

Streptomycin Inhibits Quorum Sensing in *Acinetobacter baumannii*

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Streptomycin at subinhibitory concentrations was found to inhibit quorum sensing in *Acinetobacter baumannii*. Conditioned medium prepared by growth of *A. baumannii* in the presence of subinhibitory concentrations of streptomycin exhibited reduced activation of two quorum-sensing-regulated genes, *abaI*, encoding an autoinducer synthase, and A1S_0112. The reduced expression of *AbaI* resulted in greatly decreased levels of 3-OH-C₁₂-HSL as confirmed by direct analysis using thin-layer chromatography. The effect on acyl-homoserine lactone (AHL) signal production was specific to streptomycin, as gentamicin and myomycin had no significant effect at subinhibitory levels.

Acinetobacter baumannii is an aerobic Gram-negative nosocomial pathogen possessing mechanisms of resistance to all classes of antibiotics and is responsible for various life-threatening infections, including those of the lung, skin and soft tissue, urinary tract, and bloodstream (1–5). The ability of *A. baumannii* to survive under adverse conditions, resist desiccation, and form biofilms further complicates treatment and allows these organisms to colonize health care settings (6–11).

Many bacteria respond to their population density by controlling gene expression through quorum sensing, a phenomenon where bacteria respond to small, self-generated diffusible molecules (12, 13). Previous work in our laboratory has identified and characterized an autoinducer synthase (*AbaI*) required for production of the acyl-homoserine lactone (AHL) signal 3-OH-C₁₂-HSL in *A. baumannii* M2, where it plays a role in biofilm formation (14) and surface motility (15). Other studies have shown a similar role for *AbaI* in biofilm formation (16).

Surface motility has been described in *Acinetobacter* species (17–22). Motility has been linked to virulence in many pathogenic bacteria and is regulated by diverse mechanisms (23–25). Recent work in our laboratory has demonstrated that motility of *A. baumannii* strain M2 on low-percentage (0.2 to 4%) agar plates was dependent on quorum sensing, as an *abaI* mutant exhibited a severe reduction in motility that was rescued by the addition of 3-OH-C₁₂-HSL to plates (15). In the course of studying motility, we found that wild-type *A. baumannii* M2, with a streptomycin MIC of 16 µg/ml, exhibited a prominent defect in motility in the presence of subinhibitory concentrations of streptomycin (0.5 and 1 µg/ml) (Fig. 1A). In order to demonstrate that the reduction in motility in the presence of subinhibitory concentrations of streptomycin was not due to effects on growth rate, growth curve analysis was performed. As shown in Fig. 1B, the growth of *A. baumannii* was not affected by the presence of streptomycin at 0.5 and 1.0 µg/ml. However, to further rule out possible subtle growth effects, we selected a spontaneous mutant of M2 resistant to high levels of streptomycin (>3,200 µg/ml), designated M2-SM. The *A. baumannii* M2-SM strain also exhibited a reduction in motility in the presence of 0.5 and 1 µg/ml of streptomycin (Fig. 1C). These results indicated that the decrease in motility of *A. baumannii* M2 in the presence of subinhibitory concentrations of streptomycin was not due to a growth defect.

Subinhibitory concentrations of streptomycin inhibits quorum sensing. Previously, the motility of *A. baumannii* M2 was

shown to be dependent on quorum sensing (15). Therefore, the possibility that streptomycin decreased motility by altering quorum sensing was investigated. Previous work in our laboratory has identified two quorum-sensing-regulated genes, *abaI* and A1S_0112 (14, 15). To investigate the effect of streptomycin on quorum sensing, the expression of a transcriptional *lacZ* fusion to the A1S_0112 was assayed in various subinhibitory concentrations of streptomycin. The expression levels for A1S_0112-*lacZ* decreased with increasing concentrations of streptomycin and were reduced by 2.1- and 8.4-fold at streptomycin concentrations of 0.5 µg/ml and 1 µg/ml, respectively (Fig. 2A).

To test if the above-described effect of subinhibitory concentrations of streptomycin on quorum sensing resulted from decreased quorum-sensing signal activity, conditioned medium was prepared by growing the M2 strain to a density of A₆₀₀ of 1.0 in 30 ml LB medium alone or with streptomycin at concentrations of 1 µg/ml. Cells were pelleted by centrifugation to obtain a clear supernatant which was adjusted to a pH of 7.0 and filter sterilized. Tryptone was added to a final concentration of 0.5% to the filtrate and used as conditioned medium. Conditioned medium with gentamicin or myomycin was prepared in a similar way with concentrations of 0.1 µg/ml and 0.25 µg/ml, respectively. A 9-fold decrease in the expression of A1S_0112 was found in conditioned medium prepared in the presence of streptomycin, relative to LB only (Fig. 2B). A similar effect was also observed with the quorum-sensing-regulated *abaI-lacZ* fusion (Fig. 2B).

