ISSN 1022-7954, Russian Journal of Genetics, 2012, Vol. 48, No. 5, pp. 513–521. © Pleiades Publishing, Inc., 2012.
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GENETICS OF MICROORGANISMS

Mutants of *Burkholderia cenocepacia* with a Change in Synthesis of N-Acyl-Homoserine Lactones—Signal Molecules of Quorum Sensing Regulation

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Received September 30, 2011

Abstract—By means of plasposon mutagenesis, mutants of *Burkholderia cenocepacia* 370 with the change in production of N-acyl-homoserine lactones (AHL), signal molecules of the Quorum Sensing system of regulation, were obtained. To localize plasposon insertions in mutant strains, fragments of chromosomal DNA containing plasposons were cloned, adjacent DNA regions sequenced, and a search for homologous nucleotide sequences in the GeneBank was initiated. It has been shown that the insertion of plasposon into gene *lon* encoding Lon proteinase drastically decreases AHL synthesis. Upon insertion of plasposon into gene *pps* encoding phosphoenolpyruvate-synthase, enhancement of AHL production is observed. In mutant carrying inactivated gene *lon*, a strong decline of extracellular protease activity, hemolytic, and chitinolytic activities was observed in comparison with the original strain; lipase activity was not changed in this mutant. Mutation in gene *pps* did not affect these properties of *B. cenocepacia* 370. Mutations in genes *lon* and *pps* reduced the virulence of bacteria upon infection of mice.

DOI: 10.1134/S1022795412050213

INTRODUCTION

Bacteria can manifest sensitivity to high population density and are capable of quickly and adequately responding to it. This specific regulation type was termed Quorum Sensing (QS). QS systems include low-molecular-weight signal molecules of different chemical nature (autoinducers) and regulatory proteins interacting with signal molecules. In response to an increase in the population density, autoinducers accumulate to reach the required threshold value, often causing strong activation of transcription of some gene sets in the entire bacterial population. The QS systems are global factors of bacterial gene expression, they were shown to play the key role in the regulation of various processes of bacterial metabolism; for example, they are involved in the interaction of bacteria with higher organisms, in regulation of bacterial virulence, they control biofilm formation and regulate the expression of genes, responsible for synthesis of toxins, antibiotics, and other secondary metabolites, various enzymes, etc. [1, 2]. The best studied QS systems are those that function with participation of signal molecules N-acyl-homoserine lactones (AHL).

Bacteria of the *Burkholderia cepacia* complex are widely distributed in nature inhabiting various ecological niches; they may be isolated from infected individuals, soil, water, plant rhizosphere, and others. Some strains of this complex, including *B. cenocepacia*, cause hospital infections, mainly, in cystic fibrosis patients (mucoviscidosis), in patients with persistent granulomatosis, and in patients with a decreased immunity. Bacteria *B. cenocepacia* contain at least three systems of QS regulation involved in control of virulence: CepI/CepR, CciI/CciR (these systems use AHL as signal molecules); recently, one more QS system has been recognized that functions with participation of signal molecule of the other nature, BDSF (cis-2-dodecenoic acid) [3–6].

It was shown that in most cases, bacteria *B. cepacia* participate, along with *Pseudomonas aeruginosa*, in the infection process of cystic fibrosis patients suffering from pulmonary infections [7]. Bacteria of the *B. cepacia* complex synthesize small quantities of AHL, generally, these are N-octanoyl-homoserine lactone (C8-HSL) and N-hexanoyl-homoserine lactone (C6-HSL) that is synthesized in an essentially lower amount; bacteria *P. aeruginosa* synthesize much more AHL. When the medium in which *P. aeruginosa* culture was grown until high population density and then purified from cells was added to *B.cepacia*, synthesis of pathogenic factors increased leading to a two-

Table 1. Strains of bacteria used in this work

Strains of bacteria	Characterization of strains	Source
Burkholderia cenocepacia 370	Clinical isolate	Collection of Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences
Chromobacterium violaceum (CV026)	Biosensor to determine AHL. Violacein production. Sm^r mini-Tn5 Hg^r *cviI::Tn5 xylE* Km^r	[14]
Agrobacterium tumefaciens NT1/pZLR4	Biosensor to determine AHL. Gm^r Cb^r	[16]
Escherichia coli S17-1	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tc^R::Mu) (Km^R::Tn7) λpir	Collection of Institute of Molecular Genetics, Russian Academy of Sciences
Escherichia coli S17-1/pTn*Mod*-RKm^R	S17-1 carries plasposon Tn*Mod*-RKm^R. is used for plasposon mutagenesis	[18]

fold increase in synthesis of proteases and sevenfold increase in synthesis of siderofores. In other words, AHL synthesized in *P. aeruginosa* can be used by *B.cepacia* [3, 8–10].

