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Autoinducer-2 Triggers the Oxidative Stress Response in *Mycobacterium avium*, Leading to Biofilm Formation▽†

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Mycobacterium avium **is an environmental organism and opportunistic pathogen with inherent resistance to drugs, environmental stresses, and the host immune response. To adapt to these disparate conditions, *M. avium* must control its transcriptional response to environmental cues. *M. avium* forms biofilms in various environmental settings, including drinking water pipes and potable water reservoirs. In this study, we investigated the role of the universal signaling molecule autoinducer-2 (AI-2) in biofilm formation by *M. avium*. The addition of the compound to planktonic *M. avium* cultures resulted in increased biofilm formation. Microarray and reverse transcriptase PCR studies revealed an upregulation of the oxidative stress response upon addition of AI-2. This suggests that the response to AI-2 might be related to oxidative stress, rather than quorum sensing. Consistent with this model, addition of hydrogen peroxide, a known stimulus of the oxidative stress response, to *M. avium* cultures resulted in elevated biofilm formation. These results suggest that AI-2 does not act as a quorum-sensing signal in *M. avium*. Instead, biofilm formation is triggered by environmental stresses of biotic and abiotic origins and AI-2 may exert effects on that level.**

Mycobacterium avium is ubiquitous in the environment, occurring in natural and urban water sources, as well as in soil, but also can act as an opportunistic pathogen (21, 37). It is related to the intracellular pathogens *M. tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy. The species *M. avium* is composed of several subspecies, including *M. avium* subsp. *hominissuis*, which causes disease in humans and other mammals, and *M. avium* subsp. *paratuberculosis*, a significant pathogen of livestock with an uncertain role in the etiology of human disease (53).

Although *M. avium* mainly infects the immunocompromised, there have been increased reports of infections in people with no obvious predisposing factors (12). For example, hypersensitivity pneumonitis has been reported in otherwise healthy individuals. These infections occur due to exposure to aerosolized mycobacteria in indoor swimming pools, hot tubs, and metalworking fluids in industrial settings (1, 29, 45).

In most environmental settings, bacteria are found in biofilms, complex communities that colonize all kinds of surfaces (6). Nonparatuberculous *M. avium* is among the most commonly isolated species in biofilm samples taken from drinking water distribution systems (11). *M. avium* occupies a broad range of habitats in the environment and therefore needs to adapt to different environmental conditions. It is very important for the bacterium to sense and process the gathered information. This is accomplished by signal transduction mechanisms, enabling the bacterium to monitor pH, temperature, nutrient availability, and also the presence of other bacteria which might compete with the slow-growing mycobacteria for food and space. Bacterial adaptation to environmental changes most commonly follows a general scheme in which an environmental cue is sensed by a membrane protein in a two-component system and transferred via phosphorylation to a response regulator, or if the cue is diffusible, it may bind directly to a transcriptional regulator, which then alters gene expression (16, 50). Cues can include environmental conditions such as osmolarity, nutrient availability, temperature, or ions. In the case of the oxidative stress response, the reactive oxygen species (ROS) binds directly to the OxyR transcriptional regulator and activates gene transcription (51).

Social interactions among bacteria are more specific than interactions with the environment. The bacteria sense self-produced signaling compounds at well-defined concentrations, the so-called autoinducers (AIs). This process, termed quorum sensing, enables bacteria to monitor the environment for other bacteria and to react by changing their behavior (54). This is especially important to cell density-dependent cellular functions such as light production, virulence, sporulation, and biofilm formation. Intraspecies signal molecules are often *N*-acyl-homoserine lactones or signaling peptides. The only universal signal identified to date is AI-2 (5).

AI-2 is a collective term for the molecules derived from the precursor 4,5-dihydroxy-2,3-pentanedione (DPD), which is produced by the LuxS synthase. These molecules are in equilibrium and can convert into each other, and each molecule binds to a different receptor in different bacterial species (28). AI-2 is a metabolic by-product in the detoxification of *S*-adenosylhomocysteine in the activated methyl cycle of bacterial cells. Therefore, the question of whether AI-2 is a true specific signaling compound has been raised. It is important to distinguish between signaling, which is a social interaction between bacteria, and cues, the interaction of bacteria with the environment (22). As defined by Diggle et al., signaling occurs

