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The effect of mutation in the *clp*X gene on the synthesis of *N*-acyl-homoserine lactones and other properties of *Burkholderia cenocepacia* 370

M.A. Veselova[a], Yu. M. Romanova[b], V.A. Lipasova[a], O.A. Koksharova[a,c], Yu. V. Zaitseva[a], M.U. Chernukha[b], A.L. Gintsburg[b], I.A. Khmel[a,*]

[a] *Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov Square 2, Moscow 123182, Russia*
[b] *The Gamaleya Scientific Research Centre of Epidemiology and Microbiology, Gamaleya Str. 18, Moscow 123098, Russia*
[c] *M.V. Lomonosov Moscow State University, A.N. Belozersky Institute of Physico-Chemical Biology, Leninskie Gory 1-40, Moscow 119991, Russia*

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ABSTRACT

In order to study the regulation of *N*-acyl-homoserine lactones synthesis (AHLs, the signal molecules of Quorum Sensing regulation) in *Burkholderia cenocepacia* strain 370 we obtained mutants with increased AHL production. One of the mutants, named BC-B6, was obtained by Tn*Mod*-RKm^r plasposon mutagenesis. The plasposon insertion was located within the *clp*X gene encoding the ATPase subunit ClpX of the ClpXP protease. The mutation reduced bacterial virulence in mice intranasal infection. The results of proteomic analysis demonstrated that the expression of at least 19 proteins differed not less than 2-fold between the parental and mutant strains. 18 of the proteins were upregulated in the mutant, and one protein was downregulated. The proteins included those that involved in protein synthesis and modification, in energy production, in general metabolism, in transport and regulation. To check the effect of the *clp*X mutation on the AHL synthesis, a mutant with inactivated *clp*X gene (BC-*clp*X:Km^r) was constructed by gene replacement method. This mutant also exhibited increased AHLs production. A swarming motility of both mutants was reduced compared to the original strain. Thus, the obtained results show that the *clp*X gene was involved in the regulation of AHL production and a number of cellular processes in *B. cenocepacia* 370.

1. Introduction

Bacteria of *Burkholderia cepacia* complex (Bcc) are ubiquitously distributed in nature in different ecological niches. Strains belonging to the Bcc complex can be isolated from infected patients, soil, water, and rhizosphere of plants. Some strains of the Bcc complex are considered as opportunistic pathogens that can cause severe respiratory infections among individuals suffering from cystic fibrosis or chronic granulomatous disease, as well as among immunocompromised patients. *B. cenocepacia* strains are not only important opportunistic pathogens of humans, they can also cause infections in other species, including plants, nematodes, rodents etc. (Coenye and Vandamme, 2003; Loutet and Valvano, 2010). Such a variety of habitat conditions require the presence of finely tuned regulatory mechanisms controlling bacterial metabolism in different environmental niches. These mechanisms include Quorum Sensing regulatory systems (QS).

At least three types of chemical signals identified in *B. cenocepacia* strains are used by the bacteria in cell-to-cell communication and participate in the functioning of QS regulatory systems. CepI/CepR and CciI/CciR systems include *N*-acyl-homoserine lactones (AHLs) as signaling molecules, CepI and CciI—AHL synthases, and CepR and CciR—regulatory receptor proteins that interact with AHLs. CepI AHL-synthase produces N-octanoyl-homoserine lactone (C8-HSL) and minor amounts of N-hexanoyl-homoserine lactone (C6-HSL). CciI AHL-synthase directs the synthesis of C6-HSL and minor amounts of C8-HSL. The CepIR system is widely distributed in bacteria of Bcc complex while CciIR systems only present in *B. cenocepacia* strains containing pathogenicity island (*cci*) (Malott et al., 2005; Sokol et al., 2007; O'Grady et al., 2009; Suppiger et al., 2013). AHL-mediated QS systems control various functions of bacterial cells including biofilm formation, swarming motility, synthesis of virulence factors, such as proteases, toxins, siderophores (Huber et al., 2001; Eberl, 2006; Fazli et al., 2013; Suppiger et al., 2013;

* Corresponding author.
E-mail address: khmel@img.ras.ru (I.A. Khmel).

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Fazli et al., 2014). *B. cenocepacia* bacteria synthesize another signal molecule, *cis*-2-dodecenoic acid (BDSF) (Boon et al., 2008) involved in cell–cell communication. BDSF accumulates in a cell density dependent manner with maximum levels observed in the late stationary phase. BDSF-mediated QS system regulates expression of many genes responsible for different functions including motility, biofilm formation, exopolysaccharides production, protease synthesis, virulence, stress tolerance etc. (Deng et al., 2009).

However, the regulation of genes involved in the QS system functioning in B. *cenocepacia* has been studied insufficiently. In this work, we studied regulation of the AHL synthesis in B. *cenocepacia* strain 370. It is known that strains of the *B. cepacia* complex synthesize only small amounts of AHLs (Lewenza et al., 1999). Using plasposon mutagenesis, we obtained a mutant of *B. cenocepacia* strain 370 with increased production of AHLs. The plasposon insertion was shown to inactivate the *clp*X gene encoding the ClpX-ATPase subunit of the ClpXP protease, a global regulator of bacterial gene expression. A proteomic analysis demonstrated that the expression of at least 19 proteins differed not less than 2-fold between the parental and mutant strains. The finding that inactivation of the *clp*X gene resulted in increased synthesis of AHLs was confirmed by obtaining a *clp*X:Km^r insertion mutant. Thus, we have shown that the *clp*X gene participates in the regulation of AHL synthesis and many other processes in *B. cenocepacia* 370.

