA gene PCR results from 15 patients with advanced atherosclerosis. Each numbered lane represents a carotid arterial plaque sample from a separate patient. *P. aeruginosa* PA14 was used as a positive control, a negative control containing no 16S rRNA gene sequence is labeled BLANK, and 16S indicates 16S rRNA gene bands. PB, primer band. The DNA marker is indicated at the right. (B) Results from DGGE analysis of 5 random plaque samples taken from the original 15 patients, indicated at left. Large white arrows show 16S rRNA gene bands that were common to all 5 patients. Small white arrows indicate 16S rRNA gene bands that were found in some but not all samples. A positive control with *P. aeruginosa* PA14 16S rRNA genes is shown at the top of the gel. Note that the PA14 genomic DNA did not align with any DNA from the patient samples.

In previous work in our laboratory, we isolated and identified cultivable *P. aeruginosa* as a colonizing pathogen from a diseased carotid endarterectomy sample. In the present study, we checked for the presence of this bacterial pathogen by screening all 15 patient samples for *Pseudomonas* spp.-specific 16S rRNA genes via PCR amplification. Positive PCR product for *Pseudomonas* spp. was isolated from 6 of the 15 samples (Fig. 1, patient numbers 4, 5, 6, 7, 10, and 15). These PCR products were sequenced and analyzed for alignment to known bacterial 16S rRNA gene sequences by using BLAST (Basic Local Alignment Search Tool). Samples from patients 5, 6, 7, 10, and 15 were shown to contain sequences that aligned with *P. aeruginosa*, and the sample from patient 4 indicated the presence of *Pseudomonas* sp. (Fig. 2A). BLAST results confirmed that all strains derived from carotid artery plaque samples in the present study differed from our laboratory strain, indicating that our results were not influenced by laboratory contamination (Fig. 2B).

PNA-FISH probed samples. In order to examine atherosclerotic arteries for evidence of biofilm colonization, we prepared

A

Sample	Result	Sequence Length	Expect Value	Max Identity
4	*Pseudomonas sp.*	230	1.00*10^-93	96%
5	*P. aeruginosa*	232	6.00*10^-111	99%
6	*P. aeruginosa*	184	1.00*10^-77	98%
7	*P. aeruginosa*	234	3.00*10^-108	99%
10	*P. aeruginosa*	234	2.00*10^-105	98%
15	*P. aeruginosa*	147	5.00x10^-45	90%

B

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Lab_Strain_PA14   GAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGCAAGG-TATTAACTTACTG
Sample#4          --------GGNACGGGCTATCTNGTGGGTANGTCAAA-CAGCAAGGGTATTAACTTACTG
Sample#5          --------GGANCGG-CT-TCTG--TGGTACGTCAAA-CAGCAAGG-TATTAACTTACTG
Sample#6          --------GGNACGG-CTNTCTG--TGGTACGTCAAA-CAGCAAGGTTATTAACTTACTG
Sample#7          --------GGGGCGG-CTATCTG--TGGTN-GTCAAA-CAGCAAGG-TATTAACTTACTG
Sample#10         ---------GNACGG-CTATCTG--TGGTACGTCAAA-CAGCAAGG-TATTAACTTACTG
Sample#15         ----------GACGG-CTNTCTG--TGGTACGTCAAA-CAGCAAGG-TATTAACTTACTG
                             *     ***    *    ****** ******** **************

Lab_Strain_PA14   CCCTTCCTCCCAAACTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#4          CCCTTCCTCCCAA-CTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#5          CCCTTCCTCCCAA-CTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#6          CCCTTCCTCCCAA-CTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#7          CCCTTCCTCCCAA-CTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#10         CCCTTCCTCCCAA-CTTAAAGTGCTTTACAATCCGAAGACCTTCT-TCACACACGCGGCA
Sample#15         CCCTTCCTCCCAA-CTTAAAGTGCCNTTTAANCCGAAGACCTTCTCTCACACACGCGGCA
                  ************ **********  *  ** ************ **************

