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POST-TRANSLATIONAL REGULATION OF THE *RPOS* AND *PSRA* GENES IN *PSEUDOMONAS PUTIDA* WCS358: THE ROLE OF CLPXP PROTEASE

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Abstract — The RpoS and PsrA proteins are key transcriptional regulators that are activated in response to the stationary phase of growth in pseudomonads. This study was designed to establish whether ClpXP (ATP-dependent serine protease) regulates levels of *RpoS* and *PsrA* in *Pseudomonas putida* WCS358. Western blot analysis of *P. putida* WCS358 protein extracts from the early exponentianl, late exponential, and stationary phases of growth with antibodies against *RpoS* and *PsrA* revealed that these proteins are degraded by ClpXP in the early exponential phase of growth. The obtained results demonstrate a role for ClpXP protease in post-translational regulation of proteins encoded by the *rpoS* and *psrA* genes in Pseudomonas spp.

Key words: *Pseudomonas,* post-translational regulation*, RpoS, PsrA*

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INTRODUCTION

Gram-negative bacteria have evolved sophisticated stress response mechanisms that allow them to adapt to a broad range of stressful conditions. The central regulator during the stationary phase in *Pseudomonas* spp., as in other Gram-negative bacteria, is the stationary phase RpoS alternative sigma factor (Hengge - Aronis, 2002; Jorgensen et al., 1999; Suh et al., 1999). In *Escherichia coli*, RpoS regulates more than 100 genes involved in cell survival, cross-protection against various stresses, and virulence (Ishihama, 2000). Similarly, in pseudomonads RpoS regulates a large set of genes, as recently demonstrated using microarray transcriptome analysis (Schuster et al., 2004). The expression of RpoS in *E. coli* is carefully controlled at the levels of transcription, translation, and post-translation, and its synthesis considerably increases at the onset of the stationary phase. Regulation has been studied to a lesser extent in fluorescent pseudomonads, where – contrary to the case of *E. coli* - transcriptional regulation plays a major role (Venturi, 2003). It has been shown that the TetR family regulator designated PsrA plays a major role in positively regulating *rpoS* transcription at the entry of pseudomonads into the stationary phase (Kojic and Venturi, 2001; Kojic et al., 2002).

ClpXP is a bipartite protease responsible for the degradation of RpoS in *E. coli* (Hengge – Aronis, 2002). The ClpX component of ClpXP is a hexameric ring ATPase belonging to the Clp/Hsp100 subfamily of the AAA+ ATPases (Schirmer et al., 1996). ClpX by itself has the capacity to recognize specific substrates and to denature and/or remodel the tertiary structures of these proteins in an ATP-dependent reaction (Jones et al., 1998). The ClpP component of ClpXP is a serine peptidase with broad sequence specificity. ClpP consists of two stacked heptameric rings, which enclose a central chamber containing the enzyme's 14 active sites. In *E. coli*, ClpP also associates with ClpA, an ATPase related to ClpX, to form the ClpAP protease (Katayama et al., 1988).

In this paper, the role of ClpXP protease in post-translational regulation of the RpoS and PsrA proteins in *Pseudomonas putida* WCS358 is described for the first time.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The bacterial strains used in this study were *Pseudomonas putida* WCS358 (Geels and Schippers, 1983), *P. putida* WCS358 *clpX*::Km (Bertani et al., 2003), *P. putida* WCS358 *rpoS*::Km (Kojic and Venturi, 2001) and *P. putida* WCS358 *psrA*::Km (Kojic and Venturi, 2001). Bacteria were grown in LB medium (1% tripton, 0.5% NaCl, and 0.5% yeast extract) with addition of kanamycin (100 μg/ml) for *P. putida* WCS358 *clpX*::Km, *P. putida* WCS358 *rpoS*::Km and *P. putida* WCS358 *psrA*::Km. Agar plates were prepared by the addition of agar (1.5%, w/v) (Torlak, Belgrade, Serbia). Bacteria were grown at 30°C.

