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Interleukin-1β-induced growth enhancement of *Staphylococcus aureus* occurs in biofilm but not planktonic cultures

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Abstract

Staphylococcus aureus causes recalcitrant infections and forms resistant biofilms. Mechanisms of biofilm resistance to host defenses may include changes in gene expression that confer responsiveness to chemical mediators. In earlier studies fresh clinical isolates responded to inflammatory cytokines, but responsiveness was lost after multiple in vitro passages [Meduri et al. Cytokines IL-1β, IL-6, and TNF-α enhance the In vitro growth of bacteria. Am J Respir Crit Care Med 1999;160:961–7]. Since biofilms more closely resemble in vivo growth and are implicated in recalcitrant infections, we hypothesized that biofilms, but not planktonic cells, would respond to cytokines. Biofilms were induced by ethanol in *S. aureus* ATCC 12600. Biofilms treated with 2 ng/mL interleukin-1β (IL-1β) for 6 h contained 2.5-fold more cells than untreated biofilms, but no growth-enhancement occurred in planktonic cultures. As determined by flow cytometry, IL-β bound to 63.1% of biofilm cells, but only 11.2% of planktonic cells. Our results provide evidence of a differential response of biofilm and planktonic bacteria to chemical mediators, and suggest that biofilm bacteria may evade host defenses by growing more rapidly in response to the inflammatory mediators released by activated host defense cells.

Keywords: *Staphylococcus aureus*; Biofilm; Interleukin-1β; Cytokine; Flow cytometry

1. Introduction

Biofilms are communities of microorganisms encased in self-produced polymeric matrices and attached to surfaces, forming complex structures [1]. Biofilms are more resistant to chemical biocides, antibiotics, and host defenses than planktonic cells of the same species [2–6]. Initial explanations of increased resistance focused on limited diffusion of biocides and antibiotics through the exopolysaccharide matrix [7,8]. Other explanations include the slower metabolism of biofilm bacteria resulting in low efficacy by antibiotics or biocides which require actively growing cells, as well as the presence of persister cells [9]. Differential gene expression by biofilm cells has also been suggested to contribute to increased resistance. Beenken and colleagues identified 48 genes in *Staphylococcus aureus* whose expression was induced in biofilms compared to stationary or exponential planktonic cells, and 84 genes which had reduced expression in the biofilms [10]. The function of many of the identified genes is unknown.

Biofilms cause chronic and recalcitrant infections in medical settings. Biofilms form on tracheostomy tubes, catheters, and contact lens cases, and cells released from the biofilms can cause infections [11–13]. *S. aureus* and *S. epidermidis* have been implicated in biofilm infections of sutures, exit sites, arteriovenous shunts, central venous and Hickman catheters, mechanical heart valves, and orthopedic devices (rev. in [14]). The trademark exopolysaccharide matrix of *Staphylococcus* biofilms is also known as slime or as polysaccharide intercellular adhesin (PIA), a polysaccharide composed of β-1,6-linked *N*-acetylglucosamines with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defense and antibiotic treatment [15]. PIA is a product of the *ica* operon, which is composed of the *icaR* regulatory gene and *icaADBC* biosynthesis genes [16]. The expression of *ica* operon is complex; it is modulated by ethanol and various

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environmental conditions [17], and is influenced by the alternative sigma factor *sigB* [18] and the repressor icaR [19]. The DNA-binding protein SarA appears to be essential for *ica* gene expression and biofilm formation [10,20].

Cytokines produced by cells of the innate immune system have bidirectional effects on the elimination of invading microbes. Invading microbes trigger an inflammatory response mediated in part by the proinflammatory cytokines IL-1β, TNF-α, MIP-1α, and IL-6, largely secreted by early responding monocytes and macrophages. While generally thought to be necessary for bacterial clearance [21–23], some patients with elevated levels of cytokines have more frequent bacterial infections [24,25], suggesting that cytokines stimulate bacterial growth. However, observation of acquired impairment of neutrophil chemotaxis in patients receiving IL-2 therapy suggests that impairment of host defense cell function may be responsible for elevated bacterial levels rather than growth promoting effects of cytokines on bacteria [26].

Several in vitro studies demonstrate growth promotion by cytokines in fresh clinical isolates of *Escherichia coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Mycobacterium avium* [27–32]. Lee and co-workers [33] used the same strains of bacteria in vivo and in vitro to examine the effects of cytokines on bacterial growth promotion and for recruitment of antibacterial host cells. TNF-α increased the in vitro growth of *E. coli*. However, in vivo, TNF-$\alpha-/-$ mice had higher mortality than wild-type mice when inoculated with bacteria, due to impaired neutrophil recruitment. This suggests that while TNF-α did increase the growth of bacteria, normal neutrophil recruitment negated this effect. When animals were made leukopenic, bacterial counts were 100-fold higher in TNF$+/+$ mice than TNF$-/-$ mice, indicating that bacteria can use TNF-α as a growth factor in vivo, and that in immunocompromised patients, this bacterial growth promotion may be detrimental [33].

Studies of the effects of cytokines on growth promotion of bacteria have been hampered by the inability to consistently observe growth promotion in the in vitro systems used. While Porat and colleagues [27] originally reported the growth promotion of virulent *E. coli* by IL-1, other researchers were unable to repeat the experiments [34]. Porat and colleagues further experimented and noted a loss of IL-1 responsiveness upon passaging bacteria in serum or in broth, or upon storage at $-20\,^{\circ}C$ [35]. In the studies with fresh clinical isolates of *S. aureus, P. aeruginosa*, and *Acinetobacter* spp. [31], the authors document the loss of cytokine responsiveness after six in vitro passages. We hypothesize that the variation observed by these researchers is due to the loss of the biofilm phenotype after several in vitro passages in liquid growth media. Since biofilm conditions more closely resemble in vivo growth and are often implicated in recalcitrant infections, we hypothesize that bacterial growth enhancement in response to mammalian cytokines is dependent upon gene products selectively expressed during growth as biofilms rather than as planktonic cells. The ability of biofilms to preferentially utilize the cytokines expressed by activated inflammatory cells for their own growth promotion may contribute to the increased resistance of biofilm bacteria to host defenses in comparison to planktonic cells. Determining the in vitro conditions necessary to study bacterial responsiveness to cytokines will allow for the elucidation of the molecular mechanisms involved, and may lead to new molecular targets for the treatment of bacterial biofilm infections.

2. Results

2.1. Biofilm formation in S. aureus is strain and density dependent

Biofilm formation within single species of bacteria can vary by strain, and small genetic variations also exist between strains. Since we wished to examine cytokine-responsiveness in readily available, non-clinical isolates of bacteria of the same genetic background, we tested the environmental stressor, ethanol, which enhanced biofilm formation in *S. epidermidis* [17]. After overnight treatment of four staphylococci species in the presence and absence of 4% ethanol, adherent biofilm cells were quantitated by crystal violet staining. The results of this experiment demonstrated three aspects of biofilm formation. First, biofilm formation without ethanol addition was strain-specific (Fig. 1); little biofilm formation was observed in *S. aureus* ATCC 6538 and *S. aureus* ATCC 12600 without ethanol addition, while biofilms formed readily in *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984, a known biofilm-forming strain [15]. Second, biofilm formation in the presence of ethanol was also strain-specific. There was no effect of ethanol on biofilm formation for *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 35984, while formation was enhanced by 50–100% for *S. aureus* ATCC 25923; this change was significant at the low- and mid-cell-seeding densities ($P<0.05$). One strain, *S. aureus* ATCC 12600, showed large increases (2- to 5-fold) in biofilm formation in the presence of 4% ethanol when seeded at the hi- and mid-cell-seeding densities ($P<0.005$). Third, cell-seeding density at the initiation of the experiment affected biofilm formation. For *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984, biofilm formation was not observed, regardless of ethanol concentration, at the lowest cell seeding density (final $OD_{600} = 0.02$), even though biofilms were able to form at higher densities. Based on the observation that minimal biofilm formation occurred without ethanol treatment, and that biofilm formation was consistently increased several-fold in the presence of ethanol, *S. aureus* ATCC 12600 was chosen as an experimental strain to study differential responses to cytokines in cells growing as biofilms or as planktonic cells.