The expression of extracellular protease, chitinases, polygalacturonase, migration of bacteria on solid media (swarming), and biofilm formation were shown to be under positive regulation by the CepI/CepR QS system; this system is involved in repression of sidefore synthesis and regulation of bacterial virulence [3, 5, 11, 12]. The QS system CciI/CciR is also involved in the regulation of *B. cepacia* pathogenicity. Both QS systems mediate synthesis of C8-HSL and C6-HSL; note that the CepI/CepR QS system directs the synthesis of C8-HSL in quantity that 8-10-fold exceed that of C6-HSL. whereas the CciI/CciR system catalyzes AHL synthesis in the inverted ratio [3, 5, 6]. The regulation of the expression of genes involved in the functioning of *B. cepacia* QS systems (including *B. cenocepacia*) and the regulatory role of QS systems in cellular processes of these bacteria have been poorly studied.

In this work, plasposon mutants of *B. cenocepacia* 370 with altered production of AHL were obtained for the purpose of studying genetic control of synthesis of AHL signal molecules. In the mutant strain with drastically decreased AHL synthesis, insertion of the plasposon was localized in gene *lon* encoding Lon proteinase. Mutation leading to an increase in AHL synthesis was localized in gene *pps* encoding phosphoenolpyruvate synthase. Influence of mutations on the activity of some enzymes and the virulence of bacteria was examined.

MATERIALS AND METHODS

Strains of Bacteria and Cultivation Conditions

Bacterial strains used in this work are listed in Table 1. Bacterial cultures were grown in Luria Broth (LB), LB with 1.5% agar (LA), and M9 medium containing the required supplements [13]. A culture of fungus *Sclerotinia sclerotiorum* (from the collection of Institute of Molecular Genetics, Russian Academy of Sciences) was grown on PDA medium (Sigma). The growing of bacteria was conducted at 30°C, and of fungi, at 25°C. Antibiotics (Russia) were added at the following concentrations (μg/ml): ampicillin, 100–200; kanamycin, 100; gentamycin, 40. Tetracycline (Sigma) was used at a concentration 20 μg/ml.

Determination of AHL Production

AHL production was determined using two biosensors. The first sensor *Chromobacterium violaceum* CV026 was spread onto the surface of LA medium, intercrossed with streaks of tested cultures, and incubated for 24–48 h at 30°C. If the tested strain produced AHL, violet coloration was observed in the indicator strain (CVO26). Visual estimation of the coloration intensity was conducted [14, 15].

When the second biosensor *Agrobacterium tumefaciens* NT1/pZLR4 was used, the culture was grown on LB medium with the addition of ampicillin (100 μg/ml) and gentamycin, then incubated overnight at 30°C. The plate containing agar medium M9 with X-gal (a final concentration 80 μg/ml) was overlayed with M9 medium (3 ml) containing 0.5% agar and 0.5 ml of the overnight culture *A. tumefaciens* NT1/pZLR4. Strains tested for the ability to produce AHL were plated by an inoculating needle on the surface of the agar medium after the medium solidified, or the liquid overnight culture was placed in holes of the agar medium and incubated at 30°C for 24–48 h. The synthesis of AHL was judged from the appearance of blue zones of X-gal hydrolysis [16].

Analysis of AHL by Thin-Layer Chromatography

To identify AHL in culture extracts, thin-layer chromatography (TLC) was employed [16]. Overnight culture (200 ml) was subjected to cold centrifugation,

supernatants were mixed with ethylacetate containing 0.1% acetic acid (v/v). AHL was extracted for 30 min with shaking two times, and ethylacetate fractions were unified. The remainder of water was removed from the ethylacetate phase by an addition of Na_2SO_4. Ethylacetate was dried in rotor evaporator at 37°C, its remnant was collected, evaporated to dryness in SpeedVac, and kept at −20°C. Samples containing culture extracts or synthetic AHL standards (Sigma-Aldrich) were placed on reverse-phase TLC plates RP18 (Merck, Germany) and separated in a solvent system of 60% aqueous methanol. At the completion of chromatography, the plates were dried and coated with a layer of semi-liquid agar containing biosensors (0.7% LA for strain *C. violaceum* CV026 and 0.7% M9 containing 0.4% glucose and X-gal in the case of strain *A. tumefaciens* NT1/pZLR4). After incubation at 28°C, the localization of spots was visualized with respect to the appearance of violet coloration in the sensor strain CV026 and of blue zones of X-gal hydrolysis in the case of NT1/pZLR4.