To determine if the reduced signal activity in conditioned medium was due to a general effect of aminoglycosides on protein synthesis, the expression levels of A1S_0112 and *abaI* were examined in conditioned medium prepared in the presence of gentamicin or myomycin at 0.1 µg/ml and 0.25 µg/ml, respectively, representing concentrations just below that which resulted in growth inhibition (data not shown). A slight decrease was seen in the expression of A1S_0112-*lacZ* or *abaI-lacZ* in conditioned me-

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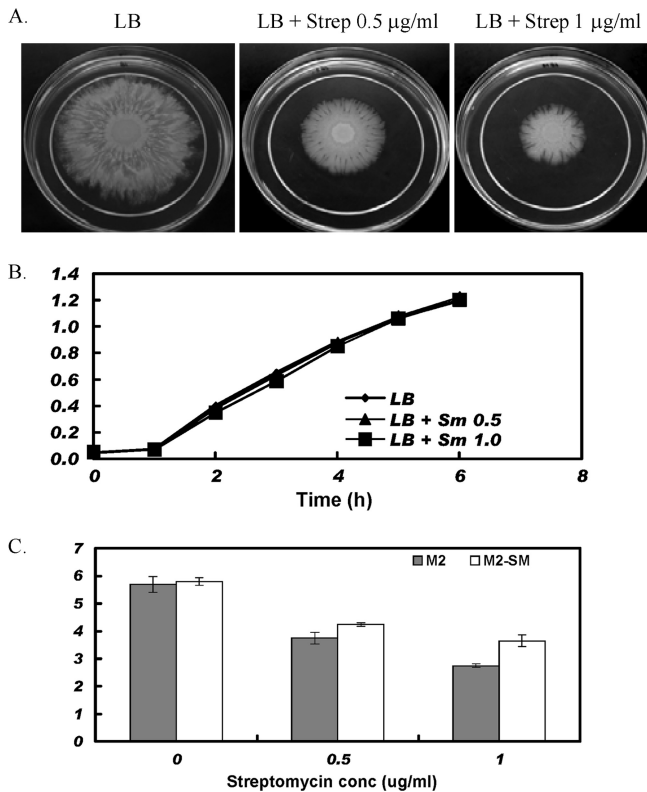


FIG 1 Motility in the presence of streptomycin. In panel A, the motility of *A. baumannii* M2 is shown on modified LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 0.3% Eiken either unsupplemented or containing streptomycin at 0.5 or 1 µg/ml. *A. baumannii* M2 was grown overnight in 3 ml of modified LB broth, and a 1-µl drop was placed on the surface of each plate. Plates were photographed when the migration on the LB-only plate reached the outer edge at 37°C. In panel B, a growth curve analysis of *A. baumannii* M2 is shown. M2 was grown overnight in 3 ml LB and adjusted to an A_{600} of 0.05 in 3 ml fresh LB only or in LB with streptomycin at concentrations of 0.5 and 1.0 µg/ml and shaken at 225 rpm at 37°C. The optical density was then monitored at hourly intervals. In panel C, the migration of wild-type M2 or the M2-SM mutant resistant to high levels of streptomycin was monitored on 0.3% Eiken agar plates. The values shown represent migration distances in centimeters. Results are the means \pm standard deviations (SD) from three independent experiments performed in duplicates. The statistical significance was determined using one-way analysis of variance (ANOVA) ($P < 0.05$).

dium prepared in the presence of gentamicin, but no effect was seen with myomycin (Fig. 2B). In addition, a control fusion, *recC-lacZ*, that was not quorum sensing regulated was used and has been described previously (26). Expression of this control *recC-lacZ* fusion was not altered in any of the conditioned medium preparations, demonstrating that the loss of activation of the quorum-sensing-regulated fusions was due to the decrease in production of quorum-sensing molecules and not by the action of residual streptomycin on protein synthesis.