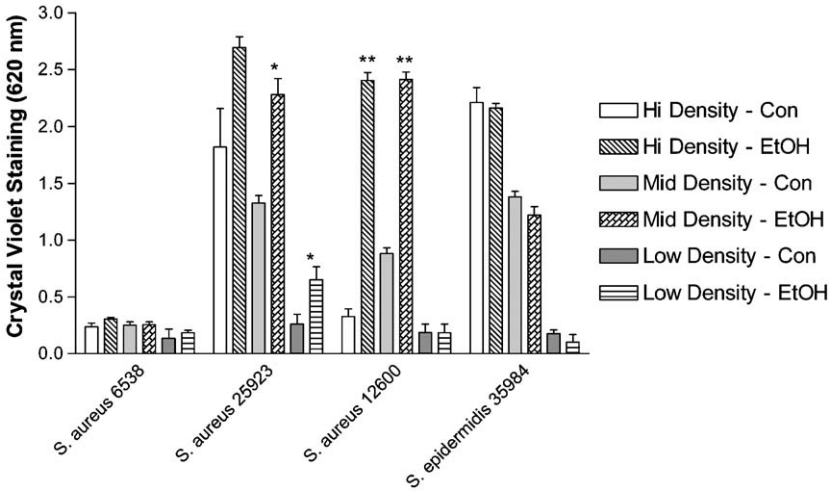

Fig. 1. Induction of Biofilm phenotype by 4% ethanol is dependent on cell seeding density and bacterial strain. Three-hour bacterial suspensions were used to seed 96-well tissue culture plates at a high density ($OD_{600} = 0.08$), medium density ($OD_{600} = 0.04$), and low density ($OD_{600} = 0.02$), using TSB medium containing 0.25% glucose. Biofilms were allowed to form overnight in the presence and absence of 4% ethanol and were quantified by crystal violet staining. Each bar represents the mean $\pm$SEM of triplicate samples. The experiment shown is representative of two experiments. A two-tailed *t*-test was used for statistical analysis to compare biofilm formation in the presence and absence of 4% ethanol. $^{*}P<0.05$. $^{**}P<0.005$.

2.2. *Expression of icaD is upregulated in biofilms of S. aureus*

Since staphylococcal biofilms are known to produce large amounts of PIA, we examined the expression of the *ica* operon and two other genes in ethanol-treated and untreated cells of the three *S. aureus* strains used in Fig. 1. For *S. aureus* ATCC 6538, biofilms do not form in the presence of ethanol, so RNA was isolated from non-adherent cells only. For *S. aureus* ATCC 12600, ethanol-treatment resulted in primarily adherent cells, and untreated cells were primarily non-adherent, so adherent and non-adherent cells were used for RNA isolation. *S. aureus* ATCC 25923 forms biofilms regardless of ethanol treatment, so RNA was isolated from adherent cells only. Purified RNA was subjected to real-time reverse transcriptase polymerase chain reaction (RT-PCR), a method that generates curves for determining the relative levels of specific transcripts present in the original RNA sample. The cycle number at which product formation exceeds a threshold value, C_t, is directly proportional to initial transcript levels. High abundance transcripts have low C_t values, and low abundance transcripts have high C_t values. So that increased expression correlates with an increased value, Table 1 shows the values expressed as the reciprocal of C_t ($1/C_t$), and all data are normalized to the constitutively expressed gene, *gyrB* [36]. The expression of *icaD* in the untreated cultures was lowest in *S. aureus* ATCC 6538 with a value of 0.68 ± 0.01, was intermediate in *S. aureus* ATCC 12600 with a value of 0.88 ± 0.02, and was highest in *S. aureus* ATCC 25923, with a value of 1.07 ± 0.04 (all differences were significant; $P<0.01$). Treatment with 4% ethanol did result in a higher *icaD* expression in *S. aureus* ATCC 6538, but the increased expression was not as high as expression in the other two strains. Treatment of *S. aureus* ATCC 12600 cultures with ethanol resulted in a significant increase in *icaD* gene expression, from 0.88 ± 0.02 to 1.20 ± 0.10 ($P<0.01$); the values from the ethanol-induced *S. aureus* ATCC 12600 were similar to the always-adherent *S. aureus* ATCC 25923. There was no significant difference in *atl* gene expression, an autolysin previously identified as involved in biofilm formation [37], in untreated cultures of the three strains. Treatment with ethanol resulted in a significant reduction in gene expression in *S. aureus* ATCC 6538, and increased expression in *S. aureus* ATCC 12600, although this increase was not considered statistically significant. Expression of a different autolysin gene, *lytM,* known to be regulated by the staphylococcal accessory regulator [38], was significantly lower in untreated *S. aureus* ATCC 6538 cultures than in the other strains ($P<0.05$). Treatment with ethanol resulted in significant increases in expression in both *S. aureus* ATCC 6538 and *S. aureus* ATCC 12600 ($P<0.05$). Products of the expected size were produced in the RT-PCR reactions, indicating that the fluorescence values observed correlate with the expected PCR product (data not shown). These gene-expression studies verify that treating *S. aureus* ATCC 12600 and *S. aureus* ATCC 6538 cells with ethanol results in increased expression of the *lytM* and the *ica* operon. In addition, cells were adherent in cultures with *icaD* expression levels 1.05 ± 0.02 or above, and cells were not adherent in cultures with *icaD* expression levels 0.88 ± 0.02 or below, suggesting that biofilm phenotype is associated with the expression of this operon, similar to what is known about *ica* expression in *S. epidermidis*. Biofilm induction of *S. aureus* ATCC 12600 by ethanol provides a useful model for studying differential gene expression in biofilm and planktonic bacteria.

Table 1
Real-time RT-PCR analysis of gene expression in cultures of *S. aureus* ATCC 6538, *S. aureus* ATCC 12600, and *S. aureus* ATCC 25923

	Gene/*gyrB* ($1/C_t/1/C_t$)				
S. aureus Strain	EtOH	Adherent	*icaD*	*Atl*	*lytM*
ATCC 6538	–	No	$0.68\pm0.01^{d,e,g,i,k}$	0.90 ± 0.04	$0.50\pm0.02^{d,e,h,j,l}$
ATCC 6538	+	No	$0.78\pm0.02^{b,e,g,i,k}$	$0.76\pm0.06^{e,h,j}$	0.60 ± 0.03^{b}
ATCC 12600	–	No	$0.88\pm0.02^{a,c,g,i,k}$	1.00 ± 0.04^{c}	$0.70\pm0.04^{a,h}$
ATCC 12600	+	Yes	$1.20\pm0.10^{a,c,e}$	1.22 ± 0.19^{d}	$0.91\pm0.16^{b,f}$
ATCC 25923	–	Yes	$1.07\pm0.04^{a,c,e}$	0.97 ± 0.02^{d}	0.69 ± 0.04^{b}
ATCC 25923	+	Yes	$1.05\pm0.02^{a,c,e}$	0.92 ± 0.01	0.65 ± 0.04^{b}

Data (mean ± SE) were obtained from two experiments performed in duplicate for *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 and from four experiments performed in duplicate for *S. aureus* ATCC 12600. Two-tailed *t*-tests were used for statistical analysis and the following significant differences were noted:

[a] $P<0.01$ and [b] $P<0.05$ compared to non-ethanol-treated *S. aureus* ATCC 6538.
[c] $P<0.01$ and [d] $P<0.05$ compared to ethanol-treated *S. aureus* ATCC 6538.
[e] $P<0.01$ and [f] $P<0.05$ compared to non-ethanol-treated *S. aureus* ATCC 12600.
[g] $P<0.01$ and [h] $P<0.05$ compared to ethanol-treated *S. aureus* ATCC 12600.
[i] $P<0.01$ and [j] $P<0.05$ compared to non-ethanol-treated *S. aureus* ATCC 25923.
[k] $P<0.01$ and [l] $P<0.05$ compared to ethanol-treated *S. aureus* ATCC 25923.