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when a cell secretes a molecule owing to the effect on the sender. If the receiving cell only benefits from the compound, it is called a cue (9). De Keersmaecker et al. suggested that there is not sufficient evidence for the assignment of a signaling role to AI-2 in all bacteria that possess the *luxS* gene (7). Given that interspecies signaling cannot always be explained from an evolutionary standpoint, it is possible that AI-2 does not always act as a signaling compound. Quorum sensing describes the bacterium's ability to cooperate, whereas a cue induces reactive behavior in a cell. Both cooperation and reaction contribute to the bacteria's fitness and ability to adapt to environmental conditions. Therefore, even if a molecule does not elicit a response from a cell that owes to the emitting cell, it still might help the receiving cell to monitor its physical and microbiological environment. In the case of *M. avium*, which is unable to produce AI-2, the molecule might act as a parainducer rather than an autoinducer. One example of parainduction was described by Duan et al.; it shows that AI-2 induces certain virulence genes in *Pseudomonas aeruginosa*, which is also unable to produce AI-2 (10). AI-2 has been shown to be involved in biofilm formation in many bacterial species. AI-2 can inhibit biofilm formation, as shown for *Bacillus cereus* (2), *Vibrio cholerae* (19), and *Eikenella corrodens* (3), and AI-2 promotes biofilm formation in *Escherichia coli* (17, 20, 38), *Streptococcus mutans* (24, 27, 55, 57), and *Aggregatibacter actinomycetemcomitans* (13, 44). Furthermore, the molecule seems to play an important ecological role in the formation of multispecies biofilms (26, 39, 57).

Because *M. avium* has been isolated from environmental biofilms, we sought to investigate the influences that favor biofilm formation, especially in the presence of AI-2. The present study shows that AI-2 increases biofilm formation in *M. avium* cultures when present at high concentrations. Microarray and reverse transcription (RT)-PCR studies show that the oxidative stress response is upregulated in response to the addition of AI-2. Hydrogen peroxide also increased biofilm formation, indicating that oxidative stress stimulates biofilm formation by this pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. avium* strain W2001 was isolated from drinking water in the Boston area. The strain was classified as *M. avium* subsp. *hominissuis* with an *hsp65* code 1 sequevar (52, 53), which is the same as that of recently sequenced *M. avium* 104.

The organism was grown at 37°C in Middlebrook 7H9 medium supplemented with 10% albumin-dextrose-catalase enrichment and 0.2% glycerol.

Assessment of biofilm formation in 96-well microtiter plates in the presence of AI-2 and hydrogen peroxide. Bacterial cultures were grown in Middlebrook 7H9 broth to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0, centrifuged, and resuspended in equal amounts of sterile milliQ deionized water. DPD, a generous gift from Peter Shoolingin-Jordan, was dissolved in water at concentrations indicated below. The microtiter plates were inoculated with 150 μl of bacterial suspension and 50 μl DPD solution, bacterial culture supernatant, or H_2O_2, respectively. The negative controls contained 50 μl water or 50 μl LB medium in the supernatant experiment. The final concentrations of DPD per well were 0.25 μM, 2.5 μM, 25 μM, 250 μM, and 2,500 μM. The final H_2O_2 concentrations were 0.05 mM, 0.5 mM, 5 mM, and 50 mM. After 10 days of incubation, the biofilm mass was assessed by an adapted version of the crystal violet (CV) staining method (34). In brief, the biofilms were stained with CV and incubated at room temperature for 45 min. Rinsing off the residual dye left only the stained cells attached to the surface of the wells. The CV was solubilized with 80% (vol/vol) ethanol, and OD_{550} readings were taken for quantification. The biofilms grown in the presence of AI-2 were observed with a Leica TCS NT confocal laser scanning microscope.

TABLE 1. Primers used for RT-PCR

Primer	Oligonucleotide sequence (5′–3′)
AhpC forward	AGCACGAGGACCTCAAGAAC
AhpC reverse	GTGACCGAGACGAACTGGAT
AhpD forward	GTACGCCAAGGATCTCAAGC
AhpD reverse	GTACTTGCCGTCCAAGAAGC
TreS forward	TACGACACCACCGACCACTA
TreS reverse	CGTGATCGTCAGAGTCGATG
MAV_4967 forward	GGATGGCAGTGGGTGACTAC
MAV_4967 reverse	CCGTAGGTGTTGAGGGACAG
MAV_2838 forward	GGATGGCACTGGGTGACTAC
MAV_2838 reverse	CCGTAGGTGTTGAGGGACAG
SigA forward	CCTCAAGCAGATCGGTAAGG
SigA reverse	AGATTCGCTTCCAGCAGATG
16S forward	GCGATATCGGGCAGACTAGAG
16S reverse	AAGGAAGGAAACCCACACCT

