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Peculiarities of the SprIR Quorum Sensing System of *Serratia proteamaculans* 94 and Its Involvement in Regulation of Cellular Processes

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Abstract—The quorum sensing (QS) system SprIR of the psychrotrophic strain *Serratia proteamaculans* 94 was investigated. A mutant was constructed with the inactivated s*prR* gene encoding the regulatory receptor protein SprR. Inactivation of this gene was shown to affect the composition of fatty acids synthesized by *S. proteamaculans* 94 and did not affect the synthesis of *N*-acyl-L-homoserine-lactones (AHL); the activities of extracellular proteases, chitinases, and hemolysins; the swimming motility of cells; and the suppression of mycelium growth of fungal plant pathogens by volatile compounds emitted by this strain. Inactivation of the *sprI* gene (but not the s*prR* gene) reduced the biofilm formation, which increased when exogenous AHL was added to the culture. The comparative proteomic analysis of cell of the parent strain and mutant strains with inactivated *sprI* and *sprR* genes showed that the expression of 30 proteins in *S. proteamaculans* 94 is affected by the SprIR quorum sensing system.

Keywords: quorum sensing, *Serratia proteamaculans*, biofilms, enzymes, fatty acids, proteomic analysis
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INTRODUCTION

Recently, researchers have focused more and more attention on the quorum sensing (QS) systems of gene expression regulation involved in bacterial communication and providing the coordinated response of bacterial populations to external factors. The QS is a special type of regulation usually functioning at high bacterial population densities. The QS systems include small signaling molecules of different chemical nature diffusing from cells into the culture medium and receptor regulatory proteins, with which the signaling molecules interact. The QS systems work as global regulation factors; they play a key role in controlling a great number of cellular processes related to different aspects of bacterial metabolism: they participate in the interaction between many bacteria and higher organisms and in the regulation of bacterial virulence and expression of the genes responsible for the synthesis of various exoenzymes, toxins, antibiotics, etc. [1–7].

The best studied systems are the LuxI/LuxR-type QS systems of Gram-negative bacteria, which use *N*-acyl-L-homoserin-lactones (AHL) as signaling molecules. The QS systems of this type can be divided into two groups depending on their functions and organization of QS gene expression.

The first group includes the classical QS system of *Vibrio fischeri*: the QS phenomenon was first discovered in this marine bacterium. The QS regulation of *V. fischeri* involves two major regulatory components: the protein LuxI—AHL synthase (catalyzes AHL synthesis) and the LuxR protein that forms a dimer and attaches AHL; as a result, its configuration changes; then this complex, binding to the promoter of the *lux* operon, activates its transcription, which leads to luciferase synthesis and light emission. When the *V. fischeri* population increases, AHL accumulate up to the threshold level sufficient for the activation of LuxR, its binding to the promoter region of the *lux* operon, and induction of this operon. Similar QS systems were found in the cells of different species of pseudomonades: *Agrobacterium tumefaciens*, *Burkholderia,* etc. [1, 3–7].

The second group includes the QS systems of bacteria from the family Enterobacteriaceae (*Pantoea*,

† Deceased.

Erwinia, *Serratia,* etc.). Regulatory R proteins in the QS systems of this group act mainly as negative regulators; they can repress the transcription of their own genes in the absence of AHL. The amount of AHL increases along with the increase in bacterial population density as the culture grows. After reaching high enough concentrations, AHL interacts with a LuxR-type protein (e.g., the EsaR protein of *P. stewartii*); the changes in its conformation occurring there prevent DNA binding, which leads to derepression [8–12].

The object of the present research was the bacterium *Serratia proteamaculans* 94 [13]. The QS systems of bacteria of this species have been little studied [11]. In the previous work [14], we showed that the strain *S. proteamaculans* 94 synthesizes two major types of signaling AHL molecules (*N*-3-oxo-hexanoyl-L-homoserine-lactone and *N*-3-hydroxy-hexanoyl-L-homoserine-lactone) and several minor AHLs. The *sprI* and *sprR* genes of this QS system, encoding AHL synthase and receptor regulatory protein SprR, respectively, were cloned and sequenced. The *sprI* and *sprR* genes were shown to undergo convergent transcription with partial overlapping of their reading frames. The study of the obtained mutant with inactivated QS *sprI* gene allowed us to elucidate the role of this gene in regulation of some cellular processes. Inactivation of the *sprI* gene resulted in the absence of AHL synthesis, the decrease in extracellular proteolytic activity, the absence of chitinolytic activity and swimming motility of cells, the absence or abrupt decrease of suppression of the mycelial growth of fungal plant pathogens by the volatile compounds emitted by the strain 94, and modification of the composition of fatty acids synthesized by this strain [14].

In the strain *S. proteamaculans* 94, we also found an AI-2 type signaling molecule and investigated the functional role of the *luxS* gene responsible for the synthesis of AI-2. Inactivation of this gene had a similar effect as inactivation of the *sprI* gene on the synthesis of exoenzymes and swimming motility and caused a decrease or absence of inhibition of fungal plant pathogens by the volatile substances emitted by *S. proteamaculans* 94 [15].

In the present work, we have obtained a mutant with inactivated *sprR* gene of the QS system of the strain *S. proteamaculans* 94 encoding the receptor regulatory protein SprR. Mutations of the gene encoding the R protein of *S. proteamaculans* have never been obtained before and there is no information about the role of this gene in the control of *S. proteamaculans* metabolism. In this work, we have studied the effect of the *sprR* gene inactivation on the regulation of cellular processes in *S. proteamaculans* 94, investigated the effect of inactivation of the *sprI* and *sprR* genes on biofilm formation by this strain, and performed a comparative proteomic analysis of protein expression in the parent and mutant strains. The findings expand our knowledge of the spectrum of cellular processes regulated with the involvement of the genes of the *S. proteamaculans* QS system.