Direct inhibition of 3-OH-C₁₂-HSL signal production by streptomycin. The above-described data suggested a direct effect of streptomycin on the production of 3-OH-C₁₂-HSL. However, it was also possible that streptomycin resulted in the production of a molecule that inhibited the activity of 3-OH-C₁₂-HSL. Therefore, to directly examine the effect of subinhibitory concentrations of streptomycin on 3-OH-C₁₂-HSL signal accumulation,

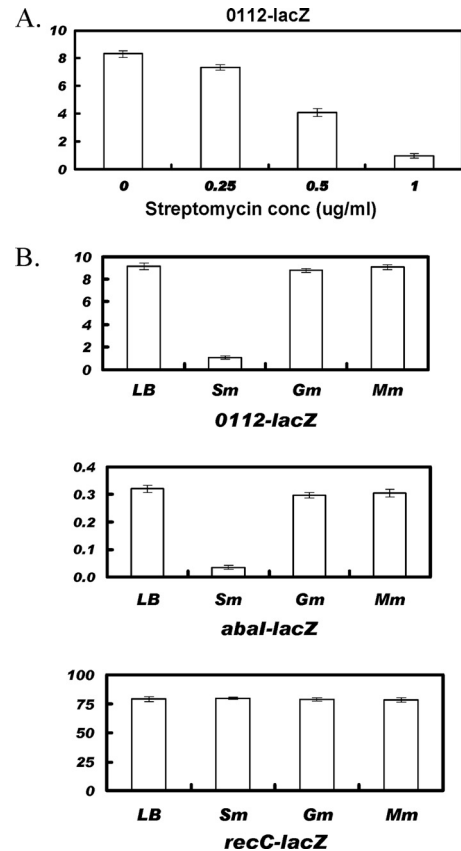


FIG 2 Streptomycin inhibits expression of quorum-sensing-regulated genes. In panel A, the expression of A1S_0112-*lacZ* in the presence of streptomycin is shown. Cells were grown overnight in 3 ml LB and adjusted to an A_{600} of 0.05 in 3 ml fresh LB containing streptomycin at concentrations of 0.25, 0.5, and 1.0 µg/ml and shaken at 37°C. The cells were harvested at an A_{600} of 1.0 and assayed for β -galactosidase activity. The expression levels were calculated in terms of Miller units. In panel B, the expression of A1S_0112-*lacZ*, *abal-lacZ*, and *recC-lacZ* in conditioned medium is shown. Conditioned medium was prepared by growing *A. baumannii* M2 in LB only or in the presence of LB plus either streptomycin (1 µg/ml), gentamicin (0.1 µg/ml), or myomycin (0.25 µg/ml) to an optical density of 1.0. *A. baumannii* M2 strains with the indicated *lacZ* fusions were grown overnight in 3 ml LB and adjusted to an A_{600} of 0.05 in 3 ml of each conditioned medium and shaken at 37°C. The cells were harvested at an A_{600} of 0.3 and assayed for β -galactosidase activity (Miller units).

conditioned medium was prepared and analyzed on a reversed-phase C₁₈ thin-layer chromatography (TLC) plate. To detect the signal, the TLC plate was dried and then overlaid with a soft agar lawn containing the *Agrobacterium tumefaciens traG-lacZ* biosensor, which can be activated by 3-OH-C₁₂-HSL, resulting in a blue spot in the agar overlay. In Fig. 3, compared to standards, the accumulation of 3-OH-C₁₂-HSL was greatly reduced in conditioned medium prepared from cells grown in the presence of 0.5 or 1 µg/ml of streptomycin.

Streptomycin acts as an antagonist of 3-OH-C₁₂-HSL. To determine if streptomycin could antagonize the ability of exogenous 3-OH-C₁₂-HSL to act as a quorum-sensing signal, *A. baumannii* M2 containing the A1S_0112-*lacZ* fusion was grown to early log phase in LB medium containing 3-OH-C₁₂-HSL at a concentration of 1 µM either with or without streptomycin at concentrations of 0.5 or 1 µg/ml. As seen in Fig. 4, the addition of strepto-