Microarray studies. Planktonic *M. avium* cultures were grown to an OD_{600} of 0.3 to 0.5 in the absence or presence of 0.5 mM AI-2. The OD was determined to ensure that AI-2 did not influence the growth of the planktonic culture. Total RNA was extracted by a phenol-chloroform extraction protocol (47). Microarray hybridization and analysis were performed as described previously (30). In brief, the RNA extracted from both AI-2-treated cultures and the negative control was labeled with Cy3 or Cy5 dUTP by reverse transcriptase (Amersham Biosciences). The 3-day induction with AI-2 was performed in triplicate and once with an induction time of 2.5 h to study the immediate change in gene expression. The labeled cDNA was hybridized to microarrays composed of oligonucleotide probes that were designed on the basis of the annotation of the *M. avium* subsp. *avium* strain 104 sequence (provided by the Institute for Genomic Research [http://www.tigr.org]). Each of the 4,158 probes (MetaBion GmbH, Martinsried, Germany) was printed in duplicate onto microarray slides (Sigmascreen; Sigma). The AI-2-versus-control comparison was performed for each dye combination (Cy3-Cy5 and Cy5-Cy3), resulting in two hybridizations per experiment and eight hybridizations in total. The hybridized arrays were scanned with Scanarray 5000XL (Perkin-Elmer, Freemont, CA), and hybridization results were quantified with Scanalyze software (http://rana.stanford.edu/software/). Array analysis was performed as previously described (4, 31). *z* scores were determined for each datum point to calculate how many standard deviations that point lies from the population mean. *z* scores for each gene were averaged, and genes with *z* scores of 2 or greater were considered up- or downregulated (see Table S1 in the supplemental material).

Real-time RT-PCR. For real-time RT-PCR, cultures were grown in the presence or absence of 0.5 mM AI-2 for 3 h. Total RNA was isolated, and residual DNA was removed by using the Ambion DNase-I kit according to the manufacturer's instructions. The real-time RT-PCR was carried out with the Corbett Rotor-Gene 3000 real-time DNA detection system and the QuantiTect Sybr green RT-PCR kit (Qiagen) in 25-μl (total volume) reaction mixtures. These mixtures contained 12.5 μl Sybr green mix, 0.25 μl QuantiTect RT mix, 2 μM primer, and 10 ng RNA. Primers were designed with the Frodo software (40) (Table 1). The reverse transcriptase reaction was carried out at 50°C for 30 min, followed by denaturation at 95°C for 15 min. This was followed by 50 PCR cycles of 15 s at 94°C, 30 s at 59°C, and 30 s at 72°C.

For analysis, the cycle threshold (C_t) values obtained for each gene were converted to linear numbers by calculating $L_t = 2^{-Ct}$ and normalized with the SigA and 16S genes. Fold changes were obtained by dividing the normalized AI-2-treated values by the normalized negative control values for each gene.

RESULTS

AI-2 influences *M. avium* biofilm formation. *M. avium* biofilms grown in microtiter plates responded to the addition of AI-2 in a concentration-dependent manner. The CV stain