MATERIALS AND METHODS

1. Bacterial Strains and Cultivation Conditions

The bacterial strains, plasmids, and PCR primers used in the present work are presented in Tables 1 and 2. Bacteria were grown in Luria Broth (LB) and on LA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and M9 media [16] with the necessary additives at 28–30°C. Domestic antibiotics were used at the following concentrations (μg/mL): ampicillin, 100–200; kanamycin, 100; gentamicin, 40. Tetracycline (Sigma) was added to the medium at a concentration: 20 μg/mL.

2. Detection of AHL Production

AHL production was detected by two biosensors. The *Chromobacterium violaceum* CV026 sensor was inoculated onto the LA surface by the streak plate technique, followed by cross-streaking with tested cultures, and incubated for 24–48 h at 30°C. When the strain produced AHL, the indicator strain CV026 became purple. The color intensity was assessed visually [17]. The second biosensor, *Agrobacterium tumefaciens* NT1/pZLR4, was grown in LB with ampicillin (100 μg/mL) and gentamicin at 30°C overnight. The plate with agarized M9 medium with the addition of X-Gal (final concentration 80 μg/mL) was flooded with 3 mL of M9 with 0.5% agar, with the addition of 0.5 mL of the overnight culture of *A. tumefaciens* NT1/pZLR4. The strains tested for AHL production were inoculated by injections onto the surface of solidified agar medium, or the liquid overnight culture was introduced into wells in the agar medium and incubated at 30°C for 24–48 h. AHL synthesis by the strains was assessed by the appearance of light blue areas of X-Gal hydrolysis [18].

3. Manipulations with DNA

Total and plasmid DNA isolation, restriction, agarose gel electrophoresis, ligation, and transformation of *E. coli* were carried out as described [19]. The PCR reaction was performed in a reaction mixture (20 μL) containing 1× buffer for *Taq* DNA polymerase (SibEnzym); dGTP, dATP, dCTP, and dTTP (SibEnzym), 250 μM each; primers (Syntol), 10 pM each; and 0.5 U *Taq* DNA polymerase (Institute of Molecular Genetics, Russian Academy of Sciences). The plasmid DNA or boiled cells from freshly grown colonies were used as PCR matrices. PCR amplification was performed in a TP4-PCR-01-Tertsik four-channel programmable thermostat (DNA-Technology).

Table 1. Bacterial strains and plasmids used in the work

Strain and plasmid	Characteristic	Source or reference
	Strain	
Serratia proteamaculans 94	Isolated from meat spoiled in refrigerator	[13]
S. proteamaculans 94 *rif*-r	Spontaneous mutant *rif*-r	[14]
Escherichia coli S17-1 (λ-*pir*)	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tc^r::Mu) (Km^r::Tn7) (λ-*pir*)	Collection of the Institute of Molecular Genetics, Russian Academy of Sciences
E. coli TG-1	K-12 *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5,* ($r_K^- m_K^-$) F' [*tra D36 proAB*$^+$ *lacI*^{q} *lac* ZΔM15]	Stratagene
Chromobacterium violaceum CV026	Biosensor for AHL determination based on violacein production, Km-r	[17]
Agrobacterium tumefaciens NT1/pZLR4	pZLR4 *traG::lacZ.* Biosensor for AHL detection based on induction of the synthesis of β-galactosidase, Gm^r Cb^r	[18]
S. proteamaculans 94 *rif*-r *sprI*::Gm	Mutant *sprI*::Gm of the strain 94 *rif*-r	[14]
	Plasmid	
p34S-Gm	Source of Gm^r-cassette	[21]
pEX18Tc	Vector for gene replacement, Tet^r oriT *sacB*	[20]
pAL-TA	Vector for cloning of PCR products, Ap^r ori pUC P_{lac}	Eurogen

Table 2. Primers used in the work

Primer	Sequence (5' → 3')
SprR-F	5'-GAGCCTGTATGTTTTCCATC
SprR-R	5'-CAACTTCCGCCATCACCTG
GM-F	5'-GGCTCAAGTATGGGCATCATT
GM-R	5'-GGCGGTACTTGGGTCGATA
M13-F	5'-GTAAAACGACGGCCAGT
M13-R	5'-CAGGAAACAGCTATGAC

4. Obtaining the S. proteamaculans 94 Mutant with Inactivated sprR Gene

The mutant with the *sprR* gene knockout was obtained by the method of gene replacement using the *sacB* strategy [20]. Previously, for cloning and sequencing the *sprI* and *sprR* genes, a 1660-bp DNA fragment containing both genes was cloned and the nucleotide sequences of these genes were determined (JX901285) [14]. The cloned *sprR* gene was obtained by PCR amplification with the primers for this gene: SprR-F and SprR-R (Table 2). PCR was performed at 94°C for 2 min, followed by 30 cycles at 94°C for 20 s, 58°C for 20 s, 72°C for 40 s, and the final stage at 72°C for 4 min. PCR products were extracted from the gel (using the Wizard SV Gel kit and PCR Clean-Up System, Promega) and ligated with the pAL-TA vector DNA. The ligase mixture was transformed in *E. coli* TG1. The cells were inoculated on LA with the addition of 200 μg/mL of ampicillin; the presence of the *sprR* gene in selected clones was detected by PCR with universal M13 primers. The constructed plasmid was designated as pAL-TA-*sprR*.

For constructing the *sprR*::Gm mutant, the DNA *Bam*HI-fragment (865 bp) of plasmid p34S-Gm [21], which carried the gentamicin resistance gene, was cloned in the *Bgl*II-site within the *sprR* gene in the pAL-TA-*sprR* plasmid. A 1700-bp DNA fragment of the obtained plasmid pAL-TA-*sprR*::Gm was cloned in the *Eco*RI site of the pEX18Tc vector [20], and the pEX18Tc-*sprR*::Gm plasmid constructed as a result was transformed in *E. coli* S17-1 (λ-*pir*), which was used as a donor for conjugative transfer of this plasmid to the *S. proteamaculans* 94 *rif*-r strain. The cells obtained were inoculated on the LA medium with

Gm, Rif, and 5% (wt/vol) sucrose; then the clones resistant to gentamicin, rifampicin, and sucrose and sensitive to tetracycline were tested by PCR with the Gm-F and GmR primers and the sprR-F and sprR-R primers. As a result, the mutant strain *S. proteamaculans* 94 *sprR*::Gm was obtained.