Lab_Strain_PA14   TGGC--TGATCAGG---TTCGCCNTTCTTNCC--------------------------
Sample#4          TGGCGTGGATCAGGCATTTCGCCCATTGTCC--AANATTCCCCACTGCTGCCTCCCGTAG
Sample#5          TGGC-TGGATCAGGC-TTTCGCCCATTGTCC--AATATTCCCCACTGCTGCCTCCCGTAG
Sample#6          TGGC-TGGATCAGGC-TTTCGCCCATTGATCCAAATATTCCCCACTGCTGCCTCCCGTAG
Sample#7          TGGC-TGGATCAGGC-TTTCGCCCATTGTCC--AATATTCCCCACTGCTGCCTCCCGTAG
Sample#10         TGGCNTGGATCAGGC-TTTCGCCCATTGTCC--AATATTCCCCACTGCTGCCTCCCGAAG
Sample#15         TGGCGTGGATCAGGACTTTCGGCCANNGNCC--AANATTCCCCAC---------------
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FIG 2 *P. aeruginosa* sequence data from carotid artery explants of study patients. (A) BLAST results for 16S rRNA gene sequences from patient samples 4, 5, 6, 7, 10, and 15, indicating probable species, sequence length, expect value (number of hits by chance when searching a database of a particular size), and maximum identity (percent similarity between the query and subject sequences over the length of the coverage area). (B) Multiple alignments between our lab strain and the sequences obtained from atherosclerotic plaque samples. It can be seen that there are various regions that did not match up, which allowed us to draw the conclusion that the strains we obtained were different from our lab strain.

two 25-μm-thick transverse sections from each of 5 carotid artery subsamples chosen from the 15 patients in the present study. The 5 subsamples were chosen due to the length of the artery, to ensure that enough of the sample was left for DNA extraction. That 10 of the samples were not included for peptide nucleic acid-fluorescent *in situ* hybridization (PNA-FISH) probing was due to the limited size of the arterial sample. Each section was analyzed by PNA-FISH with fluorescence-tagged eubacterium-specific 16S rRNA gene probes to test for the presence of eubacterial targets. Each carotid artery sample was found to be positive for the presence of bound probe ($n = 5/5$). These fluorescent deposits were arranged as microcolonies, each limited in area and comprising a few dozen to a few hundred detectable probe targets averaging approximately 1 μm in diameter and indicative of bacteria (Fig. 3). The individual probe targets showed slight separation from one another, indicating the presence of unstained matrix, and were located in areas showing a variation of tissue densities and structures, indicated by the fluorescence intensities. These areas contained locations where tissue thickness was reduced and appeared to include porous or structurally altered tissue, giving the impression that there was localized tissue damage, which is a characteristic associated with inflammation. Patient 1's sample contained red fluorescent deposits with approximately 22 and 83 PNA probe targets, respectively, the first located proximal to the internal elastic lamina and the second within the tunica externa (Fig. 3A). Increased magnification of these deposits showed these probe targets were arranged as aggregates and were confined to specific sites (Fig. 3B and C). A 3-dimensional composite image of the deposit located within the tunica externa revealed that this colonization focus was comprised of two separate microcolonies embedded within the arterial tissue (Fig. 3D). The schematic diagram (inset) indicates the locations of the various anatomical features indicated in the photomicrographs. Patient 3's sample showed extensive probe binding associated with the tunica interna at a location that appeared to have experienced a plaque rupture event due to the split in the tunica intima (Fig. 4A). The separation of individual probe targets in Fig. 4B and C was suggestive of bacteria associated with one another by being enmeshed in a nonfluorescent matrix. Patient 2's sample (see Fig. S1A in the supplemental material) contained a single isolated microcolony located