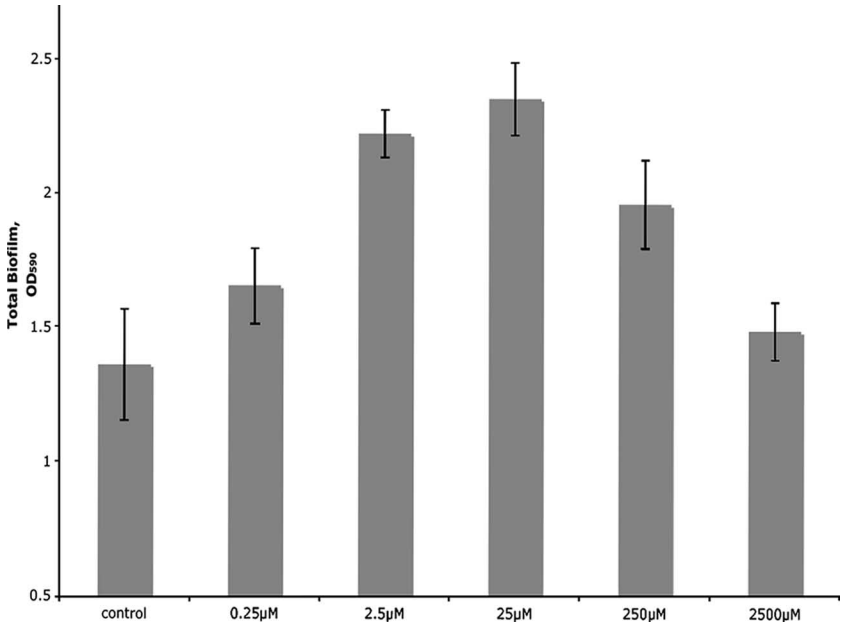

FIG. 1. CV staining of *M. avium* biofilms in 96-well microtiter plates. The addition of AI-2 increased biofilm formation in *M. avium* cultures. The optimum concentration for this induction was 25 μM; higher concentrations resulted in a lesser or no increase in biofilm biomass compared to the negative control.

showed that biofilm formation increased in the presence of 0.25 μM, 2.5 μM, and 25 μM AI-2 with 1.2-, 1.6-, and 1.7-fold increases in biofilm biomass compared to the negative control (Fig. 1). This effect was attenuated with even greater concentrations of AI-2. In the presence of 250 μM and 2,500 μM AI-2, induction decreased to 1.4- and 1.1-fold, respectively. The fact that biofilm formation occurs at an optimum concentration of AI-2 and declines at higher concentrations indicates that other environmental factors, such as starvation, are not major factors promoting biofilm formation in this study.

Confocal laser scanning microscopy confirmed these findings and showed that increased AI-2 concentrations lead to an increase in biofilm biomass and in the complexity of the three-dimensional structures of the biofilm (Fig. 2). Without AI-2, attachment is sparse and covers only a small portion of the surface (Fig. 2a). In the presence of 2.5 μM AI-2, attachment is increased and very dense cell clusters become visible (Fig. 2b). At 25 μM AI-2, the biofilm height is increased and the surface is almost fully covered. The biofilm surface appears more homogeneous (Fig. 2c).

AI-2 induces an oxidative stress response in *M. avium*. The finding that AI-2 influences the biofilm phenotype led to the question of how gene expression is affected by the molecule. Therefore, differential gene expression was studied by microarray analysis. For this, planktonic *M. avium* cultures were grown in parallel in the presence or absence of AI-2 at 37°C. OD readings after incubation were taken to demonstrate that AI-2 did not have a growth effect on the planktonic cultures compared to the negative control (data not shown). The RNA of both cultures was extracted, and microarray analysis was performed. The experiment was performed in triplicate for an incubation time of 3 days. Furthermore, an incubation time of 2.5 h was tested to assess the immediate response of *M. avium* to AI-2. Of the >4,000 genes in the *M. avium* genome and independently of the incubation time, 5 were reproducibly upregulated in response to the addition of 0.5 mM AI-2 (Fig. 3). The upregulation was confirmed by RT-PCR (Fig. 4). The fold inductions are compared and summarized in Table 2. The alkyl hydroperoxidases AhpC and AhpD, as well as the trehalose synthase TreS, are involved in the bacterial oxidative stress response. AhpC and AhpD provide antioxidant protection by removing peroxides from the environment and, upon macrophage entry, detoxify the ROS produced by the host immune system (25, 48). Trehalose is an important cell wall component

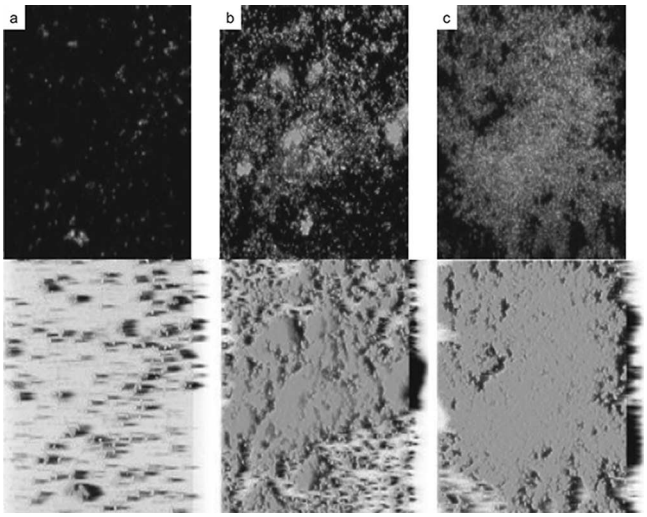

FIG. 2. Biofilms grown in the presence of AI-2. The negative control (a) shows the smallest amount of biofilm, whereas increasing the AI-2 concentration to 2.5 μM (b) or 25 μM (c) resulted in increased biofilm formation.