5. Biofilm Analysis

Fresh cultures grown on LA were inoculated into LB with the necessary antibiotics and incubated for 24 h at 30°C under aeration. Then the cultures were diluted 300-fold in LB without antibiotics. For measuring biofilm formation, the cultures were grown in polystyrene plates for 24 h under stirring on a shaker at 30°C. Biofilm formation was measured after removing the medium, washing the cells with water, and staining the attached cells with the crystal violet dye (1 g of crystal violet, REAKHIM, Russia; 2 g of phenol crystal; 90 mL of distilled water; 10 mL of 96% ethanol) for 45 min at room temperature. After staining, the liquid was poured out; the dye was extracted from the biofilms with 96% ethanol; the optical density of the solution was measured at 595 nm. The biofilm level was measured with a Model 2550 Microplate Reader (Bio-Rad, United States). The level of biofilm formation was assessed by staining intensity. Each experiment was performed in triplicate; in each variant, the culture was grown in 4–8 wells.

6. The Effect of sprR::Gm Mutations on the Properties of S. proteamaculans 94 Cells

6.1. Determination of enzyme activities

For determining extracellular enzyme activities, 3 μL of 300-fold diluted overnight bacterial culture was placed on the LA surface (1.5% agar) with the necessary additives. The experiments with all variants were performed three times, with no less than three replications in each experiment [14].

Determination of extracellular protease activity. The cells of the strains were inoculated by injection on an agarized LA medium with milk (milk with 0.5% fat was 1/3 of total volume of the medium) and incubated for 24–48 h at 30°C. The strains with the extracellular protease activity had the zones of enzymatic hydrolysis of milk casein around the colonies (clearance zones). The enzyme activity was assessed by the radius of the hydrolysis zones.

Determination of lipase activity. The cells of the tested strains were inoculated by injection on the LA medium with Tween-20 (1%) and $CaCl_2$ (0.01%) and incubated for 48 h at 30°C. The strains with the lipase activity had turbid zones around the colonies (Tween-20 was hydrolyzed with the formation of lauric acid; as a result, the insoluble salt of this acid was formed in the presence of calcium). The enzyme activity was assessed by the radius of opaque zones around the colonies and by turbidity of the medium within the opaque zone.

Determination of chitinolytic activity. The tested strains were inoculated by injection on an agarized medium (1.5% agar) containing 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \times 7H_2O$, 0.08% KH_2PO_4, 0.04% KNO_3, and 0.05% yeast extract (Difco); the medium was supplemented with 0.2% colloidal chitin. Incubation was performed for 72–96 h at 30°C. The strains with the chitinolytic activity had the zones of enzymatic hydrolysis around the colonies (clearance zones). The enzyme activity was assessed by the radius of these zones.

Determination of hemolytic activity. The cells of the tested strains were inoculated by injection into blood agar (the LA medium with 5% sterile human blood) and incubated for 3–7 days at 28°C. The hemolytic activity was assessed by the transparent zones of hemolysis around the colonies.

6.2. Determination of the effects of volatile compounds emitted by S. proteamaculans 94

Previously, we showed that *S. proteamaculans* 94 synthesizes volatile compounds (VC), including organic volatile compounds, with the maximum production of dimethyl disulfide (DMDS). It was shown that the total pool of volatile compounds emitted by this strain and individually DMDS suppress the growth of agrobacteria, fungal plant pathogens, nematodes, and drosophilae [22]. The influence of inactivation of the *sprR* gene on the VC effect on fungi was determined as follows: Petri dishes were divided into two parts with partitions not adjoining the cover to leave free space for air exchange. One half of the dish was inoculated with *S. proteamaculans* 94 of the *sprR*::Gm mutant on LA (20 μL of the overnight culture grown in LB and containing ~$4–6 \times 10^7$ cells was spread with an inoculating loop over the surface of LA). The dishes were incubated for 24 h at 28°C. Then a block with fungal mycelium was placed in the other half of the dish. Fungal blocks of 8 mm in diameter were cut out from agarized Czapek medium on Petri dishes inoculated with a loop by fungal spores from a fungal culture grown for five days at 25°C. The dishes were sealed (four times) with Parafilm M Sealing Film (Pechiney Plastic Packaging Company, Chicago, IL, United States). Fungal growth was analyzed in 3–6 days at 25°C [22].

6.3. Analysis of fatty acid composition

Overnight bacterial cultures were diluted to OD_{600} ~ 0.10, and the cells were grown for 20 h at 30°C. The samples of dry cell biomass (5 mg) were treated with 0.4 mL of 1.2 N hydrogen chloride in methanol at 80°C for 1 h (acidic methanolysis). The formed fatty acid methyl esters were extracted twice with hexane,

followed by gas chromatography and mass spectrometry. The parameters of chromatography were chosen according to the Microbial Identification System (MIS) [23, 24] with a Hewlett-Packard 5890A gas chromatograph and a Hewlett-Packard 7673A automated sampler. Gas chromatography/mass spectrometry (GS-MS) was carried out with an AT-5975 Agilent Technologies chromatograph/mass spectrometer (United States). Chromatographic separation of a sample was performed in an HP-5ms capillary column. Chromatography was performed in the temperature-programmed mode (135 to 320°C) at a rate of 7 deg/min. Data processing was performed with the operational programs of the instrument. The substances in chromatographic peaks were identified using library programs with the NIST mass spectral database.