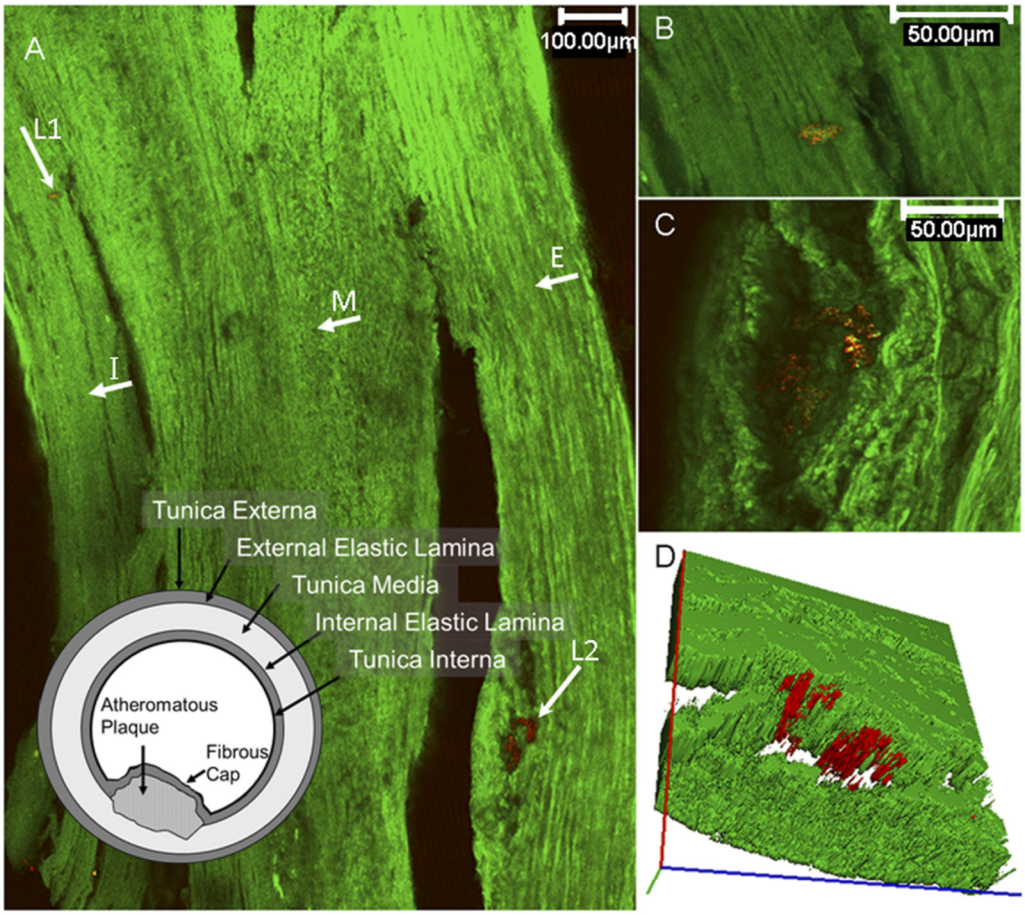

FIG 3 CSLM images of eubacterial 16S rRNA gene sequence in the carotid arterial plaque sample from patient 1, demonstrated via PNA-FISH. (A) An overview of the patient 1 sample, showing the location of 2 fluorescent deposits located proximal to the internal elastic lamina (L1) and proximal to the external elastic lamina (L2). (Inset) Anatomical features of an atherosclerotic artery in cross-section. (B) Location L1 at higher magnification. (C) Location L2 at higher magnification. (D) A 3-dimensional rendering of microcolony L2. Red fluorescence indicates eubacterial 16S rRNA gene PNA-FISH probe targets; green fluorescence indicates background.

proximal to the internal elastic lamina below the fibrous tissue. Patient 4's sample (see Fig. S1B) demonstrated extensive microcolony development within the tunica interna that was associated with a porous tissue structure that may have been due to extensive tissue damage, and patient 5's sample (Fig. S1C) showed an isolated microcolony located proximal to the internal elastic lamina. Of all microcolonies detected, 76.5% were located proximal to the internal elastic lamina, within the tunica interna and associated with fibrous tissue, while an additional 5.9% of microcolonies were associated with the external elastic lamina. Since all 5 samples contained bound probe and demonstrated a similar microcolony presence of eubacterial 16S rRNA gene sequences, no more samples were evaluated.

Transferrin-norepinephrine-induced *P. aeruginosa* PAO1 biofilm dispersion. Having shown that atheromas contain deposits of cells that bound the 16S rRNA gene eubacterial probe and that these deposits display characteristics of biofilm colonization, our next question was whether any bacteria in these deposits had the potential to undergo a biofilm dispersion response when exposed to Fe^{2+}. To test this, we grew biofilms of *P. aeruginosa*, a bacterium for which 16S rRNA gene sequences had been shown to be associated with one-third of the samples in the present study, added $FeSO_4$ to the medium, and measured the bacteria released from the biofilms, using optical density (OD) as a measure of biofilm dispersion. At a concentration of 1.78 mg and 3.5 mg Fe/liter, significant dispersion was induced and detected starting at approximately 20 min postchallenge (Fig. 5A). In control experiments, addition of up to 3.0 mg/liter free iron did not result in detectable population growth over a period of 3.5 h, indicating that the increased OD measured in the dispersion experiments was not the result of population growth over the time interval of the experiment (see Fig. S2 in the supplemental material).