2.3. IL-1β enhances S. aureus growth in biofilm but not planktonic cultures

To determine if cytokine responsiveness is dependent on biofilm growth, we used one cytokine, IL-1β, and induced biofilm formation in *S. aureus* ATCC 12600, using similar conditions to those described by Meduri [31] for cytokine studies with fresh clinical isolates of *S. aureus*. Biofilm and planktonic *S. aureus* ATCC 12600 cultures were prepared by overnight incubation of bacteria in the presence and absence of 4% ethanol. IL-1β was added to triplicate wells of both biofilm and planktonic bacteria. Previous experiments [31] using clinical isolates of *S. aureus* indicated that the maximal growth enhancing effect was observed between 1 and 10 ng/mL during a 6 h treatment with IL-1β, so 2 ng/mL was selected for use in these experiments. After 6 h the cells were harvested and enumerated using the LIVE/DEAD fluorescent stain. The results of multiple experiments comparing IL-1β-treated to untreated cultures are shown in Fig. 2. For biofilm cultures, IL-1β treatment resulted in a significant average 2.5-fold increase in the number of bacteria during the 6 h period ($P<0.01$). There was no difference in the number of bacteria in the IL-1β-treated and untreated planktonic cultures ($P=0.528$). In three experiments where bacteria were enumerated by traditional plate counts on TSA, a 6 h treatment with 2 ng/mL IL-1β resulted in a significant 3.4-fold increase in biofilm cultures (100% ± 2.4% SEM vs. 345% ± 41% SEM; $P=0.000527$), but no increase in planktonic cultures (100% ± 3.3% SEM vs. 101% ± 13% SEM; $P=0.971$).

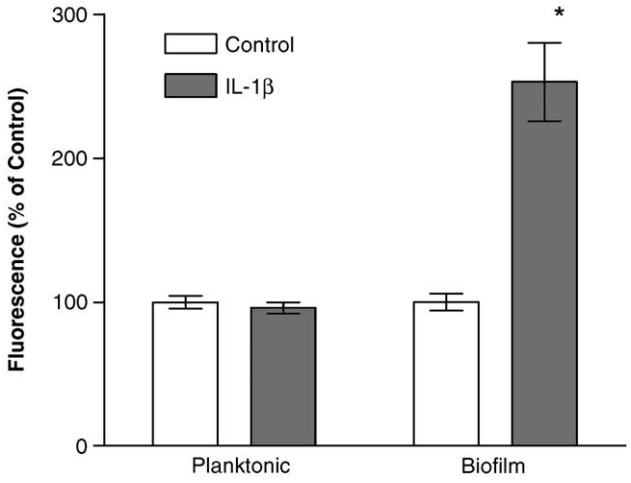

Fig. 2. Growth enhancement of *Staphylococcus aureus* biofilms by 6 h treatment with 2 ng/mL IL-1β. Biofilm and planktonic bacteria were cultured overnight in 24-well plates in TSB-0.25% glucose, the media were exchanged to RPMI-1640, and the bacteria were treated with human recombinant IL-1β for 6 h. The bacteria in each well were detached by scraping, and the bacteria were enumerated by LIVE/DEAD fluorescent staining. For each experiment the fluorescence data were normalized to the average data for the control wells. Data expressed are the mean ± SEM from 10 experiments performed in triplicate for biofilms, and from 6 experiments performed in triplicate for planktonic cells. A paired, two-tailed *t*-test was used for statistical analysis. *Denotes $P<0.01$. The range for the values from IL-1β treated cells from the multiple experiments was 120–600% for biofilm cells, and 78–112% for planktonic cells. Standard curves were prepared for each experiment and samples from curves were enumerated by standard plate counts. Converting the fluorescent data from these experiments revealed that biofilm cultures in the multiple experiments ranged from 1.6×10^{8}–1.7×10^{10} CFU/well, while planktonic cultures ranged from 1.6×10^{8}–1.1×10^{10} CFU/well. The LIVE/DEAD ratios did not differ significantly between biofilm and planktonic cells.

2.4. Anti-IL-1β treatment abrogates the growth-promoting effect of IL-1β

The growth-enhancing effect of IL-1β could be lessened by the addition of a polyclonal antibody to IL-1β. Fig. 3 shows the results of three independent experiments in which treatment of biofilms with 2 ng/mL IL-1β resulted in a 2.9-fold enhanced growth compared to the untreated cells ($P<0.000002$). Addition of an anti-IL-1β antibody prior to

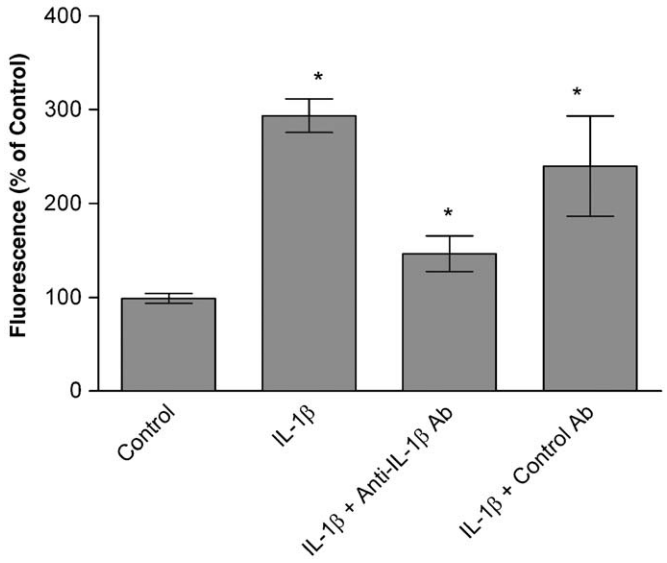

Fig. 3. The growth enhancing effect of IL-1β on Biofilm *S. aureus* is lessened by a polyclonal blocking antibody against IL-1β. Biofilm bacteria were grown for 6 h in RPMI-1640 media in the presence of a vehicle control, 2 ng/mL IL-1β, 2 ng/mL IL-1β plus 2 μg of an affinity purified antibody to IL-1β, or 2 ng/mL IL-1β plus 2 μg of a control antibody. Bacteria were enumerated with the LIVE/DEAD fluorescent stain. Data from three independent experiments performed in triplicate were normalized to the average value obtained in control wells and are expressed as means ±SEM. Two-tailed *t*-tests were used for statistical analysis; significant differences exist between vehicle control to IL-1β-treated ($P = 0.000002$), between vehicle control to anti-IL-1β antibody-treated ($P = 0.037$), between vehicle control and control antibody-treated ($P = 0.01$), and between IL-1β-treated and the anti-IL-1β antibody-treated ($P = 0.0007$).