7. Proteomic Analysis

The comparative proteomic analysis in the cells of *S. proteamaculans* 94 and two mutant strains was performed at the Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Moscow.

Protein extraction and separation. Bacterial cultures were grown at 30°C for 17 h. The cells were precipitated by centrifugation at 10000 rpm for 5 min at 4°C, washed three times with standard phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), and centrifuged. The supernatant was removed; the precipitate was frozen and stored at –80°C. For protein extraction, the precipitates of bacterial cells were resuspended in the buffer containing 7 M urea, 2 M thio-urea, 2% CHAPS, 0.5% Triton X100, 20 mM Tris-base, 2 mM $MgCl_2$, 5 mM PMSF, 65 mM DTT, and 1 mM Na_2EDTA and exposed to ultrasonic treatment. For the purpose of assessing the quality of protein extraction, each sample was exposed to analytical one-dimensional and then two-dimensional electrophoresis using the Quantity One software (BioRad, United States). The standard application was about 150 μg per 24 cm of the gel.

Two-dimensional (2D) electrophoresis. For electrophoresis in the first direction, 24 cm of IPG Strips with the applied gel at pH 3–10 (Agilent, United States) were hydrated overnight in the buffer containing 7 M urea, 2 M thio-urea, 4% CHAPS, 65 mM DTT, and 1.25% ampholytes 3-10 (Agilent). The samples in the same buffer were applied to the strips and exposed to electrophoresis in the first direction at 80 kV with an Agilent Technologies 3100 Offgel Fractionator (Agilent). The second direction of electrophoresis (SDS-PAGE) was performed in 12% polyacrylamide gel. The buffer for electrophoresis contained 25 mM Tris, 192 mM glycine, and 0.1% SDS. After gel fixation in the solution with 10% acetic acid and 20% ethanol, it was stained with silver nitrate [25].

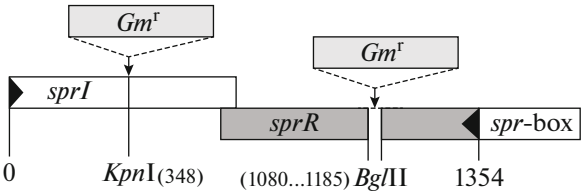

Fig. 1. Localization of the *sprR*::Gm and *sprI*::Gm mutations in *S. proteamaculans* 94. The arrows indicate the direction of gene transcription.

Image analysis of 2D gels. The stained gels were scanned with a Bio-Rad GS-800 Calibrated Densitometer. The intensity of protein spot staining was compared using Melanie III software. The spots with staining intensity differing at least 1.5-fold (expressed in percentage) were cut out from the gel and analyzed by MALDI-TOF.

Time-of-flight mass spectrometry with matrix-assisted laser desorption/ionization (MALDI-TOF MS analysis) and identification of proteins. The silver-stained protein spots cut out from the gel were treated according to Shevchenko et al. [26]. Mass spectra were recorded with an Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen (N_2) laser (O-350 nm). The list of peptide peaks was compiled in the Flex Analysis 2.4 software program (Bruker Daltonics). Protein identification according to the peptide contents was performed with Mascot 2.3 (Matrix Science, United States) corresponding to the NCBI database for the taxa of Eubacteria.

The results obtained in three independent experiments were expressed as the mean ± standard deviation (SD) and calculated using GraphPad PRISM 5.0 (GraphPad Software Inc.).

RESULTS

1. Obtaining S. proteamaculans 94 Mutant with Inactivated sprR Gene

For studying the peculiarities of function of the SprI/SprR QS system of *S. proteamaculans* 94, we previously obtained a mutant with the inactivated *sprI* gene [14]. In the present work, we used the gene replacement technique to obtain the *sprR*::Gm mutant with knockout of the second gene of the SprI/SprR QS system of this bacterium, which encodes the receptor regulatory protein SprR. The mutation was stable in *S. proteamaculans* 94 cells maintained on the medium with gentamicin (40 μg/mL). The analysis of AHL synthesis using two biosensors in Petri dishes on the LA medium showed that the mutant strain actively produces AHL. Localizations of the *sprR*::Gm and *sprI*::Gm mutations are shown in Fig. 1.

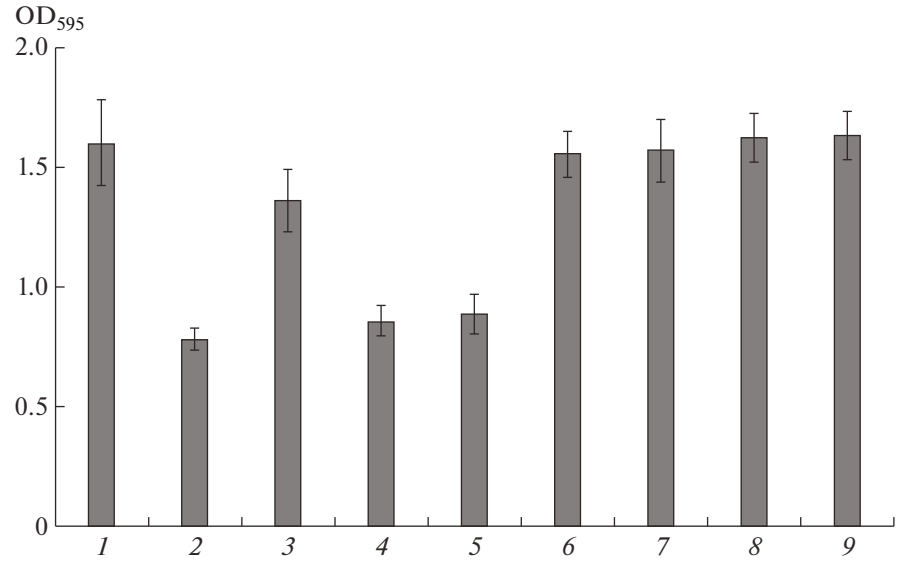

Fig. 2. Biofilm formation by the parent strain *S. proteamaculans* 94 (*1*); the mutant strain *sprI*::Gm (*2*); the mutant strain *sprI*::Gm with the addition of 3-oxo-C6-HL (*3*), C6-HL (*4*), and C4-HL (*5*); the mutant strain *sprR*::Gm (*6*); the mutant strain *sprR*::Gm with the addition of 3-oxo-C6-HL (*7*), C6-HL (*8*), and C4-HL (*9*). All AHLs were added to a final concentration of 25 nM.