Our next experiment was to determine whether a biofilm dispersion response could be induced by interaction of norepinephrine with holo-transferrin at physiologically relevant concentrations. It was subsequently demonstrated that free iron, released due to an interaction of bovine holo-transferrin (0.5 g/liter) (29) with norepinephrine (0.4 mM) (30), induced biofilm dispersion in *P. aeruginosa* cultures (Fig. 5B). The free iron concentration, measured in the medium following addition of norepinephrine, was found to be 0.267 mg/liter, compared to $<$0.02 mg/liter in medium supplemented only with transferrin. Norepinephrine-transferrin challenge resulted in a 72% increase in released cells above that with transferrin alone ($P = 5.64E-13$). The dispersion

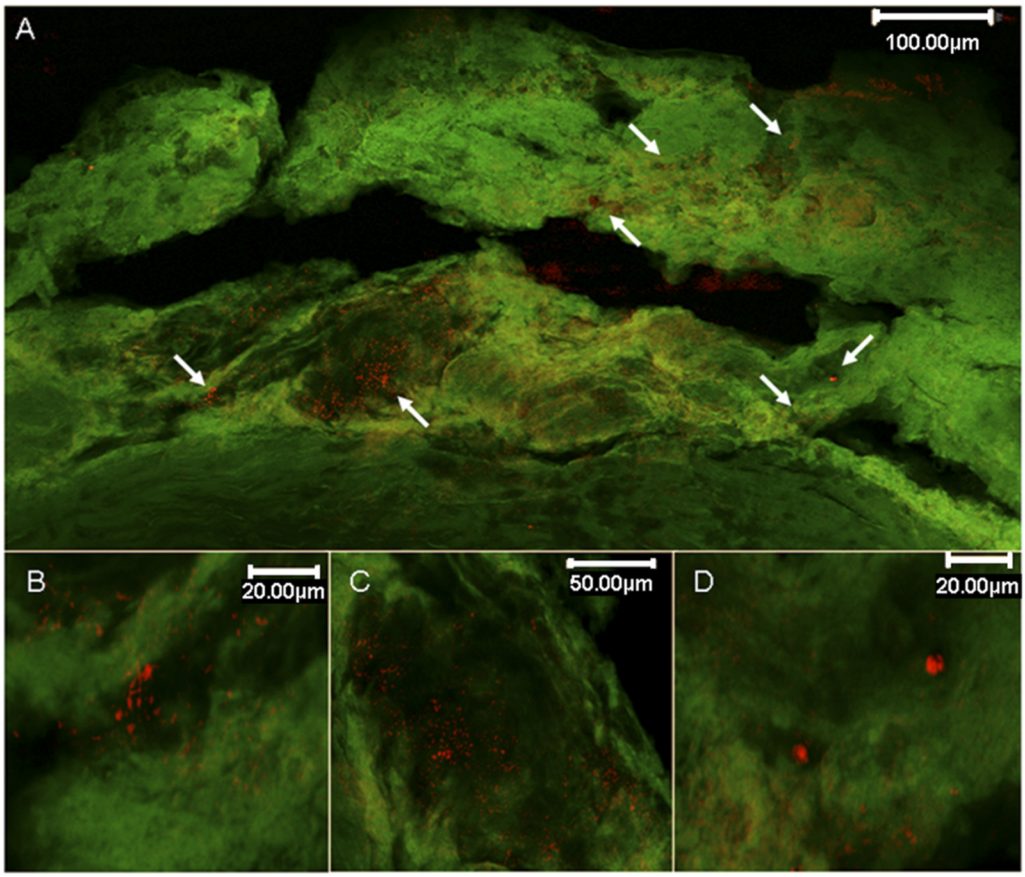

FIG 4 Confocal images of eubacterial 16S rRNA gene sequence in the carotid arterial plaque sample from patient 3, demonstrated via PNA-FISH. (A) Overall view of the location where an intima rupture appears to have occurred, with tissue damaged from severe atherosclerosis containing multiple fluorescent deposits. (B, C, and D) Magnified images of the fluorescent deposits shown in panel A. Red fluorescence indicates eubacterial 16S rRNA gene PNA-FISH probe targets; green fluorescence indicates background.

response of biofilm bacteria is known to be associated with the release of degradative enzymes. Therefore, a dispersion response *in vivo* may have important consequences for surrounding tissues.