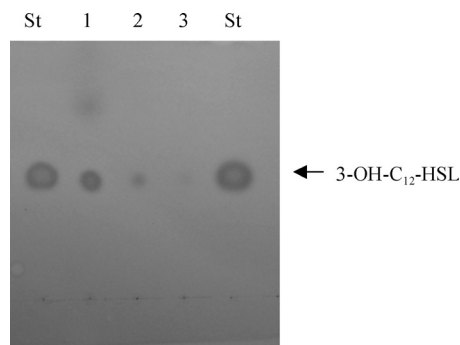


FIG 3 Bioassay for the production of 3-OH-C₁₂-HSL. The production of 3-OH-C₁₂-HSL by *A. baumannii* M2 was assayed using *Agrobacterium tumefaciens traG::lacZ* fusion as described previously by Niu et al. (14). Cell-free supernatant from growth of *A. baumannii* M2 in LB to an A₆₀₀ of 1.0 was extracted with acidified ethyl acetate (0.01%). The extract was concentrated by drying under a gentle stream of air down to 100 µl. The concentrated extract (5 µl) was applied to a C₁₈ reverse phase thin-layer chromatography plate and developed in a glass chamber with methanol-water (60:40). The developed TLC plate was overlaid with a 0.7% soft agar lawn containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the *A. tumefaciens traG::lacZ* indicator strain and was incubated at 28°C until blue spots appeared, typically 24 h. Standards of 3-OH-C₁₂-HSL at 3 µM (designated St) were added on either side of the conditioned medium samples. Lane 1 represents conditioned medium prepared from LB medium. Lane 2 represents conditioned medium prepared from LB with streptomycin (0.5 µg/ml). Lane 3 represents conditioned medium prepared from LB with streptomycin (1 µg/ml).

mycin decreased the 3-OH-C₁₂-HSL-mediated activation of AIS_0112-*lacZ* by 39% and 68% at streptomycin concentrations of 0.5 µg/ml and 1 µg/ml, respectively.

In summary, data from this study suggest that the inhibition of quorum sensing by subinhibitory concentrations of streptomycin results from decreased transcription of the *abaI* gene, encoding the autoinducer synthase responsible for 3-OH-C₁₂-HSL production (14). Currently, the mechanism responsible for the decreased *abaI* transcription is unknown. Our findings are reminiscent of those of Tateda et al., who found that subinhibitory concentrations of azithromycin inhibit quorum-sensing signal production in *Pseudomonas aeruginosa* (27). Although, like azithromycin, the aminoglycosides can alter a wide variety of cellular pathways via protein synthesis inhibition (28–32), the inhibition of quorum sensing in *A. baumannii* does not appear to be the result of protein synthesis inhibition. This is based on the finding that two other aminoglycosides, gentamicin and myomycin, when used at concentrations just below their MIC, did not significantly alter quorum sensing. Furthermore, use of myomycin, which may have the same target site as streptomycin, indicates that a unique aspect of streptomycin may be responsible for the inhibitory effects (33). The ability of streptomycin to inhibit the ability of exogenous 3-OH-C₁₂-HSL to activate quorum-sensing-dependent gene expression suggests that streptomycin itself may act as a 3-OH-C₁₂-HSL antagonist and interfere with the signal binding to the AbaR protein, which directly activates the *abaI* gene (14). Since the *abaI* gene is subject to a positive feedback activation loop via 3-OH-C₁₂-HSL and AbaR, this decreased transcription would directly lead to a reduction in quorum-sensing signal production. However, at this time, it is also a possibility that streptomycin directly inhibits the production or activity of the AbaI protein, which in turn would decrease the quorum-sensing response via reduced

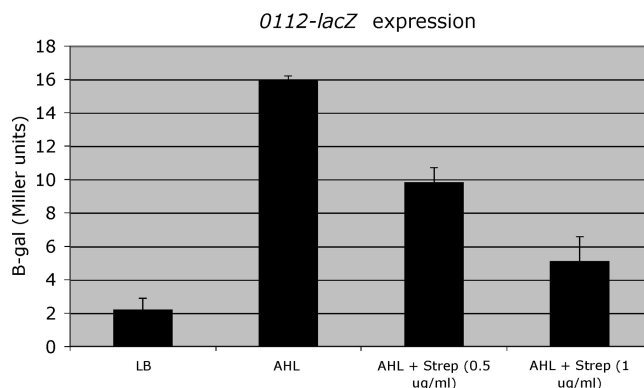


FIG 4 Expression of AIS_0112-*lacZ* in the presence of 3-OH-C₁₂-HSL. *A. baumannii* M2 with AIS_0112-*lacZ* fusion was grown overnight in 3 ml LB and adjusted to an A₆₀₀ of 0.05 in 3 ml fresh LB. AHL was added at 1 µM. Streptomycin was added to LB at the indicated concentrations. Cells were harvested at an A₆₀₀ of 0.3 and assayed for β-galactosidase activity in Miller units.

3-OH-C₁₂-HSL signal production. Regardless of the mechanism, our data point to the possible use of streptomycin in treatment therapies to inhibit quorum sensing and possibly reduce the virulence of *A. baumannii*.

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