2. Effect of the SprI/SprR QS System on Biofilm Formation in S. proteamaculans 94

The QS systems for gene expression regulation play a key role in biofilm formation by various bacteria [12, 27, 28]. The interaction between the QS systems and biofilm formation has not been studied in representatives of the species *S. proteamaculans*.

The study of effects of the *sprI* and *sprR* gene inactivation on the regulation of biofilm formation in *S. proteamaculans* 94 showed that the mutation in the *sprR* gene did not influence the level of biofilm formation (Fig. 2). As has been mentioned above, this mutation had no effect on AHL synthesis. On the contrary, in the strain 94 with mutation in the *sprI* gene, biofilm formation decreased by a factor of two compared to the level of biofilms in the parent strain. Previously, we showed that the cells of this mutant do not produce AHL [14]. The addition of exogenous 3-oxo-C6-HL, one of the two dominant AHLs synthesized by the wild-type strain *S. proteamaculans* 94 [14], restored the biofilm formation ability of the mutant almost to the level of the parent strain. The addition of other types of AHL synthesized by *S. proteamaculans* 94 cells at much lower amounts (C4-HL, C6-HL) had no such effect (Fig. 2). The findings show the dependence of biofilm formation in *S. proteamaculans* 94 on AHL synthesis; 3-oxo-C6-HL plays an important role in the control of biofilm formation.

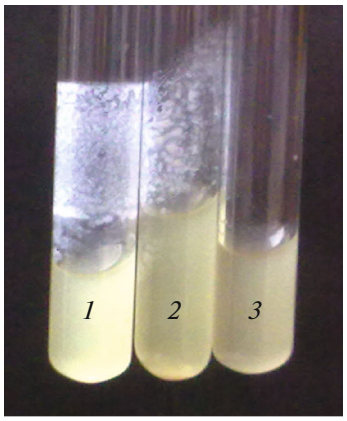

Fig. 3. Biofilm formation on the walls of glass test tubes. (*1*) *S. proteamaculans* 94; (*2*) the mutant strain *sprR*::Gm; (*3*) the mutant strain *sprI*::Gm.

The effect of mutation in the *sprI* gene on biofilm formation in *S. proteamaculans* 94 is also manifested in the experiments in glass tubes with LB. The formation of loose biofilms on tube walls was observed for the parent strain *S. proteamaculans* 94 and the *sprR*::Gm mutant, while the *S. proteamaculans* *sprI*::Gm mutant did not form biofilms on tube walls (Fig. 3). The mutation in the *sprI* gene also changed the growth pattern of *S. proteamaculans* 94 in the liquid medium. The wild-type strain and the strain mutant in the *sprR* gene are characterized by formation of multicellular aggregates and precipitate, while the *S. proteamaculans* 94 mutant in the *sprI* gene was characterized by diffuse planktonic growth with uniform turbidity of the medium.

3. Effect of sprR::Gm Mutation on the Activities of Exoenzymes Synthesized by S. proteamaculans 94

It is known that QS systems are the global regulators of bacterial gene expression. Therefore, we were interested in the role of QS in the regulation of cellular processes related to different aspects of metabolism of *S. proteamaculans* 94. The effect of knockout of the *sprR* gene of the QS system on the activities of some extracellular enzymes was studied. We showed that the protease activity in the *sprR*::Gm mutant strain actually did not differ from the activity in the parent strain, in contrast to this enzyme activity in the strain with the *sprI*::Gm mutation, where the proteolytic activity was reduced [14]. The *sprR*::Gm mutant did not differ from the parent strain in chitinolytic activity, while the *sprI*::Gm mutant strain had no chitinolytic activity. The lipase and hemolytic activities of the *sprR*::Gm and *sprI*::Gm mutant cells were not different from the activities of the parent strain *S. proteamaculans* 94 (data not shown).

4. Effects of Volatile Compounds Emitted by S. proteamaculans 94 and sprR:Gm Mutant on Fungal Plant Pathogens

We compared the effect of the total pool of volatile compounds emitted by *S. proteamaculans* 94 and *sprR*::Gm mutant on the growth of mycelium of two pathogenic fungi: *Rhizoctonia solani* and *Helminthosporium sativum*. According to experimental data, the *sprR*::Gm mutant suppresses the growth of fungal mycelium and has an inhibitory effect similar to the effect of *S. proteamaculans* 94, i.e., the *sprR*::Gm mutation apparently did not influence the genes responsible for regulation of the synthesis of volatile compounds (data not shown).

5. Analysis of the Composition of Bacterial Fatty Acids (FA)

The method of gas chromatography/mass spectrometry was used to compare fatty acid compositions in the wild-type strain *S. proteamaculans* 94 and the mutant strain *S. proteamaculans sprR*::Gm (Table 3). The samples of strains under study were shown to have saturated, unsaturated, and hydroxy acids. A total of 22 fatty acids with a chain length of 12 to 20 carbon atoms were identified in the spectra.

Nineteen fatty acids were identified in the spectrum of the wild-type strain *S. proteamaculans* 94. The major saturated acids were hexadecanoic, octadecanoic, and tetradecanoic. The predominant unsaturated acids were 9-octadecenoic, 11-octadecenoic, and 9-hexadecenoic. The fatty acid unsaturation coefficient defined as the ∑unsaturated FA/∑saturated FA ratio was 0.83 in the strain *S. proteamaculans* 94.