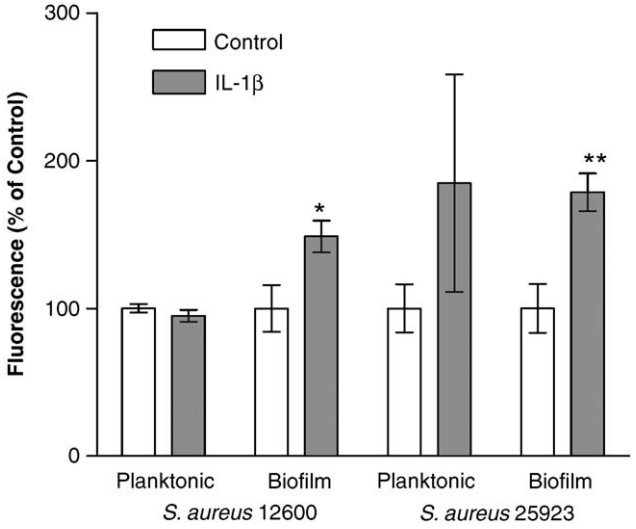

Fig. 4. Growth enhancement by IL-1β treatment also occurs in *S. aureus* strain ATCC 25923. Biofilm and planktonic bacteria were cultured overnight in 24-well plates in TSB-0.25% glucose, the media were exchanged to RPMI-1640, and the bacteria were treated with human recombinant IL-1β for 6 h. Ethanol was used for biofilm-induction in strain ATCC 12600 but not ATCC 25923. Bacteria were enumerated with the LIVE/DEAD fluorescent stain. Data from two independent experiments performed in triplicate were normalized to the average value obtained in control wells and are expressed as means ±SEM. Two-tailed *t*-tests were used for statistical analysis. *Denotes $P < 0.05$; **denotes $P < 0.01$.

the addition of the IL-1β resulted in only a 1.5-fold enhancement of growth, significantly lower than the 2.9-fold enhancement seen with IL-1β treatment alone ($P < 0.00007$). Simultaneous treatment with 2 ng/mL IL-1β and a control antibody resulted in a 2.4-fold increase in bacterial growth ($P = 0.01$), indicating that the abrogration of growth by the anti-IL-1β antibody was specific.

2.5. IL-1β-induced growth enhancement occurs in a strain of S. aureus that forms biofilms without ethanol treatment

To determine if the growth-enhancing effect of IL-1β on biofilms could be duplicated in another strain of *S. aureus* that does not require 4% ethanol to induce biofilms, the effect of IL-1β on biofilm and planktonic bacteria was studied in the induced *S. aureus* ATCC 12600, and in *S. aureus* ATCC 25923, a strain that readily formed biofilms without ethanol induction, as shown in Fig. 1. Fig. 4 shows that the growth of biofilm *S. aureus* ATCC 12600 was enhanced 1.5-fold ($P < 0.05$), while there was no effect on planktonic cells, consistent with earlier observations. In *S. aureus* ATCC 25923, there was a 1.8-fold enhancement of biofilm growth ($P = 0.01$). There was also an increase in the growth of planktonic cells, although the difference was not considered significant ($P = 0.71$). This suggests that the ability to respond to IL-1β is not specifically related to the ethanol used for induction of the biofilm phenotype, but rather that gene products which confer responsiveness to the cytokine are expressed in biofilm *S. aureus* cultures, regardless of the mechanism of biofilm induction.

2.6. Dose-dependent nature of the IL-β-induced growth enhancement of S. aureus biofilms

To determine if the growth enhancement of *S. aureus* biofilms by IL-1β is dose-dependent, we prepared overnight cultures of ethanol-induced biofilm and planktonic *S. aureus* ATCC 12600. IL-1β (0–10 ng/mL) or vehicle control was added to the cultures. After 6 h bacteria were enumerated by LIVE/DEAD fluorescent staining. Fig. 5 shows that IL-1β caused increased growth of biofilm but not planktonic bacteria at 1, 2 and 10 ng/mL, with maximum effects observed at 10 ng/mL IL-1β.

2.7. ^{125}IL-1β binds preferentially to biofilm rather than planktonic S. aureus

Initial experiments were performed with commercially available [^{125}I]IL-1β and biofilm cultures in order to determine the off-rate and the time needed to reach equilibrium binding so that appropriate binding conditions could be used. We determined that equilibrium was reached by 2 h at room temperature and that the half-life for dissociation of the [^{125}I]IL-1β from the biofilms was 2.7 min (data not shown). The very rapid off-rate required

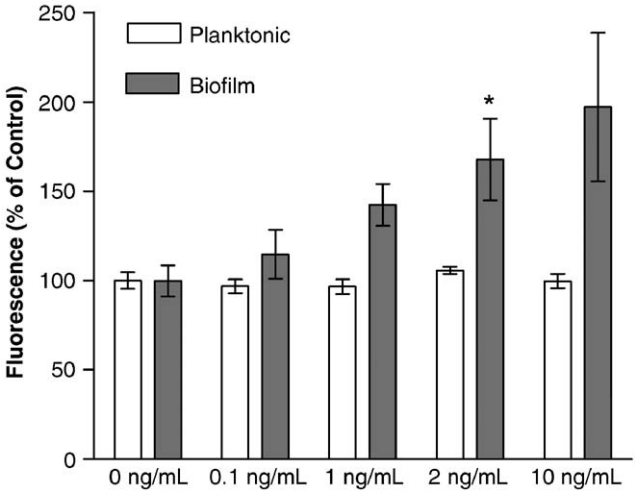

Fig. 5. Growth enhancement of *S. aureus* biofilms by IL-1β treatment occurs in a dose-response fashion. Biofilm and planktonic bacteria were cultured overnight in 24-well plates in TSB-0.25% glucose, the media were exchanged to RPMI-1640, and the bacteria were treated with vehicle control or 0.1–10 ng/mL human recombinant IL-1β for 6 h. The bacteria in each well were detached by scraping, and the bacteria were enumerated by LIVE/DEAD fluorescent staining. For each experiment the fluorescence data were normalized to the average data for the control wells. Data expressed are the mean $\pm$SEM for triplicate wells from one experiment, representative of three experiments. Paired, two-tailed *t*-test was used for statistical analysis. *Denotes $P<0.05$.

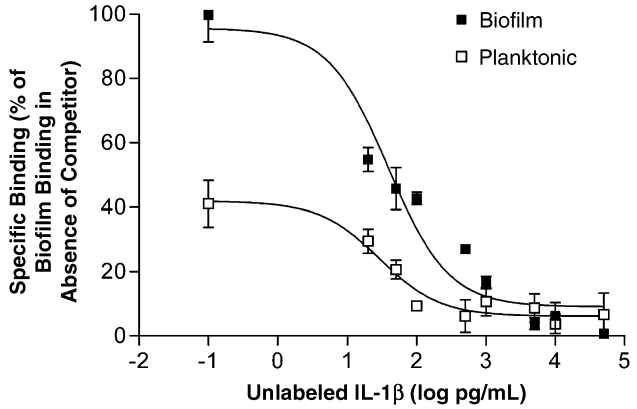

Fig. 6. High-affinity binding of ^{125}I-IL-1β to *S. aureus*. Biofilm and planktonic bacteria were cultured overnight in 12-well plates in TSB-0.25% glucose, the media were exchanged to RPMI-1640 containing 0.2% BSA, and the binding of ^{125}I-IL-1β to *S. aureus* ATCC 12600 biofilm and planktonic cells was measured in the presence of increasing concentrations of unlabeled IL-1β. Binding studies were performed at room temperature for 2.5 h, and data from triplicate wells for four independent experiments for biofilm cells and three independent experiments for planktonic cells have been normalized to the percent binding observed for biofilm cells in the absence of unlabeled competitor.

short and precise timing for separation of unbound ligand and for rinses. Binding assays were performed with iodinated IL-1β and induced biofilm cells or planktonic cells after overnight growth. The binding of [^{125}I]IL-1β was measured in the presence of increasing concentrations of unlabeled IL-1β, and Fig. 6 shows that specific binding was observed in both the biofilm and the planktonic cultures, suggesting that specific receptors were present in cells in both conditions. When the data were fit with an equation for a one-site competition model, it was noted that the concentration of unlabeled IL-1β required to reduce the [^{125}I]IL-β binding to 50% (IC_{50}) was similar for both the biofilm and planktonic (38.7 ± 1.5 and 29.1 ± 1.4 pg/mL, respectively). However, the amount of specific binding of [^{125}I]IL-1β was greater in the biofilm cells than in the density-adjusted planktonic cells, suggesting that more binding sites were present on the biofilm cells.

