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Research Article

Enhancement of Swarming and Inhibition of Prodigiosin in *Serratia marcescens* by Glyceryl Trinitrate

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I N F O

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A B S T R A C T

Introduction: *Serratia marcescens* is a gram-negative pathogen of many species. Its pathogenicity and survival are linked to its capacity to build biofilms as well as its strong inherent resistance to antimicrobials and cleaning agents.

Objectives: To analyse the impact of glyceryl trinitrate (GTN) on the gene expression of QS-related genes (*rssB*, *rsmA*, and *pigP*) of *S. marcescens*.

Methodology: The broth microdilution technique estimated the bactericidal effectiveness of glyceryl trinitrate. The presence of *rssB*, *rsmA*, and *pigP* in *S. marcescens* isolates was detected using PCR. qRT-PCR was used to assess the effect of GTN on *rssB*, *rsmA*, and *pigP* gene expression.

Results: The results demonstrated that GTN has no effect on *S. marcescens* growth. Furthermore, *rssB*, *rsmA*, and *pigP* were located in all *S. marcescens* isolates and their gene expression was downregulated due to GTN presence.

Conclusion: GTN can act as a promising anti-quorum sensing agent in reference to *S. marcescens*.

Keywords: *rssB*, *rsmA*, *pigP*, Repurposing, Glyceryl trinitrate, *Serratia marcescens*

Introduction

Serratia marcescens is a gram-negative, opportunistic, and motile nosocomial pathogen of the Enterobacteriaceae family. It produces the characteristic red pigment known as prodigiosin.[1]

This capacity is often related to a pathogen's ability to release multiple virulence factors and affect the host defences. Many studies have found that quorum sensing (QS) dependent virulence factors are critical for the formation of bacterial infection in laboratory animals. QS is a critical global gene regulation network in bacteria, which enables distinct bacteria to synchronise their pathogenicity activities in a cell density-dependent way via self-produced signalling molecules known as autoinducers.[2]

The RssA-RssB (RssA-sensor kinase and RssB-response regulator) system is a two-component system that inhibits

the *S. marcescens* swarming behaviour. The immediate interaction of RssB with the promoter of *flhDC* inhibits transcription of the *flhdDC* operon, resulting in reduced hemolysin production as well as flagellar motility.[3] According to Ang et al.,[4] the *rsmA* upregulation in *S. marcescens* affects swarming alongside prodigiosin production.

In the QS mechanism, *pigP* is the master transcriptional regulator in *S. marcescens* that governs the regulation of prodigiosin biosynthesis. RssB is linked specifically to the *pig* operon promoter, inhibiting prodigiosin biosynthesis.[5]

Glyceryl trinitrate (GTN) is an organic nitrate that acts as a vasodilator.[6] GTN possesses anti-microbial characteristics in addition to its anti-hypertensive effect. It has the potential to suppress *Candida albicans* and *P. aeruginosa* planktonic growth.[7] It also exhibits anti-microbial capabilities in addition to its antihypertensive action.[8] What's more, GTN significantly inhibited biofilm, staphyloxanthin, and oxidative stress tolerance in *S. aureus*.[9]

With reference to the aforementioned facts, it is noteworthy to enlighten the influence of GTN on the gene expression of QS-related genes (*rssB*, *rsmA*, and *pigP*) of *S. marcescens*.

Materials and Methods

Isolation and Identification

The present study was a randomized controlled trial conducted during the period from February 2022 to June 2022. A total of ten *S. marcescens* isolates were taken from the microbiology lab at the Department of Biology, College of Science, University of Anbar. These isolates were originally collected from patients sent to Al-Ramadi Hospital and re-identified using VITEK 2 Compact (bioMérieux, France).

Determination of GTN Minimal Inhibitory Concentration (MIC)

To determine the MIC of GTN, a broth microdilution test was followed[10] using serial double concentrations (0.01 through 0.175 mg/ml) of GTN.

Effect of GTN on Prodigiosin Production

Prodigiosin's production by *S. marcescens* was quantified in the presence and absence of GTN. A total of five isolates were grown in LB broth for 18 hours and normalised to OD_{600} of 0.4. They were inoculated in 2 ml of fresh LB broth. Then, they were incubated at 28 °C for a duration of 18 hours. Centrifugation was done for 8 minutes at 14000 g to collect the cells. Acidified ethanol (4% 1M HCl in ethanol) was used in order to extract prodigiosin. Biotek Spectrophotometer (Biotek, USA) was used to measure the absorbance at 534 nm, after which determination of the degree of inhibition was done. The experiment was made in triplicate.[11]

Effect of GTN on Swarming

Swarming LB agar plates (0.5% agar) with 0.175 mg/ml GTN and control plates were point inoculated with 5 µl of the prepared suspension. The plates were then incubated for a duration of 20 hours at 28 °C. Measurement of the zones of swarming was done. The experiment was made in triplicates.[12]

Detection of *rssB*, *rsmA*, *pigP* and *rplU*

DNA Extraction

ABIOpure™ Total DNA (ABIOpure, USA) procedure was used to extract genomic DNA from the bacterial isolates.