The fatty acid composition of the *sprR*::Gm mutant was substantially different from the fatty acid composition of the parent strain *S. proteamaculans* 94 (see, e.g., nos. 10, 14, 16, 17, 18). A mutation in this gene could have led to both a decrease and increase in the relative content of fatty acids. The fatty acid content of the mutant was characterized by a lower unsaturation coefficient compared to the parent strain (0.45) The fatty acid spectrum of the *sprR*::Gm mutant differed from the spectrum of the wild-type parent strain 94 in a dramatically increased content of cyclopropane heptadecanoic and 3-hydroxy-tetradecanoic acids (50- and 15-fold, respectively). Several fatty acids (7, 11–13, 20–22) were absent in the mutant strains as compared to *S. proteamaculans* 94. Three fatty acids (2, 3, 5) absent in the strain 94 were found in the mutant strain in the amount of less than 1%. The above data thus show that inactivation of the *sprR* gene of *S. proteamaculans* 94 had a considerable effect on the fatty acid composition of the bacterium.

6. Comparison of the Proteomic Maps of S. proteamaculans 94 and Mutant sprI::Gm and sprR::Gm Cultures

The mutants obtained in the present work were characterized by the proteome analysis of proteins synthesized by these strains compared to proteins of the parent strain. More than 600 protein spots were revealed in each of the 2D gels; among them, 30 protein spots differed in staining intensity, which represented substantial quantitative changes in the proteomes of wild type and mutant bacteria (1.5-fold and more).

In case of the *sprI*::Gm mutant, the expression of 20 proteins changed in comparison to the wild type strain: the expression of 11 proteins considerably increased, while the expression of 9 proteins decreased. In case of the *S. proteamaculans sprR*::Gm mutant, the expression of 14 proteins changed in comparison to the wild type strain: the expression of 2 proteins considerably increased, while the expression of 12 proteins decreased (Table 4).

The protein spots revealed by the difference in staining intensity were analyzed by the MALDI-TOF mass spectrometry. Eventually, we successfully identified eight proteins (Table 5). Both mutants demonstrated the enhanced expression of proteins associated with antibiotic resistance of the strains. The presence of aminoglycoside-(3)-*N*-acetyltransferase (596) in the mutant strains, which is absent in the wild type strain, determines their resistance to gentamicin. Aminoglycoside-(3)-*N*-acetyltransferase is encoded by the *accC1* gene carried by the gentamicin cassette from p34S-Gm [21], which was used to obtain the mutants. In the *sprR*::Gm mutant, the expression of β-lactamase (447) was significantly higher. Both mutants showed a substantial decrease in the expression of FMN reductase (195). In the *sprI*::Gm mutant, there was enhanced expression of the proteins involved in cellular defense against stresses (619, 617, 540). In

Table 3. Fatty acid composition in the strains *S. proteamaculans* 94 (A) and mutant *S. proteamaculans spr*R::Gm (B)

No.	R.T.	Fatty acid	Fatty acid content, % of total area of all chromatographic peaks	
			A	B
1	6.983	Dodecanoic	0.5 ± 0.1	0.9 ± 0.1
2	9.495	9-Tetradecenoic	0	0.4 ± 0.0
3	9.578	11-Tetradecenoic	0	0.6 ± 0.1
4	9.873	Tetradecanoic	2.7 ± 0.3	5.5 ± 0.2
5	10.120	2-Hydroxy-dodecanoic	0	0.7 ± 0.1
6	11.305	Pentadecanoic	0.6 ± 0.1	1.3 ± 0.3
7	12.379	7-Hexadecenoic	0.4 ± 0.1	0
8	12.449	9-Hexadecenoic	3.4 ± 1.2	20.7 ± 0.8
9	12.797	Hexadecanoic	37.2 ± 1.7	36.1 ± 1.6
10	12.839	3-Hydroxy-tetradecanoic	0.6 ± 0.1	8.8 ± 0.5
11	13.570	*iso*-Heptadecanoic	0.2 ± 0.1	0
12	13.694	*anteiso*-Heptadecanoic	0.3 ± 0.1	0
13	13.764	Heptadecenoic	0.2 ± 0.0	0
14	13.888	Cyclopropane-heptadecanoic	0.2 ± 0.1	10.2 ± 0.4
15	14.071	Heptadecanoic	0.8 ± 0.1	1.9 ± 0.2
16	15.144	9-Octadecenoic	33.2 ± 1.3	8.2 ± 0.5
17	15.186	11-Octadecenoic	4.6 ± 0.2	0.4 ± 0.1
18	15.427	Octadecanoic	11.0 ± 0.3	3.4 ± 0.2
19	16.170	Octadecadienoic, conjugated	1.3 ± 0.1	0.9 ± 0.2
20	17.556	9-Eicosenoic	1.5 ± 0.2	0
21	17.597	11- Eicosenoic	0.9 ± 0.2	0
22	17.845	Eicosanoic	0.5 ± 0.1	0
Fatty acid unsaturation coefficient			0.83	0.45

The mean ± standard deviation (SD) data of the analysis of two independently growing cultures of each variant are presented. R.T. is the retention time.

the *sprR*::Gm mutant strain, there was a decrease in the level of expression of succinyl-CoA synthetase. Thus, mutations in the genes the products of which were involved in QS regulation changed the expression of proteins essential for the metabolism of *S. proteamaculans* cells.

DISCUSSION

The phenomenon of QS regulation currently arouses great interest of researchers working in different fundamental and applied fields of biology, medicine, and biotechnology. It has been shown that this type of regulation is widespread in bacteria from various taxonomic groups.