2.8. Preferential binding of biotinylated IL-1β to biofilm S. aureus is demonstrated by flow cytometry

Due to the low specific activity of commercially available IL-1β (15 000 cpm/100 pg), and the fast ligand off-rate, we also examined IL-1β binding to *S. aureus* using a flow cytometry system that allowed for higher signal-to-noise ratios and rapid removal of unbound ligand. Since fluorescence is associated with individual events and cell-bound ligand can be distinguished from unbound ligand based on size, unbound ligand does not need to be removed prior to analysis. Binding of IL-1β to planktonic and biofilm *S. aureus* was analyzed by flow cytometry using biotinylated IL-1β and avidin-FITC. Fig. 7A shows a greater percentage of cells with increased fluorescence in biofilm cells (63.1%) compared to planktonic cells (11.2%). In order to verify the specificity of this binding, a protein biotinylated to the same extent as IL-1β was used as a negative control, and Fig. 6B shows that only 9.8% of the biofilm cells bound the negative control protein, compared to 63.1% that bound IL-1β. An anti-IL-1β antibody blocked the binding of IL-1β to biofilm cells, while a control antibody did not block binding (Fig. 7B). In four independent experiments the percentage of cells binding IL-1β was significantly higher ($P=0.002$) in biofilms ($51\%\pm6\%$; SEM) compared to planktonic cells ($9\%\pm2\%$; SEM), and the percentage of biofilms binding IL-1β ($51\%\pm6\%$; SEM) was significantly higher ($P=0.006$) than the number of biofilms binding the negative control protein ($11\%\pm1\%$; SEM).

2.9. Other pro-inflammatory cytokines influence growth of biofilm S. aureus

Since the pro-inflammatory cytokine IL-β increased the growth of biofilm *S. aureus*, we wanted to determine if other cytokines that are also released from activated immune system cells would also differentially affect the growth of biofilm and planktonic *S. aureus*. *S. aureus* ATCC 12600 bacteria were grown overnight as planktonic or induced biofilm cultures, and the media were exchanged

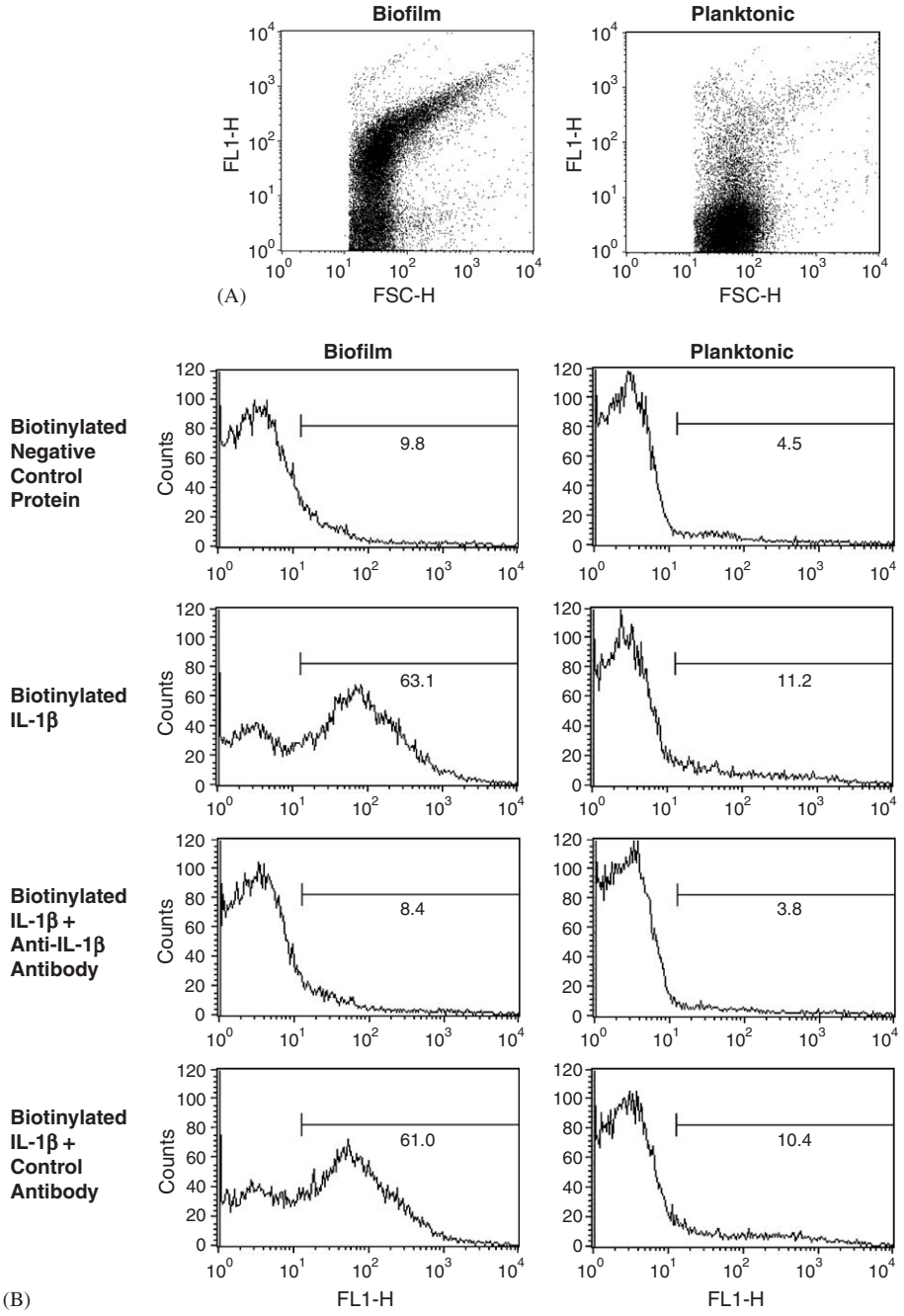

Fig. 7. Binding of biotinylated IL-1β to biofilm *S. aureus* cells. Biofilm and planktonic cells (2.5×10^6) were incubated with biotinylated IL-1β or biotinylated soybean trypsin inhibitor (negative control protein) in the presence or absence of anti-IL-1β antibody or a control antibody, followed by incubation with avidin-FITC. Flow cytometry was used to analyze 25 000 events. Panel A shows a dot plot of the binding of IL-1β to biofilm and planktonic cells, while Panel B shows histograms including the percentages of cells binding the biotinylated proteins. Data shown is representative of four independent experiments; the binding of IL-1β was significantly higher in biofilm compared to planktonic cells ($P<0.01$) and the binding of IL-1β to biofilm cells was significantly higher than the binding of the negative control protein to these cells ($P<0.01$).

to RPMI-1640. The cultures were treated with 2 ng/mL IL-1β, macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), or with heat-denatured IL-1β for 6 h. Fig. 8 shows that after 6 h treatment with 2 ng/mL of the various cytokines, there was a 1.5-fold increase in cell number for IL-1β, a 1.9-fold increase with MIP-1α, and a 1.9-fold increase with TNF-α, while IL-6 and heat-denatured IL-1β did not significantly increase growth. Since IL-6 did not promote bacterial growth, but IL-1β, MIP-1α, and TNF-α did, it appears that there is some level of specificity in the interaction of cytokines and biofilm bacteria. The lack of growth-promotion by heat-denatured IL-1β indicates that the bacterial growth promoting effect of cytokines is dependent on active cytokines. None of the indicated cytokines increased the growth of planktonic cells.