AHL markers were used at the following concentrations: 0.01 mg/ml (C6-HSL) and 0.1 mg/ml in the case of N-butanoyl-homoserine lactone (C4-HSL), N-(3-oxo)-hexanoyl-homoserine lactone (30C6-AHL), C8-HSL. For biosensor *C. violaceum* CV026, AHL markers were placed on the plate in the following quantity (μl): 5, (C6-HSL); 7, (C4-HSL); (3-OC6-HSL), 5; (C8-HSL), 2; in the case of biosensor *A. tumefaciens* NT1/pZLR4, 5 for (C6-HSL); 1, (C4-HSL); 1, (30C6-AHL) and 1, (C8-HSL).

The final volume of supernatant extracts was 160 μl for *B. cenocepacia* 370 and mutant B2; for mutant B10, it amounted to 20 μl (i.e., it was concentrated eight times). Extracts were used in the following amounts: 2 μl for *B. cenocepacia* 370, 4 for mutant B2, 2 μl for B10 (when using biosensor *C. violaceum* CV026), and 1, 2 and 1 μl, respectively, when using biosensor *A. tumefaciens* NT1/pZLR4.

Manipulations with DNA

Isolation of total DNA, plasmid DNA, restriction, agarose gel electrophoresis, ligation, and *Escherichia coli* transformation were conducted by methods described in [17] with minor modifications adopted in our laboratory. Sequencing of DNA was conducted in the GENOM Interinstitute Center for collective use of Institute of Molecular Biology, Russian Academy of Sciences (http:www.genome-centre.narod.ru). Nucleotide sequences were compared with sequences in GenBank by means of the program BLAST. PCR was performed in 20 μl of the reaction mixture containing a 1 × PCR buffer for *Taq* DNA polymerase (Sibenzyme Corporation), 250 μM dGTP, dATP, dCTP, and dTTP (Sibenzyme Corporation), 10 pM of each primer (Research and Production Association Syntol), 0.5 U *Taq* DNA polymerase (Institute of Molecular Biology, Russian Academy of Sciences). As a matrix for PCR, plasmid DNAs or boiled cells isolated from newly-grown colonies were used. Reaction of PCR amplification was run in a TP4-PCR-01-Tercic four-channel automated thermostat (Corporation NPF DNA Technologia).

Plasposon Mutagenesis and Localization of Plasposon Insertions

Mutants were obtained as in [18]. Overnight cultures of recipient (*B. cenocepacia* 370) and donor (*E. coli* S17-1/pTnMod-RKm^R) strains were diluted 1 : 50 in LB and grown for 1 h. Equal volumes of cultures (500 μl) were mixed, centrifugated (1 min, 5000 rpm), and the precipitate was resuspended in 20 μl of LB. The resulting suspension was placed on membrane filter and spread to the LA surface in Petri dish, then incubated overnight at 30°C; during these processes, conjugation occurred, and plasmid pTnMod-RKm^R passed by conjugation into cells of *B. cenocepacia* 370. The grown cultures were washed off with 1 ml of LB. The resulting suspension (0.1 ml) was plated on selective medium with kanamycin and ampicillin (200 μg/ml). The grown colonies were tested for the ability to synthesize HSL using biosensor *C. violaceum* CV026. We selected clones in which synthesis of HSL was more enhanced or decreased, compared to synthesis in the original strain (with respect to the coloration intensity in CVO26).

Localization of Mutations. For localizing mutations, we cloned regions of chromosomal DNA in mutant strains that contain plasposon insertions. Total bacterial DNA was isolated and cut with endonuclease *Sal*I. After restriction, the reaction mixture was analysed by electrophoresis, DNA fragments varying in size from 1500 to 8000 bp were isolated from agarose gel. The isolated fragments were self-ligated. From many circular molecules obtained, only plasmids containing the plasposon were capable of replication. The mixture of plasmids isolated after ligation was transformed into *E. coli* S17-1 strain. Cells obtained after transformation were plated on selective medium with kanamycin for isolating clones with recombinant plasmids. From these clones, plasmid DNAs were obtained, the presence of gene for kanamycin resistance was determined by PCR with primers Km-R (5'-GGGAAACGTCTTGCTCGAGG) and Km-F (5'-ACAGGCCAGCCATTACGCTC). PCR conditions: primary DNA denaturation at 94°C for 3 min, subsequent 30 cycles at 94°C for 20 s, at 56°C for 20 s, at 72°C for 20 s, a final cycle proceeded at 72°C for 1 min. Determination and analysis of nucleotide sequence in the region of bacterial DNA directly adjacent to the insertion site of plasposon were conducted by sequence analysis of plasmid DNA. Experiments on sequencing were reproduced two times with primer KM-END (5'-CTGGTATGAGTCAGCAACA), the primer was chosen at the end of kanamycin resistance gene in plasposon (accession number AF061930). A comparison of the resulting sequences with nucleotide

sequences in in GeneBank was conducted by means of the program BLAST. As a result, we localized genes containing plasposon insertions.