DISCUSSION

This study has shown that bacterial association with arterial plaque can be extensive and involve a genetically diverse community. Microscopic examination of these colonizing organisms indicated that they form biofilm structures within the atheroma and associated tissues. To our knowledge, this is the first direct observation of biofilm bacteria within a carotid arterial plaque deposit. This is important, because biofilm bacteria display resistance and physiological characteristics that are distinct from their planktonic counterparts and manifest unique behaviors, such as biofilm dispersion (8, 14). Thus, biofilm infections require targeted therapeutic approaches if they are to be managed successfully. It has been demonstrated by Parsek and Singh that biofilm infections display 4 characteristics by which they may be identified (31). According to these criteria, biofilm bacteria are (i) adherent to some substratum or are surface associated, (ii) aggregated in cell clusters encased in a matrix, (iii) confined to a particular site in the host, and (iv) are difficult or impossible to eradicate with antibiotics, despite the fact that the responsible organisms are susceptible to killing in the planktonic state. The biofilm nature of the carotid arterial plaque-associated bacteria detected in the present study is supported by criteria i, ii, and iii of Parsek and Singh. We did not collect data on prior antibiotic use by patients (criterion 4).

The presence of bacteria in a biofilm structure implies that known biofilm behaviors may be associated with arterial plaque colonization, notably, the ability to respond to extracellular signals to induce a dispersion event. Biofilm dispersion is significant because in the process of evacuating a biofilm structure, the dispersing bacteria must release a wide range of degradative enzymes to digest the matrix within which they are enmeshed. Such an event within an atheroma may have the potential to cause collateral damage to proximal tissues and negatively impact plaque stability. Thus, we believe there is the potential that stimulation of a biofilm dispersion response in patients with advanced atherosclerosis may be a predisposing factor in thrombogenesis.

It is significant that *P. aeruginosa*, which was identified in 5 of the 15 plaque samples analyzed, was shown in this study to undergo biofilm dispersion when challenged with elevated levels of free iron. Biofilm dispersion by this microorganism was also shown here to be inducible by the addition of norepinephrine to transferrin-containing culture medium. Thus, under laboratory conditions, an *in vitro* spike in hormone concentration was shown to induce biofilm dispersion. It is unclear at this time whether a

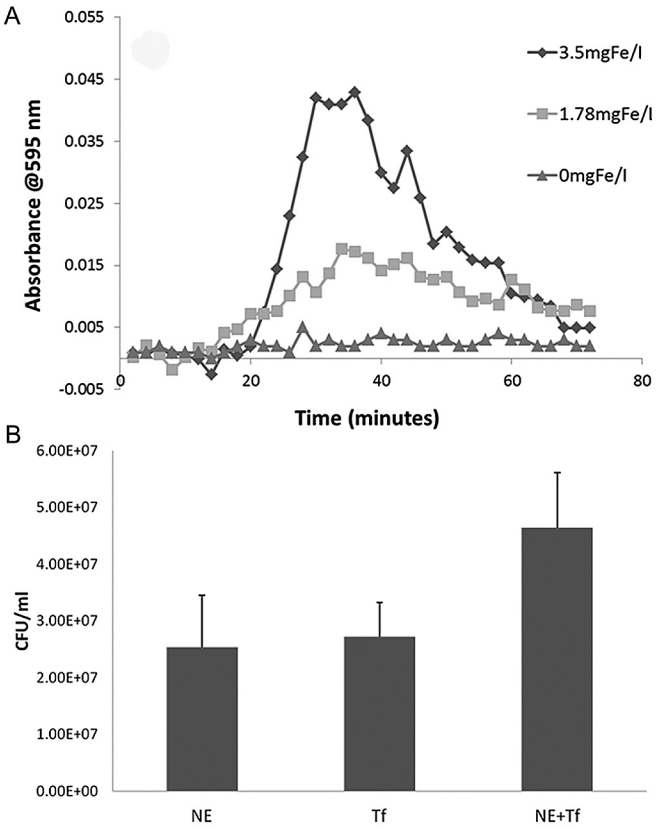

FIG 5 *P. aeruginosa* response to challenge with free iron and norepinephrine. (A) Dispersion of biofilms grown for 7 days in continuous culture and challenged with Fe^{2+} or the carrier control. Each line represents the average of 3 replicate experiments. Onset of dispersion was observed at approximately 20 min. (B) Results for 7-day *P. aeruginosa* biofilm cultures challenged with 0.4 mM norepinephrine (NE) in the presence and absence of 0.5 g/liter of holo-transferrin (Tf) at 37°C ($n = 38$ for each test; error bars represent standard deviations).

biofilm dispersion response is inducible *in vivo*. For instance, sequestration of biofilm deposits within atheromas may have a mitigating effect on the ability of norepinephrine to induce iron release in the vicinity of the infecting bacteria. Furthermore, the association of degradative enzyme release during the biofilm dispersion response with collateral tissue damage is speculative on our part. We have no direct evidence that this occurs *in vivo*; however, we believe that the potential for additional damage to surrounding tissues due to bacterial enzyme release may be an additional significant factor contributing to thrombogenesis.