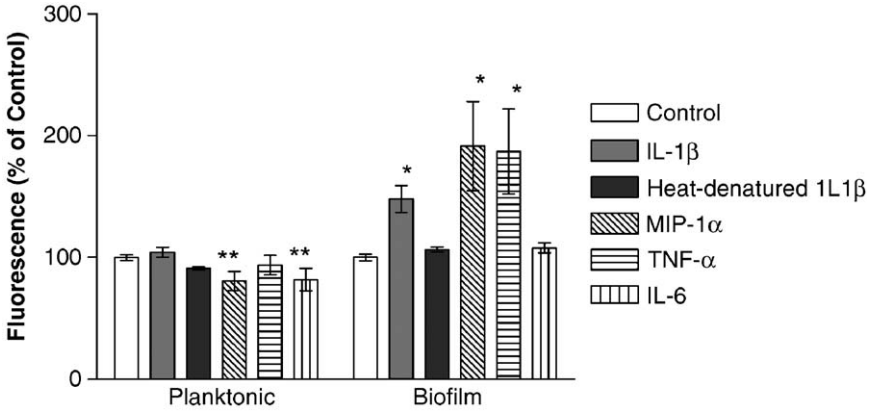

Fig. 8. Effect of various cytokines on the growth of *S. aureus* ATCC 12600 biofilm and planktonic cells. Biofilm and planktonic bacteria were cultured overnight in 24-well plates in TSB-0.25% glucose, the media were exchanged to RPMI-1640, and the bacteria were treated with 2 ng/mL of various cytokines for 6 h. The bacteria in each well were detached by scraping, and the bacteria were enumerated by LIVE/DEAD fluorescent staining. For each experiment the fluorescence data were normalized to the average data for the control wells. Data expressed are the mean ±SEM from two experiments performed in triplicate. A paired, two-tailed *t*-test was used for statistical analysis. *Indicates $P<0.01$; **indicates $P<0.05$.

3. Discussion

Our data demonstrate that proinflammatory cytokines increase the growth rate of biofilm, but not planktonic *S. aureus*. This suggests that gene products that are selectively expressed during growth as biofilms are responsible for the responsiveness to cytokines. Our data provide a potential explanation for the loss of cytokine-responsiveness after in vitro passaging of clinical isolates that was observed by previous researchers. Biofilm conditions more closely resemble in vivo growth and are often implicated in recalcitrant infections. The fresh clinical isolates used by Porat et al. [27] and Meduri et al. [31] may have likely been biofilm bacteria, and therefore responsive to cytokines. After in vitro passaging of the clinical isolates in liquid media, the biofilm phenotype may have been lost and the planktonic cells would no longer express the gene products responsible for cytokine-responsiveness. Our data demonstrating that growth of *S. aureus* as biofilms is necessary for cytokine-responsiveness will allow for further progress in understanding the molecular mechanisms that are used by bacteria to increase their growth in response to cytokines. This understanding will allow for the elucidation of the molecular mechanisms involved, and may lead to molecular targets for the treatment of bacterial biofilm infections.

Greater binding of IL-1β to biofilm cells than planktonic cells was also demonstrated. Other researchers have demonstrated cytokine binding to virulent *E. coli* [27], *M. avium* [39], *S. flexneri* [40], *S. aureus* [41], and *Yersinia pestis* [42]. Using ^{125}IL-1β with a specific activity $\approx$100 000 cpm/pg, Porat et al. [27] reported the specific binding to virulent but not avirulent *E. coli*, with saturation at 20 pg/mL and 20 000–40 000 sites per bacterium. The concentration of unlabeled IL-1β required to reduce the [^{125}I]IL-β binding to 50% (IC_{50}) was 150 pg/mL. However, Kanangat et al. [41] used 100 000 cpm (specific activity not stated) per data point and reported an IC_{50} value of $\approx$10 000 pg/mL for the binding and competition of IL-1β in *S. aureus*. The difference in IC_{50} values reported by Porat and Kanangat may be due to differences in binding between Gram-positive and Gram-negative bacteria, as has been reported by Luo et al. [40], but may also reflect technical difficulties in the binding experiments, particularly the fast ligand off-rate. Our IC_{50} values of 38.7 and 29.1 pg/mL, determined in experiments using one precise 3 min rinse, more closely align with the studies by Porat. We note that although binding of IL-1β to planktonic *S. aureus* did occur, the level of binding was significantly less than binding observed in biofilm *S. aureus*. This significantly lower level of binding was observed in both equilibrium binding studies and in flow cytometry experiments. Since growth enhancement in response to IL-β was not observed in planktonic cultures, the low level of binding to planktonic *S. aureus* suggests that the gene responsible for cytokine responsiveness is expressed at low levels in planktonic cells, but that higher expression levels and cytokine binding are needed to affect a physiological growth response.

It must be noted that in addition to observing a growth enhancement of biofilm cells, we also observed a growth enhancement of planktonic cells by IL-1β when *S. aureus* ATCC 25923 was used, although the growth enhancement was not considered to be statistically significant. We hypothesize that since *S. aureus* ATCC 25923 naturally forms biofilms without induction (see Fig. 1), the genes that are responsible for biofilm formation and for cytokine responsiveness may be constitutively expressed in this strain. It is also difficult to obtain a sufficient quantity of planktonic cells after a 6 h incubation of *S. aureus* ATCC 25923, which results in lower absolute cell numbers for planktonic cells and higher variation in this experiment.

We observed an average 2.5-fold enhancement of cell growth by the addition 2 ng/mL IL-1β to induced biofilms

of *S. aureus* after 6 h, with a range of 1.2- to 6-fold. This is less than the 10-fold growth enhancement of fresh clinical isolates of *S. aureus* by the addition of 1 ng IL-1β [31]. Perhaps the lower growth enhancement we observed was due to differences in strain (clinical isolates vs. ATCC 12600) or due to additional phenotypic modulation of bacteria by the growth factors and other mediators present in the host infection site. Additionally, the difference could be due in part to differences in bacterial enumeration methods. Because we were concerned with inadequate dispersal of biofilms to single cells when performing plate counts, we routinely used a LIVE/DEAD® *Bac*Light fluorescence technique that relies on membrane integrity to enumerate cells. We would expect that traditional plate counts might underestimate numbers of biofilm bacteria, since the likelihood of many cells contributing to one colony is more likely, but our data showing less growth enhancement than was observed by Meduri et al. do not support this hypothesis. When we used traditional plate counts to enumerate bacteria in three independent experiments, we observed an average 3.4-fold enhancement of growth after a 6 h IL-1β treatment of biofilm cells compared to untreated cells, which is higher than the 2.5-fold average enhancement that we observed in ten independent experiments enumerated by the fluorescence technique (Fig. 2). However, the 3.4-fold enhancement observed by plate counts is still within the 1.2- to 6-fold range observed in multiple experiments enumerated by the fluorescence technique.