Assay of Enzymatic Activities

Determination of extracellular protease activity. Cells of tested strains were plated by an inoculating needle on the surface of LA medium with milk (milk with 0.5% fat corresponds to one-third of the whole volume of the medium) and incubated for 24–48 h at 30°C. When strains possessed extracellular protease activity, we observed distinct zones that appear around colonies due to enzymatic hydrolysis of milk casein. The enzymatic activity was judged from the radius of hydrolysis zones.

Determination of lipase activity was carried out as in [19, 20]. Cells of the tested strains were plated by an inoculating needle on LA medium with the addition of Tween-20 (1%) and $CaCl_2$ (0.01%) and incubated for 48 h at 30°C. If the tested strains possessed lipase activity, turbid zones were observed around colonies (Tween-20 segregated to give lauric acid, which led to the appearance of insoluble salt of this acid in the presence of calcium). The enzymatic activity was judged from the radius of turbid zones around colonies and from the extent of medium turbidity within the zones.

Determination of chitinolytic activity. The tested strains were plated by an inoculating needle on agar medium (1.5% agar) of the following composition (%): $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.03; KH_2PO_4, 0.08; KNO_3, 0.04, yeast extract (Difco), 0.05; 0.2% of colloid chitin was added to the medium. Strains were incubated at 30°C for 72–96 h. If the tested strains possessed chitinolytic activity, we observed clear zones around colonies that due to enzymatic hydrolysis. The enzymatic activity was judged from the radius of zones.

Determination of hemolytic activity. Cells of the tested strains were plated by an inoculating needle on blood agar, incubated at 28°C for 3–7 h. The hemolytic activity was judged from transparent zones of hemolysis around colonies.

Assay of Antagonistic Activity of B. cenocepacia 370 toward Sclerotinia sclerotiorum

Suspension of the examined bacteria was placed to PDA medium by spreader. After incubation for two days at 30°C, agar blocks with diameter 8–10-mm were cut from the agar medium. Petri dish containing 15 ml PDA medium was inoculated with spores of fungus *S. sclerotiorum* (100 spores calculated per 1 ml of PDA medium). Blocks with the bacteria were placed on the medium surface. Zones of fungus growth inhibition around blocks were analyzed 3–7 days later.

Assay of the Virulence of Bacteria

Virulence properties of the original strain *B. cenocepacia* 370 and of mutants were compared in the experimental model for inflammation of bredless mice (males) with the weight of 18–20 g. The compared culture suspensions were administered to animals intranasally or intraabdominally at a dose of LD_{50} determined in advance. For intranasal administration, the LD_{50} dose was 2×10^9 COE/mouse, and it was 5×10^9 for intraabdominal injection. For each experimental variant, ten animals were used.

