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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 inhibit 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-
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, 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-
mycin decreased the 3-OH-C\textsubscript{12}-HSL-mediated activation of A1S\_0112-lacZ by 39\% and 68\% at streptomycin concentrations of 0.5 \mu g/ml and 1 \mu g/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 \textit{abaI} gene, encoding the autoinducer synthase responsible for 3-OH-C\textsubscript{12}-HSL production (14). Currently, the mechanism responsible for the decreased \textit{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 \textit{Pseudomonas aeruginosa} (27). Although, like azithromycin, the aminoglycosides, gentamicin and myomycin, when used at concentrations just below their MIC, did not significantly alter quorum sensing in \textit{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-C\textsubscript{12}-HSL to activate quorum-sensing-dependent gene expression suggests that streptomycin itself may act as a 3-OH-C\textsubscript{12}-HSL antagonist and interfere with the signal binding to the AbaR protein, which directly activates the \textit{abaI} gene (14). Since the \textit{abaI} gene is subject to a positive feedback activation loop via 3-OH-C\textsubscript{12}-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 AbaR protein, which in turn would decrease the quorum-sensing response via reduced 3-OH-C\textsubscript{12}-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 \textit{A. baumannii}.

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