Preparation of cellular protein extracts

LB medium (200 ml) was inoculated with over night cultures (1% finally) of *P. putida* WCS358, *P. putida* WCS358 *clpX*::Km, *P. putida* WCS358 *rpoS*::Km and *P. putida* WCS358 *psrA*::Km. Bacterial cells were obtained by centrifugation (5000 rpm, 15 min, 4°C) in a microfuge from Eppendorf (Hamburg, Germany) at optical density (OD) of 0.7, 1.5, and 2.5 at 600 nm for all four strains. Pellets were resuspended in 1 ml of the reaction buffer [40 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 60 mM KCl, 0.5 mM DTT, 0.4 mM EDTA (pH 8), and 3.4% glycerol in water], transferred to microtubes, and kept on ice. Cell suspensions were sonicated on ice five times for 10 sec at 10 mA with a 30-sec pause between each sonication. After sonication, cell suspensions were centrifuged for 1 h at 13 000 rpm and 4°C in the above-indicated microfuge. The resulting supernatant (500 μl) were transferred to Ti50 ultracentrifuge vials and centrifuged in a model L7-55 ultracentrifuge (Ti50 rotor) from Beckman Coulter (Fullerton, USA) for 2.5 h at 25 000 rpm and 4°C. Finally, 300 μl of supernatant was transferred to clean cold microtubes and mixed with the sample-loading buffer [125 mM Tris-HCl (pH 6.8) 10 mM EDTA (pH 8) 4% SDS, 25% glycerol, 5% 2-mercaptoethanol and 0.07% bromphenolblue] in a 1:1 volume ratio. Prior to loading, samples were heated at 100°C for 5 min and analyzed by SDS-PAGE on 15% gels. Cellular protein extracts from *rpoS* and *psrA* mutants of *Pseudomonas putida* WCS358 were used as a negative control for analysis of *rpoS* and *psrA* expression in further experiments.

Western blot analysis

Transfer of proteins from SDS-PAGE gels to a PVDF membrane (Millipore, Bedford, USA) was done with a semi-dry fast blot (Fastblot B43, Biometra, Goettingen, Germany) according to the manufacturer's protocol. PVDF membranes were air-dried after protein transfer, then incubated for 1 h in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4, in 1 l H_2O) containing 0.1% Tween 20 and 5% milk powder. After saturation, membranes were incubated in PBS-0.1% Tween 20 with rabbit *rpoS* and *psrA* polyclonal antibodies in a volume ratio of 1:2000 for 1 h. The membranes were washed in PBS-0.1% Tween 20 and then incubated in PBS-0.1% Tween 20 with rabbit immunoglobulins conjugated with HRP (DAKO A/S, Glostrup, Denmark) in a volume ratio of 1:4000 for 1 h, after which they were washed with PBS-0.1% Tween 20. The signal was detected with SIGMA *FAST*™ 3,3'-diaminobenzidine tablets (Sigma Aldrich Chemie, Germany).

RESULTS AND DISCUSSION

In order to determine the role of ClpXP protease in post-translational regulation of the *rpoS* and *psrA* genes of *Pseudomonas putida* WCS358 (wild type), cellular protein extracts were isolated from this strain and its *clpX*, *rpoS*, and *psrA* mutants. Cellular levels of RpoS and PsrA in protein extracts of the early exponential, late exponential, and stationary phases of bacterial culture growth were then investigated using Western blot analysis with RpoS and PsrA polyclonal antibodies.

Western blot analysis of wild type strain and *clpX* mutant strain protein extracts revealed that in the early exponential phase of growth (culture $OD_{600\ nm}$ =0.7) both RpoS and PsrA protein levels were significantly higher in the *clpX* mutant strain than in the wild type (for RpoS, see Fig. 1, lanes 1 and 2; for PsrA, see Fig. 2, lanes 1 and 2). This result supports

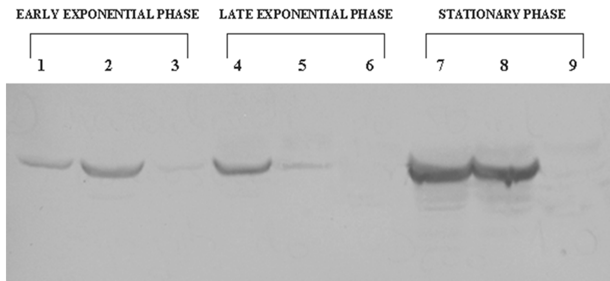

Fig. 1. Western blot analysis of protein extracts from *P. putida* WCS358, *P. putida* WCS358 *clpX*::Km, and *P. putida* WCS358 *rpoS*::Km with antibodies against *RpoS*. Lanes 1, 4, and 7: RpoS levels in protein extract of *P. putida* WCS358 grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively; Lanes 2, 5, and 8: RpoS levels in protein extracts of *P. putida* WCS358 *clpX*::Km grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively; Lanes 3, 6, and 9: RpoS levels in protein extracts of *P. putida* WCS358 *rpoS*::Km grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively – negative control.