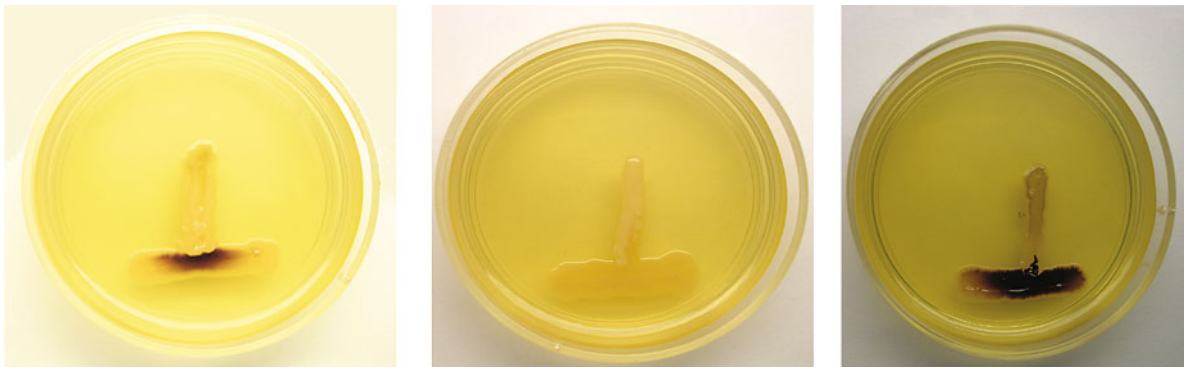
RESULTS

Strain *B. cenocepacia* 370 is a hospital isolate; it was identified in State Research Center of Antibiotics and in Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences. With the use of biosensors *C. violaceum* CV026 and *A. tumefaciens* NT1/pZLR4, this strain was shown to synthesize AHL. Genes of the Quorum Sensing system *cepI* (gene of AHL synthase) and *cepR* (gene encoding receptor regulatory protein) were identified in cells of this strain. The strain manifested potential factors of pathogenesis: hemolytic activity, extracellular protease activity, lipase activity, and also chitinolytic activity [21]. Synthesis of AHL was very weak in strain 370 (Fig. 1), which is a characteristic feature of bacteria belonging to this species.

Obtaining Mutants with Changed AHL Synthesis and Localization of Mutations

To study the regulation of the functioning of genes responsible for the QS system of *B. cenocepacia*, we undertook the work on obtaining mutants with altered AHL synthesis. With this purpose, we used plasposon mutagenesis [18]. Plasposons are derivatives of mini-transposons, which allow to rapidly localize genes with insertions, because they contain a separate origin of replication. Therefore, it was not obligatory to clone into the vector those DNA fragments that carry plasposon insertions; it is sufficient to conduct ligation of restriction fragments and transformation of *E. coli* cells, resulting in selection for antibiotic resistance, the gene of which resides in the plasposon. Plasposon Tn*Mod*–RKm^R used in this work includes Tn5 inverted repeats, the origin of plasmid R6K replication, the cassette with resistance to kanamycin, single sites of restriction near each IR element, oriT of plasmid RP4 [18].

We planned to obtain mutations in genes of QS system or in regulatory genes involved in the operation of *B. cenocepacia* 370 QS system. Clones with the altered AHL production were selected using biosensor *C. violaceum* CV026. As a result of several experiments, the mutant strain with the enhanced AHL synthesis (*B. cenocepacia* 370-B2) and strains in which AHL synthesis was absent (*B. cenocepacia* 370-B10 and *B. cenocepacia* 370-B11) were scored (Fig. 1).



B. cenocepacia 370 *B. cenocepacia* 370-B10 *B. cenocepacia* 370-B2

Fig. 1. AHL synthesis in cells of *B. cenocepacia* 370 and mutants (biosensor *C. violaceum* CV026). The lower line of bacterial growth represents *C. violaceum* CV026. The line oriented perpendicularly to the lower line represents growth of strains tested for the ability to synthesize AHL, the original strain *B. cenocepacia* 370, mutants *lon*$^-$ and *pps*$^-$. If the tested strain produces AHL, strain CVO26 produces violet pigment violacein.

While determining AHL synthesis in mutant strains with the use of biosensor *A. tumefaciens* NT1/pZLR4, we revealed AHL synthesis in mutants B10 and B11, but it was drastically decreased in comparison with synthesis in the wild-type strain *B. cenocepacia* 370 (Fig. 2). The production of AHL in mutant B2 was only slightly higher than in the wild-type strain.

For identifying genes that were disturbed by insertion of plasposon, we cloned fragments of chromosomal DNA in mutant strains containing plasposon insertions. After sequencing of DNA regions adjacent to the site of plasposon insertion, these sequences were compared with sequences in GenBank by means of the program BLAST. In mutants B10 and B11, 419- and 281-bp sequences, respectively, were sequenced in genes with plasposon insertion. It was shown that the gene *lon* encoding ATP-dependent Lon proteinase (La-protease) was destroyed in these mutants. Mutants B10 and B11 were obtained in one experiment; analysis of nucleotide sequences revealed that it is the same mutation. The mutation was located in the region corresponding to N-terminal domain of the gene.

Lon is an ATP-dependent serine proteinase that functions as homopolymer, proteolytic and ATPase centers of this enzyme are located in the single polypeptide chain. In cells of bacteria, Lon proteinase degrades defective proteins and cleaves many short-lived regulatory proteins. Lon proteinase can bind to DNA [22–25].

The resulting nucleotide sequence was homologous to sequences of *lon* genes in a large number of bacteria, including: *B. cenocepacia* (several strains) with a 93% identity; other species of *Burkholderia*, 86–93% identity; *Ralstonia solanacearum*, 85–86% identity; *Rhodopseudomonas palustris*, 75–77% identity; *Rhizobium leguminosarum*, 76%; *Brucella* (various species), 74%; *Klebsiella pneumoniae*, 74–75%.