In addition to the potential role of bacteria in the destabilization of arterial plaque, other factors have been identified that could contribute to thrombogenesis. Dietel et al. (32) assessed atherosclerotic plaques based on fibrous cap thickness (FCT) and the lipid core ratio ([LCR]; the lipid core area divided by the plaque area) and classified them as vulnerable (FCT $<$ 100 μm, LCR $>$ 50%) or stable (FCT $>$ 100 μm, LCR $<$ 50%). Increased transcription of gamma interferon (IFN-γ) and interleukin-17α (IL-17α) along with increased infiltration of mature dendritic cells (DCs) were found in vulnerable atherosclerotic plaques compared to stable atherosclerotic plaques. Production of IL-17 by Th17 cells is commonly associated with Gram-negative bacterial infection (33) and has been shown to have a proatherogenic inflammatory role in promotion of monocyte/macrophage recruitment, as has been demonstrated for the aortic arterial wall (34). Additionally, IFN-γ has been shown in a murine model to induce a Th1 immune response, resulting in the release of T-cell cytokines and maximization of macrophage bactericidal activity (35). Moser et al. demonstrated a decrease in the level of granulocyte-macrophage colony-stimulating factor (GM-CSF) and an increase in granulocyte colony-stimulating factor (G-CSF) in cystic fibrosis patients that had *P. aeruginosa* lung infections (36). The ratio of GM-CSF to G-CSF was shown to be positively correlated with the IFN-γ response. Since there was a direct correlation between *P. aeruginosa* infection, GM-CSF/G-CSF ratio, and IFN-γ activity in the lungs, we postulated that this system may also play a role in atherosclerotic lesions and explain the observed levels of increased transcription of IFN-γ in vulnerable versus stable plaques. We suspect that transport of immune system components across the fibrous cap is facilitated by its reduced thickness and may be stimulated by the presence of these bacterial biofilm infections, resulting in an increase in proinflammatory factors, such as IFN-γ and IL-17α. Maturation of DCs occurs when they phagocytize pathogens. According to Dietel et al., the increased presence of mature DCs in vulnerable plaques suggests a particular involvement of mature DCs in the process of plaque destabilization, though what factors may be stimulating immature DCs to become mature was not addressed in their study (32). It is possible that the biofilm infections observed in this study could provide the pathogenic link for the change from an immature to mature DC, and as mentioned previously, diffusion across the fibrous cap could be streamlined by its decreased thickness.

An additional factor that likely contributes to inflammation and tissue damage within atherosclerosis is C-reactive protein (CRP). Meuwissen et al. demonstrated a positive correlation between levels of CRP and severity of coronary atherosclerotic disease. CRP promotes the formation of foam cells within atherosclerotic plaque deposits (37). Tissue injury has been shown to stimulate hepatocytes to produce CRP. It could be that tissue damage caused by biofilm dispersion events causes an increase in serum CRP levels. While all of these factors are derived from the host, it appears that plaque instability is derived from a complex system of the host immune response misbehaving, resulting in tissue inflammation and damage. Biofilm infections within atherosclerotic arteries could provide the answer as to why we see an increase in proinflammatory cytokines and host-derived tissue damage.

The results from this study add another potentially significant contributing factor to the biology of the arterial plaque environment. This environment is already understood to be a complex association of interacting factors with the potential to destabilize atheromatous lesions and contribute to thrombogenesis. The involvement of biofilm bacteria within this environment may contribute to this destabilization in a number of ways. Our *in vitro* results hint at the possibility that biofilm-associated bacteria within an atheroma are induced to disperse when norepinephrine levels become elevated. Such a dispersion event would likely be associated with the release of degradative enzymes that have the potential to induce collateral tissue damage. However, whether norepinephrine has the ability to induce transferrin (or other iron chelators) to release free iron into the environment of a plaque lesion is unknown at this time. Furthermore, the release of degra-

dative enzymes from bacteria in a biofilm dispersion response within an atheroma is unproven *in vivo*. The ability of such degradative enzymes to contribute to thrombogenesis is also unproven. However, the ability of norepinephrine to stimulate biofilm dispersion suggests a potential mechanism whereby the hormonal state of an individual may contribute to arterial plaque destabilization.