The present work is devoted to investigation of the QS systems of Gram-negative bacteria *Serratia proteamaculans*. We were interested in studying the role of QS systems in regulation of some metabolic processes essential for the vital activity of these bacteria. Bacteria of this species are characterized by adaptation to different ecological niches: they use various substrates for nutrition, inhabit the soil and plant rhizosphere, and participate in spoilage of agricultural produce [11, 13, 29]. The QS systems of *S. proteamaculans* bacteria have been little studied; prior to our works [14, 15], there was only one article with the sufficiently investigated QS regulation system in *S. proteamaculans* [11]. As a model object, we chose the strain *S. proteamaculans* 94 isolated from meat that was spoiled in the cold storage room of a meat processing plant. The strain can hydrolyze collagen at low temperatures and is a producer of several extracellular proteinases [13].

In the present work, we have shown that AHL synthesis in *S. proteamaculans* 94 does not depend on the SprR protein. Organization of genes of the SprI/SprR system was convergent [14]. Previously, it was shown that expression of the *luxI*-type synthase gene in bacteria with convergent organization of the QS genes usually did not depend on the R protein [8–10, 12].

Table 4. Protein spots demonstrating statistically reliable differences and their relative intensities

No.	%V		No.	%V	
	S. prot. 94	*sprI*::Gm		*S. prot.* 94	*sprR*::Gm
175	0.15 ± 0.00	0.09 ± 0.02	64	0.03 ± 0.00	0
187	0.37 ± 0.01	0.23 ± 0.02	131	0.11 ± 0.01	0.06 ± 0.00
195	**1.00 ± 0.11**	**0.36 ± 0.17**	142	0.41 ± 0.06	0.18 ± 0.02
232	0.15 ± 0.01	0.08 ± 0.00	187	0.37 ± 0.01	0.15 ± 0.05
304	0.06 ± 0.00	0	**190**	**0.31 ± 0.00**	**0.06 ± 0.01**
477	**0.59 ± 0.07**	**1.05 ± 0.07**	**195**	**1.00 ± 0.11**	**0.09 ± 0.02**
487	0.14 ± 0.01	0.24 ± 0.02	237	0.08 ± 0.01	0
495	0.46 ± 0.08	0.69 ± 0.02	376	0.06 ± 0.00	0
524	0.14 ± 0.00	0.22 ± 0.01	391	0.04 ± 0.01	0
540	**0.31 ± 0.08**	**0.69 ± 0.06**	**406**	**0.20 ± 0.01**	**0.09 ± 0.01**
546	0.07 ± 0.01	0.17 ± 0.01	408	0.14 ± 0.03	0
560	0.11 ± 0.03	0	**447**	**0.03 ± 0.01**	**0.94 ± 0.16**
595	0.15 ± 0.01	0.43 ± 0.01	**596**	**0**	**1.77 ± 0.41**
596	**0**	**1.97 ± 0.11**	635	0.04 ± 0.01	0
610	0.11 ± 0.00	0.23 ± 0.02			
617	**0.26 ± 0.01**	**0.54 ± 0.04**			
619	**0.30 ± 0.05**	**1.16 ± 0.27**			
621	0.31 ± 0.02	0.08 ± 0.02			
631	0.14 ± 0.03	0			
642	0.61 ± 0.07	0.05 ± 0.07			

Soluble proteins from the cells of *S. proteamaculans* 94 and two mutants (*sprI*::Gm and *sprR*::Gm) were analyzed by separation on two-dimensional gels (2D gels); in the first direction, by the total charge of proteins at pI 4–7; in the second direction, by the relative molecular weight in the range of 75–10 kDa. Each strain is represented by three independent biological replications. Nine gels altogether were analyzed (three per each sample). Identified proteins are in bold. 0 is the absence of a spot on the gel in the given coordinates.

Table 5. Proteins identified in *S. proteamaculans* 94

No.	Protein name	Estimated Mw/pI	NCBI number
	Tricarboxylic acid cycle		
406	Succinyl-CoA synthetase (α-subunit)	29.8/5.9	157369513
190	Succinyl-CoA synthetase (β-subunit)	41.3/5.3	157369512
	Redox reactions		
195	FMN reductase	26.2/5.0	157368508
	General stress response proteins		
540	Heat shock protein GrpE	21.4/4.9	157371919
	Protection from oxidative stress		
619	Thioredoxin 2	15.6/4.9	157371981
617	Thioredoxin-dependent thiol peroxidase	17.3/5.0	157371748
	Antibiotic resistance		
596	Aminoglycoside (3)-*N*-acetyltransferase	19.4/5.8	239721
447	β-Lactamase	31.7/5.7	46129905

This is different from the major concept of QS regulation, where the activation of AHL of the LuxR protein is needed for expression of the AHL synthase gene (in case of the classical QS system of *Vibrio fischeri*). The nucleotide sequence analysis of the promoter region upstream of the *sprI* gene did not reveal the presence of a *lux*-box type regulatory element [14], which binds the LuxR/AHL complex in the system of *V. fischeri*,

leading to further activation of transcription of the *lux* operon. Thus, the above data indicate that the autoinduction of AHL synthesis observed in *V. fischeri* is atypical of the studied strain *S. proteamaculans* 94. As we have shown in the present work, the promoter region of the *sprR* gene contains a homolog of the *lux* box, a *spr* box, which overlaps with the —10 site in this promoter. Therefore, the binding of the SprR protein to the *spr* box can inhibit transcription of the *sprR* gene, preventing RNA polymerase binding. Such regularity has been shown for the regulation of transcription of some R-type proteins in *Serratia* and *Pantoea* [14]. The findings demonstrate the similarity between the regulatory SprI/SprR QS system of *S. proteamaculans* 94 and the QS systems of other bacteria of the family Enterobacteriaceae.

In the present work, we have studied the role of QS systems in the regulation of cellular processes related to different aspects of metabolism of *S. proteamaculans* 94. Enterobacteria of the genus *Serratia* are producers of a great number of extracellular enzymes, which are used for utilization of high-molecular compounds of the ambient medium and as virulence factors during invasion and colonization of other organisms. We have studied the effects of mutations in the genes of the QS system of *S. proteamaculans* 94 on the synthesis of proteases, lipases, chitinases, and hemolysin. In our previous work, it was shown that inactivation of the *sprI* gene led to a decrease in the exoprotease and chitinolytic activities and did not cause any changes in the lipase and hemolytic activities [14]. The mutation in the *sprR* gene did not exert any noticeable effect on enzyme activities under study.