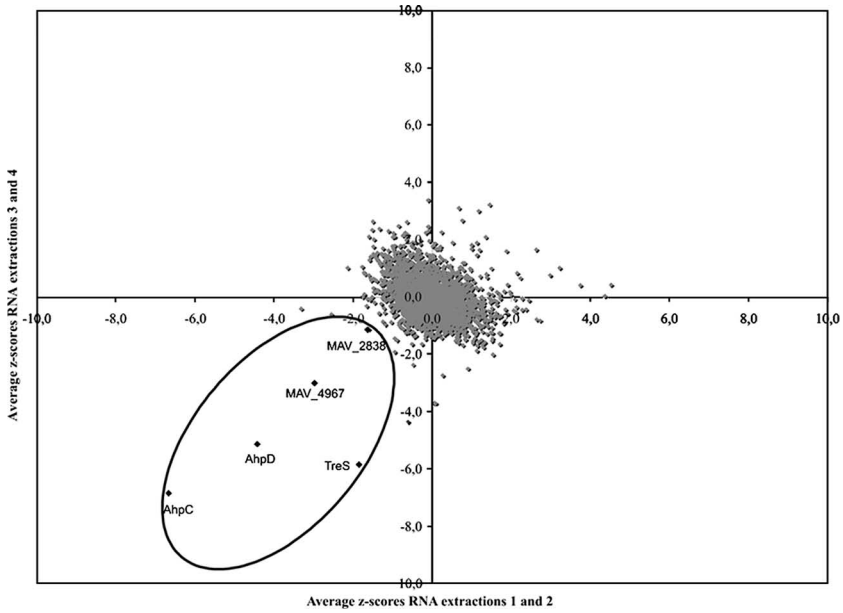

FIG. 3. Transcriptional profile of AI-2-treated cells versus the negative control determined by microarray analysis. Genes with negative *z* scores are upregulated in the AI-2-treated samples.

in mycobacteria since it increases impermeability and therefore prevents toxic compounds from entering the cell (15, 36).

The three genes are in direct proximity and divergently transcribed from the transcriptional regulator MAV_2838. MAV_2838 is annotated as a homologue to the OxyR transcriptional regulator, based on 38% identity and 53% similarity at the peptide level by BLASTP analysis. MAV_2838 exhibited a modest degree of upregulation, as seen with many transcriptional regulators. In *Mycobacterium* spp., OxyR is both a sensor of oxidative stress and a transcriptional activator by induction of the *ahpC* and *ahpD* genes (51). OxyR has been shown to be inactive in *M. tuberculosis* due to numerous deletions and frameshift mutations but is functional in *M. avium* (46). The fifth gene, MAV_4967, is a conserved hypothetical protein of unknown function.

Hydrogen peroxide induces biofilm formation in *M. avium*. The microarray study suggested that AI-2 triggers an oxidative stress response in *M. avium*. Therefore, hydrogen peroxide, known to induce the same genes (35, 46), was used to test the influence of oxidative stress on *M. avium* biofilm formation. The CV stain of biofilms grown in the presence of 5 mM and 50 mM hydrogen peroxide showed increased biofilm biomass, while hydrogen peroxide at 0.5 mM and 0.05 mM did not influence this parameter (Fig. 5).