Our data are consistent with and build on previous reports of growth enhancing effects of eukaryotic cytokines on bacteria. Since previous researchers have demonstrated responsiveness to cytokines in both Gram-positive [31] and Gram-negative [27] bacteria when tested as fresh clinical isolates, we predict that other species of bacteria that cause biofilm infections, such as *P. aeruginosa* in cystic fibrosis infections, may also be responsive to cytokines when the bacteria are grown as biofilms.

The ability of biofilm cells to grow more rapidly when confronted with mediators released from activated immune system cells may provide a mechanism that contributes to the ability of biofilm bacteria to resist destruction by host defenses. Earlier mechanistic explanations for increased resistance to host defenses included an inability of leukocytes to penetrate the biofilm, but Leid et al. [43] have demonstrated that leukocytes can adhere to and penetrate biofilms formed under shear conditions. These researchers provide evidence that leukocyte phagocytosis, but not cytokine production, was inhibited by biofilm cultures of *S. aureus* [43]. Vuong and colleagues demonstrated that deacetylation of PIA helps *S. epidermidis* resist polymorphonuclear leukocyte phagocystosis and killing by cationic peptides [44]. Our data suggest an additional mechanism that may contribute to the ability of biofilm bacteria to evade host defenses. If host leukocytes are able to penetrate biofilms and produce cytokines upon activation, and if bacteria that are growing as biofilms express genes that allow responsiveness to cytokines, then increased bacterial growth by the biofilm allows time for a larger nidus of bacteria to develop, as well as provide increased numbers of cells that may escape the biofilm and be dispersed elsewhere to form distant infections. Beenken and colleagues examined transcriptional profiles of all *S. aureus* genes under biofilm and planktonic conditions and noted that 48 genes were induced at least 2-fold under biofilm conditions [10]. The functions of several of these genes are unknown. We hypothesize that one or more proteins that are induced in *S. aureus* biofilms bind(s) to IL-1β and participate(s) in a regulatory pathway that results in an increased growth rate.

4. Conclusions

Our results provide evidence that IL-1β binds preferentially to *S. aureus* biofilms in comparison to planktonic cultures. Additionally, enhanced growth of *S. aureus* in response to cytokine treatment is observed in biofilm but not planktonic cultures. Our data suggest that genes enabling cytokine-responsiveness are upregulated in biofilms. Among other mechanisms, biofilms may evade host defenses by growing more rapidly in response to the inflammatory mediators released by activated host defense cells.

5. Materials and methods

5.1. Bacterial strains and biofilm induction

The bacteria used in this study were *S. aureus* ATCC 12600, *S. epidermidis* ATCC 35984, *S. aureus* ATCC 6538, and *S. aureus* ATCC 25923. The identity of bacterial strains was confirmed by the use of API Staph tests (bioMerieux, Durham, NC). Bacterial strains were grown for 3 h in 5 mL Nutrient Broth in a shaking water bath at 37 °C at 200 rpm. For biofilm studies, the OD_{600} of the 3 h cultures was determined, and aliquots were used to inoculate the wells of multi-well tissue culture-treated plates (Corning Costar, Cambridge, MA). The bacteria were cultured for 15–16 h (overnight) at 23 °C without shaking in Tryptic Soy Broth (TSB; DIFCO laboratories) containing 0.25% glucose. To test biofilm induction, some wells contained 4% NaCl or 4% ethanol in addition to the TSB-0.25% glucose [17]. Control wells contained TSB-0.25% glucose without the addition of bacteria. Biofilm induction by 4% ethanol or 4% NaCl was also tested using RPMI-1640 as growth medium instead of TSB containing 0.25% glucose.

5.2. Quantitation of biofilm formation by crystal violet staining

Biofilm formation was quantitated using previously reported methods [45]. Media and non-adherent cells were removed from each well by aspiration, and wells were

rinsed with 0.85% NaCl. One percent Crystal Violet was added to each well and the dishes were incubated for 10 min at 23 °C. Excess Crystal Violet was removed by aspiration and the wells were rinsed three times with 0.85% NaCl. The wells were allowed to air-dry in the inverted position and were photographed. For quantitation, the dye was solubilized with ethanol and the crystal violet absorbance was measured in a multi-well plate format spectrophotometer (Finstruments™ Multiskan MCC340 MKII 347, MTX Lab Systems, Inc.) at 620 nm. The absorbance values obtained from control wells that received media only at the time of cell seeding were subtracted from the experimental values for each well.

5.3. Treatment of planktonic and biofilm bacteria with cytokines and antibodies

Bacteria were seeded in multi-well tissue culture plates (Corning Costar, Corning, NY) at a final OD_{600} of 0.05 and were grown overnight at 23 °C in multi-well tissue culture plates in TSB-0.25% glucose or in TSB-0.25% glucose containing 4% ethanol in order to culture planktonic and biofilm bacteria, respectively. The defined medium, RPMI-1640, containing 2 mM L-glutamine and 4 mM sodium pyruvate was used for experiments with cytokines [31]. For wells containing biofilms, the non-adherent cells and media were aspirated, the biofilm layers were rinsed with 0.85% NaCl, and RPMI-1640 medium containing 4% ethanol was added to the wells. For wells containing planktonic cells, cells and media were transferred to microcentrifuge tubes and cell pellets were obtained by centrifugation at 5000*g* for 3 min in a microcentrifuge. The cell pellets were rinsed once with 0.85% NaCl, resuspended in RPMI-1640 medium, and transferred to an empty well. Recombinant human cytokines interleukin-1β, MIP-1α, TNF-α, IL-6, or anti-human IL-1β (Peprotech, Rocky Hill, NJ) were resuspended in sterile water and added to triplicate wells of either biofilm or planktonic bacteria. Control wells received an identical volume of sterile water. The plates were incubated at 23 °C for 0–9 h. Following cytokine treatment, all wells were scraped to detach adherent cells, and all of the cells in each well were transferred to separate microfuge tubes for bacterial enumeration. For antibody treatment, 2 μg anti-human IL-1β was incubated with 2 ng IL-1β for 15 min prior to addition to the bacterial cultures. For some experiments, IL-1β was heat denatured for 20 min at 95 °C.

5.4. Bacterial enumeration

Bacteria were enumerated either by standard plate counts on Tryptic Soy Agar plates, or by fluorescence using the LIVE/DEAD® *Bac*Light Bacterial Viability Kit (Molecular Probes, Eugene, OR), in accordance with the manufacturer's instructions. This kit uses two stains to differentiate between live and dead bacteria based on membrane integrity. Live bacteria stain with SYTO 9 dye and were measured with a fluorometer (Packard FluoroCount) using Excitation of 485 nm and Emission of 530 nm. Dead bacteria stain with propidium iodide, and an Excitation of 485 nm and Emission at 620 nm were used. For each experiment, standard curves were prepared from the experimental bacterial samples and were stained with the LIVE/DEAD® stain. Duplicate plate counts were also performed on one of the standard curve samples so that the fluorescence data could be related to colony forming units (CFU) measured by plate counts. Fluorescence standard curves have been linear over a two-log range.