Polymerase Chain Reaction

The extracted DNA and primers (Table 1) were mixed in PCR master mix with 20 µl as the total volume (Table 2).

PCR reaction tubes containing the mixture were placed in the thermocycler, and DNA was amplified as in the conditions indicated in Table 3. The temperature and time of the PCR programme were optimised using gradient PCR. Thereafter, amplicons were resolved in agarose gel (1.5%).

Table 1.Primers used in this Study[2]

Primer Name		Sequence (5´–3´)	Product Size (bp)
pigP	F	GAACATGTTGGCAATGAAAA	207
	R	ATGTAACCCAGGAATTGCAC	
rssB	F	TAACGAACTGCTGATGCTGT	172
	R	GATCTTGCGCCGTAAATTAT	
rsmA	F	TTGGTGAAACCCTCATGATT	173
	R	GCTTCGGAATCAGTAAGTCG	
rplU	F	GCTTGGAAAAGCTGGACATC	192
	R	TACGGTGGTGTTTACGACGA	

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Table 2.Components of Conventional PCR Reaction

Component	Volume (µl)
Master mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Nuclease free water	6
Template DNA	2
Final volume	20

Table 3.PCR Amplification Programme[2]

Step	Temperature (°C)	Minute:Second	Cycles
Initial denaturation	95	10:00	1
Denaturation	95	00:45	40
Annealing	57	00:45	
Extension	72	00:50	

Table 4.Components of qRT-PCR

Master Mix Components	Volume (µl)
qPCR master mix	5
RT mix	0.25
$MgCl_2$	0.25
Forward primer	0.5
Reverse primer	0.5
Nuclease free water	1.5
RNA	2
Total volume	10

Gene Expression

RNA Extraction

RNA was extracted from the selected isolates with and without GTN treatment at 0.175 mg/ml using TRIzol™ Reagent conforming to the instructions of the manufacturing company.

qRT-PCR

The primers shown in Table 1 were used in qRT-PCR. Table 4 summarises the reaction combination. Furthermore, following multiple trials, the thermocycler procedure was refined; the resulting program have been shown in Table 3.

Gene Expression Calculation

Relative quantitation was used to determine expression levels of *rssB*, *rsmA*, and *pigP*. The fold changes were assessed between the treated groups and each gene's calibrators.[13] These values were normalised to *rplU* as shown below:

$$\text{Folding} = 2^{-\Delta\Delta CT} \quad \ldots\ldots (1)$$

$$\Delta\Delta C_t = \Delta C_{t\ \text{Treated (T)}} - \Delta C_{t\ \text{Untreated (C)}} \quad \ldots\ldots (2)$$

$$\Delta C_t = C_{t\ \text{of target gene}} - C_{t\ \text{of housekeeping gene}} \quad \ldots\ldots (3)$$

A fold change of less than twofold was deemed insignificant.[14] A melting curve with temperature varying between 72 °C and 95 °C at 0.3 °C/s was plotted.

Statistical Analysis

Experiments were carried out three times alongside three distinct replicates (n = 3). In regard to the normality test, Shapiro-Wilk and Kolmogorov-Smirnov tests were employed. Data were presented as mean ± standard deviation. The significance level was chosen at $p < 0.05$. Data were analysed using GraphPad Prism 9 software one-way ANOVA. Dunn's test was performed as a post-hoc test.

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Results

GTN Minimal Inhibitory Concentration

GTN did not affect the growth of all tested isolates ($p > 0.05$).

Effect of GTN on Prodigiosin Production

As depicted in Figure 1, GTN significantly ($p < 0.01$) inhibited the production of prodigiosin in all tested isolates.

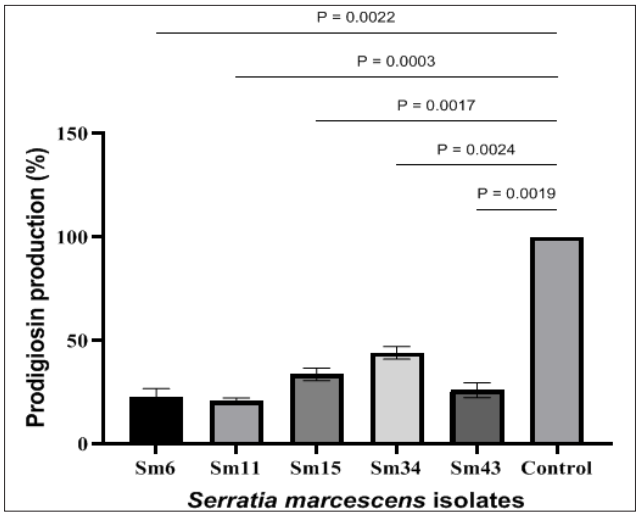

Figure 1.Effect of GTN on Prodigiosin Production in *Serratia marcescens*. Error bars represent standard deviation