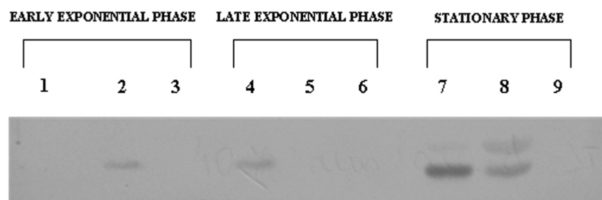

Fig. 2. Western blot analysis of protein extracts from *P. putida* WCS358, *P. putida* WCS358 *clpX*::Km, and *P. putida* WCS358 *psrA*::Km with antibodies against PsrA. Lanes 1, 4, and 7: PsrA levels in protein extract of *P. putida* WCS358 grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively; Lanes 2, 5, and 8: PsrA levels in protein extracts of *P. putida* WCS358 *clpX*::Km grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively; Lanes 3, 6, and 9: PsrA levels in protein extracts of *P. putida* WCS358 *psrA*::Km grown to $OD_{600\ nm}$ = 0.7, 1.5, and 2.5, respectively – negative control.

our presumption that ClpXP protease degrades RpoS and PsrA when these proteins are not needed for cell survival in non-stress conditions (such as the exponential phase of growth). Thus, regulation of *rpoS* and *psrA* gene expression at the level of protein stability in *Pseudomonas* is preventive. The response to stress renders cells broadly stress-resistant in such a way that damage is avoided rather than needing to be repaired. Surprisingly, in the late exponential phase of growth (culture $OD_{600\ nm}$ =1.5), the presence of RpoS and PsrA was shown in wild type but not in *clpX* mutant strain protein extracts (for RpoS, see Fig. 1, lanes 4 and 5; for PsrA, see Fig. 2, lanes 4 and 5). The experiments were performed in triplicate with the same outcome, confirming almost complete degradation of RpoS and PsrA in the late exponential phase of growth. The absence of detectable amounts of these proteins in late exponential phase of growth of *P. putida* WCS358 *clpX*::Km could be explained in the light of the associating potential of ClpP in *E. coli*. It is known that in addition to ClpX, ClpP interacts with other ATP-dependent molecular chaperones of ClpA in *E. coli* (Katayama et al., 1988). ClpA serves as a substrate-specifying adapter for ClpP peptidase in the ClpAP protease complex. Taking into account this possibility, we analyzed the sequenced genome of *Pseudomonas putida* KT2440 *in silico* looking for the presence of a gene coding for a ClpA-like protein and found a gene that codes for a protein showing 65% amino acid identity with the *E. coli* ClpA protein. The ClpA levels in *E. coli* increase during the late exponential and early stationary phases resulting in an increase of ClpAP activity (Katayama et al., 1990). Knowing that *P. putida* harbors ClpA ATPase, we could speculate that in the late exponential phase of growth, in the absence of ClpX, ClpP forms a complex with ClpA and degrades RpoS and PsrA. This degradation results in the absence of RpoS and PsrA in *P. putida* WCS358 *clpX*::Km during the late exponential phase. When cells enter the stationary phase of growth, PsrA activates transcription of the *rpoS* gene and the RpoS synthesized enables the cell to cope with starvation stress. Thus, equal levels of RpoS and PsrA were expected in both *P. putida* WCS358 and *P. putida* WCS358 *clpX*::Km in the stationary phase of growth (culture $OD_{600\ nm}$ =2.5) (for RpoS, see Fig. 1, lanes 7 and 8; for PsrA, see Fig. 2, lanes 7 and 8) and corresponded with previous reports for the RpoS protein in *E. coli* (Hengge - Aronis, 2002).

In summary, we found that *rpoS* and *psrA* expression is regulated at the post-translational level by ClpXP protease during the early exponential phase of growth in *Pseudomonas putida* WCS358. In view of the fact that RpoS and PsrA proteins are very important global transcriptional regulators involved in the response to the stationary phase, production of extracellular molecules, and quorum sensing (Kojic and Venturi, 2001; Schuster

et al., 2004, Kojic et al., 2005, Girard et al., 2006, Chatterjee et al., 2007), the results presented in this work will provide additional insight into the regulation networks in pseudomonads.

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ПОСТТРАНСЛАЦИОНА РЕГУЛАЦИЈА ЕКСПРЕСИЈЕ *RPOS* И *PSRA* ГЕНА У *PSEUDOMONAS PUTIDA* WCS358: УЛОГА CLPXP ПРОТЕАЗЕ

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RpoS и PsrA протеини су кључни транскрипциони регулатори који се код псеудомонада активирају у одговору на стационарну фазу раста. Циљ ове студије био је утврђивање улоге ClpXP (ATP зависна серин протеаза) у стабилности RpoS и PsrA протеина током различитих фаза раста у *Pseudomonas putida* WCS358. "Western blot" анализа протеинских екстраката *P. putida* WCS358 и *P. putida* WCS358 *clpX*::Km из ране експоненцијалне, касне експоненцијалне и стационарне фазе раста, са антителима на RpoS и PsrA, показала је да ClpXP деградује RpoS и PsrA у раној експоненцијалној фази раста.