In mutant *B. cenocepacia* 370-B2, 417-bp sequence was analyzed in the gene carrying plasposon insertion. A comparison of this sequence with sequences in GenBank showed that plasposon insertion occurred in *pps* gene (codes for phosphoenolpyruvate-synthase). This synthase is a very important metabolic enzyme of bacteria. The enzyme catalyses phosphorylation of pyruvate in the first stage of

Fig. 2. AHL synthesis in cells of *B. cenocepacia* 370 and mutants (biosensor *A. tumefaciens* NT1/pZLR4). *1, 2*, *B. cenocepacia* 370; *3*, mutant of *B. cenocepacia* 370-B2; *4*, of *B. cenocepacia* 370-B10. Biosensor was plated on agar medium containing X-gal to obtain a lawn of cells. Overnight cultures of tested strains were placed in holes of semi-liquid agar medium. Blue zones of X-gal hydrolysis appear around colonies of AHL-producing strain.

Table 2. Influence of mutations in genes *lon* and *pps* on enzymatic activities of *B. cenocepacia* 370

Strains of *B. cenocepacia*	Extracellular protease activity*	Lipase activity*	Hemolytic activity**	Chitinolytic activity*
370	3	7	3	3
B2 *pps*$^-$	3	7	3.5	2.5
B10 *lon*$^-$	0.5	7	0	0

Notes: * Radius of hydrolysis zone (from the edge of bacterial growth) mm.
** Radius of hemolysis zone (from the edge of bacterial growth) mm.

converting pyruvate into glucose; the conversion of pyruvate and ATP into phosphoenolpyruvate, AMP and phosphate takes place. The identified sequence was highly homologous to the corresponding sequences of strains *B. cenocepacia* (a 87–91% identity); to sequences of other *Burkholderia* species (83–89% identity); *Ralstonia solanacearum*, 82–84% identity; *Ralstonia eutropha*, 83%.

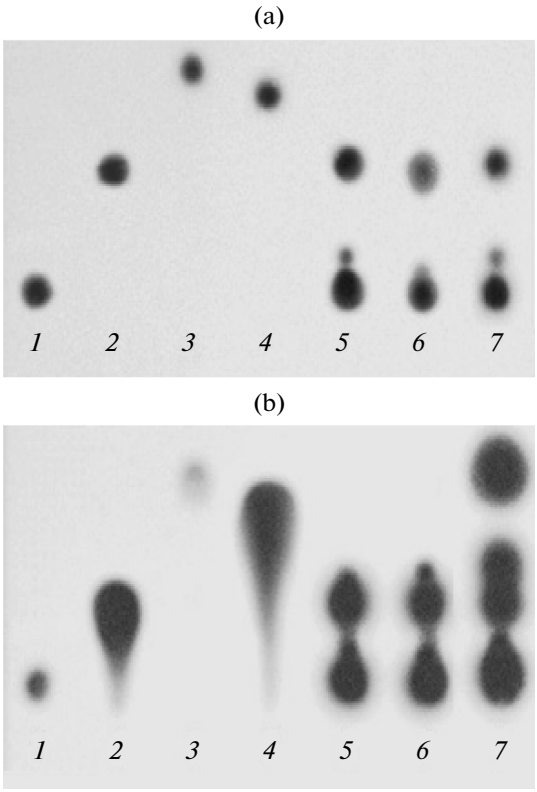


Fig. 3. Identification of AHL in culture extracts of *B. cenocepacia* 370 and mutant strains. *1*, C8-HSL; *2*, C6-HSL; *3*, C4-HSL; *4*, 30C6-AHL; *5*, AHL extract from *B. cenocepacia* 370; *6*, AHL extract from *B. cenocepacia* 370-B2(pps$^-$); *7*, AHL extract from *B. cenocepacia* 370-B10(lon$^-$). As biosensor, strain *C. violaceum* CV026 was used (a). As biosensor, strain *A. tumefaciens* NT1/pZLR4 was used (b). Quantities of extracts and AHL markers that were placed on plates are given in Materials and Methods.

Synthesis of Various Kinds of AHL in Mutant Strains

As shown above, strains *B. cenocepacia* mainly synthesize two kinds of AHL: C8-HSL and C6-HSL. To clarify synthesis of which AHL is regulated upon participation of genes *lon* and *pps*, AHL were identified by TLC in extracts of supernatants obtained in strain *B. cenocepacia* 370 and in mutant strains (Fig. 3). Note that ethylacetate extract of supernatant obtained for the strain carrying mutation at gene *lon*, had been concentrated eight times, in comparison with extracts of the wild-type strain 370 and the strain containing mutation in *pps*, as this mutant was characterized by drastically decreased AHL synthesis.