It was interesting to investigate the effects of mutations in the QS *sprI* and *sprR* genes on the regulation of biofilm formation in *S. proteamaculans* 94—the process depending on QS regulation in many bacteria [12, 27, 28]. It was shown that mutation in the *sprR* gene had no effect on the level of biofilm formation. In the strain with the mutant *sprI* gene, biofilm formation decreased by a factor of two compared to the level of biofilm in the parent strain. At the same time, the addition of exogenous 3-oxo-C6-AHL (the dominant AHL in *S. proteamaculans* 94) restored the biofilm formation ability almost to the level of the wild type strain. The results show that biofilm formation in *S. proteamaculans* 94 is positively regulated by the 3-oxo-C6-HL-dependent QS system. These data are in agreement with the results of analogous studies in *S. liquefaciens* and *S. marcescens* [28, 30]. According to the data of these authors, the biofilm formation ability of AHL-deficient mutants decreased as compared to the wild type strain.

Interestingly, in another *Serratia* species that we have studied, *S. plymuthica* (strain HRO-C48), inactivation of the AHL synthase *splI* gene caused a 2- to 2.5-fold increase in the level of biofilm formation, while introduction of a plasmid with the cloned *splI/splR* genes into the cells of this mutant decreased biofilm formation to the normal level. In this case, biofilm formation in *S. plymuthica* was negatively regulated by the SplIR QS system. In another strain of this species, *S. plymuthica* IC1270, the QS system SplIR also had a negative effect on biofilm formation. As a result of introduction of a recombinant plasmid carrying the cloned *splI/splR* genes from *S. plymuthica* HRO-C48 into *S. plymuthica* IC1270 cells, its biofilm formation decreased twofold [31]. These data show that the QS regulation of biofilm formation in the genus *Serratia* can be species-specific.

Successful biofilm formation is associated with the adhesive capacity of bacteria. We showed that the mutation in the *sprI* gene changed the growth pattern of *S. proteamaculans* 94 cells in a liquid medium and drastically reduced the ability of cells to adsorb on a glass surface. It could have been due to modifications in the structure of bacterial cell walls. Hence, we studied the composition of fatty acids, many of them being components of bacterial cell membranes, in *S. proteamaculans* 94 and in the mutant strains ([14] and the present work).

The comparative analysis showed significant alterations in fatty acid composition of the mutant strains compared to the wild type strain: the content of some fatty acids changed by more than an order of magnitude; the saturated/unsaturated fatty acid ratio varied considerably. Compared to the wild type strain *S. proteamaculans* 94, the fatty acid composition of the mutants with the knockout of the *sprI* [14] and *sprR* genes was characterized by a lower unsaturation, which is evidence of the denser packing of LPS on the cell surface. This is probably one of the mechanisms of cell adaptation to unfavorable environmental conditions, which increases membrane tolerance to stress factors. It is known that the saturated/unsaturated fatty acid ratio in the LPS structure influences the physical properties of bacterial membranes [32, 33]. The phase of liquid-crystal structure of the lipid layer of the membrane and the degree of membrane fluidity, in turn, exert quite a significant effect on the viscous and elastic properties of the cells, their deformation ability, the activity of membrane-bound enzymes, and the permeability of membranes.

A comparative analysis of parent strain and mutant cells was performed for the overall estimate of the amount of proteins with QS regulation-dependent synthesis. The findings demonstrate that the expression of more than 30 proteins is affected by the SprIR QS system. Previously, it was shown that at least 39 proteins in *S. proteamaculans* B5a were under the control of 3-oxo-C6-HL-dependent QS system [11]. Thus, our data are in agreement with the results of analogous studies of other authors.

In the present work, we first identified some proteins with different levels of expression in the wild type and mutant strains. Both mutants were shown to have

changes in the synthesis of proteins essential for bacterial metabolism. For example, there was a considerable decrease in the expression of FMN reductase, one of the components of bacterial respiratory chain, in both mutants: by almost a factor of 3 for the *sprI*::Gm mutant and by a factor of more than 10 for the *sprR*::Gm mutant. The *sprI*::Gm mutant strain demonstrated enhanced expression of proteins involved in cellular defense from stresses: thioredoxin 2, thioredoxin-dependent thiol peroxidase, and heat shock protein GrpE.

In the *sprR*::Gm mutant strain, there was a decrease in the level of expression of two subunits of succinyl-CoA synthetase: by more than a factor of 2 for the α-subunit and a factor of 5 for the β-subunit. Succinyl-CoA synthetase is one of the key enzymes of the citric acid cycle, which catalyzes the production of free succinate. This reaction is accompanied by formation of energy-rich ATP (GTP) from ADP and mineral phosphate with the involvement of succinyl-CoA. The changes caused by mutation in the *sprR* gene could result in the deficiency of ATP and the total energy level in a cell.

In addition, the *sprR*::Gm mutant showed a considerable increase in the expression of β-lactamase: more than 30-fold compared the parent strain. These enzymes are responsible for bacterial resistance to β-lactam antibiotics, including carbapenems. The synthesis of carbapenems was revealed in bacteria of the genus *Serratia* [12]. Though we have no data on the synthesis of carbapenem in *S. proteamaculans* 94, we can suppose that this antibiotic is also produced by the strain 94. In this case, repression of the synthesis of β-lactamase in the wild type strain is useful for the bacterium, because β-lactamase degrades carbapenem, which can play a significant role in competition with other bacteria.

The data obtained from the proteome analysis show that the identified proteins, the expression of which varies as a result of mutations in the QS genes, are components of important cellular processes.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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