MATERIALS AND METHODS

Acquisition of arterial plaque samples. Carotid artery explants from 15 patients with advanced atherosclerosis were collected in sterile Ringer's solution following extraction by standard carotidectomy and held at 4°C until transport to the laboratory for analysis within 24 h. No patient data were provided.

PCR amplification of 16S rRNA genes and *Pseudomonas aeruginosa* screening. Amplification of bacterial 16S rRNA genes was carried out according to the methods of McBain et al., using primers HDA1 (5′ GAC TCC TAC GGG AGG CAG CAG T 3′) and HDA2 (5′ GTA TTA CCG CGG CTG CTG GCA C 3′) (38). Amplification of *P. aeruginosa* 16S rRNA genes was performed on DNA extracted from carotid artery explants by using the primers PA-SS–F (5′ GGGGGATCTTCGGACCTCA 3′) and PA-SS–R (5′ TCCTTAGAGTGCCCACCCG 3′), developed by Spilker et al. (39). A full description of DNA extraction, PCR amplification, and *P. aeruginosa* 16S rRNA gene sequencing is provided in the supplemental material.

DGGE. PCR amplification and DGGE analysis were carried out according to the methods of Muyzer et al. (40, 41), using the primers HDA1-GC (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T 3′) and HDA2 (5′ GTA TTA CCG CGG CTG CTG GCA C 3′). Samples were stored at −20°C until analyzed (41). Additional information for the DDGE analysis is provided in the supplemental material.

Fluorescent in situ hybridization. A universal bacterial PNA-FISH kit (AdvanDx, Woburn, MA) for bacterial 16S rRNA genes was used to label eubacteria present within arterial plaque thin sections. A Zeiss 510 Meta (Zeiss, Jena, Germany) confocal laser scanning microscope (CLSM) was used to detect *in situ* bacteria. A full description of the PNA-FISH protocol is provided in the supplemental material.

Bacterial strains and media. All biofilm and dispersion studies were performed using *Pseudomonas aeruginosa* strain PAO1 grown aerobically in minimal medium as described by Sauer et al. (42).

Biofilm growth and dispersion in continuous culture. Biofilms were grown in continuous culture on the luminal surface of silicon tubing (internal diameter of 1.6 mm and length of 81.3 cm) at a flow rate of 0.1 ml/min according to the method of Sauer et al. (42). After 7 days of biofilm growth, minimal medium was supplemented with known concentrations of iron as ferrous sulfate ($FeSO_4 \cdot 7H_2O$), pH 7.0. Biofilm reactor effluent was collected in 96-well microtiter plates at intervals of 2 min for 72 min. Cell densities were determined based on the absorbance at 595 nm by using a Fisher Scientific Multiskan MCC reader. Data were obtained from 3 replicate lines and averaged. Dissolved iron was determined spectrophotometrically by using a FerroMo iron test kit (Hach Co., Loveland, CO).

Microtiter plate biofilm dispersion assay. Biofilms were grown in acetone-etched 96-well microtiter plates following transfer of 150 μl of 0.1% *P. aeruginosa* PAO1 preculture into wells containing 0.5 g/liter bovine holo-transferrin (MP Biomedicals, Solon, OH) and incubated at 37°C with shaking at 220 rpm for 24 h. Spent medium was exchanged with fresh, sterile medium every 24 h for 5 days, followed by two additional exchanges after 12 h and a wash step after 2 h. Samples were supplemented with 0.4 mM norepinephrine (Sigma, St. Louis, MO) for test samples or carrier for control samples and incubated for an additional 90 min. Dispersion was determined by recording the OD at 595 nm of culture supernatants by using a Fisher Scientific Multiskan MCC reader (Thermo Electron Corporation, Vantaa, Finland). Data were obtained from 3 replicate experiments of 13 samples for each treatment ($n = 39$) and averaged. A 2-tailed *t* test was performed on the data.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01206-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 11.9 MB.
Figure S2, TIF file, 1.5 MB.

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