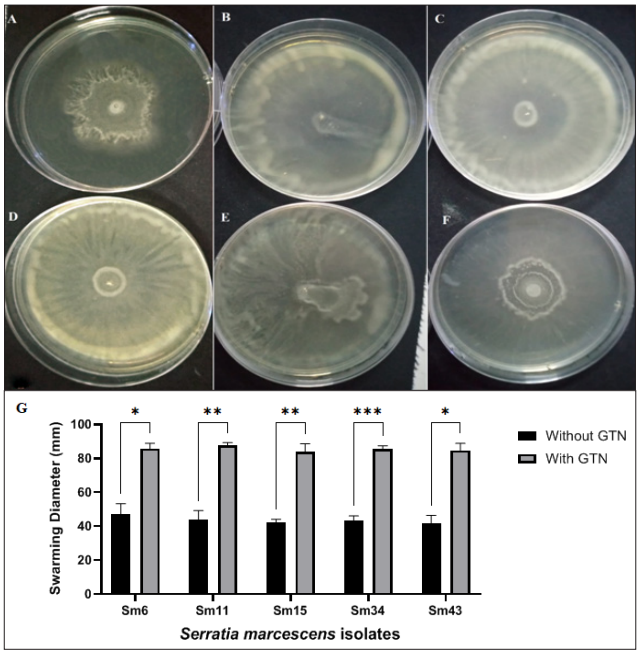

Figure 2.GTN Effect on Swarming of *Serratia marcescens*. Plates A, B, C, D, E, and F demonstrated control plate, *S. marcescens* isolates Sm6, Sm11, Sm15, Sm34, and Sm43, respectively. Plate Diameter = 9 cm

Effect of GTN on Swarming

Treating *S. marcescens* with GTN significantly ($p < 0.05$) increased the swarming diameter in comparison to the control (Figure 2).

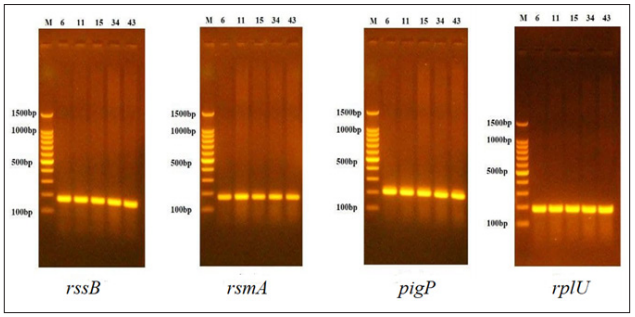

Figure 3.*rssB*, *rsmA*, *pigP* and *rplU* Amplicons of Selected *Serratia marcescens* Isolates at 172, 173, 207, and 192 bp, respectively run in Ethidium Bromide-containing Agarose Gel (1.5%) at 5 V/cm. M denotes 100 bp DNA Ladder

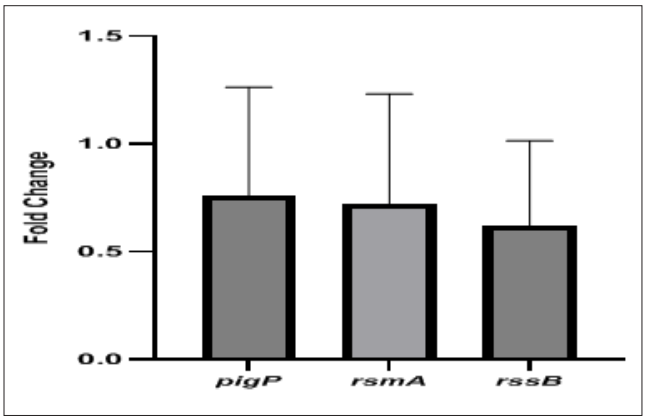

Figure 4.Effect of GTN on the *rssB*, *rsmA*, and *pigP* Expression. Error Bars denote Standard Deviation

rssB, *rsmA*, *pigP* and *rplU* Detection

PCR amplification revealed the presence of *rssB*, *rsmA*, *pigP* and *rplU* genes in all *S. marcescens* isolates at the assigned molecular size; 172, 173, 207, and 192 bp, respectively (Figure 3).