Results of TLC conducted with two sensor strains (*C. violaceum* CV026 and *A. tumefaciens* NT1/pZLR4) showed that the strain *B. cenocepacia* 370 possesses two principal HSL: C6-HSL and C8-HSL, together with two minor components the functional significance of which has not yet been understood. In strain with mutation at gene *pps*, the same types of AHL were synthesized as in the original strain. Mutation *lon* leads to significant general decrease in AHL synthesis. Moreover, one more type of AHL appears in cells of this mutant, and its nature is still unclear; the zone on the chromatogram coresponding to this AHL is located at the level that approximately corresponds to C4-HSL.

Enzymatic Activities of Mutant Strains

We showed that extracellular protease activity was virtually absent in mutant *lon* of *B. cenocepacia* B10, in contrast to wild-type cells. It has been detected [3] that the production of extracellular proteases is under positive regulation by the CepI/CepR QS system in strains *B. cepacia*. Since the *lon* mutant (B10) is characterized by drastically decreased AHL synthesis, the regulation of these two processes may be associated. Lipase activity of mutant B10 was retained at the level of strain *B. cenocepacia* 370 (Table 2).

No changes of these enzymatic activities were observed in mutant strain B2, in comparison with the original strain 370. A lack of hemolytic activiy was shown for the strain containing mutation in *lon* gene (B10), whereas the zones of hemolysis virtually similar

to those in strain *B. cenocepacia* 370 were observed in *pps* mutant (B2).

Thus, inactivation of gene *lon* causing a drastic decrease in production of AHL, signal molecules of the QS system in *B. cenocepacia* 370, also leads to a decrease in synthesis of pathogenic factors in this bacterium, i.e., extracellular proteases and hemolysines.

As noted above, bacteria *B. cenocepacia* may exist under varying environmental conditions, including soil and rhizosphere of plants. Under these conditions, chitinolytic activity may be useful for them. The strain examined in this work manifests this activity. We demonstrated that the mutation causing inactivation of gene *lon* led to the absence of chitinolytic activity (Table 2). One can assume that the observed effect of the lack of chitinolytic activity in mutant *lon* may be explained by the influence of this mutation on AHL synthesis and, hence, on the functioning of QS system in *B. cenocepacia* 370.

Synthesis of chitin is one of the factors that underlie the antagonistic activity of many bacteria toward phytopathogenic fungi. We found that *B. cenocepacia* 370 inhibits the growth of fungus *Sclerotinia sclerotiorum*; noticeable zones of growth inhibition appeared on the lawn of fungus around blocks cut from the lawn of *B. cenocepacia* 370. However, mutation in gene *lon* virtually does not affect the bacterial antagonistic activity toward *S. sclerotiorum* (data not shown). This finding indicates that the antagonistic activity of *B. cenocepacia* 370 is not specified by synthesis of chitinolytic enzymes; this activity may be connected with production of the substance, synthesis of which does not essentially depend on QS system that functions with participation of AHL. Mutation in gene *pps* has no effect on either chitinolytic activity or the action of *B. cenocepacia* 370 on *S. sclerotiorum*.

Influence of Mutations in Genes lon and pps on Virulence of B. cenocepacia 370

The effect of obtained mutations on virulence of *B. cenocepacia* 370 was examined. This strain manifests extremely weak virulence properties; therefore, mice (bredless) were infected with high doses of bacteria (LD_{50} was 2×10^9 COE/ml upon intranasal infection and 5×10^9 upon intraabdominal injection). The conducted experiments demonstrated that mutations in genes *lon* and *pps* significantly decreased the virulence of bacteria, and it was especially clear upon intranasal infection (Table 3).

Table 3. Influence of mutations in genes *lon* and *pps* on virulence of *B. cenocepacia* 370

Strain	% of dead mice
Intraabdominal model	
B. cenocepacia 370	80
B. cenocepacia – B2 *pps*$^-$	20
B. cenocepacia – B10 *lon*$^-$	20
Intranasal model	
B. cenocepacia 370	60
B. cenocepacia – B2 *pps*$^-$	0
B. cenocepacia – B10 *lon*$^-$	0

DISCUSSION

As we already noted, bacteria of the *B. cepacia* complex may exist under varying environmental conditions and in various hosts. This diversity suggests the presence of many genes ensuring the survival of these bacteria in various ecological niches. However, these genes may be needed only under specific conditions and may not be necessarily connected with the existence of bacteria under other conditions. Apparently, the effective mechanisms for regulating expression of such adaptive genes in bacteria must be involved. Regulators of QS system can be involved in this regulation. In the case of the examined strain *B. cenocepacia* 370, synthesis of hemolysines, exoprotease and lipase activities are considered to be factors of pathogenesis important for the existence of bacteria in the human organism or in animals, whereas synthesis of chitinases and the antagonistic activity toward phytopathogenic fungi may serve as instruments for competitive *B. cenocepacia* 370 interactions in soil and plant rhizosphere.