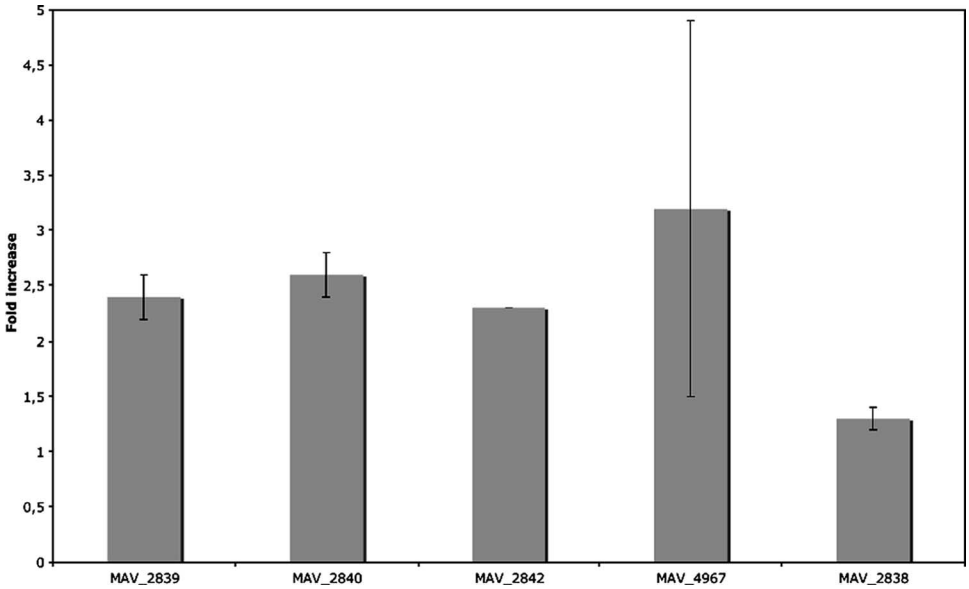

FIG. 4. Fold inductions determined by quantitative RT-PCR.

TABLE 2. Genes upregulated in the presence of AI-2

Gene	Product	Fold induction	
		Microarray	RT-PCR
MAV_2839 (*ahpC*)	Alkyl hydroperoxide reductase C	8.1 ± 2.4	2.4 ± 0.2
MAV_2840 (*ahpD*)	Alkyl hydroperoxide reductase D	5.8 ± 2.3	2.6 ± 0.2
MAV_2842 (*treS*)	Trehalose synthase	4.0 ± 1.6	2.3 ± 0.0
MAV_4967	Conserved hypothetical protein	2.5 ± 0.8	3.2 ± 1.7
MAV_2838	Transcription regulator	1.8 ± 0.9	1.3 ± 0.1

DISCUSSION

The environmental bacterium *M. avium* is able to adapt to a wide variety of external conditions and can be found in water, soil, and animal sources. The bacterium can be found aerosolized, in aqueous suspension, or attached to surfaces as biofilms. Due to its enhanced natural resistance to chlorination and heat (33), the bacterium has been isolated from drinking water, hot tubs, sauna walls, shower heads, and swimming pools, where it represents a health risk to the immunocompromised and others.

Microorganisms in the environment live predominantly in biofilms, which enables them to withstand harsh conditions and increases their resistance to antimicrobial agents. It has been shown that environmental signals and bacterial interactions are very important for biofilm formation (49). Therefore, we studied the influence of the universal quorum-sensing signal AI-2 on *M. avium* biofilm formation. The addition of AI-2 to *M. avium* biofilms led to increased biofilm formation, indicating that *M. avium* is able to sense the molecule. The concentration of the added AI-2 was important to this effect. The optimum concentration was between 2.5 and 25 μM, whereas the addition of 2,500 μM did not induce biofilm formation. The concentration-dependent effect of AI-2 on biofilm formation has been shown for the oral bacteria *Actinomyces naeslundii* and *Streptococcus oralis*, where the optimal concentration lies between 0.08 and 0.8 nM (39). One possible explanation for this enormous difference in concentration optima is that AI-2 does not act as an AI in *M. avium*, which does not have the LuxS synthase. Instead, AI-2 produced by a different bacterial species might act as a cross-species signal or a parainducer.

Microarray analysis revealed a set of five genes that are consistently upregulated upon the addition of AI-2. *ahpC*, *ahpD*, and *treS* are situated immediately adjacent to each other in the genome. The gene for the transcriptional regulator MAV_2838 is situated in close proximity to these three genes and may regulate their transcription. The conserved hypothetical protein MAV_4967 does not cluster with these genes. *ahpC* and *ahpD* encode alkyl hydroperoxidases that contribute to the mycobacterial oxidative stress response by detoxifying ROS, especially organic peroxides and hydrogen peroxide. This defense mechanism is crucial for survival and persistence inside macrophages. The upregulation of *ahpC* and *ahpD* upon the addition of AI-2 suggests that these proteins may also have important roles outside the mammalian host.