5.5. RNA isolation

Total RNA was prepared from *S. aureus* cultures. *S. aureus* cultures were grown overnight in 6-well tissue culture plates (Corning Costar, Corning, NY) planktonically in TSB-0.25% glucose or as a biofilm in TSB-0.25% glucose containing 4% ethanol. The medium was exchanged to RPMI-1640 (planktonic cells) or RPMI-1640 containing 4% ethanol (biofilm cells), as described earlier. After 6 h incubation at 22 °C in RPMI-1640 media, the planktonic cells were transferred to microcentrifuge tubes, and cell pellets were obtained by centrifugation at 10,000*g* for 2 min. The supernatant was removed and 1 mL TRI-reagent (Sigma, St. Louis, MO) was added to the cell pellet for RNA isolation. For biofilm cells, the media and non-adherent cells were removed, the biofilm cell layers were rinsed once with 0.85% NaCl, and 1 mL TRI-reagent was added directly to the wells and cells were detached by scraping. The biofilm cells and TRI-reagent were transferred to microcentrifuge tubes for RNA isolation. Glass beads (0.4 g; 0.1 mm diameter; Biospec, Bartlesville, OK) and 25 μL of a saturated solution of hexa-decyl-trimethylammonium bromide [46] were added to the tubes containing TRI-reagent, and the cells were lysed with a 3-min pulse in a mini Bead Beater (Biospec). Total RNA was isolated from the cell lysate following the manufacturer's instructions. The RNA concentration was determined by measuring the OD_{260}. Ten micrograms of total RNA were treated with DNase using a DNA-free™ kit (Ambion, Austin, TX), and the concentration of DNA-free RNA was determined by measuring the OD_{260} and verified by ethidium bromide staining on agar.

5.6. Transcription analysis by real-time reverse transcriptase PCR (RT-PCR)

RT-PCR was carried out using the Access RT-PCR System (Promega, Madison, WI) and 0.5 μg total RNA per sample tube. Master mixes were prepared following the manufacturer's instructions, using a final concentration of each primer of 0.8 μM. Primers were obtained from The Midland Certified Reagent Company (Midland, TX) and the sequences were as follows: for *gyr* (GenBank accession no. **D10489**, nt 219–536), Forward 5′ TTA TGG TGC

TGG GCA AAT ACA, Reverse 5′CAC CAT GTA AAC CAC CAG ATA; for *icaD* (GenBank accession no. **AF086783**, nt 3532–3729), Forward 5′ATG GTC AAG CCC AGA CAG AG, Reverse 5′AGT ATT TTC AAT GTT TAA AGC AA; for *lytM* (GenBank accession no. **L77194**, nt 55–759), Forward 5′CAT GCA TTA GCG TTG AAT CG, Reverse 5′CTC GCG TCT GGA CCT ACA TT; for *atl* (GenBank accession no. **D17366**, nt 2326–2915), Forward 5′GCC TGT TGC AAA GTC AAC AA, Reverse 5′CAC CGA CAC CCC AAG ATA AG. Real-time PCR was carried out on a SmartCycler (Cepheid, Sunnyvale, CA) using 25 μL sample volume and a 45 min reverse transcriptase step at 48 °C, a 2 min denaturation step at 94 °C, and 40 cycles of PCR (94 °C for 30 s, 57 °C for 30 s, 68 °C for 70 s). Product formation was monitored at the end of each cycle via the fluorescence of the double-stranded DNA dye, SYBR green. Product formation was verified by electrophoresis of the PCR products on 1.0% agarose gels and visualization by ethidium bromide staining.

5.7. Binding of [^{125}I]interleukin-1β to S. aureus biofilms and planktonic cells

[^{125}I]Interleukin-1β (2250 Ci/mmol) was used for equilibrium binding studies (Amersham, Piscataway, NJ). In preliminary experiments it was determined that equilibrium was reached by 2 h of incubation at 22 °C. Biofilm cell layers that were grown overnight in TSB-0.25% glucose containing 4% ethanol were rinsed once with 0.85% NaCl, and the medium was replaced with Complete RPMI-1640 containing 0.2% bovine serum albumin (BSA) and 4% ethanol (0.5 mL per well in 12-well tissue culture plates). For comparison purposes, the cell density in biofilm wells was measured by harvesting the cells from similarly seeded biofilm wells, resuspending them in 1 mL Complete RPMI-1640, and measuring the optical density at 600 nm. Planktonic cells that were grown overnight in TSB-0.25% glucose were transferred to Eppendorf tubes, collected by centrifugation, rinsed with 0.85% NaCl, and resuspended in Complete RPMI-1640 containing 0.2% BSA at a similar density to the biofilm cells. At precise time intervals, [^{125}I]IL-1β (0.1 ng, 15 000 cpm, diluted in RPMI-1640 containing 0.2% BSA) was added to individual wells or tubes. For the determination of non-specific binding, unlabeled IL-1β (0.02–50 ng/mL) was added to some wells or tubes prior to the addition of the [^{125}I]IL-1β. The cells were incubated at 22 °C for 2.5 h. The free [^{125}I]IL-1β was separated from the bound [^{125}I-IL]1β by removing the binding medium from biofilm cell layers, or by centrifugation at 10 000 rpm for 2 min followed by aspiration for planktonic cells. The biofilm cell layers and planktonic cells were rinsed once by adding 0.5 mL 0.85% NaCl and removing the rinse after 3 min. Fresh medium (0.25 mL) was added to the wells or tubes, and adherent cells were detached from the wells by scraping and were transferred to scintillation vials. Planktonic cells were resuspended and transferred to scintillation vials. To lyse cells, 0.25 mL of a solution containing 0.85% NaCl, 0.2% BSA and 0.5% Triton X-100 was added to the cells. Bound [^{125}I]IL-1β was quantitated by adding 5 mL Ultima Gold^{TM} scintillation fluid (Sigma, St. Louis, MO) and counting in a scintillation counter (Beckman LS 6500) for 10 min. Total CPM from one well of a 12-well plate averaged 500 cpm. Values obtained for binding of [^{125}I]IL-1β to an empty well or an empty microcentrifuge tube were subtracted from the biofilm and planktonic experimental values, respectively, and the data from multiple experiments are expressed as the percent binding of biofilm cells in the absence of unlabeled competitor.

5.8. Flow cytometry

Flow cytometry was carried out using a FACSCalibur flow cytometer equipped with 488 and 635 nm lasers and FACS-Flow sheath fluid (BD Biosciences, San Jose, CA). Using CellQuest Pro software (Becton Dickinson), data were acquired and analyzed for forward scatter (FSC-H), side scatter (SSC-H), and FITC fluorescence (FL1-H). Instrument settings were set to maximize discrimination of bacteria from debris, and included a Forward Scatter setting of EO1, log amplification and threshold of 300; Side Scatter setting of 420, log amplification and threshold of 320; and FL1 setting of 570 with log amplification. A total of 25 000 events were collected using low flow rate. Planktonic or biofilm bacteria from 15 to 16 h overnight cultures were rinsed once with PBS and then fixed with 1% formaldehyde, 1% BSA, 0.01% EDTA in PBS for 2 h at 4 °C. A total of 1×10^8 cells (as determined by optical density and comparison to standard curve) were centrifuged for 2 min at 10 000 rpm, and the pellet was resuspended in 1 mL Dulbecco's PBS. A Fluorokine Kit for Human Interleukin-1β (R&D Systems, Catalog Number NFLB0; Minneapolis, MN) was used for flow cytometric analysis of IL-1β binding sites on bacteria. Biofilm or planktonic bacteria (25 μL; 2.5×10^6 cells) were mixed with biotinylated-IL-1β or biotinylated soybean trypsin inhibitor (negative control) for 1 h at 4 °C, followed by a 30 min incubation at 4 °C with avidin-FITC, according to the manufacturer's instructions. Cells were diluted to 1 mL with kit-provided wash buffer and used immediately for flow cytometry analysis. For specificity testing, kit-provided polyclonal goat IgG anti-human IL-1β blocking antibody or control antibody (polyclonal goat IgG; ICN/CAPPEL, Aurora, OH) were used following kit instructions.

5.9. Statistical analysis

The paired Student's *t*-test was used for the statistical analysis of data. Graphs were created using GraphPad Prism 4.03.

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