Effect of GTN on the Gene Expression of *rssB*, *rsmA*, and *pigP*

Figure 4 indicated that GTN caused a reduction in gene expression of *rssB*, *rsmA*, and *pigP* by 0.62 ± 0.39, 0.72 ± 0.50, and 0.75 ± 0.50 - fold, respectively.

Discussion

S. marcescens is a significant human opportunistic pathogen, which is implicated in a wide spectrum of nosocomial infections like bacteraemia, respiratory tract infections, eye infections, and, most significantly, urinary tract infections. It uses a signal-mediated QS system to release a variety of virulence agents and create a biofilm.[2]

Since GTN has a relatively short half-life (1 to 3 minutes), any clinical consequences are quite temporary. Oral dosages of

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200 g of GTN were well-accepted and showed no clinically relevant negative consequences. According to the findings of Rosenblatt et al.,[15] these transitory effects are often short, non-clinically noteworthy hypotensive incidents of 10 mmHg or less. Also, numerous doses of GTN may be provided successively without difficulty in various clinical investigations. When used to relax the uterus during a complicated delivery, it reduces the risks of complications for both mother and foetus.

GTN at 0.175 mg/ml efficiently ($p < 0.05$) reduced the production of prodigiosin in all tested isolates (Figure 1). Such inhibition could be attributed to the downregulation of *pigP* and upregulation of *rssB* due to GTN treatment (Figure 4). The production of pigment prodigiosin in *S. marcescens* under the QS mechanism is regulated by the master transcriptional regulator, *pigP*. Moreover, RssB is linked specifically to the *pig* operon promoter, inhibiting prodigiosin biosynthesis.[5]

Swarming motility is a pattern of movement in which many bacterial pathogens proliferate and spread quickly across a surface. This reduces bacterial cell competition for resources while also hastening their development.[16] This usual virulent *S. marcescens* behaviour is critical in catheter-associated urinary tract infections. Swimming and swarming motility are connected with QS in this bacterial species. As a result, an attempt was undertaken to investigate the QS inhibition potential of GTN in preventing the swarming of *S. marcescens*. Unfortunately, the current investigation found significant swarming motility in the untreated *S. marcescens* control plate; however, the 0.175 mg/ml GTN treatments increased the swarming diameter (Figure 2). This increase might be owing to *rssB* and *rsmA* downregulation-related mechanisms (Figure 4); given that these genes negatively regulate swarming in *S. marcescens*.

Bacterial diminution is among the alternative methods for combating bacterial pathogenicity by targeting its QS.[17] The fundamental reason for tackling QS is to reduce the burden on bacterial proliferation that increases resistance, as well as to develop a new treatment approach in light of *S. marcescens* high antibiotic resistance. QS is a key regulator of pathogenicity in many bacteria. Inhibiting it may effectively lower virulence and facilitate the treatment of infectious illnesses caused by resistant bacterial strains.[18]

In this field, several researchers explored the capacities of numerous compounds as anti-QS and anti-biofilm agents.[19,20] Nitrogenous heterocyclic compounds like pyrazine dicarboxylic acid derivatives are examples of substances that could be utilised for the modulation of the QS of *Vibrio cholerae*; they primarily targeted LuxO, the global response regulator. Pyrazine derivatives inhibited vibrio's adhesion and penetration into intestinal cell lines by acting as anti-biofilm agents.[21]

Interfering with QS may limit *S. marcescens* virulence factor production. The ability of GTN to significantly reduce the production of the QS-regulated violacein pigment in the biosensor strain *Chromobacterium violaceum* CV026 corroborated this idea. GTN has also been found as a QS agonist in the *P. aeruginosa* PAO1 strain.[22]

Triazoles are among the nitrogenous heterocyclic moieties which were extensively used in the production of antibiotics such as cefatrizine and tazobactam.[23] Several triazole moiety-containing medicines might bind to a wide range of biological receptors via hydrogen bonding and dipole interactions.[23,24] Surprisingly, triazole substances with natural N-acyl homoserine lactone, isoxazole, and thymidine structures which significantly reduced bacterial QS and might be used as alternate constituents in the development of effective QS inhibitors demonstrated anti-QS activity.[25]

Finally, QS has been recognised as a mechanism in pathogenic and opportunistic bacteria that controls biofilm, motility, secreted toxins, enzymes, and many virulence factors. Since bacterial growth is unrestricted, addressing bacterial pathogenicity was considerably less probable to end up in resistance. Instead, virulence attenuation diminishes infections while increasing the immune system's ability to eliminate them.[26]

Conclusion

The current findings showed that GTN had no influence on the growth of *S. marcescens*. Besides, *rssB*, *rsmA*, and *pigP* gene expression was suppressed by the presence of GTN. Consequently, GTN may be regarded as a potential anti-quorum sensing agent.

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Conflict of Interest: None

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