The trehalose synthase TreS converts maltose to trehalose and is capable of catalyzing the reaction in both directions (8). Trehalose is a nonreducing disaccharide and has a protective effect on proteins and biological membranes exposed to environmental stresses by increasing cell wall impermeability. Trehalose is a major constituent of many glycolipids in the mycobacterial cell wall, such as trehalose 6,6′-dimycolate (cord factor) (15). Furthermore, the sugar plays a role in mycolic acid transport during cell wall biogenesis.

The gene for the transcriptional regulator MAV_2838 is a homologue of the *oxyR* gene, the mycobacterial equivalent of the central regulator of the oxidative stress response in gram-negative bacteria. OxyR is a peroxide-sensing positive regula-

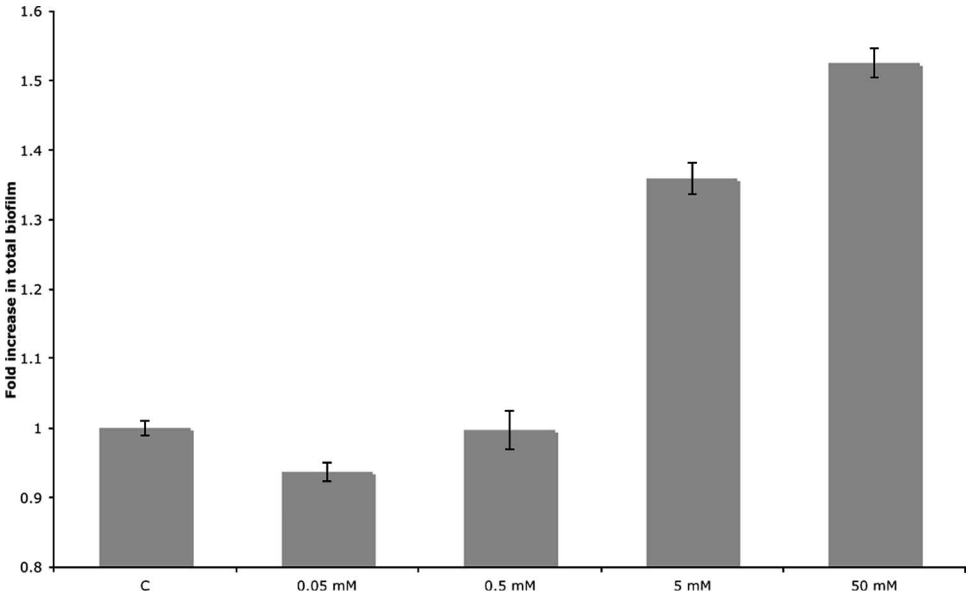

FIG. 5. Influence of H_2O_2 on *M. avium* biofilm formation.

tor, and its gene is tightly linked to and divergently transcribed from *ahpC* (18). These five genes are not biofilm specific, since the array studies were performed with planktonic *M. avium* cultures. However, the results show that AI-2 triggers an oxidative stress response in *M. avium*, suggesting that AI-2 acts not as a quorum-sensing signal per se but as an environmental cue. This model is supported by the fact that addition of hydrogen peroxide also resulted in increased biofilm formation. Kovacic (23) suggested that AI-2 could act as a ROS. Analysis of the chemical structure of DPD, the AI-2 precursor, suggests a possible role in electron transfer, a process generating ROS. Therefore, it is possible that the OxyR regulator senses AI-2 and triggers the oxidative stress response. Biofilm formation has been shown to be a response to oxidative stress in the mammalian host, as well as in the environment. The oxidative stress response is crucial for the survival of the pathogen within the host. A recent study correlated the ability to form biofilms with the invasiveness of *M. avium* cells (56). This may be associated with the morphotypic expression of cell wall structures (14); however, the oxidative stress response may also play an important role.

A study by Seib et al. (43) shows that OxyR is necessary for biofilm formation in *Neisseria gonorrhoeae*. The link between biofilm formation and oxidative stress has been shown in a number of bacteria, including *E. coli* (42), *Helicobacter influenzae* (32), *Campylobacter jejuni* (41), and *Streptococcus mutans* (55). These examples show that biofilm formation can be linked to oxidative stress as a reaction to changing environmental conditions.

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