We obtained *B. cenocepacia* 370 mutants with altered AHL synthesis; a mutant with drastically decreased AHL synthesis (*lon*$^-$) and a mutant with enhanced AHL synthesis (*pps*$^-$). Mutations in these genes were identified in *B. cenocepacia* for the first time.

The fact that mutation in gene *pps* (encoding phosphoenolpyruvate synthase) leads to an increase in AHL production suggests the relationship (probably, indirect) between this enzyme and the QS system of regulation in *cenocepacia* 370, more likely, its participation in AHL synthesis. There are no data in the literature suggesting such a relationship in *B. cenocepacia* and other Gram-negative bacteria. Mutant cells, as well as cells of the original strain, synthesized all four types of AHL. Mutation in gene *pps* did not affect the examined enzymatic activities and antagonistic activity in *B. cenocepacia* 370 (Table 2).

Mutation in gene *lon* led to a total drastic decrease in AHL synthesis and to the occurrence in cells of additional AHL, possibly, C4-HSL. The nearly complete absence of exoprotease, hemolytic, and chitinolytic activities was observed in mutant strains carrying the inactivated *lon* gene as opposed to the original strain 370. Collectively, the data on a decrease of AHL production and on a decrease of these three enzymatic activities suggest that the QS system and/or Lon pro-

teinase play a positive role in the regulation of synthesis of the indicated enzymes in *B. cenocepacia* 370.

It is still unclear how inactivation of gene *lon* affects the operation of QS system in *B. cenocepacia* 370. LuxR homologs are known to be substrate of Lon proteinase, for instance, LuxR of *Vibrio fischeri* [26, 27], TraR of *A. tumefaciens* [28]; proteins of this type comprise proteins CepR and CciR found in *B. cenocepacia*. Hence, one could expect that inactivation of receptor regulatory proteins will lead to an increase in AHL synthesis. This was in fact recorded in the case of QS system LuxI/LuxR of *V. fischeri* in *E. coli* cells [26, 27] and PpuI/PpuR QS system of *Pseudomonas putida* [29]; the amount of synthesized AHL increased in cells of *lon* mutants. However, mutation in gene *lon* did not affect synthesis of N-3-oxo-C12-HSL in *Pseudomonas aeruginosa* [29]; according to other authors [30], this mutation enhances the production of C4-HSL and C6-HSL.

Studies of the role of proteinase Lon in the QS system regulation were not conducted in *B. cenocepacia* (and other species of *Burkholderia*); it is still unknown whether this enzyme causes degradation of receptor proteins CepR and CciR. We did not observe enhanced AHL production in cells of *lon* mutant; on the contrary, it was markedly decreased in this mutant. This effect can have the following explanation. It is likely that substrates of Lon proteinase in *B. cenocepacia* 370 include, apart from receptor R proteins (by analogy to other QS systems), also some repressor or repressors of the QS system or systems. Lon proteinase almost completely degrades repressor and partially CepR, CciR proteins. Therefore, the original strain *B. cenocepacia* 370 manifested weak AHL synthesis: the repressor is inactivated, but there are few receptor R proteins. In mutants for gene *lon*, R proteins do not undergo degradation, the repressor is extremely active, and as a result, AHL synthesis is inhibited in cells. This asuumption requires special experimental investigation.

In this work, we showed the necessity of functioning the genes *lon* and *pps* for the virulence of *B. cenocepacia*. The effect of mutation in gene *lon* might result from the lack of AHL production or direct inactivation of Lon proteinase, which led to the inhibition of cellular processes needed for the virulence of bacteria, such as synthesis of potential factors of pathogenesis: extracellular proteases and hemolysines. It was found that Lon protease is involved in the regulation of expression of genes related to the virulence of other bacteria, for example, *Salmonella enterica*, *S. typhimurium*, *Yersinia pestis*, and *Y. pseudotuberculosis* [31].

Because mutation in gene *pps* enhanced AHL production, on which virulence of *B. cenocepacia* 370 is dependent, it may well be that decreased virulent properties in the mutant are directly connected with disturbing of functions of genes, the expression of which is essential for the virulence of bacteria.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (projects nos. 06-04-48585 and 09-04-00422).

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