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are shown in Table 1. Bacteria were grown in liquid Luria Broth medium (LB, Sigma), on agarized (1,5%) LB (LA) medium and in M9 medium (Miller, 1972) with required supplements at 30 °C. Fungus *Sclerotinia sclerotiorum* (Collection of the Institute of Molecular Genetics, RAS) was grown on PDA (Sigma) medium at 25 °C.

Appropriate antibiotics were added at the following concentrations: ampicillin (Ap, "Biochemist", Russia), 200 μg/ml; tetracycline (Tc, Sigma-Aldrich), 20 μg/ml; kanamycin (Km, "Synthesis", Russia), 100–200 μg/ml; gentamicin (Gm, KRKA, Slovenia), 40 μg/ml; trimethoprim (Sigma-Aldrich), 50–100 μg/ml.

2.2. Assays of AHL production

AHLs bioassays were performed on plates using pigment violacein-reporter strain of *Chromobacterium violaceum* CV026 producing pigment violacein and β-galactosidase-producing reporter strain of *Agrobacterium tumefaciens* NTL4/pZLR4 (McClean et al., 1997; Shaw et al., 1997; Cha et al., 1998).

To detect AHLs by TLC analysis, ethyl acetate extracts of cell culture supernatants were used, according to the procedures described (Shaw et al., 1997). Briefly, 200 ml of an overnight culture (17 h of growth) were centrifuged at 4 °C, and the supernatant was mixed with ethyl acetate containing 0.1% acetic acid (v/v). After AHL extraction, the water was removed from the ethyl acetate phase by using Na_2SO_4, and ethyl acetate was evaporated. Samples of ethyl acetate extracts of the tested strains and AHL standards (Fluka) were spotted onto glass-backed RP18 F_{254S} reverse-phase TLC plates (Merck), and separated in methanol/water (60% v/v) solvent. TLC plates were then overlaid with biosensor strain *A. tumefaciens* NTL4/pZLR4 in soft agar (0.6% M9 medium supplemented with glucose 0.2% and X-gal 40 μg/ml). After incubation overnight at 30 °C, AHLs were identified visually as blue spots.

2.3. DNA manipulations

Isolation of total genomic DNA and plasmids, restriction enzymes digestions of DNA, ligation, agarose gel electrophoresis, PCR, and transformation of *Escherichia coli* cells with plasmid DNA were generally performed according to standard procedures (Ausubel et al., 1994). Restriction enzymes and bacteriophage T4 DNA ligase were purchased from MBI Fermentas (Vilnius, Lithuania) and used according to the manufacturer's instructions. DNA sequencing was performed with ABI PRISM® BigDye™ Terminator v. 3.1 at the Applied Biosystems 3730 DNA Analyzer (Center for Collective Use "Genome"). Comparative sequence analysis was performed with BLAST software.

PCR reactions were performed in a total volume of 20 μl containing a buffer for Taq DNA polymerase ("Sylex", Russia), 250 μM each of the four deoxynucleoside triphosphates, 10 pM of each PCR primer ("Syntol", Russia), and 0.5 U of Taq DNA polymerase (Institute of Molecular Genetics, Moscow, Russia). Plasmid DNA or boiled cells obtained from the fresh-grown colonies were used as templates for PCR.

2.4. Plasposon mutagenesis

Plasposon mutagenesis was performed as described (Dennis and Zylstra, 1998). In brief, the plasposon pTn*Mod*-RKm^r was transferred during conjugation between the donor *E. coli* strain S17-1 and *B. cenocepacia* 370, and transconjugants were selected by screening in LA medium containing 200 μg/ml Km and 200 μg/ml Ap (w/v). We obtained more than 1500 clones containing a plasposon (Km-r). All clones carrying plasposon were analyzed for the synthesis of AHL. As a result, the only one mutant BC-B6 with greatly increased AHL synthesis was obtained in this work; the plasposon insertion was located in a *clpX* gene.

Cloning of the DNA fragment of BC-B6 mutant strain containing plasposon was performed to localize the plasposon insertion. After *Sal*I digestion of chromosomal DNA the reaction mixture was analyzed by gel-electrophoresis and the DNA fragments of 1500–8000 bp in size were isolated and subjected to self-ligation. *E. coli* S17-1 cells were transformed by ligation mixture, and bacterial cells were plated on selective LA medium supplemented with kanamycin, 100 μg/ml.

Plasmids from Km-r clones were isolated, and the presence of the Km-r gene was confirmed by PCR using the primers Km-R 5′-GGGAAACGTCTTGCTCGAGG and Km-F 5′-ACAGGCCAGCCATTACGCTC. PCR conditions were: 94 for 3 min, then 30 cycles at 94 °C for 20 s, 56 °C for 20 s, 72 °C for 20 s, and 72 °C for 1 min. The region of chromosomal DNA adjacent to the insertion of plasposon was twice sequenced using the primer KM-END 5'-CTGGTATGAGTCAGCAACA (accession number AF061930). The obtained nucleotide sequences were compared with those in the GeneBank. The plasposon insertion was located within the *clp*X gene.

2.5. Construction of a clp*X* mutant by gene replacement method

2.5.1. Construction of a pEX18Tp plasmid

A 794-bp *Ehe*I fragment of the Tc-r gene of the plasmid pEX18Tc (Hoang et al., 1998) was replaced by a 633-bp *Sma*I fragment that contained the trimetoprim resistance encoding gene of a p34S-Tp plasmid (Dennis and Zylstra, 1998). The resulting plasmid (6188 bp) was named pEX18Tp.

2.5.2. Construction of a BC-clpX:Km^r mutant

A 926-bp *B. cenocepacia* 370 chromosomal DNA fragment containing part of the *clp*X gene was amplified by PCR using the primers CLPX-F 5′- GTGTACAACCACTACAAGCG and CLPX-R 5′-